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Camden Gowler April 4, 2013

Parasite Resistance and the Potential Longevity Costs of a High Cardenolide Diet in the Monarch Butterfly

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ABSTRACT

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Monarch butterflies, *Danaus plexippus*, have adapted over time to utilize milkweed as a larval food source. Milkweed species contain varying amounts of toxic steroids known as cardenolides, which the larvae sequester as an anti-predation technique. Higher cardenolide concentrations have been associated with shorter adult lifespans in healthy monarchs, but the chemicals are beneficial under certain circumstances. Cardenolides confer resistance to the *Ophryocystis elektroscirrha* parasite, which infects monarch butterflies and reduces the host's lifespan. In most cases, high cardenolide diets increase both resistance and longevity of infected monarchs. However, recent evidence shows that after a certain concentration threshold is crossed, the benefits of increased resistance may be outweighed by the negative physiological cost of cardenolide consumption. While *Asclepias physocarpa* contains nearly three times the typical amount of cardenolides, the infected monarchs reared on it demonstrate lower longevities than a linear relationship would predict. The experiment presented here tests the potential costs of a prolonged diet on *A. physocarpa* in comparison to *A. incarnata*, a low cardenolide species. Monarchs were reared on varying diets of each species and analyzed for differences in pupal score, spore load and longevity. Treatment groups reared on *A. physocarpa* after infection had higher longevities than expected, and infected individuals reared on *A. physocarpa* during the early larval stages showed lower than predicted longevities. Therefore, the negative effects of a high cardenolide diet are most pronounced during the early stages of larval development. To see if smaller larvae are more susceptible to the costs of cardenolides, a new experiment is being developed where the larval diet will be varied for the first three days after hatching.

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BACKGROUND

Monarch Butterfly

The Monarch butterfly, *Danaus plexippus*, is quite possibly the most recognizable insect in the world and for good reason. Identified by elegant orange and black wings, monarchs are famous for their yearly migration from the Eastern United States to overwintering sites in Mexico. This incredible journey of up to 2,500 miles is just one of many amazing monarch abilities (Zhan et al. 2011). Monarch butterflies inhabit most of North and Central America, but have colonized countries as far as the Philippines, Australia, Hawaii, and Spain (Smith et al. 2005). They eat enormous quantities of milkweed plants, which are toxic to most herbivores, and use the plant's chemicals for protection from predators and parasites. The interactions between monarchs, milkweed, and parasites set the stage for some of the most intriguing studies in modern biology.

A monarch's life begins when a female butterfly lays eggs on a milkweed plant, which serves as the exclusive food source for the young. After about four days have passed, larvae hatch from the eggs. Although only a couple millimeters long at first, they grow very quickly by feeding on the milkweed leaves. In two weeks, the larvae are many times greater than their original size. At this point, metamorphosis occurs, and the larvae become pupae. After about a week and a half in the pupal stage, an adult butterfly emerges from the chrysalis, and the cycle continues.

Milkweed

A monarch's life is tied closely to the milkweed plant. Monarch caterpillars consume vast amounts of plant matter, but the milkweed protects itself from most other herbivores. To do so,

milkweed plants use toxic steroids known as cardenolides as a form of defense (Sternberg et al. 2012). Cardenolides cause a negative reaction in many herbivores, deterring them from eating the milkweed. Through adaptation to the toxic food source, monarchs became specialized to feed on milkweed (Agrawal et al. 2012). As monarchs became better adapted to eating toxic milkweed, their overall diet became less varied (Agrawal et al. 2012). This specialization to milkweed helps the monarch by limiting the number of competitors on the same food source.

After feeding on milkweed as larvae, monarchs sequester the cardenolides in their bodies (Brower et al. 1968). Larvae carry out this process very efficiently; the cardenolide content in the monarch can be even higher than that found in the plant itself (Zalucki et al. 2012). Once acquired from the plant, the larvae use the cardenolides to their own advantage. Monarchs store the concentrated cardenolides through the adult stage for protection from predators (Zalucki et al. 2012). When vertebrate predators feed on the butterflies, they get an adverse reaction from the cardenolides (Brower et al. 1968). The extent of this reaction is related to the type of host plant, likely correlated with the amount of cardenolides (Brower et al. 1968). Through exposure to the unpalatable taste, predators learn to avoid eating them, and the bright orange color of their wings makes for easy identification.

Parasitism in the Monarch

Monarchs have more to worry about than predators. The protozoan parasite, *Ophryocystis elektroscirrha*, is exclusively found in monarch and queen butterfly (*D. gilippus berenice*) populations (McLaughlin and Myers 1970). *O. elektroscirrha* is a member of the phylum *Apicomplexa*, which contains many parasitic species, including those responsible for causing malaria (Altizer and Oberhauser 1999). *O. elektroscirrha* was first discovered on queen butterfly

specimens in Florida during 1966 (McLaughlin and Myers 1970). Now the parasite has been found in every known monarch population.

The impact of the parasite on monarch populations varies by geographical location, from less than 5% of the population infected in parts of the Eastern United States to as high as 80% in Southern Florida (De Roode et al. 2008). With more limited ranges, non-migrating populations like those in Southern Florida typically have higher levels of parasitism. Additionally, the yearly migration to overwintering spots in Mexico may partially account for the differences in prevalence of *O. elektroscirrha* on the East coast. Infected monarchs have reduced flight ability and may not be able to travel very far north (Altizer and Oberhauser 1999). This selectively limits the percentage of infected monarchs at higher latitudes.

O. elektroscirrha infects the monarch larvae shortly after the eggs hatch (Lefèvre et al. 2010). Infected female butterflies carry dormant spores on their abdomens and spread the spores to the eggs and larval plant (De Roode et al. 2011). Upon hatching, the larvae consume the eggshell and surrounding milkweed plant, ingesting a portion of the spores (De Roode et al. 2011). Each spore contains several parasites, and once inside the caterpillar's digestive system, the spores release their contents (Sternberg et al. 2012). The infectious parasite cells, classified as sporozoites, make their way from the gut to the area just below the skin known as the hypoderm (Sternberg et al. 2012). For smaller larvae in particular, this process can cause considerable damage to the host. Infections in the first instar stage can be more detrimental because of damage to the intestinal wall (Altizer and Oberhauser 1999).

The parasitic replication process takes place in several stages and is correlated with the monarch's lifecycle. First, the parasite completes asexual reproduction during the monarch's larval stage (Lefèvre et al. 2012). After the larva pupates, the parasite begins to reproduce

sexually (Altizer and Oberhauser 1999). During the sexual stage of replication, the parasite prepares for transmission to the next host by producing spores, and it remains in the spore stage until it infects a new host (Lefèvre et al. 2012). Towards the tail end of the pupal stage, the spores are visible through the chrysalis. Asymmetrical black patches of spores on the pupae appear roughly two days before the adult emerges (Altizer and Oberhauser 1999). This marks the end of the internal replication, since only dormant spores are present on adults. Infected adults will emerge with spores covering the outside of their bodies but none inside. Most of the spores are confined to the abdomen, but they can also be found on the legs, head, and thorax (Altizer and Oberhauser 1999). With spores coating the outside of their body, a butterfly will scatter spores through contact. Spreading spores on milkweed plants or eggs increases the chance of parasite transmission.

