Chemical defense expression and growth in three specialist aphid herbivores of the common milkweed, *Asclepias syriaca*

Winter 2008

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A paper submitted in partial fulfillment
of the requirements for the degree of
Bachelor of Arts at
Kalamazoo College

Acknowledgements

Thanks to Pierce Cedar Creek Institute for providing housing, sample sites, and funding for this research. Thanks should also be given to the Career Development Center at Kalamazoo College for its financial and logistical support during off-site field work. I appreciate the support of Western Michigan University for allowing me to access laboratory and field materials. Thanks to the NSF-REU program at Western Michigan University for support during the summer of 2006. Thank you to Dr. Steve Malcolm for advising me through the process of this research, providing laboratory materials for my project, and forcing me to think critically. Thank you to Natalie Hickman for helping me conduct field work throughout the course of this project. Special thanks to Allister Malcolm, Ned Malcolm, Sean Smith, Anne Wohlford, and Kerry McMartin, without whom the cardenolide extraction could not have been performed. Thanks to Ann Fraser, Ariane Reister, Ellen Chenoweth, Jackie Howard, Stefano Crescentini, Cat Bartnik, and Kelly Houseal for critically reviewing manuscripts.

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Abstract

It is known that the aposematic aphid, *Aphis nerii* sequesters toxic cardenolides from its host plants in the family Apocynaceae. *A. nerii* is a conspicuous herbivore of the common milkweed, *Asclepias syriaca*, in Michigan where it competes for phloem resources with two additional aphid species, *Aphis asclepiadis* and *Myzocallis asclepiadis.* While these two aphids are also specialists they differ from *A. nerii* in that they are not brightly colored and little is known about them. I contrast the defenses of these three aphid species as "fight", "run" and "hide" strategies against their natural enemies and hypothesize that tradeoffs should maximize sequestration in *A. nerii* which "fights" with aposematism. In contrast, both *A. asclepiadis* and *M. asclepiadis* appear to use crypsis to "hide" from predators and *M. asclepiadis* is also very active and appears to be able to "run" from predators as well as "hide." In field experiments with aphids constrained on *A. syriaca* for replicated time treatments I found that the aphids did not differ significantly in terms of reproductive rate on *Asclepias syriaca*, but were significantly different in average aphid mass. Additionally, *A. asclepiadis* and *M. asclepiadis* were found to sequester cardenolides at levels significantly lower than those of *A. nerii*. I also found that both *A. nerii* and *A. asclepiadis* were strongly aggregated on milkweeds, but *M. asclepiadis* was widely dispersed. In addition, *A. nerii* had only one conspicuous, bright yellow morph, while both *A. asclepiadis* and *M. asclepiadis* varied in coloration with time. Such polymorphism associated with low, but measurable cardenolide sequestration could suggest a mechanism for the evolution of aposematism, with toxicity and developing conspicuousness coupled in an insect herbivore.

Introduction

Investment in defense against natural enemies is an important component of all life histories as evidenced by the extraordinary diversity of defensive adaptations in plants and animals (Cott, 1940; Edmunds, 1974). This diversity has been conveniently summarized by Malcolm (1992) who suggested that all defenses function as "fight", "run" or "hide" strategies against natural enemies and that they vary according to their cost:benefit tradeoffs. While no defense is absolutely effective, there are circumstances in which one or another of these three basic strategies are favored. Thus the insect herbivores of milkweeds are almost all brightly colored, or aposematic, and their strategy is to "fight" against natural enemies with their sequestered chemical defenses (Weiss & Dickerson, 1921; Price & Willson, 1976; Malcolm, 1991). They sequester these defenses from plants of the milkweed genus *Asclepias*, which produce these steroidal chemical defenses for their own protection against herbivores. These steroids are known as cardiac glycosides, or more specifically as cardenolides, and are characterized by a five membered lactone ring and 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 (Holzinger and Wink, 1996). Thus, these toxins disrupt the ionic balance of cells in organisms that consume milkweeds (Malcolm, 1991).

Among the specialist insect herbivores of milkweeds are three aphid species that vary in their defensive strategies against natural enemies. We know that *Aphis nerii* (B. de F.) is bright yellow with black legs and siphunculi because it sequesters cardenolides from milkweeds and uses these chemical defenses effectively against a range of natural enemies (Malcolm, 1981; 1986; 1989; 1990; 1992; 1995). This aphid is a common herbivore of milkweeds worldwide. In the Great Lakes region of North America, *A. nerii* occurs on milkweeds with two additional aphid specialists, *Aphis asclepiadis* (Fitch) and *Myzocallis asclepiadis* (Monell) both of which appear cryptically colored green on milkweed leaves. Such co-occurrence of the three specialist aphids led me to question whether their chemical defenses differ and whether there are differences in performance among the aphids associated with defensive life history trade-offs. Here I test the null hypothesis that there is no difference in cardenolide sequestration among the three aphids and that there is no difference in performance, as measured by biomass accumulation with time. My results have implications for the operation of defense as well as the debate about the evolution of conspicuousness and aposematism (Ruxton et al. 2004).

Field Observations

Milkweeds (*Asclepias spp.*) get their common name from the fact that they 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 (Woodson 1954; Price and Willson 1979), and exhibit different defensive characteristics (Malcolm, 1991, Agrawal and Fishbein 2006). The common milkweed, *Asclepias syriaca* is found throughout North America and is known to produce cardenolides (Malcolm et al. 1989). *A. syriaca* is the most modeular milkweed known and has an underground stem system that connects individual above-ground stems, or ramets within the genet and such modularity may influence its defense (Woodson 1954, Hoch 1961, Price and Willson 1979, Malcolm et al. 1989).

