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Eleanore Sternberg Date

Studies of local adaptation and ecological determinants of infection in a monarch butterfly-parasite interaction

By
Eleanore Sternberg
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Population Biology, Ecology, and Evolution

_____
Jacobus de Roode, Ph.D.
Advisor

_____
Berry Brosi, Ph.D.
Committee Member

_____
Nicole Gerardo, Ph.D.
Committee Member

_____
Todd Schlenke, Ph.D.
Committee Member

_____
Michael Zwick, Ph.D.
Committee Member

Approved:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

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By

Eleanore Sternberg

B.S. University of California, Berkeley, 2005

Advisor: Jacobus C. de Roode

An abstract of

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Abstract

Selective pressures are spatially heterogeneous and many adaptations are specific to the local environment. By comparing multiple populations, we can gain unique insights into how species interact with their environment, and how this ultimately results in adaptation. Such studies are common in host and parasite species, because the reciprocal and antagonistic nature of their interaction is thought to frequently result in local adaptation. As expected, a number of studies have found evidence of local adaptation in host-parasite system. However there are also a substantial number of studies that have failed to find such evidence. The numerous studies that fail to find local adaptation suggests that the interaction between host and parasite genotypes may be insufficient to explain expressed infection phenotype and ultimately, coevolution. In this dissertation, I have examined infectivity, virulence, and parasite burden in three populations of monarch butterflies and their protozoan parasites, to explore how these traits vary between populations and to test for local adaptation. I have then quantified the effect of environmental factors on host and parasite fitness in this system. Specifically, I examine the effects of monarch food plant and the effects of competing parasites. When quantifying host fitness in different populations and on different food plants, I have distinguished between the ability of a host to resist infection and the ability to tolerate infection without limiting parasite transmission. I find that there are large differences between the three study populations and I also find that infection phenotype is often modulated by environmental variables. Because the environment has a significant effect on host and parasite fitness, I suggest that these differences may be explained by differences in the ecologies of the three

populations and I emphasize that future studies of local adaptation should include the important components of the environment which I have identified in this dissertation.

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Chapter 1

Introduction

The following dissertation focuses on two major fields of study: local adaptation and environmental variation. Consequently, the subsections of this introduction provide the necessary backgrounds for these fields of study. There is a third major component to this dissertation however, which is the study of host-parasite interactions. Parasitism - defined broadly as any organism living on or in a host and causing damage - is not a distinct component but rather the context of this work. Because parasitism provides a unifying theme, it is necessary to first justify the study of host-parasite interactions.

According to the World Health Organization (2011), there were 9.04 million human deaths due to infectious diseases in 2008, which makes the case emphatically for studying parasites from a public health perspective. From the perspective of evolutionary ecology however, there are also compelling reasons for the study of parasitism. J.B.S. Haldane (1949) recognized this, when he wrote that infectious diseases are "a very important evolutionary agent and some of its results have been rather unlike those of the struggle for life in its common meaning." Virtually all organisms experience the selective pressures of parasitism (Windsor 1998; Combes 2001) and these selective pressures result in the evolution of both host and parasite species. These kinds of species interaction can lead to an evolutionary arms race, as described by the Red Queen hypothesis (Van Valen 1973), where there is continual coevolution but no long-term increase in the relative fitness of either species. Such coevolutionary dynamics in host-parasite systems result from negative, frequency-dependent selection where the most common host genotypes are more vulnerable to infection (Hamilton 1980; Bell and

Maynard Smith 1987; Hamilton et al. 1990; Dybdahl and Lively 1998; Lively and Dybdahl 2000). Parasite-driven frequency dependent selection can maintain biodiversity (Laine 2009), for example by reducing the probability of spatial aggregation of plant species in temperate and tropical forests (Augspurger 1983; Packer and Clay 2000; Clay 2006). Parasites can regulate host population size (Ebert et al. 2000) and this includes producing cyclical host population crashes (Hudson et al. 1998; Pedersen and Greives 2008). Parasitism can modulate other species interactions, such as predation (Packer et al. 2003), competition (Tompkins et al. 2000), and mutualism (Dunn et al. 2008), and parasites are an often ignored but important component of food webs (Lafferty et al. 2006). Because the influences of parasitism are so widespread, Hudson et al (2006) have argued that parasite communities are not only a marker but drivers of the stability, productivity, and resilience that characterizes a healthy ecosystem. As evolutionary ecologists, we should study host-parasite interactions because they represent one of the most common species interactions on earth and understanding the effects of such interactions is imperative to understanding the ecology and evolution of all species.

1.1 Local adaptation

Local adaptation, where organisms gain higher relative fitness in their native environment, arises from the same processes that drive all evolutionary change. By measuring local adaptation in multiple populations experiencing divergent selective pressures, we can better understand how natural selection produces adaptive change (Reznick and Ghalambor 2001). For example, research by Reznick and colleagues has focused on local adaptation in Trinidadian guppies (*Poecilla reticulate*) inhabiting isolated pools, resulting

in naturally produced replicate populations. By quantifying morphological and reproductive traits in multiple guppy populations in the presence of different predatory species, Reznick et al. demonstrated that the presence of larger predators resulted in selection for earlier and more frequent reproduction in smaller guppies (Reznick and Endler 1982; Reznick et al. 1990; Reznick et al. 1997). Larger predators preferentially prey on the larger adult guppies and these results support life history theories which predict that high rates of early mortality will select for greater reproductive output earlier in life (Gadgil and Bossert 1970; Law 1979).

Empirical tests of local adaptation like those carried out by Reznick et al. generally fall into two broad categories: transplant and common garden. Transplant experiments measure the fitness of an organism in either its native habitat or after translocation to a foreign environment. This approach captures all aspects of the environment which may be important for local adaptation; however, an overabundance of environmental variation in the field may mask variation that is indicative of local adaptation (Laine 2007). Additionally, for logistical reasons a transplant approach may be unfeasible particularly when studying mobile species. In contrast, common garden experiments are conducted under uniform conditions, such as in a laboratory or greenhouse, and the fitness of the study organism is measured in response to a single factor. This approach is often used to study local adaptation in species interactions such as parasitism (e.g. Lively 1989; Imhoof and Schmid-Hempel 1998; Oppliger et al. 1999; Ganz and Washburn 2006). When a common garden (i.e. reciprocal cross-infection) design is applied to host-parasite interactions, local adaptation in the system will be revealed by the results in figure 1.1. Because transplant experiments include all potential

local variation and common garden experiments include only one controlled source of variation, the results of transplant and common garden experiments should yield identical results only when local adaptation is entirely due to the interaction between focal species (Nuismer and Gandon 2008). The interaction between hosts and parasites generate strong selective pressures, and the fitness of hosts and parasites is often a result of the interaction between genotypes (Restif and Koella 2003; Lambrechts et al. 2006), thus the assumption that local adaptation is driven by a single species interaction is more likely to be satisfied in host-parasite systems. As a result, many studies of local adaptation have been carried out in host-parasite systems (Kawecki and Ebert 2004).

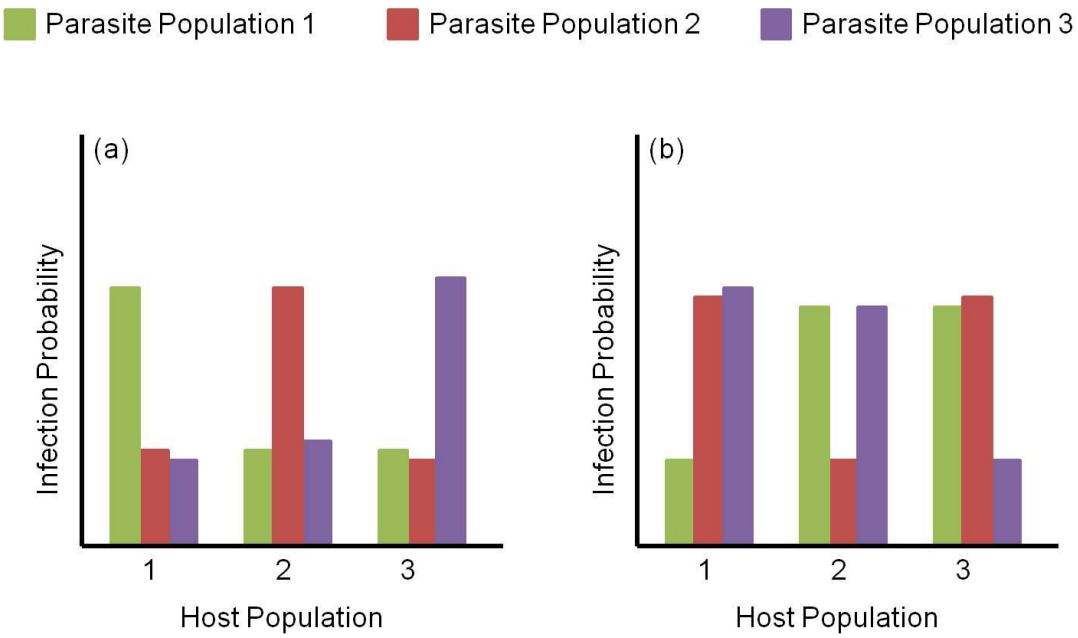

Figure 1.1 Expected results of a reciprocal cross-infection experiment for three locally adapted populations. (a) Increased infection probability in sympatric hosts indicates that parasite is locally adapted. (b) Decreased infection probability in sympatric host indicates that parasites are locally maladapted, or that hosts are locally adapted to parasites.

Again, like the studies of guppy populations conducted by Reznick et al, local adaptation studies in host-parasite systems have served to test key hypotheses in

evolutionary biology, for example regarding the maintenance of sexual reproduction. The problem with sexual reproduction from an evolutionary perspective is that it is costly endeavor. This includes the two-fold cost of sex, where a female producing offspring through sexual reproduction will produce only half as many daughters, and consequently half as many grand-offspring, as a female reproducing asexually (Maynard-Smith 1978). Thus, unless there is a strong benefit to sexual reproduction, we would expect asexual organisms to quickly outcompete sexual organisms, but this is clearly not the case. One of the suggested benefits of sexual reproduction is that it results in genetic recombination, which accelerates host evolution and is therefore beneficial in the evolutionary arms race between hosts and parasites (Jaenike 1978; Hamilton 1980; Hamilton et al. 1990; Ebert and Hamilton 1996; Kersters et al. 2012). This hypothesis has been dubbed the Red Queen hypothesis, and it predicts that common host genotypes will be more susceptible to parasite infection due to parasite driven negative frequency-dependent selection. This prediction has been tested in multiple populations of the snail *Potamophyrgus antipodarum* and trematode parasites (*Microphallus* sp). Reciprocal cross-infection experiments find that parasites are locally adapted, and that this local adaptation is a result of increased parasite infectivity in the more common snail genotypes (Lively 1989; Lively and Dybdahl 2000). Experimental evolution of the snails and trematodes found that parasites became increasingly infective in the initially most common host genotype and the frequency of this genotype declined with time, indicating negative frequency-dependent selection (Koskella and Lively 2009). Direct sampling from wild populations of snails and trematodes also finds that infection prevalence is higher than expected in common genotypes for some lakes, but lower than expected in other lakes (Dybdahl and

Lively 1995). Populations also exhibited changes in host genotype frequencies over a five year period that were consistent with time lagged negative frequency-dependent selection (Nee 1989; Dybdahl and Lively 1998; Kaltz and Shykoff 1998) and thus provide support for the Red Queen hypothesis. Complementary results supporting the Red Queen hypothesis were also found in field studies of multiple sexual and asexual populations of topminnows (*Poeciliopsis monacha*), where the most common asexual fish genotypes were found to be more frequently infected with trematodes (*Uvulifer* sp) when compared to the most common sexual genotypes (Lively et al. 1990).

Another evolutionary insight derived from the study of local adaptation is that natural selection may act to increase the virulence of a parasite. The fact that parasites rely on their host for survival and transmission, as well as striking examples of highly virulent and recently introduced parasites (Smith 1904), originally led to the "conventional wisdom" (May and Anderson 1983) that parasites evolve to be less virulent to their hosts. Extensive theoretical work has since demonstrated that virulence should evolve to maximize parasite fitness, and therefore selection can maintain or increase virulence depending on aspects of host and parasite biology (Levin and Pimentel 1981; May and Anderson 1983; Bull 1994; Frank 1996; Alizon et al. 2008). This issue was addressed empirically in a local adaptation study of water fleas (*Daphnia magna*) infected with the protozoan parasite *Glugoides intestinalis* (formerly *Pleistophora intestinalis*), which is horizontally transmitted via spores shed in host feces prior to host mortality (Ebert 2005). The study found that geographic proximity was associated with increased infection probability, indicating local adaptation, as well as with increased spore production and virulence (Ebert 1994). Because parasites that were locally adapted

were also found to be more virulent, these results support that hypothesis that coevolved parasites can continue to be virulent in their hosts. Additional studies since Ebert (1994) have similarly found a positive relationship between virulence and transmission in a variety of systems (e.g. Paul et al. 2004; Salvaudon et al. 2005; Wickham et al. 2007).

Given that local adaptation studies provide a unique opportunity to test evolutionary hypotheses, it is not surprising that the field is substantive. The focus on host-parasite interactions is understandable, both because parasitism is an important and widespread source of selection and because host-parasite systems are well suited to the study of local adaptation. Arguably the most common approach to studying local adaptation is to use a laboratory-based reciprocal cross-infection experiment, with one meta-analysis finding over 54 studies that included this approach (Greischar and Koskella 2007). Although a great deal has been learned from reciprocal cross-infection experiments, they are common garden experiments and therefore require that local adaptation is determined entirely by the interaction between host and parasite genotype. As discussed in the following section, this can be a problematic assumption.

1.2 Environmental variability and host-parasite interactions.

For both hosts and parasites, there is extensive evidence that infection-related traits such as resistance and virulence can be genetically determined (Mead-Briggs and Vaughan 1975; Collins et al. 1986; Little and Ebert 1999; Mackinnon and Read 1999; Salvaudon et al. 2007). Infection phenotype (see figure 1.2) and ultimately the epidemiology of an infectious disease can therefore be determined by the interaction between host and parasite genotypes (Restif and Koella 2004; Fellous et al. 2012). Because heritability is a

prerequisite for natural selection to act on a trait, the well-supported assumption that there are genetic bases to infection traits is central to our understanding of host and parasite coevolution.

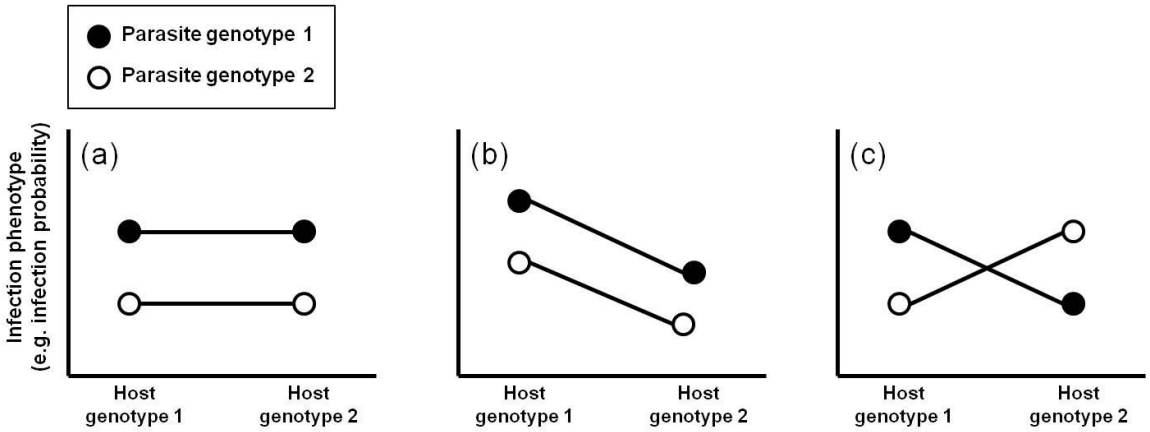

Figure 1.2 Infection phenotype determined (a) entirely by the parasite genotype, (b) both the host and parasite genotypes, or (c) the interaction between host and parasite genotypes.

Even basic models of infection depend on the assumption that infection outcome depends on the interaction between host and parasite genotypes. These models (depicted in figure 1.3) include gene-for-gene, where a given parasite genotype can infect any host unless the host possesses resistance alleles specific to the parasite genotype (Flor 1956), and matching alleles, where a given parasite genotype can only infect hosts with a specific, complementary set of alleles (Grosberg and Hart 2000).

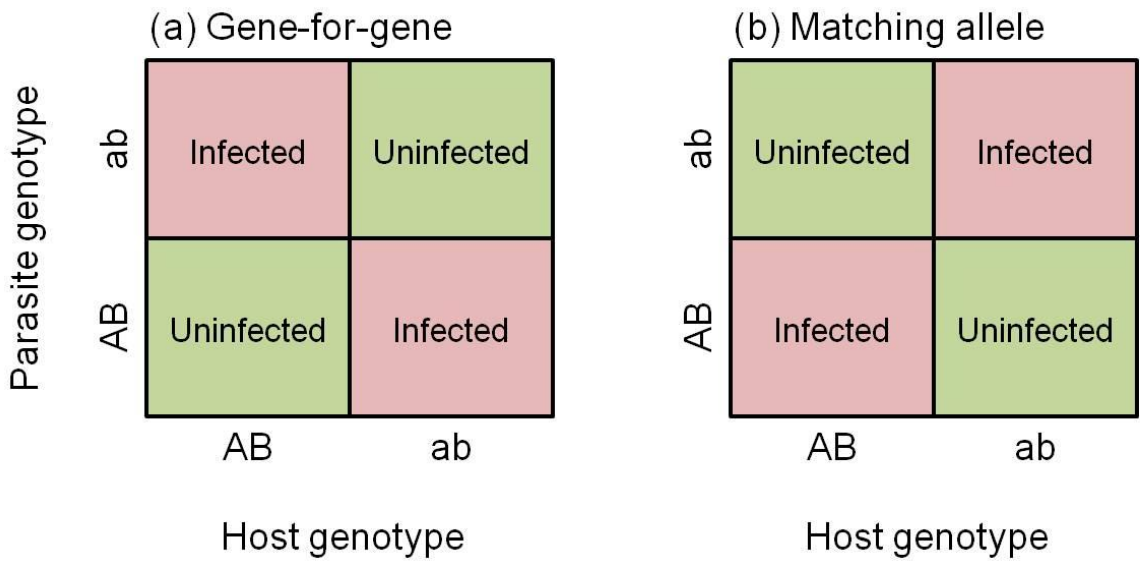

Figure 1.3 Basic models of infection include (a) gene-for-gene and (b) Matching allele.

Although gene-for-gene and matching alleles are treated as two distinct models, realistically they represent extremes on a continuum of genotypic interactions that determine infection probability (Agrawal and Lively 2002; Rolff and Siva-Jothy 2003).

While the importance of host and parasite genotypes in determining infection phenotype is indisputable, focusing on genotype captures only part of the variation in infection phenotype. In natural populations, environmental factors can also introduce variation, either through direct effects on infection phenotype or through interactions with host and parasite genotypes (figure 1.3).

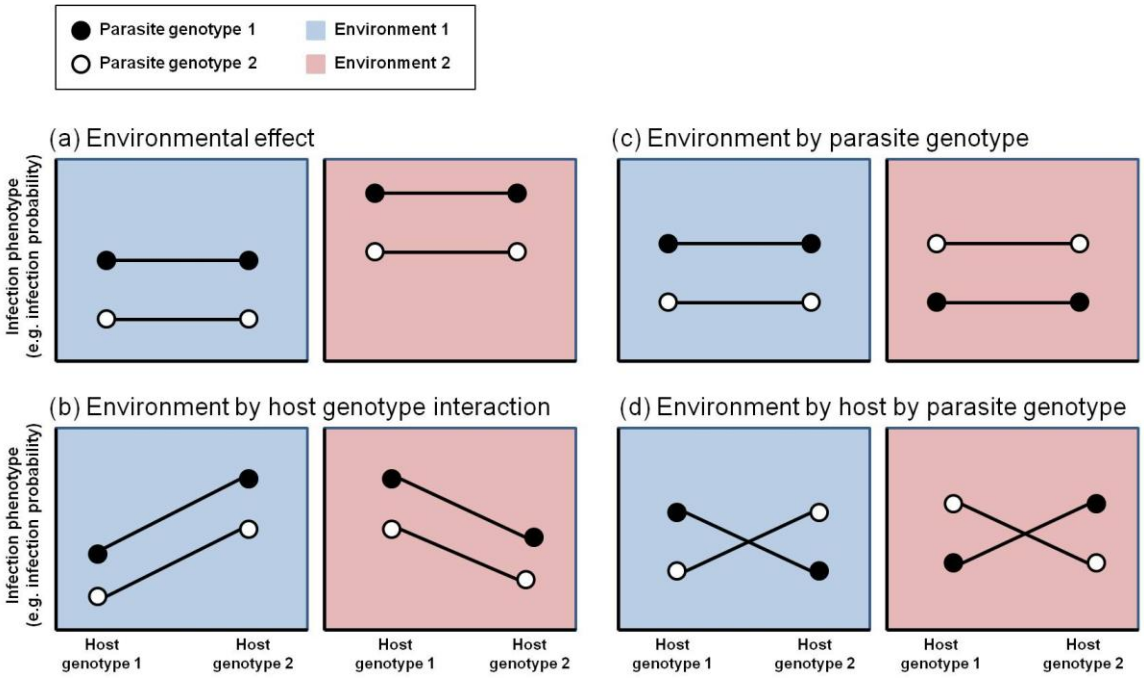

Figure 1.4 Interactions between host and parasite genotypes, and environment where (a) environment has an effect on the magnitude of infection phenotype for two parasite genotypes, but there is no interaction, (b) rank order of parasite genotype changes in different environments, indicating an interaction between parasite genotype and environment, (c) infection phenotype depends on host genotype and environmental conditions, but the rank order of parasite genotypes does not change indicating an interaction between host genotype and environment, and (d) environment modifies the interaction between host and parasite genotype.