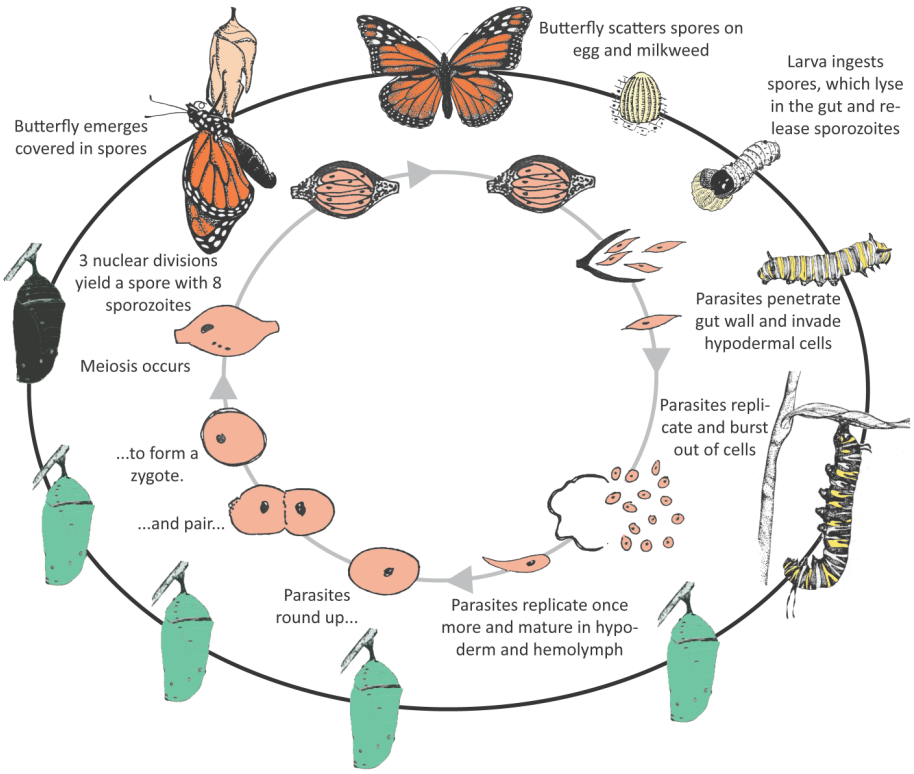

Figure 1 Life cycle of *O. elektroscirrha* in comparison to that of the monarch butterfly. Image courtesy of Dr. Jacobus de Roode.

Since the spores are no longer replicating, the parasite number is greatest right after the adult emerges. A single spore can generate millions of parasites within the newly infected host (De Roode et al. 2009). The transfer of parasites from one generation to the next, such as mother to offspring, is called vertical transmission (Altizer and Oberhauser 1999). New infections can only result from larvae ingesting spores, and uninfected adults cannot become infected themselves (Altizer and Oberhauser 1999). Therefore, vertical transmission is the primary method of parasite proliferation, and infected females can do little to prevent the parasite from spreading to the next generation.

In theory, vertical parasite transmission is associated with lower levels of virulence. Transmission occurs during oviposition, so host mortality prior to reproduction does not benefit the parasite. If the host does not live to reproduction, the parasite will die along with the host. This leads to a trade off between parasite replication and damage to host fitness (De Roode, Yates, and Altizer 2008). Light infections ensure parasite transmission, but fewer parasites are produced. On the other hand, heavy infections produce many more parasites, which causes more of a burden to the host. In heavy infections, this increased burden could compromise parasite transmission (Altizer and Oberhauser 1999). Because host survival is crucial for parasite proliferation, vertically transmitted parasites usually have less of an effect on host fitness compared to other types of parasites (Altizer and Oberhauser 1999).

Parasite replication via spore production directly affects the monarch's biological fitness, which is defined by an individual's ability to survive and reproduce (Lefèvre et al. 2010). This cost is an unintentional outcome of parasite reproduction within the host. In monarchs, the parasite burden causes significantly shorter adult lifespans (De Roode et al. 2011). Furthermore, the reduced growth in infected individuals can lead to lower body masses and smaller wingspans

(Altizer and Oberhauser 1999). The weakened wings may limit flight and mating capabilities, posing a significant disadvantage to infected adults (Lefèvre et al. 2010). The physical burdens and reduction in lifespan limit the amount of time to find a mate and for the female to lay eggs.

Parasite Resistance

Because parasites generate a cost to the host's fitness, evolution would favor means of reducing this cost. Among infected individuals, those that are better at reducing parasite growth will have a selective advantage. Hosts can limit their parasite burden in a variety of ways, such as resistance and tolerance. Resistance limits the burden to the host by effectively reducing parasite fitness (Sternberg et al. 2012). This process usually involves a reduction in the chance of infection or a reduction in the growth of parasites within the host (Sternberg et al. 2012). In a different way, tolerance alleviates the symptoms of parasitism in the host without reducing the total number of parasites (Sternberg et al. 2012).

Natural selection favors more resistant hosts because they are more likely to survive and pass on their genes. With less of a parasite burden, resistant individuals will have more time and energy to contribute offspring to the next generation. Through smaller infection sizes or reduced parasite growth, resistance increases the host's fitness while decreasing that of the parasite. Reduced parasite reproduction through host resistance can affect the parasite prevalence in a population (Sternberg et al. 2012). Higher levels of resistance in a population could lead to fewer parasites in a given area over time.

Cardenolides and Resistance

Natural mechanisms of resistance exist for the monarch. The cardenolides in milkweed can potentially provide resistance to *O. elektroscirrha* (De Roode et al. 2008). Across the 27 different species of milkweed that the monarchs use for food, the plant's cardenolide levels vary greatly (De Roode et al. 2008). It is the diversity and polarity of the host plant's cardenolides responsible for the parasite resistance (Sternberg et al. 2012). Typically, each milkweed species will produce either non-polar or polar cardenolides, with little mixing of the two types (Agrawal et al. 2012). Non-polar cardenolides are more toxic and likely contribute more to resistance than polar ones (Sternberg et al. 2012). Also, non-polar molecules may be more useful for defense because they can move across cellular membranes more easily than polar molecules (De Roode et al. 2008). This mobility increases the chance of the molecules interacting with the parasite.

Although it is challenging to prove the direct mechanism of resistance, the associations between cardenolide concentrations in the host plant and parasite resistance are well documented. De Roode et al. (2008) compared parasite resistance by plant species. Using *Asclepias incarnata* and *A. curassavica* as larval feeding plants, the researchers analyzed the parasite burden of infected monarchs. They quantified the abundance of each cardenolide type using High Performance Liquid Chromatography (HPLC) of milkweed leaf material (De Roode et al. 2008). This technique detects the exact structure of each molecule, allowing one to quantify each type of cardenolide present in the milkweed. In this case, *A. incarnata* contains a few, mostly polar cardenolides, while *A. curassavica* contains many cardenolides of varying polarities. The following graph from Lefèvre et al. (2010) helps illustrate the differences between the two species.

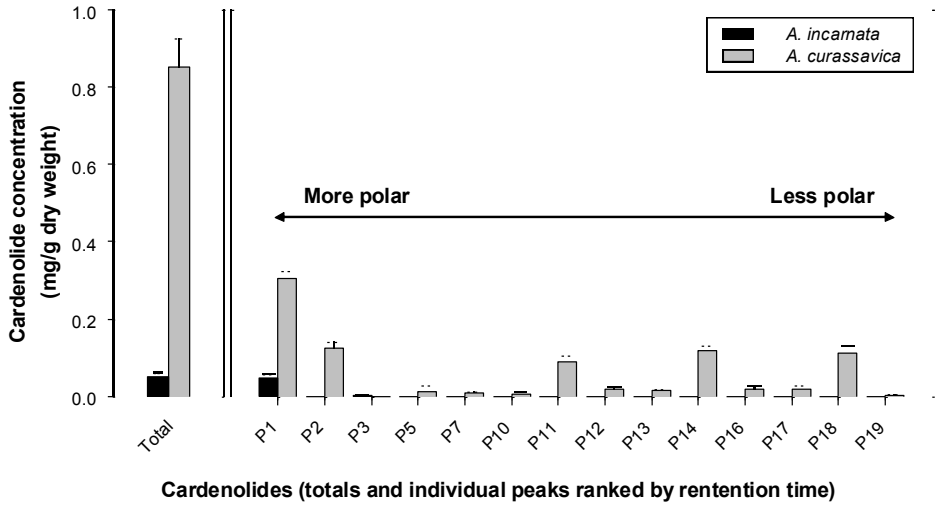

Figure 2 Cardenolide content by plant type. Each bar represents the amount of a specific type of cardenolide molecule present in the milkweed plant. *A. curassavica*, denoted by gray, has many more distinct cardenolide types and a greater total concentration of cardenolides. (Lefèvre et al. 2010)