While milkweeds photosynthesize energy and store nutrients, their herbivore parasites are a sink for these nutrients that they use for their own growth and defense. In addition to acting as a nutrient sink, many of the herbivores also sequester milkweed chemical defenses for use in their own defense against natural enemies in the third trophic level. For example, the aphid *Aphis nerii* uses plant cardenolides for its own defense by sequestering them from the internal phloem of milkweeds (Rothschild et al. 1970, Botha et al. 1976). *Aphis nerii* is bright yellow aphid and is aposematic. We observed it to be densely aggregated in the field. We also observed *A. nerii* to exhibit only one bright yellow morph, without any polymorphism. Because of these observations, *A. nerii* seems to be employing a fighting defense against its natural enemies. By examining natural history clues we can make predictions about the defense strategy of *A. nerii.*

While much is known about the cardenolide sequestration of *A. nerii*, much less is known about that of *A. asclepiadis.* It is known that *A. asclepiadis* is also commonly found on *A. syriaca* in nature (Weiss and Dickerson 1921). We observed *A. asclepiadis* to be polymorphic and that it can range in color from olive green to dark green with tiny white dots. We observed winged adult *A. asclepiadis* to be black in color. We also saw wingless adults but they maintained the typical green colors. We observed *A. asclepiadis* to be densely aggregated on plants in the field and that it does not use an abandoning (running defense) tactic similar to 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*.

Like *A. asclepiadis*, little is known about *M. asclepiadis.* We observed aphids of this species to be cryptic, often having a translucent green color with later instars, or stages of postembryonic development, exhibiting a pattern of light red spots late in the season. All adults of *M. asclepiadis* were observed to be winged. These aphids were observed to be highly mobile in the field and tended to be widely dispersed. When we disturbed *M. asclepiadis* with light, they mobilized and early instars 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. The cardenolide handling of *M. asclepiadis* has not been documented. Again, by examining the life history of this species as well as its cardenolide handling, we may be able to elucidate the mode of defense that it uses.

Materials and Methods

In order to test these hypotheses we placed aphids of *A. nerii*, *A. asclepiadis*, and *M. asclepiadis* on the common milkweed for varying lengths of time in controlled environments and then measured life history traits and cardenolide levels. The two independent variables that we manipulated were thus aphid treatment and time treatment. A combination of these two variables represents a trial (e.g. *A. asclepiadis*, 2 day). We ran 10 replicates of each trial. The natural history measurements that we recorded included nearest neighbor distance, aphid reproduction, total aphid mass and individual aphid mass. We then measured the aphid and plant cardenolide levels per mass.

Insect Culture

Aphid cultures of *A. nerii* and *A. asclepiadis* were established from natural populations feeding on *A. syriaca* in Kalamazoo County, Michigan in June of 2007. Cultures were created from the same original population and placed on milkweeds at Pierce Cedar Creek Institute in Hastings, Michigan and Western Michigan University in Kalamazoo, Michigan. 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 able to be isolated in a culture prior to experimental trials and instead were removed from naturally occurring *A. syriaca* prior to each trial.

Plant Selection

Naturally occurring *A. syriaca* plants were used from sites on the Western Michigan University campus and at Pierce Cedar Creek Institute. Plants selected were at least 1 meter in height and without initial evidence of major herbivore feeding. Trial plants were selected at random from each cluster of plants (genetic individual or genet) and individual plants were used for only one trial. One trial of each aphid species and time interval was used in each cluster selected, with each replicate being located in a different cluster. There were thus 10 plant clusters used in the study, each one representing a replicate group. Each cluster, then, hosted several trials on different individual plants.

Aphid Density Sampling

Aphid density sampling was done for the three aphid types in one of two ways on sites on the Western Michigan University campus in the summer of 2006. For the aggregated *A. nerii* and *A. asclepiadis*, density measurements were taken in the laboratory using a dissecting microscope connected to a computer with MetaMorph digital imaging software. We broke off an entire leaf containing naturally occurring *A. nerii* or *A. asclepiadis* and placed it under 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. For the widely dispersed *M. asclepiadis*, nearest neighbor data were taken by measuring the distance from one aphid to the nearest neighboring aphid using a metric ruler in the field. This was done because of the flight response that we observed in *M. asclepiadis* in response to light disturbance (Winged adults flew while wingless individuals ran and dropped from the leaf). When we placed *M. asclepiadis* on leaves under the microscope the bright light caused the aphids to disperse and we were unable to measure nearest neighbor distances.

Clip Cage Construction

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 polyvinyl chloride (PVC) rings held together so that leaves could be placed in the center. Each cage had a 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 of a given 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 ramets (from here written as plants) in each cluster that contained no aphids. The trial durations 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 with a metric ruler. The total number of leaf pairs on each plant was also recorded. Evidence of other herbivory was noted upon completion of each trial and categorized as sucking or chewing herbivory.

Post-Trial Measurements

Once trials were completed, leaves with clip cages attached were removed from the plants and 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. Dry aphid mass and leaf mass were taken for all samples.

Chemical Analysis

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 pellet was 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 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, Airgas© 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 two times to remove any remaining cardenolides. The syringe and filter were blotted to remove remaining acetonitrile. Each 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, 996 diode array detector and Millennium^{32}™ 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 213.5 and 223.5 nm. Cardenolide concentration for each peak (µg/0.1g sample DW) was calculated from a calibration curve with the cardenolide standard digitoxin (Sigma). Only cardenolide peaks reported by Millennium software as consistently pure were considered for analysis.

