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CARDENOLIDES AND CATERPILLARS: the effects of cardenolides on the longevity of monarch butterflies, *Danaus plexippus*, infected with the protozoan parasite *Ophyrocystis elektroscirrha*.

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An abstract of
A thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

CARDENOLIDES AND CATERPILLARS: the effects of cardenolides on the longevity of monarch butterflies, *Danaus plexippus,* infected with the protozoan parasite *Ophyrocystis elektroscirrha*.

By Junjian Huang

Cardenolides are secondary metabolites synthesized by milkweed plants. The functions of secondary metabolites are not fully understood but there have been studies that suggest milkweed plants use cardenolides as a form of protection from generalist herbivores. Monarch larvae are specialist herbivores that feed only on milkweeds and the larvae are able to sequester these toxic compounds into their bodies, which protects them from predation. In addition to anti-predator properties, it has been hypothesized that these cardenolides may possess antiparasitic properties as well. The focus of these three experiments is to study the role of cardenolides in the host-parasite system of monarch butterflies, *Danaus plexippus*, and its parasite, *Oprhyocystis elektroscirrha*. We found that latex from *A. curassavica*, which contains a wide range and high concentration of cardenolides, reduced the parasite load of infected monarchs; however, the cardenolides digitoxin and ouabain did not show significant effects of reducing parasite load in infected monarchs. This result suggests that cardenolides may be involved since they are the only organically active compounds discovered thus far in milkweeds. However, further tests are necessary before we can draw any definite conclusions regarding the anti-parasitic properties of cardenolides.

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1. Introduction

Medication is not a foreign phenomenon when it comes to humans. We use medicine daily to solve problems ranging from the viruses that cause the common cold to protozoan parasites that cause malaria. Like humans, animals have deleterious parasites that cause them to become ill and perhaps die. However, animals do not have research institutions such as the NIH and CDC to come up with novel treatments for diseases. Instead they have to use what is provided to them through their environment, which includes the food source for that particular animal. In fact, there have been recent studies that suggest the fitness of insect parasites, such as insect nematodes, are affected by larval host plant choice of the insect host (Bezemer *et al* 2005).

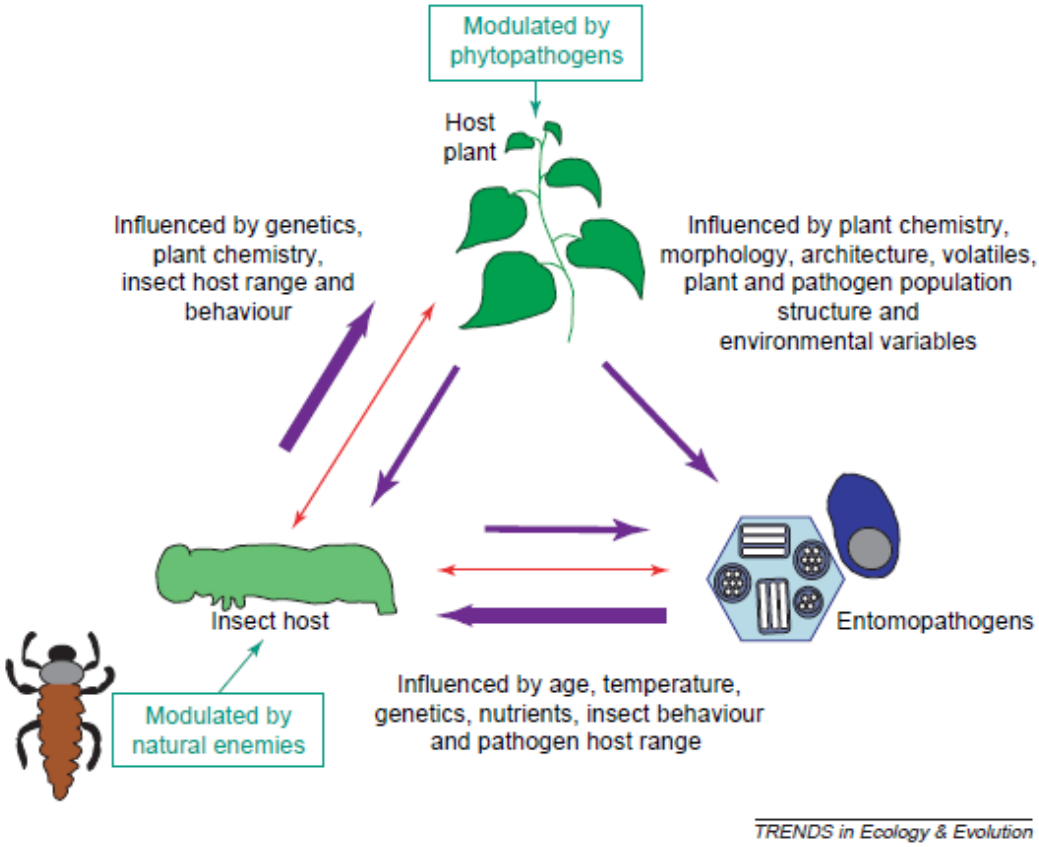

Figure 1 The theoretical methods by which insects, plants, and entomopathogens interact (Cory *et al* 2006).

Tritrophic interactions between insects, their larval host plants, and entomopathogens are shown in figure 1. Two theories have been proposed to explain how host plants influence parasite fitness (Cory *et al* 2006). The *direct effect* of the host plant is that host plant chemicals or toxins interact with the parasite directly to alter parasite fitness. The *indirect effect* of host plants is that host plant chemicals alter parasite fitness through host plant effects on the insect host. For example, the nutritional benefit of feeding on a particular host plant does nothing to the parasite directly; however, by providing caloric energy and cofactors to the insect host, the insect host could develop a better immune system to defend itself against the parasite, thus lowering the parasites fitness in that host(Cory *et al* 2006). The host insect can impact the evolution of the host plant because the insect uses the host plant as its food source. The parasite can affect range and grazing behavior of the insect host, thereby affecting the host plant. These interactions drive the evolution of tritrophic systems.

Recent studies on tritrophic interactions involving pathogens, phytophagous insects, and their larval host plants have already emphasized the role of host plants on parasite virulence (Cory *et al* 2006). We are using the tritrophic system of monarch butterflies, *Danaus plexippus*, one of their protozoan parasites, *Ophryocystis elektroscirrha* (McLaughlin *et al* 1970), and their host plants, milkweeds of the genus *Asclepias*, to study the effects of host plants on host-parasite evolution.

