Plant Quality Mediates the Response of Disease to Global Environmental Change

By

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Dedication

To my family: Christa, Bill and Kayleigh.
Thank you for always picking up the phone.

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Abstract

A major challenge for ecology rests in understanding how direct and indirect effects of abiotic and biotic drivers combine to influence organisms under rapid environmental change. The local environment of both hosts and parasites can have profound impacts on disease dynamics, but the major mechanisms underlying these changes remain largely unresolved. In this dissertation, I combine a series of manipulative experiments to assess the effects of an ongoing and pervasive driver of global environmental change, elevated CO_2, on a plant-phytophagous host-parasite system. In Chapter II, I investigated the effects of elevated CO_2 on the defensive and nutritional chemistry of milkweeds and the subsequent impacts of those phytochemical changes on monarch tolerance and parasite virulence. I found that high-cardenolide milkweeds lost their medicinal properties under elevated CO_2; monarch tolerance to infection decreased, and parasite virulence increased. Declines in foliar medicinal quality were associated with declines in foliar concentrations of lipophilic cardenolides. In Chapter III, I examined how those same phytochemical changes induced by elevated CO_2 influence the defensive phenotype of monarchs against predation, e.g. toxin sequestration and flight ability. I found that monarchs maintained the concentration and composition of cardenolides that they sequestered despite changes in the phytochemistry of milkweed under elevated CO_2. Additionally, feeding on high cardenolide milkweed was associated with the formation of rounder, thinner wings, which are less efficient at gliding flight. In Chapter IV, I evaluated changes in monarch cellular and humoral immunity in response to phytochemical shifts induced by elevated CO_2. I found that the immune enzyme activity of early-instar monarchs declined under parasite infection but was "rescued" by consuming foliage grown under elevated CO_2. Additionally, infection and a diet of foliage from elevated CO_2 increased the hemocyte concentrations of early-instar monarchs. In late-instar monarchs, the immune response against parasitoids declined on "medicinal" milkweed, suggesting a potential tradeoff between resistance against parasitoids and resistance against agents of disease. Finally, in Chapter V, I examined how elevated CO_2 might alter plant resistance traits and regrowth tolerance and the subsequent relationship between these defense

strategies. I found that elevated CO_2 altered the resistance of regrowth foliage in a species-specific manner. However, the tradeoff between resistance and regrowth tolerance varied only among milkweed species. Taken together, my research illustrates that anthropogenic changes in abiotic and biotic factors operate in complex combinations, and at multiple scales, to influence the outcome of host-parasite interactions in our changing world.

Chapter 1 : Introduction

1.1 Global Environmental Change and Ecological Interactions

Species interactions are intricately connected to the surrounding environment. Both abiotic and biotic conditions influence the physiology, phenology, and behavior of organisms with important implications for population dynamics and trophic interactions (Tylianakis *et al.* 2007) Critically, these environmental conditions are changing at an accelerating rate.

Over the next 100 years, Earth surface temperatures are projected to rise nearly 4°C, and precipitation patterns will become more variable (IPCC 2013). Increases in atmospheric greenhouse gas concentrations like methane, nitrous oxide and carbon dioxide have driven much of this recent warming and combine with other effects of human activity to alter biogeochemical cycles. The rate at which atmospheric CO_2 concentrations are increasing remains unrivaled in the last 800,000 years and expected to rise from roughly 400 ppm today to 700 ppm over the next 100 years. Additionally the anthropogenic fixation and deposition of reactive nitrogen will continue to accelerate (Galloway *et al.* 2008) along with increased habitat loss and fragmentation (Sala *et al.* 2000).

Because ecological interactions are context dependent, global environmental change alters the composition of communities through both direct and indirect effects. Variable environmental factors directly impact the phenotype of some organisms (Parmesan & Yohe 2003; Altizer *et al.* 2013; Chu *et al.* 2016), which, in turn, may generate cascading indirect effects on other community members (Parmesan 2006; Gilman 2017; Gunderson *et al.* 2017). For example, reactive nitrogen deposition enhances the growth and floral display of an Andean Aster, which, in turn, increases pollinator visitation rate and overall fitness (Muñoz *et al.* 2005). Conversely, the same global change driver (N deposition) reduces the performance of another member of the Andean plant community because N deposition reduces the prevalence of

beneficial fungal mutualists in the surrounding soil (Dean *et al.* 2014). Therefore, a major challenge for ecology rests in understanding how direct and indirect effects of abiotic and biotic drivers combine to influence organisms under environmental change (Tylianakis *et al.* 2008; Gilman *et al.* 2010; Gunderson *et al.* 2017).

Host-parasite interactions are ubiquitous within ecosystems and critical to their function. Diseases influence the abundance, distribution, evolution and extinction of not only the hosts and parasites, but other members of the surrounding community (Hudson *et al.* 1998, 2002; Hochachka & Dhondt 2000; Torchin *et al.* 2003; De Castro & Bolker 2005). Therefore, understanding the factors that govern the strength of host-parasite interactions is critical to predicting future community composition and ecosystem function.

Our knowledge of disease stems from a long history of pairwise studies centered on interactions between hosts and their parasites. However, the abiotic and biotic environment may profoundly shape the outcome of these dynamics. Environmental conditions affect both the host (e.g. density, immunity, resistance, etc.) and the parasite (e.g. density, replication and transmission, etc.), with important consequences for disease dynamics (Harvell *et al.* 2002; Keesing *et al.* 2006; Wolinska & King 2009; Altizer *et al.* 2013; Civitello *et al.* 2013, 2015). For example, increases in sea-water temperature simultaneously stimulate the production of anti-fungal compounds by the seafan, *Gorgonia ventalina*, and the growth of its fungal pathogen, *Aspergillus* spp*.,* resulting in an overall increase in infection prevalence (Ward *et al.* 2007). Likewise, changes in biotic environmental factors influence disease dynamics, such as the exclusion of large vertebrate herbivores decreasing overall pathogen prevalence in grasses around central Californian oak savannahs (Borer *et al.* 2009).

Moreover, anthropogenic sources of variability in abiotic and biotic factors regularly operate in combination and at multiple scales to influence the outcome of host-parasite interactions (Duffy *et al.* 2011; Aalto *et al.* 2014; Raffel *et al.* 2015). For instance, eutrophication resulting from nutrient enrichment causes a predator-induced shift in snail communities, favoring the prime intermediate host of *Ribeiroia ondatrae*, a pathogen that induces malformations in frog populations (Johnson & Chase 2004). Such a shift in the pool of available hosts increases the

prevalence of disease in the surrounding frog population. Therefore, predicting the effects of environmental change on host-parasite interactions is complex and requires a thorough knowledge of not only host and parasite ecology, but also of the surrounding ecological community.

1.2 Milkweed Chemistry, Monarchs, Parasites, and Other Natural Enemies

The effects of global change should be particularly apparent in herbivorous host-parasite interactions, because food-plant quality is plastic with respect to environmental conditions (Hunter 2001; Bidart-Bouzat *et al.* 2005; Robinson *et al.* 2012) and herbivore performance is tightly linked to the nutritional and defensive chemistry of their food plants (Mattson 1980; Hunter 2016). My dissertation centers around one such interaction between a phytophagous specialist insect, its obligate host plant and a sub-lethal, but debilitating parasite.

The interaction between the monarch butterfly, *Danaus plexippus*, and its protozoan parasite, *Ophryocystis elektroscirrha* depends heavily on the local biotic environment (de Roode *et al.* 2007, 2008a; Tao *et al.* 2015). Monarchs become infected with *O. elektroscirrha* after ingesting parasite spores on the surface of egg chorea and milkweed (*Asclepias*) tissues (Leong *et al.* 1997a, b). Spores lyse within the larval gut, sporozoites penetrate the larval hypoderm and replicate over the course of the monarch's development (Mclaughlin *et al.* 1970). Infected adult monarchs emerge covered in dormant parasite spores and experience reduced lifespan, decreased fecundity, and limited flight ability (Altizer & Oberhauser 1999; Bradley & Altizer 2005; de Roode *et al.* 2008b, 2009).

In addition to the nutritional benefits that milkweeds provide for monarchs, milkweed plants also produce a group of toxic steroids, known as cardenolides, which are medicinally active against *O. elektroscirrha* (Gowler *et al.* 2015). Monarchs utilize the diversity of milkweed species that they encounter throughout their range as larval host plants (Vickerman & de Boer 2002). Milkweed vary substantially in the composition and concentration of cardenolides in their foliar tissues (Rasmann & Agrawal 2011; Agrawal *et al.* 2012). Feeding on high-cardenolide (hereafter medicinal) milkweed ameliorates the fitness costs of harboring each additional parasite, a form

of defense known as host tolerance (Sternberg *et al.* 2012; Råberg 2014). Additionally, medicinal milkweeds reduce infection probability, and the number of parasites produced in adult butterflies (an inverse measure of host resistance) (de Roode *et al.* 2008a, 2011; Gowler *et al.* 2015). Because medicinal milkweed improve both monarch tolerance and resistance to disease, feeding on these plants also reduces the inherent damage (virulence) that parasites cause to their hosts (de Roode *et al.* 2008a).

We know very little about the mechanism by which cardenolides provide protection to monarchs against *O. elektroscirrha.* Cardenolides disrupt the function of Na^+/K^+-ATPase in the sodium-potassium channels of animal cells (Agrawal *et al.* 2012), rendering them toxic to most animals. The biological activity of cardenolides is determined, in part, by the polarity of the different sugar moieties attached to steroid skeleton of the compound (Rasmann & Agrawal 2011; Agrawal *et al.* 2012). Because animal cell membranes are outwardly hydrophobic, lipophilic (nonpolar) cardenolides are thought to be the most toxic (Sternberg *et al.* 2012; Tao *et al.* 2016). Critically, monarchs that feed on milkweed containing more lipophilic cardenolides experience enhanced protection against the parasite to a certain point, after which the plants become too toxic even for monarch specialists (Sternberg *et al.* 2012). The cellular membranes of a close relative of *O. elektroscirrha* possess these same ion channel enzymes vulnerable to the action of cardenolides (Felibertt *et al.* 1995). Furthermore, medicinal milkweeds provide increased protection against the parasite when consumed immediately before or during ingestion of spores (de Roode et al. 2011). This critical period of medicinal action suggests that milkweed cardenolides influence the effective dose of parasites that monarchs initially experience. Thus, cardenolides could potentially be directly toxic to the parasite.

Alternatively, monarchs possess the well-characterized innate immune response of Lepidoptera. Namely, monarchs encapsulate foreign antigens with specialized immune effector cells (hemocytes) which adhere, harden and die around the surface of the invader (Lavine & Strand 2002; Beckage 2008). Within adhering hemocytes and the surrounding cytoplasm, Phenoloxidase (PO) enzymes catalyze the production of melanin which combines with the encasement of dead and dying cells to both asphyxiate and poison the antigen ("encapsulation" (Rolff & Reynolds 2009)). Given the strong biological activity of cardenolides within the insect,

it is also possible these toxins may either stimulate or suppress the monarch immune response to *O. elektroscirrha*.

Monarchs also face attack from parasitoids and predators that may be vulnerable to the toxicity of cardenolides. Monarchs begin to mount an immune response after oviposition by the multiple species of tachinid flies and other parasitoids that attack mid to late instar monarch larvae (Arnaud 1978; Stenoien *et al.* 2015). However, we know very little about general rates of parasitoid survival within monarchs (Hunter *et al.* 1996; Sternberg *et al.* 2011), and monarch immune defense against parasitoid infection. Due to the intimate nature of parasitoid development within the monarch larvae, the nutritional and medicinal quality of the monarch host diet likely influences parasitoid success (Oberhauser *et al.* 2015). However, cardenolides may impact the strength of the immune response that monarchs can mount against parasitoids. In other herbivorous insect systems toxic secondary metabolites reduce insect immune defense against parasitoid attack (Smilanich *et al.* 2009; Richards *et al.* 2012; Singer *et al.* 2014).

Monarchs defend themselves against predators by sequestering cardenolides from ingested milkweed foliage (Reichstein *et al.* 1968; Malcolm & Brower 1989). The composition and concentration of cardenolides sequestered by monarchs is correlated tightly with milkweed chemistry (Malcolm 1990, 1994; Agrawal *et al.* 2015). Besides making themselves unpalatable to predators, adult monarchs can also physically evade capture through flight. Monarchs are particularly famous for their nearly 4500 km yearly migration to overwintering sites in Mexico (Urquhart & Urquhart 1978; Brower & Malcolm 1991). Butterflies with larger and more elongated wings and higher wing loading values are more successful flyers (Altizer & Davis 2010; Li *et al.* 2016). Wing development can be affected by the environmental conditions experienced by larvae in their final instars and during pupation (Speight *et al.* 1999). Therefore, environmental factors, such as diet (Johnson *et al.* 2014), and parasite infection (Bradley & Altizer 2005), may influence the ability of monarchs to fly. However, no study to date has examined the effects of varying cardenolide concentrations on monarch wing morphology.

Thus, monarchs participate in a diversity of interactions, all influenced by milkweed chemistry. Critically, one ongoing and pervasive global environmental change driver, elevated CO_2, reduces

the medicinal and nutritional quality of milkweed foliage (Vannette & Hunter 2011; Zavala *et al.* 2013). Given the strong dependence of monarch-enemy interactions on host plant chemistry, any changes in the phytochemical quality of milkweed should have important implications for monarch populations. The research here examines the potential effects of elevated CO_2 on the multitrophic interactions, mediated by phytochemistry, in which monarchs participate.

1.3 Summary of Dissertation Chapters

My dissertation is divided into four chapters. Chapters II-IV focus on the indirect impacts of elevated CO_2 on aspects of monarch defense against parasitism and predation. Chapter V focuses on potential effects of elevated CO_2 on plant defense against herbivory. Broadly, Chapter II investigates the effects of elevated CO_2 on the defensive and nutritional chemistry of milkweeds and the subsequent impacts of those phytochemical changes on monarch tolerance and parasite virulence. In Chapter III, I examine how those same phytochemical changes induced by elevated CO_2 compound to influence the defensive phenotype of the monarch against predation, e.g. toxin sequestration and flight ability. In Chapter IV, I evaluate changes in monarch cellular and humoral immunity in response to phytochemical shifts induced by elevated CO_2. Finally, in Chapter V, I examine how elevated CO_2 might alter plant resistance traits and regrowth tolerance and the subsequent relationship between these defense strategies.

Chapter II: Elevated Atmospheric Concentrations of Carbon Dioxide Reduce Monarch Tolerance and Increase Parasite Virulence by Altering the Medicinal Properties of Milkweeds.

Hosts combat their parasites using mechanisms of resistance and tolerance, which together determine parasite virulence (Råberg *et al.* 2007). Environmental factors, including diet, mediate the impact of parasites on their hosts, with diet providing both nutritional and medicinal properties (Sternberg *et al.* 2012; Tao *et al.* 2015; Zeller & Koella 2017). For herbivorous insect hosts, plant quality substantially influences the deleterious effects of parasites (Cory & Hoover 2006; Shikano 2017) and plant quality varies markedly in response to environmental change (Hunter 2001; Robinson *et al.* 2012; Jamieson *et al.* 2017). In this chapter, I explore how elevated CO_2 alters the medicinal quality of milkweeds and, in turn, influences monarch host tolerance and parasite virulence.

We reared monarch larvae on four milkweed species (*A. incarnata* (low cardenolide), *A. speciosa, A. syriaca* (both medium cardenolide), and *A. curassavica* (high cardenolide)) grown under either elevated (760ppm) or ambient (400ppm) CO_2. We also infected a subset of those monarchs with *O. elektroscirrha* to measure how monarch resistance and tolerance, and parasite virulence, changed under future atmospheric conditions. We predicted that eCO_2 would reduce the production of diverse and lipophilic cardenolides, and decrease foliar nutrient quality, thereby decreasing monarch performance and increasing parasite virulence.

***Chapter III**: Managing Migration and Defense in a Changing World.*

As I demonstrate in Chapter II, higher trophic levels are affected by CO_2-induced shifts in plant quality (Hentley *et al.* 2014; Ode *et al.* 2014). Typically, the effects of elevated CO_2 on natural enemies are mediated through shifts in prey nutritional quality and growth rate (Roth & Lindroth 1995; Holton *et al.* 2003; Chen *et al.* 2005; Klaiber *et al.* 2013). However, elevated CO_2 may also inhibit the defense and escape capabilities of herbivores. In this chapter, I examine the indirect effects of elevated CO_2 on toxin sequestration and flight morphology of the monarch butterfly, mediated by plant quality.

We measured cardenolide sequestration and wing morphology of the monarchs reared from Chapter II on the same four milkweed host plant species grown under ambient or elevated CO_2. A portion of these monarchs were also infected with the parasite, to understand how infection and environmental change combine to alter herbivore defense, including wing traits associated with migratory escape from parasites. Phytochemistry influences insect sequestration patterns (Malcolm 1990, 1994; Agrawal et al. 2015), therefore, we expected sequestration profiles to mirror changes in plant chemistry induced by elevated CO_2. In terms of butterfly morphology, we predicted that phytochemical changes would cause declines in the quality of the insect flight phenotype: smaller, thinner and rounder wings with lower wing loading values. Finally, we expected that the metabolic costs of parasitic infection would exacerbate any deleterious effects of elevated CO_2 on toxin sequestration or wing morphology.

Chapter IV: Effects of CO_2 on Environmentally Mediated Immunity in a Specialist Herbivore

The mechanisms underlying the impacts of anthropogenic environmental change on host-parasite interactions remain largely unresolved. The strength and activity of the host immune response is a central line of defense against infection and varies with environmental context (Rolff & Siva-Jothy 2003; Lazzaro & Little 2009; Brock *et al.* 2014). Importantly, insect immunity is strongly determined by the nutritional and phytochemical quality of the diet (Singer *et al.* 2014). In this chapter, I explore the plant-mediated effects of elevated CO_2 on monarch immunity in response to infection by *O. elektroscirrha* and to challenge by simulated parasitoid attack.

We fed monarchs foliage from two species of milkweed, *A. curassavica* (medicinal), and *A. incarnata* (non-medicinal), grown under ambient and elevated CO_2. Larvae were either infected with *O. elektroscirrha* or left as uninfected controls. We then measured critical aspects of the monarch humoral (*in vitro* PO activity) and cellular (*in vitro* hemocyte concentrations and types) (Beckage 2008; Strand 2008) immune response. We also measured foliar secondary metabolites and nutritional quality, to understand the mechanisms underlying monarch immunity under future atmospheric conditions. Because immunity is costly (Sheldon & Verhulst 1996; Kraaijeveld *et al.* 2002; Schmid-Hempel 2003) and dependent on host condition, we predicted two alternative effects of milkweed cardenolides on insect immunity: 1) cardenolides would suppress immunity by either directly inhibiting immune function or reducing the number of infective spores through direct toxicity to the parasite; 2) alternatively, cardenolides could stimulate monarch immunity within the nutritional constraints of the insect host.

Chapter V: Variation Among Individual Milkweed Species, Not Elevated CO_2, Influences the Relationship Between Plant Resistance and Tolerance.

Similar to the monarch host, milkweed defense against attack from enemies (herbivores) may take two broad forms: regrowth after defoliation (hereafter regrowth tolerance) (Strauss & Agrawal 1999) and chemical resistance (Rhoades 1985). Because plant resources are finite, a trade-off may exist between these two strategies of defense, but this relationship is ultimately complex and context dependent (Coley & Chapin 1985; van der Meijden *et al.* 1988; Fineblum & Rausher 1995). Changing environmental conditions, including elevated atmospheric concentrations of CO_2, alter resource availability and thereby influence the defensive strategies of plants.

In this study, we investigated the effects of elevated CO_2 on the chemical resistance and regrowth tolerance traits of the four milkweed species used in Chapters II and III. We first measured plant growth rate and chemical resistance before damage occurred. We then simulated herbivory by cutting plants at the base of the stem. Following three weeks of growth in a common ambient CO_2 environment, we measured regrowth tolerance and plant resistance traits. We then examined four potential trade-offs among these traits: a) a tradeoff between plant growth rate and chemical resistance before damage occurs, b) a tradeoff between plant growth rate before damage and the chemical resistance of regrowth tissues, c) a tradeoff between chemical resistance before damage and regrowth tolerance after damage, and d) a tradeoff between regrowth tolerance and the chemical resistance of regrowth tissues.

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Chapter 2 : Elevated Atmospheric Concentrations of Carbon Dioxide Reduce Monarch Tolerance and Increase Parasite Virulence by Altering the Medicinal Properties of Milkweeds

2.1 Abstract

Hosts combat their parasites using mechanisms of resistance and tolerance, which together determine parasite virulence. Environmental factors, including diet, mediate the impact of parasites on their hosts, with diets providing both nutritional and medicinal properties. Here, we present the first evidence that ongoing environmental change decreases host tolerance and increases parasite virulence through a loss of dietary medicinal quality. Monarch butterflies use dietary toxins (cardenolides) to reduce the deleterious impacts of a protozoan parasite. We fed monarch larvae foliage from four milkweed species grown under either elevated or ambient CO_2, and measured changes in resistance, tolerance, and virulence. High-cardenolide milkweeds lost their medicinal properties under elevated CO_2; monarch tolerance to infection decreased, and parasite virulence increased. Declines in medicinal quality were associated with declines in foliar concentrations of lipophilic cardenolides. Our results emphasize that global environmental change may influence parasite-host interactions through changes in the medicinal properties of plants.

2.2 Introduction

When facing parasite infection, hosts utilize two fundamentally different avenues of defense: resistance and tolerance (Råberg *et al.* 2007; Best *et al.* 2008). Resistance mechanisms reduce the probability and degree of parasitic infection, and subsequent parasite replication (e.g. parasite fitness)(Best *et al.* 2008). In contrast, tolerance describes the ability of hosts to ameliorate the negative fitness impacts of infection for a given pathogen load (Råberg *et al.* 2009; Kutzer & Armitage 2016a). While host resistance reduces parasite fitness, host tolerance does not. Therefore, the two defense strategies should engender different co-evolutionary outcomes for host-parasite dynamics (Roy & Kirchner 2000; Restif & Koella 2004).

Resistance and tolerance evolve to combat the inherent damage (virulence) that parasites cause to their hosts. Virulence differs among parasite and host genotypes (Lambrechts *et al.* 2006; Råberg *et al.* 2007) and with host ecology and condition (Thomas & Blanford 2003; Boots 2011; Howick & Lazzaro 2014). For example, hosts that consume high nutrient diets express increased immune function, reducing virulence (Lee *et al.* 2006; Povey *et al.* 2009). Together, host resistance and tolerance influence the rate at which parasites replicate and damage the host, combining to govern the severity of virulence (de Roode *et al.* 2008a, b; Tao *et al.* 2015).

Understanding variance in resistance has long been a focus of disease ecology. Host genotype, physiology and environment all contribute to parasite resistance (Lambrechts *et al.* 2006; Wolinska & King 2009). In contrast, our understanding of host tolerance derives in large part from the study of pests that attack plants (*reviewed in* Baucom & De Roode 2011). Recent work investigating host tolerance in animal systems has focused largely on host genotype under laboratory conditions (Råberg *et al.* 2007; Rohr *et al.* 2010; Jackson *et al.* 2014; Regoes *et al.* 2014). However, we miss important factors that contribute to variation in tolerance (Lefèvre *et al.* 2011) by isolating host-parasite interactions from the complex community of organisms or environmental conditions within which they normally interact (Sternberg *et al.* 2012; Hayward *et al.* 2014; Tao *et al.* 2015; Debes *et al.* 2017; Zeller & Koella 2017). For example, variation in the nutritional quality of host diet combines with genotype to affect the tolerance of mice and fruit flies to parasites (Clough *et al.* 2016; Kutzer & Armitage 2016b).

Global environmental change directly affects the ecology and evolution of host-parasite interactions (Tylianakis *et al.* 2008; Altizer *et al.* 2013; Becker *et al.* 2015). In many cases, environmental change increases the distribution and prevalence of parasites in host populations (Garamszegi 2011; Zamora-Vilchis *et al.* 2012). Host resistance can increase or decrease in response to the direct effects of environmental change on parasite life cycle and host physiology (Bruno *et al.* 2003; Adamo & Lovett 2011; Paull & Johnson 2014). However, surprisingly little work has investigated how future environmental conditions may impact host tolerance of disease.

Moreover, indirect effects of environmental change on host-parasite interactions remain largely unexplored. Future environmental conditions will alter the composition and traits of the surrounding biotic community (Tylianakis *et al.* 2008; Gunderson *et al.* 2017), which may lead to additional shifts in host resistance and tolerance, and parasite virulence. Phytophagous insect-parasite systems are excellent models with which to study the indirect effects of global change on host-parasite interactions in the context of their communities. Host-plant quality influences the effects of parasites on phytophagous insects (Cory & Hoover 2006; Shikano 2017), and plant nutritional and defensive chemistry vary substantially in response to environmental change (Bidart-Bouzat & Imeh-Nathaniel 2008). For instance, elevated concentrations of atmospheric CO_2 can induce the accumulation of foliar carbohydrates, reduce leaf nitrogen concentrations, and change secondary metabolite production (Hunter 2001; Robinson *et al.* 2012; Zavala *et al.* 2013). Such changes in host plant secondary chemistry and nutrient content can alter herbivore performance against parasites through changes in host immunity or by directly affecting parasite performance (Cory & Hoover 2006; Shikano *et al.* 2010; Lampert 2012). In essence, global environmental change alters plant quality, which can affect the interactions between herbivores and their parasites.

Here, we assess the impact of a pervasive driver of environmental change, elevated atmospheric CO_2 concentration (eCO_2), on the interaction between monarch butterflies, *Danaus plexippus,* and their protozoan parasite, *Ophryocystis elektroscirrha* (Mclaughlin *et al.* 1970). *Ophryocystis elektroscirrha* infection reduces adult monarch lifespan, fecundity, and flight ability (Bradley & Altizer 2005; de Roode *et al.* 2008b, 2009). Monarchs become infected by consuming dormant parasite spores on the surface of egg chorea and leaf tissue. During monarch development, parasites replicate within the insect and adult butterflies emerge covered in dormant parasite spores. The specialist monarchs lay eggs on their milkweed, *Asclepias,* host-plants (Malcolm & Brower 1989), thereby contaminating foliage and eggs with spores.

Certain milkweed species are strongly medicinal, reducing the probability of infection, growth rate, and virulence of *O. elektroscirrha* (de Roode *et al.* 2008a). The medicinal qualities of milkweed are related to concentrations of cardenolides, toxic steroids produced in a majority of milkweed species (Gowler *et al.* 2015). Larvae that feed on plants with high cardenolide

concentrations, or a high diversity of lipophilic cardenolides, suffer lower rates of infection, maintain higher fitness at a given parasite load, and produce fewer new parasites (de Roode *et al.* 2011b; Sternberg *et al.* 2012; Gowler *et al.* 2015). Additionally, high foliar nutrient concentrations can increase monarch tolerance to their parasites (Tao *et al.* 2015). Thus, foliar cardenolides and nutrients combine to mediate the resistance and tolerance of monarchs to their parasites. Recent work shows that eCO_2 causes decreases in cardenolide concentrations, changes in the composition of cardenolides, and declines in nutrient concentrations of milkweed (Vannette & Hunter 2011; Matiella 2012). Therefore, increasing atmospheric CO_2 concentrations may influence milkweed-mediated interactions between monarchs and their parasites

Together these data motivate the overarching question of this study: Will monarch resistance and tolerance and *O. elektroscirrha* virulence change with milkweed phytochemistry under future atmospheric CO_2 concentrations? We performed a field mesocosm experiment to explore how eCO_2 alters the foliar chemistry of four milkweed species. We then measured the CO_2-mediated effects of altered food-plant chemistry on three aspects of monarch and parasite performance: 1) the spore load of infected monarchs; 2) the tolerance of monarchs, expressed as the rate of decline in longevity with increasing spore load; and 3) the virulence of *O. elektroscirrha*, calculated as the decline in adult monarch lifespan due to infection. We hypothesized that the presence of diverse and lipophilic cardenolides, in conjunction with foliar nutrient quality, dictates the effects of eCO_2 on monarch-parasite interactions.

2.3 Materials and Methods

We performed the experiment in two temporal blocks during the summers of 2014 and 2015. General experimental procedures were the same for both blocks, with some minor differences noted below. The experiment was fully factorial, with milkweed species, parasite treatment (infected or uninfected), and CO_2 treatment (ambient or elevated) as main effects (Table S1).

Plant Materials

We grew four species of milkweed under current ambient (400 ppm, aCO_2) and future (760 ppm, eCO_2) concentrations of atmospheric CO_2 at the University of Michigan Biological Station (45.5587° N, 84.6776° W). Plants grew in a mesocosm array of 40 chambers, 20 maintained at

aCO_2 concentrations, and 20 at eCO_2 concentrations from dawn until dusk (Drake *et al.* 1989). We chose milkweed species that differed in their cardenolide concentrations and diversity, on a gradient of anti-parasitic effects from low to high: *A. incarnata* (low), *A. speciosa, A. syriaca* (both medium) and *A. curassavica* (high) (Sternberg et al. 2012). All four milkweed species occur in sympatry in North America (Woodson 1954; Malcolm & Zalucki 1996). Seeds were obtained from commercial sources (Butterfly Encounters, CA in 2014 and Prairie Moon Nurseries, MN in 2015). After six weeks of cold stratification (for all but tropical *A. curassavica*), seeds were germinated and planted on 5/3/14 and 5/5/15 in deepots ™ containing Metromix 360 and Osmocote 16:16:16 controlled release fertilizer. Seedlings were watered daily and kept in a glass house for two weeks following germination to avoid frost damage. We transferred seedlings outside to their assigned chambers on 5/24/14 and 5/23/15.

Monarch caterpillars can consume three entire plants as larvae. Due to space limitations in 2014, only two plants of the assigned milkweed species and CO_2 treatment were grown for each larva. This made for 16 experimental plants in total in each chamber (4 species of milkweed x 2 parasite treatments x 2 plants per monarch). Once larvae had consumed both assigned plants, they were fed cuttings from *A. tuberosa*, a milkweed with negligible cardenolides. Milkweed chemistry influences parasite infection success and severity just before and during consumption of parasite spores on plant material (de Roode *et al.* 2011a). Therefore, switching to an almost cardenolide-free host-plant just before pupation should have no effect on monarch-parasite interactions. In 2015, each chamber held enough plants to feed all larvae for their entire larval periods (4 species of milkweed x 2 infection treatments x 3 plants per monarch = 24 plants per chamber) with 20 aCO_2 and 20 eCO_2 chambers as before.