Statistical Analysis

Results were analyzed using JMP statistical analysis software. Two-way analysis of variance (ANOVA) was used to measure differences in trials by time interval and aphid treatment. Significant differences were determined by *P* values less than 0.05. Post-hoc analyses were performed using Tukey HSD.

Results

There was a significant difference among nearest neighbor distances for the 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).

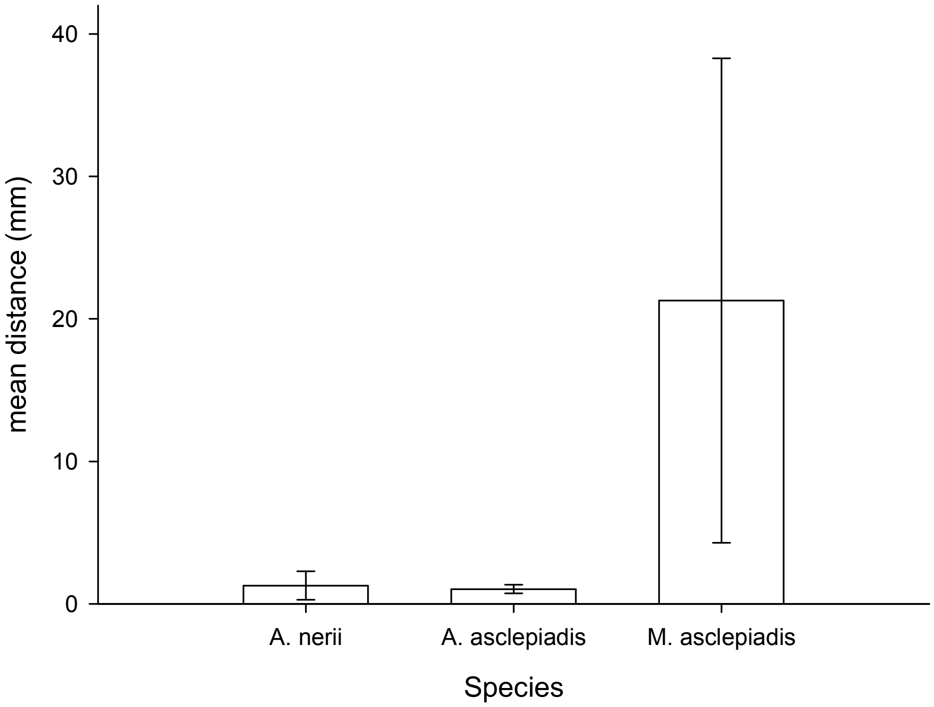

Figure 1. Nearest neighboring aphid distances for each aphid species shown in mm as mean ± SE showing the comparatively broad distribution of *M. asclepiadis* to *A. nerii and A. asclepiadis*. ($F_{2,224} = 68.7$, $P<0.0001$).

The leaf area change measured before and after trials showed a significant difference by time, but not by aphid treatment. A two-way analysis of variance (ANOVA) showed a significant effect of time treatment on leaf growth ($F_{(3,\,154)} = 7.38$, $P<0.0001$), indicating that longer time intervals produced larger leaf area change. Aphid treatment and control did not show significant differences on plant growth ($F_{(3,\,154)} = 1.75$, $P= 0.16$). There was no significant interaction between time and aphid treatment ($F_{(9,\,154)} = 0.82$, $P= 0.60$). These results are summarized in Figure 2.

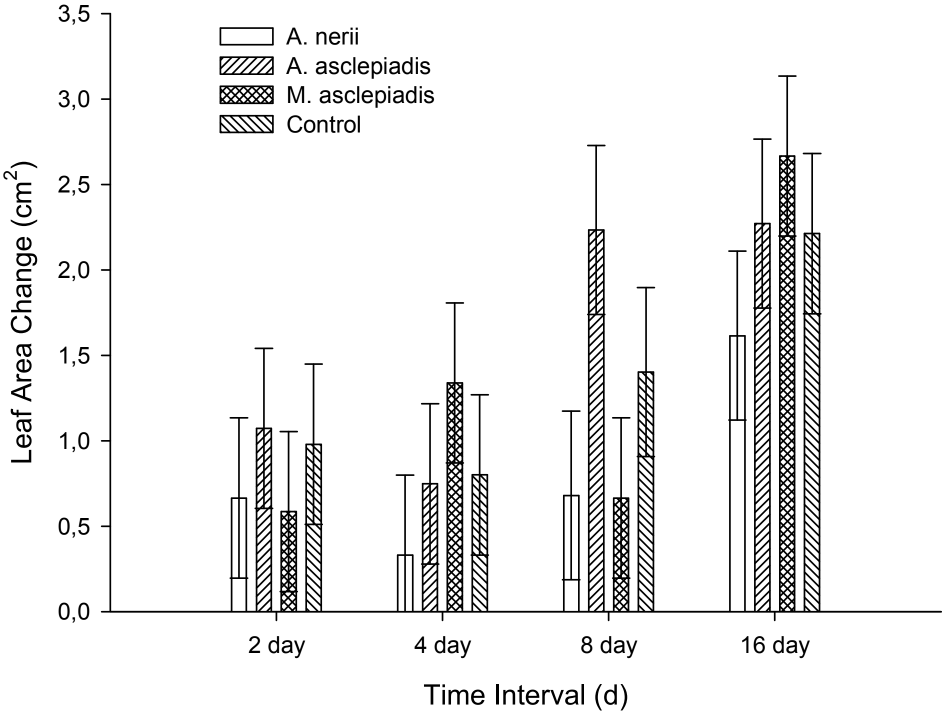

Figure 2. Graphical summary of the leaf area changed grouped by the four time treatments and aphid treatments used. Data represented in cm^2 as mean ± SE.