Because environment can introduce phenotypic variation, it has the potential to alter selection, particularly if the relative fitness of different genotypes depends on the surrounding environment (Mostowy and Engelstädter 2011). For example, multiple studies of *Daphnia magna* infected with the sterilizing bacterial pathogen *Pasteuria ramosa* found evidence that the fitness of different host genotypes changed with temperature, and overall the fitness cost to the host from infection depended on the ambient temperature (Mitchell et al. 2005; Vale et al. 2008b; Vale et al. 2011). Even if an entire generation experiences constant environmental conditions, additional work in *D. magna* and *P. ramosa* has revealed a significant effect of maternal environment (Mitchell

and Read 2005; Hall and Ebert 2012). Such environmentally induced polyphenism implies that there is no universal "best genotype" and therefore, it provides one hypothesis for the maintenance of genetic polymorphisms in host and parasite populations (Byers 2005; Summers et al. 2007; Lazzaro and Little 2009; Wolinska and King 2009). The general ability of environment to maintain polymorphisms in a population has been demonstrated, for example in a long-term study of the Soay sheep of St. Kilda which found that genetic variance and selection changed with environmental conditions, and the selection differential on birth weight was negatively related to environmental quality (Wilson et al. 2006). Another study of the Soay sheep suggests that variation in environmental quality can specifically maintain heterogeneity in host immune response, as increased immune responsiveness was negatively associated with reproduction under normal conditions but positively associated with offspring survival under harsh conditions (Graham et al. 2010).

An alternative, but not mutually exclusive, hypothesis for the maintenance of genetic polymorphisms is that organisms experience tradeoffs, for example between host fecundity and immune maintenance as demonstrated empirically in a number of systems (e.g. Boots and Begon 1993; Kraaijeveld and Godfray 1997; Fellowes et al. 1998; Webster and Woolhouse 1999; reviewed in Zuk and Stoehr 2002; Schmid-Hempel 2005). A negative relationship between fecundity and immune maintenance is not always observed however (e.g. Lazzaro et al. 2008), and this can occur either because the testing environment is not appropriate for measuring such a trade-off, indicating that the trade-off is context dependent, or again because the environment itself is responsible for maintaining polymorphisms in resistance (Sandland and Minchella 2003).

The effect of temperature in the *Daphnia-Pasturia* system is not an isolated example, as there are a number of studies across a variety of taxa which find similar results (reviewed by Thomas and Blanford 2003). For example, recent studies have demonstrated the importance of temperature in mediating relative host and parasite fitness in the pea aphid *Acyrthosiphon pisum* and the fungal pathogen *Erynia neoaphidis* (Blanford et al. 2003), in chestnut blight (*Cryphonectria parasitica*) and the virus *Cryphonectria* hypovirus-1 (Bryner and Rigling 2011), and in the ciliate *Paramecium caudatum* and the bacterial pathogen *Holospora undulata* (Duncan et al. 2011). As suggested by these examples, a survey of the relevant literature makes it clear that temperature is one of the most commonly studied aspect of environmental variation (Wolinska and King 2009). The prominence of temperature in the literature is not surprising, given that temperature varies spatially and as well as temporally, and seasonal temperature fluctuations have already been studied extensively in relation to infectious disease epidemiology (reviewed by Altizer et al. 2006).

In addition to temperature, another frequently examined aspect of the environment is nutrient availability. One recent study in bumblebees (*Bombus terrestris*) infected with the trypanosome parasite *Crithidia bombi* found that glucose concentration had a significant effect on infection intensity and there was a significant, three-way interaction between food quality, host genotype, and parasite genotype (Sadd 2011). Similarly, when *Anopheles stephensii* mosquitoes were infected with the rodent malaria parasite, *Plasmodium chaubaudi* there was a significant interaction between parasite genotype and glucose availability in determining mosquito mortality (Ferguson and Read 2002a). Such results suggest that environment, in this case nutrient availability, could

explain why there are a number of conflicting reports regarding the virulence of *Plasmodium* in its mosquito vector and more broadly, explain why parasite virulence in a vector species remains an open question (Ferguson and Read 2002b).

Although these studies show the importance of environmental variation, in natural populations food quality is a complex function of species interactions and the wider ecosystem. For example, in the wild, bumblebees and mosquitoes do feed on plant nectars that provide glucose at varying concentrations, but these nectars can also contain different secondary metabolites that affect infection. One study found that secondary metabolites in nectar affected *C. bombi* infection in bumblebees (Manson et al. 2010). In general, hosts that eat plants and plant products (e.g. nectar and seeds) experience diverse and even conflicting effects from the nutritional content and the secondary metabolites present in their food (Singer et al. 2004; Haviola et al. 2007a).

The conclusion that infection occurs within an ecosystem can, and should, be extended beyond the interaction with food species. Given the vast number of parasites present on earth, all ecosystems will also include interactions between coinfecting parasite strains and species (Lafferty 2010). There can even be interactions between nutrient availability and the effect of coinfection, such as in the mosquito *Aedes aegypti* where the cost of being infected with both microsporidia and protozoa depends on the interaction between infectious doses and food availability (Fellous and Koella 2010). Even in the absence of any additional environmental variation, the outcome of infection in coinfected hosts is determined by at least three genotypes, and potentially more (Pedersen and Fenton 2006). In a study examining multiple strains of two species of fish eye flukes (*Diplostomum* sp), the relative benefit and cost of coinfection to one fluke

species was strongly affected by the genotypes of coinfecting species (Seppälä et al. 2009). Like the variation in host resistance observed at different temperatures, if the fitness of a parasite depends on the genotype of coinfecting parasites then coinfection can potentially maintain genotypic variation in parasite populations.

As a whole, the literature on environmental variation and parasitism serves as a precaution against oversimplification. Although isolating hosts and parasites does produce a more tractable experiment, it can also tell an incomplete story. Furthermore, as evolutionary biologists we are rightfully preoccupied with variation, because it provides the material on which natural selection can act. Because environmental factors introduce phenotypic variation, and could ultimately maintain genotypic variation, the role of environment in host-parasite interactions is of general interest and importance.

1.3 Monarch butterflies and *Ophryocystis elektroscirrha* as a model system

In this dissertation, local adaptation and environmental effects are studied in the monarch butterfly (*Danaus plexippus*). Monarchs are susceptible to infection by the protozoan parasite *Ophryocystis elektroscirrha* and transmission of the parasite is exclusively from adults to larvae (McLaughlin and Myers 1970). Transmission often occurs when females scatter spores on their eggs or the surrounding leaf, which serve as food for the newly hatched larvae (Altizer et al. 2004). When larvae ingest the spores, they lyse in the gut, penetrate the intestinal wall, and then undergo asexual followed by sexual reproduction in the larvae's hypodermal tissues. During the pupal stage, parasites form spores around the scales of the developing monarch such that when monarchs emerge as adults, they are covered with the dormant spores (figure 1.4).

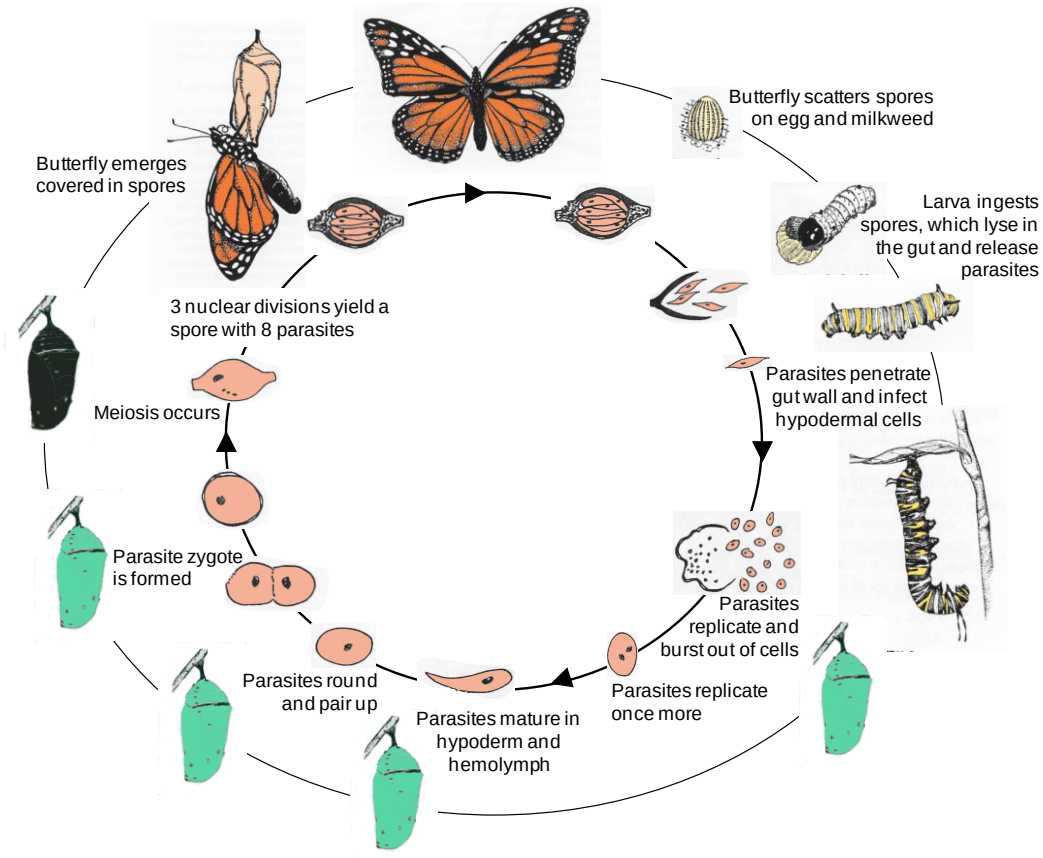

Figure 1.5 The life cycle of the monarch butterfly and the protozoan parasite *Ophryocystis elektroschirra*. Modified from figure created by Jacobus de Roode.

Monarchs and *O. elektroscirrha* share a broad distribution, including eastern and western North America, Hawaii, and south Florida (Altizer et al. 2000). The Hawaiian and south Florida monarchs are non-migratory, but the eastern North American monarchs are well known for their impressive annual migration to Mexico. The western monarchs also migrate, but in smaller numbers and over a shorter distance (Brower 1995). Microsatellite analysis has revealed that there is extensive gene flow between the eastern and western North American monarchs, despite their divergent migratory habits, and that the Hawaiian population is genetically isolated from the North American population

(Lyons et al. 2012). Parasite prevalence differs dramatically between these monarch populations, with less than 8% infection reported in eastern North American monarchs to greater than 70% in south Florida monarchs (Altizer et al. 2000). Like parasite prevalence, parasite virulence has been found to differ between isolates collected from eastern and western populations (De Roode et al. 2008b; De Roode and Altizer 2009), despite extensive host gene flow between these populations.

Because of their wide distribution and spectacular migration, monarchs are a well-known and charismatic species. Yet another component of the monarch's appeal is undoubtedly the striking coloration exhibit by both adults and larvae (figure 1.5).

Figure 1.6 An adult monarch (left) and second instar monarch larva (right).

These bright colors are aposematic and serve to advertise to potential predators that the monarchs are distasteful and potentially toxic (Rothschild 1973). The source of the monarch's toxicity is the cardiac active glycosides or cardenolides, which the monarchs sequester from their food plants (Brower and Glazier 1975; Malcolm and Brower 1989). Monarchs feed exclusively on plants in the family *Asclepiadaceae*, commonly referred to as milkweeds, but this is not a uniform group of plants with milkweed species differing

extensively in their geography and in the concentration, polarity, and diversity of cardenolides that they produce. Different milkweed species are available to different monarch populations and in North America alone, monarchs are known to feed on at least 27 different species of milkweed (Woodson 1954; Ackery and Vane-Wright 1984; Hickman 1993). The cardenolides present in a monarch's tissues are specific to the plant on which it feeds during larval development, but monarchs can preferentially sequester the cardenolides such that the relationship between monarch cardenolides and milkweed cardenolides is not necessarily linear (Malcolm and Brower 1989).

As a natural host-parasite system, there are a number of relevant ecological factors that affect the interaction between monarchs and *O. elektroscirrha*. For monarchs, as with other herbivorous insects, food plant is generally an important environmental factor (reviewed in Cory and Hoover 2006), and there is prior evidence that food plant species exerts an effect on infection in the monarch-parasite system. Research focused on two species of milkweed, *Asclepias curassavica* (tropical milkweed) and *Asclepias incarnata* (swamp milkweed), found that infected monarchs inoculated and reared on *A. curassavica* had reduced parasite growth compared to infected monarchs inoculated and reared on *A. incarnata* (De Roode et al. 2008a). Moreover, *A. curassavica* consumed exclusively post-infection did not have an effect on parasite growth, indicating that consumption at the time of infection is necessary for food plant species to have an effect (De Roode et al. 2011a).

Coinfection is also important in this system, for example the parasitoid fly *Lespesia archippivora* commonly occurs in monarch populations including in North American and Hawaii with varying prevalence (Etchegaray and Nishida 1975;

Oberhauser et al. 2007). *L. archippivora* can strongly affect the fitness of both monarchs and the protozoan *O. elektroscirrha*, as it kills the monarch prior to adulthood, and thus prior to transmission of *O. elektroscirrha* (Prysby 2004). Overall, the widespread, between-population variation in ecology (e.g. available food plant species and coinfection prevalence) makes this system well suited for detailed studies of how environment affects infection, which may ultimately serve to explain observed variation in infection traits between populations.

Chapter 2

Patterns of infection probability, virulence, and parasite burden in three populations of monarch butterflies and a naturally occurring protozoan parasite

Introduction

The concept of geographic variation in natural selection is longstanding in evolutionary biology (Williams 1966), as is the understanding that selection is modulated by the environment (Haldane 1946; Falconer 1952). Comparing geographically isolated populations experiencing different environmental conditions, and thus different selective pressures, provides a uniquely tractable approach for studying adaptation (Reznick and Ghalambor 2001). Adaptation to the local environment has been predicted and demonstrated empirically in many populations, and these studies of local adaptation have informed our understanding of diverse and important topics from life history trait evolution (Reznick and Endler 1982; Reznick et al. 1997) to the maintenance of sexual reproduction (Ebert and Hamilton 1996; Lively and Dybdahl 2000).

While local adaptation studies are undoubtedly important in evolutionary biology, experimental design is crucial for interpreting the results. Common garden experiments are conducted under uniform conditions, such as in a laboratory or a greenhouse, and the fitness of organisms is measured in response to a single factor. This approach is often used to study local adaptation in species interactions, where local adaptation is expected to be driven primarily by variation in such interactions (Nuismer and Gandon 2008). Reciprocal cross-infections are essentially common garden experiments specifically using host and parasite species. Such experiments occur frequently in the local adaptation

literature because the conditions necessary for local adaptation are likely to be met in the host-parasite interaction (Kawecki and Ebert 2004; Greischar and Koskella 2007). These studies have yielded a variety of results, including increased infectivity in sympatric host-parasite combinations suggestive of local adaptation (Lively 1989; Koskela et al. 2000; Laine 2005). Other studies have found decreased infectivity in sympatric combinations, which has been referred to as parasite maladaptation (Imhoof and Schmid-Hempel 1998; Kaltz et al. 1999; Oppliger et al. 1999). This could also represent host local adaptation but, because parasites are typically thought to have an evolutionary advantage over hosts, local adaptation tends to be examined from the perspective of the parasites. A third possible result is no pattern of local adaptation (Dufva 1996; Mutikainen et al. 2000; Prugnolle et al. 2006). The absence of local adaptation has spurred extensive discussion and theory, which has primarily focused on aspects of population genetics that impact host and parasite coevolution (Gandon et al. 1996; Gandon et al. 1998; Gandon and Michalakis 2002; Kawecki and Ebert 2004). An alternative, but not mutually exclusive, hypothesis is that environmental variation drives more complex patterns of local adaptation (Kaltz and Shykoff 1998); however, this hypothesis is much less well developed both theoretically and empirically.

In addition to experimental design, another crucial aspect of testing for local adaptation in host-parasite systems is choosing appropriate measures of infection outcome. Most experiments focus on infection probability, because higher infection probability is necessarily positively correlated with parasite fitness and usually negatively correlated with host fitness. A measure such as virulence (i.e. the damage a parasite causes to its host) is more difficult to interpret in relation to parasite fitness (Dybdahl and

Storfer 2003). Increased virulence may be adaptive for some parasites, but this depends on the relationship between virulence and parasite transmission. For example, castrating or obligate killer parasites transmit only from sterile or dead hosts and therefore experience maximum fitness with maximum virulence (Ebert et al. 2004). In contrast, parasites that transmit from living hosts over a number of days may obtain highest fitness at an intermediate level of virulence. This is because parasite replication within the host does increase the number of transmissible stages of the parasite but it also damages the host on which the parasite relies. As a result, parasites may experience a trade-off between the costs of damaging their host (i.e. being virulent) and the increased transmission (Levin and Pimentel 1981; May and Anderson 1983; Read 1994; Frank 1996; Alizon et al. 2008). Such a trade-off has been supported empirically in a small number of studies, including myxomatosis in rabbits (Bolker et al. 2010), HIV-1 in humans (Fraser et al. 2007), and protozoan parasites in monarch butterflies (De Roode et al. 2008b).

Like virulence, attempting to identify local adaptation on the basis of parasite burden (a measure of within-host replication) is difficult because of the complex relationship between within-host replication, virulence, and parasite fitness. Importantly, parasite burden is generally a shared trait of both the parasite and the host (Restif and Koella 2003; Lambrechts et al. 2006) and thus, may reveal insights into local adaptation of the host as well at the parasite. Decreased parasite burdens in sympatric hosts may not indicate parasite maladaptation but rather, host local adaptation in the ability to limit parasite growth (i.e. quantitative resistance). Such quantitative host resistance can select for increased parasite virulence. (Gandon and Michalakis 2000; Gandon et al. 2001; De

Roode et al. 2011a). Because of its importance in host-parasite coevolution, a number of studies have attempted to measure local adaptation in quantitative resistance, despite the difficulty in interpreting results (Imhoof and Schmid-Hempel 1998; Koskela et al. 2000; Mutikainen et al. 2000; McCoy et al. 2002).

When experiments fail to find local adaptation in either qualitative or quantitative resistance, another possible explanation is that hosts have evolved to tolerate their local parasites. Tolerance encompasses host defense mechanisms that limit the costs of parasitism without limiting the infection itself, which is distinct from resistance where hosts limit parasite replication and transmission (Boots 2008; Schneider and Ayres 2008; Råberg et al. 2009; Svensson and Råberg 2010; Baucom and De Roode 2011; Medzhitov et al. 2012). Such a distinction is crucial, because resistance and tolerance have divergent effects on host-parasite coevolution. Tolerance does not have a negative effect on parasite fitness and therefore, theory predicts that tolerant hosts will increase parasite transmission which will in turn increase selection for host tolerance, creating a positive feedback that will ultimately fix tolerance in the population. In contrast, resistance decreases parasite transmission which reduces selection for resistance, which is assumed to be costly to the host. With decreasing resistance in a population, parasite prevalence should increase and ultimately lead to cycling in host resistance and parasite prevalence (Roy and Kirchner 2000; Miller et al. 2006; Boots 2008). Despite the important distinction between disease resistance and tolerance, both serve to protect the host from the cost of parasitism. Hosts should not invest resources in redundant, and costly, defense mechanisms and therefore, a trade-off between resistance and tolerance is expected (Baucom and Mauricio 2008; Castella et al. 2008; Simone et al. 2009). Such a trade-off has been demonstrated in some

studies of tolerance (Fineblum and Rausher 1995; Råberg et al. 2007), but not all (Carr et al. 2006; Lefèvre et al. 2011). Generally, tolerance is a well-established concept in the study of plant-parasite and plant-herbivore interactions, but it is a relatively new concept in the study of animal-parasite interactions. With the growing recognition that tolerance is an important mechanism of host defense in animals (Corby-Harris et al. 2007; Råberg et al. 2007; Ayres and Schneider 2009; Blanchet et al. 2010; Rohr et al. 2010; Lefèvre et al. 2011; Soler et al. 2011; Sternberg et al. 2012), it is becoming clear that studies of local adaptation should address tolerance as well as resistance.