In this case, *A. curassavica* had essentially eight times the amount of cardenolides by concentration. Each individual peak represents a different type of cardenolide compound, most of which come from *A. curassavica*. With more distinct and non-polar cardenolides, *A. curassavica* is expected to contribute more to resistance (De Roode et al. 2008). As shown in the graph below, the researchers found a significantly lower parasite spore load in the infected monarchs reared on *A. curassavica* compared to those reared on *A. incarnata* (De Roode et al. 2008). Spore load trends were consistent across several parasite clones, suggesting that host plant has more of an effect than parasite genotype (De Roode et al. 2008). The reduction in parasite number supports the notion that cardenolides contribute to parasite resistance and benefit the infected host.

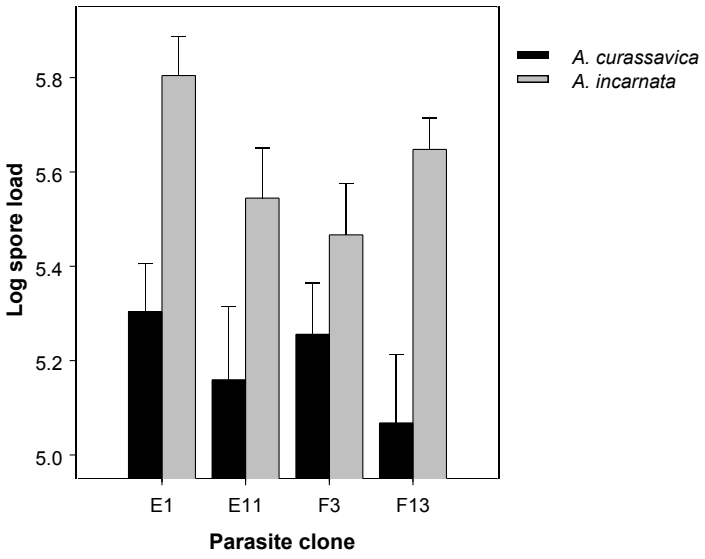

Figure 3 Log spore load for monarchs infected with different parasite clones. Monarchs were reared on either *A. incarnata* (gray) or *A. curassavica* (black). The spore load is consistently higher for those reared on *A. incarnata*. Monarchs were infected with four genetically different parasite clones and the results for each clone varied slightly. (De Roode et al. 2008)

On a molecular level, cardenolides disrupt Na^+/K^+ ATPases in the cell membrane. The ATPase transports K^+ into the cell and Na^+ out (Agrawal et al. 2012). Under normal conditions, this process creates an ion gradient, which can be used for transportation of molecules and other cellular functions (Agrawal et al. 2012). Without the ATPases functioning properly, the ion balance across the cell membrane is lost, and many cellular functions are disrupted. For vertebrate predators of monarchs this disruption can cause ill effects in nerve and muscle tissue, especially the heart (Ackery 81). Although ATPases have not been found in *O. elektroscirrha*, they have been found in similar types of parasites (McLaughlin and Myers 1970). There exists a possibility that these ATPases are also found in *O. elektroscirrha*. If so, the cardenolides ingested by the larvae could negatively affect the ATPases in *O. elektroscirrha* and reduce parasite growth in the host.

The toxic nature of cardenolides does not deter the monarch. Larvae consume incredible amounts of milkweed, but little is known about the exact adaptations that make this feasible. One possibility is that high levels of K^+ in the monarch's circulatory system dilute the effects of cardenolides (Agrawal et al. 2012). With higher amounts of ions already present, the ion imbalance may be less significant. An alternative hypothesis is that the Na^+/K^+ ATPases within the monarch are not sensitive to cardenolides (Agrawal et al. 2012). Recent studies have supported this idea, finding a reduction in binding of cardenolides to the monarch's ATPases (Dobler et al. 2012). The substitution of a new amino acid in the ATPase protein alters the molecular structure at a specific binding site (Dobler et al. 2012). The new conformation restricts binding of cardenolides to the complex, thereby preserving the ion balance in the cell membrane.

Despite this adaptation to the toxic effects, there is evidence that consuming cardenolides still comes at a cost to the monarch (Agrawal et al. 2012). Work by Vaughn and Jungreis supports the idea of a physiological burden due to cardenolides intake (Ackery 82). They found a "slight but significant sensitivity of the Na^+/K^+ ATPase in *D. plexippus*" to cardenolides (Jungreis and Vaughan 1977). The sensitivity to cardenolides, no matter how small, may lead to negative effects in the monarch. Monarch larvae consume so much milkweed that completely eliminating the negative effects is likely impossible.

Cost of Cardenolides

Even with the specialized adaptation to their host plant, monarchs still incur a cost from consuming the toxic chemicals. The negative consequences to the host depend on the concentration of cardenolides in the plant. Multiple experiments have demonstrated that the growth of first instar larvae is limited by high cardenolide diets (Zalucki et al. 2001, Zalucki et

al. 2012). This reduction in growth puts uninfected larvae reared on high cardenolide milkweed at a distinct disadvantage. To combat this, females in nature have been observed to avoid high cardenolide plants and instead lay their eggs on milkweed containing an average number of cardenolides (Zalucki, Brower and Malcolm 1990).

Interestingly, Lefèvre et al. (2010) showed that infected females preferentially choose to lay eggs on high cardenolide plants, but uninfected individuals do not. The cardenolides in *A. curassavica* benefit the offspring of infected monarchs through parasite resistance, but do not aid uninfected monarchs. As evident by the control lifespan results, uninfected monarchs perform better when reared on low cardenolide milkweed (Lefèvre et al. 2010).