The reproduction per day measurements showed statistical significance in two-way ANOVA by time treatment (Figure 3, $F_{(3, 119)} = 4.95$, P=0.003), but not by aphid species ($F_{(2, 119)} = 0.77$, P=0.46), and there was no interaction between the terms ($F_{(6, 119)} = 0.67$, P=0.67) (Figure 4). Presence of other herbivores at the end of trials was analyzed against aphid reproduction rate and shown to have no significant differences ($F_{(2, 119)} = 1.0199$, P=0.3146).

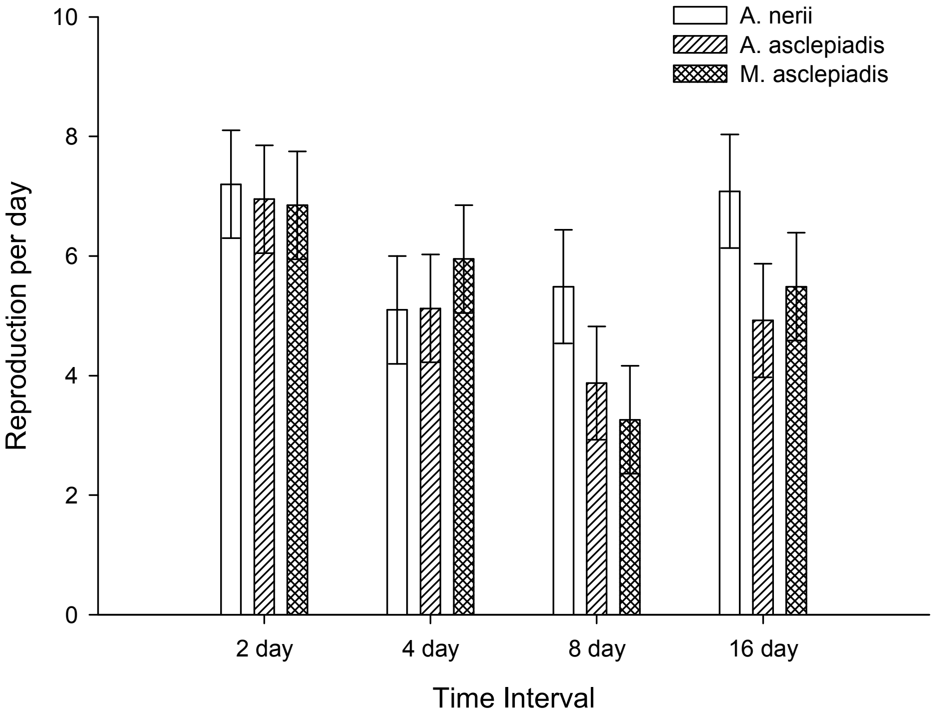

Figure 3. Graphical representation of aphid reproduction in aphids per day plotted against time in days shown in individuals present per trial length as mean ± SE.

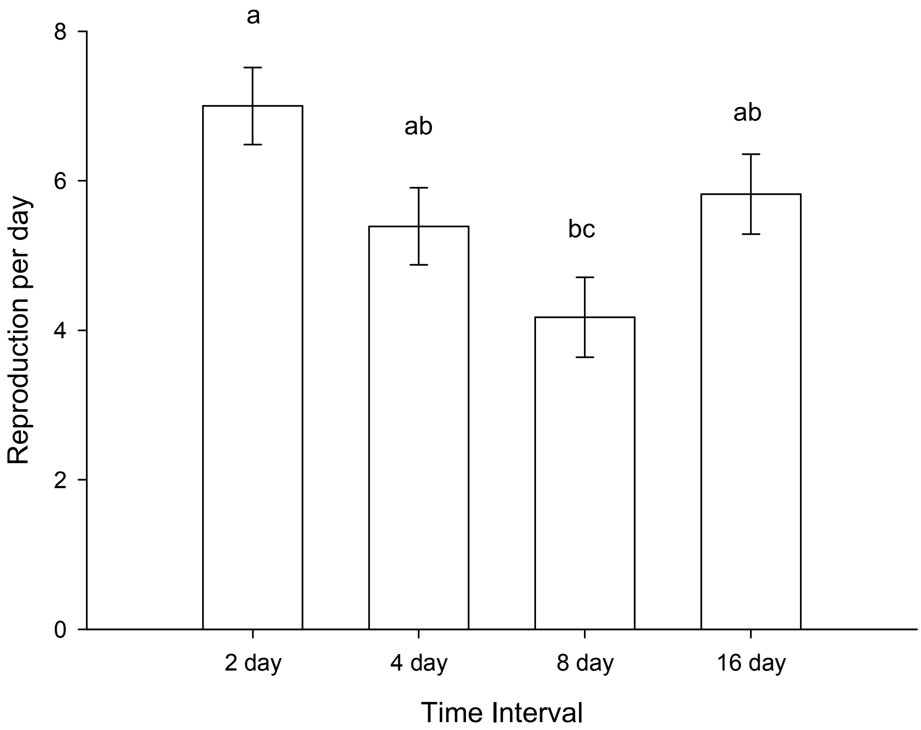

Figure 4. Summary of aphid reproduction per day measurements grouped by time treatment shown in individuals present per trial length as mean ± SE. Different letters represent significant difference.

Aphid mass was found to vary by aphid species and by time interval. Two-way ANOVA showed a significant difference in aphid mass per day by aphid species ($F_{(2, 112)} = 19.1055$, $P<0.0001$), as well as by time treatment ($F_{(3, 112)} = 5.47$, $P=0.002$). There was also a significant interaction between these terms ($F_{(3, 112)} = 5.34$, $P<0.0001$). These results are summarized graphically in Figure 5.