In this study, we investigate local adaptation using four different infection traits, including tolerance, at a population level in monarch butterflies infected with the naturally occurring protozoan parasite *O. elektroscirrha*. The populations we focus on are the non-migratory Hawaiian and South Florida populations, and the eastern North American population famous for its annual, long-distance migration to overwintering sites (Urquhart and Urquhart 1976; Brower 1995). Analysis of microsatellite markers has established that the Hawaiian and eastern North American populations are genetically distinct (Lyons et al. 2012). Chemical analysis of S. Florida monarchs found that the majority of monarchs sampled had fed on a S. Florida food plant species as larvae, while a small number of monarchs sampled in the fall had fed on a northern plant species (Knight and Brower 2009). These results strongly suggest that S. Florida monarchs constitute a stable, continually-breeding population that occasionally incorporates eastern North American individuals during their annual, southward migration. Additional analysis of wing morphology supports phenotypic differentiation between the non-migratory South Florida monarchs and the migratory eastern North American monarchs

(Altizer and Davis 2010). These populations are also known to differ dramatically in parasite prevalence, typically ranging from less than 8% in eastern North America to greater than 70% in the S. Florida population (Altizer et al. 2000).

Monarchs can only become infected by the parasite as larvae, when they consume dormant spores that have been passively transferred by their mother on their egg shells or the surrounding food plant. These spores lyse in the gut, penetrate the intestinal wall, and then undergo asexual followed by sexual reproduction in the hypoderm. Parasites form spores around the scales of the developing butterfly during the pupal stage so that when monarchs emerge as adults, they are covered with dormant spores (McLaughlin and Myers 1970). Because parasites do not replicate in adult monarchs, quantifying the spore load of a monarch butterfly provides a measure of the total within-host replication of the parasites. Increasing parasite spore load has previously been associated with decreasing adult longevity, as well as other measures of host fitness, and there is evidence of a trade-off between virulence and transmission in this system (De Roode et al. 2008b). Because of their relevance to host and parasite fitness, we have quantified adult longevity and spore load in addition to infection probability and tolerance in our three populations. To test for local adaptation, we have examined main effects of host and parasite source populations, as well as the interaction between populations.

Methods and Methods

Host and Parasite Source

In the fall of 2009, adult and larval monarchs were collected from three Hawaiian Islands (Oahu, Kauai, and Maui). Adult monarchs were also collected from the eastern

North American population during their annual migration through St. Marks, Florida. In the fall of 2010, adults were again collected from the eastern North American population in St. Marks, Florida. Until S. Florida monarchs could be collected, in the spring of 2011, eastern monarchs were maintained under temperature and light conditions consistent with those found at Mexican overwintering sites. The non-inbred progeny of these wild-caught monarchs were used for experiments.

Ten clonal lines from the eastern North American population, six clonal lines from the Hawaiian population, and five clonal lines from the Miami population were used in the experiments. The name, collection site, and date of each clonal line are provided below in table 1.

Eastern population	**Origin**	**Date collected**
E14	St Marks, FL	10/2009
E17	St Marks, FL	10/2009
E18	St Marks, FL	10/2009
E20	St Marks, FL	10/2009
E21	St Marks, FL	10/2009
E23	St Marks, FL	10/2010
E25	St Marks, FL	10/2010
E26	St Marks, FL	10/2010
E27	St Marks, FL	10/2010
E28	St Marks, FL	10/2010
Hawaiian population	**Origin**	**Date collected**
H13	Maui, HI	11/2009
H15	Hawaii	11/2009
H17	Oahu, HI	11/2009
H18	Hawaii	11/2009
H19	Kauai, HI	11/2009
H20	Kauai, HI	11/2009
South Florida population	**Origin**	**Date collected**
F18	Miami, FL	4/2011
F19	Miami, FL	4/2011
F20	Miami, FL	4/2011
F21	Miami, FL	4/2011
F22	Miami, FL	4/2011

Table 1 Site and date of collection

The parasite clones used in the experiments were generated from isolates taken from infected, wild-caught adult monarchs collected at the same time as the parental monarch lineages. Individual haploid spores from the infected monarchs were used to inoculate larvae in the laboratory, creating single genotype infections. The larvae were reared to adulthood and then used as a source of clonal parasite lineages for the experimental inoculations (De Roode et al. 2007; De Roode et al. 2009).

Experimental design

Two reciprocal cross-infection experiments were carried out: the first compared eastern North American and Hawaiian populations (2010) and the second compared eastern North American and S. Florida populations (2011). In both experiments, nine replicate larvae from each family line were infected with one of the parasite clones. For every nine infected larvae, one uninfected larvae from the same family was also reared as a control. In the first experiment, there were ten monarch family lines and eleven clonal parasite lines, plus an extra 61 individuals from a sixth family line (N = 1161) and in the second experiment, there were eight monarch family lines and ten clonal parasite lines (N = 800).

For both experiments, mated females were provided with greenhouse-grown *Asclepias incarnata* (swamp milkweed) for oviposition. Hatching larvae were removed from the plants and pooled by hatching date. Two days after hatching, larvae were placed in individual Petri dishes containing moist filter paper and an *A. incarnata* leaf disc on

which 10 parasite spores had been manually deposited (De Roode et al. 2007; De Roode et al. 2008a). Control larvae were provided with clean leaf discs. After consuming their leaf discs, larvae were reared individually in 1L plastic containers, maintained at 26 ºC on a 16 L: 8D light cycle, and fed fresh greenhouse-grown *A. incarnata* cuttings as needed until pupation.

One week after pupation, the pupae were transferred into a separate room maintained under the same conditions to prevent transmission of spores from emerging adults to larvae. Pupae were checked daily for discoloration indicative of parasite infection (De Roode et al. 2009). Upon emergence, adult monarchs were transferred into individual glassine envelopes and maintained at 14 ºC. To measure the difference between emergence and death date (referred to as adult longevity), monarchs were checked daily. This provided a combined measure of longevity and starvation resistance, which correlates with the effect of parasitism on monarch longevity under more natural conditions (De Roode et al. 2009).

After the monarchs died, infection status was confirmed and parasite burden quantified by vortexing the bodies in 5 mL of water to shake off parasite spores. These spores were then counted using a hemocytometer (De Roode et al. 2007; De Roode et al. 2008b). Because spores do not replicate after the adult monarch emerges, spore load provides a measure of lifetime parasite replication and can serve as a measurement of parasite fitness.

Statistical analysis

We began our analysis by testing for local adaptation in the three populations of hosts and parasites. Logistic regression by generalized linear mixed model (GLMM; binomial errors, logit link) was used to assess the effect of parasite inoculation on larval survival, with inoculation as a fixed effect and monarch and parasite lineages as random effects. GLMM with binomial errors were also used to assess the effect of monarch and parasite source population on infection probability, with monarch and parasite populations as a fixed effect and monarch and parasite lineages as random effects. A significant interaction between monarch and parasite population would indicate local adaptation. GLMM with normal error distributions were used to assess the effect of population on monarch adult longevity (a measure of host fitness) and parasite spore load (a measure of parasite burden). For the subset of data that represented only sympatric infections, we used an ANOVA to test whether monarch and parasite lineages differed significantly in adult monarch longevity and parasite spore load.

For each of the four host populations, we measured tolerance as the slope of a regression line between spore load and monarch adult longevity (Mauricio et al. 1997; Simms 2000; Råberg et al. 2009; Lefèvre et al. 2011; Sternberg et al. 2012). Using a linear mixed effects model with monarch population as a fixed effect and lineage as a random effect, a significant interaction between population and parasite spore load indicates variation in tolerance. An additional analysis was carried out including sympatry status (yes/no) as a fixed effect. A significant interaction between sympatry status and spore load indicates local adaptation in tolerance. Uninfected control monarchs were included in this analysis to distinguish between tolerance and general vigor, but monarchs that were inoculated but failed to become infected were excluded. We also

included a quadratic term for parasite burden to test a possible non-linear relationship between parasite burden and host fitness, as suggested in previous work (Tiffin 2000; Råberg et al. 2007; Blanchet et al. 2010).

All analyses were carried out in R v. 2.7.1. The lme4 package was used for all mixed effect models. Minimal models were derived by step wise model simplification followed by model comparison. Terms were retained in models if their removal significantly ($p < 0.05$) reduced the explanatory power of the model. Throughout our analyses, variables were transformed as necessary to ensure compliance with model assumptions (Crawley 2007).

Results

Eastern North America and Hawaii comparison (2010)

In the eastern/Hawaii comparison, 725 monarchs survived to adulthood. There was no significant effect of inoculation on the probability of monarchs surviving to adulthood (GLMM with binomial error distribution; $\chi^2 = 2.3$, d.f. = 1, $p = 0.129$). Because the transmissible life stage of the parasite is only present in adult monarchs, and because the costs of infection are only evident in adult monarchs, we restricted all further analysis to monarchs that survived to adulthood (De Roode et al. 2007; De Roode et al. 2009).

For the inoculated monarchs, particularly those inoculated with Hawaiian parasite, infection was high overall (figure 1a; eastern monarchs: 95% infection with Hawaiian parasite compared to 89% infection with eastern parasite; Hawaiian monarchs: 82% infection with Hawaiian parasite compared to 53% infection for the eastern parasite). This demonstrates that infectiousness and resistance was highest in the Hawaiian parasite

and host populations respectively. Overall, there was a significant effect of host (figure 1; GLMM with binomial error distribution; $\chi^2 = 19.0$, d.f. = 1, $p < 0.001$) and parasite ($\chi^2 = 9.08$, d.f. = 1, $p = 0.003$) source populations on the probability of infection. We did not find a significant interaction between monarch and parasite populations.

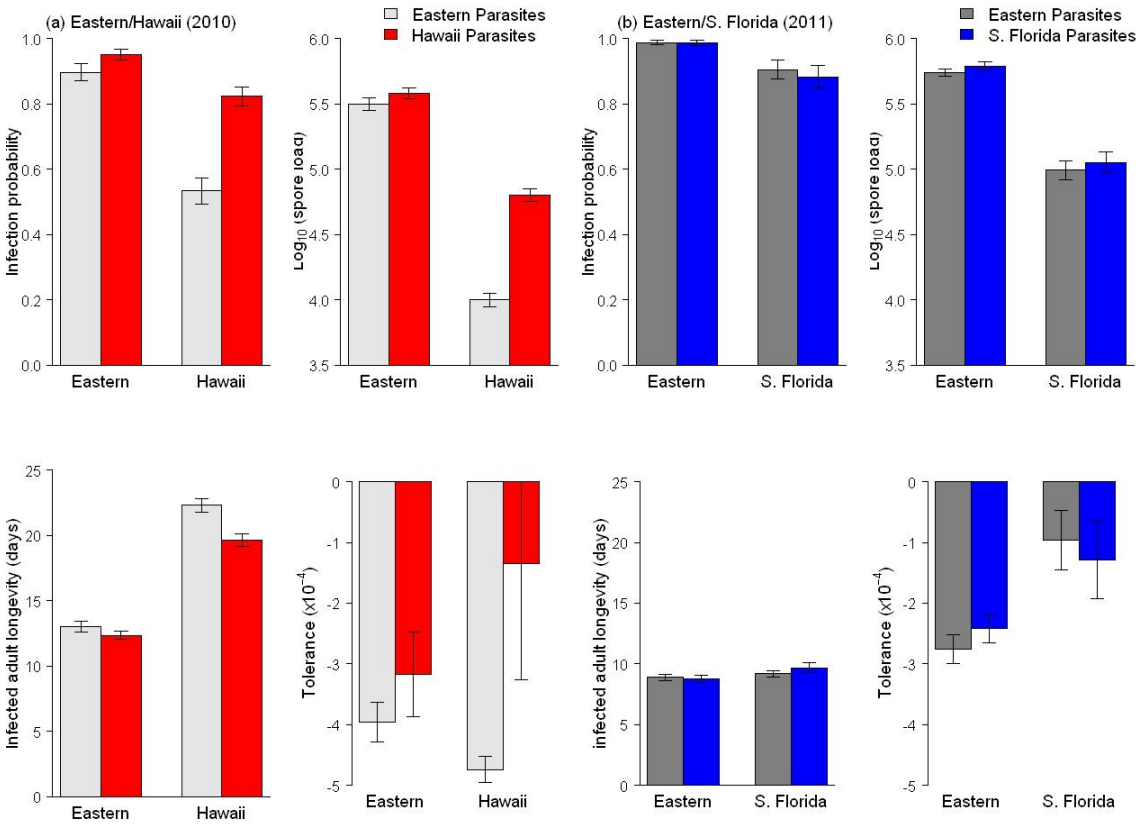

Figure 1 Infection probability, parasite spore load, monarch adult longevity and tolerance in (a) eastern/Hawaii comparison and (b) eastern/S. Florida comparison. Note that tolerance measures the slope of a regression line between parasite spore load and monarch adult longevity. Thus a value closer to zero indicates a shallower slope and greater tolerance.

Of the monarchs that became infected (N = 513), there was again a significant effect of monarch population (figure 1a; GLMM with normal error distribution; $\chi^2 = 34.5$, d.f. = 1, $p < 0.001$) and parasite population ($\chi^2 = 14.9$, d.f. = 1, $p < 0.001$) on spore load. There

was also a significant interaction between monarch and parasite source populations (χ^2 = 58.0, d.f. = 1, $p < 0.001$). In addition to being more infectious, Hawaiian parasites also showed higher mean spore loads, indicating increased within-host replication. In Hawaiian monarchs mean $\log_{10}$(spore load) was 4.80 ± 0.05 (mean ± standard error) when infected with Hawaiian parasites, compared to 4.00 ± 0.05 when infected with eastern parasites. Mean $\log_{10}$(spore load) was highest in eastern monarchs (5.58 ± 0.04 when infected with Hawaiian parasite; 5.50 ± 0.05 when infected with eastern parasite), indicating that eastern monarchs suffer greater parasite burdens when infected.

Our results for monarch adult longevity were largely consistent with our results for spore load, given that there is a well-established negative relationship between parasite burden and host fitness in this system. In addition to increased infection probability and increased spore loads, monarchs infected with Hawaiian parasites had decreased longevities when compared to monarchs infected with eastern parasites (figure 1a). The more resistant Hawaiian monarchs had a mean longevity of 19.6 ± 0.45 days when infected with Hawaiian parasites, compared to 22.3 ± 0.52 days when infected with eastern parasites. There was a significant effect of both monarch and parasite source population on adult longevities (χ^2 = 18.7, d.f. = 1, $p < 0.001$ and χ^2 = 10.6, d.f. = 1, p = 0.001 respectively). We also found a significant interaction between monarch and parasite populations (χ^2 = 6.57, d.f. = 1, p = 0.010).

When we restricted our analysis to the subset of monarchs infected with sympatric parasites, we found evidence of both host and parasite lineage effects on parasite spore load and monarch adult longevity in the Hawaiian populations. For spore load, we found a significant effect of both monarch and parasite lineage (figure 2; $F_{5, 114}$ = 2.60, p =

0.0286 and $F_{5, 114} = 2.59$, $p = 0.0293$ respectively. We also found a significant interaction ($F_{20, 114} = 1.74$, $p = 0.0371$) between monarch and parasite lineages, indicating a host genotype by parasite genotype (G_h x G_p) interaction. For monarch adult longevity, we found a significant effect of monarch lineage ($F_{5, 114} = 8.31$, $p < 0.001$) but we did not find a significant effect of parasite lineage. In the eastern population we found no significant effects of lineages on either adult longevity or spore load.

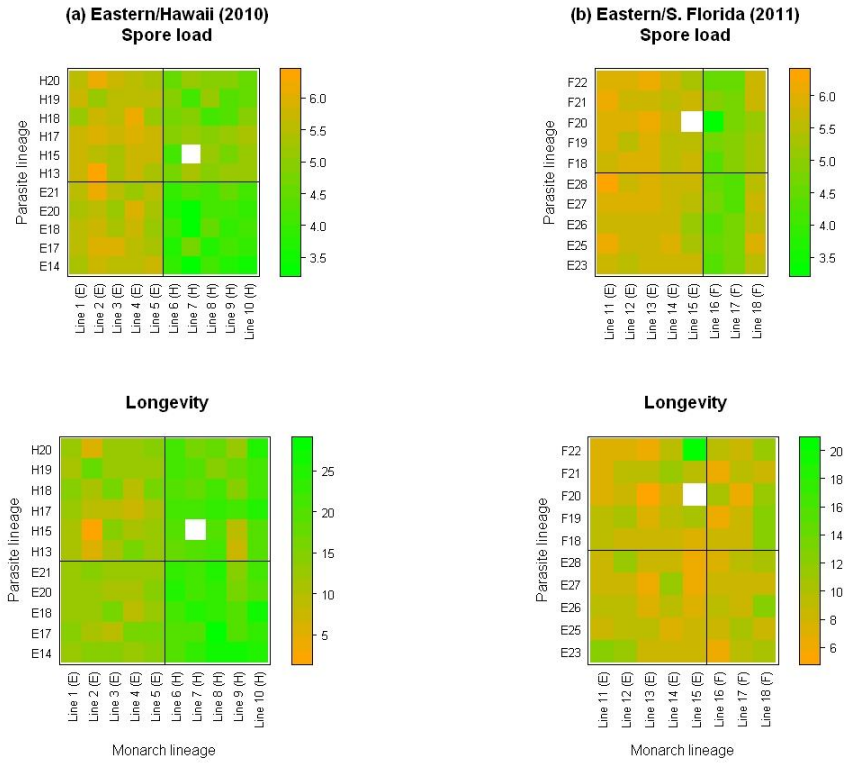

Figure 2 Heat map showing grid of host and parasite genotypes for infection probability, parasite burden, and adult longevity in (a) eastern/Hawaii comparison and (b) eastern/S. Florida comparison. Each square indicates mean longevity or spore load for the given parasite and host lineage combination. The white square indicates a parasite/host lineage combination where no monarchs survived to adulthood. Lines indicate division between source populations.

Lastly, we examined the relationship between spore load and host fitness (i.e. tolerance) in the two populations of monarchs. We found a significant interaction between spore load and monarch populations (figure 3a; $\chi^2 = 5.23$, d.f. = 1, p = 0.022), as well as spore load and parasite populations ($\chi^2 = 6.16$, d.f. = 1, p = 0.013). When we examined the same relationship in sympatric versus allopatric infections we found no significant interaction (figure 3b; $\chi^2 = 0.06$, d.f. = 1, p = 0.810), indicating that monarchs inoculated with sympatric parasites were no more or less tolerant than monarchs inoculated with allopatric parasites.

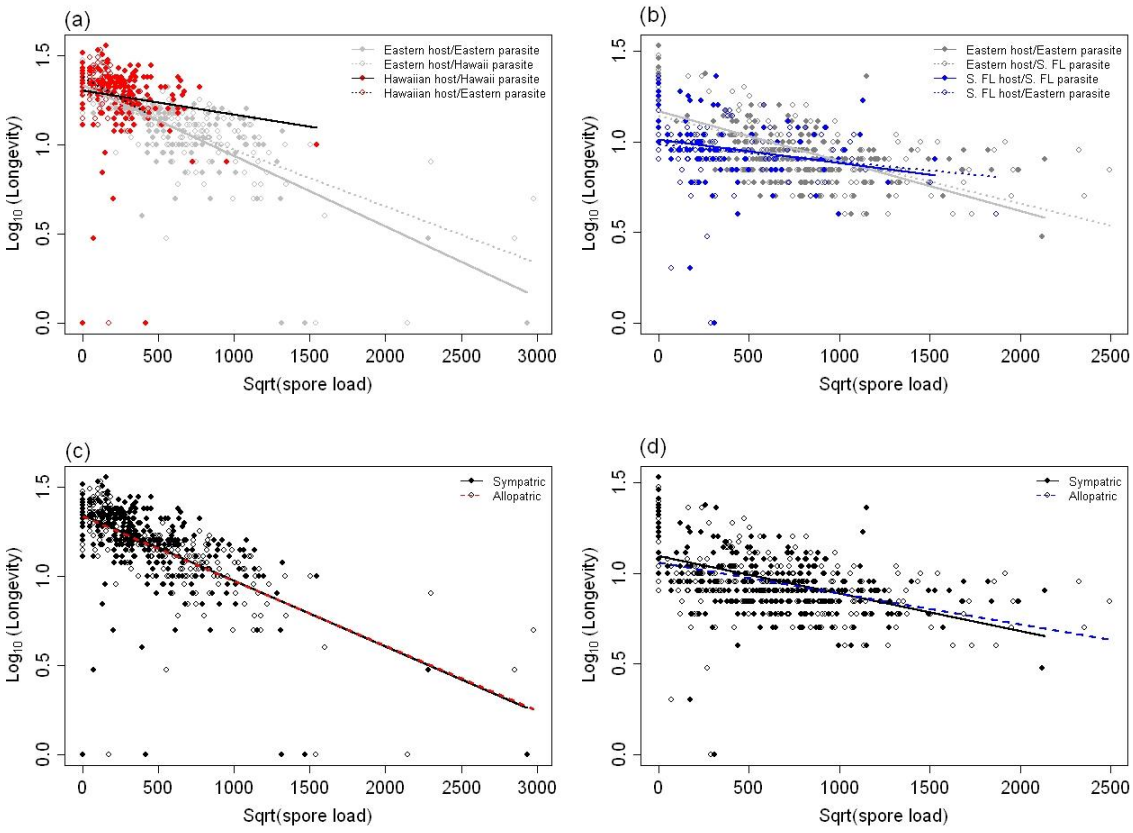

Figure 3 Regression of spore load and longevity in (a) eastern North America and Hawaii populations, (b) eastern North America and S. Florida populations, (c) combined sympatric versus allopatric infections in eastern North American and Hawaii monarchs, (d) combined sympatric versus allopatric infections in eastern North American and S. Florida monarchs.