CO_2 concentrations were monitored continuously during daylight hours in all eCO_2 chambers and one ambient chamber using a LI-COR 320 IRGA (LI-COR, Lincoln, NE, USA). The concentrations of CO_2 were adjusted throughout the day to maintain target concentrations in each elevated chamber. Air temperatures within the chambers were monitored throughout the experiment using iButton dataloggers (IbuttonLink, Whitewater, WI, USA). Elevated CO_2 chambers averaged 21.03 (±0.03)ºC, and ambient CO_2 chambers averaged 21.24 (± 0.04)ºC which were roughly 2ºC higher than the outside average temperature of 18.93 (± 0.04)ºC and fell

well within those temperatures experienced by monarchs in eastern North America. Plants were maintained in their chambers for 61 days in 2014 and 42 days in 2015 before experimental trials began.

Monarch Sources and Rearing Methods

Monarchs were F_1 offspring of eight (2014) and seven (2015) genetic crosses between eastern North American lineages. Darkened monarch eggs (those about to hatch) were placed individually on milkweed cuttings taken from plants grown in the array. Only one larva from each treatment group (4 milkweed species x 2 levels of parasite infection = 8 treatments) was reared on plants from each chamber (Table A1). We kept larvae individually in 0.64L plastic containers under ambient conditions on foliage transferred daily from the appropriate atmosphere to avoid any possible confounding direct effects of CO_2 on insect performance (although such effects are considered negligible (Bale *et al.* 2002)).

Each year, we infected monarchs with a single parasite genotype cultured from spores collected from an eastern North American butterfly. Hatchlings fed for three days on their assigned leaf tissue before inoculation with *O. elektroscirrha*. To infect larvae, 10 parasite spores were transferred to a 70.6 mm^2 leaf disk taken from each larva's assigned host plant. The leaf disk was placed in a petri dish containing moist filter paper and the assigned larva. Control larvae received spore-free leaf disks. Immediately after disks were punched from plants, foliage was collected for chemical analysis (below). Petri dishes containing disks and larvae were kept in an incubator held at 26°C with 16-hour daylight. Once each larva had consumed its entire leaf disk (and therefore all spores) it was returned to its assigned container and fed foliage *ad libitum* from its designated plants until pupation.

Measures of Monarch Performance

Upon emergence, butterflies were sexed, transferred to individual glassine envelopes, stored at 14°C and inspected daily until death. Lifespan under these conditions correlates strongly with monarch lifetime reproduction (fitness) (De Roode *et al.* 2008b). Parasite virulence was measured as the magnitude reduction in lifespan of infected monarchs when compared to control monarchs. After death, spore loads were measured from adults following established methods

(de Roode *et al.* 2008a). Wings were removed and each monarch body was placed in a scintillation vial with 5 mL of deionized water. The mixture was vortexed for five minutes, and 10 μL aliquots were deposited into 4 wells in a hemocytometer for counting. Spore load represents the inverse of monarch resistance. Tolerance to *O. elektroscirrha* was measured as the slope of the relationship between spore load and longevity, with a separate line (slope estimate) for each milkweed species by CO_2 treatment. The slopes of the tolerance lines are an index of the fitness cost suffered per spore in each treatment.

Plant Defense Measurements

On the same day as inoculations, we sampled foliage to measure cardenolide and nutrient concentrations using established methods (Zehnder & Hunter 2009; Tao & Hunter 2012). Six 424 mm^2 leaf disks were taken from one leaf of the fourth leaf pair, deposited in 1 mL methanol and stored at -10°C for cardenolide analysis. Cardenolides were extracted, separated and quantified by reverse-phase high-performance liquid chromatography (HPLC) on a Waters Acquity UPLC with PDA detector (Waters Coperation, Milford, MA, USA) with 0.15mg/mL digitoxin internal standard (Sigma Chemical Company, St. Louis, Missouri, USA). Peaks with symmetrical absorbance between 217-222 nm were identified as cardenolides. Another six disks from the opposite leaf in the leaf pair were collected, weighed, dried, and reweighed to provide estimates of sample dry mass. Remaining foliage from the two punched leaves was collected, oven dried, and analyzed using a TruMac CN Analyzer (Leco Corporation, St. Joseph, MI) to estimate foliar carbon (C) and nitrogen (N) concentrations.

Statistical Methods

Analyses were carried out in R (version 3.3.2). In all of the linear mixed effects models (lme4 package, LMMs) that follow, we included experimental year and chamber identity as random effects, to account for unintended temporal and spatial variation. We also included monarch genotype as a random effect in all models of monarch performance.

Monarch performance

Our analyses are limited to only those monarchs that survived to adulthood, including successfully infected monarchs, and uninoculated (control) monarchs (Sternberg *et al.* 2012).

Analyses of parasite burden were restricted to infected monarchs, but analyses of tolerance included both infected and uninfected monarchs.

To investigate effects of our treatments on monarch tolerance, we modeled adult longevity (square-root-transformed) as a function of spore load (square-root-transformed) (Sternberg et al. 2012), including milkweed species and CO_2 treatment as additional fixed effects. To investigate the effects of CO_2 treatment and milkweed species on parasite virulence, we used monarch life span (square-root-transformed) as the dependent variable and parasite treatment as an additional fixed effect. Finally, to estimate monarch resistance, we included monarch spore load (square-root-transformed) as the dependent variable, with milkweed species and CO_2 treatment as fixed effects. Analysis of monarch resistance was only conducted on infected individuals. For all models, homoscedasticity of variance was tested using Levene's Tests (car package in R).

To test for a trade-off between host tolerance and resistance, we associated monarch tolerance with resistance to *O. elektroscirrha* using a LMM with year as a random effect (Råberg *et al.* 2009). We assessed any relationship between the 16 tolerance slope values of each treatment group in each year (4 milkweed species x 2 CO_2 treatments x 2 years) and the mean resistance values (1/spore load) of those treatment groups.

Milkweed chemistry and elevated CO_2

We explored the responses to our CO_2 treatments of (a) total foliar cardenolide concentration (log-transformed), (b) cardenolide diversity, (c) cardenolide polarity, (d) C:N ratio, and (e) foliar N concentration using LMMs. Cardenolide diversity was calculated using the Shannon diversity index borrowed from the biodiversity literature: $H=-\text{sum}(P_i \log[P_i])$ where P_i is the relative amount of a cardenolide peak compared to the total amount of cardenolides in an individual plant (Rasmann & Agrawal 2011). We excluded *A. incarnata* plants from the LMM exploring effects of CO_2 treatments on cardenolide diversity because only 2 individuals produced more than a single cardenolide peak. Following Rasmann & Agrawal (2011), we calculated an index of cardenolide polarity $P=\text{sum}(P_i\ RT_i)$, where RT_i is the retention time of the *i*th peak in the individual. The polarity index values that result range from 0 (highly polar) to 1 (highly lipophilic).

We also compared cardenolide composition among milkweed species and CO_2 treatments using permutational multivariate analysis of variance (PerMANOVA) (Anderson 2001) following Bray-Curtis ordination. The analysis was performed using the Adonis procedure of the Vegan package in R. Using metaMDS we performed Nonmetric Multidimensional Scaling (NMDS) (McCune & Grace 2002) reducing the dimensions of the model with 999 permutations per model run and a maximum of 500 runs per dimension. We ultimately used a three-dimensional model in subsequent analyses (model stress = 0.119) (McCune and Grace 2002).

Within the assemblage of cardenolides available to monarchs, two specific cardenolide peaks (RT585 and RT650) have been associated previously with the medicinal efficacy of milkweed species against *O. elektroscirrha* (de Roode *et al.* 2011b). In our experiment, we found these two cardenolides only in the foliage of *A. curassavica,* with the exception of one *A. syriaca* plant grown under aCO_2. Given the established importance of these two compounds, and the losses that we observed in the medicinal activity of *A. curassavica* under elevated CO_2 (see Results, below), we ran separate LMMs with each of these cardenolides as dependent variables and CO_2 treatment as a fixed effect.

Milkweed chemistry and monarch performance

We observed significant effects of elevated CO_2 on monarch tolerance and parasite virulence on only one milkweed species, *A. curassavica* (see Results). We used LMMs to assess any individual and interactive effects of *A. curassavica* traits on monarch tolerance and parasite virulence. All analyses were restricted to those *A. curassavica* plants for which we had measures of cardenolide diversity and corresponding C and N data (N=77). We also performed a PerMANOVA on the cardenolide communities produced in *A. curassavica* alone.

We used Akaike's information criterion (AIC) scores to select chemical traits that were associated with virulence or tolerance. We planned to add additional traits (and interactions) to each model only if the AIC scores improved by two points and if the significance level of the new variable was below 0.05 (Burnham & Anderson 2002). However, in no case was more than one independent variable included in any model.

(i) Chemistry and Tolerance. Using the procedure described above, we assessed associations between *A. curassavica* traits and monarch tolerance (slope of fitness decline) by investigating effects of spore load (log10-transformed) and plant traits on lifespan (square-root-transformed).

(ii) Chemistry and Virulence. Likewise, we measured associations between individual A. *curassavica* traits and parasite virulence by investigating the effects of infection treatment and plant traits on lifespan (square-root-transformed). Because we were interested in how plant traits could influence the fitness consequences of infection, we also performed analyses on infected butterflies only (N=32) using only plant traits that produced significant and marginally significant interactions with infection treatment.

2.4 Results

Monarch tolerance and parasite virulence

Monarch tolerance to *O. elektroscirrha* declined by 77% under eCO_2 for individuals reared on the medicinal milkweed, *A. curassavica* (see slope relating monarch longevity to parasite spore load in Fig. 1). The tolerance of monarchs feeding on less medicinal milkweed species remained unchanged under eCO_2 (spore load *species*CO_2: $F_{3,315}$= 4.50, p= 0.00415, Fig. 2.1). These results suggest that eCO_2 reduces the protective properties of *A. curassavica* to the same levels as those of less-medicinal milkweeds (Fig. 2.1).

Consistent with effects on tolerance, the virulence of *O. elektroscirrha* increased under eCO_2 in those monarchs reared on *A. curassavica*, and remained unchanged on the lower cardenolide milkweed species (infection*species*CO_2: $F_{3, 308}$= 4.44, p= 0.0045, Fig. 2.2a). Essentially, eCO_2 made *A. curassavica* non-medicinal, magnifying the reduction in fitness caused by infection to values similar to those of those monarchs feeding on the other three milkweed species. The magnitude of the reduction in lifespan between control and infected monarchs feeding on *A. curassavica* increased from 1.8 days to 7.2 days under eCO_2 (Fig. 2.2b). As expected, all infected monarchs had shorter lifespans than uninfected monarchs (infection: $F_{1, 314}$=263.55, p<0.0001, Fig. 2.2b).

In contrast to their effects on monarch tolerance and parasite virulence, we found no effects of eCO_2 ($F_{1,129}$=1.71, p=0.1931), host plant species ($F_{3,137}$=1.28, p=0.2845), or their interaction ($F_{3,138}$=1.35, p=0.2596) on monarch resistance to the parasite as measured by spore load. Additionally, we found no tradeoff between monarch tolerance and resistance to *O. elektroscirrha* ($F_{1, 15}$ =0.91, p=0.3548).

Milkweed chemistry and elevated CO_2

Foliar cardenolide concentrations were twelve times higher in *A. curassavica* than in *A. syriaca,* the next highest milkweed species (milkweed species: $F_{3,166}$ =192.31, p<0.0001, Fig. 2.3a). Cardenolide concentrations declined under eCO_2 across all milkweed species (CO_2: $F_{1,166}$ =5.77, p=0.0174, Fig. 2.3a), and there was no interaction between milkweed species and CO_2 treatment ($F_{1,166}$=0.48, p=0.6963). The diversity of cardenolide molecular forms was four times higher in *A. curassavica* than in the other milkweed species (milkweed species: $F_{3, 109}$= 47.11, p<0.0001, Fig. 2.3b) and declined under eCO_2 in all milkweed species but *A. incarnata*, a species which rarely produces more than one cardenolide (CO_2: $F_{1, 33}$=5.63, p=0.02362). There was no interaction between milkweed species and CO_2 treatment on cardenolide diversity (milkweed species*CO_2: $F_{2,141}$= 0.54, p = 0.58274). The average polarity of *A. curassavica* cardenolides was marginally reduced by eCO_2 treatments, while the average polarity of cardenolides increased in *A. speciosa* and decreased in *A. syriaca* (species*CO_2: $F_{3, 153}$=2.99, p=0.03281, Fig. 2.3c).

Across all four species, foliar N concentrations (an estimate of plant nutritional quality) declined under eCO_2 (CO_2: $F_{1,48}$= 12.33, p= 0.00098, Fig. 2.3d). Milkweed species also varied in their foliar N concentrations from 1.42% in *A. curassavica* to 1.14% in *A. syriaca* ($F_{1,137}$ = 4.43, p= 0.0052, Fig. 2.3d).

Beyond simple measures of cardenolide polarity and diversity, milkweed species differed in the composition of cardenolides in their foliage (PerMANOVA; milkweed host plant species: $F_{3, 171}$= 20.02, p= 0.001, R^2=0.26, Fig. A1). Additionally, the effects of CO_2 treatment on cardenolide community composition varied among milkweed species (PerMANOVA; milkweed species*CO_2: $F_{1, 171}$= 2.12, p= 0.001, R^2=0.027, Fig. A1). In *A. curassavica* alone, the communities of cardenolides produced by individual plants differed between CO_2 treatments

(CO_2: $F_{1, 76} = 2.80$, p= 0.03, R^2=0.036, Fig. 2.4a). NMDS1 was associated with declines in the concentrations of lipophilic cardenolides (decline in polarity index) (p<0.0001, R^2=0.47, Fig. 2.4b).

Concentrations of both RT585 and RT653, the two cardenolides with established medicinal activity (de Roode et al. 2011b), declined in *A. curassavica* under eCO_2. Concentrations of RT585 declined by 25% under eCO_2 ($F_{1, 61}$= 5.36, p= 0.02401, Fig. 2.5a). Far fewer *A. curassavica* individuals produced RT653, making for a very small sample size (N=6). Nonetheless, we detected a large (65%), marginally nonsignificant, decline in RT653 concentration under eCO_2 ($F_{1, 4}$= 5.92, p= 0.0717, Fig. 2.5b).

A. curassavica *chemistry and monarch performance*

Tolerance

A significant interaction between spore load and a given plant trait on monarch lifespan indicates a correlation between that trait and tolerance to *O. elektroscirrha*. Monarch tolerance correlated positively with the expression of lipophilic cardenolides in leaves (spore load* polarity: $F_{1, 72}$ =4.10, p= 0. 04665, Fig. 2.6a). No other individual plant trait or combination of traits correlated with monarch tolerance.

Virulence

A significant interaction between a plant trait and parasite treatment on monarch lifespan indicates a relationship between that trait and parasite virulence. As with tolerance, there was a positive relationship between the expression of liphophilic cardenolides and the lifespan of infected individuals (declining virulence) but it was marginally non-significant (parasite treatment*polarity: $F_{1, 72}$= 3.32, p= 0.0726, Fig. 2.6b); only infected monarch lifespans increased significantly with increasing expression of lipophilic cardenolides ($F_{1,23}$= 14.41, p= 0.0009). No other individual plant trait or combination of traits correlated with monarch virulence.

2.5 Discussion

Monarch butterflies benefit from the medicinal properties of milkweeds when combating their parasites (de Roode *et al.* 2008a, 2011a; Sternberg *et al.* 2012; Gowler *et al.* 2015), and infected females can actively choose the most medicinal milkweeds for oviposition in laboratory choice tests (Lefèvre *et al.* 2010, 2012). Here, we show that a medicinal milkweed species, *A. curassavica*, loses its protective abilities under eCO_2. Our results suggest that rising concentrations of atmospheric CO_2 will reduce the tolerance of monarch butterflies to their common parasite, *Ophryocystis elektroscirrha*, and will increase parasite virulence. Ongoing changes in water availability (Andrews 2015), ambient temperature (Couture *et al.* 2015) and soil nutrient loading (Zehnder & Hunter 2009; Tao *et al.* 2014) have already been shown to influence the cardenolide chemistry of milkweeds, with potential consequences for parasite-monarch interactions. Here, we add elevated concentrations of atmospheric gases to the list of drivers that may alter parasite-host interactions of monarch butterflies in a changing world.

We observed the lowest tolerance values in those monarchs feeding on *A. syriaca* grown under eCO_2 and the highest tolerance values in those monarchs feeding on *A. curassavica* grown under aCO_2. However, monarchs feeding on the same species of milkweed that once conveyed a tolerance advantage under aCO_2 (*A. curassavica*), experienced a 77% reduction in their tolerance levels under eCO_2. In parallel, parasite virulence also depended on both milkweed species and CO_2 concentrations. Parasites caused the most virulence when monarchs fed on *A. incarnata* under aCO_2, reducing host lifespan by nearly 8 days (see Fig. 2.2b). Parasites caused the least virulence in those monarchs feeding on *A. curassavica* grown under aCO_2, reducing mean lifespan by only 2 days. Importantly, monarchs feeding on this same species, *A. curassavica*, under eCO_2 experienced virulence of comparable values to non-medicinal species like *A. incarnata*, suffering a reduction in lifespan of 7 days due to infection. To our knowledge, these results are the first to show that there can be effects of environmental change on host tolerance to parasites and the virulence of those parasites as a result of indirect effects mediated by community members.

A growing number of studies stress the importance of understanding the indirect mechanisms by which disease will respond to changing environmental conditions (Harvell *et al.* 2002; Tylianakis *et al.* 2008; Altizer *et al.* 2013; Gunderson *et al.* 2017). Indirect effects of environmental change on host-parasite interactions emerge from additional members of ecological communities (Keesing *et al.* 2006; Wolinska & King 2009; Vuong *et al.* 2017). Associated predators, competitors and symbionts are all subject to the effects of environmental change, which may alter their interactions with host-parasite pairs (Ritchie 2006; Gherlenda *et al.* 2016). Here, we provide a previously unrecognized indirect mechanism by which environmental change can act on disease: the loss of medicinal compounds in host diet, contributing to reductions in host tolerance and increases in parasite virulence.

Changes in host tolerance and parasite virulence under future environmental conditions have important evolutionary implications. Theory predicts that reductions in resistance will lesson antagonistic coevolution between host and parasite (Roy & Kirchner 2000; Råberg *et al.* 2009; Rohr *et al.* 2010). However, we are less certain what changes in host tolerance could mean for host-parasite dynamics (Best *et al.* 2008; Schneider & Ayres 2008). Because tolerance helps to maintain host fitness when infected, less tolerant hosts should suffer shorter infections due to increased mortality, thereby potentially decreasing transmission and the prevalence of parasites in the host population (Miller *et al.* 2006). In our study, reduced tolerance was also accompanied by increased parasite virulence. In some cases, increases in virulence may lead to local extinction (Kutzer & Armitage 2016a; Wilber *et al.* 2017). We expect parasites that cause higher virulence to be selected against when host tolerance is also reduced because the risk of premature host mortality is higher. Early host death reduces parasite fitness and thus, induces selection on parasite virulence to decrease to a new optimum (Little *et al.* 2010). Given the reductions in host tolerance, we predict that future environmental conditions may select for less virulent parasites. But it remains unclear how potential feedback mechanisms between host defenses and parasite transmission will ultimately shape disease dynamics under global change regimes (Metcalf *et al.* 2017).

Our results add to a substantial body of work that emphasizes the role of environmental factors in phytophagous host-parasite interactions (Cory & Hoover 2006; Myers & Cory 2016; Shikano

2017). The largest declines in tolerance and increases in virulence occurred in monarchs feeding on *A. curassavica*, a species in which cardenolide production declined by nearly 25% when grown under eCO_2. However, total cardenolide concentrations did not correlate with changes in tolerance. Rather, reductions in cardenolide concentration under eCO_2 occurred in concert with changes in cardenolide community composition and declines in the expression of lipophilic cardenolides (Figs. 2.4, 2.5). Because the polarity of cardenolides partially determines their biological activity (Agrawal *et al.* 2012), the loss of lipophilic cardenolides under eCO_2 compromises the anti-parasitic properties of milkweed foliage. Infected monarchs that consume lipophilic cardenolides live longer than do those infected monarchs consuming polar cardenolides (Sternberg *et al.* 2012; Tao *et al.* 2016). Previous work has shown that declines in the concentrations of two key lipophilic cardenolides, RT585 and RT653, increase parasite virulence (de Roode *et al.* 2011b). We also observed reductions in these two cardenolides in our populations of *A. curassavica* that were exposed to eCO_2, which likely led to the observed increases in parasite virulence.

Surprisingly, the declines in tolerance to *O. elektroscirrha* that we observed in monarchs feeding on *A. curassavica* under elevated CO_2 were unrelated to host-plant nitrogen concentrations, our proxy for nutritional quality. Recent studies of environmentally-determined host tolerance have reported that diets high in nutrients generally increase host tolerance to parasite infection (Clough *et al.* 2016; Kutzer & Armitage 2016b; Miller & Cotter 2017; Zeller & Koella 2017). While foliar nitrogen concentrations often limit herbivore performance (Mattson 1980), there can be complex, non-linear relationships between milkweed nitrogen concentrations and herbivore performance (Zehnder & Hunter 2009; Tao *et al.* 2014). We may need more comprehensive evaluations of nutritional quality before we can establish the effects of dietary quality on *O. elektroscirrha* tolerance in monarchs. Nonetheless, our study supports previous work (Sternberg et al. 2012) suggesting that secondary metabolites, not just nutrients, influence the tolerance of herbivores to their parasites.

By demonstrating that tolerance to parasite infection can be altered by environmental change, we reinforce the idea that tolerance is not solely determined by intrinsic host factors but relies additionally on environmental conditions including interactions with other community members.

As the environmental factors that mediate host tolerance and parasite virulence continue to change, further empirical studies are sorely needed to explore the interplay between multiple global change drivers and host-parasite interactions embedded within diverse ecological communities.

Data Accessibility

Data will be made available in the Dryad Digital Repository:

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2.7 Tables & Figures

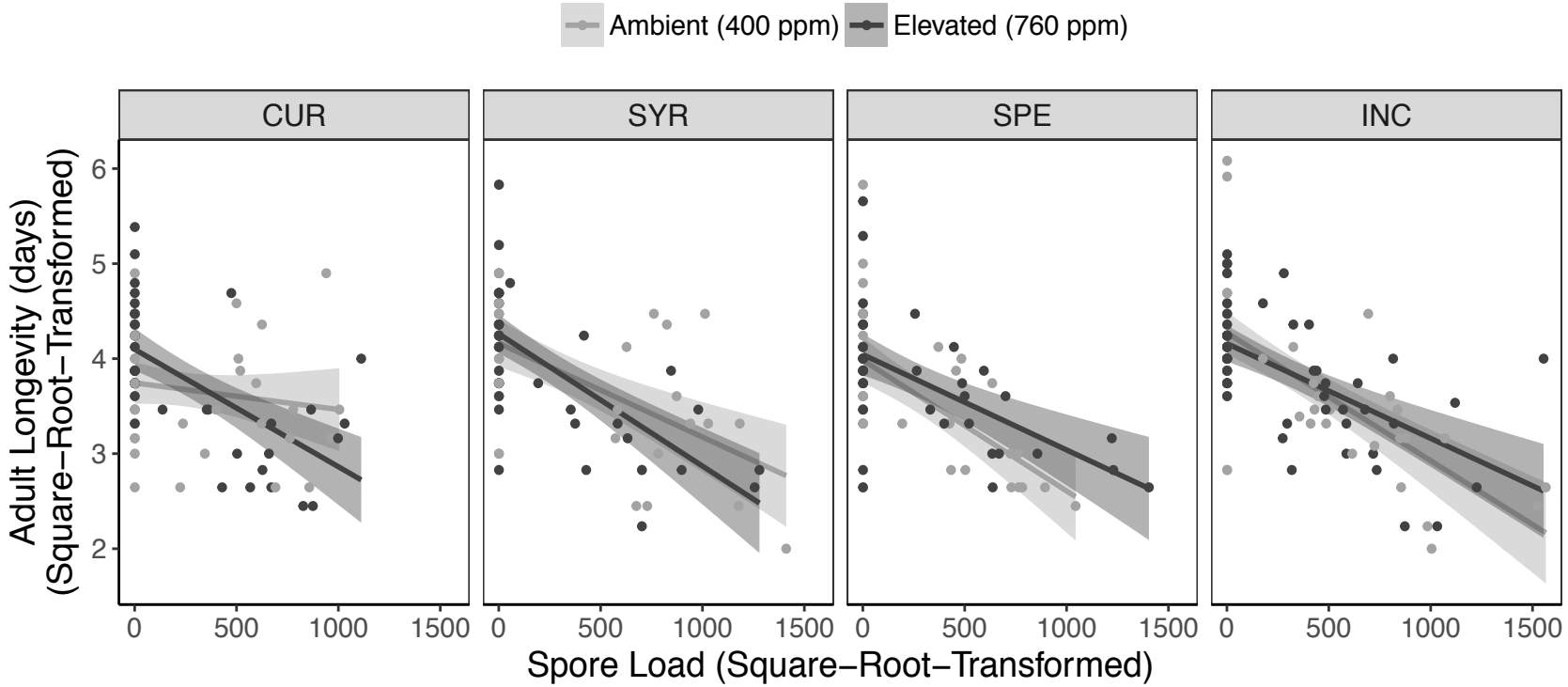

Figure 2.1. Monarch tolerance to *O. elektroscirrha* infection as a function of milkweed species and CO_2 treatment.
Light gray lines and points correspond to tolerance slopes of monarchs reared on plants grown under ambient CO_2 (400 ppm) and dark gray lines and points correspond to tolerance slopes of monarchs reared on plants grown under elevated CO_2 (760 ppm). Tolerance slopes are presented by milkweed species: CUR =*A. curassavica*, SYR=*A. syriaca*, SPE=*A. speciosa*, INC=*A. incarnata.*

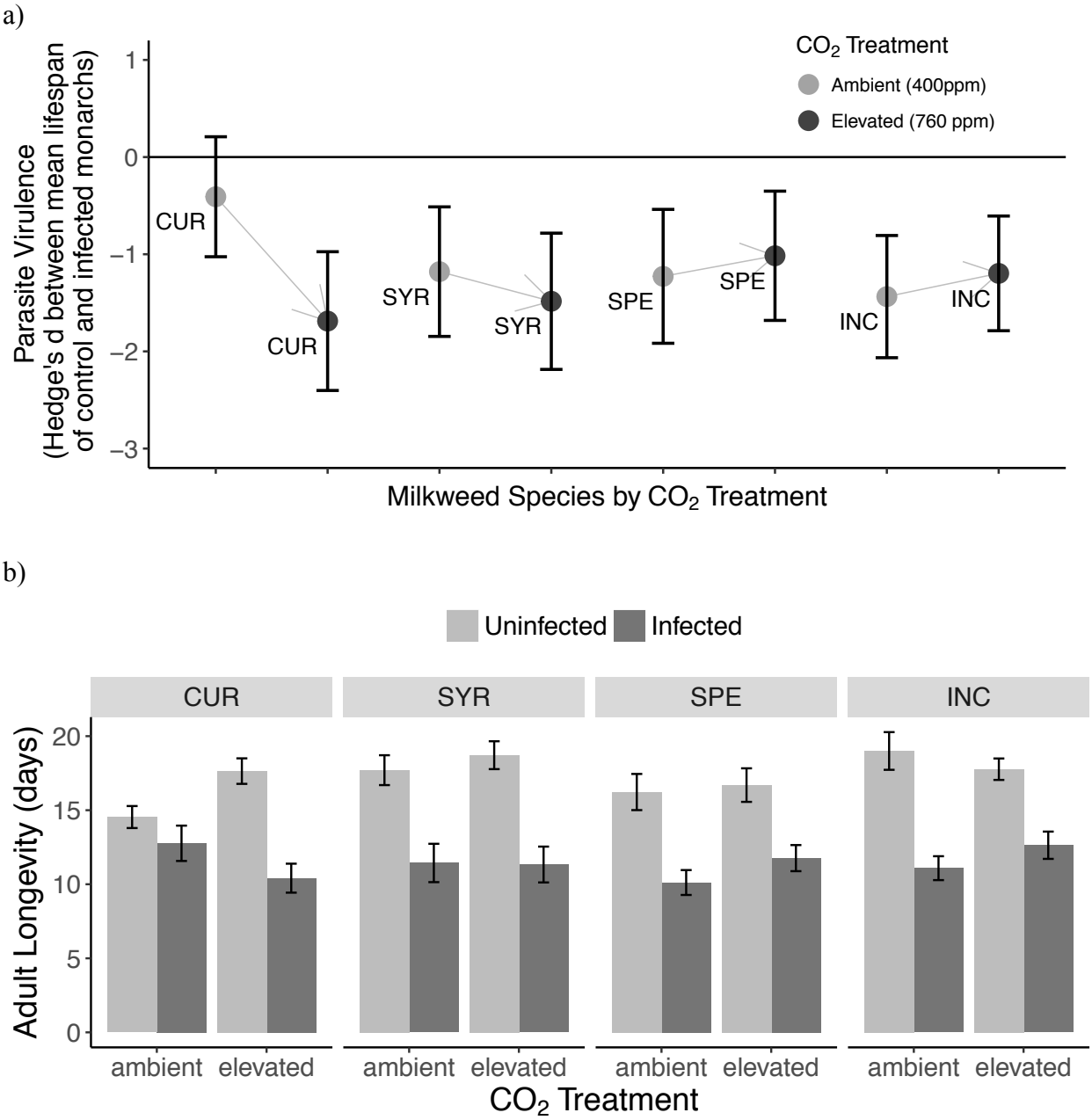

Figure 2.2: The virulence of *O. elektroscirrha* parasites increases under elevated CO_2 when monarch larvae feed on *A. curassavica*. Virulence is measured as the magnitude of the reduction in host fitness resulting from infection. In (a), points represent the standardized difference (Hedge's d ± 95% CI) in mean lifespan between uninfected and infected monarchs fed different species of milkweed under ambient CO_2 (400 ppm, light gray) and elevated CO_2 (760 ppm, dark gray). In (b), we show mean longevity of parasite-infected (dark gray bars) and uninfected (light gray bars) monarchs used to calculate the Hedge's d values shown in (a). Longevities were transformed to approximate normality of errors before statistical analyses but are presented here as untransformed values for ease of interpretation. Milkweed species codes match those presented above

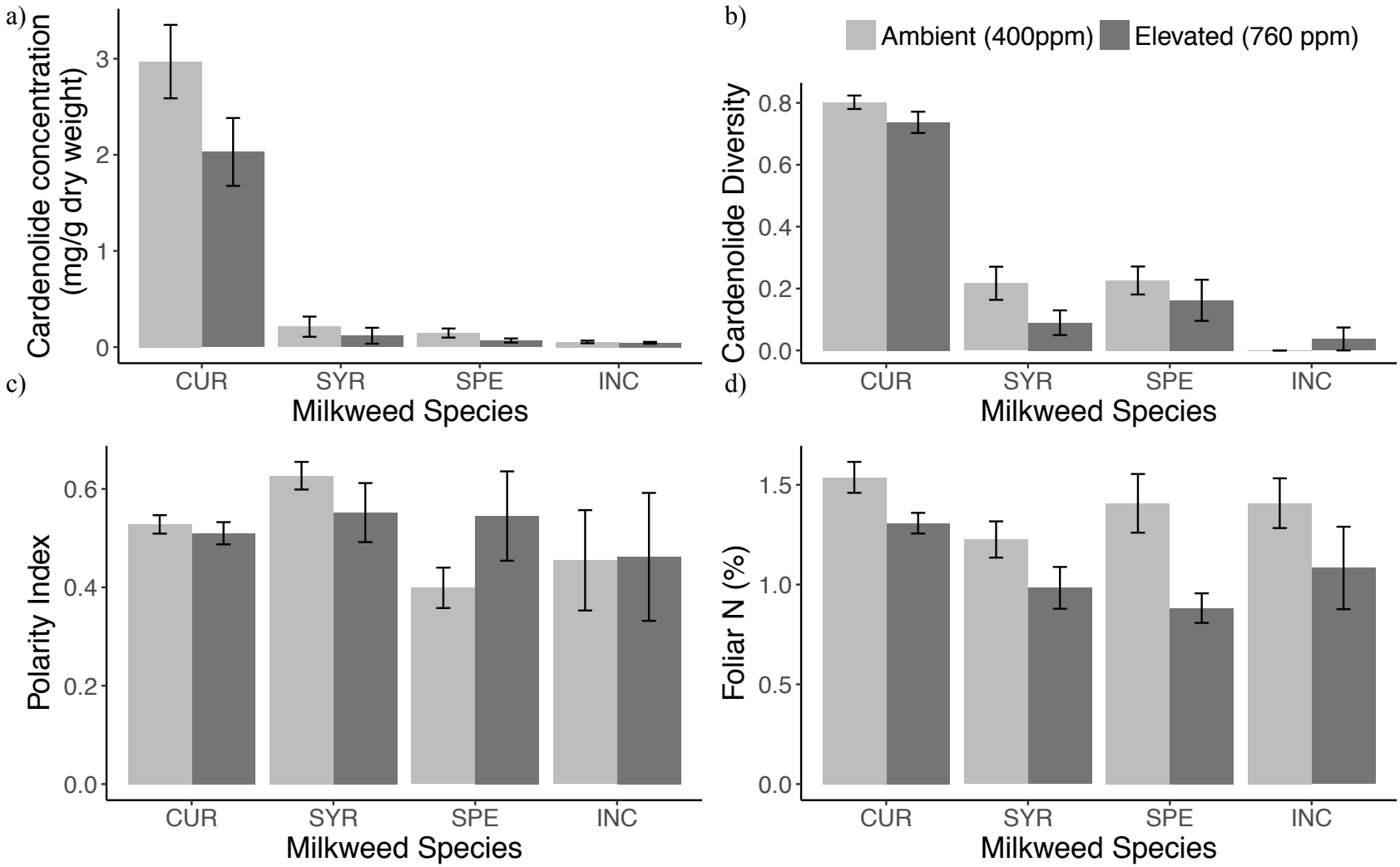

Figure 2.3: Effects of elevated CO_2 on foliar cardenolide concentrations (mg/g dry mass, a), cardenolide diversity (b), cardenolide polarity index (c), and foliar nitrogen concentration (%N) (d) of four milkweed species. Trait values were transformed to approximate normality of errors before analyses but are presented here in their untransformed values for ease of interpretation. Light gray bars represent plants grown under ambient CO_2 and dark gray bars are those from elevated CO_2. Milkweed species codes match those presented above.