Recent studies have shown that the particular host plant, on which monarchs are reared, can affect the virulence the protozoan parasite *O. elektroscirrha* (De Roode *et al* 2008). Virulence is the cost to fitness an infected organism suffers when it becomes infected by a parasite. The costs, in regard to the host, can come in the form of the inability to mate, failure to emerge properly into adult hood, and decreased longevity. The severity of these consequences is

proportionally relative to parasite load. Elevated parasite load leads to increased virulence and transmission of the parasite (De Roode *et al* 2008). However, the mechanism that the parasite utilizes to transmit between hosts (see parasite section of intro) is largely dependent upon host survival and procreation. In trade off theory (Anderson *et al*1982) transmission probability is positively correlated with fitness, however, mortality is negatively correlated with fitness. Thus, there should be an optimal amount of virulence at which the parasite has maximum fitness. This phenomenon has also been observed in the monarch system. The fact that host diet has a strong effect on parasite fitness suggests that the host plant species may drive the ecology and evolution of parasite virulence in this particular host-parasite system (De Roode *et al* 2008).

1.1 Monarch Butterflies

There are at least two distinct populations of North American Monarchs, the Eastern and Western populations. The Eastern population migrates between northern Mexico, their overwintering site, and Southern Canada/Northern US; however, the Western population is thought to migrate thousands of miles, from areas west of the Rocky Mountains, to overwinter along the coasts of California(Malcolm *et al* 1987,1993).

The monarch life cycle in nature is shown in Figure 2. Basically, an egg, once laid by a female, hatches in about 4 days into a larva. Then, the larva eats the host plant that it hatched on and grows for about 2-3 weeks, at which time the larva undergoes pupation and becomes a pupa. It takes about two weeks for the monarch inside to undergo metamorphosis.

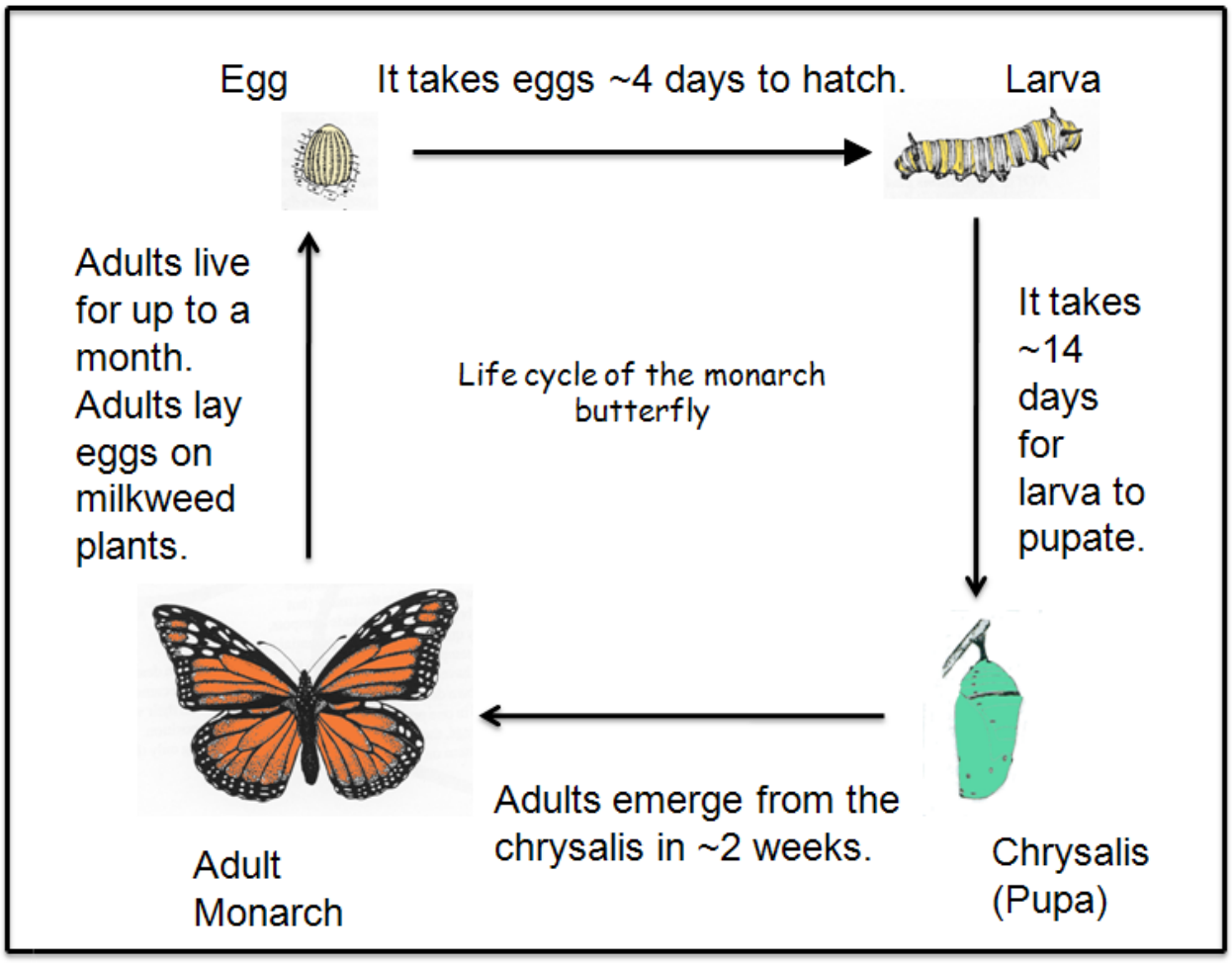

Figure 2 **The life cycle of the monarch butterfly, *Danaus plexippus*.** Images courtesy of Dr. Jacobus de Roode.

The mature monarchs live for up to a month. The developmental time from egg to adult is sped up slightly under laboratory conditions because the monarchs are nurtured at their optimal temperature of 26 ^{o}C. The developmental time from egg to larva generally takes less than 72 hours in the lab; larvae become pupae in about 11 days; pupae emerge into adults in under 10 days. However, the lifespan of adult monarchs do not change under laboratory conditions.

1.2 The parasite

Ophryocystis elektroscirrha is an apicomplexan protozoan parasite that infects monarch butterflies ubiquitously in certain populations of monarchs, such as the population from South

Florida, and not so much in others, such as the North American population (Leong *et al* 1997; Altizer *et al* 2000). Ingestion of the spores of *Ophryocystis elektroscirrha* from contaminated eggs or leaves of the host plant initiates the infection. The spores lyse in the gut of the caterpillar and disseminate into the larval hypoderm where they undergo asexual replication.