Eastern North American and S. Florida comparison (2011)

In the eastern/S. Florida comparison, 586 monarchs survived to adulthood. There was no significant effect of inoculation on monarch survival (GLMM with binomial error distribution; $\chi^2 = 0.002$, d.f. = 1, p = 0.969). Further analysis was again restricted to the monarchs that survived to adulthood.

Infection probability was high for all inoculated monarchs (infected N = 505), ranging from 88% in S. Florida monarchs infected with S. Florida parasites to 99% in eastern monarchs infected with eastern parasites. There was a significant effect of monarch source population on susceptibility to infection ($\chi^2 = 7.16$, d.f. = 1, p = 0.007), with S. Florida monarch being more resistant to infection than eastern North American monarchs; however, there was no significant effect of parasite population nor an interaction between host and parasite population. These results indicate that, while monarch populations differ in susceptibility to infection, this does not depend on the parasite source population. Such results indicate an absence of local adaptation in hosts and parasites.

For infected monarchs, mean $\log_{10}$(spore load) ranged from 5.79 ± 0.03 in eastern monarchs infected with S. Florida parasites to 4.99 ± 0.07 in S. Florida monarchs infected with eastern parasites. As with infection probability, there was a significant effect of monarch source population ($\chi^2 = 8.03$, d.f. = 1, p = 0.005) but there was no significant effect of parasite source population. Thus, monarchs from the S. Florida population appear to experience more limited parasite growth (i.e. they are more resistant), but this is not dependent on the parasite source population.

Notably, despite the effect of monarch population on parasite spore load, neither monarch nor parasite source population had a significant effect on monarch adult longevity ($\chi^2 = 0.3044$, d.f. = 1, $p = 0.5812$ and $\chi^2 = 0.9157$, d.f. = 1, $p = 0.6326$ respectively). When we restricted our analysis to sympatric infections, monarch lineage had a significant effect on longevity in both S. Florida (Figure 2; $F_{2, 61} = 6.76$, $p = 0.002$) and eastern populations ($F_{4, 150} = 4.58$, $p = 0.002$). In the eastern population, there was also a significant effect of parasite lineage on longevity ($F_{4 ,150} = 3.47$, $p = 0.01$) as well as a significant interaction between monarch and parasite lineage ($F_{16, 150} = 1.80$, $p = 0.035$). In the S. Florida population, monarch lineage again had a significant effect on parasite spore load ($F_{2, 61} = 24.6$, $p < 0.001$), as did monarch and parasite lineage in the eastern population ($F_{4, 150} = 4.78$, $p = 0.001$ and $F_{4, 16} = 2.45$, $p = 0.048$ respectively). There were no significant interactions in the analysis of spore load.

When we examined tolerance in the S. Florida and eastern populations from 2010, we did not find a significant interaction between spore load and monarch population ($\chi^2 = 0.224$, d.f. = 1, $p = 0.224$) nor between spore load and parasite population ($\chi^2 = 0.128$, d.f. = 1, $p = 0.720$) indicating no differences in tolerance. An analysis examining differences between sympatric and allopatric infections similarly revealed no significant interaction between spore load and infection type (figure 3d; $\chi^2 = 0.711$, d.f. = 1, $p = 0.128$). This is consistent with the results presented for the Hawaiian and eastern comparison, suggesting that monarchs are not better or worse at tolerating infection by a local parasite.

Discussion

Based on infection probabilities and parasite burdens, we found that Hawaiian monarchs were more resistant to infection when compared to eastern North American monarchs. Theory predicts that when hosts reduce within-host parasite replication, this will select for increased virulence in coevolving parasites (Gandon and Michalakis 2000; Gandon et al. 2001; De Roode et al. 2011a). Consistent with this prediction, we found that Hawaiian parasites were more virulent in both Hawaiian and eastern hosts when compared to eastern parasites. We also found that in the Hawaiian monarchs, Hawaiian parasites were more infective than eastern parasites. This suggests that the Hawaiian parasites have become adapted to the more resistant, local hosts. However, because the Hawaiian parasites were also more infective in eastern monarchs, these results are not strictly consistent with the common definition of local adaptation (Kawecki and Ebert 2004). To address whether monarchs were locally adapted in terms of tolerance, instead of resistance, we also examined differences in reaction norms for parasite burden and host fitness in sympatric and allopatric infection (i.e. tolerance). We found that Hawaiian monarchs were more tolerant of infection than eastern monarchs, which suggests that populations can be both highly resistant and tolerant. We did not find an interaction with sympatry, which suggests that monarchs are no more tolerant of local parasites.

In the comparison between eastern North American and S. Florida populations we again found that S. Florida monarchs were more resistant than eastern North American hosts, but we found no differences between parasite populations. This indicates that, unlike the Hawaiian population, increased resistance in the S. Florida monarchs has not selected for increased parasite virulence. In addition, we did not find a significant difference in tolerance between populations. This is notable, because of the high infection

prevalence in the S. Florida population (Altizer et al. 2000). Theory predicts that, because of the positive feedback between tolerance and parasite prevalence, high parasite prevalence should be associated with greater tolerance (Roy and Kirchner 2000; Miller et al. 2006). One explanation for this inconsistency is that gene flow between the eastern North American and S. Florida populations could prevent parasite local adaptation to increased host resistance in the S. Florida population, or host local adaptation to increased parasite prevalence. While there is evidence of occasional monarch migration into S. Florida from eastern North American (Knight and Brower 2009), further work directly measuring admixture in the parasite population will be necessary to address this hypothesis.

Across all populations, the absence of a strong signal of local adaptation is not unusual. Indeed, one meta-analysis of local adaptation in host-parasite systems found a lack of local adaptation or maladaptation in 30 of 54 studies (Greischar and Koskella 2007). Our results are also consistent with a previous study comparing eastern and western North American populations (De Roode and Altizer 2009), which found no evidence of local adaptation. Recent genetic analysis has found that the eastern and western North American monarchs represent a single, admixed population (Lyons et al. 2012) and thus the absence of local adaptation in De Roode and Altizer (2009) may have been a result of extensive gene flow between populations. Hawaiian and eastern North American monarchs were found to be genetically distinct, however, and therefore it is unlikely that homogenizing gene flow is preventing local adaptation in these populations. Alternatively, it may be parasite migration rate relative to host migration rate that is not conducive to local adaptation. While very high migration rates can homogenize

populations, lower levels of migration can introduce and maintain genetic variation which in turn increases the evolutionary potential of parasites and allows for local adaptation (Gandon et al. 1996; Gandon et al. 1998; Lively 1999; Gandon and Michalakis 2002; Morgan et al. 2005; Hoeksema and Forde 2008). The importance of relative migration rate has been demonstrated previously, for example through mathematical models and experimentally in the bacterium *Pseudomonas fluorescens* infected with bacteriophage, where higher relative migration in the parasites was necessary for parasite local adaptation (Morgan et al. 2005). However, in a field experiment using the mosquito *Ochlerotatus sierrensis* and its protozoan parasite *Lambornella clarki*, parasite local adaptation was still observed despite hosts having higher relative migration rates (Ganz and Washburn 2006). Clearly, quantifying gene flow between parasite populations is a necessary next step for studying local adaptation in the monarch system.

Our study did find significant effects of host and parasite lineages on parasite spore load and adult monarch longevity, indicating that genotype is partially responsible for determining infection phenotype. However, given that we did not find a consistent signal of local adaptation, genotypic interactions between hosts and parasites are not sufficient to drive local adaptation in this system. This raises the issue that reciprocal cross-infection experiments carried out in a laboratory environment may fail to capture environmental conditions that are important for the interaction of host and parasites. For example, one study of ribwort plantain and its fungal parasite found that both the strength and direction of local adaptation depended on experimentally controlled temperature (Laine 2008). Similarly, in a cross-fostering experiment using sea birds, sympatric ectoparasites differed from allopatric ectoparasites only under resource-poor conditions

(McCoy et al. 2002). In herbivorous insect host-parasite systems, such as the monarch system, food plants have been shown repeatedly to be important components of the environment during infection (reviewed in Cory and Hoover 2006). Milkweeds, the larval food plants of monarchs, are known to affect disease resistance (De Roode et al. 2008a; Sternberg et al. 2012) and tolerance (Sternberg et al. 2012). Because monarchs can obtain protection from parasitism through their food plant, selection may be weak for increased physiological resistance against local parasites. In addition, female monarchs can preferentially lay eggs on anti-parasitic milkweed species (Lefèvre et al. 2010), and such behavioral resistance to parasitism is not captured in reciprocal cross-infection experiments. Thus, we suggest that future experiments incorporate ecologically relevant factors such as food plant species when testing for local adaptations.

In conclusion, we show that in isolated populations, host resistance against parasite replication (i.e. quantitative resistance) is associated with increased parasite virulence. In contrast, this was not demonstrated in more closely connected populations. Furthermore, we show that the hosts from the more quantitatively resistant population also have a lower probability of infection (i.e. higher qualitative resistance) and increased tolerance, indicating that such defense mechanisms are not mutually exclusive. Despite the occurrence of G_h x G_p interactions, we did not find patterns of local adaptation in either of our two cross-infection experiments. Although gene flow may be responsible for this in the eastern North America/S. Florida comparison, we suggest that environmental factors, such as the food plants that monarchs use as larvae, may disguise patterns of local adaptation and should be considered in future studies.

Chapter 3

Food plant-derived disease tolerance and resistance in a natural butterfly-plant-parasite interaction.

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Introduction

Because parasites pose a major threat to free-living species, natural selection should strongly favor the evolution of host defenses to limit parasite-induced fitness loss (Combes 2001). Hosts can in principle evolve two distinct defense mechanisms: resistance and tolerance (Råberg et al. 2007; Boots 2008; Råberg et al. 2009). Resistance encompasses behavioral, physiological and genetic mechanisms that reduce infection probability or parasite growth upon infection. In contrast, tolerance mechanisms do not reduce parasite infection or growth, but instead alleviate the fitness consequences of parasite infection. Both types of defense limit fitness costs to the host from parasitism but they vary critically in their effects on parasites. Specifically, resistance limits parasite fitness while tolerance does not (Boots 2008; Svensson and Råberg 2010).

These varying effects have important consequences for the long-term coevolution of hosts and parasites (Boots and Bowers 1999; Roy and Kirchner 2000; Rausher 2001; Restif and Koella 2004; Miller et al. 2005; Miller et al. 2006; Svensson and Råberg 2010). Theoretical models of the evolution of host defenses predict that genetic variation in

resistance will be maintained but tolerance mechanisms will become fixed (Boots and Bowers 1999; Roy and Kirchner 2000; Miller et al. 2006; but see Best et al. 2008). The reason for this difference is that resistance results in a negative epidemiological feedback where parasite infection selects for resistant hosts and this reduces parasite prevalence in the population. Assuming that resistance is costly, low parasite prevalence then reduces selection for resistance and susceptible hosts are favored. In contrast, tolerance evolution results in positive feedback where parasite infection selects for tolerant hosts. Tolerant hosts increase parasite transmission, which results in greater parasite prevalence and continuing selection for tolerant hosts. Because tolerance does not reduce parasite infection or transmission, it has been suggested that disease treatments based on tolerance are less likely to select for countermeasures in parasites than are treatments based on disease resistance (Roy and Kirchner 2000; Rausher 2001; Schneider and Ayres 2008). It has also been suggested that increased host tolerance may lead to increased parasite virulence (Restif and Koella 2004; Miller et al. 2006), and additional work will be necessary to determine how tolerance affects host-parasite coevolution dynamics (Little et al. 2010).

The distinction between resistance and tolerance has long been recognized in plants that suffer attack from herbivores (e.g., Fineblum and Rausher 1995; Mauricio et al. 1997; Tiffin and Rausher 1999; Simms 2000) and parasites (e.g., Simms and Triplett 1994; Koskela et al. 2002; Kover and Schaal 2002; Carr et al. 2006). That animals also show both resistance and tolerance to enemies has received attention only recently (e.g. Corby-Harris et al. 2007; Ayres and Schneider 2009). Since tolerance per se is difficult to measure (Råberg et al. 2007; Boots 2008; Råberg et al. 2009) studies have mainly

investigated whether host genotypes vary in their levels of tolerance, usually measured as variation in the slopes of the relationships between host fitness and parasite burden (Råberg et al. 2007; Blanchet et al. 2010; Rohr et al. 2010; Lefèvre et al. 2011; Soler et al. 2011). Although these studies are a noteworthy step forward, they are entirely focused on tolerance as a genetically determined trait. This is a major limitation because, in addition to varying genetically, hosts and parasites in nature interact within a larger ecological community (Lafferty et al. 2006). Interacting species can affect traits such as host resistance and parasite virulence (Wolinska and King 2009; De Roode et al. 2011b; Parker et al. 2011; Sternberg et al. 2011) and it is possible that tolerance is also affected by such interactions. By isolating hosts and parasites from their environment, we may erroneously conclude that hosts do not use tolerance as a defense or that there is no variation in this trait.

Here we explicitly test how the environment in which hosts and parasites interact can provide hosts with tolerance and resistance to their parasites. We focus on monarch butterflies (*Danaus plexippus*) and their naturally occurring protozoan parasite *Ophryocystis elektroscirrha* (McLaughlin and Myers 1970). In this system, infections occur when larvae ingest parasite spores on eggs or milkweed plants (genus *Asclepias*). Spores lyse in the gut and parasites penetrate the intestinal wall to undergo asexual and sexual replication in the hypoderm; parasites then form spores around the scales of the developing butterfly, such that adult monarchs emerge covered with dormant spores on the outsides of their bodies (McLaughlin and Myers 1970). Parasites do not replicate on adults, and spores must be ingested by larvae to cause new infections. Most parasite

transmission occurs from infected butterflies to their offspring, when females scatter spores on eggs and milkweed during oviposition (Altizer et al. 2004).

The monarch-parasite system is ideally suited for testing the effect of environment on host resistance and tolerance because monarchs and their parasites have an obligate interaction with milkweed plants, which monarchs use as their larval food plants (Ackery and Vane-Wright 1984). Previous work has shown that certain milkweed species reduce infection and growth of *O. elektroscirrha* in monarch larvae, most likely due to the presence of milkweed toxic secondary chemicals known as cardenolides (De Roode et al. 2008a; Lefèvre et al. 2010; De Roode et al. 2011a; De Roode et al. 2011b). Here we infected and reared monarch larvae on twelve species of milkweed, and we quantified the cardenolides present in milkweed foliage. We show that there is a gradient of resistance to *O. elektroscirrha* conferred by the twelve milkweed species, and that the cardenolide composition of the milkweed plants affects the fitness of both infected and uninfected monarchs. Importantly, we show that milkweed species provide disease tolerance to monarch butterflies, and that this tolerance is associated with milkweed cardenolides. Hence, we demonstrate that an environmental variable can confer disease tolerance to an animal host.

Methods

Experimental procedure

The monarchs used in this experiment were the non-inbred grand-progeny of monarchs collected from Pismo Beach, CA, USA These monarchs are part of a large panmictic genetic population inhabiting North America (Lyons et al. 2012). Mated females were

provided with *Asclepias incarnata* for oviposition and eggs were manually transferred to leaves from one of twelve food plant species. The species of plants used were: *A. curassavica*, *A. eriocarpa*, *A. erosa*, *A. fascicularis*, *A. incarnata*, *A. physocarpa*, *A. purpurascens*, *A. speciosa*, *A. sullivantii*, *A. syriaca*, *A. tuberosa*, and *A. verticillata*. With the exception of *A. physocarpa*, all of these species are widely distributed throughout North America (Woodson 1954; Hickman 1993), thus making them ecologically relevant species for the North American monarch population (Malcolm and Brower 1989). All plants used in this experiment were grown under uniform conditions in a climate-controlled greenhouse, from seeds obtained from Butterfly Encounters, CA.

Upon hatching, larvae were randomly assigned a single unique plant and transferred to petri dishes with leaves from their plant. Two days after hatching, larvae were transferred into fresh petri dishes containing leaf discs from their assigned plants. Larvae were inoculated by manually depositing ten parasite spores on the leaf disc, while control monarchs received clean discs (De Roode et al. 2007; De Roode et al. 2008a). The parasite spores used for inoculation came from a clonal line (denoted C1C10-P2-3) originally isolated from monarchs collected in California, USA.

After consuming their leaf discs, larvae were placed in individual plastic containers with florist tubes holding cuttings from their assigned plants. These containers were kept in a climate-controlled room at 26°C on a 16L:8D light cycle, and checked daily until pupation. Fresh cuttings of each larva's assigned plant were provided as needed. If the individual plant was not big enough to feed the monarch until pupation, randomly selected cuttings of the same species were used. Although some monarchs consumed foliage from multiple individuals, previous studies have shown that the

milkweed fed after infection has no effect on adult monarch longevity or parasite burden (De Roode et al. 2011a).

Monarchs were transferred to a new room (also held at 26°C, 16L:8D) six days after pupation to prevent parasite contamination of the larval rearing room by emerging infected adults. When the monarchs eclosed, they were sexed, then placed in individual glassine envelopes held at 12°C and checked daily for death. The difference in days between eclosion and death under these conditions provides a combined measure of longevity and starvation resistance (referred to as adult longevity). Previous experiments have shown that the effects of infection and parasite burden on monarch longevity under starvation conditions are similar to the effects under more natural, non-starvation, conditions, and that adult longevity is an important component of monarch fitness (De Roode et al. 2008b; De Roode et al. 2009).

After the monarchs died, we quantified their parasite burden (referred to as spore load) by vortexing their bodies for 5 minutes in 5 mL of water to shake off the parasite spores, and then counting the spores using a haemocytometer (De Roode et al. 2007).

Collecting and measuring cardenolides

To assess effects of plant chemistry on parasite infections, we quantified the foliar cardenolides of the plants assigned to infected monarchs. When leaves were collected for inoculations, we also obtained samples for chemical analysis. Six leaf discs were collected into methanol and stored at -80ºC until analysis, as described previously by Vannette and Hunter (2011). Six additional leaf discs were oven-dried overnight to estimate sample dry weights. The cardenolides were analyzed using reverse phase high-

performance liquid chromatography (HPLC). Digitoxin was used as an internal standard, and absorbance spectra were recorded from 200 to 300 nm. Peaks were detected by diode array at 218 nm and those with symmetrical absorbance maxima between 217 and 222 nm were considered to be cardenolides (Malcolm and Zalucki 1996). The concentration of each peak was calculated relative to the internal standard and the total cardenolide concentration of each plant was the sum of the peaks.

In addition to assessing total cardenolide concentration, we calculated two additional measures of the chemical community present in the milkweed: diversity, and relative polarity. Diversity was quantified by adapting the Shannon-Wiener index H, taken from the biodiversity literature (as described by Rasmann and Agrawal 2011). This index measures the number of different cardenolides present in a plant as well as the evenness of their distribution, and it is calculated as $-\text{sum}(P_i \log[P_i])$, where P_i is the relative amount of a given cardenolide in a plant. Polarity was calculated using $\text{sum}(P_i RT_i)$, where RT_i is the retention time of a given peak, weighted by the relative amount of the peak (P_i) (Rasmann and Agrawal 2011). Under reverse phase HPLC, cardenolide retention time increases as polarity decreases; therefore, our polarity index increases with the presence of more non-polar cardenolides. More non-polar cardenolides are thought to be an important mediator of food plant effects on other species due to their increased toxicity (e.g. Fordyce and Malcolm 2000; Zehnder and Hunter 2007).

Statistical analysis

Logistic regression by generalized linear model (GLM with binomial error distribution, logit link) was used to assess effects of food plant species on monarch survival to

adulthood and infection probability. Generalized linear models with normal error distributions were used to examine effects of food plant species on the $\log_{10}$-transformed parasite spore load of infected monarchs, the effects of food plant species on the longevity of all monarchs, and the effects of parasite spore load on the longevity of infected monarchs. Tolerance was measured as the slope of a regression line between square-root-transformed spore load (a measure of parasite burden) and monarch longevity (a measure of host fitness) in infected and control monarchs (Mauricio et al. 1997; Simms 2000; Råberg et al. 2007; Blanchet et al. 2010; Lefèvre et al. 2011). We included the interaction between spore load (square-root-transformed) and food plant species in our model, to investigate whether tolerance varied in monarchs reared on different food plant species. We also included a quadratic term for square-root-transformed spore load (i.e. untransformed spore load) in our model to investigate the possibility of a non-linear relationship between spore load and host fitness (Tiffin 2000; Råberg et al. 2007; Blanchet et al. 2010; Lefèvre et al. 2011). Linear regression was used to test for a relationship between tolerance and resistance (measured as the inverse of mean spore load) (Råberg et al. 2007).