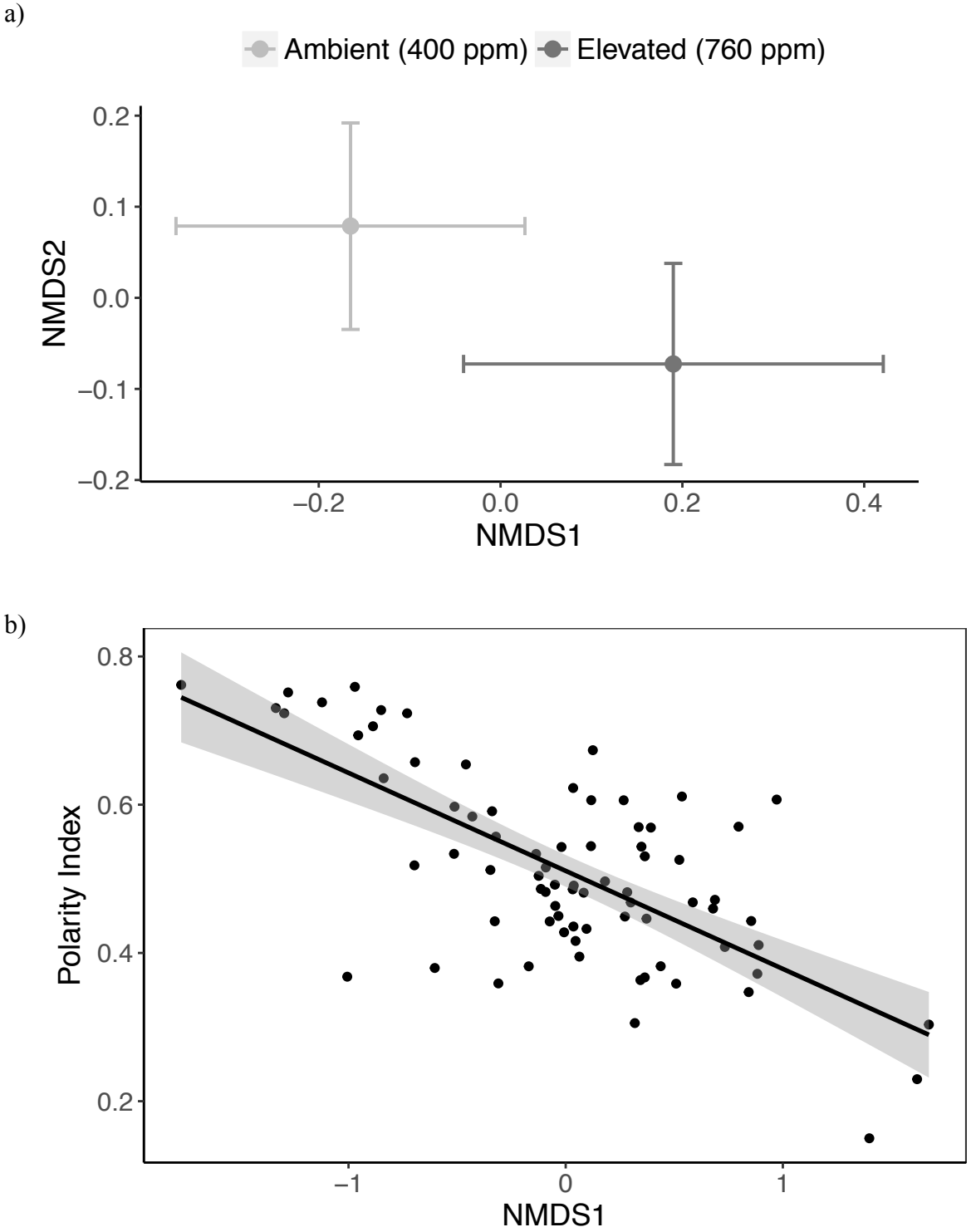

Figure 2.4. *A. curassavica* plants differed in the composition of cardenolides that they produced under the different CO_2 treatments. In (a) light gray points represent plants grown under ambient CO_2 (400ppm) and dark gray points represent plants grown under elevated CO_2 (760 ppm). In (b), we illustrate the negative association between NMDS axis 1 and the occurrence of lipophilic cardenolides.

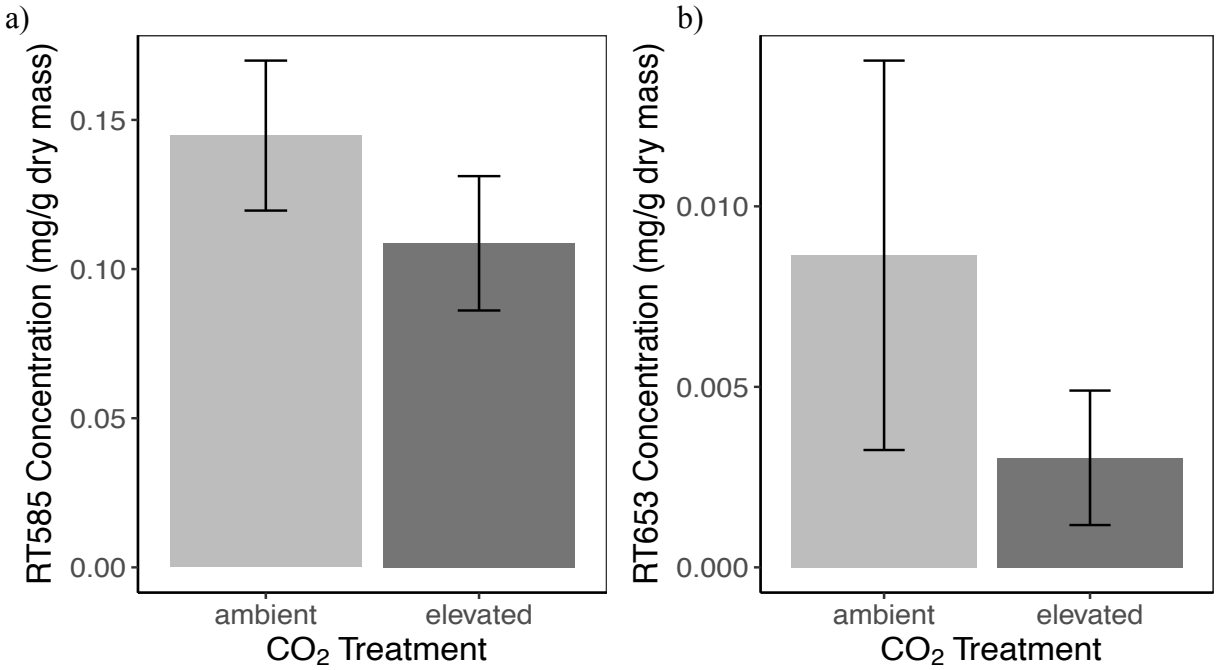

Figure 2.5. Effects of elevated CO_2 on the concentration of two medicinal cardenolides: (a) RT585 and (b) RT653 in *A. curassavica*. Light gray bars represent foliar samples taken from plants grown under ambient CO_2 and dark gray bars are those from elevated CO_2.

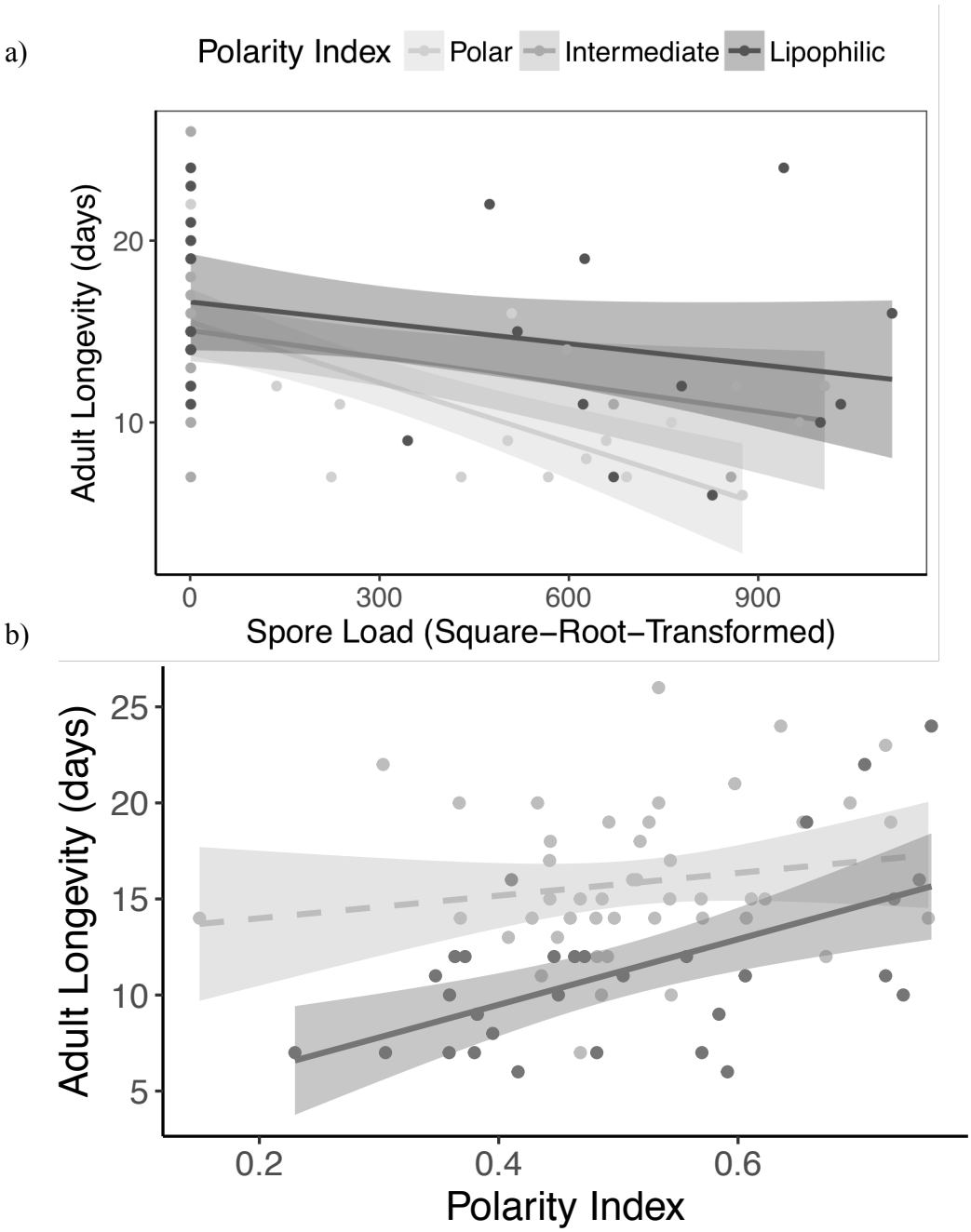

Figure 2.6. The effects of cardenolide polarity on (a) monarch tolerance to infection by *O. elektroscirrha* and (b) the lifespan of infected and uninfected monarchs. A high polarity index reflects greater expression of lipophilic cardenolides. The slopes of the lines in (a) indicate monarch tolerance to infection, with steeper slopes representing lower tolerance. For visual simplicity, we have binned butterflies in (a) by the polarity of the cardenolides that they consumed as larvae. However, the analysis was performed with un-binned polarity data, and binning was used purely as a simplified alternative to a 3D graph. In (b), light gray points and lines indicate uninfected (Control) monarchs, and dark gray points and lines indicate infected monarchs. Only the lifespan of infected monarchs was positively correlated with the index of cardenolide polarity.

Chapter 3 : Defense and Evasion from Predators in A Changing World

3.1 Abstract

Environmental change has the potential to influence trophic interactions by altering the defensive phenotype of prey. For example, changing environmental conditions may influence prey resistance to disease, prey palatability, or the ability of prey to escape from areas of high predation risk. Here we present the first study to examine the effects of a major environmental change driver, elevated CO_2, on toxin sequestration and flight morphology of a specialist herbivore. We fed monarch butterfly larvae, *Danaus plexippus*, foliage from four milkweed, *Asclepias*, host plant species of varying chemical defense profiles grown under either ambient or elevated CO_2. We also infected a subset of these herbivores with a protozoan parasite, *Ophryocystis elektroscirrha*, to understand how infection and environmental change combine to alter herbivore defenses. We measured changes in phytochemistry induced by eCO_2 and assessed subsequent toxin sequestration and morphology of butterflies. Our results demonstrate that 1) monarchs compensate for lower plant toxin concentrations under elevated CO_2 by increasing toxin sequestration rate; they maintain the same composition and concentrations of cardenolides in their wings under the two CO_2 treatments. 2) Flight morphology, including wing shape, wing loading, and wing density vary by elevated CO_2, milkweed species, infection status, and sex. 3) Feeding on high cardenolide milkweed is associated with the formation of rounder, thinner wings, which are less efficient at gliding flight. We suggest that changes in the rate of sequestration under elevated CO_2 are a byproduct of compensatory feeding aimed at maintaining a nutritional target in response to declining dietary quality. Ingesting larger amounts of foliage from milkweed high in cardenolides may come at a cost to the monarch. Such costs may manifest as lower quality flight phenotypes: rounder, thinner wings with lower wing loading values. Small changes in wing morphology may have important consequences for flight ability and migration success. Energetic costs due to alterations in sequestration and morphology may, therefore, have important consequences for monarch defense in a changing world.

3.2 Introduction

Global environmental change alters the composition of ecological communities through both direct and indirect effects. For example, variable environmental factors directly impact the phenotype of some organisms (Parmesan & Yohe 2003; Altizer *et al.* 2013; Chu *et al.* 2016), which, in turn, may generate cascading indirect effects on other community members (Parmesan 2006; Gilman 2017; Gunderson *et al.* 2017). Therefore, a major challenge for ecology rests in understanding how direct and indirect effects of abiotic and biotic drivers combine to influence organisms under environmental change (Tylianakis *et al.* 2008; Gilman *et al.* 2010).

The rising concentration of carbon dioxide (CO_2) in Earth's atmosphere is one of the most pervasive global change drivers. Elevated CO_2 typically causes reductions in the nutritional quality of plants, increasing the concentration of nonstructural carbohydrates in relation to nitrogen-based compounds (Drake *et al.* 1997; Ainsworth & Long 2005; Robinson *et al.* 2012; Bazzaz *et al.* 1992). Because herbivore growth is often limited by nitrogen (Mattson 1980), elevated CO_2 generally causes herbivores to increase the amount of foliage that they consume (Docherty *et al.* 1996; Johnson *et al.* 2014b). Along with diluting nutritional quality, elevated CO_2 also alters the defensive chemistry of plants with important implications for herbivores (Hunter 2001; DeLucia *et al.* 2012; Robinson *et al.* 2012; Zavala *et al.* 2013; Facey *et al.* 2014; Ode *et al.* 2014; Jamieson *et al.* 2017). Elevated CO_2 changes both the composition and concentration of plant secondary metabolites (PSMs) depending on compound class (Bidart-Bouzat *et al.* 2005; Ryan *et al.* 2010; Klaiber *et al.* 2013a; Jia *et al.* 2016). Because detoxifying or catabolizing PSMs is energetically costly, changes in PSM concentrations affect the performance, abundance and distribution of herbivores (Hunter 2016). A number of authors have reviewed the plant-mediated effects of elevated CO_2 on plant-herbivore interactions (most recently Jamieson *et al.* 2017). Generally, herbivore growth rates decline under elevated CO_2, accompanied by decreases in fecundity, survival, and abundance in several insect orders including Lepidoptera (Robinson *et al.* 2012), although responses are context dependent.

Higher trophic levels are also affected by CO_2-induced shifts in plant quality (Ode 2006; Ode & Crompton 2013; Hentley *et al.* 2014a; Ode *et al.* 2014), with the effects of elevated CO_2 on

natural enemies mediated mainly through shifts in prey nutritional quality and growth rate (Roth & Lindroth 1995; Holton *et al.* 2003; Chen *et al.* 2005; Klaiber *et al.* 2013b). For example, elevated CO_2 reduces alfalfa nutritional quality, which increases the development times of armyworm caterpillars. Delayed development results in asynchrony between caterpillars and parasitoid wasps, and ultimately reduces parasitoid fitness (Dyer *et al.* 2013). However, changes in prey nutritional quality and growth rates are not the only means by which elevated CO_2 may affect the natural enemies of herbivores. Elevated CO_2 may also inhibit the defense and escape capabilities of herbivores. For example, elevated CO_2 impairs aphid escape from predator attack by disrupting conspecific chemical alarm signaling (Hentley *et al.* 2014b). Hence, assessing the indirect influence of elevated CO_2 on defensive strategies of herbivores in the context of natural enemies remains an important goal.

In response to the threat of predators and parasitoids, many specialist herbivores have evolved mechanisms to co-opt PSMs for their own defense (Dyer & Deane Bowers 1996; Nishida 2002; Ode 2006; Opitz & Müller 2009; Petschenka & Agrawal 2016). Sequestration by insect herbivores is an active process involving the modification, transfer, and storage of toxic compounds, often at a significant metabolic cost to the insect (Opitz *et al.* 2010). This process may come at an additional cost to herbivores if the presence of toxic PSMs reduces the insect's ability to mount a strong immune response against other natural enemies such as parasites (Smilanich *et al.* 2009; Greeney *et al.* 2012). The concentration and composition of PSMs sequestered depends on both the amount of tissue consumed by the insect, and insect sequestration efficiency, defined as the proportion of PSMs ingested that are retained (Bowers & Collinge 1992; Camara 1997). Host plant chemical profiles strongly influence the concentration and composition of PSMs sequestered by herbivores (Malcolm 1990, 1994; Agrawal *et al.* 2015). Therefore, environmental factors that alter phytochemistry could also influence herbivore sequestration (Prudic *et al.* 2005; Tao & Hunter 2015) with important consequences for herbivore susceptibility to predation and parasitism (Duffey 1980; Malcolm & Brower 1989a; Stamp & Bowers 1995; Dyer & Deane Bowers 1996). Despite the well-demonstrated phytochemical changes induced by elevated CO_2, we are unaware of any study to date that has examined the effects of CO_2 on toxin sequestration by insects.

Changes in herbivore morphology may also mediate herbivore-enemy interactions under elevated CO_2. Diet and stress influence the development and morphology of herbivores, with significant ecological consequences (Wainwright & Reilly 1994; Koehl 1996; Stoks 2001; Bernabò *et al.* 2013; Stoks *et al.* 2016). Therefore, phytochemical changes induced by elevated CO_2 may alter aspects of herbivore morphology that directly influence escape from predators on both small and large scales. Escape over large spatial scales includes the phenomenon of "migratory escape", in which prey may move long distances annually to mitigate any local build-up of predators and parasites (Altizer *et al.* 2011, 2015). In flying animals, foraging, courtship, predator escape, and migratory ability are strongly influenced by wing size, shape and wing loading, the ratio between body mass and wing area (Wootton 1992; Berwaerts *et al.* 2002; Dudley 2002). Subtle changes in wing size and shape can affect drag, lift, and ultimately, flight behavior (Srygley & Thomas 2002). To maximize energy use efficiency during flight, animals typically employ a combination of gliding and active propulsion (Park *et al.* 2010; Kovac *et al.* 2012). Larger, more elongated wing shapes, where the ratio is high between wing length and width (Aspect Ratio), result in optimal gliding flight (Kerlinger 1989). Indeed, many migratory animals have bigger, more elongated wings (higher aspect ratios) with narrower tips that reduce drag and, thus, improve long distance flight performance (Lockwood *et al.* 1998; Leisler & Winkler 2003; Vágási *et al.* 2016). Wing loading also influences flight efficiency and behavior. Flying insects with higher wing loading values tend to possess larger energy reserves for stronger powered flight (Srygley & Kingsolver 2000; Dudley & Srygley 2008). Migration and habitat use impose strong selection on wing size, shape (Altizer & Davis 2010; DeVries *et al.* 2010; Chazot *et al.* 2015; Li *et al.* 2016), and wing loading (Buler *et al.* 2017). However, despite an extensive body of literature detailing the importance of dietary chemistry for insect fitness (Awmack & Leather 2002; Chown & Sue Nicolson. 2004), only a handful of studies have explored the effects of diet on wing morphology and consequent flight ability (Boggs & Freeman 2005; Pellegroms *et al.* 2009; Johnson *et al.* 2014a).

Natural enemies themselves may also influence toxin sequestration and morphology of herbivores. Despite our knowledge of how sequestration jeopardizes immune defense against parasitoids and parasites (*reviewed in* Greeney *et al.* 2012), no study to date has examined how infection itself alters sequestration. In contrast, parasites have well-studied impacts on host

morphology (Johnson *et al.* 2002; Cunningham *et al.* 2005). In insects, disease may cause severe wing deformations ultimately inhibiting flight performance (Genersch *et al.* 2006; de Roode *et al.* 2008b; Villacide & Corley 2008; Dorhout *et al.* 2011). Infection and environmental factors may also combine to influence host morphology (Kristan & Hammond 2000), with the potential to alter defensive phenotypes. However, we do not yet know how the presence of natural enemies combines with environmental change to alter host defense.

Here, we investigate the effects of elevated CO_2 on the defense and susceptibility of a specialist Lepidopteran herbivore to its natural enemies. We fed larvae foliage from four host plant species with varying chemical defense levels grown under either ambient or elevated CO_2. We also infected a subset of these herbivores with a parasite, to understand how infection and environmental change combine to alter herbivore defense, including wing traits associated with migratory escape. We measured changes in phytochemistry induced by elevated CO_2 and assessed subsequent toxin sequestration and morphology of butterflies. Because phytochemistry influences insect sequestration patterns (Malcolm 1990, 1994; Agrawal *et al.* 2015), we expected sequestration profiles to mirror changes in plant chemistry induced by elevated CO_2. In terms of butterfly morphology, we predicted that changes in PSMs and reductions in the nutritional quality of larval host plants grown under elevated CO_2 (Robinson *et al.* 2012) would cause declines in the quality of the insect flight phenotype: smaller, thinner and rounder wings with lower wing loading values. Finally, we expected that the metabolic costs of parasitic infection would exacerbate any deleterious effects of elevated CO_2 on toxin sequestration or wing morphology.

3.3 Materials and Methods

Study System

Monarch butterflies, *Danaus plexippus,* are best known for two ecologically important behaviors relevant to defense against higher trophic levels: the sequestration of toxic PSMs and long-distance migration. Some of the earliest studies of sequestration detail the monarch's ability to store toxic steroids, cardenolides, derived from the foliage of their milkweed, *Asclepias,* host plants (Reichstein *et al.* 1968; Malcolm & Brower 1989b). Cardenolides disrupt the function of

Na^+/K^+-ATPase in the sodium-potassium channels of animal cells (Agrawal *et al.* 2012) and render monarchs bitter tasting, well-defended prey (Reichstein *et al.* 1968; Malcolm & Brower 1989b). The polarity of cardenolides determines, in part, their biological activity (Rasmann & Agrawal 2011; Agrawal *et al.* 2012), whereby the most lipophilic (nonpolar) cardenolides are the most toxic, even reducing monarch performance (Sternberg *et al.* 2012; Tao *et al.* 2016). Monarchs utilize the diversity of milkweed species that they encounter throughout their range as larval host plants (Vickerman & de Boer 2002). Milkweed species vary substantially in the composition and concentration of cardenolides in their foliar tissues (Rasmann & Agrawal 2011; Agrawal *et al.* 2012). In turn, the composition and concentration of cardenolides sequestered by monarchs is correlated tightly with milkweed chemistry (Malcolm 1990, 1994; Agrawal *et al.* 2015). Importantly, monarchs can selectively sequester moderately lipophilic cardenolides in their tissues (Malcolm & Brower 1989b; Tao & Hunter 2015). Despite a growing body of work illustrating the effects of environmental change on milkweed chemistry (Vannette & Hunter 2011; Matiella 2012; Tao *et al.* 2014; Andrews 2015), we know very little about how monarch sequestration will respond to future environmental conditions (Tao & Hunter 2015).

Monarchs exist in both migratory and non-migratory (resident) populations distributed across the globe (Ackery & Vane-Wright 1984). In eastern North America, monarchs migrate up to 4500 km from their summer breeding grounds to overwintering sites in Mexico every Fall (Urquhart & Urquhart 1978; Brower & Malcolm 1991; Flockhart *et al.* 2017). Previous work comparing flight phenotype (wing size, shape, and loading) among different geographic populations of monarchs established that eastern N. American monarchs have larger and more elongated wings than their non-migratory conspecifics (Altizer & Davis 2010; Li *et al.* 2016). Migratory monarchs also have higher wing loading values. Within the eastern N. American population of monarchs, wing size and shape also vary with migration timing; earlier migrants have larger more elongated wings than do later migrants (Satterfield & Davis 2014). Wing development can be affected by the environmental conditions experienced by larvae in their final instars and during pupation (Speight *et al.* 1999). Therefore, morphological variation within populations may reflect the influence of environmental factors, such as diet, on flight phenotype. Extreme food restriction reduces monarch wing size (Johnson *et al.* 2014a); however, no study to date has examined the effects of varying cardenolide concentrations on monarch wing morphology.

Throughout their range, monarch populations suffer infection by a debilitating, protozoan parasite, *Ophryocystis elektroscirrha,* (Mclaughlin *et al.* 1970; Leong *et al.* 1997a; Altizer *et al.* 2000). Infection by *O. elektroscirrha* reduces monarch lifespan, decreases fecundity, and limits flight ability (Altizer & Oberhauser 1999; Bradley & Altizer 2005; de Roode *et al.* 2008b, 2009). Monarchs become infected as early-instar larvae by ingesting spores on the surface of egg chorea and leaf tissue. During monarch development, parasites undergo both asexual and sexual replication. Ultimately, adult monarchs emerge covered in dormant parasite spores and heavily infected individuals are less able to migrate long distances (Altizer *et al.* 2000, 2015). Because heavily infected monarchs migrate poorly and are more likely die in transit ("migratory culling"), monarchs that do arrive to overwintering grounds experience a much lower prevalence of the pathogen ("migratory escape" (Altizer *et al.* 2011)). Thus, the migration behavior itself reduces the prevalence of infected individuals in the population seasonally (Bartel *et al.* 2011). For monarchs suffering light to moderate infection, there is no clear link between the impaired flight ability resulting from disease and morphological changes induced by parasitic infection (Bradley & Altizer 2005). *Ophryocystis elektroscirrha* more likely depletes the energy reserves necessary for flight (Altizer *et al.* 2015). However, additional stressors, such as reductions in diet quality induced by elevated CO_2, may influence the impact of parasitic infection on wing morphology.