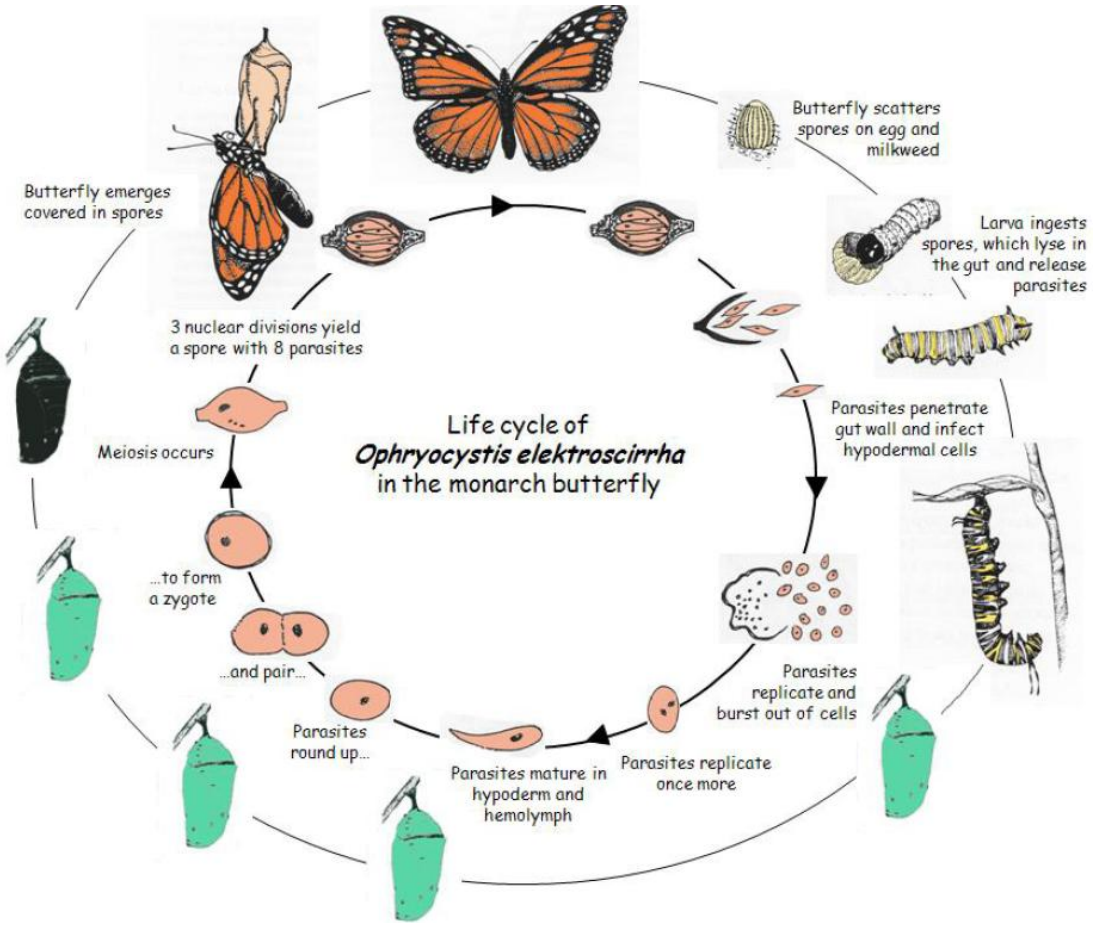

Figure 3 Life cycle of *Ophryocystis elektroscirrha* along with that of *Danaus plexippus.* Image courtesy of Dr. Jacobus de Roode.

The infection is not qualitatively noticeable during the caterpillar phase, rather, it manifests itself during pupation as dark splotches within the chrysalis. Sexual reproduction of the parasite within the host during the pupal stage and the subsequent spore formation results in the dark splotches on the normally verdant chrysalis. The spores of the protozoan are pervasive on the integument of the adult monarch, especially on the abdomen. The spores do not replicate and

must be ingested to cause new infections (De Roode *et al* 2008). This parasite is known to reduce larval survival, butterfly mass, adult longevity (De Roode *et al* 2008, Altizer *et al* 1999), fecundity, and mating ability (De Roode *et al* 2009) in monarchs.

1.3 The Monarch-Host plant system

Monarch larvae feed exclusively on milkweeds and there are circa 115 species of milkweeds that fall under the *Asclepias* genus in North America and the Caribbean (Malcolm *et al*. 1992; Malcolm 1994). Monarchs are able to utilize at least 27 species of milkweeds as host plants (Ackery *et al* 1984; Malcolm *et al* 1989; Vickerman *et al* 2002). It is known that the level of cardenolides and latex differs between species of milkweeds. For example, *A. incarnata* and *A. curassavica* have vastly differing cardenolide concentrations (Malcolm *et al* 1986, Zalucki *et al* 1990) and different quantities of latex (Zalucki *et al* 2001).

The plant latex acts as a deterrent for herbivores and contains a higher concentration of cardenolides than plant epidermal cells (Zalucki *et al* 2001). Cardenolides are secondary chemicals produced by milkweeds and used to deter herbivores (Brower *et al* 1968; Malcolm *et al* 1989; Malcolm 1991). Secondary chemicals are metabolites or compounds that a plant produces but does not utilize in its catabolic pathways. Some secondary chemicals have been shown to affect the metabolism of organisms that ingest them and may confer immunity against parasitic invaders of that organism (Malcolm 1989).

It has been known that cardenolides are acquired throughout the life of the monarch caterpillar (De Roode, *et al* 2008) and these cardenolides have anti-predator effects (Malcolm *et al*, 1989). Coincidentally, the milkweeds that have been shown to reduce parasite growth and virulence(Figure 3, 4) harbor a higher concentration of cardenolides compared to the milkweeds that do not elicit such effects(De Roode, *et al* 2008).

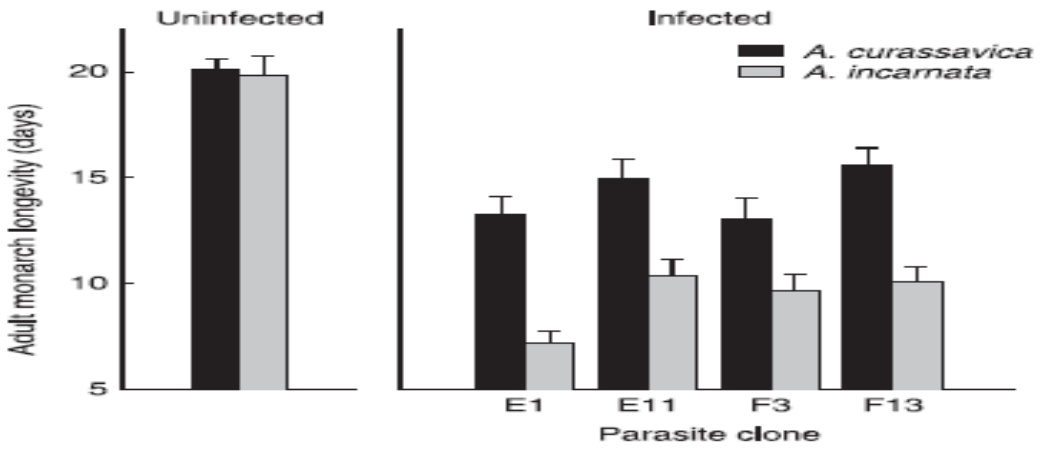

Figure 4 The left graph shows the average adult monarch longevity in uninfected monarchs reared as larvae on *A curassavica* and *A. incarnata* whereas the right graph shows the adult longevity of the monarchs infected with 10 spores(De Roode *et al* 2008). The parasite clone represent the various genetypes of parasite used. What is important to notice is that the infected monarchs raised on *A curassavica* lived longer than those reared on *A. incarnata* while the uninfected monarchs showed no difference(De Roode *et al* 2008). Error bars represent standard error.