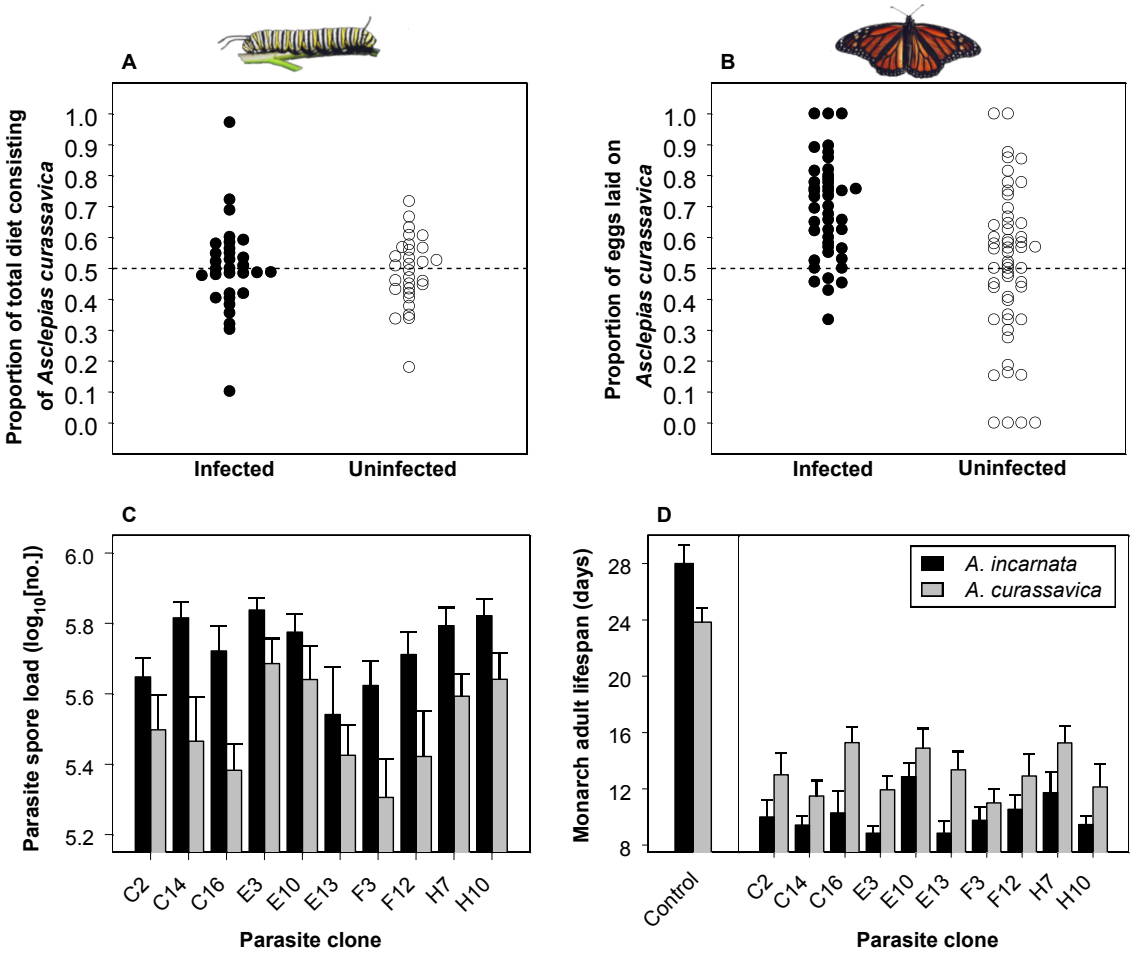

Figure 4 A) The proportion of *A. curassavica* milkweed consumed by larvae. There is no preference based on infection status. B) The proportion of eggs laid on *A. curassavica* by either an infected or uninfected female. This proportion is significantly greater when the monarch is infected. C) Each group of bars shows the spore load for monarchs infected with a different parasite clone. Infected monarchs reared on *A. curassavica* have lower spore loads in general. D) Infected monarch longevity by parasite clone and milkweed diet. For almost all, longevity is greater when reared on the anti-parasitic *A. curassavica*. Uninfected monarchs are the only ones that live significantly longer on *A. incarnata*. (Lefèvre et al. 2010)

Because there is a slight reduction in adult longevity when uninfected monarchs feed on *A. curassavica*, it is only beneficial for infected individuals to seek out high cardenolide plants (Lefèvre et al. 2010). Without a cost associated with the medicinal host plant, all monarchs should lay a greater proportion of eggs on high cardenolide plants regardless of their infection status. Although *O. elektroscirrha* exists in every population, not all monarchs lay eggs on high cardenolide plants. The negative effects of cardenolides on longevity may deter females.

The negative cost of cardenolides is thought to apply equally to both infected and uninfected individuals. However, in infected individuals, the anti-parasitic benefits of cardenolides can potentially outweigh the negative costs of the diet. Without a parasite burden, there is no incentive to take in the extra cardenolides. Cardenolides negatively impact all monarchs, but the effect is more easily detectable in uninfected monarchs. The longevities of uninfected monarchs isolate the costs of each diet without the parasitic interactions blurring the effects. For this reason, uninfected monarchs are almost always included as a control.

Asclepias physocarpa

Since cardenolide content varies by milkweed species, each species can confer different levels of parasite resistance. Sternberg et al. (2012) compared the longevity of uninfected and infected monarchs reared on twelve different milkweed species, representing a wide range of cardenolide concentrations. As shown below, some milkweed species benefited uninfected monarchs more than others. Several of the species containing high total cardenolide levels, such

as *A. curassavica* and *A. physocarpa*, led to considerably lower longevities for uninfected monarchs (Sternberg et al. 2012). On the other hand, monarchs reared on low cardenolide milkweed such as *A. incarnata* lived very long when uninfected. This provides evidence for a negative fitness cost when uninfected monarchs consume cardenolides.

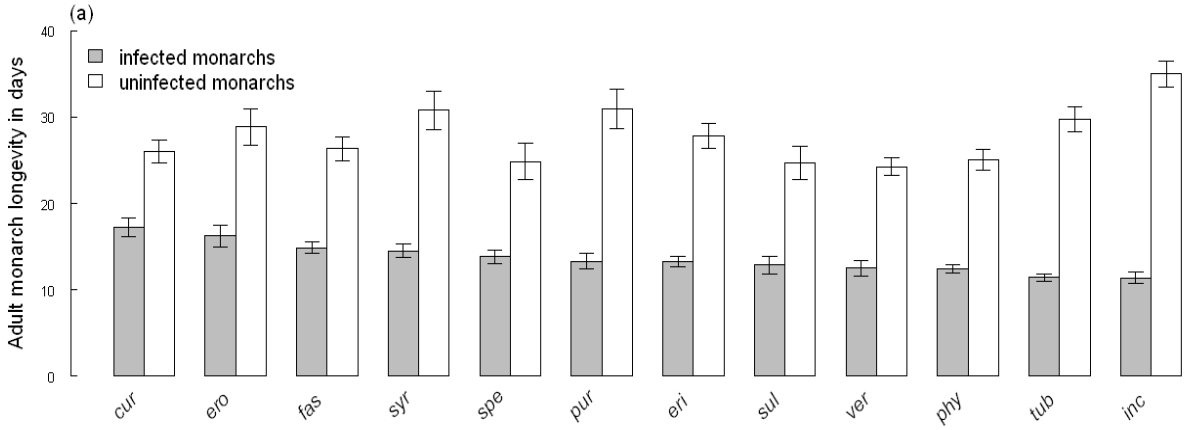

Figure 5 A comparison of infected and uninfected monarchs reared on twelve different plant types. The results are ordered by descending levels of infected longevity. Infected monarchs have significantly shorter lifespans. Both uninfected and infected longevities vary significantly by host plant type. (Sternberg et al. 2012)

Sternberg et al. (2012) also analyzed the total cardenolide concentrations for each of the host plants. They compared these values to the average longevity of infected monarchs. The following graph shows the relationship between cardenolides and longevity by host plant type. Each point represents a species of milkweed.

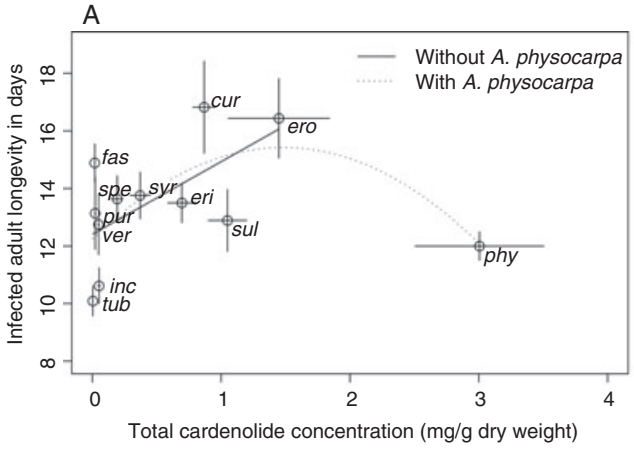

Figure 6 A plot showing the adult longevities for infected monarchs reared milkweed with varying cardenolide content. Without including *A. physocarpa*, the longevity of infected monarchs increases as the cardenolide content of the host plant increases. Those reared on *A. physocarpa* experience reduced longevity, which does not fit the linear relationship for the other groups. The results for *A. physocarpa* suggest a curvilinear relationship where the highest infected longevity values are found when larvae consume milkweed with intermediate cardenolide concentrations. (Sternberg et al. 2012)