Milkweed and Monarch Source Materials

We analyzed the wings of monarch butterflies reared on milkweeds grown under ambient (400 ppm) or elevated (760 ppm) CO_2 at the University of Michigan Biological Station (UMBS). We provide full details of the UMBS CO_2 array in Decker *et al.* (2018). Briefly, during the summer of 2015, we grew four species of milkweed in a 40 chamber mesocosm array (Drake *et al.* 1989), with 20 chambers maintained at ambient and 20 at elevated concentrations of CO_2. Within those chambers, we grew milkweed species that varied substantially in their cardenolide concentrations, ranging from high to low: *A. curassavica, A. syriaca, A. speciosa,* and *A. incarnata*. We purchased *A. curassavica* and *A. speciosa* seeds from Prairie Moon Nurseries, MN and collected *A. syriaca* and *A. incarnata* seeds from wild populations growing near Pellston, MI in 2014. We surface sterilized seeds in a 5% bleach solution and germinated them on damp filter paper. Germinated seedlings were planted in deepots ™ containing Metromix 360

and Osmocote 16:16:16 controlled release fertilizer and were watered daily. After planting, we let the seedlings establish in the UMBS glass house for three weeks and then transferred them outside into the chamber array for the remainder of the experiment (below), where they were watered daily. Each chamber contained at least six individual plants of each species, with three designated for a single parasite-infected monarch and the other three for an uninfected (control) monarch. Monarchs are voracious herbivores and will consume up to 3 fully-grown milkweed plants as larvae, which is why we grew at least 3 plants for each monarch in each chamber (3 plants x 4 species x 2 parasite treatments x 40 chambers = 960 plants total).

The monarchs used in this study were the F_1 offspring of seven genetic crosses between monarch lineages from eastern North America (St Marks, FL). Individual monarchs were assigned to one of 16 treatments (2 parasite treatments x 4 host plant species x 2 levels of CO_2) making for 320 monarchs reared in total (2 parasite treatments x 4 host plant species x 2 CO_2 concentrations x 20 replicates of each). However, not all monarchs survived to adulthood, with mortality notably higher among monarchs infected with parasites. Moreover, some inoculated monarchs resisted infection, thereby inflating the sample size of uninfected monarchs. Final sample sizes for each treatment are shown in Table 3.1.

After 42 days of growth in the chamber array, cuttings were excised from plants, placed in individual 0.64L plastic containers, and kept under ambient CO_2 conditions. A darkened monarch egg (darkening indicates eggs just about to hatch) was attached to a leaf on each cutting to ensure that neonate monarchs consumed only tissue from their assigned plant before inoculation with parasite spores. Three days after hatching, monarchs were inoculated following the methods of de Roode *et al.* (2008), whereby 10 spores were deposited onto one 70.6 mm^2 leaf disk taken from the assigned plant on which they hatched. Spores originated from a single genetic parasite lineage collected directly from an eastern North American, wild-caught butterfly. Uninfected control monarchs were fed clean leaf disks of the same size with no parasite spores. Foliar chemistry samples were taken from each plant at the same time as inoculations following the methods detailed below; the chemistry of milkweed foliage just before and during inoculation influences parasite infection success and severity (de Roode *et al.* 2011a). We only measured the chemistry of the individual plants used at inoculation, and assume that their chemistry reflects

adequately that of the other two plants per treatment in each chamber, which larvae consumed in later instars.

Monarch larvae were fed cuttings from their assigned host-plants *ad libitum* until pupation. 24-hours after adult eclosion, monarchs were sexed and weighed to obtain adult wet mass. For the remainder of their adult lives, monarchs were kept in 5.75 x 9.5 cm glassine envelopes at 15°C without food to estimate lifespan under starvation conditions (an estimate of parasite virulence)(de Roode *et al.* 2007).Three weeks after death, the wings were carefully removed from each monarch body with forceps. The spore load of each monarch was measured by individually vortexing bodies in 5mL of DI water for 5 minutes and then counting the number of spores found in 10 μL aliquots of that solution using a KOVA glasstic hemocytometer (KOVA International Inc., CA) (de Roode *et al.* 2008a). Monarch left forewings were stored at -20°C for up to three months and then scanned using an HP scanJet 6300C. The left forewing of each monarch was then weighed and deposited in 1mL centrifuge tubes for cardenolide analyses.

Cardenolide Chemical Analysis

We quantified cardenolides in both milkweed foliage and monarch left forewings (wing cardenolide concentrations correlate tightly with body cardenolide concentrations (Fink & Brower 1981) following well-established methods (Zehnder & Hunter 2009; Vannette & Hunter 2011; Tao & Hunter 2012). Samples were ground in 1 mL of methanol for 3 minutes, sonicated for 1 hour at 60°C, and centrifuged for 6 minutes. The supernatant was then transferred to a new 1 mL ependorff tube and evaporated under vacuum at 45°C until dry. We then resuspended the samples with 150 μL of methanol spiked with 0.15 mg/mL digitoxin internal standard and separated compounds of interest using ultra performance liquid chromatography (UPLC, Waters Inc., Milford, MA, USA) with an Acquity BEH C18 column (1.7 μm, 2.1 x 50 mm, Waters Inc., Milford, MA, MA, USA). We eluted each 2 μL sample injection for 9 minutes under a gradient of 20% acetonitrile (ACN): 80% water for 3 minutes followed by a linear gradient to 45% ACN: 55% water over the remainder of the run with a constant flow rate of 0.7 mL per minute. Peaks were detected by absorption at 218 nm using a diode array detector. Only those peaks that absorbed symmetrically around maxima between 216-222 nm were considered cardenolides.

We calculated three descriptive measures of cardenolide chemistry for both foliar and wing tissues: total cardenolide concentration of the sample, cardenolide diversity, and cardenolide polarity. Cardenolide concentrations were calculated as the sums of all separated peak areas, corrected by the concentration of the internal digitoxin standard and estimated by the dry sample mass. Cardenolide diversity was calculated using the Shannon diversity index borrowed from biodiversity literature: $H=-\text{sum}(P_i \log[P_i])$ where P_i is the relative amount of a cardenolide peak produced in an individual plant compared to the total amount of cardenolides in that same individual. We calculated cardenolide polarity $P=\text{sum}(P_i\ RT_i)$, where RT_i is the retention time of the *i*th peak in the individual following Rasmann & Agrawal (2011). Finally, we subtracted the cardenolide concentration in plant tissues from the cardenolide concentration sequestered in monarch wings for a measure of the magnitude of difference in cardenolide concentration between monarch wings and plant tissues.

Wing Morphometrics

In our analysis of monarch wing morphology, we concentrated on monarch forewings alone for two reasons: first, monarchs position their forewings to cover their hindwings during soaring flight (Altizer & Davis 2010). Therefore, forewing size and shape should have the largest influence on flight ability. Second, preliminary work established that variation in milkweed chemistry only affects forewing morphology (Berns *et al.* 2014). Therefore, we scanned the left forewing of each specimen next to a ruler for scaling of the image.

Forewing Size

We calibrated Adobe Photoshop to calculate distance measures based on a pixel-to-millimeter ratio. We then took four basic measures of forewing morphology: (1) length of the butterfly wing from wing apex to thorax insertion (mm), (2) width of the forewing at the longest axis perpendicular to the length measurement (mm), (3) total forewing area (mm^2), and (4) wing perimeter (mm) (see Figures 3.1a,b). When minor damage to wings occurred, we estimated wing edges to create a complete outline; butterflies with substantial wing damage were discarded from all analyses.

Forewing Shape

We calculated two traditional metrics of forewing shape using the wing measures taken in Adobe Photoshop: 1) wing aspect ratio (length divided by width), and 2) roundness (area to perimeter ratio: 4π area/perimeter^2) (Altizer & Davis 2010). We also calculated wing loading (wet body mass/wing area) (Altizer & Davis 2010), a common aeronautical measure indicative of maneuverability and performance in flight. Finally, we created a metric that examined butterfly wing density, which we termed, specific wing area (wing area/wing mass).

Statistical Analysis

Basic Model Structure

We used linear mixed models (R version 3.3.2.; package: lme4) to test for effects of our treatments on plant chemistry, toxin sequestration, and monarch morphology. Because each of the 40 chambers contained plants from multiple treatments (plant species and monarch infection status), we included chamber identity as a random effect in all models to account for the lack of independence of samples within chambers (Littell *et al.* 2002; Vannette & Hunter 2011). Additionally, for models with monarch traits as response variables, we also included monarch genotype as a random effect. Variables were transformed when necessary, and model homoscedasticity of variance was tested using Levene's Tests from the car package in R.

Milkweed Host-Plant Chemistry

To determine the effects of CO_2 treatment and milkweed species on foliar chemical traits, we ran models with square-root-transformed total concentration, log-transformed diversity, and polarity index of cardenolides as response variables, and CO_2 treatment and milkweed species as fixed effects. *Asclepias incarnata* tended to produce only one cardenolide compound and therefore had cardenolide diversity values of 0 for most plants. We therefore excluded *A. incarnata* from analyses of cardenolide diversity.

Cardenolide Sequestration by Monarchs

We used similar models to test for effects of CO_2 treatment, milkweed host-plant species and infection status on monarch wing cardenolides (square-root transformed total concentration, diversity, and polarity). In addition, we used the same model structure to test for treatment effects on the magnitude of difference in cardenolide concentration between monarch wings and

plant tissues. We used Tukey Post-Hoc tests to compare means among treatments (significance reported at the 0.05 level) when significant effects were detected in our models.

Infection status is complicated because some monarchs exposed to *O. elektroscirrha* can resist infection and are spore-free as adults. Therefore, before conducting the analyses described above, we compared the wing cardenolides of control monarchs (never exposed to the parasite) with those of monarchs that were exposed to the parasite but had resisted infection (zero spore loads). There were no significant differences between cleared monarchs and control monarchs in the sequestration of total cardenolides ($F_{1,190} = 0.90$, $p = 0.3446$), cardenolide diversity ($F_{1,190} = 0.02$, $p = 0.8773$) or cardenolide polarity ($F_{1,190} = 0.13$, $p = 0.9101$). Therefore, in all subsequent analyses of sequestration, we grouped these two monarch treatments into one "uninfected" status. We followed a similar procedure to determine whether or not monarch sex played a role in sequestration chemistry and found no effect of monarch sex on total sequestered cardenolide concentrations ($F_{1,250} = 0.24$, $p = 0.6237$), diversity ($F_{1,250} = 0.39$, $p = 0.5336$) or polarity ($F_{1,250} = 0.13$, $p = 0.9101$). Therefore, we did not include monarch sex in the models that explored treatment effects on butterfly sequestration.

In addition to the analyses above, we used permutational multivariate analysis of variance (PerMANOVA) (Anderson 2001) to compare the effects of CO_2 treatment, milkweed host-plant species and, in the case of butterfly cardenolides, infection status on the assemblage (identity and relative abundance) of cardenolide compounds produced in milkweed and sequestered by monarchs. For the PerMANOVA, we used the Bray-Curtis ordination with the Adonis package in R 3.3.2.

Monarch sequestration in relation to host plant chemistry

Previous studies of cardenolide sequestration by monarchs have revealed a positive correlation between the concentration of cardenolides that monarchs consume in milkweed foliage as larvae and the total amount of cardenolides sequestered in their bodies as adults (Malcolm & Brower 1989). Therefore, we tested whether CO_2 treatment or infection status might alter the slope of this relationship by including them in a linear mixed model with plant cardenolide concentration as an independent variable and total wing cardenolide concentration as the dependent variable.

As before, both the chamber number of the plant and monarch genotype were included as random effects in the model.

Wing Morphology

Because of collinearities among the different size and shape metrics of monarch wings, we followed the methods of Altizer & Davis (2010) and used Principal Component Analysis (PCA) to reduce our morphology measures into one PCA axis explaining forewing size (PCA-size) and another PCA axis explaining forewing shape (PCA-shape). Specifically, forewing length, width, area and perimeter were used to create the PCA-size axis that explained 99.6% of the total variance, while forewing area and roundness were used to create the PCA-shape axis that explained 95.2% of the total variance. High values of PCA-size correspond to larger wings and low values correspond to smaller wings. High values of PCA-shape represent monarchs with more elongated wings while low values of PCA-shape represent monarchs with blunt, rounder wings. We then ran models with these PCA axes as response variables; CO_2 treatment, milkweed host-plant species, infection status and monarch sex were fixed effects. Because some treatments had small sample sizes (see Table 3.1) we could not include the four-way interaction in any of our full models.

Wing Loading and Specific Wing Area

To examine effects of treatments on monarch wing loading and specific wing area, we ran models with wing loading (wet body mass/wing area) and specific wing area (wing area/wing mass) as response variables. CO_2 treatment, milkweed host-plant species, infection status and monarch sex were fixed effects. Because we found effects of infection, sex, and host-plant on specific wing area (see below), we then tested whether wing mass, wing area, or both varied among treatments.

3.4 Results

In total, 252 monarchs survived to adulthood, sequestered cardenolides in their wings, and were used in our analyses. The *O. elektroscirrha* variant used in our study was extremely virulent (Decker *et al.* 2018), and 43 of the 68 butterflies not included in this study died due to infection. The remaining 19 either did not survive the experiment, had wings that were too badly

malformed or damaged to perform morphometric measures, or did not sequester measurable cardenolides in their wings.

Milkweed Host-Plant Chemistry

Of the 252 milkweeds that supported surviving monarchs, only 114 produced measurable cardenolides, and we have restricted our chemical analyses to those plants. Within those 114 plants, there were 65 *A. curassavica*, 19 *A. syriaca*, 18 *A. speciosa,* and 12 *A. incarnata* individuals. Elevated CO_2 changed the total concentration of foliar cardenolides produced by milkweed in a species-specific manner (species*CO_2: $F_{3,106} = 3.047$, $p = 0.032$, Figure 3.2a). Under elevated CO_2 there was a 52% decline in the foliar cardenolide concentrations of *A. curassavica* ($F_{1,36} = 13.43$, $p = 0.0008$, Figure 3.2a). Concentrations in *A. syriaca* ($F_{1, 13} = 1.0847$, $p = 0.32$), *A. speciosa* ($F_{1, 13} = 0.76$, $p = 0.399$) and *A. incarnata* ($F_{1, 11} = 0.01$, $p = 0.910$) remained unaffected by elevated CO_2. Across CO_2 treatments, *A. curassavica* produced the highest cardenolides concentrations, while *A. incarnata* produced the lowest ($F_{3, 92} = 19.92$, $p < 0.001$, Figure 3.2a).

We present mean values of foliar cardenolide diversity and polarity in Table B1. *Asclepias curassavica* plants produced nearly 6 times the diversity of next highest species *A. syriaca,* followed closely by *A. speciosa* ($F_{2, 96} = 60.94$, $p < 0.0001$). There was no effect of CO_2 treatment on foliar cardenolide diversity ($F_{1, 96} = 0.19$, $p = 0.6656$) and no interaction between CO_2 and milkweed species ($F_{2, 96} = 0.15$, $p = 0.8653$) on foliar cardenolide diversity. *Asclepias syriaca* plants produced cardenolides with the highest polarity values (most lipophilic), followed by *A. curassavica* then *A. speciosa* ($F_{3, 96} = 13.30$, $p < 0.0001$). *Asclepias incarnata* produced foliar cardenolides with the lowest polarity index values (most polar). There was no main effect of CO_2 treatment ($F_{1, 55} = 1.01$, $p = 0.3189$) on foliar cardenolide polarity, and no interaction between CO_2 and milkweed species ($F_{3, 96} = 1.42$, $p = 0.2415$, Table B1).

As expected (Sternberg et al. 2012), milkweed species varied in the assemblage of cardenolides that they produced (PERMANOVA, species: $F_{3, 110} = 24.16$, $R^2 = 0.39$, $p = 0.001$). In addition, the effect of CO_2 treatment on cardenolide composition varied among milkweed species but

explained relatively little of the variation in cardenolide community composition (PERMANOVA, CO_2*species: $F_{3, 110} = 2.26$, $R^2 = 0.037$, $p = 0.004$).

Monarch Wing Chemistry

Monarchs maintained the same cardenolide concentrations in their wings between the two CO_2 treatments ($F_{3, 214} = 1.60$, $p = 0.1909$, Figure 3.2b) despite the decline in foliar cardenolide concentration in *A. curassavica* induced by elevated CO_2 (Figure 3.2a). Monarchs were able to increase the rate at which they sequestered cardenolides from their host plants under elevated CO_2 (CO_2*plant cardenolide $F_{1, 110} = 12.41$, $p = 0.0006$, Figure 3.2c). In other words, monarchs feeding on milkweed foliage grown under elevated CO_2 sequestered more cardenolides per unit cardenolide available in their larval host plants (see difference in slopes in Figure 3.2c). Consequently, the difference between the cardenolide concentrations sequestered in the butterflies and those available in the plants varied by CO_2 treatment in a species-specific manner (CO_2*species: $F_{3, 93} = 2.80$, $p = 0.04402$, data not shown). The largest difference between butterfly and plant cardenolide concentrations was found in those monarchs feeding on *A. curassavica* under elevated CO_2. Monarchs feeding on *A. incarnata* grown under elevated CO_2 sequestered concentrations of cardenolides that were the most similar to the concentrations of their host plants.

When feeding on *A. syriaca*, monarchs infected with parasites sequestered nearly 20% less cardenolide in their wings than did uninfected monarchs (Infection*Species $F_{3, 232} = 2.84$, $p = 0.0385$, Figure 3.3). However, the spore load of infected monarchs was unrelated to the concentration ($F_{1, 58} = 2.12$, $p=0.1505$), diversity ($F_{1, 58} = 2.23$, $p=0.1405$), or polarity ($F_{1, 58} = 0.72$, $p= 0.4007$) of cardenolides sequestered in the wings. Monarchs feeding on other milkweed species showed no such parasite-induced decline in sequestration (Figure 3.3). CO_2 treatment, milkweed species and infection status did not interact to influence the concentration of cardenolides sequestered by monarchs ($F_{1, 225} = 0.83$, $p = 0.4803$).

Monarchs that fed on *A. incarnata* grown under elevated CO_2 sequestered a lower diversity of cardenolides than did those that fed on *A. incarnata* under ambient CO_2 (Species*CO_2 $F_{3, 211} = 4.44$ $p = 0.005$, Figure 3.4). There was no interaction between CO_2 treatment, milkweed host-

plant species and infection status on the diversity of cardenolides sequestered by monarchs ($F_{3,219} = 1.92$, $p = 0.1271$. The cardenolides sequestered by monarchs fed *A. curassavica* had the highest polarity index values (most lipophilic), while those sequestered by monarchs fed *A. speciosa* had the lowest (most polar) ($F_{3, 243} = 287.20$, $p < 0.0001$, data not shown). In contrast, the polarity of the cardenolides in monarch wings was generally unresponsive to CO_2 treatment ($F_{1, 243} = 0.024$, $p = 0.8758$) or parasite infection ($F_{1, 242} = 0.036$, $p = 0.7593$). Moreover, there were no significant interactions among treatments.

Despite the interactive effects of CO_2 treatment and milkweed species on the composition of cardenolides produced in the milkweed host-plants, monarchs themselves sequestered a consistent assemblage of cardenolides in their wings among treatments (PERMANOVA, CO_2*species: $F_{3, 247} = 1.41$, $R^2 = 0.006$, $p = 0.149$). Further, the composition of wing cardenolides was also unaffected by CO_2 treatment alone (PERMANOVA, CO_2: $F_{1, 250} = 2.37$, $R^2 = 0.003$, $p = 0.073$). However, just as in the foliar tissue, milkweed host-plant species strongly influenced the composition of cardenolides sequestered by monarchs (PERMANOVA, species: $F_{3, 247} = 157.00$, $R^2 = 0.65$, $p = 0.001$).

Monarch Wing Morphology

Size

Monarch sex was the only factor to affect wing size in our study. Male monarch wings were slightly larger than were those of female monarchs ($F_{1, 231} = 3.50$, $p = 0.064$), corroborating the findings of Altizer *et al.* (2010). Monarch wing size was unaffected by CO_2 treatment ($F_{1, 31} = 0.21$, $p = 0.6525$), milkweed host plant species ($F_{1, 203} = 2.39$, $p = 0.070$), infection status ($F_{1, 225} = 2.78$, $p = 0.097$) and the interaction between these three treatments ($F_{1, 212} = 0.66$, $p = 0.576$).

Shape

In contrast to the weak effects of our treatments on wing size, monarch wings were more angular and elongated (higher values of PCA-Shape) when larvae had fed on milkweed grown under elevated CO_2 ($F_{1, 214} = 15.82$, $p < 0.0001$, Figure 3.5a) or when larvae had consumed *A. syriaca* or *A. incarnata* ($F_{1, 212} = 3.78$, $p = 0.0113$, Figure 3.5c). Additionally, the wings of female

butterflies were more angular and elongated than were those of males ($F_{1, 214} = 15.50$, p = 0.0001, Figure 3.5b).

While *O. elektroscirrha* infection had no independent effect on forewing shape ($F_{1, 212} = 0.86$, p=0.3550) a finding consistent with previous work (Bradley & Altizer 2005), infection influenced the response of monarch wing shape to elevated CO_2; infected monarchs from plants under ambient CO_2 had rounder wings than did butterflies from other treatments (infection*CO_2: $F_{1, 212} = 9.46$, p = 0.0024, Figure 3.6a). Moreover, effects of infection on wing shape varied among milkweed species (infection*species: $F_{1, 212} = 4.54$, p = 0.0041, Figure 3.6b). Specifically, infected monarchs had rounder wings than uninfected monarchs when feeding on *A. curassavica, A syriaca*, and *A. incarnata*, but had more elongated wings than uninfected monarchs on *A. speciosa* (Figure 3.6b). Finally, there were some differences between male and female butterflies in the way that their wing shapes responded to plant species and infection (sex*infection*plant $F_{3, 212} = 2.96$, p = 0.0331, Figure B1). However, the three-way interaction term explained only a small portion of variance in the model when compared to the strength of the main effects reported above. Wing aspect ratio correlated most strongly with PCA-shape ($r = 0.999$, N = 237, $p < 0.0001$) and therefore partially represents the changes in wing shape found in our study. We summarize the model results for wing shape in Table 3.2 along with corresponding mean values of wing aspect ratio (wing length (mm)/ wing width (mm)) by treatment for ease of interpretation.

Wing Loading

Flying animals with higher wing loading values tend to exhibit faster more powerful flight. Therefore, we examined how our treatments influenced this aspect of the flight phenotype in monarchs. Male monarchs had 5% higher wing loading (wet body mass/wing area) values than female monarchs ($F_{1,16} = 17.12$, p = 0.0008, Figure 3.7a), corroborating the findings of Altizer *et al.* (2010). Notably, wing loading also varied with larval diet ($F_{3,16} = 4.77$, p = 0.0152, Figure 3.7b) such that monarchs reared on *A. syriaca* had a 5% higher wing loading than did those reared on other milkweed species. Wing loading was unaffected by CO_2 treatment, parasite infection, or their interactions with other treatments.

The wings of monarchs infected with *O. elektroscirrha* were 7% less dense than were the wings of uninfected monarchs (specific wing area: $F_{1, 221} = 20.65$, $p < 0.0001$, Figure 3.8a). This decline in wing density in response to infection was likely due to the 11% reduction in forewing mass induced by infection ($F_{1, 221} = 16.18$, $p < 0.0001$), because the area of monarch wings did not respond to infection ($F_{1, 231} = 3.42$, $p= 0.0656$). Female monarch wings were 6% more dense than male monarch wings ($F_{1, 228}= 15.74$, $p < 0.0001$, Figure 3.8b). In this case, the difference in wing density between sexes was due to the fact that females had smaller wing areas ($F_{1, 237}= 3.78$, $p = 0.0530$) because there was no difference in wing mass between monarch sexes ($F_{1, 234}= 1.82$, $p = 0.1780$). Finally, milkweed host-plant species influenced monarch specific wing mass as well. Monarchs fed *A. curassavica* had the least dense wings while those fed *A. syriaca* had the densest wings ($F_{3, 199}= 2.66$, $p = 0.0492$, Figure 3.8c). The influence of milkweed host-plant species on wing mass ($F_{3, 201}= 2.95$, $p = 0.0340$) partially explains the differences in specific wing mass observed by species. Monarchs fed *A. curassavica* had the lightest wing masses in our study, while those fed *A. syriaca* had the heaviest wing masses.

3.5 Discussion

Rapid environmental change has the potential to influence trophic interactions by altering the defensive phenotype of prey. Here we present the first study to examine the effects of a major environmental change driver, elevated CO_2, on toxin sequestration and morphology of monarch butterflies. Our results demonstrate that 1) monarchs maintain the concentration and composition of cardenolides that they sequester despite changes in the phytochemistry of milkweed under elevated CO_2. 2) Aspects of monarch morphology important to flight ability such as wing shape, wing loading, and wing density are influenced by elevated CO_2, milkweed host plant species, parasite infection, and sex. 3) Feeding on high cardenolide milkweed is associated with the formation of rounder, thinner wings, which are less efficient at gliding flight. We suggest that changes in the rate of sequestration under elevated CO_2 are a byproduct of compensatory feeding aimed at maintaining a nutritional target in response to declining dietary quality. Ingesting larger amounts of foliage from milkweed high in cardenolides may come at a cost to the monarch. Such costs may manifest as lower quality flight phenotypes: rounder, thinner wings with lower wing

loading values. Critically, small changes in wing morphology can have important consequences for migration success (Bradley & Altizer 2005), which includes migratory escape from parasites. Changes in sequestration and morphology may, therefore, have negative consequences for monarch defense and migration in a changing world.

Monarchs increase the rate of sequestration under elevated CO_2

We demonstrate that monarchs increase their rate of cardenolide sequestration under elevated CO_2 (Figure 3.2a). Consequently, the concentration and composition of cardenolides that monarchs sequester from one species of milkweed, *A. curassavica*, remain the same despite both a 52% reduction in the concentration of cardenolides and changes in the composition of those cardenolides induced by elevated CO_2 (Figure 3.2a-d). Monarchs have been found previously to maintain constant concentrations of sequestered cardenolides in their tissues in response to changes in the chemical quality of *A. curassavica* (Tao & Hunter 2015). Herbivores can regulate sequestration by altering both the total amount of foliage consumed and sequestration efficiency (Camara 1997). Arthropods are well known for their ability to track target ratios of carbohydrates to protein in their diet through behavioral shifts in consumption (Simpson *et al.* 2015). Therefore, it is possible that the monarchs in this study increased the total amount of foliage that they consumed to compensate for the characteristic reduction in nutritional value of plants grown under elevated CO_2 (Lincoln *et al.* 1984; Docherty *et al.* 1996; Hunter 2001; Zavala *et al.* 2013; Johnson *et al.* 2014b).

In addition to increasing the amount of tissue monarchs consumed, elevated CO_2 may have also lowered the energetic requirements of sequestration by changing the community of cardenolides consumed by monarchs. Sequestration is an active process during which toxic metabolites are either absorbed, metabolized, or excreted with significant energetic costs to the insect (Nishida 2002; Hartmann 2004; Opitz & Müller 2009). The new cardenolide communities produced by milkweed under elevated CO_2 may have included compounds that are easier to modify, transport, and store in biological tissue (Nishida 2002).

Increased consumption rates and sequestration efficiency may come at an ecological cost to monarchs in the context of other natural enemies. Monarchs become infected with *O.*

elektroscirrha by ingesting spores on the surface of milkweed foliage (Leong *et al.* 1997b; de Roode *et al.* 2008a). If monarchs increase the amount of leaf tissue consumed under elevated CO_2, they may also increase the probability of ingesting dormant spores and becoming infected. Yet, certain cardenolides provide medicinal protection to monarchs against *O. elektroscirrha*, reducing infection probability, severity and fitness costs (de Roode *et al.* 2008a, 2011b; Sternberg *et al.* 2012; Gowler *et al.* 2015). However, elevated CO_2 decreases concentrations and changes the composition of cardenolides produced by *A. curassavica* (Decker *et al.* 2018). If monarchs cannot also ingest enough protective cardenolides to negate increased spore consumption, then they may suffer more prevalent and intense infections under future atmospheric conditions.

Elevated CO_2, milkweed species, infection, and sex influence monarch morphology

We found that diet quality influences aspects of monarch wing morphology important to both aerial maneuverability and long-distance flight (wing shape, wing $loading_{mass,}$ and wing density) (Berwaerts *et al.* 2002; Ortega Ancel *et al.* 2017). Notably, the concentration of atmospheric CO_2 under which the plants grew and the species of milkweed on which the monarchs fed as larvae influenced adult wing shape contingent upon infection status (Figure 3.6a, 3.6b, Table 3.3b). Under ambient CO_2, infection induced rounder wings, which are less efficient for gliding flight. However, under elevated CO_2 both infected and uninfected monarchs developed elongated wings better suited for gliding (Figure 3.6a). Additionally, monarchs that fed on low cardenolide milkweed, *A. incarnata,* also produced elongated wings regardless of infection status (Figure 3.6). Pointed forewings are thought to improve the flight efficiency of long-distance migratory species (Lockwood *et al.* 1998; Leisler & Winkler 2003; Vágási *et al.* 2016) because elongated wing tips minimize drag (Kerlinger 1989). Therefore, our data suggest that future environmental conditions, in combination with milkweed species, may induce the formation of wing shapes that improve the efficiency of monarch flight.

This is the first study to report effects of milkweed species on monarch wing shape. Changes in wing morphology in response to different host plants have been reported in other flying insects, such as *Drosophila* utilizing different species of cacti (Soto *et al.* 2008). Furthermore, in some tropical moths, certain larval host plant species can induce increased wing shape asymmetry

(Benítez *et al.* 2015). In the eastern N. American monarch population, wing shapes vary temporally throughout the migratory period: earlier migrants have more elongated wings than do later migrants (Satterfield & Davis 2014). In addition to temporal changes in shape, earlier migrants have redder pigmentation as compared to later migrants (Satterfield & Davis 2014). Wing redness predicts monarch flight distance and endurance (Davis *et al.* 2012; Hanley *et al.* 2013) and is loosely linked to diet (Johnson *et al.* 2014a). Perhaps, changes in monarch wing shape and reductions in redness throughout the migration period result from changes in the abundance of different milkweed species that vary in chemical and nutritional quality (Flockhart *et al.* 2012).

Additionally, we found that wing loading varied among milkweed species: monarchs fed *A. syriaca* had 6% higher wing loading values than monarchs fed other milkweed species (Figure 3.7b). During migration, monarchs actively propel themselves within the boundary layer and utilize upward convection currents of air and prevailing winds for gliding to reduce energy loss (Gibo 1986). Higher wing loading values are beneficial to this flight pattern, as they typically produce faster powered flight (Srygley & Kingsolver 2000; Dudley & Srygley 2008). Heavier monarchs with larger energy reserves tend to exhibit higher wing loading values as well. Our data suggest that eastern N. American monarchs feeding on the most prevalent species of milkweed in that region, produce higher, more beneficial wing loading values.