To assess effects of total cardenolide concentration, diversity, and non-polarity on the longevities of infected monarchs, we used generalized linear models with normal error distributions. We included a quadratic term in all models to test for a non-linear relationship between the measures of cardenolide community and the longevity of infected monarchs. Also using generalized linear models, we assessed the effects of our measures of cardenolide chemistry on monarch tolerance to parasitism by associating

tolerance with the log-transformed mean cardenolide concentration of each milkweed species.

We also compared cardenolide composition among milkweed species using permutational multivariate analysis of variance (PerMANOVA) (Anderson 2001) following Bray-Curtis ordination. Analysis was conducted using the Adonis procedure of the Vegan package in R v 2.7.1. We used metaMDS in Vegan for Nonmetric Multidimensional Scaling (NMDS) (McCune and Grace 2002), stepping down from a six dimensional model to a one-dimensional model, with 999 permutations per model run and a maximum of 20 runs per dimension. Inspection of the scree plot illustrated that model stress declined rapidly from a one-dimensional to two-dimensional model, declining only slightly thereafter. We therefore used a two-dimensional model in subsequent analysis (model stress = 13.60, well within the range of 10 to 20 that is typical of ecological data (McCune and Grace 2002). We used both NMDS axes as independent variables in generalized linear models (Poisson error distribution, log link function) to associate milkweed cardenolide composition with monarch longevity.

Throughout our analyses, variables were transformed as necessary to ensure compliance with model assumptions and Fligner-Killeen tests were used to confirm homogeneity of variance (Crawley 2007). Minimal models were derived by removing terms, followed by model comparisons. Terms were retained in the model if their removal significantly ($p < 0.05$) reduced the explanatory power of the model (Crawley 2007). All analyses were carried out in R v. 2.7.1.

Results

Host fitness, parasite replication, and food plant species

A total of 463 out of 520 (89%) monarchs survived to adulthood, with 366 out of 409 (89%) inoculated monarchs surviving and 97 out of 111 (87%) control monarchs surviving. Inoculation with the parasite had no effect on the probability of larvae surviving to adulthood (GLM with bionomial error distribution, likelihood ratio chi-square; $\chi^2 = 1.45$, d.f. = 1, p = 0.23) whereas food plant species did ($\chi^2 = 26.1$, d.f. = 11, p = 0.01); larval survival ranged from 80% on *A. purpurascens* to 100% on *A. verticillata*. The total number of surviving monarchs per plant ranged from 24 (inoculated = 20, control = 4) out of 30 larvae on *A. purpurascens*, to 49 (inoculated = 39, control = 10) out of 50 larvae on *A. physocarpa*. All subsequent analyses were restricted to monarchs that survived to adulthood. Analyses of parasite burden were restricted to infected monarchs, but analyses of tolerance included both infected and uninfected monarchs (Råberg et al. 2009; Svensson and Råberg 2010; Baucom and De Roode 2011).

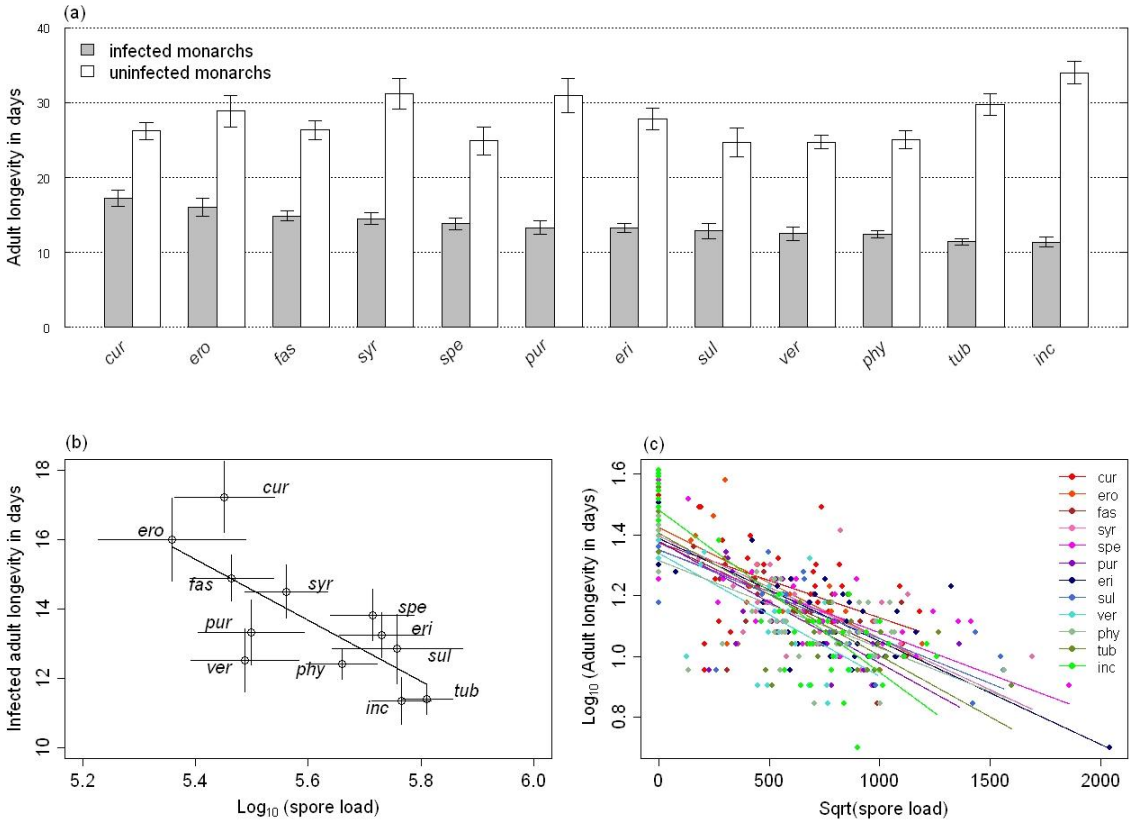

Figure 1. Effects of milkweed food plant species on parasite spore load and monarch adult longevity. (a) Adult longevity of infected (gray) and uninfected monarchs (white) reared on the twelve milkweed species. Bars show mean longevity ± 1 SE. **(b)** Adult longevity and spore load of infected monarchs on the twelve milkweed species. The x axis indicates parasite burden (i.e., the inverse of resistance) for each group of monarchs. Points indicate means for each plant species ± 1 SE; line indicates regression line. **(c)** Monarch adult longevity as a function of parasite spore load. Lines indicate species-specific regression lines. The differences in slopes of these lines indicate variation in tolerance. Data points indicate individual monarchs. (Three letter abbreviations used for plant species names; cur = *A. curassavica*, ero = *A. erosa*, fas = *A. fascicularis*, syr = *A. syriaca*, spe = *A. speciosa*, pur = *A. purpurascens*, eri = *A. eriocarpa*, sul = *A. sullivantii*, ver = *A. verticillata*, phy = *A. physocarpa*, tub = *A. tuberosa*, inc = *A. incarnata*).

Parasite infection did significantly reduce adult longevity in monarchs that survived to adulthood (Fig. 1a; $F_{1, 407} = 867$, $p < 0.001$). Adult longevity also varied among milkweed species (Fig. 1a; $F_{11, 407} = 4.48$, $p < 0.001$) for both infected and uninfected monarchs. Moreover, the effect of plant species on longevity differed between

infected and uninfected monarchs (interaction between infection and plant species $F_{11, 407} = 5.86$, $p < 0.001$). This interaction between infection status and plant species is clearly illustrated by comparing monarchs reared on *A. incarnata* and *A. curassavica* (Fig. 1a). Uninfected monarchs reared on *A. incarnata* lived longer as adults than those reared on *A. curassavica*; in contrast, infected monarchs had longer adult life spans when reared on *A. curassavica*, indicating that *A. curassavica* mitigates the reduction in monarch fitness due to parasitism.

Overall infection probability was high in all monarchs exposed to parasites, ranging from 23 out of 25 monarchs infected (92%) on *A. verticillata* to 100% on *A. eriocarpa* (35 monarchs), *A. physocarpa* (36 monarchs), *A. purpurascens* (19 monarchs), *A.sullivantii* (20 monarchs), and *A. tuberosa* (25 monarchs). We found no significant effect of plant species on the probability of infection ($\chi^2 = 0.447$, d.f. = 11, $p = 0.95$). In monarchs that became infected, however, there was a significant effect of food plant on parasite spore load (Fig. 1b; $F_{11, 312} = 3.27$, $p < 0.001$), as well as an effect of monarch sex ($F_{1, 312} = 5.70$, $p = 0.02$). Some plant species (e.g. *A. curassavica* and *A. erosa*) exhibited anti-parasitic effects such that monarchs reared on these species had a lower mean spore load than did monarchs reared on less anti-parasitic plant species (e.g., *A. incarnata* and *A. tuberosa*). These results indicate that milkweed species can confer resistance (i.e. a reduction of parasite growth) to monarch butterflies. Because of a significant negative effect of spore load on infected adult longevity ($F_{1, 312} = 3.82$, $p < 0.001$), the mean longevity of infected monarchs was negatively correlated with mean parasite burden across all food plant species (Fig 1b; $F_{1, 10} = 12.92$; $R^2 = 0.56$, $p = 0.005$).

Critically, in addition to effects on disease resistance, we also observed effects of plant species on monarch tolerance to parasite infection. Specifically, the negative relationship between monarch longevity and parasite spore load varied significantly among plant species (Fig. 1c; plant species by spore load interaction $F_{11, 387}$= 2.66, p = 0.003). This variation in slopes indicates that monarchs reared on different milkweed species vary in their ability to maintain fitness with increasing parasite loads, and thus indicates variation in tolerance. We also found a significant effect of the quadratic term for spore load ($F_{1, 387}$ = 56.6, p < 0.001), suggesting a non-linear relationship between spore load and host fitness. We did not find evidence of a correlation, either negative or positive, between milkweed-conferred tolerance (measured as the slope of the regression of adult longevity and spore load) and resistance (measured as the inverse of spore load) ($F_{1, 10}$ = 0.05; R^2 = 0.005, p = 0.8). In combination with the observed effect of plant species on the longevity of uninfected monarchs and on parasite spore load, these results indicate that food plant species are crucial in determining host and parasite fitness via effects on tolerance and resistance.

Food plant chemistry and host fitness

We began our analyses of plant chemistry with total cardenolide concentration as a straightforward measure of individual plant chemistry and we found no simple linear ($F_{1, 315}$ = 0.03, p = 0.870) or quadratic ($F_{1, 315}$ = 2.48, p = 0.116) relationship between the total concentration of cardenolides present in the plant and the longevity of infected monarchs reared on the plant. However, we noted that the average cardenolide

concentration in *A. physocarpa* was over two-fold higher than that in any other *Asclepias* species (Fig. 2a).

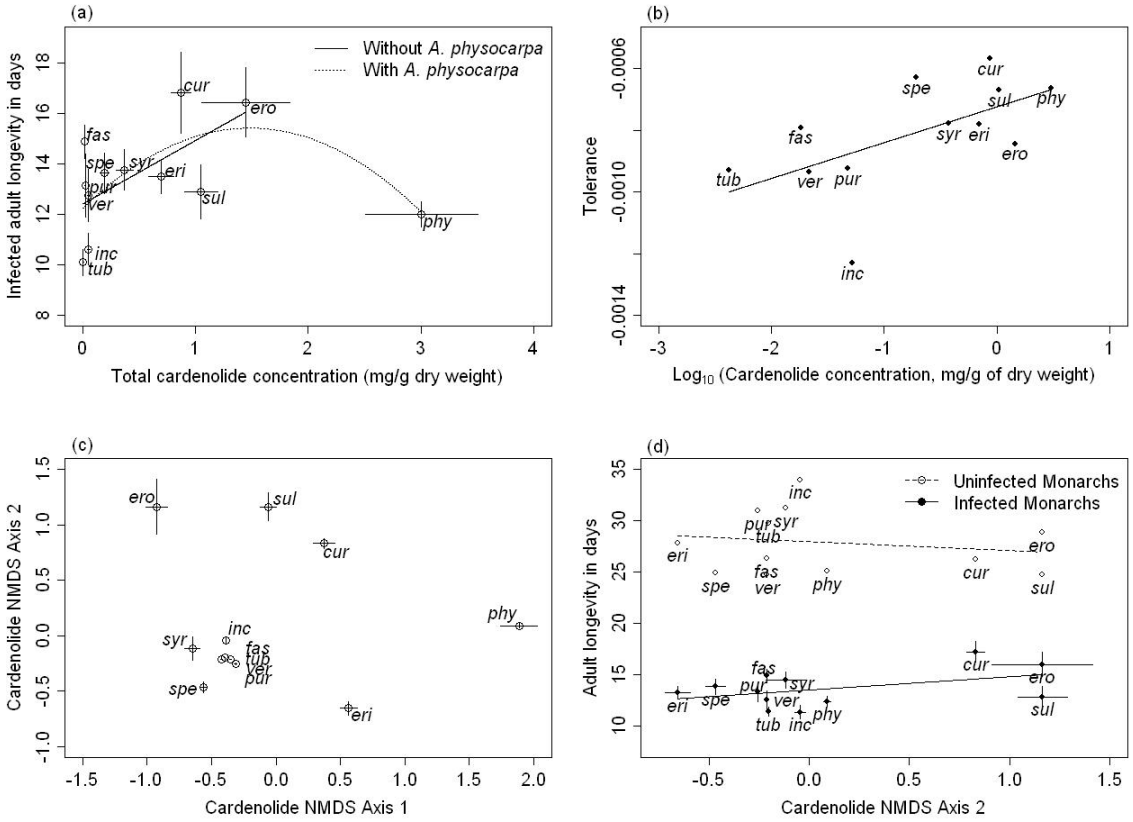

Figure 2. Associations between milkweed cardenolide chemistry and the fitness of infected monarch butterflies. (a) Average total cardenolide concentrations correlated linearly with monarch adult longevity (a fitness measure) when the outlier *A. physocarpa* was excluded, and non-linearly when *A. physocarpa* was included. **(b)** The tolerance of monarchs to parasites was associated with foliar cardenolide concentration. Data points indicate milkweed species means, bars indicate ± SE; lines indicate least-squares regression lines. **(c)** Milkweed species differed in the composition of cardenolides that they contained, separating in two-dimensional NMDS analysis. **(d)** NMDS axis 2 tended to associate positively with longevity of infected monarchs and negatively with the longevity of uninfected monarchs. This association was significant across all infected monarchs, but not significant for the mean longevities of infected and uninfected monarchs.

The principle of hormesis predicts that plant toxins can have conflicting effects so that a smaller dose of toxins increases herbivore fitness while a larger dose decreases fitness

(Kaiser 2003; Forbey and Foley 2009). There is some preliminary evidence for hormesis in our results based on the observation that monarchs fed on plants with intermediate levels of cardenolides exhibited increased longevity compared to monarchs that received either very little or large doses of cardenolides. We found a significant quadratic relationship between the mean longevity of infected monarchs and the mean cardenolide concentrations of their milkweed food (Fig. 2a; $F_{2, 9} = 3.01$, linear term: $p = 0.037$; quadratic term: $p = 0.041$, $R^2 = 0.40$); the relationship is linear when *A. physocarpa* is removed ($F_{1, 9} = 5.50$, $p = 0.044$, $R^2 = 0.38$). We also found a significant association between monarch disease tolerance (i.e., the slope of the regression of adult longevity and spore load) and average milkweed cardenolide concentration (Fig. 2b; $F_{1, 10} = 2.25$, $p = 0.047$, $R^2 = 0.34$). Tolerance was also associated with cardenolide diversity ($F_{1, 10} = 2.98$, $p = 0.014$, $R^2 = 0.47$) but not with cardenolide polarity ($F_{1, 10} = 1.46$, $p = 0.175$, $R^2 = 0.176$). Neither diversity nor polarity was retained in a model that accounted first for the effect of cardenolide concentration ($p = 0.169$ and $p = 0.540$, respectively).

In addition to our analyses using the concentrations of cardenolides we found that milkweed species differed dramatically in their cardenolide compositions (PerMANOVA; $F_{11, 306} = 67.81$, $p < 0.001$, $R^2 = 0.71$). These differences were plotted using an ordination technique, Nonmetric Multidimensional Scaling (NMDS) (McCune and Grace 2002), with a two dimension model separating most milkweed species by their cardenolide compositions. The exceptions were a cluster of four milkweed species with extremely low cardenolide concentration (Fig. 2c). NMDS axis 2 was positively associated with the longevity of infected monarchs across all milkweed plants (Fig. 2d; $F_{1, 315} = 5.60$, $p = 0.02$). Because we did not measure the cardenolide chemistry of plants upon which

uninfected monarchs were reared, we used the mean NMDS scores of each host plant species to compare mean responses in longevity of infected and uninfected monarchs. There was a non-significant trend with the longevity of infected monarchs increasing and the longevity of uninfected monarchs decreasing with increases in NMDS axis 2 (Fig. 2d; $F_{1, 20} = 3.29$, $p = 0.085$). These results support the hypothesis that the interaction between monarchs and certain foliar cardenolides is contingent upon whether or not the monarchs are infected with *O. elektroscirrha*.

Lastly, we examined cardenolide diversity (a composite index of the number and relative abundance of cardenolides present) and relative non-polarity (a measure that is inversely proportional to the average polarity of cardenolides), in relation to the longevity of infected monarchs. Unlike total cardenolide concentrations, the relationship between mean longevity of infected monarchs and mean diversity was not significant, and neither was the relationship between mean longevity and mean non-polarity. For both measurements, analyses on individual monarchs found significant linear (Fig. 3a and 3b; $F_{1, 315} = 8.00$, $p = 0.005$ and $F_{1, 315} = 15.2$, $p < 0.001$ respectively) and quadratic terms ($F_{1, 315} = 11.3$, $p < 0.001$ and $F_{1, 315} = 16.4$, $p < 0.001$ respectively).

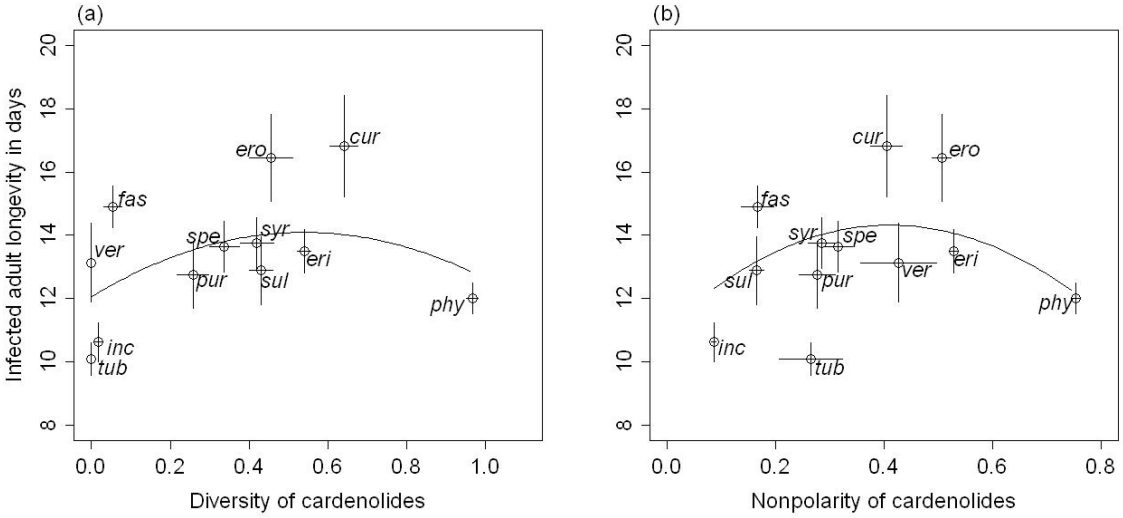

Figure 3. Associations between milkweed cardenolide composition and the performance of infected monarch butterflies. Both (a) the diversity and (b) the average non-polarity of cardenolides in milkweeds were associated with adult longevity of infected monarchs, with monarchs experiencing greatest longevity when reared on milkweed species with intermediate cardenolide diversity and polarity.

This suggests that the polarity and diversity of cardenolides present in milkweeds may be important for understanding the effects of milkweed chemistry on infection, but additional species will be necessary to determine whether the relationship holds true among milkweed species. The observation that infected monarchs experience the highest longevity on plant species with intermediate levels of cardenolide diversity and polarity is again consistent with a trade-off between the anti-parasitic effect of cardenolides and the physiological cost to monarchs from the cardenolides (e.g. Fig. 2c). However, of the 12 species in this experiment, *A. physocarpa* appears to be the only one beyond the threshold where the physiological cost of cardenolides outweighs the anti-parasitic effect.