One would predict that as milkweed cardenolide levels increase, parasite resistance would also increase. This prediction holds true for the majority of the plants in this experiment, and there is a positive linear trend when *A. physocarpa* is excluded. If this trend were true for all species, larvae fed *A. physocarpa* would demonstrate the highest parasite resistance because the plant has nearly three times as many cardenolides as other species (Sternberg et al. 2012). However, when infected larvae were reared on *A. physocarpa*, the average lifespan resembled that of milkweed with low cardenolide levels (Sternberg et al. 2012). The monarchs fed *A. physocarpa* lived slightly longer than those fed *A. incarnata*, but not nearly as long as those fed milkweed of an intermediate content. This result suggests that after a certain concentration threshold, the cardenolides contribute a detrimental fitness cost that outweighs the benefits of parasite resistance. Sternberg et al. (2012) believe this is an effect of hormesis, where toxins act beneficially at small doses, but reduce overall fitness at larger doses. According to this hypothesis, once a certain cardenolide threshold is crossed, the negative physiological costs outweigh the positive anti-parasitic effects.

If cardenolides do generate a fitness cost at high levels, then the ideal concentration for parasite resistance will be at some intermediate value. Milkweed with very few cardenolides cannot provide adequate resistance, but milkweed with too many hurt the host directly. There is likely a trade-off between the resistance and the physiological cost of the cardenolides (Sternberg et al. 2012). The extremely high cardenolide levels in *A. physocarpa* make it a suitable host plant for testing this hypothesis.

By feeding monarchs varying levels of *A. physocarpa* and *A. incarnata* at different larval stages, one can analyze the costs and benefits of a high cardenolide diet. If the cardenolides come at a cost to the host, uninfected monarchs will show decreasing longevities as the amount of *A. physocarpa* in the diet increases. For infected monarchs, a diet with a balanced amount of *A. physocarpa* and *A. incarnata* will maximize parasite resistance and minimize the physiological cost. In terms of resistance, the cardenolides provide the greatest benefit when consumed during the time of infection, and cardenolides ingested after infection will provide little if any resistance effects (De Roode et al. 2011). Thus, high cardenolide milkweed consumed after the infection stage will contribute more costs to fitness than benefits. Infected monarchs fed *A. physocarpa* during the infection stage and *A. incarnata* for the remainder of the time are predicted to show the best overall fitness. These monarchs may potentially receive the anti-parasitic benefits and avoid most of the costs, leading to greater longevity.

Objectives

I investigated: the anti-parasitic effects of *A. physocarpa* in relation to *A. incarnata*, the potential costs of prolonged consumption of cardenolides via *A. physocarpa*, and the tradeoff between these costs and benefits in both infected and uninfected monarchs.

MATERIALS AND METHODS

Treatment Groups

Much of the experimental procedure was modeled after the study by De Roode et al. (2011). The goal was to assess spore load and adult longevity by varying the type of milkweed food source during the larval stage. Groups of uninfected and infected larvae were fed either *A.*

incarnata or *A. physocarpa* during three different development stages: pre-infection, infection, and post-infection. The pre-infection stage encompasses the time larvae hatch up to the second instar stage, which is at two days old. The infection stage includes only second instar larvae and lasts one day. After infection, the rest of the larval development represents the post-infection stage.

Five treatment groups were used. Each group was composed of 30 infected and 30 uninfected larvae. The host plant for each stage is designated by either an "I" for *A. incarnata* or a "P" for *A. physocarpa*. For example, "IIP" were reared on *A. incarnata* during pre-infection and infection stages and *A. physocarpa* during post-infection. The table below details the different groups.

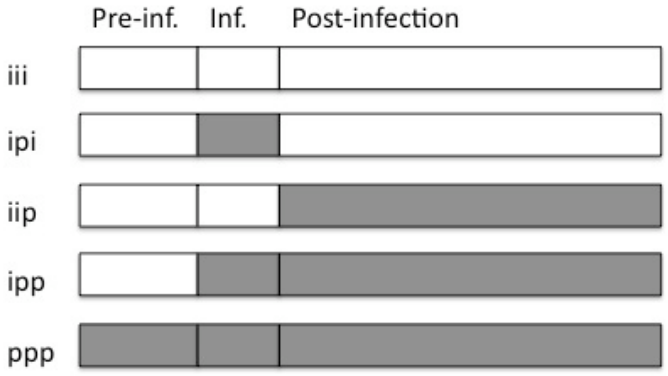

Figure 7 The milkweed diets of the five treatment groups for each larval stage. The white bars show *A. incarnata* diets and the gray bars show *A. physocarpa* diets.

The III and PPP treatments were designed to serve as controls. They are the same type of treatments used by Sternberg et al. (2012), allowing for direct comparison of results. The middle three groups all test for varying combinations of resistance and fitness costs. The IPI group was expected to receive only the benefits of resistance, and the IIP group was expected to incur a

high physiological cost without any benefits. A combination of the previous two, IPP was predicted to receive both the benefits of resistance and the post-infection costs.

Milkweed

A. incarnata and *A. physocarpa*, representing low and high cardenolide plants respectively, were grown from seeds purchased through Butterfly Encounters. The milkweed plants were grown in a greenhouse with supplemental lighting and weekly fertilization. To account for variation in cardenolide content among plants of the same species, several different plants were used during each stage of feeding.

Pre-infection

Monarchs from six different genetic lineages were reared on diets consisting of *A. incarnata* and *A. physocarpa*. Separated by lineage, larvae were placed in plastic containers and provided a surplus of cuttings from either milkweed plant for feeding. After three days, healthy larvae were transferred to individual petri dishes for inoculations.

Infection

During the infection stage, second instar larvae were inoculated with ten parasite spores. The spores were collected from the most recent experiment to ensure their viability. After swabbing an infected monarch's body, the spores were smeared onto a glass slide in a petri dish. A Bunsen burner was used to melt a glass capillary tube and create a small glass bulb on the end. The glass bulb was used to pick up and transfer a portion of the spores from the petri dish. For larvae in the infected treatments, ten spores were placed on a 0.8cm diameter leaf disk from the

appropriate host plant. A leaf disk of the same size without spores was used for feeding the uninfected treatment groups. Only larvae that consumed the entire leaf disk were used for the experiment.

Post-infection

Post-infection, the larvae were transferred to individual plastic containers and kept indoors at room temperature. By placing plant cuttings in florist tubes within the containers, larvae were fed a surplus of milkweed until pupation. Upon pupation, the date was recorded and all vegetation was removed from the container.

Pupal Score

To help access parasite burden, the pupae were examined using the pupal score method. Pupae were checked every day and examined for asymmetrical discolorations, which are indicative of parasite spores. They were judged on a 0 to 5 infection scale, with 0 representing uninfected pupae and 5 representing heavily infected pupae. While a more subjective measurement, pupal scores provide an accurate way of assessing the number of parasite spores. Pupal score results show a strong correlation with other methods of quantifying infection.

Longevity

After the pupal stage, the sex and date of emergence was recorded for each monarch. Upon emerging, butterflies were transferred to glassine envelopes and stored at 12º Celsius. Monarchs were not fed while in the adult stage; starvation resistance helps provide an accurate measure of overall fitness. Adults were checked every day for mortality, and the date of death for

each was recorded. Adult longevity was measured in terms of the number of days the monarchs survived past pupation. The longevity data helps uncover any negative fitness costs associated with the treatments. In infected monarchs, reduced longevity is correlated with a higher parasite load.