Both milkweed host plant species and infection by *O. elektroscirrha* influence the specific wing area of monarch wings (wing density). Monarchs fed *A. curassavica* have less dense wings when compared to monarchs feeding on the other three milkweed species tested (Figure 3.8c). Additionally, uninfected monarchs have denser wings than their infected counterparts (Figure 3.8a). Both infection and feeding on *A. curassavica* caused reductions in wing mass with no change in wing area. Therefore, though wings may experience more lift due to reduced mass, they may also be more prone to the deleterious effects of extended flight such as tearing or splitting.

Small differences in wing morphology that may affect the efficiency of flight could have large consequences for monarch migration success (Bradley & Altizer 2005). Eastern N. American

monarchs migrate up to 4,500 km through a combination of both soaring and active flight (Urquhart & Urquhart 1978; Gibo 1986; Brower & Malcolm 1991). Like other migratory insects, monarchs must take shelter during adverse weather conditions, utilize tail and head winds effectively, and cross large expanses of unsuitable habitat (Gibo & Pallett 1979; Srygley & Kingsolver 2000; Garland & Davis 2002). Therefore, any factor that causes monarchs to remain grounded during beneficial flying conditions or reduces the amount of time monarchs may stay aloft over unsuitable habitat will significantly reduce migration success. Here, we suggest that diet quality, mediated by milkweed species and CO_2 concentrations, in combination with infection and sex, influence monarch flight ability. Our data support previous findings, wherein infection by *O. elektroscirrha* did not alter wing shape or size directly (Bradley & Altizer 2005; Satterfield & Davis 2014). However, our data reveal changes in wing shape due to infection in combination with milkweed species, CO_2 treatment, and sex. Infection by *O. elektroscirrha* reduces monarch flight endurance (Bradley & Altizer 2005), and may, therefore, combine with these subtle changes in wing shape to influence overall migration success. Any influence of diet on the flight phenotype of monarchs may alter the effectiveness of migratory culling, whereby heavily infected monarchs have poor migration success (Altizer *et al.* 2000, 2015). In our data, elevated CO_2 eliminates the shape difference between infected and uninfected individuals, inducing more elongated wings in both groups (Figure 3.6a). If infected individuals become more efficient gliders under environmental change, this might jeopardize the migratory escape phenomenon that reduces pathogen prevalence seasonally in the N. American monarch population (Altizer *et al.* 2011; Bartel *et al.* 2011).

It should be noted that, although our treatments affected monarch wing shape, we detected no effect of diet or infection on wing size. All of the butterflies used in this study originated from the same migratory eastern N. American population. Therefore, strong selection for larger wings imposed by migration distance within this population may explain the consistency of wing size among treatment groups (Altizer & Davis 2010; Li *et al.* 2016; Yang *et al.* 2016; Flockhart *et al.* 2017).

Conclusions

Monarchs face multiple threats from anthropogenic environmental change (Malcolm 2017). Our data reveal the potential for elevated CO_2 to alter interactions between monarchs and their natural enemies mediated through changes in plant quality. We demonstrate that monarchs maintain consistent levels of cardenolide sequestration, despite changes in milkweed chemistry under elevated CO_2. Surprisingly, monarch wing shapes become more favorable for long-distance flight under elevated CO_2, but infection and milkweed host plants also influence monarch wing shape, loading, and density. Ultimately, feeding on high cardenolide milkweed reduces the quality of the flight phenotype, but further studies are needed to test the effects of diet chemical and nutritional quality on monarch flight and its implications for migration success.

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3.7 Tables & Figures

Table 3.1. Sample sizes of 252 surviving monarchs whose forewings were used to explore the effects of milkweed species, elevated CO_2 and infection by a protozoan parasite, *Ophryocystis elektroscirrha*, on toxin sequestration and wing morphology.

Milkweed Species	Infection Status	CO_2 Treatment	N total	Female	Male
A. curassavica	infected	ambient	7	3	4
		elevated	9	6	3
	uninfected	ambient	26	9	17
		elevated	27	12	15
A. incarnata	infected	ambient	11	4	7
		elevated	8	5	3
	uninfected	ambient	24	10	14
		elevated	19	9	10
A. speciosa	infected	ambient	9	2	7
		elevated	6	6	0
	uninfected	ambient	21	9	12
		elevated	24	13	11
A. syriaca	infected	ambient	5	1	4
		elevated	5	1	4
	uninfected	ambient	26	16	10
		elevated	25	9	16

Table 3.2. The main effects of treatments on mean monarch wing Aspect Ratios (wing length/wing width), a component of monarch wing shape. Values are considered to be equal if they do not significantly differ from each other using p = 0.05.

CO_2 treatment	$F_{1, 214} = 15.82$	$p < 0.0001$
	Aspect Ratio	
Ambient	1.95 ± 0.002	
Elevated	1.96 ± 0.002	
Butterfly Sex	$F_{1, 214} = 15.50$	$p < 0.0001$
	Aspect Ratio	
Female	1.97 ± 0.002	
Male	1.93 ± 0.002	
Milkweed Species	$F_{3,16} = 4.77$	$p = 0.0152$
	Aspect Ratio	**Tukey Results**
A. curassavica	1.94 ± 0.003	A
A. incarnata	1.96 ± 0.003	B
A. speciosa	1.95 ± 0.004	B
A. syriaca	1.96 ± 0.003	AB

Table 3.3. The two-way interactions between treatments on mean monarch wing Aspect Ratios (wing length/wing width), a component of monarch wing shape. Values are considered to be equal if they do not significantly differ from each other using p = 0.05.

Infection*CO₂	$F_{1, 212} = 9.46$	p = 0.002		
Infection Status	**CO_2 treatment**	**Aspect Ratio**	**Tukey Results**	
Infected	Ambient	1.93 ± 0.004	infected ambient > infected elevated	
	Elevated	1.97 ± 0.005	uninfected elevated = infected elevated	
Uninfected	Ambient	1.95 ± 0.002	uninfected ambient = uninfected elevated	
	Elevated	1.96 ± 0.002	uninfected ambient > infected ambient	
Infection*Species	$F_{3, 212} = 4.61$	p = 0.004	**Tukey Results**	
Infection Status	**Milkweed Species**	**Aspect Ratio**	**Infected**	**Uninfected**
Infected	*A. curassavica*	1.93 ± 0.008	CUR = INC	CUR < INC
	A. incarnata	1.95 ± 0.005	CUR < SPE	CUR = SPE
	A. speciosa	1.97 ± 0.006	CUR = SYR	CUR = SYR
	A. syriaca	1.94 ± 0.0102	INC = SPE	INC > SPE
Uninfected	*A. curassavica*	1.95 ± 0.003	INC = SYR	INC = SYR
	A. incarnata	1.97 ± 0.003	SPE = SYR	SPE = SYR
	A. speciosa	1.94± 0.004		
	A. syriaca	1.96 ± 0.003		

Table 3.4. The three-way interaction between treatments on mean monarch wing Aspect Ratios (wing length/wing width), a component of monarch wing shape.

Monarch Sex*Infection Status*Milkweed Species		$F_{3, 212} = 2.96$	$p = 0.033$
Infection Status	**Sex**	**Milkweed Species**	**AR**
Infected	Female	*A. curassavica*	1.959 ± 0.011
		A. incarnata	1.960 ± 0.008
		A. speciosa	1.979 ± 0.011
		A. syriaca	1.970 ± 0.003
	Male	*A. curassavica*	1.899 ± 0.010
		A. incarnata	1.938 ± 0.004
		A. speciosa	1.955 ± 0.007
		A. syriaca	1.930 ± 0.012
Uninfected	Female	*A. curassavica*	1.960 ± 0.004
		A. incarnata	1.991 ± 0.003
		A. speciosa	1.972 ± 0.005
		A. syriaca	1.974 ± 0.003
	Male	*A. curassavica*	1.939 ± 0.003
		A. incarnata	1.952 ± 0.004
		A. speciosa	1.918 ± 0.006
		A. syriaca	1.949 ± 0.005

Figures

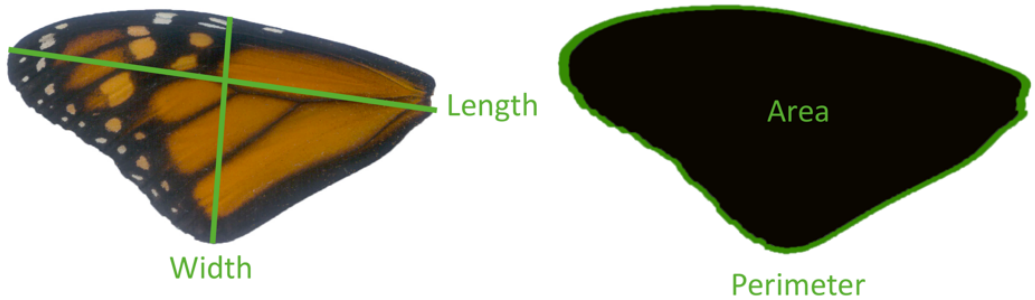

Figure 3.1. A scanned monarch butterfly forewing. A) & B) illustrate four basic morphometric measures taken in Adobe Photoshop: length, width, area and perimeter.

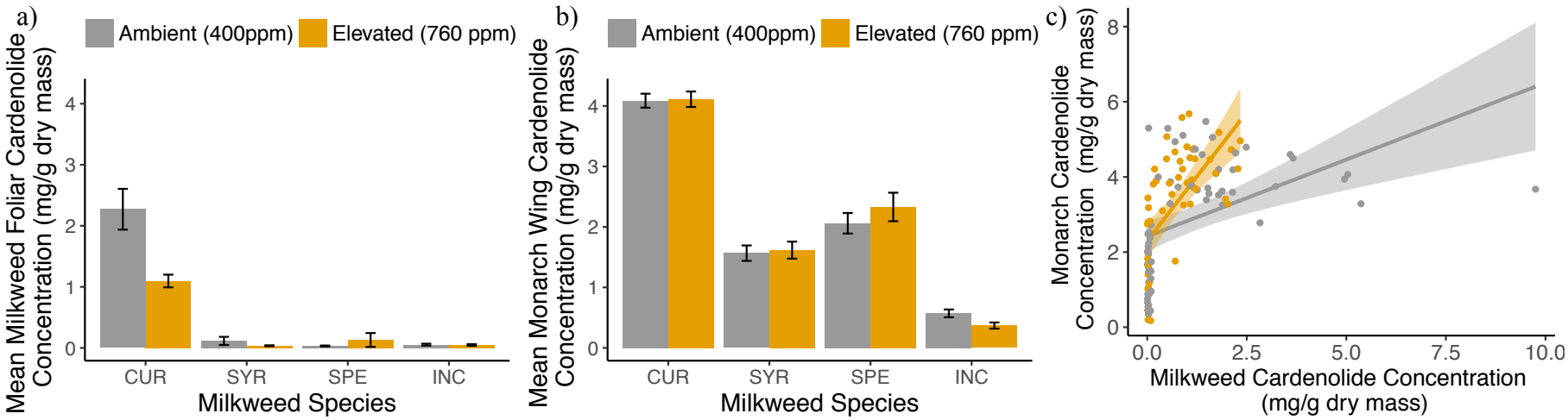

Figure 3.2 Effects of elevated CO_2 on (a) foliar cardenolide concentrations (mg/g dry mass), (b) monarch wing cardenolide concentrations (mg/g dry mass), and (c) the relationship between the cardenolide concentrations of plants and the concentrations sequestered by monarch. Bars represent mean values ±1 SE. Traits were transformed to approximate normality of errors before analyses but are presented here as untransformed values for ease of interpretation. Grey bars represent plants grown under ambient CO_2 and orange bars are those from elevated CO_2 or the monarchs that fed on those plants. Milkweed species codes: CUR = *A. curassavica*, SYR = *A. syriaca*, SPE = *A. speciosa*, INC= *A. incarnata*.

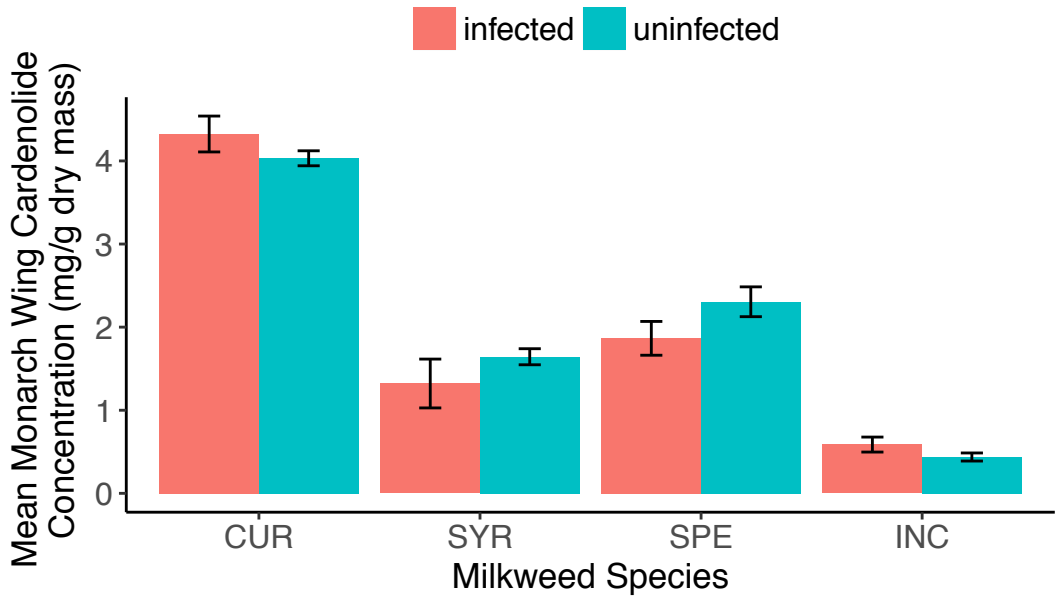

Figure 3.3. Effects of infection by *Ophryocystis elektroscirrha* on the total concentration of cardenolides sequestered by monarchs feeding on four species of milkweed. Red bars represent mean sequestration of infected monarchs and blue bars represent mean sequestration of uninfected monarchs ±1 SE. Milkweed species codes are the same as above.

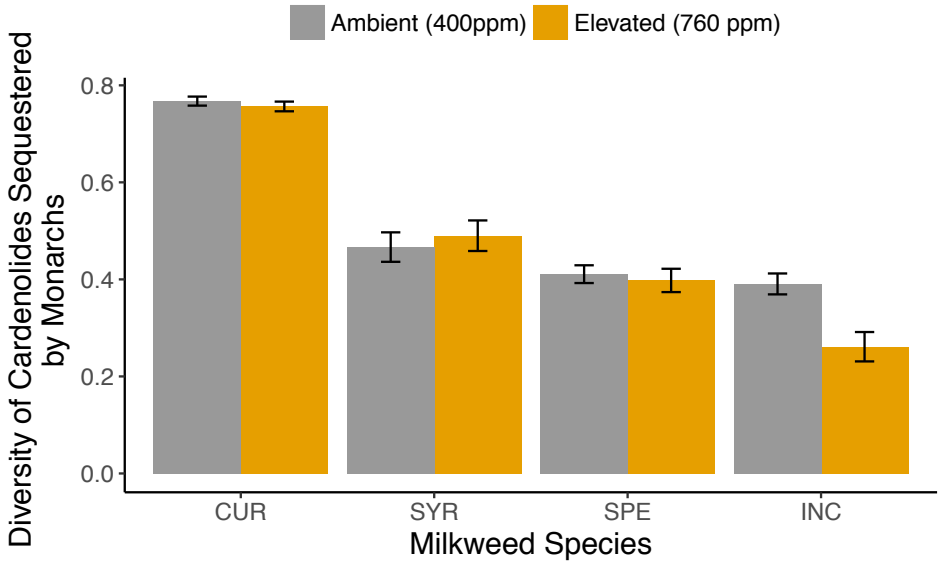

Figure 3.4. Effects of elevated CO_2 on the diversity of cardenolides sequestered by monarchs. Grey bars represent mean diversity values ±1 SE of cardenolides sequestered by monarchs that fed on plants grown under ambient CO_2 and orange bars are those from elevated CO_2. Milkweed species codes are the same as above.

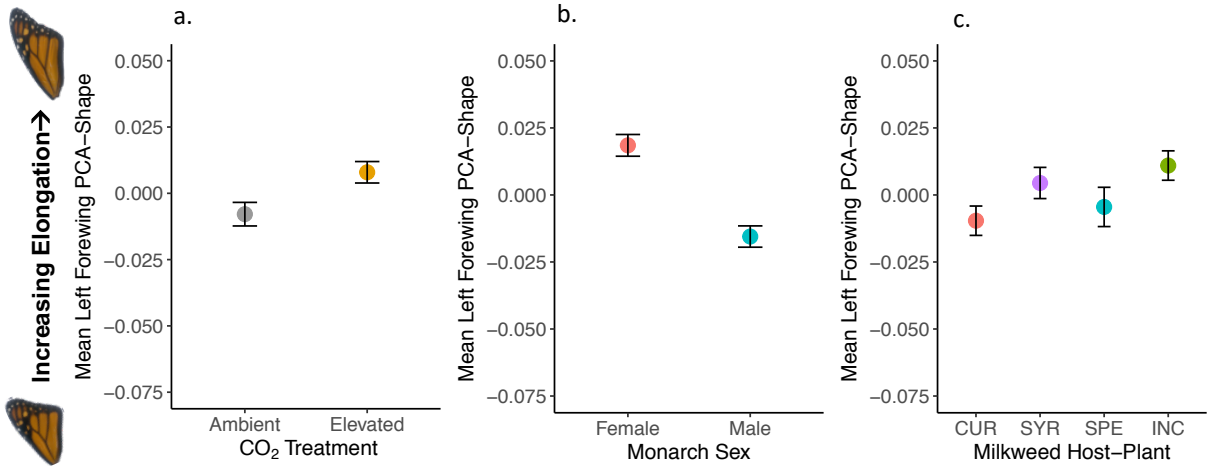

Figure 3.5. The effects of (a) CO_2 treatment, (b) sex and (c) milkweed host-plant species on a composite measure of monarch forewing shape. Points represent mean PCA-shape values ±1 SE. With increasing PCA-shape values wings become more elongated and angular. Milkweed species codes are the same as above.

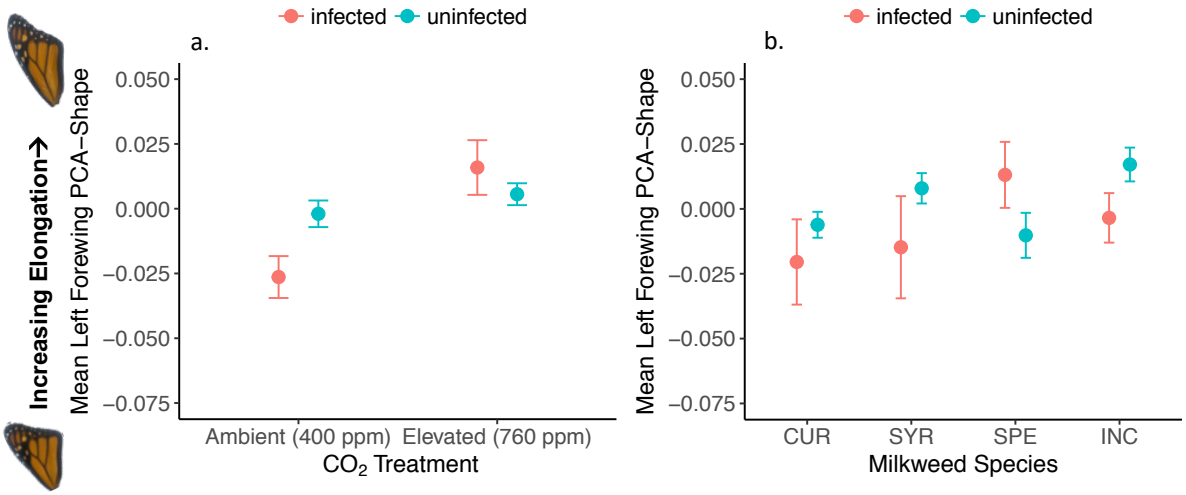

Figure 3.6. The interactions between (a) CO_2 treatment and infection by *Ophryocystis elektroscirrha*, and (b) milkweed host-plant species and infection on a composite measure of monarch forewing shape. Points represent mean PCA-shape values ±1 SE. Red points indicate mean shape values of infected monarchs while, blue points represent uninfected monarchs. With increasing PCA-shape values wings become more elongated and angular. Milkweed species codes are the same as above.

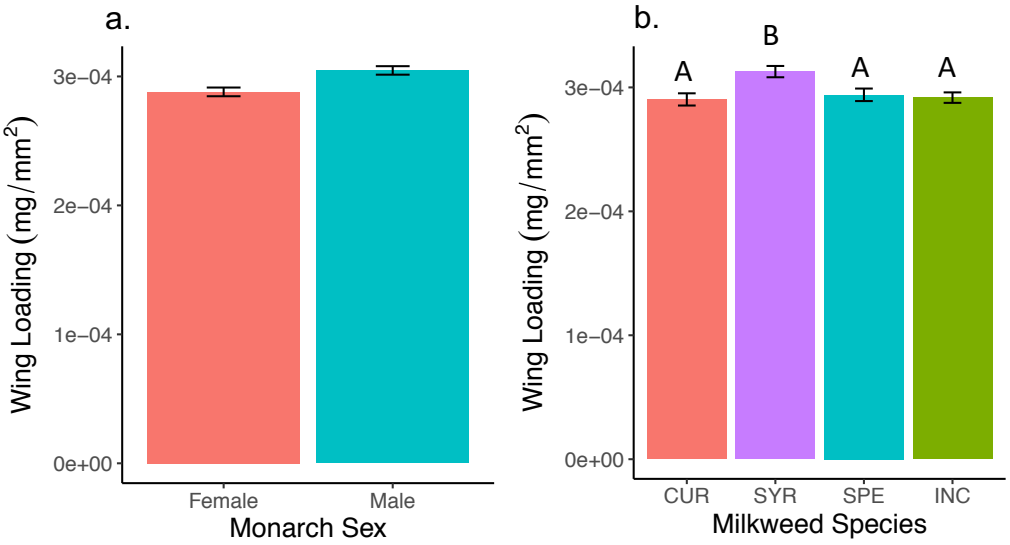

Figure 3.7. The effects of (a) monarch sex and (b) milkweed host-plant species on monarch wing loading (wet body mass/wing area). Bars represent mean values ±1 SE. Milkweed species codes: CUR = *A. curassavica* (red), SYR = *A. syriaca* (purple), SPE = *A. speciosa* (blue), INC= *A. incarnata* (green).

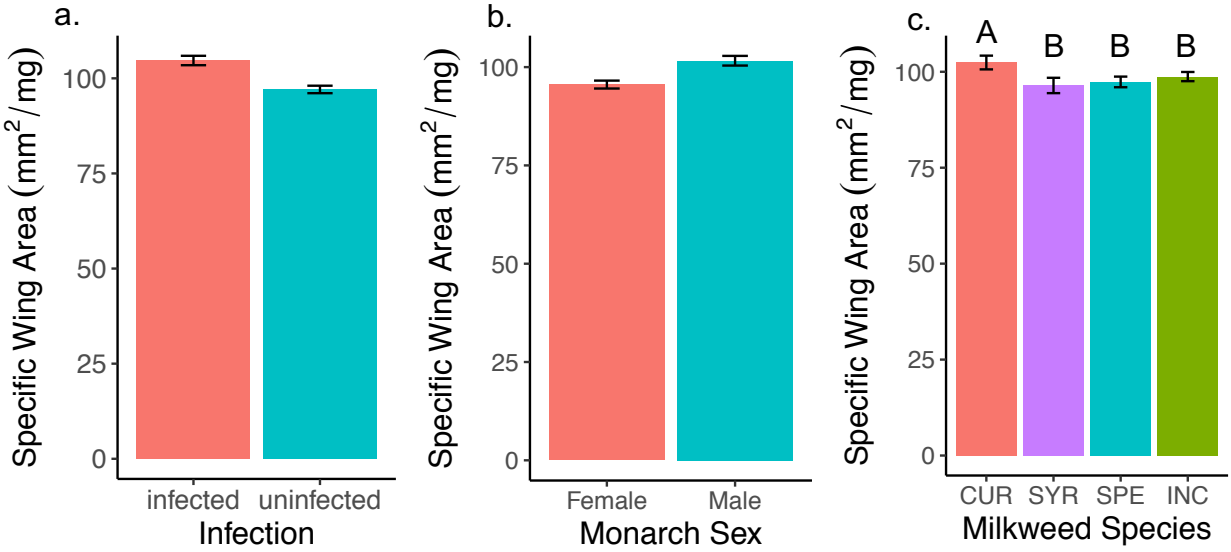

Figure 3.8. The effects of (a) *Ophryocystis elektroscirrha* infection, (b) monarch sex and (c) milkweed host-plant species on monarch specific wing area (wing area/wing mass), a measure of wing density. Bars represent mean specific wing area values ±1 SE. Higher specific wing area values indicate wings that are less dense. More dense wings will have lower specific wing area values.

Chapter 4 : Effects of CO_2 on Environmentally Mediated Immunity in a Specialist Herbivore

4.1 Abstract

Hosts use diverse immune responses to protect themselves from parasite attack. Understanding how and why the efficacy of immune responses varies with environmental conditions is important, especially given current rates of environmental change. Hosts must balance energetic investment in the immune response with investments in other life history traits. Therefore, any factor that generates further energetic deficits for the organism may lead to compromised immune function. Here, we investigate the influence of elevated CO_2 on the immune response of the monarch butterfly, *Danaus plexippus*, to infection by a sub-lethal, protozoan parasite, *Ophryocystis elektroscirrha* and simulated parasitoid attack. Certain species of milkweed host plants with high concentrations of toxic steroids known as cardenolides protect monarchs from infection by the parasite and reduce the fitness costs of infection. Yet we know very little about how plant secondary metabolites influence the monarch's immune response. To investigate the effects of cardenolides on monarch immunity and determine how elevated CO_2 will alter this relationship, we fed monarchs foliage from two species of milkweed- *A. curassavica* (medicinal), and *A. incarnata* (non-medicinal) - grown under ambient and elevated concentrations of CO_2. We then measured critical aspects of the monarch immune response, along with foliar secondary metabolites and nutritional quality, to understand the mechanisms underlying monarch immunity under future atmospheric conditions.

We demonstrate that elevated CO_2 influences the immune response of monarch hosts to infection by *O. elektroscirrha*. The immune enzyme activity of early-instar monarchs declined under parasite infection but was "rescued" by consuming foliage grown under elevated CO_2. Additionally, infection and a diet of foliage from elevated CO_2 increased the hemocyte concentrations of early-instar monarchs. In late-instar monarchs, the immune response against

parasitoids declined on "medicinal" milkweed, suggesting a potential tradeoff between resistance against parasitoids and resistance against agents of disease. With an improved understanding of immune mechanisms underlying host-enemy interactions, we can begin to make more powerful predictions about alterations in trophic cascades and emerging infectious diseases.

4.2 Introduction

Hosts must defend themselves against attack from parasites while embedded within complex communities and ecosystems. The local abiotic and biotic environment can have profound impacts on the outcomes of host-enemy interactions (Tylianakis *et al.* 2008; Wolinska & King 2009; Altizer *et al.* 2013; Dyer *et al.* 2013). However, the primary mechanisms underlying variation in host-enemy interactions often remain unresolved. Ecoimmunology is a burgeoning field that concentrates on the importance of environmental context in determining the strength, activity and variability of the host immune response (Rolff & Siva-Jothy 2003; Lazzaro & Little 2009; Brock *et al.* 2014). Central to this field is the idea that organisms must dynamically balance energetic investment in the immune response with investments in other life history traits (Stearns 1992) such as growth, survival, and reproduction (Sheldon & Verhulst 1996; Kraaijeveld *et al.* 2002; Schmid-Hempel 2003). Because immunity is costly and dependent on the host condition, environmental variability that generates further energetic deficits for the organism may lead to compromised immune function. Therefore, anthropogenic environmental change has the potential to influence host immunity through both direct physiological impacts and through changes in patterns of resource allocation by hosts.

A handful of studies have begun to explore the effects of environmental change on host immunity, and have thus far yielded variable results (*reviwed in* Martin *et al.* 2010; Jolles *et al.* 2015; Gherlenda *et al.* 2016). For example, increased ambient temperatures stimulate the immune enzyme activity and subsequent resistance of crickets, *Gryllus texensis* to bacteria (Adamo & Lovett 2011). In contrast, higher more variable ambient temperatures reduce frog immunity against infection by *Batrachochytrium dendrobatidis* (Bd) (Raffel *et al.* 2006). Much of the research on immunity and environmental change focuses on the direct physiological effects of warming temperatures and pollutants on host immune function, e.g. enzymatic activity (Martin *et al.* 2010; Bauerfeind & Fischer 2014; Richard *et al.* 2015; Wojda 2017). However,

other aspects of the host environment including stress, population density, and diet quality can shift in response to environmental change and consequently impact host immune function (Kraaijeveld *et al.* 2002; Schmid-Hempel 2003, 2005).

The growth, survival, and reproduction of insect herbivores are all vulnerable to changes in the nutritional and defensive chemistry of their food plants (Mattson 1980; Hunter 2016). Insects also face regular challenges from parasites and parasitoids and, as such, have well characterized immune responses. Insect immunity primarily targets foreign entities within the haemocoel, e.g. parasitoids, parasites and pathogens (Beckage 2008), and can be subdivided into humoral and cellular defenses (*reviewed in* Strand 2008). Humoral defenses encompass soluble effector molecules such as antimicrobial peptides that act on the membranes of pathogenic microbes (Kavanagh & Reeves 2007), take part in melanin formation and regulate clotting (Theopold *et al.* 2004; Rolff & Reynolds 2009). In contrast, cellular immunity consists of cellular defenses such as phagocytosis and encapsulation originating from the rapid synthesis of immune cells known as hemocytes (Eslin & Prévost 1998; Kraaijeveld *et al.* 2001; Wilson *et al.* 2003; Kavanagh & Reeves 2007; Strand 2008; Kacsoh & Schlenke 2012; Triggs & Knell 2012). While insects employ phagocytosis and immune effector molecules against smaller parasites and pathogens, the encapsulation response targets multicellular invaders, such as parasitoid eggs and parasites.

Despite the categorical subdivisions of immunity, both humoral and cellular immune defenses generally combine to produce the encapsulation response. In fact, many humoral molecules influence hemocyte function and many hemocytes produce humoral factors which all contribute to the strength of encapsulation (Lavine & Strand 2002). Encapsulation begins with the identification of a foreign antigen by recognition proteins which then activate specialized hemocytes (Rolff & Reynolds 2009). These hemocytes attach to the surface of the antigen and form layers of cells that eventually die and harden, surrounding the object. As the hemocytes undergo apoptosis, prophenoloxidase (proPO), an inactive dimer, is activated into polyphenoloxidase (PO), an enzyme critical to the cascade that produces melanin and other cytotoxic molecules (Hagen *et al.* 1994; Nigam *et al.* 1997; Reeson *et al.* 1998; Wilson *et al.* 2001; Cotter *et al.* 2004). Antigens typically die from simultaneous asphyxiation and poison exposure as result of both encasement by dead, melanized cells (encapsulation) and toxins

produced in the oxidative reactions taking place during melanization (Strand 2008). For insects, encapsulation and melanization are immediate and effective defenses against parasitoids such as wasps and flies, and against some parasites (Gillespie and *et al.* 1997; Lavine & Strand 2002; Beckage 2008).