It has been shown that monarchs infected with *O. elektroscirrha* had a longer adult lifespan when reared on *A. curassavica* than those reared on *A. incarnata* (Figure 4) (De Roode *et al* 2008). *A. incarnata* has a much lower concentration of cardenolides than *A. curassavica* as shown in Figure 5 (Malcolm *et al* 1986, Zalucki *et al* 1990, De Roode *et al* 2008); furthermore, *A. incarnata* contains fewer types of cardenolides than *A. curassavica*.

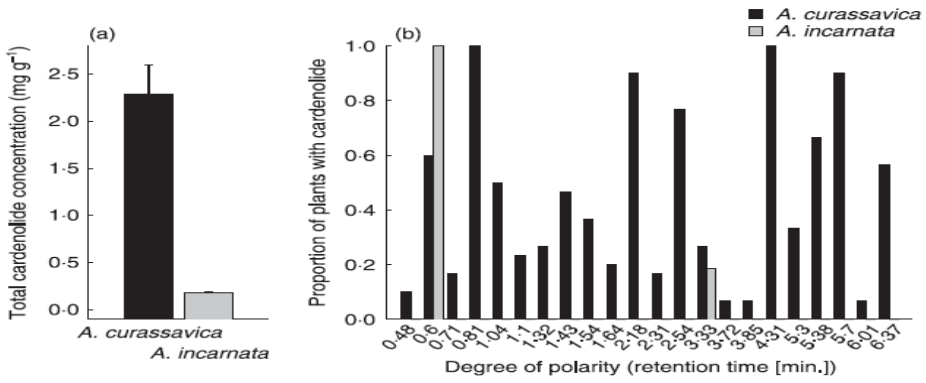

Figure 5 (a) The total concentration of cardenolides in *A. curassavica* and *A. incarnata*. Error bars represent standard error. (b) Relative polarity of the cardenolides; each retention time represents a distinct cardenolide. Black bars are for *A. curassavica* while grey bars are for *A. incarnata*(De Roode *et al* 2008).

Cardenolides are the only organically active chemical compounds discovered thus far in milkweeds. It is possible that these secondary chemicals are responsible for the palliative effects. The goal of this thesis is to explore whether or not cardenolides are responsible for the reduction in fitness of the protozoan parasite, *Ophryocystis elektroscirrha*, in its host, the monarch butterfly, *Danaus plexippus*. It is hypothesized that this result will lead towards greater understanding the effect of milkweed host plants on the evolution and ecology of this host-parasite system.

2. Materials and Methods

2.1 Cardenolides and Latex Digitoxin and ouabain were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Natural cardenolide extracts were provided by Dr. Mark Hunter, University of Michigan.

2.2 Leaf Discs The milkweed leaves were grown in the Emory greenhouse. Leaf discs of area $1cm^2$ were made from milkweed leaves by punching holes in the leaf with a hole-puncher. The circular pieces of leaf had an area of $1cm^2$.

2.3 Parasite Spores Parasite spores were provided by the De Roode laboratory.

2.4 Spore Deposition

Step1: Glass needles were exposed to open flames to form a glass bulb at one end.

Step2: The bulb-end of the glass-blown needle was used to pick up parasite spores from the petri dish.

Step3: The spores were inoculated under a microscope onto the leaf surface.

Step4: Leaf discs with the appropriate treatment and parasites are called inocula (control inocula were leaf discs without parasites), and they are ready for immediate use.

2.5 Caterpillar Rearing

Step1 : Caterpillars were picked up individually from the milkweed filled plastic boxes, where they were fed for the first 2 days, and placed gently into their own individual petri dish. They

were placed into a climate room and were monitored periodically until they had eaten their inoculum completely.

Step2 : The larvae were transferred to individual 470 cc plastic cylindrical tubes following complete ingestion of the inocula. The caterpillars were provided with a stalk of *A. tuberosa* in a 10 mL florist tube containing a moist paper towel. The tube was then capped with a mesh top to prevent caterpillar escape and allow air circulation. The caterpillars were checked daily for mortality or pupation and florist tubes were refilled to the brim each morning. *A. tuberosa* was added whenever the caterpillar finished the stalk inside the tube.

Step3: The caterpillars generally pupated on the mesh lid of the tubes. After pupation, the pupa was transferred into a 240 cc cylindrical plastic cup that contained a dry paper towel. The pupae were put into a different room, away from the larvae, to avoid contamination.

Step 4: Once the pupae emerged as adults, they were placed into translucent envelopes that were labeled with the appropriate number, pupation date, and emergence date. These monarchs were then put into a container and stored in a 14 ^{o}C refrigerator. These adults were checked daily for mortality.

2.6 Pupal scoring Pupal scoring is a quantitative method to assess the infection status of an individual monarch. Starting on the 6th or 7th day of pupation, the parasite will manifest itself as dark, irregular patches. These patches and their distribution indicate the degree of infection. The pupal score range is between 0-5. A score of 0 represents the lowest degree of infection whereas a score of 5 signifies the highest degree of infection. Pupal scores have been strongly correlated with spore load.

2.7 Vortexing The dead body of an adult monarch was vortexed for 5 minutes at maximum speed in 50 mL tubes containing 5 mL of water. This process removed the parasites from the body of the insect for quantification of the parasite.

2.8 The effects of digitoxin and ouabain on the fitness of monarch butterflies infected with *O. elektroscirrha*

Digitoxin and ouabain (Sigma Chemical Company) were used to test whether or not these two cardenolides could decrease parasite virulence when ingested by larvae. These particular cardenolides were chosen due to their commercial availability and chemical nature (digitoxin is nonpolar whereas ouabain is very polar) (Tracqui *et al* 1997). *A. tuberosa* was chosen to be the host plant for this experiment because it has little to nil cardenolides (Seiber 1982).

The cardenolides were dissolved in 1mL of 100% methanol in 1.5 mL Eppendorf tubes. The cardenolide solutions in this experiment were clear. The concentration of the stock solutions was 100 mM. The stock solution was further diluted into 4 working solutions at concentrations of 10 mM, 2 mM, 1 mM, and 0.2 mM, respectively.

The experiment was designed in a way such that the treatment (digitoxin, ouabain, or methanol) and infection status (infected or uninfected) were factors. The larvae were divided randomly into 9 treatment groups. There were 20 larvae in each group resulting in a total of 180 larvae for the experiment. In each group, 8 of the larvae did not receive parasite spores while the other 12 received 10 spores each. The larvae were transferred to *Asceplias tuberosa* immediately upon hatching. Two days after hatching, they were individually transferred to 10 cm petri dishes for parasite inoculation. These dishes contained leaf discs from *A. tuberosa.* and a moistened filter paper.