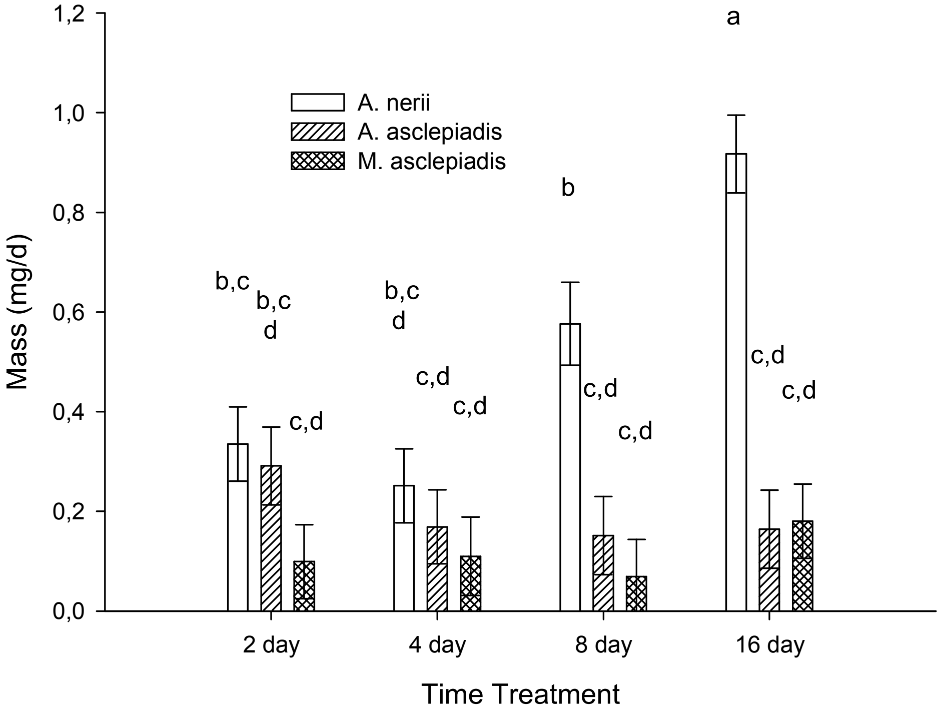

Figure 5. Graphical summary of aphid mass change per day shown as mean ± SE, showing the increasing mass per day of *A. nerii* with time. Mass is represented in mg per day and time is shown in days.

The cardenolide analysis of trial leaves showed significant differences by aphid species ($F_{(3,\ 139)} = 2.74$, P=0.0458), but not by time ($F_{(3,\ 139)} = 2.04$, P=0.1121), nor by the interaction of species and time treatments ($F_{(9,\ 139)} = 1.48$, P=0.1611). The cardenolide levels of leaves grouped by species can be found in Figure 6. A Tukey HSD post hoc test found no significant differences by species, time, or the interaction of these two variables.

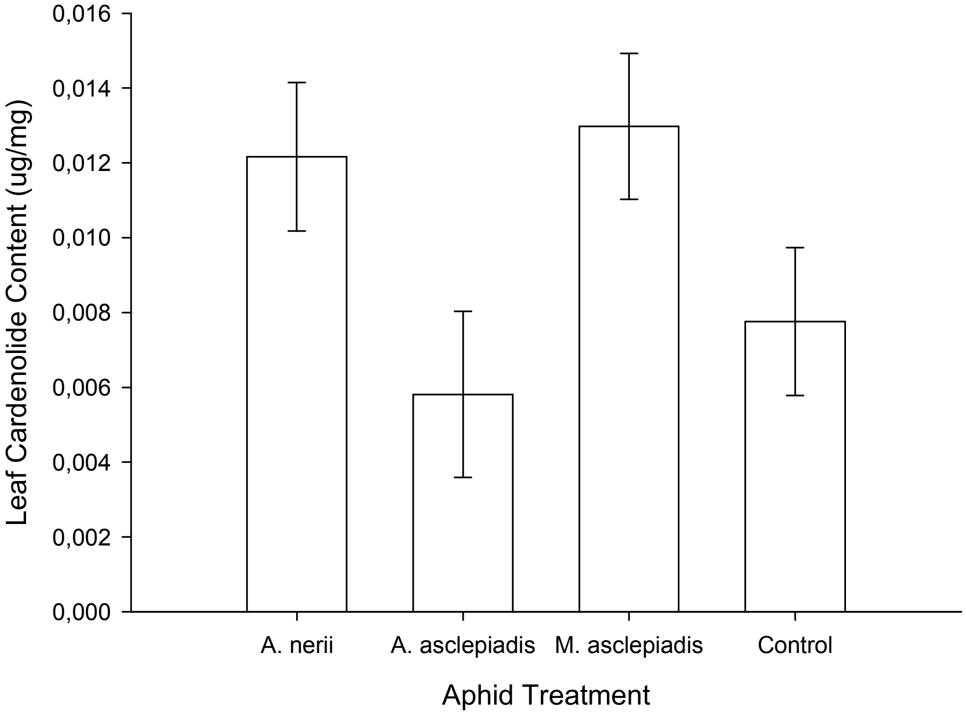

Figure 6. Summary of leaf cardenolide content (µg/mg) by aphid treatment shown as mean ± SE.