Insect immunity depends strongly on diet quality (*reviewed in* Singer *et al.* 2014). Because immune defenses are tightly modulated by host energetic constraints, the ratio of protein to carbohydrate concentrations (nutritional quality) of host food-plants is well-known to influence immunity (Klemola *et al.* 2008; Srygley *et al.* 2009; Cotter *et al.* 2011). For instance, the immune response of a generalist caterpillar, *Spodoptera littoralis,* increases substantially when larvae are fed diets high in protein (Lee *et al.* 2008). Alternatively, hosts may starve themselves to reduce the intake of food molecules such as lipids that require costly enzymatic machinery to digest, and thereby detract from the resources allocated to immune function (Adamo *et al.* 2008, 2010). The concentration of plant secondary metabolites (PSMs) within the host diet can also alter immune function (Smilanich *et al.* 2009, 2011; Richards *et al.* 2012; Trowbridge *et al.* 2016). PSMs are inherently toxic, thus, certain concentrations and combinations of plant toxins may reduce insect immune function (Haviola *et al.* 2007; Smilanich *et al.* 2009; Greeney *et al.* 2012; Hansen *et al.* 2016). For example, the presence of dietary catalpol, an iridoid glycoside, reduces the melanization response of a Sphingid moth larva (Lampert & Bowers 2015). Alternatively, PSMs can sometimes enhance the insect immune response, such as the increased immunity expressed by a generalist Arctiid when consuming foliage high in antioxidants (Ojala *et al.* 2005). Given the large diversity of PSMs, and the many modes of their chemical action on insect performance, it is still unclear how plant secondary metabolism and nutritional quality combine to influence insect immunity.

More comprehensive methods for quantifying the diversity of plant secondary metabolites have recently been developed, refined, and associated with insect performance on plants (Krishnan *et al.* 2005; Macel *et al.* 2010; Bose *et al.* 2014; Dyer *et al.* 2014; Richards *et al.* 2015). Using ^{1}H-NMR (nuclear magnetic resonance) recent studies have begun to describe the structural diversity of secondary metabolites in plant foliage, not just calculate diversity indices based on compound

identities. Here, we utilize this technology to better associate the holistic plant "metabolome" with insect immunity.

The monarch butterfly, *Danaus plexippus*, is a specialist insect herbivore known to utilize the secondary chemistry of its host plants, *Asclepias,* as a defense against infection by a sub-lethal, protozoan parasite, *Ophryocystis elektroscirrha* (Lefèvre *et al.* 2010, 2012). Monarchs become infected with *O. elektroscirrha* after ingesting parasite spores on the surface of egg chorea and milkweed (*Asclepias*) tissues (Leong *et al.* 1997a, b). Spores lyse within the larval gut, sporozoites penetrate the larval hypoderm and replicate over the course of the monarch's development (Mclaughlin *et al.* 1970). Infected adult monarchs emerge covered in dormant parasite spores and experience reduced lifespan, decreased fecundity, and limited flight ability (Altizer & Oberhauser 1999; Bradley & Altizer 2005; de Roode *et al.* 2008b, 2009).

The chemical quality of the host plants that monarchs consume influences *O. elektroscirrha* infection. Certain milkweed species with high concentrations of toxic steroids known as cardenolides reduce infection probability, parasite growth rate, and parasite virulence in monarch larvae (de Roode *et al.* 2008a, 2011a; Gowler *et al.* 2015). Feeding on high-cardenolide (hereafter medicinal) milkweed also ameliorates the fitness costs of harboring each additional parasite, a form of defense known as host tolerance (Sternberg *et al.* 2012). Yet, despite a decade of research centered around milkweed medicinal protection and *O. elektroscirrha* infection (de Roode *et al.* 2008a, 2011b; Sternberg *et al.* 2012; Gowler *et al.* 2015), we know very little about how PSMs influence the monarch's immune response. Importantly, medicinal milkweeds provide increased protection against the parasite when consumed immediately before or during ingestion of spores (de Roode *et al.* 2011a). This critical period of medicinal action suggests that milkweed chemistry influences the effective dose of parasites that monarchs initially experience. However, we do not know if the secondary metabolites in milkweed are interfering directly with parasites within the midgut or promoting a strengthened anti-parasite immune response.

Monarchs also face attack from other enemies vulnerable to the insect immune response. Several microbial parasites and pathogens including a nuclear polyhedrosis virus (NPV), gram-positive bacteria such as *Bacillus*, gram-negative bacteria such as *Pseudomonas* and a microsporidian

Nosema species infect monarchs (Oberhauser *et al.* 2015b). Additionally, there are 12 species of tachinid flies, one brachonid wasp and one chalcid wasp known to parasitize monarchs (Arnaud 1978; Stenoien *et al.* 2015). The tachinid fly, *Lespesia archippivora*, has received the most attention (Smithers 1973; Borkin 1982; Prysby 2004; Oberhauser *et al.* 2009; Oberhauser 2012). This parasitoid attacks middle to late instar larvae and pupae, and may parasitize anywhere from 10-90% of monarchs in a given population (Oberhauser 2012). Due to the intimate nature of parasitoid development within the monarch larvae, the nutritional and medicinal quality of the monarch host diet likely influences parasitoid success (Oberhauser *et al.* 2015a). However, we know very little about general rates of parasitoid survival within monarchs (Hunter *et al.* 1996; Sternberg *et al.* 2011), and monarch immune defense against parasitoid infection. Interestingly, *O. elektroscirrha* infection reduces the mortality caused by the tachinid parasitoid, *L. archippivora* to late-instar monarch larvae (Sternberg *et al.* 2011). We need additional studies to understand the complex interactions among co-infection, monarch immunity, and diet chemistry.

Critically, global change drivers like temperature, rainfall, and elevated atmospheric concentrations of carbon dioxide (CO_2) have direct effects on plant physiology, which manifest themselves in plant nutritional quality and defensive chemistry (Bidart-Bouzat & Imeh-Nathaniel 2008, Hunter 2001; Robinson *et al.* 2012; Zavala *et al.* 2013). For example, elevated CO_2 causes substantial reductions in the cardenolide concentrations and nutritional quality of milkweed leaves (Vannette & Hunter 2011; Matiella 2012).

In this study, we examined how one global change driver, elevated CO_2, alters monarch immune function through changes in the medicinal (secondary chemistry) and nutritional (carbon, and nitrogen) quality of milkweed. We fed monarchs foliage from two species of milkweed, *A. curassavica* (medicinal), and *A. incarnata* (non-medicinal), grown under ambient and elevated concentrations of CO_2. Larvae were either infected with *O. elektroscirrha*, or left as uninfected controls. We then measured critical aspects of the monarch humoral (*in vitro* PO activity) and cellular (*in vitro* hemocyte concentrations and types) immune response, along with foliar secondary metabolites and nutritional quality, to understand the mechanisms underlying monarch immunity under future atmospheric conditions.

Because we still lack a complete understanding of the interaction between plant secondary metabolites, *O. elektroscirrha*, and monarch immunity, we approached this study with two contrasting predictions. Prediction 1: monarchs feeding on medicinal plants will produce a lower immune response. Rationale 1: If the mechanism of plant protection against the pathogen is through direct toxicity of cardenolides to the parasite, then monarchs will encounter lower effective doses of the parasite. Given that immunity is costly, monarchs feeding on medicinal plants that encounter lower spore loads will not invest energy into the immune response.

Alternatively, we generated prediction 2a: medicinal milkweed may improve insect immunity. Rationale 2a: certain secondary metabolites may have stimulating effects on insect immunity (Ojala *et al.* 2005). However, ecoimmunology teaches us that the increased energetic investment needed for this immune stimulation may be dependent on the nutrient concentrations of the milkweed foliage. We, thus, additionally generated prediction 2b: under conditions of low resource availability, e.g. reduced nutritional quality of foliage induced by elevated CO_2, monarchs may exhibit suppressed immune function despite feeding on medicinal host plants. Rationale 2b: despite the positive effects of dietary secondary metabolites for immunity, monarchs may still experience nutrient limitation in the immune response.

4.3 Materials and Methods

We performed a fully factorial manipulation with milkweed species (*A. incarnata* and *A. curassavica*), CO_2 treatment (ambient or elevated), and *O. elektroscirrha* treatment (infected or uninfected) as fixed factors. We then ran immune assays from two groups of caterpillars reared under the factorial treatments: (i) an immune assay, measuring PO activity and hemocyte counts of early-instar larvae, and (ii) a filament assay, measuring encapsulation activity of late-instar larvae. A third group of caterpillars (assay controls) were reared to adulthood to estimate the effects of our factorial treatments on monarch resistance and tolerance to parasite infection (Table 4.1).

Milkweed Sources and Growing Conditions

We obtained the seeds of both milkweed species from commercial vendors (*A. curassavica*: Victory Seed, OR and *A. incarnata*: Lupine Gardens, WI). Seeds were surface sterilized using a

5% bleach solution and only *A. incarnata* seeds were cold stratified for six months prior to planting. Seedlings were germinated on moist, sterilized paper towels and planted on 5/1/17 in deepots ™ containing Metromix 360 (SunGro Horticulture, Vancouver, BC) and Osmocote 16:16:16 controlled release fertilizer (ICL Specialty Fertilizers, Dublin, OH). We grew and watered seedlings daily in the glass house at the University of Michigan Biological Station (UMBS, 45.5587° N, 84.6776° W) for two weeks before transferring them outside into the CO_2 array.

On 5/28/17 we distributed plants evenly into 40 open-top controlled atmosphere chambers in the field at UMBS. The CO_2 array was comprised of 20 chambers maintained at ambient CO_2 (410 ppm) and 20 chambers maintained at elevated CO_2 (810 ppm) from dawn until dusk (Drake *et al.* 1989). We monitored CO_2 concentrations in all 20 elevated CO_2 chambers and one ambient CO_2 chamber during daylight hours using a LI-COR 320 IRGA (LI-COR, Lincoln, NE, USA). Additionally, we recorded air temperatures within the chambers using iButton dataloggers (IbuttonLink, Whitewater, WI, USA). The average air temperature inside elevated CO_2 chambers was 20.81 (± 0.05) ºC, and 20.80 (± 0.05) ºC inside ambient CO_2 chambers. These temperatures fall well within temperatures typically experienced by monarchs in eastern North America (Couture *et al.* 2015; Faldyn *et al.* 2018).

Within each chamber, we grew 3 plants of each treatment group (2 milkweed species x 2 parasite treatments x 3 assay groups = 12 treatments), making for 36 plants per chamber. We planned to rear one caterpillar per treatment group from each chamber (20 replicate larvae per treatment), using the three plants per treatment to rear each individual caterpillar. However, final replicate numbers were smaller based on some mortality during rearing (Table 4.1). Plants were watered at least once a day, sometimes twice depending on the weather. We began taking cuttings of plants for the different assays after approximately 1 month of growth in the array (6/27/17). Cuttings were placed individually in 710 mL plastic containers containing one monarch each.

Monarch Sources and Rearing Methods

A darkened monarch egg (darkening indicates close proximity to hatching) was randomly assigned and attached to the leaf surface of each cutting. The monarchs used in this study were

the grand-offspring of lab-reared butterflies collected from St. Marks, FL and Lawrence, KS. We distributed monarchs from five full-sib family lines evenly across our experimental treatments. Three days after neonates hatched on their assigned plant cuttings, we began the inoculation process. Each larva was transferred to a petri dish containing a 95 cm^2 piece of moist filter paper and a 70.6 mm^2 leaf disk taken from the larva's assigned host plant, cleaned with a 5% bleach solution and rinsed thoroughly with water. For those larvae designated as inoculated, we placed 10 parasite spores on the surface of the leaf disk, while uninoculated larvae received spore-free leaf disks. Immediately after the leaf disk was taken from the plant for inoculation, we collected foliage for chemical analyses (detailed below). The petri dishes containing larvae and leaf disks were kept in an incubator maintained at 26°C with 16-hour daylight. Upon consuming the entire leaf disk (and therefore all spores), larvae were returned back their cleaned original containers with new plant cuttings. We continued to feed monarchs in the control and filament treatments *ad libitum* until pupation or until filament insertion (see below), replacing tissue and cleaning each monarch container every 2-3 days. Monarchs designated for the immune assays (see below) were sacrificed 48 hours following inoculation to determine the initial immune response to *O. elektroscirrha* infection. We chose this period because most larvae finished feeding on the entire leaf disk around 24-36 hours after being transferred to a petri dish, thus, to ensure that all larvae had completed inoculation and had adequate time to mount an immune response (Beckage 2008), we waited for a complete 48 hours to pass.

Monarch Performance Measures (only Assay Controls)

Approximately 24 hours following the formation of the chrysalis, we removed all frass and remaining foliage from each container so that monarchs could pupate in a clean container. We sexed monarchs 24 hours after eclosion and transferred them to pre-weighed 5.75 x 9.5 cm glassine envelopes. To obtain adult wet mass, we weighed each butterfly within its envelope. Monarchs were then stored in an incubator kept at 15°C with 16-hour daylight for the remainder of their adult lives. We checked each monarch daily and recorded date of death. The lifespan of monarchs under these starvation conditions correlates strongly with lifetime reproductive fitness (de Roode *et al.* 2008b). Therefore, we estimated parasite virulence as the reduction in the lifespan of infected monarchs as compared to uninfected monarchs (de Roode *et al.* 2007, 2008a). Two months after death, we quantified the spore loads of each monarch following well-

established methods (de Roode *et al.* 2007, 2008a, b). We removed wings from the bodies of deceased monarchs and transferred each monarch body into a 10 mL scintillation vial with 5 mL of deionized water. We vortexed the mixture for 5 minutes, and then transferred 10 µL aliquots into 4 wells in a glasstic hemocytometer (KOVA International, Inc., Garden Grove, CA) for counting. The tolerance of monarchs to the parasite was measured as the slope of the regression between spore load and longevity, with separate regressions for each milkweed species by CO_2 treatment (Sternberg *et al.* 2012, 2013; Tao *et al.* 2015).

Immune Assays to Determine Monarch Immune Response

As in most insects, the immune defenses of monarchs include both humoral and cellular responses (Lindsey & Altizer 2009; Satterfield *et al.* 2013). We performed two major groups of immune assays on the hemolymph of those monarchs designated for the immune assay treatment group (Table 4.1): phenoloxidase (PO) activity (humoral immunity) and hemocyte counts (cellular immunity). Monarchs use the PO enzyme to orchestrate melanization and ultimately encapsulation. We used a colorimetric assay to determine the activity of free, naturally active PO (PO activity), PO proenzymes (proPO) and the total PO activity (total PO activity) in monarch hemolymph following published methods (Adamo 2004; Smilanich *et al.* 2017; Dhinaut *et al.* 2018). We made incisions in the larval cuticle above the final proleg in the A6 abdominal segment using a hand-pulled Pasteur pipette needle (Smilanich *et al.* 2009, 2017). With a micropipette, we took 2 µL of hemolyph from each larva and deposited it into 50 µL of chilled phosphate-buffered saline (PBS) solution in a 1.0 mL Eppendorf tube and vortexed the mixture. We then incubated two 50 µL aliquots of the hemolymph-PBS mixture with 300 µL of L-DOPA (g L-DOPA in mL deionized water) in two wells of a 96-well plate for 20 minutes at room temperature. To the second designated well of each sample, we also added 17 µL of 10% Cetylpyridinium chloride monohydrate (CPC) to activate any proPO in the hemolymph. Using an ELx800 Absorbance Microplate Reader (BioTek) we measured absorbance of the samples at a wavelength of 490 nm every 30 seconds for 180 minutes. In our analyses, we used the slope of the linear portion of the absorbance curve (30-106 minutes) as our measure of PO, and total PO activity. We calculated the activity of proPO by subtracting free PO activity from total PO activity.

Counting hemocytes present in the hemolymph provides an additional measure of insect immunity. The density and frequency of different hemocytes can indicate insect encapsulation ability (Eslin & Prévost 1998; Kacsoh & Schlenke 2012; Triggs & Knell 2012). At the same time samples were extracted for the PO analysis, we took an additional 4 µL of hemolymph and added it to 8 µL of chilled anticoagulant solution (0.684 g EDTA, 0.346 g citric acid dissolved in 180 mL PBS). Within 24 hours of taking the sample, we performed counts using a Neubauer Bright-Line hemocytometer (Cambridge Instruments, Inc.) and 10 µL of the sample. We counted the total number of hemocytes present in the entire central gridded area and recorded the different hemocyte types present in the hemolymph following the descriptions of Strand (2008) and Vogelweith *et al.* (2016). Monarchs produce four major hemocyte types: plasmocytes, granulocytes, oenocytoids and spheroulocytes. Plasmocytes are the most prevalent hemocyte type in Lepidoptera (Strand 2008) and are cells involved with aggregation and encapsulation. Granulocytes are cells involved primarily in phagocytosis and encapsulation. Oenocytoides are thought to be associated with PO synthesis and production and we are unsure of the exact function spheroids have in monarch immunity (Altizer & de Roode 2015).

Filament Assay to Determine Monarch Encapsulation Response

To measure the immune defense of 5th instar larvae designated for the Filament treatment (Table 4.1), we inserted an artificial "parasite egg" into monarchs following Klemola *et al.* (2008). Our simulated parasite eggs were 2 mm long pieces of nylon, which we rubbed with sandpaper, knotted at one end (for ease of handling), sterilized with pure ethanol, and dried before inserting into larvae. Similar to the hemolymph extraction protocol, we made a small incision into the larval cuticle of the A6 abdominal segment just above the final proleg. We then inserted the filament into the larval haemocoel parallel to the abdomen, taking care not to perforate the intestine. Larvae were returned to their cleaned, original containers and allowed 24 hours to mount an immune response. We removed the implanted filaments using forceps, deposited the filaments into a 70% ethanol solution and stored samples at – 20 ºC for three months before further analyses. Incisions, insertions and removals were made by the same person every time.

To quantify filament melanization (our estimate of encapsulation response) we photographed filaments under a dissecting microscope using an iPhone 6 (Apple Inc.) with an iDu LabCam

Microscope Adapter (iDu, Detroit, MI, USA) in a dark room. We calibrated Adobe Photoshop to calculate distance measures based on a pixel-to-millimeter ratio. We then quantified the mean gray value (MGV, 0 = black to 255 = white) of a roughly 0.500 mm^2 rectangle selected from the tip of the filament that was directly inserted into the insect.

Foliar Chemical Analyses

Cardenolide extraction and analysis

To quantify milkweed foliar cardenolides, we followed well-established methods (Zehnder & Hunter (2009); Vannette & Hunter (2011); Tao & Hunter (2012)). At the same time that we inoculated monarchs with *O. elektroscirrha* (above), we punched 6 leaf disks from each monarch's assigned plant into 1 mL of methanol and stored samples at -10°C until processing. We took another 6 disks and recorded wet and dry mass of these disks to obtain the approximate dry mass of each foliar cardenolide sample. To extract cardenolides, we ground foliage for 3 minutes, sonicated the sample for 1 hour at 60°C, and centrifuged the sample for 6 minutes. We then transferred the supernatant to a new 1 mL ependorff tube and evaporated the sample under vacuum at 45°C until dry. Samples were resuspended in 150 μL of methanol spiked with 0.15 mg/mL digitoxin internal standard. We separated cardenolide compounds of interest using ultra performance liquid chromatography (UPLC, Waters Inc., Milford, MA, USA) with an Acquity BEH C18 column (1.7 μm, 2.1 x 50 mm, Waters Inc., Milford, MA, MA, USA). Each 2 μL sample injection was eluted for 9 minutes with a constant flow rate of 0.7 mL per minute under a mobile phase of 20% acetonitrile (ACN): 80% water for 3 minutes followed by a gradient that increased to 45% ACN: 55% water over the remainder of the run. Cardenolides were quantified using a diode array detector scanning between 200 and 300 nm; peaks that absorbed symmetrically with maxima between 216-222 nm were considered cardenolides.

We calculated three descriptive measures of cardenolide chemistry from our milkweed foliar samples: total cardenolide concentration of the sample, cardenolide diversity, and cardenolide polarity. Cardenolide concentrations were calculated as the sums of all separated peak areas, corrected by the concentration of the internal digitoxin standard and estimated by the dry sample mass. Cardenolide diversity was calculated using the Shannon diversity index borrowed from biodiversity literature: $H=-sum(P_i log[P_i])$ where P_i is the relative amount of a cardenolide peak

produced in an individual plant compared to the total amount of cardenolides in that same individual. The biological activity of cardenolides is determined, in part, by the polarity of the different sugar moieties attached to steroid skeleton of the compound (Rasmann & Agrawal 2011; Agrawal *et al.* 2012). Because animal cell membranes are outwardly hydrophobic, the most lipophilic (nonpolar) cardenolides are thought to be the most toxic (Sternberg *et al.* 2012; Tao *et al.* 2016). We calculated cardenolide polarity P=sum(P_i RT_i), where RT_i is the retention time of the *i*th peak in the individual following Rasmann & Agrawal (2011).

NMR-extraction and analysis

At the same time that we removed leaf disks for cardenolide analysis, we harvested three additional leaves for NMR sampling. We dried these leaves in a 30°C drying oven and ground the tissue to a fine powder. We then transferred 200 mg of each sample to a centrifuge tube and added 3 mL of deuterated extraction buffer (25% KH_2PO_4 90 mM, pH 6 in D_2O, 75% CD_3OD with Tetramethylsilane). We vortexed each sample for 30 seconds, sonicated and then centrifuged each sample for 15 minutes each. We filtered the supernatant into an NMR tube and prepared the sample for NMR analysis on a 400 MHz Varian Instrument (Aligent Technologies). We processed the NMR spectral data using MestReNova software (Mestrelab Research) and aligned sample spectra using the solvent peak. Sample spectra were then baseline-corrected, phase-corrected, and normalized to the total area of 100, and binned every 0.04 ppm from 0.5 to 14 ppm. As an estimate of whole-plant chemical diversity we calculated the Simpson diversity index ($D = 1 - \sum (n/N)^2$) of chemical shifts (approximations of secondary metabolites) where n is the integral of a specific binned frequency range, and N is the total number of binned frequency ranges measured in the sample (Richards *et al.* 2015).

C:N extraction and analysis

Remaining dried foliar tissue was ground to a fine powder and then analyzed using a TruMac CN Analyzer (Leco Corporation, St. Joseph, MI) to provide estimates of foliar carbon (C) and nitrogen (N) concentrations. Examining the foliar C:N ratio is a simple approximation of the nutritional quality of the plant (Mattson 1980).

Statistical Analyses

For all of our analyses, we used either linear mixed models (LMMs; lme4 package) or generalized linear mixed models (GLMMs; lme4 package) always including chamber identity and monarch genotype (when applicable) as random effects. We implemented all statistical tests in R version 3.3.2 (R Development Core Team, 2018) and all variables were transformed to best achieve normality of error.

Monarch Immunity, Milkweed Species and Elevated CO_2

To investigate the effects of CO_2 treatment, infection by *O. elektroscirrha*, and milkweed species on the PO activity of larvae, we ran LMMs with these three treatments and their interactions as fixed effects and a) total-PO activity (square-root transformed), b) proPO activity (square-root transformed), and c) free PO activity (log transformed) as response variables. To all of the PO mixed models we also included plate ID as an additional random effect to account for any unintended variance from plate to plate.

We used LMMs to assess the effects of our treatments on a) total hemocyte concentration (log-transformed), b) granulocyte (log-transformed), c) oenocytoid (log-transformed), d) spherule cell (square-root transformed), and e) plasmocyte (log-transformed) concentrations. In each of these LMMs, CO_2 treatment, infection by *O. elektroscirrha*, milkweed species, and their interactions were fixed effects.

We assessed the encapsulation response of late-instar monarchs as a measure of Mean Gray Value (MGV) over a standardized area of filament by running an LMM with CO_2 treatment, infection by *O. elektroscirrha*, milkweed species, and their interactions as fixed effects.

Within the Assay Control group (Table 4.1), we investigated the effects of our treatments on monarch performance. To determine if monarch tolerance to infection varied by CO_2 treatment and milkweed species, we used an LMM with the lifespan of infected monarchs as the response variable and spore load (log_{10}-transformed), CO_2 treatment, and milkweed species as fixed effects. Any significant interaction between either CO_2 treatment or milkweed species and spore load indicates effects on monarch tolerance to infection. To test for any effects of CO_2 treatment, and milkweed species on parasite virulence, we ran an LMM with monarch lifespan as the

response variable and parasite treatment, CO_2 treatment, and milkweed species as fixed effects. Similar to tolerance, an interaction of either of these treatments with infection indicates a difference in the magnitude reduction in lifespan (fitness) induced by infection (our definition of a change in virulence). To estimate any treatment effects on monarch resistance, we used an LMM with spore load (log_{10}-transformed) as the response variable and CO_2 treatment, and milkweed species as fixed effects. Lastly, we used GLMMs with binomial error distributions and logit link functions to assess the effects of CO_2 treatment, and milkweed species on the binary response variables of monarch survival and infection probability.

We assessed the effects of milkweed species and CO_2 treatment on a) total foliar cardenolide concentration (log-transformed), b) cardenolide polarity, c) cardenolide diversity (log-transformed), d) whole-plant secondary metabolite diversity detected with H^1-NMR, and e) foliar C:N ratio (log-transformed) using LMMs.

To explore the effects of foliar chemical traits on monarch immunity we used LMM's with all of the immunological traits that we measured as response variables and our five foliar traits (cardenolide concentration, cardenolide diversity, cardenolide polarity, H^1NMR diversity, and C:N ratio (log-transformed)) as fixed effects. To avoid detecting spurious correlations that result from differences between plant species, we originally included milkweed species as a fixed effect in these models. However, the species term was never significant, and we have removed it from the models presented here. We report only those models that showed significant effects of foliar quality: a) total PO activity, b) total hemocyte concentration (log-transformed), c) oenocytoids concentration (log-transformed), and d) encapsulation.

4.4 Results

Early-instar monarch PO activity declines under parasite infection but is "rescued" by consuming foliage grown under elevated CO_2

Infection by *O. elektroscirrha* suppressed the total PO response of monarch larvae by 25% when larvae were reared on milkweed grown under ambient CO_2 conditions; feeding on foliage grown under elevated CO_2 eliminated the immune suppression caused by parasite infection (infection*CO_2: $F_{1, 134} = 5.80$, $p= 0.0174$, Figure 4.1a). Similarly, the ProPO activity of infected

monarchs was 35% lower than that of uninfected monarchs when larvae were reared on milkweed grown under ambient CO_2 conditions; once again, feeding on foliage grown under elevated CO_2 eliminated this immune suppression (infection*CO_2: $F_{1, 134} = 8.34$, p= 0.0045, Figure 4.1b). Surprisingly, milkweed species had no effect on total PO activity (milkweed species: $F_{1, 135} = 0.04$, p= 0.8423) or proPO activity (milkweed species: $F_{1, 133} = 0.01$, p= 0.9141). Further, there was no interaction between milkweed species, CO_2 treatment and infection on total PO activity ($F_{1, 133} = 2.64$, p = 0.1066) or proPO activity ($F_{1, 133} = 1.86$, p = 0.1750).

Infection and a diet of foliage from elevated CO_2 increased the hemocyte concentrations of early-instar monarchs

Circulating hemocytes aid in the recognition and phagocytosis of microbial parasites and encapsulation of parasitoids. Monarchs typically produce four differentiated hemocyte types: phagocytic granulocytes, capsule-forming plasmocytes, oenocytoids that contain components of the PO cascade, and spherule cells that potentially contain cuticular components (Strand 2008). We first present the effects of our treatments on total hemocyte concentrations circulating in monarch hemolymph followed by the responses of each hemocyte type.

Infected monarchs that fed on foliage grown under elevated CO_2 had 48% higher total concentrations of hemocytes circulating in their hemolymph than uninfected monarchs fed foliage from the same CO_2 treatment (infection*CO_2: $F_{1,107}$= 5.57, p = 0.0201, Figure 4.2a). Put differently, feeding on milkweed grown under future concentrations of CO_2 and being infected with a parasite induced the strongest hemocyte response in monarch larvae. There was no main effect of infection ($F_{1,105} = 0.29$, p = 0.5924), CO_2 treatment ($F_{1,105} = 2.27$, p = 0.1353) or an interaction among infection, CO_2 treatment and milkweed species on total hemocyte concentrations ($F_{1,105} = 0.65$, p = 0.4217).

Parallel to the pattern found across all hemocyte types, the concentration of granulocytes (phagocytic cells) in infected monarchs fed foliage grown under elevated CO_2 was 89% higher than uninfected monarchs fed the same foliage (infection*CO_2: $F_{1,95} = 4.26$, p = 0.0418, Figure 4.2b). CO_2 treatment ($F_{1,95} = 1.96$, p = 0.1651), infection ($F_{1,95} = 1.66$, p = 0.20) and milkweed

species ($F_{1,95} = 1.42$, $p = 0.2370$) had no main or interactive ($F_{1,95} = 0.33$, $p = 0.5671$) effects on granulocyte concentrations.

Oenocytoids are much rarer in lepidopteran hemolymph but are thought to be directly involved in PO production (Strand 2008; Altizer & de Roode 2015). The concentration of oenocytoids circulating in monarch hemolymph was markedly higher in those infected monarchs feeding on *A. curassavica* grown under ambient CO_2 (infection*milkweed species*CO_2: $F_{1,54} = 4.60$, $p = 0.0364$, Figure 4.2c).

Though we are unsure the exact immunological function of spherule cells, they do contain cuticular components necessary for clotting after a wound (Altizer & de Roode 2015). Parasite infection increased the concentration of spherule cells circulating in larvae by 71% on those larvae fed *A. incarnata* (infection *milkweed species: $F_{1,77} = 4.02$, $p = 0.0485$, Figure 4.2d). Additionally, across infection and milkweed species, elevated CO_2 induced a 60% increase in the concentration of spherule cells in monarchs (CO_2: $F_{1,77} = 5.69$, $p = 0.0195$, Figure 4.2e). There was no main effect of milkweed species ($F_{1,77} = 0.61$, $p = 0.4363$), or parasite treatment ($F_{1,77} = 2.09$, $p = 0.1526$) on spherule cell concentration, nor was there an interaction among our three treatments ($F_{1,77} = 0.01$, $p = 0.9234$).