Discussion

Our results show that milkweed species can affect relative levels of resistance and tolerance to parasite infection in monarch butterflies. Across the twelve species of milkweed that we tested, monarch butterflies experienced highest resistance (i.e. lowest parasite spore loads) on *A. erosa* and lowest resistance on *A. tuberosa* (Fig. 1b). Highest tolerance (i.e. smallest reduction in adult longevity with increasing parasite spore load) was observed in monarchs reared on *A. curassavica* and lowest tolerance in monarchs on *A. incarnata* (Fig. 1c). We found no significant relationship between milkweed-conferred resistance and tolerance, suggesting that milkweed species do not simultaneously confer greater resistance and tolerance to monarchs and that there is no trade-off between milkweed-conferred resistance and tolerance. Because our experiment used only a single parasite genotype for infection, follow up studies will be necessary to examine the effect of food plants on tolerance across parasite genotypes. However, the effect of food plants on host resistance has previously been confirmed using multiple parasite genotypes (De Roode et al. 2008a; Lefèvre et al. 2010). Our results are an important addition to the growing number of studies indicating that environmental factors are important modulators of host-parasite interactions (reviewed in Wolinska and King 2009). Until now, these studies have focused primarily on resistance, but as illustrated by our results, environmental factors – including interacting species – can also significantly affect tolerance.

Demonstrating that tolerance can be environmentally determined has important implications for the study of host-parasite systems. It suggests that, when hosts are removed from their natural environments, researchers may erroneously conclude that hosts have not evolved tolerance. As a case in point, our own previous study of tolerance

in monarch butterflies revealed no genetic variation and concluded that monarch butterflies either had not evolved tolerance or that it had become fixed at a maximum level (Lefèvre et al. 2011). However, in that study, we reared monarchs on a single species of milkweed, thus excluding the possibility of measuring tolerance conferred by milkweed species. In our current experiment, we have tested multiple species of milkweeds, most of which (11 out of 12) are found in sympatry with the monarch population represented in our experiment (Woodson 1954; Malcolm and Zalucki 1996). This includes milkweed species with overlapping distributions and as our results indicate, monarchs can obtain tolerance to infection by utilizing particular species of milkweed. This could also impact the oviposition preference of female monarchs. As we have previously shown, parasite-infected monarchs preferentially lay eggs on *A. curassavica* over *A. incarnata* in two-species choice tests (Lefèvre et al. 2010; Lefèvre et al. 2012) and this preference could provide the monarchs' offspring not only with greater effective resistance, but also greater effective tolerance (Fig. 1c). The ability to obtain tolerance to parasitism through such interactions would be missed in experiments that do not incorporate environmental variability.

Environmental variation is one potential mechanism for the maintenance of polymorphism in host resistance (Lazzaro and Little 2009; Wolinska and King 2009), and this may be true for tolerance as well. Although the majority of theoretical models have predicted a lack of genetic variation in tolerance (but see Best et al. 2008), many empirical studies have found such genetic variation, both in plants and animals (reviewed in Baucom and De Roode 2011). Authors have mostly attributed this variation to fitness costs associated with tolerance (Simms and Triplett 1994; Tiffin and Rausher 1999;

Koskela et al. 2002) and trade-offs between resistance and tolerance (Fineblum and Rausher 1995; Carr et al. 2006; Råberg et al. 2007). In some cases, however, environmental effects may explain the observed variation in tolerance when measured under natural conditions. For example, recent work on ectoparasites in fish reported a significant interaction between sampling site and parasite burden, suggesting that the environment is influencing tolerance (Blanchet et al. 2010). Conversely, environmental factors may negate genetic variation in tolerance observed under standard laboratory conditions. For example, if different host genotypes are subject to different environmental factors in the wild, and if those factors affect tolerance, it is possible that the observed variation in the laboratory is not actually expressed in nature.

In addition to contributing to our understanding of resistance and tolerance, our findings add to a growing body of evidence that food plants are major determinants of fitness in phytophagous hosts and their parasites (reviewed in Cory and Hoover 2006). With this type of tritrophic interaction, understanding the role of plant chemistry, including nutrient content (Lee et al. 2006) and defensive or allelopathic chemicals (Felton and Duffy 1990; Keating et al. 1990), is essential for predicting how plants will influence infection. Diet quality can have profound effects on the immune system (Bhaskaram 2002; Wintergerst et al. 2007; Ponton et al. 2011) and this may contribute to the dietary-based tolerance that we observed in our experiment. However, plant chemistry can impose conflicting effects on hosts (e.g., Singer et al. 2004; Haviola et al. 2007b), likely resulting in the interactions between infection status and plant chemistry in our study (Figs. 1a & 2d). This interaction is illustrated by *A. curassavica* which, relative to other milkweed species, depresses adult longevity in the absence of the parasite and

promotes adult longevity in the presence of the parasite. We also found a significant, curvilinear relationship between the mean concentration of cardenolides present in milkweed species and the mean longevity of infected adults, and between the proportion of non-polar cardenolides in individual milkweed plants and the longevity of the infected adult monarchs reared on these plants. A curvilinear relationship is consistent with the general predictions of a pharmacological approach to plant-herbivore interactions, wherein herbivores are expected to respond to plant chemical variation in a dose-dependent fashion (Forbey and Foley 2009). The curvilinear relationships are also consistent with the specific biology of this system, where non-polar cardenolides are thought to be more toxic than polar cardenolides (Fordyce and Malcolm 2000; De Roode et al. 2011b). This is apparent in the adult longevity of infected monarchs reared on *A. physocarpa*, a milkweed species with over forty distinct cardenolides, including many highly non-polar cardenolides present at high concentrations. Because adult longevity is a measure of the combined effect of the plant on the parasite and on the monarch, the cardenolides present in *A. physocarpa* may have direct negative effects on monarch health that outweigh any negative effect on the parasite (Fig. 2a). Given the complexity of plant chemistry and the capacity for direct and indirect effects on monarch health, we emphasize that there are no universally beneficial milkweed or cardenolides. Rather, the effects of food plants on monarchs depend on multiple aspects of plant chemistry and the prevalence of parasites.

It is clear that environmental factors vary within and among natural populations, both spatially and temporally, and the idea that environmental variability can affect selection has been present in the literature for over half a century (Haldane 1946;

Falconer 1952). It is only recently, however, that this concept has been extended specifically to infectious diseases (Lazzaro and Little 2009; Wolinska and King 2009). As our results show, environmental factors – such as interacting species in a food web – can have an important effect on host tolerance to infection. This suggests that environmental factors need to be investigated to obtain a complete picture of host-parasite coevolution. Moreover, by identifying the chemical and physiological mechanisms that provide hosts with tolerance, studies on environmentally induced tolerance may aid in the development of disease therapies that are less likely to be circumvented by parasite evolution than are therapies based on resistance (Roy and Kirchner 2000; Rausher 2001; Schneider and Ayres 2008).

Chapter 4

A Virulent Parasite can Provide Protection Against a Lethal Parasitoid

Modified from: E.D. Sternberg, T. Lefèvre, A.H. Rawstern, J.C. de Roode (2011). *Infection, Genetics and Evolution*. 11; 399-406.

Introduction

In laboratory studies of infectious diseases, experimental infections typically consist of a single parasite species or strains. However, infection of a host with multiple parasite strains or species occurs frequently outside of the laboratory (Petney and Andrews 1998; Cox 2001; Rigaud et al. 2010). In natural populations, the parasites infecting a host can range from multiple strains of the same species (Lord et al. 1999; Bharaj et al. 2008) to different species with varying degrees of taxonomical distance (Petney and Andrews 1998; Cattadori et al. 2007; Craig et al. 2008; Rutrecht and Brown 2008). Previous work has demonstrated that the effect of co-occuring parasites on the host is not necessarily the additive effect of each single infection (Malakar et al. 1998; Thomas et al. 2002; Druilhe et al. 2005; Haine et al. 2005; Pedersen and Fenton 2006). For example, infection with multiple parasite strains or species may benefit the host if the parasites use similar host resources, thus resulting in the competitive suppression of parasite growth (Berchieri and Barrow 1990; Dobson and Barnes 1995; Read and Taylor 2001; Ishii et al. 2002). Alternatively, the host may incur a greater cost from infection with multiple parasites strains or species, for example due to collateral damage from intense competition between parasites (Roper et al. 1998; Read and Taylor 2001).

From the parasite's perspective, there can be benefits to infecting a host with other parasites, e.g. if one of the parasites dampens the host immune response (Su et al. 2005; Cattadori et al. 2007; Graham 2008) or if one of the parasites facilitates infection or transmission of the other parasite (Friedli and Bacher 2001; Poulin et al. 2003; Hughes and Boomsma 2004). However, there can also be costs if the parasites overlap extensively in their host resource use (Hochberg 1991; Ishii et al. 2002) or if one species alters the host environment in a way that makes it inhospitable to another parasite, for example through the activation of the host immune response (Dobson and Barnes 1995; Lello et al. 2004). Negative interactions can occur between parasitoids and microparasites (e.g., bacteria and viruses), for example when parasite-induced host death occurs too quickly for the second parasite to develop fully, thereby blocking the transmission of the second parasite (Chilcutt and Tabashnik 1997; Escribano et al. 2000). In these examples, the amount of time between infections often determines whether the second parasite is able to transmit upon host death. In addition to the temporal spacing of infection, the order of infection can modify the effect of multiple infections on host and parasites. Simultaneous infections may have a different outcome than sequential infections and in some systems (but not all: (Lohr et al. 2010), the effect of multiple infection on the parasites depends on which parasite has prior residency in the host (De Roode et al. 2005).

All of the examples above illustrate that the outcome of infection with multiple parasites can diverge greatly from that of single infections, and that the different biological characteristics of the host and parasite species determine the outcome of infection. Furthermore, theoretical work has shown that the impact of co-occuring parasites extends to the evolution of hosts and parasites (Rigaud et al 2010), for example,

by affecting the selection pressures that drive parasite virulence (Van Baalen and Sabelis 1995; Brown et al. 2002; Choisy and De Roode 2010), facilitating the emergence of novel pathogen strains (Lloyd-Smith et al. 2008), and altering the antagonistic co-evolutionary feedback between host and parasite (Mostowy et al. 2010).

Even with increasing awareness of both the ubiquity and importance of multi-parasite infections, there are relatively few examples of studies in systems where transmission of one parasite is completely blocked by the successful transmission of a second parasite. In the literature that does exist on this subject, the examples predominantly come from systems in which one parasite is transmitted trophically (through predation by a definitive host on the intermediate host) while a second parasite is either not trophically transmitted or requires a different definitive host species (Cezilly et al. 2000; Thomas et al. 2002; Haine et al. 2005; Rigaud and Haine 2005). In these cases, there is evidence that parasites can rescue their hosts from the potentially lethal effects of a second parasite if host survival is required for transmission of the first parasite (Cezilly et al. 2000; Haine et al. 2005). For example, the amphipod *Gammarus roeseli* serves as a host for both the trophically transmitted acanthocephalan *Polymorphus minutus* and the vertically transmitted (from parent to offspring) microsporidium *Dictyocoela* sp. (roeselum). *P. minutus* induces behavioral changes in *G. roeseli* to increase predation by its definitive host; however, in the presence of the microsporidium, this behavioral manipulation is reduced (Haine et al. 2005).

Based on these results, we expect to find a similar outcome in other systems where transmission of one parasite is blocked by a second parasite causing extensive damage to their shared host. This type of interaction occurs between the monarch

butterfly (*Danaus plexippus*) and two of its most common parasites, the virulent protozoan *Ophryocystis elektroscirrha* and the lethal parasitoid fly *Lespesia archippivora*. Transmission of the protozoan *O. elektroscirrha* occurs exclusively through the transfer of spores from adult butterflies to larvae, and hence depends on the survival of infected monarchs to the adult stage (McLaughlin and Myers 1970). In contrast, the fly *L. archippivora* lays eggs onto monarch larvae, after which the eggs hatch and the maggots penetrate the larvae, consume them from the inside out, and emerge at the monarchs' pre-pupal or pupal stage, killing the monarchs in the process. These differences in life cycle would suggest that *L. archippivora* prevents transmission of *O. elektroscirrha* when it kills the host during the pre-adult stages. However, as an alternative hypothesis, it is possible that the protozoan parasite reduces the infection success of the lethal fly, and thereby alleviates its own fitness loss as well as that of the host. Here, we address these hypotheses by studying single and multiple infections of these two parasite species in laboratory experiments.

Methods

The Host-Parasites System

The protozoan *O. elektroscirrha* is a parasite that infects monarch butterflies across their natural range (McLaughlin and Myers 1970; Leong et al. 1997; Altizer et al. 2000). Infection occurs when adult female butterflies shed parasite spores on their eggs or milkweed foliage during oviposition, after which these spores are ingested by hatching larvae; as a result, transmission occurs often from mother to offspring but may also occur from adult butterflies to unrelated larvae (McLaughlin and Myers 1970; Altizer et al.

2004). Upon ingestion, parasite spores lyse in the larval gut to release sporozoites that invade the hypoderm, replicate asexually, and then form sexual spores on the outside of the developing butterfly. The production of these spores reduces monarch adult lifespan, mating ability and fecundity (Altizer et al. 2004; De Roode et al. 2007; De Roode et al. 2009). *O. elektroscirrha* does not continue to replicate once the adult monarch emerges, so the number of spores present on a newly emerged adult monarch represents the entire transmission potential of that infection. Importantly, *O. elektroscirrha* requires its host to reach the adult stage, when the host can lay eggs and transfer these spores to hatching caterpillars (De Roode et al. 2009).

The parasitoid fly (*L. archippivora*) co-occurs with *O. elekroscirrha* in the monarch populations inhabiting North America and Hawaii (Etchegaray and Nishida 1975; Leong et al. 1997; Altizer et al. 2000; Oberhauser et al. 2007). Female *L. archippivora* deposit their eggs on the cuticle of larval monarchs and when these eggs hatch, the maggots burrow into the host. Previous work on *L. archippivora* indicates that this species typically limits its brood size to one to three offspring per host (Etchegaray and Nishida 1975; Stapel et al. 1997; Oberhauser et al. 2007). When the host reaches its final instar or soon after it pupates, the fly maggots kill the host as they emerge to form pupae (Stapel et al. 1997; Stireman et al. 2006; Oberhauser et al. 2007). Thus, in contrast with the protozoan *O. elektroscirrha*, the parasitoid fly *L. archippivora* kills the host during the pre-adult stage.

Host and Parasite Collection and Care

This study consisted of two experiments conducted approximately one year apart. All of the monarchs belonged to the migratory eastern North American population, and all of the parasites were isolated from wild-caught monarchs belonging to this population. Thus, both experiments used sympatric host and parasite combinations. The protozoan parasite used for both studies (denoted C1E3-P3-1) was isolated from a monarch belonging to the eastern North American population (Cape May, New Jersey, 2001). A clonal line of the parasite was used to prevent mixed-genotype infections and to provide consistency in protozoan parasite genotype across experiments. To establish the line, a monarch was inoculated with a single spore to produce an infection with genetically identical parasites. The parasite was then passaged through three monarchs and held at 12°C between infections. Experimental designs were similar, except for some minor differences as outlined below.

The monarchs used in this study were the grand-progeny of monarchs collected either as overwintering adults in Central Mexico (March 2008; experiment 1) or as larvae in Georgia, USA (September 2009; experiment 2). Unrelated females and males were mated in a design that produced independent families of half- or full siblings. Adults were held in mesh cages at 26 °C on a 16L:8D cycle and fed with a 10% honey water solution. After mating, males were removed and females were provided with greenhouse-grown *Asclepias incarnata* (swamp milkweed) for ovipositing. The plants were checked daily and those with eggs were replaced with fresh plants. Hatching larvae were pooled by hatch date and family, and transferred into plastic containers (739 mL) with fresh *A. incarnata* cuttings. Individuals from each of the families were randomly distributed across all treatment groups for both experiments.

The parasitoid flies used for this study came from a laboratory colony descended from maggots that emerged from monarchs collected as larvae in Ohio, USA (June 2008; experiment 1) or Georgia, USA (September 2009; experiment 2). Upon emergence from the monarch larvae, the maggots were transferred either into 1L plastic containers (experiment 1) or into a 50.8 cm x 27.9 cm x 33.0 cm glass terrarium fitted with a screened lid (experiment 2). When adult flies eclosed, they were provided with sugar, dehydrated milk and a moistened cotton ball for water. To establish and maintain the colony, flies were given ≥ 24 hours to mate, then provided with 3rd instar monarch larvae for ovipositing. Once the monarch larvae had been attacked by flies, they were pooled into plastic containers and provided with fresh *A. incarnata* cuttings. When maggots emerged from these monarch larvae, they were collected and added to the existing colony.

Experiment 1

Monarchs (n = 292) received one of four treatments: (1) uninfected control (n = 18); (2) exposure to only the protozoan parasite *O. elektroscirrha* (n = 38); (3) exposure to only the parasitoid fly *L. archippivora* (n = 117); and (4) exposure to both the protozoan parasite and the parasitoid fly (n = 119). Larger sample sizes were used for the monarchs that were exposed to the parasitoid fly so that differences in low survival rates could be detected, even in the presence of the highly lethal parasitoid.

To infect monarchs with the protozoan parasite, 2-day old 2nd instar monarch larvae were placed in individual 10 cm petri dishes with moist filter paper and a disc of *A. incarnata* leaf (0.8 cm in diameter) on which 10 *O. elektroscirrha* spores had been deposited manually (De Roode et al. 2009); uninfected controls and monarchs that were

infected with the parasitoid fly only were fed a leaf disk without parasite spores. To infect monarchs with the parasitoid fly, we placed 4-day old 3rd instar monarch larvae in the plastic containers housing the parasitoid flies and observed until a fly was seen approaching a larva to perform ovipositing behavior. At this point the larva was removed and examined under a dissecting microscope for fly eggs. If fly eggs were not observed, the larva was returned to the container with flies. Once eggs were visible, the number of eggs present was recorded and the larva was not returned to the plastic container with the flies. In the multiple infection treatment, monarchs were first inoculated with the protozoan parasite (2 days post-hatching) and then exposed to the parasitoid (4 days post-hatching).

After treatment, monarch larvae were transferred into individual plastic containers covered with mesh tops. They were provided with fresh cuttings of greenhouse-grown *A. incarnata* in florist tubes. These containers were kept in a climate-controlled room (26 °C, 16L:8D) and checked daily. Fresh plant cuttings were added as needed. Monarch larvae that died prior to pupation were monitored for signs of parasitoid maggots. If maggots emerged, the number of maggots per host was recorded. If the monarch larvae survived to pupation, they were transferred into clean plastic containers and kept in the climate controlled room for an additional 7 days. Monarch pupae were also checked daily for signs of fly maggots. After 7 days, the monarch pupae were transferred to a separate room to prevent cross-contamination with spores from the protozoan parasite. When the adult monarchs eclosed, they were sexed and weighed and then transferred into individual glassine envelopes and kept at 12 °C. Adult monarchs were checked daily for mortality to measure post-eclosion longevity. This measure of lifespan provides a

combined index of adult monarch life span and starvation resistance and responds to parasite infection and increasing parasite numbers in a similar way as lifespan under more natural conditions (Crawley 2007).

Experiment 2

As with experiment 1, monarchs (n = 351) received one of four treatments: (1) uninfected control (n = 25); (2) exposure to only the protozoan parasite *O. elektroscirrha* (n = 32); (3) exposure to only the parasitoid fly *L. archippivora* (n=155); and (4) exposure to both the protozoan parasite and the parasitoid fly (n = 139). Again, larger sample sizes were used for groups exposed to the parasitoid fly to enable the detection of differences in the low survival rates of fly-exposed monarchs.

Procedures for experiment 2 were similar to those for experiment 1, except for the following. First, to infect monarchs with parasitoid flies, 3rd instar monarch larvae were placed in the terrarium housing the *L. archippivora* colony and observed until a fly was seen approaching a larva and performing characteristic ovipositing behavior, at which point the larva was removed from the terrarium. The larva was then examined under a dissecting microscope and the number of parasitoid fly eggs present recorded. Unlike experiment 1, larvae were not returned to the container with the flies even if there were no visible eggs. The results from experiment 1 had shown to us that we often missed eggs, as evidenced by the fact that some monarchs produced more maggots than the recorded number of eggs. This modified protocol was used to more accurately mimic the natural numbers of flies per infected monarch (in experiment 1 they exceeded those numbers). Second, larvae were reared as in experiment 1, except that *Asclepias curassavica*

(tropical milkweed) was used in addition to *A. incarnata* to feed later stage caterpillars; the use of a second species of milkweed has no effects on the protozoan parasite (De Roode et al unpublished data) and parasitoid fly post-inoculation (M. Solensky, unpublished data). The host plant species that was provided was randomly allocated across treatment groups. Third, if fly maggots emerged from monarch larvae or pupae, the number of maggots per host and the mass of each maggot once it pupated were recorded, after which the fly pupae were placed in individual plastic containers. If a fly eclosed from its pupa, it was recorded to measure the proportion that survived to adulthood. Adult flies were provided with sugar, dehydrated milk, and a moist cotton ball. Flies were checked daily for mortality to measure adult longevity. Fourth, after monarchs died, their bodies were vortexed and the protozoan parasite spore load was measured with a haemocytometer as described in De Roode et al (2007, 2008a). Since the protozoan does not replicate on the adult monarch, this was used as a measure of parasite replication and transmission potential.