The HPLC analysis of aphid cardenolides showed a significant difference across aphid species ($F_{(2, 112)} = 29.08$, $P<0.0001$), but not by time ($F_{(3, 112)} = 1.08$, $P=0.36$). The interaction of these terms produced a significant result, however ($F_{(6, 112)} = 5.01$, $P=0.0002$). A graphical summary of cardenolides grouped by aphid species is shown in Figure 7 and a detailed representation of cardenolide levels separated by time and aphid treatment is shown in Figure 8.

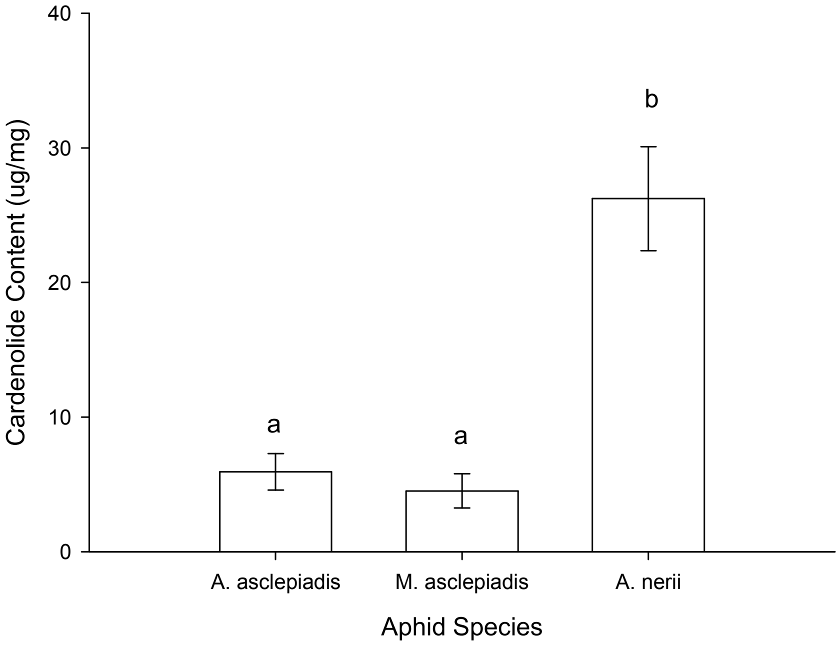

Figure 7. Cardenolide content of each aphid species in µg/mg shown as mean ± SE. *A. nerii* showed a significantly higher cardenolide content than the other two species, as signified by different letters.

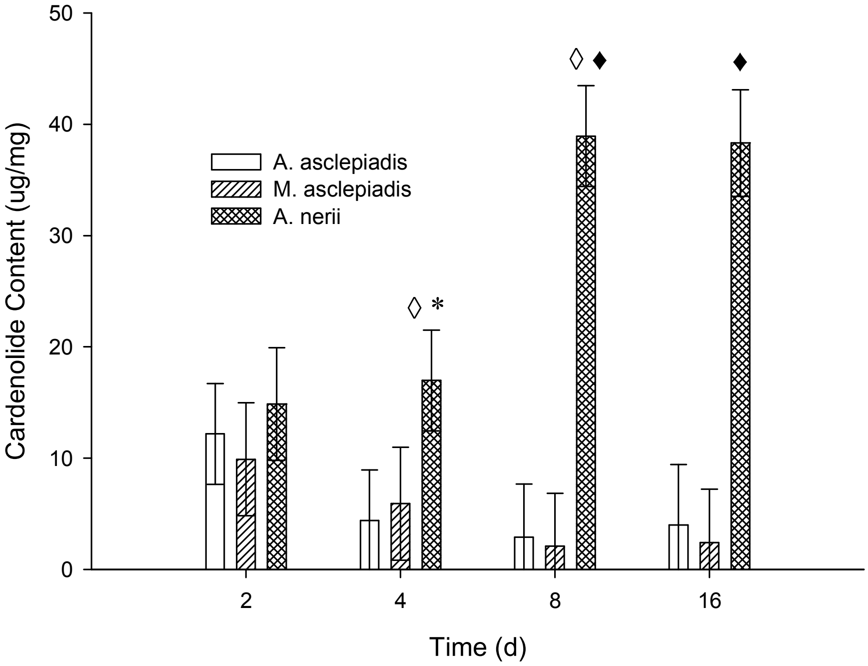

Figure 8. Aphid cardenolide levels in µg/mg separated by species and grouped by time treatment showing a statistically significant increase in *A. nerii* cardenolides through time. Data are shown as mean ± SE. Statistically significant differences are denoted by different symbols. Bars unmarked with symbols are the same as *.

Discussion

In a complex tritrophic system where tradeoffs abound for both competition and defense, it is no surprise that three milkweed specialist aphids have adapted different life histories and modes of defense. The differences in dispersion, growth, and cardenolide content that we measured in these aphids allow us to bolster our confidence in the hypothesis that they employ different defensive strategies. Because we found *A. nerii* to be aggregated, have a fast growth rate by mass, and sequester cardenolides, it appears that this species employs some type of fighting defense. We found *A. asclepiadis* to be densely aggregated, apparently cryptic but polymorphic by instar, have a slow growth rate, and have low cardenolide levels, suggesting a hiding defense. Our field observations on *A. asclepiadis* confirmed that it is readily tended by ants, which are thought to play a role in its defense (Mooney et al., 2007). Thus, the defense of *A. asclepiadis* may be a hybrid of hiding and fighting. We observed *M. asclepiadis* to be widely dispersed, have a slow growth rate by mass, and have low cardenolide levels, which in conjunction with its variably cryptic coloration and high mobility, suggests that this aphid may use a blend of fighting, running, and hiding defensive tactics.