For each test group, 5 uL of the methanol-cardenolide solution was put on all twenty leaf discs of that group. The solution was allowed to dry onto the leaf discs; 12 of the leaf discs had spores deposited onto them and we made sure the other 8 did not get contaminated by spores.

Table 1 Experimental groups for the digitoxin and ouabain experiment.

Group	Cardenolide	Concentration
1	Digitoxin	0.2mM
2	Digitoxin	1mM
3	Digitoxin	2mM
4	Digitoxin	10mM
5	Ouabain	0.2mM
6	Ouabain	1mM
7	Ouabain	2mM
8	Ouabain	10mM
9	Control (100% methanol)	

Statistical analyses for this experiment were carried out in SPSS 15.0.1 (SPSS Inc. 2006). T-tests were performed on the data set to see whether or not the cardenolide treatment groups yielded longevities that were different from those fed on methanol. Analysis was also performed to determine if there was a difference in longevity between infected monarchs and uninfected monarchs.

2.9 The effects of natural cardenolide extract on the fitness of monarchs infected with *O. elektroscirrha*

The fact that the cardenolides tested failed to yield the expected longevity results suggests that digitoxin and ouabain are not factors in this host-parasite system and not all cardenolides can improve monarch fitness in this host-parasite system. Since *A. curassavica* increased monarch fitness as described by De Roode (2008), it is possible that *A. curassavica* contains specific cardenolides that do have an effect on monarchs infected with *O.*

elektroscirrha. Cardenolides extracted from *A. curassavica* were used in this experiment to test for the effect of cardenolides on monarchs infected with *O. elektroscirrha*.

The experiment was carried out with two groups of 18 larvae. All the caterpillars were inoculated with 10 spores on *A. incarnata* leaf discs and reared on *A. incarnata.* We used *A. incarnata* with *A. curassavica* cardenolides so that we could distinguish between nutritional benefit and chemical action of the host plant. The nutritional component comes strictly from the leaf disc while the chemical component comes from the cardenolide extract. The cardenolide extract was provided by Dr. Mark Hunter, University of Michigan.

The caterpillars in this experiment were obtained from an *A. incarnata* plant that was placed into a mating cage for 24 hours. Since the **c**aterpillars all came from the same *A. incarnata* plant and lineage, they were divided up randomly into two groups of 18 and reared on *A. incarnata*. All of the caterpillars were inoculated with 10 spores. Group 1 received *A. incarnata* leaf discs with methanol control and group 2 received the cardenolide extract from *A. curassavica* (Table 2). Caterpillars were reared on *A. incarnata* instead of *A. tuberosa* as previously described.

Table 2 Experimental groups for the natural cardenolide extract experiment

Group	Cardenolide	Parasite	n=
1	Yes	Yes	18
2	No	Yes	18

2.10 The effects of latex on host-parasite interactions

The natural cardenolide experiment did not work (see discussion of the natural cardenolide experiment). The aim of this experiment was to test the effect of cardenolides from

A. curassavica on this host-parasite system and to deliver the cardenolides in a solution that would not destroy the milkweed leaf disc as happened in the natural cardenolide extract experiment. Zalucki *et al* (2001) found that the concentration of cardenolides was a thousand fold greater in plant latex than in the leaf epidermal cells. Our previous experiment showed that milkweed leaf discs were destroyed by excess amounts of methanol in the cardenolide extract. Therefore, latex was an ideal source of cardenolides since it contained high concentrations of cardenolide and was not detrimental to the plant epidermal cells.

There were 3 experimental groups in this study. The first group was comprised of 20 caterpillars that were reared on *A. incarnata* and infected with 10 parasite spores. The second group was comprised of 20 caterpillars that were reared on *A. incarnata,* infected with 10 parasite spores, and exposed to *A. curassavica* latex in the inoculum. The third group was the control group, and it was comprised of 10 caterpillars reared on *A. incarnata* without spores and without latex(Table 3). In summary, all of the caterpillars used in this experiment were reared on *A. incarnata* and the only difference between group 1 and group 2 was that group 2 received *A. curassavica* latex on the inoculum. (See caterpillar rearing and replace *A. tuberosa* with *A. incarnata*).

Table 3 The experimental groups for the latex experiment.

Group	Latex	Parasite	n=
1	No	Yes	20
2	Yes	Yes	20
3	No	No	10

The latex used in this experiment was obtained by severing the top four leafs of *A. curassavica* plants and collecting the drops of latex that surged out from the open wounds. The

drops of latex were collected into Eppendorf tubes by placing the open end of the tube below the drop of latex and then scooping the droplet into the tube. This process was repeated about 50 times in order to obtain ~500 uL of latex.

After parasites were deposited (see spore deposition), 7.5 uL of homogenized *A. curassavica* latex were spread onto leaf discs of group 2 and allowed to dry at room temperature for 2-4 hours. The pipette tip was changed each time to ensure no cross contamination between leaf discs.

Statistical analysis for this experiment was done in R version 2.4.0 (R Development Core Team 2006). Analysis of variance (ANOVA) tests were done on development time, pupal scores, and longevity data.

3. Results

3.1 The effects of digitoxin and ouabain on the fitness of monarch butterflies infected with *O. elektroscirrha*

Figure 6 shows the treatment group versus the average adult longevity for infected and uninfected monarchs. We found that the longevity of infected monarchs was not influenced by digitoxin or ouabain at the concentration range from 0.2 mM to 10 mM($P>.05$). The final group was the control series. Although it seemed that digitoxin may have been more beneficial for infected monarchs than both Ouabain and the control, the differences in longevity between the groups were not statistically significant ($P>.05$).There were no significant differences in longevity ($P>.05$) among the groups of uninfected monarchs given digitoxin, ouabain, and methanol. The difference in adult longevity between infected and uninfected individuals were significantly different within the treatment groups ($P<.005$). This result suggests that infected monarchs have reduced adult longevity regardless of treatment.

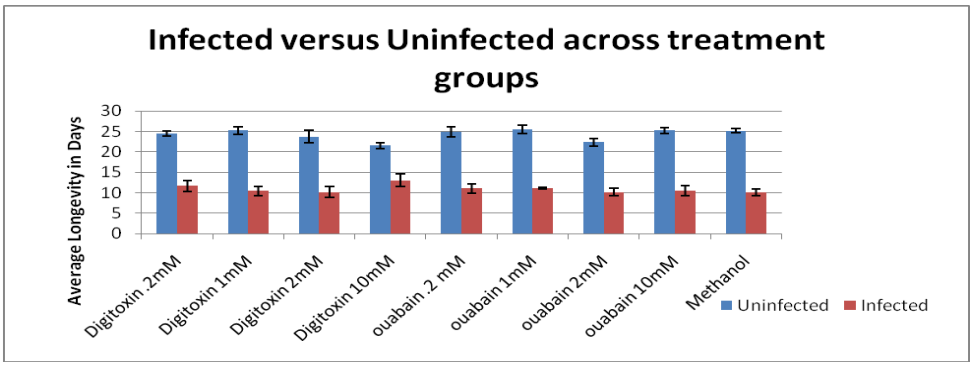

Figure 6 The blue bars represent the average longevity of the monarchs that were uninfected. The red bars represent the average longevity of monarchs that were infected. (N=12,N=8) Error bars represent standard error of the mean.