Spore Load

After the butterflies died, spore load was analyzed further by removing the wings and vortexing the individual bodies in 5 mL of water. This process displaces some of the spores and a proportion of the spores will remain in the water after the body is removed. Using this water, the number of spores was counted on a haemocytometer slide under a microscope. The spore load data provides a basis for determining the anti-parasitic effects of each treatment group.

Statistical Analysis

All results and plots were created using R version 2.15.1 (2012 Mac version) from the R Development Core Team. These results were checked with analysis of variance (ANOVA) tests, including Tukey post-hoc assessment of pair wise differences.

RESULTS

Pupal Score

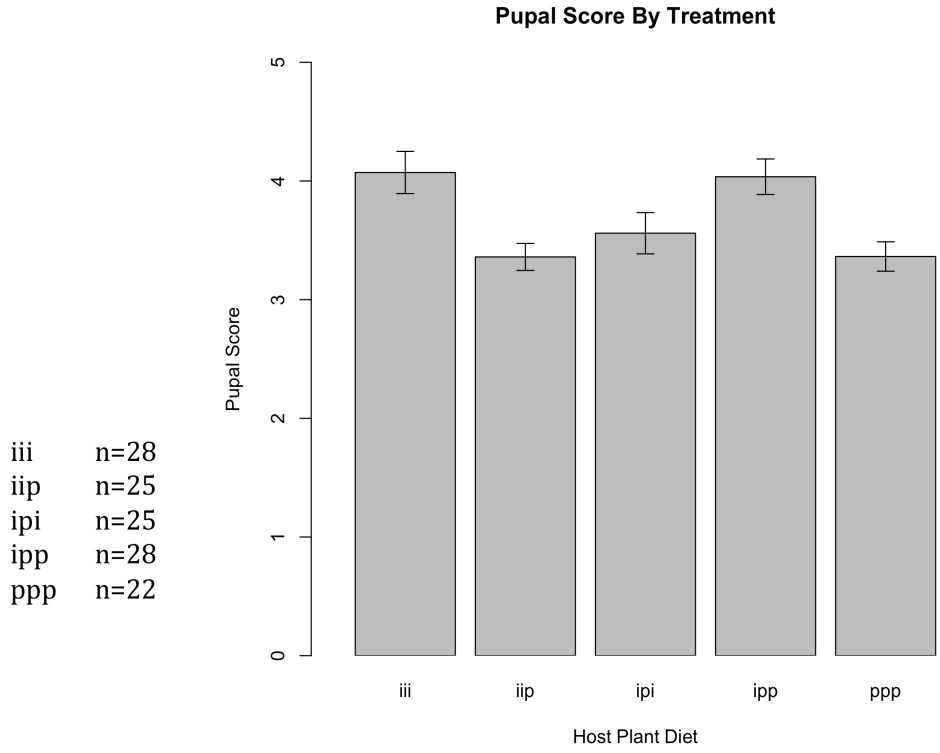

Figure 8 Average pupal scores for each treatment group. The values to the left show the total number of individuals per treatment. The error bars denote standard error.

Characterized on a 0 to 5 infection scale, each treatment group was above three. The pupal score effect was significant ($F_{4, 129}=4.61$, $p=0.002$), and two groups, III and IPP were above four. While not significantly different from one another, these two groups had heavy infections. On the other hand, the IIP, IPI, and PPP pupal scores were all significantly lower than those of III, but not statistically different from each other. Comparing the controls, III was significanly higher than PPP.

The results from the pupal scores present both expected and unexpected outcomes. The high pupal score for III is expected because of the lack of cardenolides in *A. incarnata* to provide resistance. On the other end of the spectrum, PPP had a low pupal score, demonstrating the

resistance acquired through a high cardenolide diet. Interestingly, IIP showed very low pupal scores, insignificantly different from IPI and PPP. The IIP results are especially surprising given the low cardenolide diet during infection.

Longevity

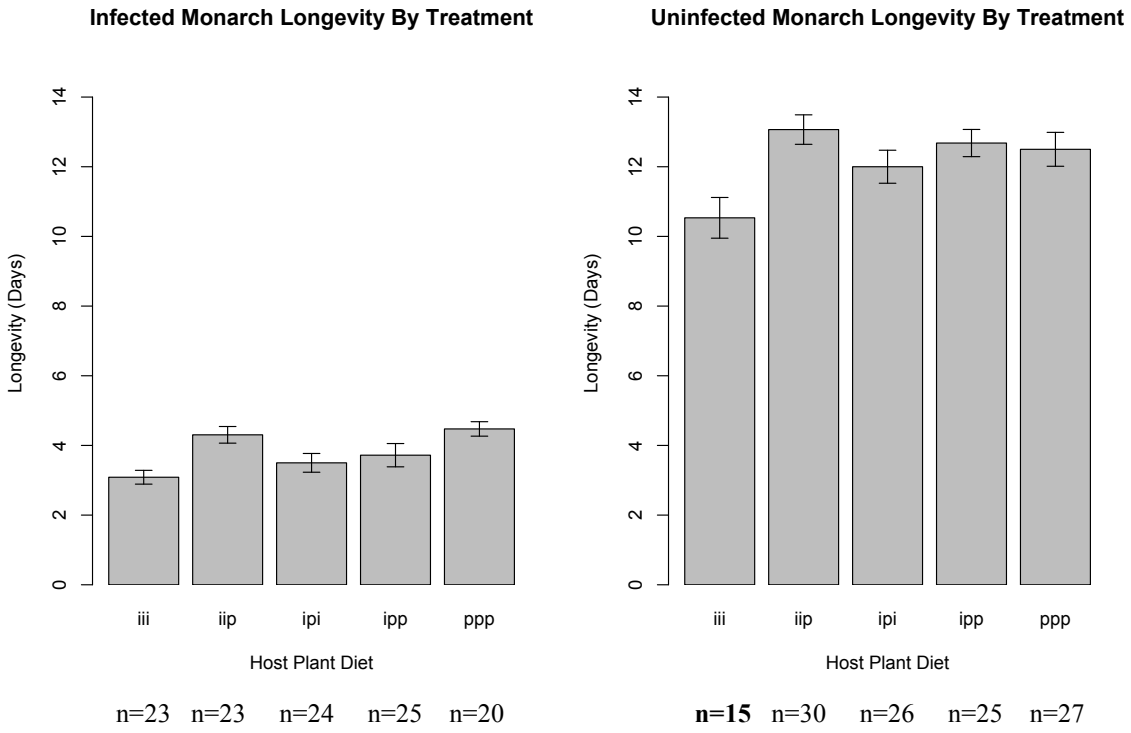

Figure 9 Adult longevity for infected and uninfected monarchs. The values below the graphs show the total number of individuals per treatment. The error bars denote standard error.

Uninfected monarchs lived roughly eight days longer than their infected counterparts, with several treatment groups surviving twelve days or more. Out of uninfected individuals, III had the shortest longevity. The results were significant for the longevity of uninfected monarchs ($F_{4, 118}=3.3$, $p=0.01$) but only for the III group. It is difficult to draw any conclusions from the longevity of III because the sample size is small at fifteen individuals. There is a nonsignificant trend in which IIP's longevity is unexpectedly higher than that of IPI.

In terms of adult lifespan, uninfected monarchs were predicted to have the greatest longevity when fed low cardenolide milkweed, but actually had the shortest longevity. The predicted cost of consuming *A. physocarpa* in the post-infection stage was not evident by the results, where the IIP group of uninfected monarchs had a surprisingly high longevity. There is no clear trend in relation to the total amount of *A. physocarpa* in the diet. If there were a strong cost based on the total amount of cardenolides consumed, IIP, IPP and PPP would have much lower longevities. Instead, IPI had a lower than expected longevity, yet this group only ate a single leaf disk of *A. physocarpa*.