General Defense Tactics

In order to further understand the way that these aphids defend themselves from their natural enemies, we should first define each defensive strategy separately. Fighting defense is one of the general models for defense which have been proposed to explain the way that organisms protect themselves from predators. A fighting defense comes in many forms and is physically manifested in spines, claws and chemical defenses. Chemical fighting defense is often coupled with aposematic coloration, which is generally defined by structures, colors or other signals that animals with dangerous attributes use to advertise their danger to predators to avoid being attacked. 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). The natural history traits that we measured in our aphids allow us to better classify their mode of defense.

A running defense includes evading predators instead of acting 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 typically signs of a running or predator evasion defense. A predator evasion defense can have apparent tradeoffs in other natural history measurements such as time to maturity and growth rate (Loose and Dawidowicz, 1994). We can examine the apparent running defense of *M. asclepiadis* using our natural history measurements.

A hiding defensive strategy does not rely on physically harming or evading predators like the fighting and running defenses, but instead relies on crypsis to avoid detection by those predators altogether. This can be achieved in different ways including physical camouflage, low population density, and living in inconspicuous places. Hiding defense is variably effective against different predators since some predators can see colors that others cannot. Hiding defense can also be variable with the background color that an organism is upon (Endler and Greenwood, 1988). The presence of a hiding defensive strategy has implications for the aggregation or dispersion of individuals (Ruxton and Sherratt, 2006).

There are apparent energy trade offs for each of these defenses. Fighting defense provides low vulnerability to predators, but has a high energy cost due to the necessity to develop mechanisms to fend off predators. Likewise although running prey 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. Organisms employing a hiding defense use a much different defensive strategy, wherein 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). Our observations indicate that these defensive strategies may be employed together or change through time.

Based on our findings *A. nerii* seems to employ a fighting defense, while *A. asclepiadis* and *M. asclepiadis* do not have a defensive strategy that fits into one of these three categories, and may instead use different hybrid defensive tactics. We will first look at each of our natural history measurements separately in terms of the aphid defense that the results imply, and then move on to examine the implications on aphid defense of our results as a whole.

Dispersion

The significant difference in nearest neighbor distances across aphid species further confirms the suspected natural 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. Cryptic individuals that are widely dispersed have a lower chance of being encountered by a predator than a large group of the same individuals (Staddon and Gendron, 1983). This method of defense could use a strategy where predators are more likely to encounter a single individual due to a dispersed nature, but encountering one individual does not sacrifice the safety of other individuals. Courtney proposed that this could be an effective defensive strategy (1986).

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 variably 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 aggregation helps alert predators of their location, and when predators encounter the aggregate they will consume an aphid and become unable to consume others (Lindström et al., 2001). Due to its cryptic nature, *A. asclepiadis* is not likely warning predators of its unpalatability like *A. nerii*. Instead, a small aggregate of aphids may have differences in ant tending from a dispersed group of the same size or from a large aggregate (Morales, 2000). As such, *A. asclepiadis* may be better able to recruit ants, which are suspected to aid in its defense (Mooney et al., 2007), by being densely aggregated in small clusters.

Plant Performance

The presence of different aphid species did not significantly affect plant growth against the control. Although this result suggests that the aphid sink does not affect the plant health in terms of leaf area, this may be due to the relatively small aphid population size for a relatively large plant with underground connections to other plants. It has been shown in similar experiments that even higher aphid densities did not elicit changes in milkweed defense compound levels from a control (Mooney et al., 2007). Since both changes in leaf growth and defense compound production are responses to herbivory, it follows that the relatively small aphid sinks used in these trials were too small to draw out a response from plants in measurable leaf size difference against controls. As one would expect however, the trial length significantly affected leaf growth, with 16 day trials growing much more than any of the other treatments. This can be expected since plant leaf surface should increase with time interval. Although the aphid presence did not have an effect on the leaf area change, time universally did.

Aphid Performance

The three aphid species also did not show significant differences in terms of aphid reproduction. This suggests that although the aphids employ different defensive strategies through their natural densities, they still produce offspring at rates that are not significantly different. Time treatment showed a significant difference in aphid reproduction, however. Not surprisingly, the time treatment that had the largest mean reproduction rate was the two day treatment. This can be explained by the initial presence of two adult aphids in an empty clip cage on a leaf that has no other aphid herbivory. It has been shown that other aphid herbivory leads to a decreased aphid reproduction rate (Sluss, 1967). The four and eight day treatments showed decreasing mean reproductive rates, which may correspond to the response noted by Sluss. The 16 day trial showed a surprising result though, 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 the aphid offspring are able to reproduce and give a second 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 aphid mass per day showed significant differences by aphid species with *A. nerii* accruing much more mass per time than the other two aphid species, which were similar to each other. The different time intervals showed significant differences because of the effect that increasing *A. nerii* mass per time had on the time means (Figure 5). Thus, it was most likely the effect of increasing *A. nerii* mass rather than the time difference itself that caused the differences by time. This helps confirm that the aphid species employ different survival strategies. The aphid mass per day results show the difference between the three aphid species in terms of energy use. Since *A. nerii* accumulates the most mass per day by far, 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 less energy to maintain. It could be that for an aposematic aphid such as *A. nerii*, it is advantageous to take up more nutrients and in turn cardenolides, in order to be as potent as possible to predators. Mooney et al. also found that *A. nerii* consumes more phloem per time than *A. asclepiadis* and suggested that this correlates to a higher degree of fitness for *A. nerii* (2007). The cryptic nature of *A. asclepiadis* and *M. asclepiadis* and the high mobility of *M. asclepiadis,* however, may cause these aphids to be better served by staying small and thus less detectable by predators (Bednekoff and Lima, 1998).