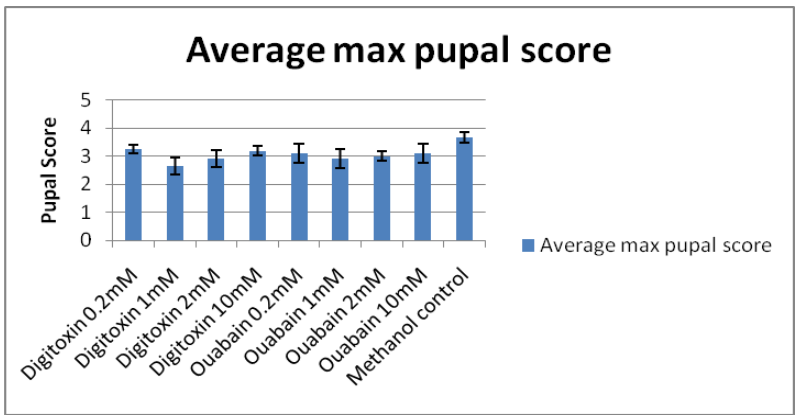

Figure 7 Average pupal scores given from the 6-9th days of the pupal stage. Bars represent the average maximum pupal scores of each group. The error bars are standard errors. (N=12 for each group)

The pupal score (Figure 7) is used to quantify the level of infection in an individual monarch. The methanol control group showed a higher pupal score ($P<0.05$) than the other groups. However, since the longevity (Figure 6) of the methanol group was not statistically different from the other groups, this pupal score result was not expected. We thought that this difference in pupal score was not real because we have always observed a strong inverse correlation between pupal score and longevity in previous experiments. We vortexed members of the methanol control group and members of the digitoxin 2 mM group and ouabain 10 mM group and counted the spores to test whether or not the difference in pupal score was real.

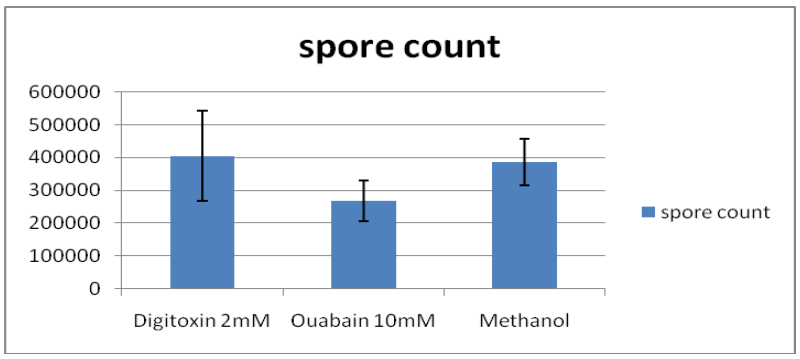

Figure 8 Spore count from Vortexing. The bars represent the average spore count of that group and the error bars are standard error.

Using this more reliable and accurate method, we found no significant difference among the three groups ($P>.05$).

3.2 The effects of natural cardenolide extract on the fitness of monarchs infected with *O. elektroscirrha*

Since the cardenolides used in this experiment were of very low concentration, large quantities ranging from 5uL to 40uL of the solution were put onto individual leaf discs. The cardenolide solution was allowed to dry before the parasites were inoculated on the leaf disc. The cardenolide solutions in this experiment were green and left a tar-like residue after the methanol solute had evaporated, which made it very difficult to put the parasites on the leaf discs. Furthermore, the excess methanol severely damaged the milkweed leaf discs almost to the point of disintegration. After about four days, most of 18 caterpillars given the cardenolides died presumably from starvation. The experiment was aborted.

3.3 The effects of latex on host-parasite interactions

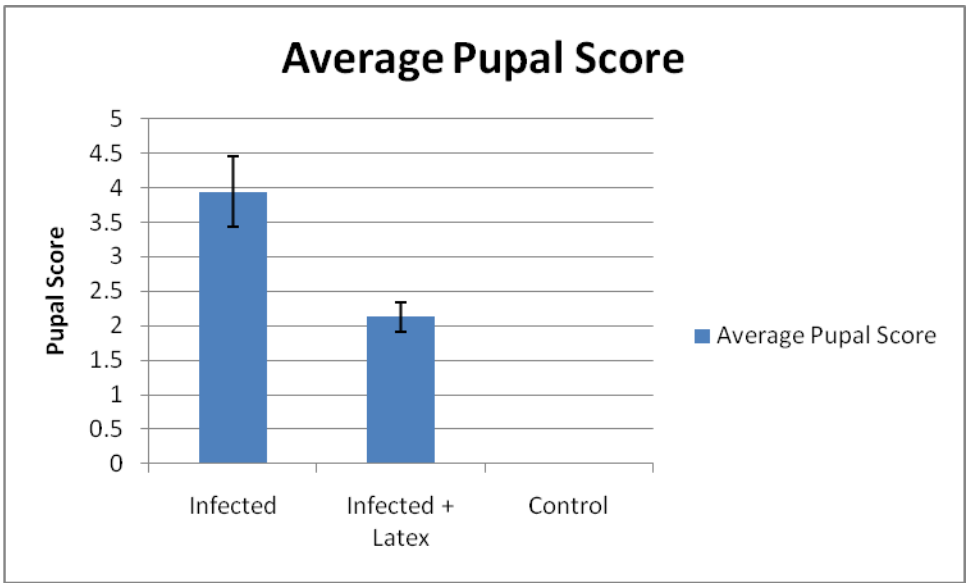

Figure 9 **Average pupal scores for the groups. The higher the pupal score means higher parasite load. Infected, Infected + Latex, and Control (N=16,16,10) error bars are for standard error.**

ANOVA tests were performed on the pupal scores (Figure 9) and we found the differences among the three groups to be highly significant (P<.005). The infected group clearly showed a higher level of infection than the group that was given both the parasite and latex from *A. curassavica*. The control group had the lowest level of infection.