Finally, monarch plasmocyte concentrations (cells involved with encapsulation) were unaffected by parasite infection ($F_{1,74} = 0.38$, $p = 0.5409$), species ($F_{1,74} = 0.04$, $p = 0.8383$), CO_2 treatment ($F_{1,35} = 0.07$, $p = 0.7976$) and their interaction ($F_{1,79} = 0.12$, $p = 0.7351$).

Late-instar monarch immunity against parasitoids declined on "medicinal" milkweed

The extent of encapsulation around an artificial antigen is an integrated measure of insect immune resistance against parasitoids. In our study, monarch encapsulation around a sterile filament (simulated antigen) was 11% lower (higher Mean Gray Values indicate less dark objects) in larvae feeding on the high-cardenolide *A. curassavica* in comparison to those fed low-cardenolide *A. incarnata* (species: $F_{1, 130} = 4.92$, $p= 0.0283$, Figure 4.3a). In other words, feeding on a milkweed species with high concentrations of toxins reduced the strength of monarch immune defense against parasitoids. Across all monarchs, inoculation with the protozoan *O.*

elektroscirrha increased encapsulation marginally by 10% (infection: $F_{1, 129} = 3.35$, p= 0.0694, Figure 4.3b). There was no effect of CO_2 treatment on monarch encapsulation (CO_2: $F_{1, 130} = 0.92$, p= 0.3404) nor was there an interaction between CO_2 treatment, parasite infection and milkweed species (species*infection*CO_2: $F_{1, 130} = 0.47$, p= 0.4927).

Tolerance declined in monarchs fed foliage grown under elevated CO_2

The tolerance of infected monarchs within the Control Assay Group (Table 4.1) declined by 70% under elevated CO_2 across infected adults fed both species of milkweed host plants ($F_{1, 40} = 1.61$, p = 0.0212, Figure 4.4). Unlike previous studies (Decker *et al. in Revision*), there was no milkweed species-specific decline in tolerance that differed between CO_2 treatments ($F_{1, 39} = 2.31$, p = 0.1369), nor were there differences in tolerance between monarchs fed distinct host-plant species ($F_{1,40} = 1.50$, p = 0.2280). Additionally, there was no effect of milkweed species (milkweed species*infection: $F_{1,115} = 0.83$, p = 0.6340), CO_2 treatment (CO_2*infection: $F_{1,115} = 0.2$, p = 0.6232), or their interaction (milkweed species* CO_2*infection: $F_{1,116} = 0.48$, p = 0.4920) on parasite virulence. Finally, the spore loads of infected monarchs did not respond to CO_2 treatment ($F_{1,23} = 0.06$, p = 0.8118), milkweed species ($F_{1,23} = 2.39$, p = 0.1357) or their interaction ($F_{1,23} = 0.74$, p = 0.3975). Monarchs fed *A. incarnata* foliage had a 3% lower survival rate than those monarchs fed *A. curassavica* as larvae (milkweed species: $\chi^2 = 4.24$, p = 0.0395). There was no effect of CO_2 treatment ($\chi^2 = 1.36$, p = 0.24321) or infection ($\chi^2 = 1.41$, p = 0.2347) on monarch survival.

Foliar chemical defenses and nutritional quality declined under elevated CO_2

Elevated CO_2 induced a 23% reduction in the foliar cardenolides produced by *A. curassavica* and a 30% reduction in *A. incarnata* cardenolide production (CO_2: $F_{1,98} = 7.88$, p = 0.006, Figure 4.5a, Table 4.2)**.** The two species of milkweed also differed substantially in the amount of cardenolides they produced, whereby *A. curassavica* produced nearly 16 times more foliar cardenolides than *A. incarnata* (species: $F_{1,265} = 622.82$, p < 0.0001, Figure 4.5a, Table 4.2). Because elevated CO_2 caused similar magnitude reductions in both species, there was no interaction between milkweed species and CO_2 treatment on cardenolide production (species* CO_2: $F_{1,265} = 1.26$, p = 0.2630). The mean polarity index of cardenolides produced by *A.*

curassavica was twice that of *A. incarnata* (species: $F_{1,264} = 104.40$, $p < 0.0001$, Figure 4.5b Table 4.2); a high polarity index indicates an abundance of lipophilic cardenolides. However, CO_2 treatment had no effect on the mean polarity value of cardenolides (CO_2: $F_{1,94} = 2.79$, $p = 0.0982$, Figure 4.5b, Table 4.2) and caused no significant interaction (species* CO_2: $F_{1,263} = 2.48$, $p = 0.1166$). Similarly, cardenolide diversity was 86% higher in *A. curassavica* than in *A. incarnata* (species: $F_{1,239} = 26.10$, $p < 0.0001$ Figure 4.5c, Table 4.2). Elevated CO_2 had no effect on cardenolide diversity (CO_2: $F_{1,239} = 2.43$, $p = 0.1205$, Figure 4.5c, Table 4.2) nor was there an interaction between milkweed species and CO_2 treatment (species* CO_2: $F_{1,239} = 2.89$, $p = 0.0903$, Figure 4.5c, Table 4.2).

Using H^1-NMR we were able to quantify the holistic diversity of secondary metabolites produced within the milkweed plants sampled using Simpson's diversity index of binned chemical shift values. The diversity of metabolites declined by roughly 2% in both species of milkweed under elevated CO_2 (CO_2: $F_{1,38} = 6.93$, $p = 0.0122$, Figure 4.5d, Table 4.2). *A. incarnata*, produced a higher diversity of metabolites than *A. curassavica* but only by about 1% (species: $F_{1,435} = 13.21$, $p = 0.0003$, Figure 4.5d, Table 4.2). Just as with cardenolide concentrations, elevated CO_2 caused similar declines in the chemical diversity of both milkweed species, and consequently had no interaction with milkweed species in the model (species* CO_2: $F_{1,435} = 1.36$, $p = 0.2437$, Figure 4.5d, Table 4.2).

Supporting two decades worth of CO_2 research, the nutritional quality of milkweed foliage declined under elevated CO_2 (CO_2: $F_{1,38} = 75.11$, $p < 0.0001$, Figure 4.5e, Table 4.2). Specifically, the foliar C:N ratio increased by 29% in *A. curassavica* and by 38% in *A. incarnata*. Across both CO_2 treatments, *A. incarnata* had 22% higher foliar C:N ratios than *A. curassavica* (species: $F_{1,437} = 41.34$, $p < 0.0001$, Figure 4.5e, Table 4.2), but there was no species-specific response of the foliar C:N ratio to CO_2 treatment (species* CO_2: $F_{1,437} = 0.10$, $p = 0.7571$, Figure 4.5e, Table 4.2).

Decreasing diet nutritional quality increased early-instar immunity, while late-instar monarch immunity was influenced by cardenolide polarity and diversity

Infected larvae increased the strength of their immune response (high total PO activity and hemocyte concentrations) with decreasing diet nutritional quality (parasite treatment*CN: $F_{1,137}$ = 4.06, p = 0.04589; $F_{1,105}$ = 5.51, p = 0.0208, Figure 4.6a & b*)*. Additionally, the concentration of circulating oenocytoids in infected monarchs responded to both diet cardenolide concentration and nutritional quality (parasite treatment*cardenolides*CN: $F_{1,105}$ = 5.51, p = 0.0208, Figure 4.6c). Infected monarchs fed high cardenolide foliage reduced the concentration of oenocytoids produced as foliar nutritional quality declined. Conversely, infected monarchs fed low cardenolide tissue maintained oenocytoid concentrations despite reductions in diet nutritional quality (Figure 4.6c).

Both foliar cardenolide polarity and cardenolide diversity were negatively correlated with the encapsulation response of uninfected monarchs (parasite treatment*cardenolide polarity: $F_{1,132}$ = 6.25, p = 0.0136; parasite treatment*cardenolide diversity: $F_{1,132}$ = 3.97, p = 0.0483, Figure 4.6d & e). Namely, there was a cost to feeding on plants with more lipophilic and diverse cardenolides for uninfected, late-instar monarchs in the form of increased vulnerability to parasitoid attack. However, being infected with the parasite eliminated the negative relationship between foliar cardenolide chemistry and encapsulation.

4. 5 Discussion

Here, we demonstrate that a single global environmental change driver, elevated CO_2, can impact the immune response of recently challenged, young monarch hosts to parasitic infection by *O. elektroscirrha*. Conversely, the immune defense of late-instar monarchs shows no response to elevated CO_2 but declines when monarchs are fed medicinal milkweed. Additionally, monarch age influenced the relationship between phytochemistry and the strength of the immune response. In infected early-instar larvae, both PO (a critical immune enzyme) activity and hemocytes (immune effector cells) increased with decreasing foliar nutrient concentrations. This increased immune response despite reductions in the nutritional quality of plants could reflect differences in young host resource allocation. The encapsulation response of uninfected late-instar monarchs was negatively correlated with cardenolide diversity and polarity. Consequently, monarchs may experience an ecological cost to feeding on toxic plant species in the form of increased vulnerability to parasitism. To our knowledge, this is the first study to investigate the

effects of elevated CO_2 on the interplay of foliar nutrients, and secondary metabolites on insect immunity against parasites and parasitoids.

Our data suggest that milkweed foliage from elevated CO_2 can stimulate monarch immune responses to *O. elektroscirrha* and relieve PO and total hemocyte suppression induced by the parasite (Figures 4.1 & 4.2). Despite our predictions, we found no evidence that cardenolides play a role in immune inhibition or induction (except in oenocytoids production discussed below). This is in contrast to the negative effects of other secondary metabolites such as iridoid glycosides on the circulating PO activity of other lepidopterans (Smilanich *et al.* 2009, 2017). Instead, the immune response of infected monarchs was positively correlated with declining foliar nutrient concentrations induced by elevated CO_2 (Figure 4.6a &b). Typically, insect immunity follows the opposite pattern, whereby immune responses decline on diets low in nutrients (Beckage 2008; Strand 2008). Dietary proteins especially have been implicated as limiting macronutrients for insect immunity (Lee *et al.* 2006; Povey *et al.* 2009; Simpson *et al.* 2015; Miller & Cotter 2017). However, in some instances, diets low in protein but high in carbohydrates (a condition commonly induced in foliage grown under elevated CO_2) may promote insect melanization induced by PO activity (Mason *et al.* 2014). Presumably, a diet high in soluble carbohydrates is easier to metabolize than one consisting of the high-energy peptide bonds within proteins. Within other insect systems, research has revealed a trade-off between lipid digestion and immunity (Adamo *et al.* 2008, 2010). Therefore, infected monarchs may have more energy to invest in their immune response because of reduced energetic requirements for digestion when feeding on foliage with higher C:N ratios. One other study tested the PO activity of Lepidoptera larvae feeding on plant foliage grown under elevated CO_2, and found that future atmospheric conditions decreased PO activity in unchallenged larvae (Gherlenda *et al.* 2015). The uninfected larvae in our study also exhibited this trend of reduced PO activity, which could reflect energetic investment into other life history traits unrelated to immunity under elevated CO_2 in the absence of parasite challenge.

The concentration of oenocytoids, a hemocyte that contains PO cascade components (Lavine & Strand 2002), circulating in early-instar monarch larvae may reflect a selective influence of plant chemistry on monarch cellular immunity (Figure 4.2c). When monarchs fed on foliage from *A.*

curassavica (the medicinal milkweed) grown under ambient CO_2, the production of this specific hemocyte type spiked. This stimulation of immunity on the highest-cardenolide plants in our study may provide weak support for hypothesis 2a: that cardenolides may stimulate monarch immunity in response to *O. elektroscirrha* infection. Additionally, the oenocytoids concentrations of infected and uninfected monarchs were related simultaneously to both nutrient and cardenolide concentrations (Figure 4.6c). Infected early-instar larvae fed foliage with intermediate to high cardenolide concentrations produced fewer oenocytoids in their hemolymph when that foliage was also of low nutritional quality. Conversely, the production of oenocytoids in uninfected monarchs feeding on the low cardenolide foliage was unrelated to nutritional quality. Taken together, this pattern supports hypothesis 2b, where monarchs may exhibit suppressed immune function despite feeding on medicinal host plants under conditions of nutrient stress.

Our results contribute to a growing body of research illustrating the cost of secondary metabolite ingestion to the melanization and encapsulation responses of hosts under parasite attack (Smilanich *et al.* 2009; Richards *et al.* 2012; Lampert & Bowers 2015; Hansen *et al.* 2016). Late-instar monarch larvae reared on high-cardenolide milkweed produced a weaker encapsulation response against a standardized antigen (filament) (Figure 4.3a). Intriguingly, the strength of encapsulation in uninfected monarchs was also negatively related to the presence of more diverse and lipophilic cardenolides in the insect diet (Figure 4.6d & e). This reduction of immune defense suggests the potential for synergistic interactions among cardenolides and the importance of molecular traits such as polarity to insect performance (Richards *et al.* 2012; Sternberg *et al.* 2012). Additionally, the lack of a reduction in encapsulation in infected monarchs despite consuming toxic metabolites lends support to the previous finding that *O. elektroscirrha* protects monarchs from mortality induced by a tachinid parasitoid (Sternberg *et al.* 2011). Our data imply that *O. elektroscirrha* infection prior to parasitoid attack increases the monarch encapsulation response despite the presence of toxic secondary metabolites (Figure 4.6 d& e). Because improving monarch host fitness also improves the parasite's own fitness, the induction of an increased encapsulation rate by *O. elektroscirrha* may be an adaptive function for the parasite. We suggest future experiments exploring variation in the protective capabilities of this parasite in relation to changing foliar chemistry, and different parasitoid attackers.

Additionally, if *O. elektroscirrha* can actually modulate the immune response of monarchs this may explain the suppression of PO and hemocytes in infected individuals under ambient CO_2.

Host age directly influences the strength of insect immunity (Butcher & Lord 2004; Laws *et al.* 2004; Zerofsky *et al.* 2005). In monarchs, we know that PO activity and total hemocyte concentrations directly increase with larval age (Altizer *unpublished data*, Altizer & de Roode 2015). Therefore, it is extremely interesting that the clearest effects of plant species on monarch immune defense occurred in late-instar larvae. Perhaps, the effects of host plant chemistry take time to develop within the insect host. Our study only examined the underlying humoral and cellular immune response of monarchs at one time point, 48 hours following inoculation. It is possible that further sampling over the course of *O. elektroscirrha* infection may reveal stronger effects of host plant species on monarch immunity.

Though our holistic secondary metabolite diversity index provides a rough estimate of the milkweed metabolome and its relationship to monarch performance, further studies investigating the importance of specific compounds or chemical structures other than cardenolides are sorely needed. Our study provides a clear example of why correlations with simple diversity indices (e.g. Simpson index) of chemical structures are insufficient in determining the importance of secondary chemistry to herbivore performance. We recommend further analyses of this dataset and future studies that employ metabolic profiling (Watrous *et al.* 2012) to detect molecular features important to biological activities such as immunity. This type of holistic approach may better detect synergies among compounds and perhaps aid in future drug discovery (Krishnan *et al.* 2005; Macel *et al.* 2010; Bose *et al.* 2014; Dyer *et al.* 2014; Richards *et al.* 2015a, b).

Monarch butterfly populations currently face multiple threats induced by anthropogenic environmental change (Malcolm 2017). Here we demonstrate enhanced monarch immunity in early-instar larvae feeding on milkweed grown under elevated CO_2. Monarchs experience very high mortality rates as neonates and other early instars (Prysby 2004). Thus, our results suggest one aspect of environmental change may promote increased protection from parasites and parasitoids at this stage. However, increased immune investment will likely come at a cost of other important life history traits (Schmid-Hempel 2005) such as growth and reproduction that

may ultimately decrease monarch fitness. Ecologists in the Anthropocene face the major challenge of determining the direction and magnitude of change in ecological interactions in response to future environmental conditions. With an improved understanding of immune mechanisms underlying host-enemy interactions, we can begin to make more powerful predictions about alterations in trophic cascades and emerging infectious diseases.

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4.7 Tables & Figures

Table 4.1. The total number of larvae used in an experiment investigating the effects of infection by *Ophryocystis elektroscirrha*, elevated CO_2 and milkweed species on monarch immunity. The table shows experimental treatments, initial sample sizes, and the number of individuals that survived to be assayed (encapsulation or PO/Hemocytes) or that survived to adulthood (Assay Controls).

Assay	CO_2 Treatment	Milkweed Species	Parasite Treatment	Surviving N	Initial N
Encapsulation	ambient	*A. curassavica*	uninfected	14	20
			infected	19	20
		A. incarnata	uninfected	15	20
			infected	12	20
	elevated	*A. curassavica*	uninfected	15	20
			infected	19	20
		A. incarnata	uninfected	18	20
			infected	12	19
PO & Hemocytes	ambient	*A. curassavica*	uninfected	18	20
			infected	20	20
		A. incarnata	uninfected	16	20
			infected	20	20
	elevated	*A. curassavica*	uninfected	20	20
			infected	19	20
		A. incarnata	uninfected	19	20
			infected	19	20
Assay Control	ambient	*A. curassavica*	uninfected	20	20
			infected	19	20
		A. incarnata	uninfected	18	20
			infected	20	20
	elevated	*A. curassavica*	uninfected	20	20
			infected	19	20
		A. incarnata	uninfected	18	20
			infected	20	20

Table 4.2. The effects of CO_2 treatment, milkweed species and their interaction on log-transformed foliar cardenolide concentration, foliar cardenolide polarity, log-transformed foliar cardenolide diversity, entire plant secondary metabolite diversity detected with H^1-NMR, and carbon (C) to nitrogen (N) ratio.

	CO_2		**Species**		**CO_2*Species**	
	F	*P*	F	*P*	F	*P*
log(Cardenolide Concentration)	**$F_{1,98}$ = 7.88**	**p = 0.006****	**$F_{1,265}$ = 622.82**	**p < 0.0001*****	$F_{1,265}$ = 1.26	p = 0.2632
Cardenolide Polarity	$F_{1,94}$ = 2.79	p = 0.0982	**$F_{1,264}$ = 104.40**	**p < 0.0001*****	$F_{1,263}$ = 2.48	p = 0.1166
log(Cardenolide Diversity)	$F_{1,239}$ = 2.43	p = 0.1205	**$F_{1,239}$ = 26.10**	**p < 0.0001*****	$F_{1,239}$ = 2.89	p = 0.0903
NMR Simspon Diversity Index	**$F_{1,38}$ = 6.93**	**p = 0.0122***	**$F_{1,435}$ = 13.21**	**p = 0.0003****	$F_{1,435}$ = 1.36	p = 0.2437
log(C:N Ratio)	**$F_{1,38}$= 75.11**	**p < 0.0001*****	**$F_{1,437}$= 41.34**	**p = 0.0003****	$F_{1,437}$ = 0.10	p = 0.7571

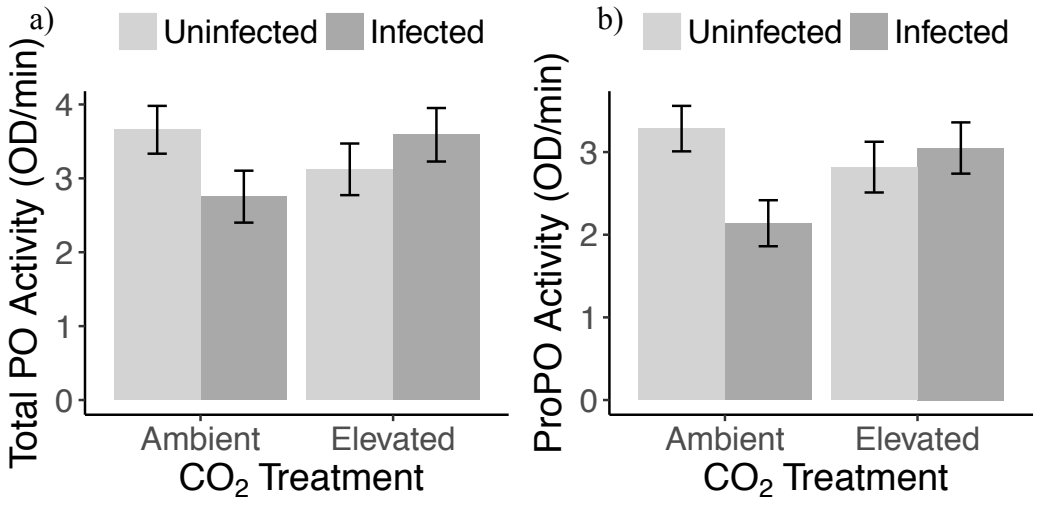

Figure 4.1. The interactive effects of infection by *Ophryocystis elektroscirrha* and CO_2 on a) total Phenoloxidase (PO) activity, and b) prophenoloxidase (proPO) activity. Error bars represent ±1 SEM. Ambient CO_2 concentrations averaged 410 ppm and elevated CO_2 concentrations averaged 810 ppm.

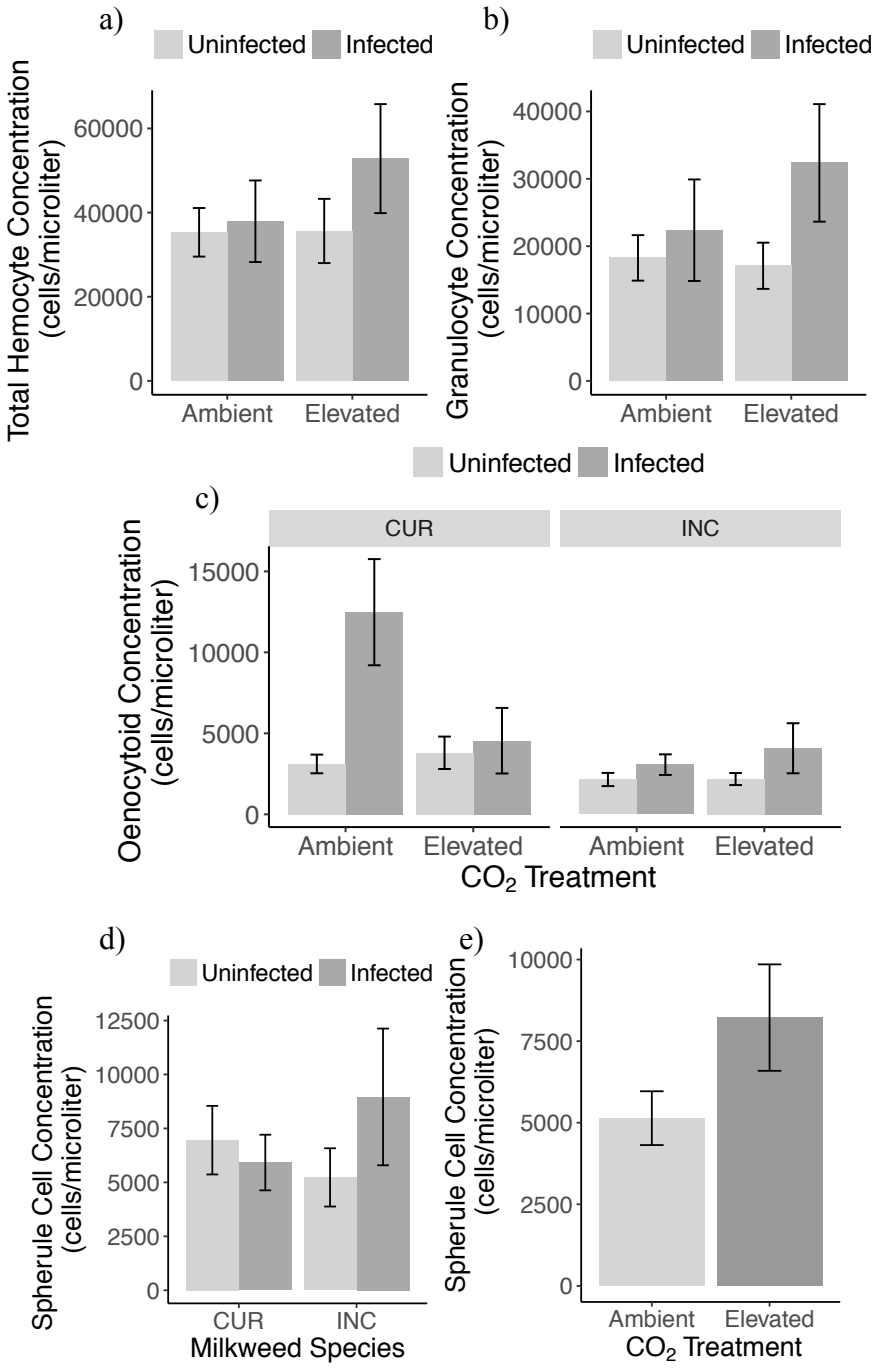

Figure 4.2. The interactive effects of infection by *Ophryocystis elektroscirrha*, and CO_2 treatment on a) total hemocyte concentration, and b) granulocyte concentration in monarch hemolymph. The c) interactive effects of infection, milkweed species and CO_2 treatment on oenocytoid concentrations. The interaction of d) infection and milkweed species and e) the main effect of CO_2 on spherule cell concentrations. Error bars represent ±1 SEM and milkweed species codes are as follows: CUR= *A. curassavica* and INC= *A. incarnata*. Ambient CO_2 concentrations averaged 410 ppm and elevated CO_2 concentrations averaged 810 ppm.

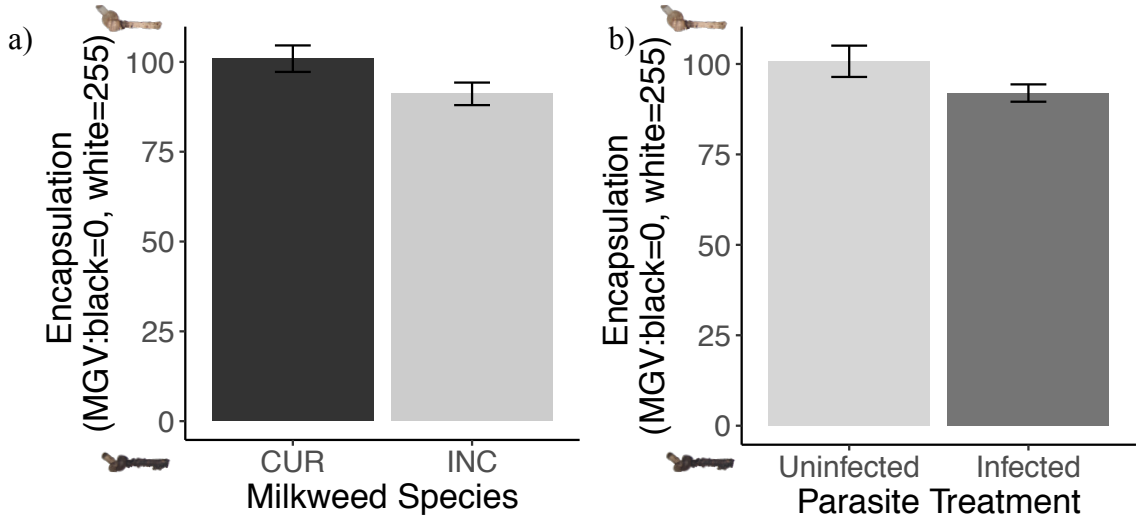

Figure 4.3. The encapsulation response of 5th instar monarch larvae to artificial antigens (sterile filaments) varied by a) the species of milkweed on which monarchs were feeding before insertion, and b) whether or not monarchs were infected with Ophryocystis elektroscirrha, a protozoan parasite. Higher Mean Gray Values (MGV) represent lighter pigmented filaments or lower encapsulation responses. Error bars represent ±1 SEM from the mean values and milkweed species codes are as follows: CUR= *A. curassavica* and INC= *A. incarnata*. Images are of actual plastic filaments inserted into monarchs that span the continuum between dark (highly encapsulated) or light (un-encapsulated).

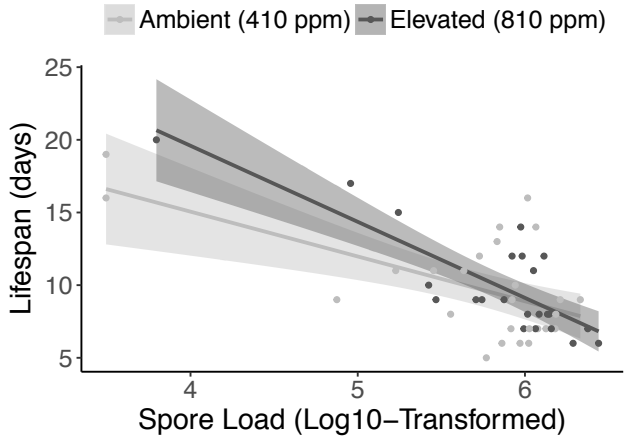

Figure 4.4. Monarch tolerance to *O. elektroscirrha* infection declined on foliage from the elevated CO_2 treatment. Light gray lines and points correspond to tolerance slopes of monarchs reared on plants grown under ambient CO_2 (410 ppm) and dark gray lines and points correspond to tolerance slopes of monarchs reared on plants grown under elevated CO_2 (810 ppm).