As a consequence of the parasite burden, the infected monarchs suffered reduced longevity and the treatment effects were significant ($F_{4, 110}=5.1$, $P=0.001$). Of the infected group, III had the shortest longevity although its value was not significantly different from that of IPI. At a value of over four days, IIP and PPP demonstrated the best longevity. Both IIP and PPP had significantly higher longevities than III.

The balance between the benefits of parasite resistance and the detrimental cost of a high cardenolide diet determines the longevity for infected monarchs. Since PPP has a higher longevity than III, the benefits of an *A. physocarpa* diet may outweigh the costs. However, the other treatments do not follow the same pattern. Predicted to incur all the costs and receive none of the benefits, IIP should have a very short longevity. Under the same principle, IPI should have the best resistance and the fewest costs. Instead, IIP outlived IPI, which does not fit the predictions of the costs and benefits model. Although the difference between the two was not significant, it still presents a very unexpected result.

Spore Load

Figure 10 Log spore load results for each treatment group. The IPI average was significantly lower than all others. The values to the left show the total number of individuals per treatment. The error bars denote standard error.

The spore load effects were not significant (F4, 124=2.1, P=0.086), but most groups showed differences in value. In general, the results for spore load appear to match up well with the pupal score data. In agreement with the pupal score results, the spore load of III was higher than PPP, confirming the anti-parasitic affects of *A. physocarpa*. Once again, III and IPP had high levels of infection and were similar to each other in value. IPI was lower in spore load than every other group, but the values were only slightly lower than those of IIP and PPP.

There are some noticeable differences in the spore load results for the individuals fed *A. physocarpa* at infection; the spore loads for IPI were the lowest, those for IPP were very high, and those for PPP were in between. For monarchs receiving the same treatment at infection,

these spore loads are rather variable. There is no common trend across the three groups and no clear explanation for the observed differences. Strangely, IIP was less infected than III for both pupal score and spore load, and the cause of this reduction is unclear.

DISCUSSION

The observed results in this experiment provide limited support for the hypothesis. In terms of adult lifespan, uninfected monarchs were predicted to have the greatest longevity when reared on a higher ratio of *A. incarnata* to *A. physocarpa*. The predictions were based on cardenolide concentration and the quantity of milkweed consumed. By sheer quantity of plant material, post-infection larvae ate the greatest amount, while infection stage larvae only consumed a single leaf disk. Therefore, larvae reared on *A. physocarpa* during the post-infection stage were predicted to demonstrate the greatest physiological cost from the cardenolides. Many of the monarchs fed *A. physocarpa* after infection had higher than anticipated longevities. The uninfected longevity of IIP was greater than that of III and IPI. Even if one excludes III because of the small sample size, the other groups do not show any discernable trend. There were no significant differences in uninfected longevity among monarchs with a diet containing *A. physocarpa*. Given the differences in cardenolide intake, these groups were expected to vary considerably from one another. The longevity of IPI was especially surprising because the consumption of *A. physocarpa* was limited to the infection stage.

Infected monarchs obtain resistance to *O. elektroscirrha* primarily in the infection stage. Any extra cardenolides consumed during the post-infection stage are thought to contribute little to resistance. Under this principle, IPI should have the greatest longevity out of the infected monarchs because they consume a high concentration of cardenolides during infection but not

afterwards. They obtain the benefits of parasite resistance without the cost of extensive feeding on high cardenolide milkweed. On the other hand, IIP would have limited parasite resistance, but a high cost from feeding on *A. physocarpa* after infection. Experimentally, the exact opposite trend occurred; IPI had a low longevity and IIP a higher one. This result strongly conflicts with the original predictions.

The controls offer the most useful evidence for the hypothesis by comparing the effects of a full diet on a single plant type. PPP had significantly lower pupal scores than III, demonstrating a reduction in parasite burden associated with the higher cardenolide content in *A. physocarpa*. With fewer parasites, PPP experienced a higher longevity than III. If the cost of consuming the excess cardenolides in *A. physocarpa* was greater than the benefit of parasite resistance, then PPP would have shorter longevities than III. This does not appear to be the case because III had a significantly lower longevity out of infected monarchs. The increased longevity in PPP provides some evidence for the benefits of resistance outweighing the costs of the cardenolides.

For the uninfected control groups, the cardenolides in the PPP group should only come at a disadvantage. Based on previous experiments (Sternberg et al. 2012), uninfected monarchs consistently perform better on lower cardenolide diets. Sternberg et al. (2012) did not find any advantage for uninfected individuals reared on *A. physocarpa*. However, in the present experiment, uninfected monarchs lived longer on the exclusively *A. physocarpa* diet. While the current experiment found contradicting results, the sample size for the uninfected III is too small to draw definitive conclusions.

Adult Longevity (Sternberg et al. 2012)

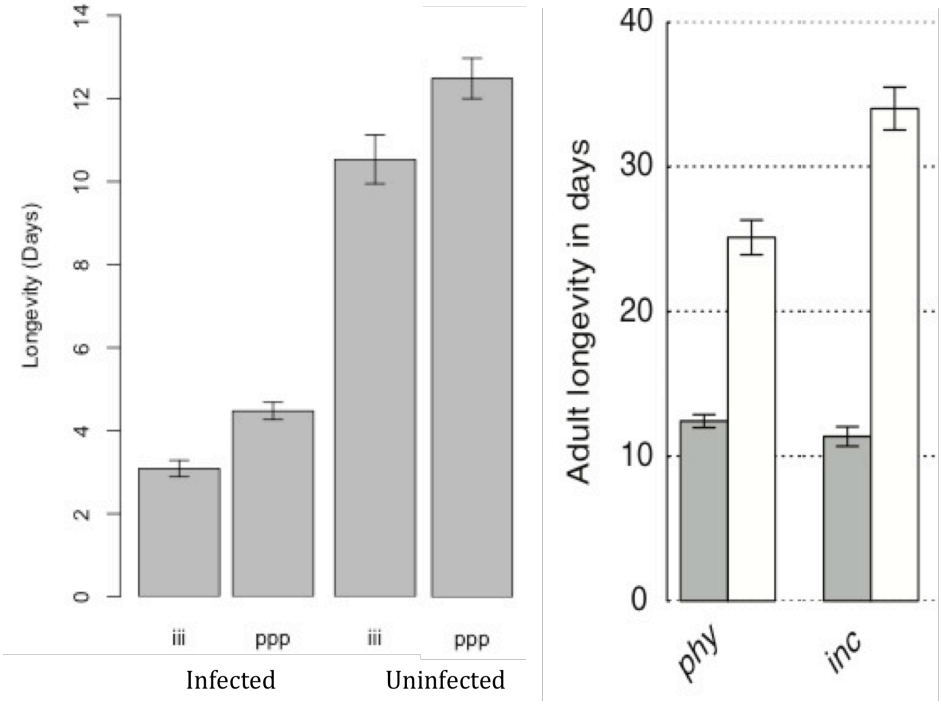

Figure 11 A comparison of this paper's longevity results and those of Sternberg et al. 2012. The data comes from the control groups with diets consisting of either *A. incarnata* or *A. physocarpa*. On the left, iii represents an *A. incarnata* diet and ppp represents an *A. physocarpa* diet. On the right, infected monarchs are shown in gray and uninfected monarchs in white.

In comparison to Sternberg et al. 2012, the longevities differed substantially in value. The Sternberg et al. 2012 longevities are much greater for both infected and uninfected monarchs. This difference is likely due to natural variations in the health of the butterflies over generations. While the values for longevity vary greatly between the two experiments, one can still compare the general trends. The contrast between infected monarchs reared on *A. physocarpa* and those reared on *A. incarnata* is especially useful.