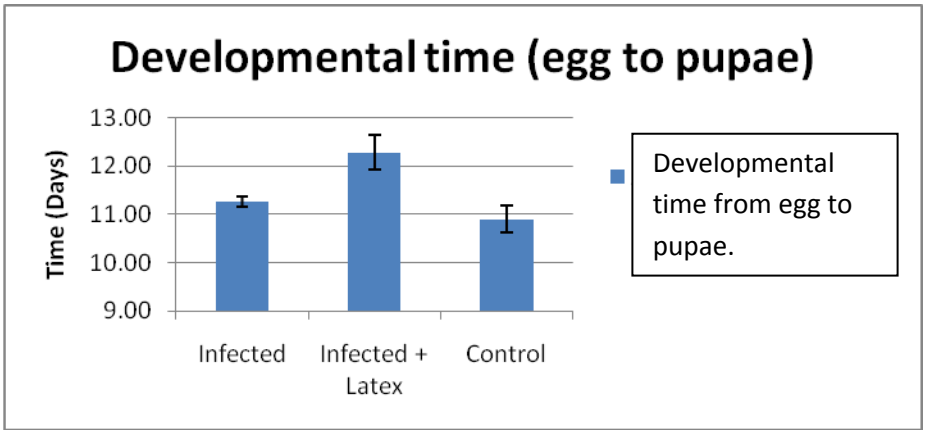

Figure 10 **Developmental time from larvae to pupae. Infected, Infected + latex, and Control(N=16,16,10). Error bars are for standard error.**

We analyzed the effect of latex on developmental time (figure 10). The infected+latex group's developmental time from egg to pupae was significantly longer (P<.05) than that of the other two groups. The developmental times of the infected and control groups were not significantly different from each other (P>.05).

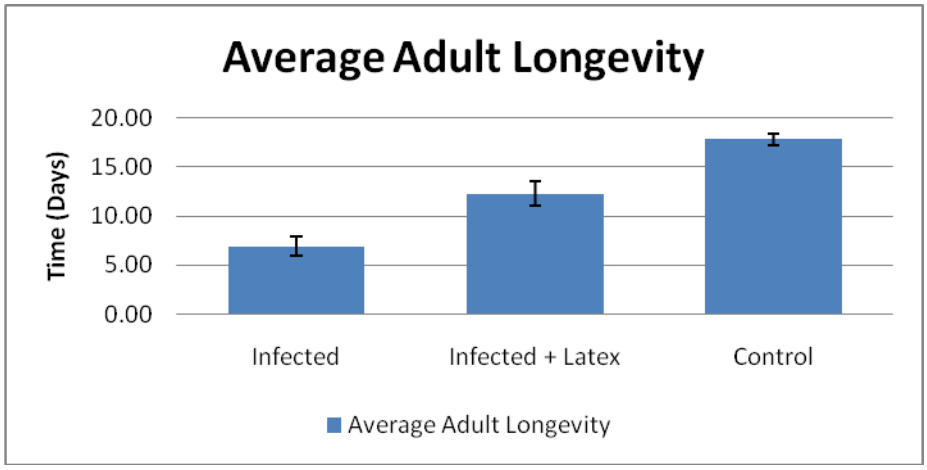

Figure 11 Adult longevety from emergence to death. Infected, Infected + latex, control (N=16,16,10). Error bars are for standard error. (n=16,16,10)

The infected group had a significantly shorter life span than both groups of infected + latex group(P<.01) and uninfected control group(P<.005) as shown in Figure 11. The average longevity of infected members was about 7 days while the average lifespan for monarchs given the latex was about 13 days, which is still significantly lower than uninfected monarchs (18 days).

Our results of pupal scores from figure 9 and average adult longevity from figure 11 agree with the previously published result that adult longevity is inversely correlated with parasite spore load (De Roode *et al* 2008). The infected group had the highest pupal score (spore load) and the shortest longevity, the infected + latex group had an intermediate pupal score along with an intermediate longevity and the control group (uninfected) had the lowest pupal score and the highest adult longevity.

4. Discussion

The cardenolides digitoxin and ouabain were chosen because of their commercial availability and opposite polarities (Tracqui *et al* 1997). We had originally expected that treatments of digitoxin and ouabain would have increased the longevity of the monarch more than the methanol control in our infected monarchs. However, the results have shown, conclusively, that these cardenolides have no distinguishable effect on the longevity of infected or uninfected monarch butterflies. Furthermore, the pupal score and spore load showed that these cardenolides have no significant effect on parasite fitness (figure 7 and 8). These results do not support our original hypothesis that digitoxin and ouabain would decrease the reproduction of the parasite and hence alleviate virulence of the parasite and increase the longevity of adult monarchs. One explanation for these results is that not all cardenolides have an effect on parasite fitness. If only some cardenolides have an effect on the parasite, then they would most likely be found in milkweeds that have been shown to have anti-parasitic effects. The next step in this query was to retry the experiment with cardenolides extracted from milkweeds.

Natural cardenolide extracts from *A. curassavica* were kindly provided from Dr. Mark Hunter, University of Michigan. There were many complications in this experiment. The caterpillar's refusal to eat the cardenolide laced leaf discs (methanol damaged) posed a problem for this experiment. The methanol damaged leaf discs if used in amounts excess of 10 uL. However, the average amount of solution placed on a leaf disc was close to 20 uL of solution due to the low cardenolide concentrations of the samples. The next step was to find a way to deliver natural cardenolides in sufficient dosages in solution that did not damage leaf discs.

Latex was chosen for its high cardenolide concentration and because it was not detrimental for the leaf discs. Latex treatment increased adult longevity (Figure 11), increased developmental time from egg to pupae (Figure 10) and reduced parasite load (Figure 9) in infected monarchs. These results showed that latex from *A. curassavica* affected the host-parasite dynamic by making the host more resilient to the infection by reducing the growth of *O. elektroscirrha*. However, the results in figure 10 indicated a strong developmental cost of being fed latex. The major discovery of this experiment was that the nutritional benefit from leaves was most likely not a big factor in the dynamics between the monarch and its parasite. Previously, it had been found that monarchs reared on *A. curassavica* grew larger than those reared on *A. incarnata*. In this experiment, the latex treatment group showed no significant difference in weight, but a substantial difference in longevity, from those that were infected but were not given latex.

This experiment was limited in many ways. It was small experiment with relatively few caterpillars and the composition of latex has not been fully documented. Therefore, more tests need to be done before any definitive conclusion regarding the role that cardenolides play in this host-parasite system can be made. A large scale experiment should be conducted next by using latex from both *A. incarnata* and *A. curassavica* to distinguish the physical and chemical effects of latex on this host-parasite system. The physical properties of latex from *A. incarnata* and *A. curassavica* are similar (Zalucki *et al* 2001) but their chemical composition differs greatly (De Roode *et al* 2008). Since latex is a complex mixture, purification of the effective component is necessary to determine which cardenolides, if any, are associated with the anti-parasitic activity.

5. Conclusion

The latex experiment suggests that host diet, and therefore ecology, plays a major factor in parasite and host fitness. It seems that latex from *A. curassavica*, regardless of what the underlying cause is, depresses the ability of this parasite to infect its host or grow. Recent studies show that infected monarchs are more likely to lay eggs on *A. curassavica* than *A. incarnata*, whereas uninfected monarchs show no preference (Lefevre *et al* submitted 2009). But the question is why adult monarchs do not always choose cardenolide rich plants, such as *A. curassavica*, over plants that contain low concentrations of cardenolides, such as *A. incarnate.* One possible explanation is that parasite virulence is a factor in host plant selection. *O. elektroscirrha* is closely related to *Plasmodium falciparum*, the parasite causing malaria. Recent studies have shown that vaccinations for malaria can lead to more virulent strains of the disease (Mackinnon *et al* 2008). One theory of why pathogens are virulent is because pathogens need to extract resources from the host in order to have the ability to transmit itself to a new host. If a vaccine can reduce parasite replication then it may also select for more virulent strains of the parasite.