Statistics

All analyses were carried out in R version 2.7.1 (R Development Core Team 2006). Logistic regression by Generalized Linear Model (GLM, binomial error distribution, logit link) was used to investigate the effect of treatment, number of parasitoid fly eggs, and experimental block (experiment 1 vs. 2) on the proportion of monarch hosts that survived to adulthood. A multiway Analysis of Variance (ANOVA) was used to analyze the effect of treatment and experimental block on monarch host longevity and in experiment 2 protozoan parasite spore load. Logistic regression (GLM, quasi-binomial distribution)

was used to assess the effect of treatment and experimental block on the proportion of monarch hosts that produced fly parasitoid maggots. A GLM with a quasi-Poisson error distribution was used to analyze the effect of treatment on the number of fly parasitoid maggots that emerged from monarch hosts. For the data from experiment 2, a Generalized Linear Mixed Model (GLMM) with a normal error distribution was used to analyze the effect of treatment (fixed effect) and monarch host (random effect) on fly parasitoid mass and longevity. An Analysis of Covariance (ANCOVA) was used to test for a relationship between fly parasitoid pupal mass, adult longevity, and for an effect of treatment on this relationship. Protozoan parasite load and monarch host longevity were Log_{10}-transformed prior to analyses and models were checked for homogeneity of variance by using the Fligner-Killeen test (Crawley 2007). In these analyses, treatment and experimental block were treated as categorical explanatory variables. Full models included treatment, experimental block and the interaction between them as explanatory variables. Minimal models were derived by removing model terms followed by model comparison. Only terms for which removal significantly ($P < 0.05$) reduced the explanatory power of the model were retained in the minimal model (Oliver et al. 2003; Scarborough et al. 2005; Vorburger et al. 2010).

Results

Monarch host larval survival and adult longevity

Parasitoid flies dramatically reduced the survival to adulthood of their monarch hosts, both in the presence of the protozoan parasite (Fig. 1; Odds Ratio (OR) = 25.0, 95% Confidence Interval (CI) = [24.1, 26.0], $p < 0.001$) and in single infections (OR = 41.9,

CI = [40.9, 42.9], $p < 0.001$). The protozoan parasite alone did not affect monarch host survival to adulthood; however, it did increase survival of monarchs that were also infected with the parasitoid fly (Fig. 1; OR = 1.8, CI = [1.28, 2.27], $p = 0.0239$). Only 12% and 17% (experiments 1 and 2 respectively) of monarchs survived to adulthood when infected with the parasitoid fly alone, but when the protozoan parasite was also present, survival increased to 18% and 27% (experiments 1 and 2 respectively).

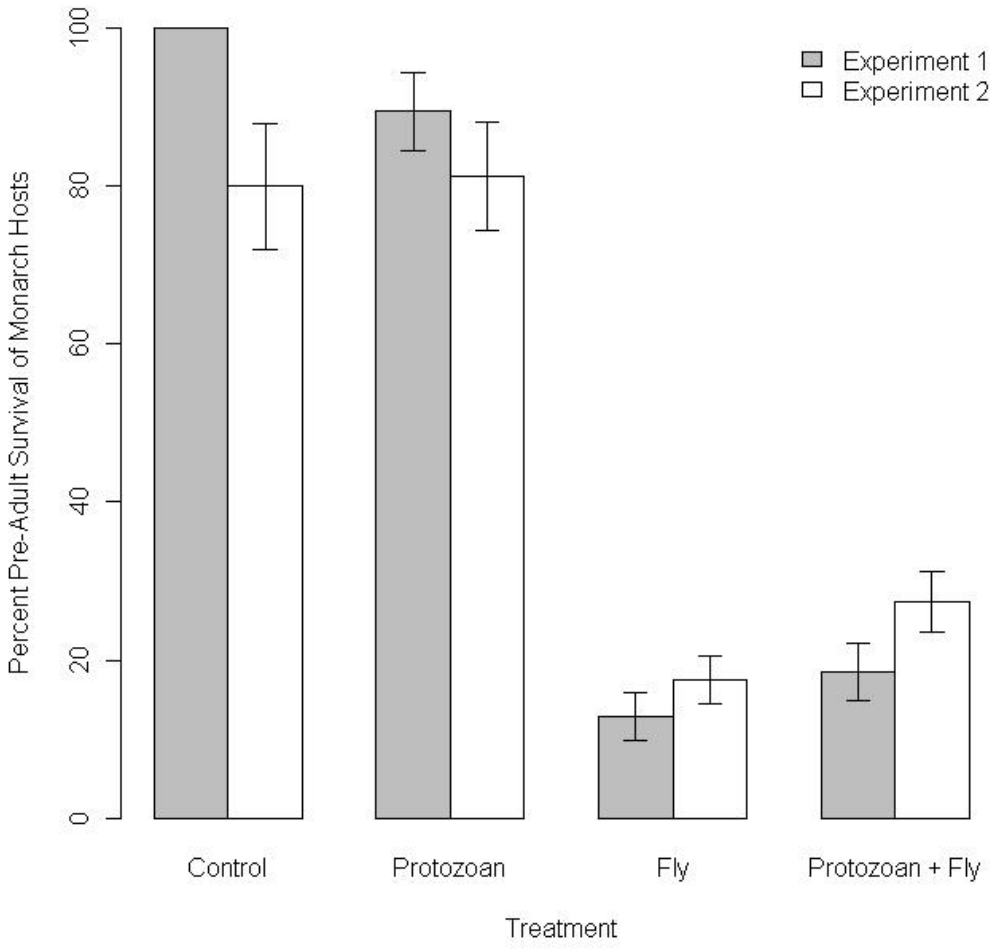

Figure 1. Monarch survival to adulthood by experiment and treatment group. The presence of the parasitoid fly resulted in a large decrease in survival for both the single and multiple infected monarchs, but there was a higher percent survival for monarchs

infected with the fly and the protozoan together compared to those that were infected with the fly only. Data are presented as percent survival ± SE.

The number of eggs laid by the parasitoid fly also had a significant effect on survival in monarchs that were exposed to the parasitoid fly (OR = 4.12, CI = [3.72, 4.52], $p < 0.001$), with higher numbers resulting in lower survival. There were no significant interactions between the number of eggs and the treatment group. Overall, monarch pre-adult survival differed significantly between experiments (OR = 1.9, CI = [1.34, 2.50], $p = 0.0281$) but there were no significant interactions between experiment and treatment or the number of eggs present.

Analysis of monarch host longevity was performed on the monarchs that survived to adulthood. For experiment 1, there were 89 surviving monarchs (18 of 18 in the control group, 34 of 38 in the protozoan only group, 15 of 117 in the fly only group, and 22 of 119 in the protozoan and fly group). For experiment 2, there were 111 surviving monarchs (20 of 25 in the control group, 26 of 32 in the protozoan only group, 27 of 155 in the fly only group, and 38 of 139 in the protozoan and fly group).

The presence of the protozoan parasite had a significant effect on mean longevity (Fig. 2; $F_{1, 185} = 695$, $p < 0.001$): both singly protozoan parasite-infected and co-infected monarchs lived much shorter as adults than control monarchs and monarchs that had survived single infection with the parasitoid fly. There was no significant effect of larval exposure to the parasitoid fly on adult longevity across all treatment groups ($F_{1, 186} = 1.2$, $p = 0.271$). A significant interaction between experiment and treatment ($F_{1, 185} = 62.0$, $p < 0.001$) arose from the fact that the protozoan parasite reduced adult monarch lifespan more strongly in experiment 1 than 2.

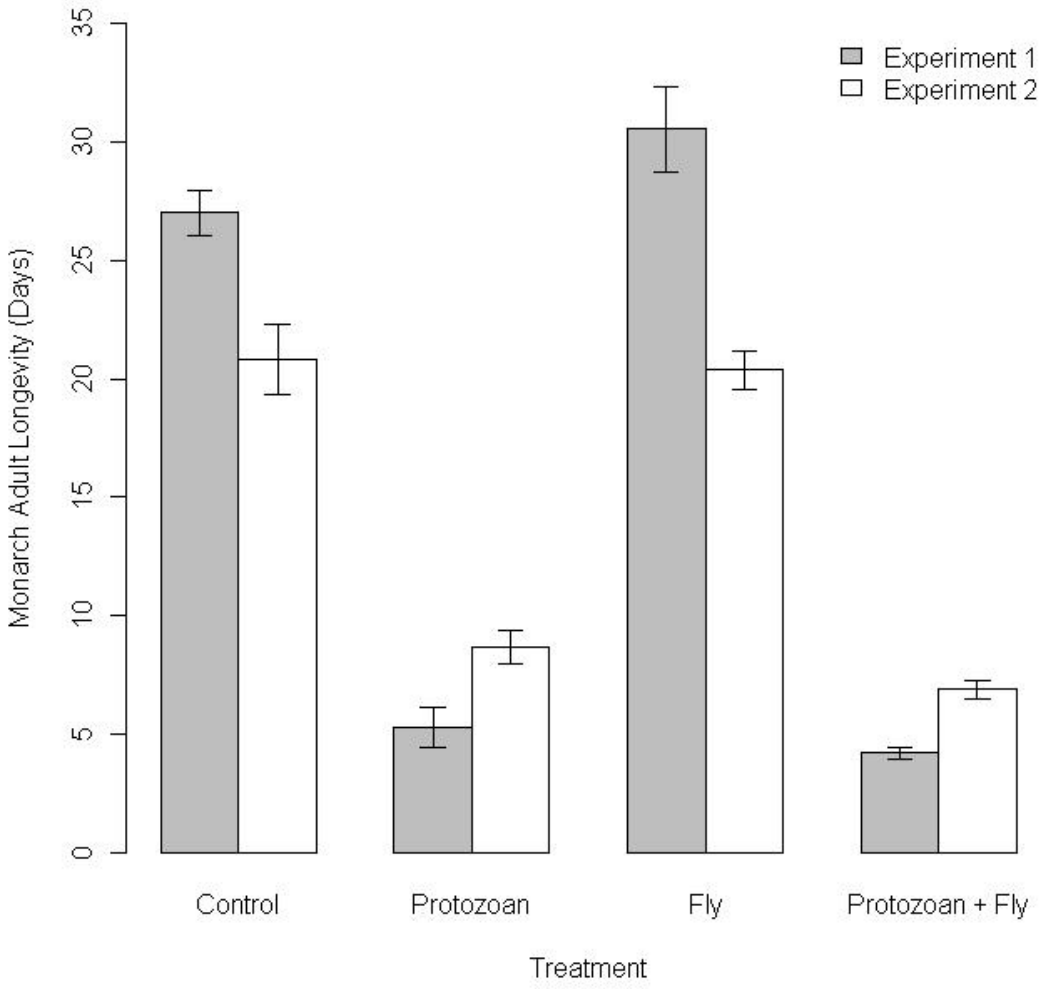

Figure 2 Mean adult longevity in days for monarchs that survived the larval stage, by experiment and treatment group. The presence of the protozoan parasite had a significant effect on host longevity in both single and multiple infection groups; however, in monarchs that survived attack by the parasitoid fly there was no additional decrease in adult longevity. Data are presented as mean adult longevity ± SE.

Protozoan parasite spore load

Analysis of the protozoan parasite spore load was carried out for the monarchs in experiment 2 that survived to adulthood (n = 64). All of the surviving monarchs that were inoculated with the protozoan parasite were infected. There was no significant difference

in the mean spore loads of monarchs that survived attack by the parasitoid fly compared to monarchs that had been exposed to the protozoan parasite only ($F_{1, 62} = 0.036$, $p = 0.85$).

Parasitoid fly emergence, pupal mass, and adult longevity

Although the protozoan parasite lowered the pre-adult mortality of monarchs infected with the parasitoid fly, this did not result in a significant reduction of parasitoid fly fitness. Thus, although slightly fewer fly-infected monarchs produced viable maggots when the protozoan parasite was present (Fig. 3; single infection vs. multiple infection: 82% vs. 78% in experiment 1 and 58% vs. 54% in experiment 2), these reductions were not significant (OR = 1.11, CI = [0.71, 1.52], p = 0.602). As expected (based on lower inoculation doses in experiment 2), fewer monarchs produced maggots in experiment 2 (OR = 2.45, CI = [1.51, 3.38], p = 0.0609). There was a significant interaction between number of eggs and experiment, indicating that the proportion of monarchs that produced maggots increased more quickly with increasing numbers of eggs in experiment 1 than experiment 2 (OR = 3.78, CI = [3.06, 4.50], $p < 0.001$). The interaction between treatment and experiment was not significant.

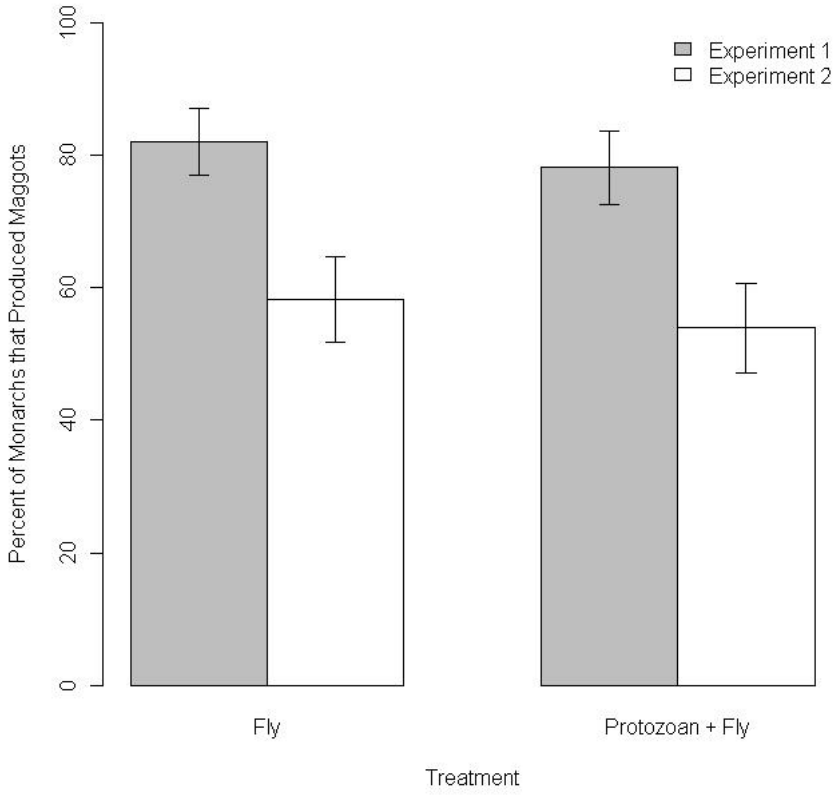

Figure 3 Percent of monarchs attacked by flies that produced maggots, by experiment and treatment group. There was a small trend with fewer monarchs producing maggots when also infected with the protozoan parasite, but this trend was not significant. Data are presented as percent of monarchs that produced at least one maggot ± SE.

As with the proportion of monarchs that produced maggots, the protozoan parasite did not significantly reduce the numbers of maggots that emerged from monarchs exposed to the parasitoid fly (Fig. 4; $F_{1, 525} = 0.423$, $p = 0.52$). As expected, however, monarchs produced fewer maggots in experiment 2 than experiment 1 ($F_{1, 526} = 231$, $p < 0.001$). There was no significant interaction between treatment and experiment.

Data on fly pupal mass and adult longevity were obtained for 249 flies (142 maggots from monarchs singly infected with the fly and 107 maggots from co-infected

monarchs); 3 maggots in the protozoan and fly multiple infection group were removed from analysis due to incomplete data.

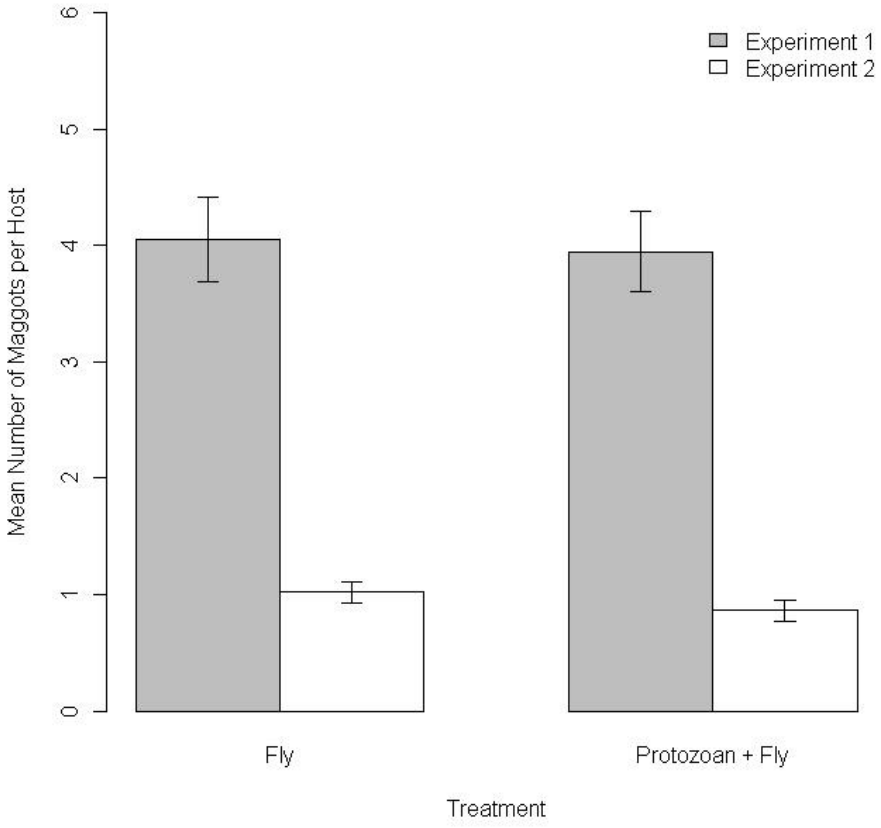

Figure 4 Mean number of maggots emerging per host for all monarchs exposed to the parasitoid, by experiment and treatment group. There was no significant difference between treatments in the mean number of maggots that emerged from monarchs. There was a significantly higher parasitoid burden in experiment 1 compared to experiment 2. Data is presented as mean number of maggots per host ± SE.

Comparison between fly single infections and protozoan and fly multiple infections showed that the protozoan parasite had no effect on the mean fly pupal mass (mean ± SE; 26.2 ± 1.1 and 27.0 ± 1.2 mg in single and multiple infections respectively; d.f. = 1, p = 0.851), on the proportion of fly pupae that successfully eclosed as adults (73.9% and 78.8% from single and multiple infection respectively; $X^2 = 0.517$, d.f.=1, p = 0.472), or

on the adult longevity of flies that eclosed successfully (18 ± 1 days for single and multiple infections; d.f. = 1, p = 0.83). Higher fly pupal mass resulted in greater fly adult longevity (Fig. 5; $F_{1,185} = 22.4$, $R^2 = 0.103$, $p < 0.001$) but there was no significant interaction with treatment group.

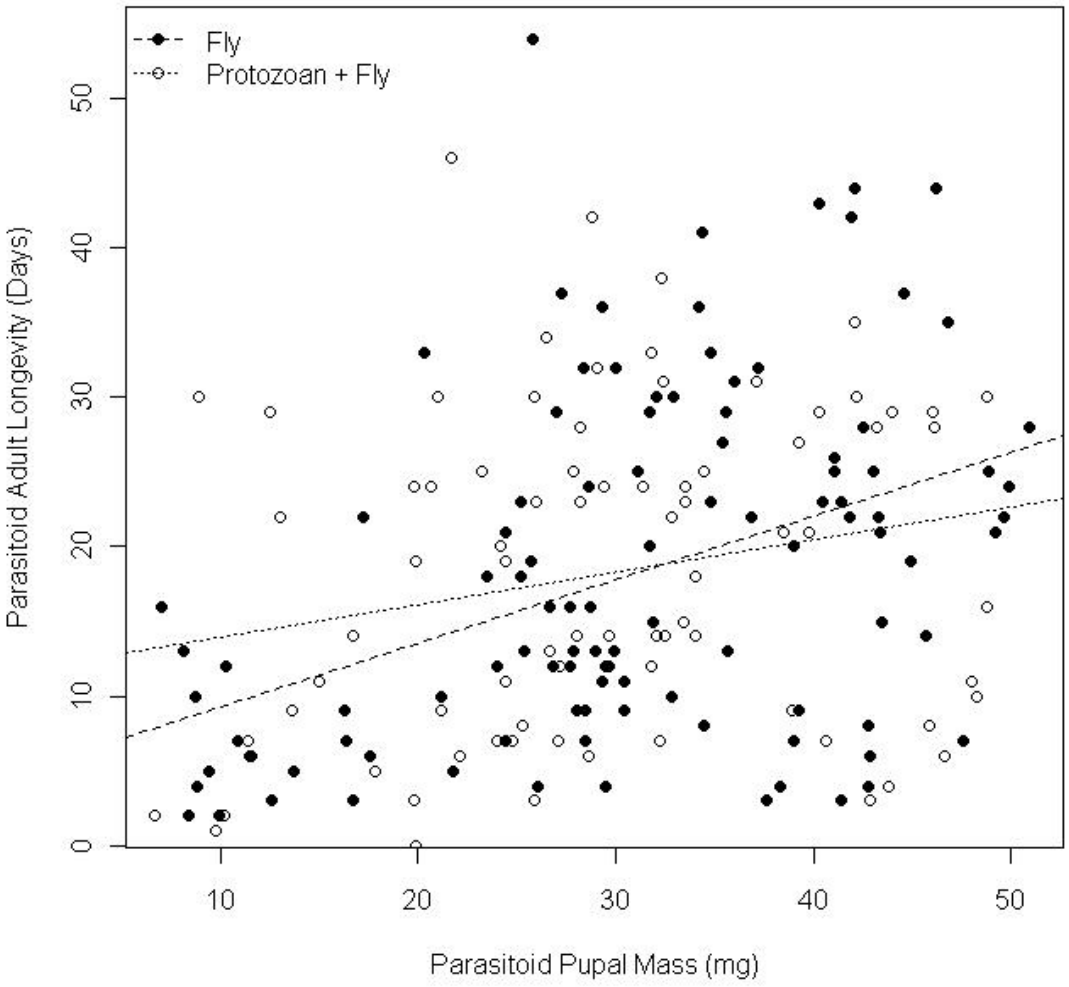

Figure 5 Relationship between parasitoid fly pupal mass and parasitoid adult longevity; experiment 2 only. Data points represent individual animals. There was a significant positive relationship between pupal mass and longevity; however, this relationship was not significantly different between single and multiple infection groups.