The original hypothesis does little to explain many of the observed longevity results. There is minimal evidence that higher quantities of *A. physocarpa* in the diet lead to reduced longevities, or that there is a clear trade-off between costs and benefits of such a diet. Some of

the strongest evidence against the hypothesis comes from the results for IIP and IPI. The cardenolide interactions in monarchs appear to be complicated, and several alternative hypotheses could provide answers to the unexpected results. One alternative is that the physiological cost of cardenolides may be less significant than predicted. While unlikely, this would explain why many of the longevity values for uninfected monarchs reared on *A. physocarpa* are similar. It would also explain why the longevity of IIP is so high.

A more probable explanation is that cardenolides have different physiological costs at different larval stages. Most researchers acknowledge a negative effect of consuming cardenolides for the monarch. Nevertheless, the detrimental effects of such a diet may vary depending on the larval size at which the milkweed is consumed. Early studies by Erikson (1973) found no trend between larval growth and host plant cardenolide concentration (Zalucki, Brower and Alonso 2001). Unfortunately, this study did not capture the entire picture. A key flaw was the use of fourth instar larvae, which may be too large in body size to be significantly affected by the toxins (Zalucki, Brower and Alonso 2001). The results provide little insight into the effect of cardenolides during early larval development. Because of the extreme size differences across the range of instars, the early instars must be studied as well.

More recent research has suggested the size of the larvae is important for determining cardenolide effects. Zalucki, Brower, and Alonso (2001) observed lower growth rates for first instar larvae reared on high cardenolide milkweed. Furthermore, another study by Zalucki et al. (2012) confirmed these results, demonstrating that "early stage survival was negatively correlated with plant cardenolide level." Taken together, the Zalucki and Erikson studies suggest that the effect of cardenolides on larval growth depends on the size of the larvae. Whereas first instar larvae fed high cardenolide milkweed suffer reduced growth, fourth instar larvae do not.

Clearly, first instar larvae cannot entirely compensate for the toxic nature of the milkweed. Proportional to body size, cardenolides could have a significantly different effect on first instar larvae compared to fourth instar.

The size-specific effects of cardenolides pose a significant challenge for this experiment. The potential cost of the *A. physocarpa* diet may not be determined by the exact quantity consumed during a monarch's lifetime. In designing the treatment groups, the post-infection larvae reared on *A. physocarpa* were expected to incur the highest cost because they would feed on the greatest amount of high cardenolide milkweed. However, the post-infection larvae are much larger and may be less affected by cardenolides. Despite consuming a larger total milkweed mass, post-infection larvae may be better equipped to handle the toxic effects.

If the excess cardenolides do indeed create the most fitness costs during the early instar stages, then the predictions for longevity must be readjusted. The larval diet during the pre-infection and infection stages contributes more to longevity than the post-infection diet. While IIP was initially expected to have one of the shorter lifespans when uninfected, under the new assumption, IIP would live longer than IPI, IPP, and PPP. Of the larvae fed *A. physocarpa* during their development, IIP should receive fewer negative effects due to cardenolides. In the post-infection stage, the IIP larvae are more capable of handling the chemicals.

Judging by the results for the IIP group in particular, there is evidence for this "early cost hypothesis". In both the infected and uninfected groups, IIP had one of the highest longevities. There was a nonsignificant trend in the uninfected longevities of IIP and IPI, where the longevity of IIP appears higher than that of IPI. While unexpected under the original hypothesis, this trend makes perfect sense if cardenolides are most costly during the earlier larval stages. Even though the total cardenolide intake of IPI was drastically lower than that of IIP, the timing makes all the

difference. IPI consumed a smaller amount of *A. physocarpa*, but it did so at a time when the negative effects would be most pronounced.

The early cost hypothesis may also explain the unexpectedly low longevity of IPI when infected. Even with the high cardenolide diet during infection, the longevities of the infected IPI group overlapped with that of III. These two groups were predicted to be on opposite ends of the spectrum, because the IPI diet was thought to have more benefits than costs. There are significant differences in pupal score between III and IPI, indicating that the *A. physocarpa* consumed during inoculation does convey parasite resistance. IPI had a low parasite burden, which normally leads to a high longevity. Therefore, the lower than expected longevity for IPI, can be attributed to the cost of cardenolides because the longevity would likely be much greater were there not this cost. The drop in longevity for IPI for infected individuals supports the idea of a physiological cost related to early cardenolide intake.

The comparison of IPI and IIP also provides evidence for the early cost hypothesis. Although the differences between the two were not significant, the values were so far from the expected results that they provide important details. Once again, IPI had the lower spore load because of the high cardenolide diet during infection. Despite the reduction in spores, IPI still had a lower longevity compared to IIP. This is incredible considering that IIP received no anti-parasitic milkweed in the infection stage and had a higher spore load. In this case, the cost of consuming cardenolides early in development may be greater than the benefits of resistance. Only when the size of the larvae is taken into account do the longevity results for IPI begin to make sense.

No hypothesis is perfect, but the idea that cardenolides cause the most detrimental effects in the early instar larvae fits these results better than the original hypothesis. The unpredicted

results involving IIP and IPI pose significant problems for the original hypothesis but not the early cost hypothesis. Regardless, some treatment groups do not fit the newly proposed model. For example, PPP has one of the highest infected longevities, yet this group consumed more *A. physocarpa* during the early instars. Under the early cost hypothesis, PPP would incur the greatest cost by consuming cardenolides from the moment the larvae hatch. This should lead to a low longevity, but the results showed otherwise. PPP did have relatively low spore loads, which could partly explain the increase in adult lifespan.

All things considered, the early cost hypothesis gives the best available explanation. This hypothesis offers solid reasoning for several unexpected results: the low infected and uninfected longevities of IPI, the high infected and uninfected longevities of IIP, and the fact that IIP has a greater spore load than IPI but also a greater longevity than IPI. None of these results match up with the initial predictions because the original hypothesis was developed under the premise that the total quantity of cardenolides in the diet determined the cost. Other research supports the notion of an early cost, as several different authors have confirmed that cardenolides disproportionately affect smaller larvae (Zalucki et al. 2012). Combined with these previous studies, the results of this experiment give important insight into the role of cardenolides in monarch butterflies.

FUTURE PROJECTS

In the original experiment, the treatments were not organized in a way to assess accurately if the cost of cardenolides is directed mostly to first and second instars. The *A. physocarpa* post-infection diet was assumed to contribute the most substantial cost. If this is not

the case, then further experiments must carefully analyze the effects of cardenolides on early instar larvae.

A follow up experiment is currently being developed to test the early cost hypothesis. It will analyze the effects of an *A. physocarpa* diet during the initial days of larval development. For the first three days after hatching, larvae will be fed either *A. physocarpa* or *A. incarnata*. On the third day, larvae will be inoculated with parasite spores and subsequently reared exclusively on *A. incarnata*. With each letter symbolizing the daily diet for the first three days, the treatment groups are as follows: III, PII, IPI, IIP, and PPP. If the cardenolides' negative effects are greater at smaller larval sizes, then larvae that consume *A. physocarpa* earlier in life will have reduced longevities. For example, PII will have a shorter longevity than IIP because PII consumes *A. physocarpa* much sooner. This experiment will also uncover which day of feeding is most important for conferring parasite resistance. Most previous work has shown that cardenolides consumed during infection determine the extent of resistance. Therefore, larvae fed *A. physocarpa* closer to the day of infection may demonstrate the lowest parasite burden and highest longevity.

Future experiments, like the one proposed here, will provide more insight into the complex interactions between the monarch butterfly and its larval host plant. There is still much to be uncovered regarding the benefits of parasite resistance and the physiological cost of consuming cardenolides. Using previous studies and the results of this paper as a foundation, future research may discover even more interesting connections between monarchs and cardenolides.

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