Cardenolide Sequestration

In conjunction with the role of size and growth rate on aphid defense, defense compound production by milkweeds allows some aphids to be chemically defended. 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 milkweeds 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 the different aphid species feeding on milkweeds. The significant differences in plant cardenolides by aphid species suggest that the aphids differentially affect milkweed defense compound production; however our post-hoc analysis was not able to determine where those differences lie. Although Mooney et al. (2007) found no change in plant cardenolides with similar aphid densities to those used in this study, Martel and Malcolm (2004) showed that aphids can induce and reduce milkweed cardenolide production. It is known that *A. nerii* feeds preferentially from the internal phloem of milkweeds (Malcolm, 1981), but where *A. asclepiadis* and *M. asclepiadis* feed is not well understood. Differences in the feeding sites of these aphid species may cause the plant to differentially produce defensive compounds, but further studies must be done to elucidate those differences.

Differences in defensive compound sequestration across aphid species helps confirm the hypothesis that these herbivores use differing modes of defense. The significant decrease in cardenolide content from *A. nerii* to the other two aphid species, *A. asclepiadis* and *M. asclepiadis,* shows that the differences in natural history of these aphid species not only are found in aphid density and mass, but also defense compound sequestration. It should be noted, though, that both *A. asclepiadis* and *M. asclepiadis* were found to contain a certain amount of cardenolides. This may be due to defense compounds from the plant material in the gut of these aphids. Even if *A. asclepiadis* and *M. asclepiadis* do not actively sequester cardenolides, having these compounds in their guts could allow these aphids to decrease their vulnerability to natural enemies, especially in later instars which we observed to exhibit red spots for *M. asclepiadis* and black and white spots for *A. asclepiadis*. Although much work has been done on crypsis as a defense mechanism, little is known about the visibility of *A. asclepiadis* and *M. asclepiadis* to the vast array of natural enemies they encounter. We suspect, however, that instar polymorphism of these aphids allows some instars to be less visible to predators than others. Since red spots on *M. asclepiadis* and black coloration of *A. asclepiadis* cause these aphids to have a different color than the leaf background, these markings may alert predators to the location of the aphids and it follows that these aphids would be appropriately chemically defended. Future studies should be done to analyze the various instars of *A. asclepiadis* and *M. asclepiadis* independently for cardenolide content to elucidate cardenolide sequestration variance across the life cycle of these aphids. Since *A. asclepiadis* and *M. asclepiadis* show some level of cardenolides, though, this may be a glimpse at the evolution of aposematism as noted by Rothschild and Reichstein (1974), where aposematic coloration develops after the presence of defensive compounds.

In order to put the natural history differences that we measured in context, we must give thought to the ecological complexity of the three trophic levels involved in this system. Aphid predators, the third trophic level, have a positive impact on plants since they remove 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 and have been termed 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).

Thus, this is a complex system where the dynamics of competition and predation may influence the fitness of herbivores.

Aphid natural enemies, the third trophic level, vary widely temporally, spatially, and in diet specialization, and this affects the aphid community (Malcolm, 1992, Snyder and Ives, 2001, 2003, Sunderland and Vickerman, 1980). The variation in predators is often due to generalist predators disrupting the ability of specialists to affect predation rates (Snyder and Ives, 2001). As predator populations are variable, we suggest that these three aphid species are variably able to defend themselves from predators and the predator and prey population dynamics affect the survival of each species. Thus, although we measured that *A. nerii* had a higher growth rate and cardenolide content than the other aphid species, its defense strategy may only be the most effective against a subset of the natural enemies to these aphid species with the other species being better defended against other natural enemies. Bristow (1991) found that *A. nerii* may have an association with ants, but other research has suggested that *A. nerii* is relatively much less likely to be tended by ants than *A. asclepiadis* (Mooney et al., 2007). There is a mutualism between *A. asclepiadis* 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). This defense could contribute to the reduced cardenolide content of *A. asclepiadis* that we measured compared to *A. nerii,* and could enhance the fitness of *A. asclepiadis* against some natural enemies. Against other enemies, the dispersed nature of *M. asclepiadis* that we measured as well as its observed fleeing response may be the most effective. Further studies should be done to confirm that the defenses of these aphids are variably effective against differing natural enemies, but there is almost certainly no universally effective defense. In part, it is for this reason that there can be such a great diversity of herbivores to milkweeds.

Conclusions

The null hypothesis that the three aphid species would not vary in terms of reproduction and growth was supported for reproduction, but not for aphid growth. For growth measured as mass change per day, *A. nerii* had significantly higher rates than *A. asclepiadis* and *M. asclepiadis*. This may be due to the aposematic nature of *A. nerii* and the variably cryptic nature of *A. asclepiadis* and *M. asclepiadis.*

The null hypothesis, that each of the three aphid species would sequester the same amounts of cardenolides was rejected by the significant aphid cardenolide differences across species. I found that *A. nerii* sequesters significantly more cardenolide than the other two aphid species. Although I found these species to contain some cardenolides, I cannot confirm that they are being sequestered, since they could have been present residually in the guts of the aphids analyzed. Nevertheless, since both *A. asclepiadis* and *M. asclepiadis* were found to contain cardenolides, they may be unpalatable or toxic to predators, which may influence their defense and the possible evolution of aposematism (Malcolm 1986, 1989). Further study should be done to examine the effect of instar on cardenolide content of these aphids.

Overall, these findings support the observation that these aphid species employ different modes of defense. These defenses may be differentially effective against the various natural enemies to aphids. Also, the polymorphism of *A. asclepiadis* and *M. asclepiadis* by instar may play a role in the evolution of aposematism.

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