Discussion

Our results clearly demonstrate that infection of hosts with multiple parasite species may have important consequences for host and parasite fitness. Overall, the parasitoid fly *Lespesia archippivora* had strong detrimental effects on its monarch host as well as on the co-occurring protozoan parasite *Ophryocystis elektroscirrha*. *L. archippivora* caused dramatic pre-adult mortality of monarch butterflies and thereby strongly reduced *O. elektroscirrha*'s fitness as the latter parasite requires the monarch to reach adulthood for its transmission. However, despite the parasitoid's overwhelming effects, we found that the protozoan parasite reduced the mortality caused by the parasitoid fly, with more monarchs surviving multiple infection with the protozoan and fly than infection with the fly alone (Fig. 1). This effect is beneficial to the host and resembles the protective effects that mutualists and commensals can confer. For example, previous research has shown that mutualistic bacterial symbionts of aphids can protect their host against parasitoid wasps and pathogenic fungi (Oliver et al. 2003; Scarborough et al. 2005; Vorburger et al. 2010), that *Wolbachia* bacteria can protect *Drospophila melanogaster* against viral infections (Hedges et al. 2008) and that *Spiroplasma* bacteria confer protection against a sterilizing nematode in *D. neotestacea* (Jaenike et al. 2010). Like symbionts, transmission of the monarch's protozoan parasite depends on the survival of its host but unlike these bacterial symbionts, *O. elektroscirrha* is highly virulent to monarchs, reducing adult longevity, mating ability, fecundity and flight ability (De Roode et al. 2007; De Roode et al. 2008b; De Roode et al. 2009). Thus, our results suggest that infection with a virulent parasite can be beneficial for a host when it confers protection against a parasite that is even more detrimental. However, this protection appears to be weaker than that provided by beneficial symbionts, perhaps because parasites face different constraints than

symbionts. For instance, it is likely that a beneficial symbiont and host cooperate to resist lethal parasitoids; in contrast, although it may be beneficial to one parasite to reduce the virulence induced by another, it is in the host's interest to resist both.

The increased survival of monarchs infected with both parasites is not only beneficial to the host, but also to the protozoan parasite, suggesting that this effect may be an adaptive parasite trait. Parasites are well known to change the phenotypes of their hosts in ways that serve a specific adaptive function for the parasite (Moore 2002; Thomas et al. 2005; Lefèvre et al. 2009; Poulin 2010). For example, parasites often modify the behaviors of their host to prevent infection with a competing parasite (Brodeur and McNeil 1992), to prevent predation (Grosman et al. 2008), or to increase transmission to the definitive host (Lagrue et al. 2007). Based on these studies, it is possible that the increase in host survival that we observed may also serve an adaptive function for the protozoan parasite due to the protozoan parasite's requirement that the monarch host survive to adulthood for transmission to occur. If this is the case, we would expect that the parasite's ability to increase host survival is selected for in populations where the parasitoid occurs and is highly prevalent. Since this study tested the effect of a single genotype of the protozoan parasite, future experiments are necessary to determine if there is variation among protozoan parasite genotypes in their protective effect. However since all genotypes of the protozoan parasite require host survival to the adult stage for transmission to occur, we expect to see a similar effect with other parasite genotypes.

One challenge to understanding the protozoan parasite's protective effect will be to explain how the protozoan reduced monarch pre-adult mortality without also affecting

the parasitoid fly's fitness. Although the protozoan reduced the mortality of fly-infected monarchs (Fig. 1), this did not reduce the proportion of fly-infected monarchs that produced maggots (Fig. 3), nor did it reduce the average number of maggots produced per monarch (Fig. 4). It is known that in some infections, fly maggots do not develop successfully into pupae, but still end up killing the monarch butterfly larva (Oberhauser et al 2007). This finding, in combination with our results, suggests that the protozoan parasite reduced monarch mortality caused by unsuccessful, rather than successful, parasitoid flies. There are two potential explanations for such a scenario. First, the protozoan parasite may enhance the monarch's clearance of dying parasitoids inside its body. Previous research on multiple infections has suggested that parasites can limit the growth and transmission of a competing parasite by eliciting a host immune response (Lello et al. 2004; Pedersen and Fenton 2006; Råberg et al. 2006; Meister et al. 2009), and it is possible that the protozoan parasite increases the immune response against the fly parasitoid. It is certainly likely that the monarch host mounts an immune response against the fly since in our experiments there were several cases in which monarchs were infected with the fly, yet did not produce any maggots. As an alternative explanation, it is possible that the protozoan parasite increases host survival by dampening the host immune response, and that this reduced immune response results in a lower amount of immunopathology. Immunopathology occurs in many vertebrate and invertebrate species (Shi et al. 2001; Brandt et al. 2004; Graham et al. 2005; Sadd and Siva-Jothy 2006) and may be especially relevant in systems where an insect host must defend itself against insect parasites, such as parasitoid flies. If the parasitoid fly elicits a monarch immune response that damages both monarch and fly, and if the protozoan parasite decreases this

response, then this may result in increased host survival. Clearly, we must advance our understanding of the monarch's immune system to test these hypotheses.

Our results showed that multiple infection with the protozoan and parasitoid fly affected host and parasite fitness only on the basis of parasite interactions in the monarch's larval stage, and that these interactions did not lead to further effects during the adult stage. From the monarch host's perspective, adult longevity was not significantly different between uninfected monarchs and monarchs that survived attack by the parasitoid fly, and there was no difference in longevity between monarchs infected with the protozoan alone or those who were also exposed to the parasitoid fly. Thus, the parasitoid fly does not appear to have a long-lasting effect on the monarch if the monarch is able to successfully defend itself against fly infection.

As for the protozoan parasite, infecting a host that was later infected with the parasitoid fly severely reduced its fitness, since most monarchs were killed before they reached adulthood and transmitted the protozoan. However, we found no significant differences between the mean spore loads of surviving monarchs infected only with the protozoan compared to monarchs that were also attacked by the parasitoid fly. Spore load is a measure of replication for the protozoan and it is also associated with virulence and transmission probability, which makes it an appropriate measure of parasite fitness (De Roode et al. 2008b). These results further support the conclusion that if the monarch succeeds in defending itself against the parasitoid, there are no long-term costs to either the host or the protozoan parasite resulting from exposure to the parasitoid fly. Finally, we also found no evidence for an effect of multiple infection on the parasitoid fly beyond its development inside of the host. When we compared the flies that developed in the

presence of the protozoan to those that developed alone, we found no significant difference in the pupal mass of the flies, the proportion of flies that successfully eclosed or the longevity of the flies that survived to adulthood. We did find a positive correlation between pupal mass and longevity in the parasitoid fly, but this relationship did not differ between flies that developed from monarchs infected or not infected with the protozoan parasite (Fig. 5). Thus, while the protozoan parasite can reduce the host mortality caused by exposure to the parasitoid fly, it does not appear to do so in a way that affects any of the fly fitness components that we measured.

Overall, our results underscore the need to consider infection with multiple parasites as a major determinant of parasite and host fitness, particularly in systems where one parasite's success results in a loss of transmission of another one. We emphasize the importance of the host and parasite life cycles and biological characteristics in determining how multiple infection differs from single infection. Finally we suggest that in systems where one parasite is extremely deadly, the host may actually benefit from a second, more benign parasite if that parasite can increase its own transmission by increasing host survival. As such, our results support the view that parasitism is a context-dependent phenomenon (Michalakis et al. 1992; Thomas et al. 2000; Vale et al. 2008a; Fellous and Salvaudon 2009; Wolinska and King 2009), and that parasites can act as mutualists in the presence of more deadly natural enemies.

Chapter 5

Summary and conclusions

5.1 Local adaptation

The first aim of this dissertation was to test for local adaptation and characterize infection phenotypes in three populations of monarch butterflies and the protozoan parasite *O. elektroscirrha*. This aim is addressed in chapter 2, using reciprocal cross-infection experiments with parasites and monarchs isolated from eastern North American, Hawaii, and South Florida. Specifically, I quantified infection probability, parasite burden, and host longevity in monarchs infected either with sympatric or allopatric parasite genotypes. I found that sympatric host-parasite combinations did not differ consistently from allopatric pairs, such that sympatric parasites were on average no better than allopatric parasites at infecting hosts across all three populations. However, I did find that Hawaiian parasites were better able to infect and replicate in their more resistant local hosts when compared to eastern monarchs and parasites, and Hawaiian parasites had a larger effect on host fitness. These results are consistent with increased host resistance selecting for increased parasite virulence, as predicted by previous theoretical work (Gandon and Michalakis 2000; Gandon et al. 2001; De Roode et al. 2011a). Although S. Florida monarchs were also more resistant to infection when compared to eastern monarchs, I did not find a significant difference between populations in the parasites. When I examined variation in the relationship between parasite burden and host fitness (i.e. tolerance) in the different populations, and in sympatric versus allopatric infections, I found that the Hawaiian population of monarchs were more tolerant to infection. This indicates that highly resistant populations can also be tolerant. Because of positive epidemiological

feedback, increased parasite prevalence should be associated with increased host tolerance (Boots and Bowers 1999; Roy and Kirchner 2000; Miller et al. 2005) but despite an extremely high parasite prevalence (Altizer et al. 2000), I did not find a difference in tolerance in the S. Florida population. In both population comparisons, I did not find an effect of sympatry on tolerance, indicating that host populations are not more tolerant of local parasites. Because local adaptation in tolerance has not been previously examined, additional studies in other host-parasite systems will be necessary to determine whether this is a general result.

The experiments described in chapter 2 are examples of reciprocal cross-infection experiments under uniform laboratory conditions, which is a typical approach to studying local adaptation in host-parasite systems. Underlying these studies is the assumption that the interaction between host and parasite genotypes leads to coevolution, which leads to increasing specialization of parasites to locally common host genotypes. However, as demonstrated in chapter 2, and in a substantial number of other studies (Kaltz and Shykoff 1998; Greischar and Koskella 2007), this approach often does not yield the expected results.

To understand why empirical studies fail to detect parasite local adaptation, there are generally two approaches. The first approach focuses on the population genetics of host and parasite populations, particularly in relation to evolutionary potential. For example, relative migration rate is expect to be a predictor of local adaptation because while very high migration rates can homogenize populations, lower levels of migration can introduce and maintain genetic variation which increases evolutionary potential (Gandon et al. 1996; Gandon et al. 1998; Lively 1999; Gandon and Michalakis 2002;

Morgan et al. 2005; Hoeksema and Forde 2008). Consistent with theoretical predictions, parasite migration was necessary for local adaptation in bacteriophages infecting laboratory populations of the bacterium *Pseudomonas fluorescens* and, in the absence of migration, parasites tended to be maladapted to local hosts (Morgan et al. 2005). The focus on evolutionary potential exists because greater evolvability predicts which species has the advantage in a coevolutionary arms race. Because parasites tend to have shorter generation times, larger population sizes, and higher rates of mutation and migration, they are assumed to have greater evolutionary potential compared to host species. Thus parasites are expected to be locally adapted to coevolved host genotypes more frequently than hosts are adapted to their local parasites (Gandon and Michalakis 2002). Caution is required however, as host and parasite genotypes are expected to cycle in such a coevolutionary arms race. As a result, local adaptation may be an average phenomenon, requiring repeated sampling to detect (Nee 1989; Morand et al. 1996; Dybdahl and Lively 1998; Kaltz and Shykoff 1998; Lively 1999).

A second approach to understanding complex patterns of local adaptation is to integrate the environmental context of infection. Although this approach is less well developed theoretically, there is recent, promising empirical evidence to support it. For example, in experimentally coevolved bacterium and phage populations, the nutrient concentration of the media had a strong and predictable effect on infection and the signal of phage local adaptation was strongest when comparing populations evolved on different media (Pascua et al. 2012). In a similar experiment utilizing wild populations of bacterium and phage isolated from tree leaves, phage were generally more infective in bacteria isolated from the same tree; however, phage isolated from within the leaves were

more infective in local bacteria than phages isolated from the leaf surface. These results suggest that the within-leaf environment is more conducive to the evolution of local adaptation (Koskella et al. 2011). In general, such studies demonstrate the potential for local ecology to produce coevolutionary "hot spots" where the intensity of selection varies spatially (Thompson 1999; Gomulkiewicz et al. 2000) which could explain why some populations show signatures of local adaptation while others do not (Lively 1999).

Given that environmental variation affects local adaptation, the next logical question is how to incorporate relevant variation in future studies. One approach is to use transplant experiments which captures the environment as a whole, instead of using reciprocal cross-infection experiments. However in addition to being logistically challenging when studying mobile species, this approach may fail because the stochasticity of field-based experiments decreased sensitivity to detect local adaptation. For example in ribwort plantain (*Plantago lanceolata*) infected with the fungus (*Podosphaera plantaginis*) local adaptation was detected in a laboratory based reciprocal cross-infection experiment but not in a field based transplant experiment (Laine 2007). A good compromise may be to incorporate critical environmental variables into more controlled laboratory based experiments (Nuismer and Gandon 2008). Using such an approach, further studies of ribwort plantain and its fungal parasite found that both the strength and direction of local adaptation depended on the experimentally controlled temperature (Laine 2008). Another approach could be to pair laboratory studies with field experiments. For example, laboratory-based studies of wheat cultivars infected with the fungus *Puccinia striiformis* f.sp. *tritici* found a significant interaction between the parasite population of origin and temperature. Concurrent field studies in northern and

southern France found that southern fungal strains outcompeted northern strains in all cases, but this effect was particularly pronounced in the southern field sites. Combined, these experiments provide support for the hypothesis that the fungal parasite can become locally adapted to climate, as well as to host genotypes (Mboup et al. 2012).

5.2 Environmental variability

As discussed in the previous section, there is no consistent signal of local adaptation in the monarch-parasite system. The absence of local adaptation in host-parasite systems is a topic of broad interest and there are a number ways to approach this topic, including assessing the role of environmental variation in host-parasite interactions. With this approach, identifying important environmental variables is the first step for any approach aimed at incorporating ecology into local adaptation studies. Therefore, the second aim of this dissertation was to identify specific environmental conditions that strongly affect host and parasite fitness in the monarch-parasite system. The results I presented in chapter 3 clearly indicate that food plant is an important environmental variable in the monarch system. Specifically, my results show that monarch butterflies experience different levels of tolerance to parasitism depending on the species of milkweed that they feed on, with some milkweed species providing over two-fold greater tolerance than others. Resistance was also affected by milkweed species, consistent with previous results (De Roode et al. 2008a), but there was no relationship between milkweed-conferred resistance and tolerance. Together, these results demonstrate that environmental factors – such as interacting species in ecological food webs – are important drivers of disease resistance and tolerance. The importance of food plant in the

monarch-parasite system is entirely consistent with the relevant literature, which shows that food plant generally modulate the interactions between herbivores and their parasites (Cory and Hoover 2006). One study of nucleopolyhedrovirus in the western tent caterpillar (*Malacosoma californicum pluviale*) found that speed of kill varied on different food plants and in two of the three sampled populations, viruses killed hosts more quickly when reared on the local plant species (Cory and Myers 2004). The relationship between speed of kill, virus productivity, and ultimately transmission was not predictable in this study however, and thus it is unclear if the increased rate of kill on the local food species truly indicates adaptation by the virus.

In the monarch-parasite system, the relationship between measures such as adult monarch longevity, spore load, transmission, and host and parasite fitness, has been well documented (De Roode et al. 2007; De Roode et al. 2008b; De Roode and Altizer 2009; De Roode et al. 2009; De Roode et al. 2011a). Given that the range of many milkweed species is already characterized (Woodson 1954; Hickman 1993), and given that chemical analyses can reveal the milkweed species used by monarchs as larval food plants (e.g. Malcolm and Brower 1989; Knight and Brower 2009), it is plausible to investigate the effect of milkweed species on local adaptation. For example, sampling from wild monarch and parasite populations would allow us to test whether there is an association between plant use and parasite virulence. If monarch populations feeding on antiparasitic plant species are also infected with more virulent parasites, this would support the hypothesis that limiting within-host parasite replication will select for increasing parasite virulence (Gandon and Michalakis 2000; De Roode et al. 2011a). This could also be tested experimentally, by passaging parasites for several generations

through monarchs reared on more or less antiparasitic food plant species. Additionally, reciprocal cross-infection experiments in the laboratory could incorporate sympatric or allopatric species of milkweed, which would test the hypothesis that monarchs and their parasites are locally adapted to food plants rather than to each other.

Also presented in chapter 3 are analyses of milkweed chemistry which suggest that milkweed cardenolides are responsible for the effect of food plant species. Specifically, I show that infected monarchs obtain the highest fitness when reared on milkweeds with an intermediate concentration, diversity, and polarity of the toxic secondary plant chemicals known as cardenolides. Conversely, uninfected monarchs obtain the highest fitness when reared on milkweeds with low cardenolide concentrations. In the future, this research should be extended through experimental manipulation of cardenolide concentration and diversity. This could be achieved either by inducing cardenolide production in the plant via herbivore damage, or by adding cardenolides directly to the leaves consumed by monarchs. Such studies are highly relevant to previous research that demonstrated latitudinal differences in cardenolide toxins production by milkweeds (Rasmann and Agrawal 2011). It is also hypothesized that parasitoid diversity is unusually low in the tropics, because plants in this region produce more toxic secondary compounds and thus the herbivorous hosts of parasitoids are also more toxic (Gauld and Gaston 1992). Given that my results show cardenolide content is strongly indicated as a driver of the milkweed species effect, it is reasonable that geographic patterns in plant chemistry could explain geographic patterns of host and parasite coevolution in this system.

Lastly, in chapter 4 I studied the effect of another ecological interaction on infection outcome, namely coinfection. I showed that when monarch butterfly larvae are inoculated with the virulent protozoan parasite *O. elektroscirrha* and then attacked by the lethal parasitoid fly *Lespesia archippivora*, survival is higher than when the larvae are exposed to the parasitoid only. The findings demonstrate how a non-lethal parasite can play a protective role for its host, depending on the presence of other parasites. In the context of this dissertation, these results again emphasize that infection occurs within an ecosystem. Interestingly, my results show that increased monarch survival does not result in decreased survival of larval parasitoids, which suggests that *O. elektroscirrha* is preventing mortality in monarchs infected with otherwise unsuccessful parasitoids. Given this observation, the effect of *O. elektroscirrha* during coinfection may be due to the suppression of a damaging host immune response. Such suppression could be a mechanism through which *O. elektroscirrha* evades the host immune response and the observed protective effect could be merely a byproduct of this function. Alternatively, because *O. elektroscirrha* requires host survival for transmission, the observed protective effect could represent an adaptive trait in the protozoan parasite that is selected for in the presence of the parasitoid. If the latter hypothesis is correct, then future studies in populations with high parasitoid prevalence should reveal increased protection against host mortality when compared to populations where the parasitoid is absent. Additionally, the ability to protect the monarch host may come at a cost to other infection related traits, such that less protective *O. elektroscirrha* may outcompete more protective *O. elektroscirrha* in the absence of the parasitoid fly.

To summarize the first aim of this dissertation in two words, "populations differ" and likewise, the second aim can be summarized as "environment matters". In combination, these four words provide a solid foundation for incorporating environmental variation in future local adaptation studies. Such studies are crucial, because they will allow us to better understand how and why there is variation in infection related traits both within and between populations. Selection on infection related traits can also potentially affect other aspect of an organisms' phenotype, for example through trade-offs or pleiotropy. Once again, this emphasizes that parasitism is unusual in both the depth and breadth of its impact and thus, studies such as the ones discussed in this dissertation have the potential to yield results that are widely applicable towards understanding the ecology and evolution of all species.

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