The results from the latex experiment further showed that latex from *A. curassavica* reduced the pupal score of infected individuals, a measure very closely correlated with spore load. Spore load is increased through parasite replication. Higher levels of parasite replication increase virulence and transmission. If latex from *A. curassavica* reduces the parasite's ability to reproduce then it effectively reduces the virulence and transmission rate of the parasite. If that is the case then it is plausible that higher virulence levels will provide optimal fitness for the

parasite and will be selected for through natural selection, thus leading to a population of parasites with higher virulence.

In summary, our experiments suggest that host plant species may influence the ecology and evolution of this monarch-parasite system. Specifically, the latex experiment concludes that latex is an important factor in this process and further investigation is needed to clarify whether or not the cardenolides within the latex are the cause.

6. References

Ackery, P. R. & Vane-Wright, R.I. 1984. Milkweed Butterflies: Their Cladistics and Biology. Cornell University Press, Ithaca, NY.

Altizer, S.M., & K. S. Oberhauser. 1999. Effects of the protozoan parasite *Ophryocystis elektroscirrha* on the fitness of monarch butterflies (*Danaus plexippus*). *Journal of Invertebrate Pathololog*y, 74:76-88

Altizer, S.M., Oberhauser, K.S., & Brower, L.P. 2000. Associations between host migration and the prevalence of a protozoan parasite in natural populations of adult monarch butterflies. *Ecological Entomology*, 25:125-139

Anderson, R. M. & May, R. M. 1982. Coevolution of hosts and parasites. *Parasitology*, 85:411-426

Bezemer, T. *et al.* 2005. Soil community composition drives aboveground plant-herbivore-parasitoid interactions. *Ecology letters*, 8: 652-661

Brower, L.P., Ryerson, W.N., Coppinger, L. & Glazier, S.C. 1968. Ecological chemistry and the palatability spectrum. *Science*, 161: 1349–1351.

Cory & Hoover 2006. Plant-mediated effects in insect-pathogen interactions. *Trends in Ecology and Evolution*, 21: 278-286

De Roode, JC., Chi J., Rarick, R. 2009. Strength in numbers: high parasite burdens increase transmission of a protozoan parasite of monarch butterflies (*Danaus plexippus*). *Oecologia*, 161: 67-75

De Roode, Pederson, Hunter, and Altizer. 2008. Host plant species affects virulence in monarch butterfly parasites. *Journal of Animal Ecology*, 77:120-126.

De Roode, Yates, Altizer. 2008. Virulence-transmission trade-offs and population divergence in virulence in a naturally occurring butterfly parasite. *PNAS*, 105, 21:7489-7494

Leong, K.L.H., Yoshimura, M.A. & Kaya, H.K. 1997. Occurrence of a neogregarine protozoan, *Ophryocystis elektroscirrha* McLaughlin and Myers, in populations of monarch and queen butterflies. *Pan-Pacafic Entomologist*, 73:49-51

Lefevre, T. , Oliver, L., Hunter, M., De Roode, JC. 2009. Transgenerational medication in the monarch butterfly. Submitted 2009

Mackinnon, Gandon, and Read. 2008. Virulence evolution in response to vaccination: The case of malaria. *Vaccine*, 26:42-52

Malcolm, S. B. 1992. Milkweeds, monarch butterflies, and the ecological significance of cardenolides. *Chemoecology*, 5:101-117

Malcolm, S. B. 1989. Cardenolide-Mediated Interactions between Plants and Herbivores. Herbivores: Their Interactions with Secondary Plant Metabolites, 251-296.

Malcolm, S.B., B.J. Cockrell, and L.P Brower. 1987. Monarch butterfly voltinism: Effects of temperature constraints at different latitudes. *Oikos,* 49:77-82

Malcolm, S.B., B.J. Cockrell, and L.P Brower. 1993. Spring recolonization of eastern North America by the monarch butterfly: successive brood or single sweep migration? In Malcolm, S.B., and M.P. Zalucki, eds., Biology and conservation of the monarch butterfly, 253-267.

Malcolm & Brower. 1989. Evolutionary and ecological implications of cardenolide sequestration in the monarch butterfly. *Cellular and Molecular Life Sciences,* 45 284-295.

Malcolm, S.B & Brower, L.P. 1986. Selective oviposition by monarch butterflies(*Danaus plexippus* L.) in a mixed stand of *Asclepias curassavica* L. & *A. incarnata* L. in South Florida. *Journal of the Lepidopterists' Society*, 40:255-263

Malcolm, S.B., R.A. Martin, S.P. Lynch, L.P. Brower, S.B., and T. Van Hook. 1992. Cardenolide content, emetic potency, and thin-layer chromatography profiles of monarch butterflies, *Danaus plexippus*, and their larval host-plant milkweed, *Asclepias humistrata*, in Florida. *Chemoecology,* 3:1-13

McLaughlin, R.E. Myers, J. 1970. *Ophryocystis elektroscirrha* sp.n., a neogregarine pathogen of monarch buttefly *Danaus plexippus* (L.) and the Florida queen butterfly *D. gilippus berenice* Cramer. *Journal of protozoology*, 17:300-305

R Development Core Team (2006) *R: a Language and Environment for Statistical Computing*. R Foundation for statistical Computing, Vienna, Austria. Available at http://www.R-project.org.

Seiber, J.N. 1982. Analysis of toxicants in agricultural environments. *Basic Life Sciences*, 21:219-34

Tracqui, A., Kintz, P., Ludes, B., & Mangin P. 1997. High-performance liquid chromatography-ionspray mass spectrometry for the specific determination of digoxin and some related cardiac glycosides in human plasma. *Journal of Chromatography B: Biomedical Sciences and Applications*, 692:101-109

Vickerman, D.B. & de Boer, G. 2002. Maintenance of narrow diet breadth in the monarch butterfly caterpillar: response to various plant species and chemicals. *Entomologia Experimentalis et Applicata*, 104:255-269

Zalucki, M.P., Brower, L.P. & Malcolm, S.B. 1990. Oviposition by *Danaus plexippus* in relation to cardenolide content of 3 *Asclepias* species in the southeastern USA. *Ecological Entomology*, 15:231-140

Zalucki, M.P., Brower, L.P. & Alonso-M. A. 2001. Detrimental effects of latex and cardiac glycosides on survival and growth of first-instar monarch butterfly larvae *Danaus plexippus* feeding on the sandhill milkweed *Asclepias humistrata. Ecological Entomology*, 26: 221-224