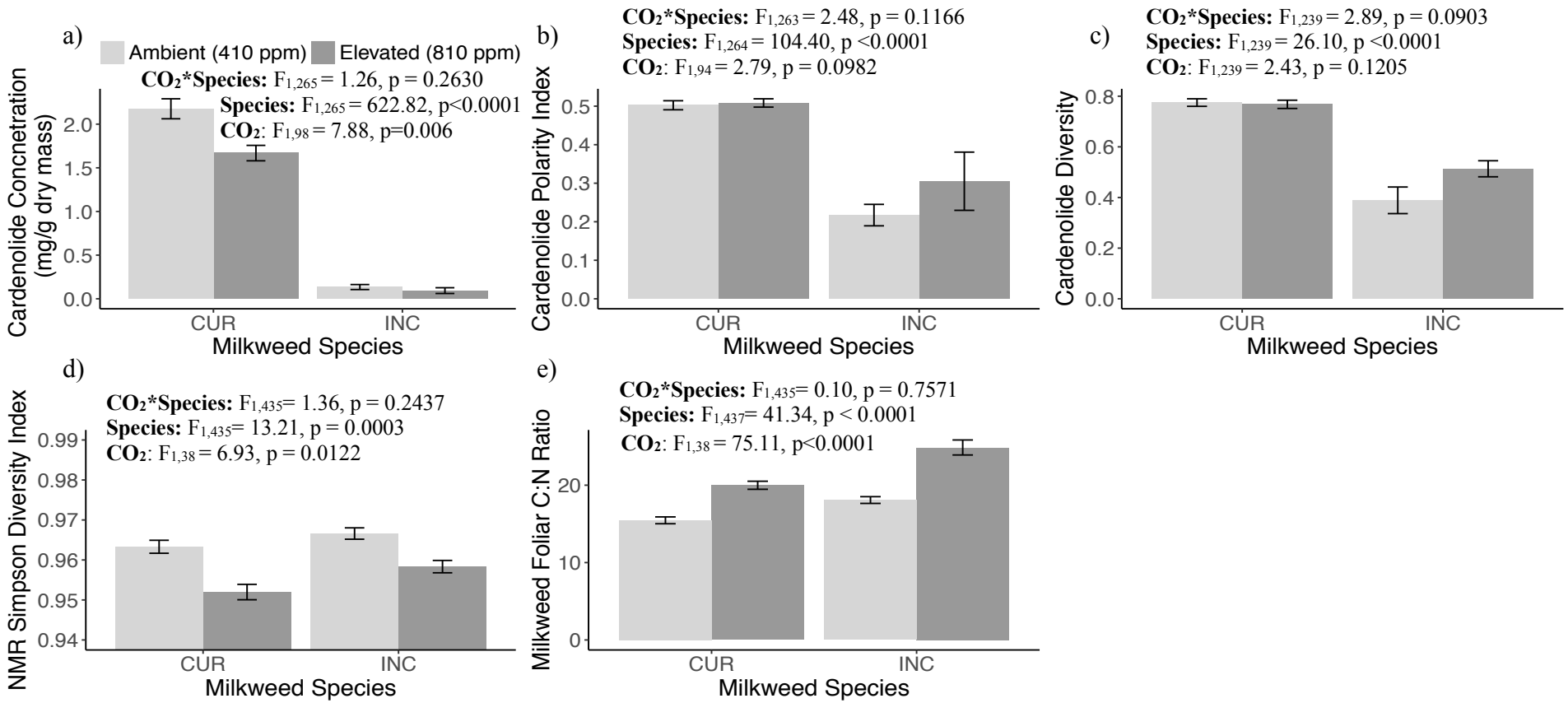

Figure 4.5. The effects of CO_2 treatment and milkweed species on a) initial foliar cardenolide concentrations (mg/g dry mass), b) the polarity index of foliar cardenolides, c) the diversity of foliar cardenolides, d) the diversity of the entire assemblage of foliar secondary metabolites detected with H^1-NMR and e) the ratio of foliar carbon (C) to nitrogen (N). ANOVA statistics are presented with each graph; although all interactions were not significant, we present the data in this fashion for ease of comparison. Error bars represent ±1 SEM from the mean values and milkweed species codes are the same as in Figure 1. Dark gray bars represent plants grown under elevated CO_2 and light gray bars are those grown under ambient CO_2.

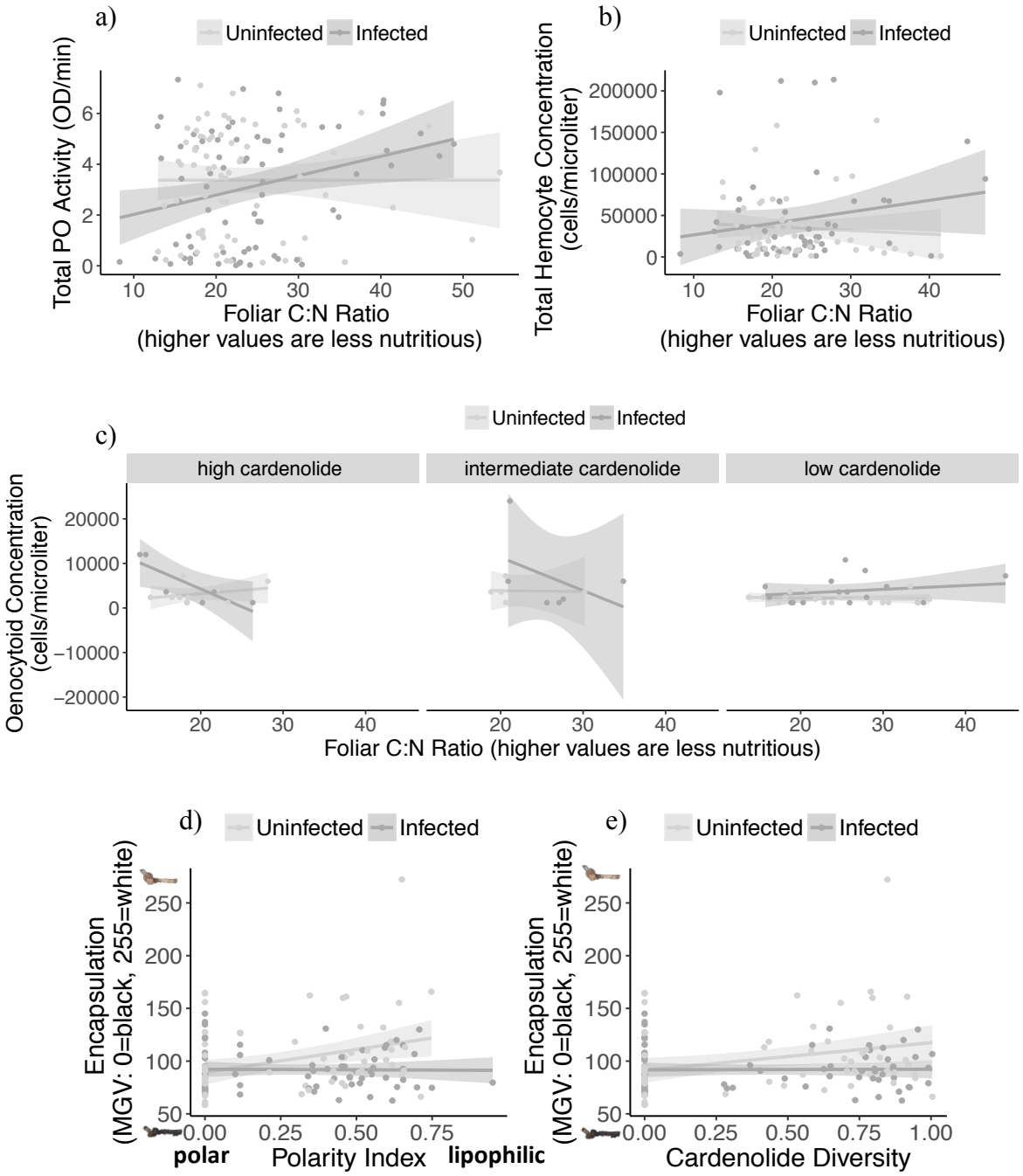

Figure 4.6. The relationship between diet nutritional quality (C:N ratio), infection, and a) total PO activity and b) total hemocyte concentrations. c) The combined relationship among infection, diet nutritional quality, cardenolide concentration and oenocytoid concentrations in larval hemolymph. The relationship between infection by *O. elektroscirrha,* d) diet cardenolide polarity index and e) diet cardenolide diversity and monarch encapsulation. A high polarity index reflects greater expression of lipophilic cardenolides. For visual simplicity, we have binned larvae in (c) by cardenolide concentrations of the foliage that they consumed. However, the analysis was performed with un-binned data, and binning was used only as a simplified alternative to a 3D graph.

Chapter 5 : Variation Among Individual Milkweed Species, Not Elevated CO_2, Influences the Relationship Between Plant Resistance and Tolerance

5.1 Abstract

Regrowth after defoliation (hereafter regrowth tolerance) and chemical resistance are two major forms of defense that plants employ against herbivory and that jointly influence plant fitness. Because plant resources are finite, a trade-off may exist between these two strategies of defense, but this relationship is ultimately complex and context dependent. Predicting the conditions under which defense trade-offs manifest is therefore of considerable importance, particularly under global environmental change. Changing environmental conditions, including elevated atmospheric concentrations of CO_2, alter resource availability and thereby influence the defensive strategies of plants. In this study, we investigated the effects of elevated CO_2 on the chemical resistance and regrowth tolerance traits of four milkweed species (*Ascelpias*). Specifically, we examined the effects of elevated CO_2 on four potential tradeoffs: a) a tradeoff between plant growth rate and chemical resistance before damage occurs, b) a tradeoff between plant growth rate before damage and the chemical resistance of regrowth tissues, c) a tradeoff between chemical resistance before damage and regrowth tolerance after damage, and d) a tradeoff between regrowth tolerance and the chemical resistance of regrowth tissues.

We found support for only one of the four potential trade-offs described: within regrown plants, there was a trade-off between plant tolerance (regrowth rate) and resistance traits (foliar cardenolide concentrations). This trade-off varied substantially among milkweed species but was unaffected by previous exposure to elevated CO_2. In all tests of potential trade-offs, milkweed species identity was by far the most important factor determining plant growth and resistance. Previous exposure to elevated CO_2 did alter chemical resistance of regrowth tissue in a species-specific fashion. Our data add to a growing body of work that demonstrates the complex nature of plant growth and resistance relationships in the context of changing resource availability.

5.2 Introduction

Plants use a suite of defensive traits to deter herbivory. These defenses often occur in concert and together constitute the plant defensive phenotype (Agrawal & Fishbein 2006). As a result, critical factors and synergies that contribute to plant fitness in the context of herbivory may be overlooked when we study defenses in isolation (Baucom & De Roode 2011). Regrowth after defoliation (hereafter, regrowth tolerance) and chemical resistance are two major forms of defense that plants employ against herbivory that jointly influence plant fitness (Strauss & Agrawal 1999; Stamp 2003; Núñez-Farfán *et al.* 2007; Agrawal 2011; Fornoni 2011; Zas *et al.* 2011). Resistance is defined as the ability of plants to decrease herbivory through reductions in herbivore density, performance, and feeding (Rhoades 1985). Plants resist herbivore damage through physical and chemical traits such as trichomes, latex exudation, thorns, and toxic secondary metabolites that together reduce herbivore performance. In contrast, plant tolerance to herbivory is defined as the maintenance of plant fitness following damage (van der Meijden *et al.* 1988; Stowe *et al.* 2000; Baucom & De Roode 2011). Tolerance is usually achieved through simultaneous shifts in physiology and resource allocation that together increase the capacity of plants to regrow (Rosenthal & Kotanen 1994; Strauss & Agrawal 1999; Fornoni *et al.* 2003).

It is widely recognized that plant resistance and regrowth tolerance are inter-related (Stamp 2003; Agrawal 2011). Because plant resources are finite, early studies predicted that a trade-off would exist between plant growth/regrowth and resistance (Coley & Chapin 1985; van der Meijden *et al.* 1988; Fineblum & Rausher 1995). The cost of producing resistance traits directed at herbivores, such as defensive structures and secondary metabolites, may result in depressed herbicide resistance and pathogen resistance, and reductions in growth rate (Bergelson & Purrington 1996). For example, there is a genetic tradeoff between resistance and tolerance in the tall morning glory, *Ipomoea purpurea*, wherein those genotypes that exhibit high levels of resistance simultaneously express lower regrowth tolerance to herbivory (Fineblum & Rausher 1995). However, the link between the two plant defense strategies is less clearly understood in other systems (Tiffin & Rausher 1999; Leimu & Koricheva 2006; Stevens *et al.* 2007; Fornoni 2011; Oduor *et al.* 2011; Carmona & Fornoni 2013) where tolerance also depends on the type of reproductive cost incurred by herbivory, plant genetic constraints, and resource allocation during

development (Stowe *et al.* 2000; Boege *et al.* 2007; Núñez-Farfán *et al.* 2007; Scholes & Paige 2011, 2014; Siddappaji *et al.* 2013). In some instances, resistance and tolerance may correlate positively because the costs of the two defenses are mediated by factors other than resource allocation (Stowe *et al.* 2000; Fornoni *et al.* 2004; Núñez-Farfán *et al.* 2007). Additionally, plants that suffer higher herbivore pressure may benefit from high levels of both resistance and tolerance (Krimmel & Pearse 2016). Thus, the proposed tradeoff between resistance and tolerance is both complex and context dependent (Núñez-Farfán *et al.* 2007; Züst & Agrawal 2017). Predicting the conditions under which such defense trade-offs manifest remains an important goal of plant ecology and evolution.

Resource availability dictates in part the physiology of plants. A rich literature exists describing the defense syndromes of plants in the context of variable resource regimes (Coley & Chapin 1985; Strauss & Agrawal 1999; Hawkes & Sullivan 2001; Wise & Abrahamson 2007) and has led to the accumulation of numerous defense hypotheses that center around the evolution of resistance or tolerance. Notably, the Resource Availability Hypothesis (RAH) posits that plants living in environments low in resources will evolve greater energetic investment in resistance traits (Coley & Chapin 1985). While a central tenet of RAH is a tradeoff between growth and resistance, effects of environmental variation on resistance-tolerance tradeoffs remain unclear (Gianoli & Salgado-Luarte 2017). The compensatory continuum hypothesis (CCH) (Hawkes & Sullivan 2001), represents a complimentary tolerance theory that predicts higher plant tolerance under nutrient-rich environments with low competition on both ecological and evolutionary scales. However, the empirical tests of these theories provide mixed support—in some instances, plants inhabiting low nutrient environments express higher resistance and lower regrowth tolerance over evolutionary time because of a reduced capacity to replace lost tissue (Fine *et al.* 2006; Gianoli & Salgado-Luarte 2017). Alternatively, some species grown in nutrient-poor environments actually increase their regrowth tolerance in the presence of additional stressors (Hawkes & Sullivan 2001; Wise & Abrahamson 2007). Within the lifetime of individual plants, many studies have demonstrated shifts in plant resistance syndromes in response to environmental factors such as nutrient availability (Herms & Mattson 1992; Vannette & Hunter 2011; Tao *et al.* 2016a). However, we lack consensus on the relationship between resistance and tolerance allocation by plants in response to changing resource regimes across ecological time.

Elevated atmospheric concentrations of CO_2 influence both plant resistance and regrowth tolerance to herbivores. Both the composition and concentration of some plant secondary metabolites change in response to elevated CO_2 (Hunter 2001; Bidart-Bouzat *et al.* 2005; Ryan *et al.* 2010; Robinson *et al.* 2012; Klaiber *et al.* 2013; Zavala *et al.* 2013; Jia *et al.* 2016). Elevated CO_2 also positively affects plant growth rates by increasing photosynthesis, and water use efficiency (Drake et al. 1997; Ainsworth & Long 2005; Robinson et al. 2012; Bazzaz et al. 1992). However, the direct effects of elevated CO_2 on plant tolerance are generally negative (Wilsey 2001; Marshall *et al.* 2008; Lau & Tiffin 2009; Guo *et al.* 2012) partially because nutrient limitation may increase under CO_2 enrichment. Concentrations of atmospheric CO_2 may also influence the phytohormonal signaling pathways of plants, which mediate resistance and tolerance (Guo *et al.* 2012). However, studies that explore the integrated influence of elevated CO_2 on the interaction between resistance and tolerance are lacking.

Here, we investigated the effects of elevated CO_2 on the chemical resistance traits and regrowth tolerance of four milkweed species (*Ascelpias*) over ecological time. Specifically, we examined the effects of elevated CO_2 on four potential tradeoffs: a) a tradeoff between plant growth rate and chemical resistance before damage occurs, b) a tradeoff between plant growth rate before damage and the chemical resistance of regrowth tissues, c) a tradeoff between chemical resistance before damage and regrowth tolerance after damage, and d) a tradeoff between regrowth tolerance and the chemical resistance of regrowth tissues. We predicted that elevated CO_2 would: a) induce higher growth rates and regrowth rates and depress cardenolide concentrations; and b) mitigate in part any tradeoff between chemical resistance traits and regrowth tolerance in milkweed. By analyzing changes in plant regrowth and defensive chemistry, we hope to improve our understanding of how future environmental conditions may influence the defensive phenotype of plants, with implications for the herbivore communities that damage them.

Study System

Milkweeds in the genus *Asclepias* originate from North and Central America (Woodson 1954). The four milkweed species used in our study (*A. syriaca, A. speciosa, A. incarnata,* and *A.*

curassavica) support herbivores that range from phloem feeding insects such as oleander aphids (*Aphis nerii)* to chewing insects capable of removing large amounts of tissue, like monarch caterpillars (*Danaus plexippus*), and long horn beetles (*Tetraopes* spp.). Most milkweed herbivores specialize within the genus because *Asclepias* produce a well-characterized suite of defenses against herbivory.

To physically deter feeding by arthropod herbivores, milkweed plants exude latex, produce trichomes, and increase leaf toughness (Hochwender *et al.* 2000; Zalucki *et al.* 2001; Agrawal & Fishbein 2006; Agrawal & Konno 2009). However, milkweeds are best known for synthesizing a class of toxic steroids known as cardenolides that disrupt Na^+/K^+-ATPase in the sodium-potassium channels of animal cells (Agrawal *et al.* 2012). The composition and concentration of cardenolides produced by milkweed plants vary substantially among species and as well as within different parts of the plant (Rasmann & Agrawal 2011; Agrawal *et al.* 2012). Regrowth tolerance also play a prominent role in the defensive phenotype of milkweeds (Agrawal & Fishbein 2008; Tao *et al.* 2016a). Despite a growing body of work illustrating the effects of environmental change on milkweed chemistry and milkweed growth rates (Vannette & Hunter 2011; Matiella 2012; Tao *et al.* 2014; Andrews 2015), no study to date has explored the interplay between milkweed resistance traits and regrowth tolerance under future environmental conditions.

5. 3 Materials and Methods

Plant Materials

We grew four species of milkweed under ambient (400 ppm) and elevated (760 ppm) concentrations of atmospheric CO_2 at the University of Michigan Biological Station (UMBS) in northern Michigan. To manipulate atmospheric CO_2 concentrations, we used an array consisting of 40 open-top chambers, with 20 chambers maintained at ambient CO_2 and 20 chambers maintained at elevated CO_2 from May through August of 2015.

We chose *Asclepias* species for our study that vary in their foliar cardenolide concentrations. Specifically, we included *A. incarnata* (low cardenolide), *A. speciosa, A. syriaca* (both medium cardenolide), and *A. curassavica* (high cardenolide). Seeds of *A. speciosa* and *A. curassavica*

were obtained from commercial sources (Prairie Moon Nurseries, MN) and seeds of *A. incarnata* and *A. syriaca* were collected locally (Cheboygan county, MI). We surface sterilized all seeds following a six-week cold stratification period (for all but tropical *A. curassavica*), and germinated seeds on moist filter paper for 1 week. We planted seedlings in deepots ™ containing Metromix 360 (SunGro Horticulture, Vancouver, BC) and Osmocote controlled release fertilizer [N:P:K:16:16:16 ppm N (g/g)] (ICL Specialty Fertilizers, Dublin, OH) on 5/5/15. Germinated seedlings were watered daily and grown in the UMBS glass house for two weeks before they were moved to their randomly assigned chambers outside in the array. Once in the array, plants were watered a least once a day and were maintained under their CO_2 treatments for 3 months.

Within each chamber, we grew as many as 7 plants of each milkweed species. Low germination success limited the number of *A. speciosa* and *A. syriaca* used in this study, and not all milkweed species were represented in every chamber. Overall, our 8 treatments (2 CO_2 treatments x 4 milkweed species) combined for a total of 442 plants, with exact replicate numbers reported in Table 5.1 a & b. To compensate for the nested nature of plants within the 40 chambers (Table 5.1b), we used the chamber number as a random effect in all of our linear mixed models described below.

Using a LI-COR 320 IRGA (LI-COR, Lincoln, NE, USA), we monitored atmospheric CO_2 concentrations daily in the 20 chambers maintained under elevated CO_2 and in one randomly selected ambient chamber. The concentrations of CO_2 were adjusted throughout the day to maintain the target concentration of 760 ppm in each elevated chamber. The ambient temperature inside each chamber was recorded every hour using a thermochron datalogger (Thermochron, Australia). Elevated CO_2 chambers averaged 21.03 (±0.034) ºC, and ambient CO_2 chambers averaged 21.24 (± 0.038) ºC, roughly 2ºC higher than the outside average temperature of 18.93 (± 0.039) ºC.

Simulated Damage and Growth Measures

Three months following the initial transfer of plants into the array, we simulated defoliation by cutting all plants at the soil line. At our field site in northern Michigan, we have observed monarch caterpillars, milkweed tussock moths (*Euchaetes egle*), chipmunks (*Tamius striatus*),

milkweed stem weevils (*Rhyssomatus lineaticollis*) and porcupines (*Erethizon dorsatum*) all remove the entire above ground tissues of milkweed plants, and so our clipping treatment represents severe but not infrequent levels of damage. The aboveground biomass that we removed was dried at 60°C, weighed, and used to calculate growth rate prior to damage (below). Cut plants were then thoroughly watered and moved to the UMBS glass house, where all plants were maintained under identical (ambient CO_2) conditions for 21 days. We regrew plants under ambient CO_2 conditions to disentangle the resource allocation decisions made by plants before damage from the direct effects of CO_2 after damage. After that time period, the aboveground regrowth plant material was harvested, dried at 60°C, and weighed as a measure of regrowth tolerance. For a measure of growth rate prior to damage, we divided the aboveground dry biomass of the plant by 64 days (the number of days since the seedling had been transferred to soil) following Agrawal & Fishbein (2008). Similarly, to calculate plant regrowth rate following mechanical damage (our measure of tolerance) we divided the mass of the regrowth material by 21 days (the length of time plants were allowed to regrow following damage). Differences in growth rate following damage are important for the competitive success and ultimate fitness of plants (Züst & Agrawal 2017).

Immediately following our removal of regrowth tissue, we harvested the roots of plants by soaking the entire root mass in water for 48 hours and then gently washing the soil from the root with a hose. Once most of the soil had been removed, roots were dried and stored until desiccated soil particles could be removed by hand. After roots were completely free of soil, they were weighed and recorded as the belowground biomass of each sample. Due to time constraints, we could only weigh the root tissues of 293 plants. Our sample sizes (Table 5.1) were not large enough to sacrifice a subset of plants before our clipping treatments. We stress that by only sampling roots after the period of regrowth, we can only assess the combined effects of CO_2 treatment and clipping on root biomass and chemistry, and not their independent effects.

Chemical Analyses of Above and Belowground Tissues

We collected samples of the original aboveground foliage, the regrowth foliage, and the fine root tissue of each plant for cardenolide analysis using established methods (Zehnder & Hunter 2009; Vannette & Hunter 2011; Tao & Hunter 2012). Roughly 20 mg of dried plant material was

ground in a ball mill, deposited in 1 mL methanol, and stored at -10°C prior to analysis. Cardenolides were extracted, separated and quantified with a 0.15mg/mL digitoxin internal standard (Sigma Chemical Company, St. Louis, Missouri, USA), by reverse-phase high-performance liquid chromatography (HPLC) on a Waters Acquity UPLC with PDA detector (Waters Corporation, Milford, MA, USA). Peaks with symmetrical absorbance between 217-222 nm were identified as cardenolides. Cardenolide concentrations were calculated as the sums of all separated peak areas, corrected by the concentration of the internal digitoxin standard and sample dry mass.

Statistical Analyses

In all analyses that follow, we used either linear mixed models (LMMs; Lme4 package) or generalized linear mixed models (GLMMs; Lme4 package), always including chamber identity as a random effect. We performed all statistical tests in R version 3.3.2 (R Development Core Team, 2018). Variables were transformed to best achieve normality of error as tested by the Shapiro-Wilk normality test, and model homoscedasticity of variance was confirmed using Levene's Tests from the car package.

Relationships Among Milkweed Growth, Regrowth Tolerance, and Resistance Chemistry

Our analyses were designed to assess the potential for a) a tradeoff between plant growth rate and chemical resistance before damage occurs, b) a tradeoff between plant growth rate before damage and the chemical resistance of regrowth tissues, c) a tradeoff between chemical resistance before damage and regrowth tolerance after damage, and d) a tradeoff between regrowth tolerance and the chemical resistance of regrowth tissues. In all cases, we examined the effects of elevated CO_2 on the putative tradeoffs.

Specifically, to test for the potential effects of elevated CO_2 on a) the relationship between initial growth rate and initial chemical resistance, we used an LMM with log-transformed initial foliar cardenolide concentrations as the dependent variable and square-root-transformed growth rate prior to clipping, CO_2 treatment, and milkweed species as fixed effects. An interaction between growth rate prior to clipping and CO_2 indicates a difference between the two atmospheres in the extent to which growth rate correlates with the production of cardenolides.

To test for the potential effects of elevated CO_2 on b) a tradeoff between plant growth rate before damage and the chemical resistance of regrowth tissues, we used an LMM with log-transformed foliar cardenolide concentrations of the regrowth foliage as the dependent variable and square-root-transformed growth rate prior to clipping, CO_2 treatment, and milkweed species as fixed effects. An interaction between initial growth rate and CO_2 indicates a difference between CO_2 treatments in the potential trade-off between plant growth rate before damage and chemical resistance after damage.

Likewise, to test for potential effects of elevated CO_2 on c) a tradeoff between chemical resistance before damage and regrowth tolerance after damage, we ran a LMM with square-root-transformed regrowth rate as the response variable and log-transformed initial foliar cardenolide concentrations, CO_2 treatment, and milkweed species as fixed effects. An interaction between initial foliar cardenolide concentration and CO_2 would indicate a difference between atmospheres in the relationship between initial plant chemical resistance and regrowth tolerance.

Lastly, we assessed any potential effects of elevated CO_2 on d) a tradeoff between regrowth tolerance and the chemical resistance of regrowth tissues. To do this, we ran a LMM with log-transformed regrowth foliar cardenolide concentrations as the response variable and square-root-transformed regrowth rate, CO_2 treatment, and milkweed species as fixed effects. A significant interaction between CO_2 treatment and regrowth rate would signify a difference between the two atmospheres in any correlation between the two defense traits.

Elevated CO_2, Milkweed Species, and Plant Growth

To determine the effects of our treatments on plant growth rate prior to damage and regrowth rate after damage (tolerance), we used CO_2 treatment and milkweed species as fixed effects and square-root transformed growth rates (mg/day) and square-root transformed regrowth rates (mg/day) as response variables. We then examined how CO_2 treatment and species influenced the relationship between growth rate prior to damage and regrowth rate following damage, using a LMM with square-root transformed regrowth rate as the response variable and square-root transformed initial growth rate, CO_2 treatment and species as fixed effects.

Not all milkweed individuals regrew following damage. We therefore used generalized linear mixed models with binomial error distributions and logit link functions to assess the effects of plant species and CO_2 treatment on the proportion of milkweed plants that regrew following damage. For the subset of plants for which we had root biomass values (N = 293), we determined the combined effects of our treatments on belowground biomass with a LMM, with log-transformed root biomass as the dependent variable and milkweed species and CO_2 treatment as fixed effects.

Elevated CO_2, Milkweed Species, and Defense Chemistry

Similar to the models described above, we constructed LMMs, with milkweed species and CO_2 treatment as fixed effects and the cardenolide concentrations of (a) the aboveground foliage before damage, (b) the regrowth foliage after damage, and (c) the fine root tissue as response variables. Over the course of the experiment, some plants did not produce cardenolides and a few samples were mishandled. Therefore, we only analyzed the chemistry of plants for which we had both aboveground cardenolide samples before damage and matching root chemistry samples (N=299). Additionally, we used GLMMs with binomial error distributions and logit link functions to assess the effects of plant species and CO_2 treatment on the proportion of milkweed plants that produced measurable cardenolides in aboveground foliage before damage, in the regrowth foliage after damage, and in the fine roots. Because 100% of *A. curassavica* roots produced cardenolides and 100% of *A. curassavica* plants that regrew produced cardenolides, this species was excluded from these two GLMMs.

Relationships Among Root Traits, Milkweed Growth, and Resistance Chemistry

To determine if regrowth tolerance might occur at the expense of root biomass, we used a LMM with log-transformed belowground biomass as a response variable and binary regrowth response, milkweed species, and CO_2 treatment as fixed effects. Additionally, using only those plants that regrew after clipping, we also tested how root mass varied with a) regrowth rate and b) the concentration of regrowth foliar cardenolides. We ran LMMs with root mass as a dependent variable and either a) the square-root-transformed rate of regrowth or b) log-transformed foliar

cardenolides of regrowth foliage as fixed effects. In both of these models, we also included milkweed species and CO_2 treatment as additional fixed effects.

To assess any independent and interactive effects of our treatments on root cardenolide concentrations, we repeated the analyses described above for root mass, but using root cardenolide concentration as the dependent variable.

Finally, we examined relationships among the initial growth rate and chemical resistance of plants before damage, and their root mass and root chemistry after the final harvest. We created LMMs with log-transformed belowground biomass or log-transformed root cardenolides as response variables and square-root-transformed growth rate and log-transformed cardenolide concentrations as fixed effects. In all four models, we also included milkweed species and CO_2 treatment as fixed effects.

5.4 Results

Milkweed species, not elevated CO_2, influenced Tolerance-Resistance Relationships

First, we present the effects of milkweed species and CO_2 treatment on the four potential growth and defense trade-offs that motivated our study: a) a tradeoff between growth rate and chemical resistance before damage occurs, b) a tradeoff between growth rate before damage and the chemical resistance of regrowth tissues, c) a tradeoff between chemical resistance before damage and regrowth tolerance after damage, and d) a tradeoff between regrowth tolerance and the chemical resistance of regrowth tissues.

Tradeoff a): Milkweed growth rate prior to damage was unrelated to foliar cardenolide concentrations prior to damage (growth: $F_{1, 316} = 0.47$, $p = 0.4924$, Figure 1a). Growing under elevated CO_2 did not change that result significantly (growth*CO_2: $F_{1, 316} = 3.20$, $p = 0.07437$, Figure 1a). We can therefore reject our hypothesis of a tradeoff between initial plant growth rate and initial chemical resistance, and our hypothesis that the tradeoff would be mitigated under elevated CO_2. Plant species was by far the most important determinant of the foliar cardenolide concentration of undamaged milkweed (Species: $F_{3, 314} = 8.17$, $p < 0.0001$, Figure 2a). There was no significant interaction between CO_2, milkweed species and initial growth rate on foliar

cardenolide concentrations before damage (CO_2* milkweed species* growth: $F_{3, 313} = 1.78$, $p = 0.1504$).

Tradeoff b): Likewise, we found no evidence of a trade-off between growth rate before damage and the chemical resistance of regrowth tissues (growth: $F_{1, 211} = 1.57$, $p = 0.2121$, Figure 1b). CO_2 treatment also did not influence the existence of a trade-off between initial growth rate and regrowth resistance (CO_2*growth: $F_{1, 211} = 2.70$, $p = 0.1021$, Figure 1b). Similar to the foliage before damage (above), milkweed species varied substantially in the concentrations of foliar cardenolides in their regrowth foliage (milkweed species: $F_{3, 211} = 5.70$, $p < 0.001$, Figure 2b). There was no interactive effect of CO_2, milkweed species and growth rate before damage on regrowth foliar cardenolide concentrations (CO_2* milkweed species* growth: $F_{3, 210} = 0.81$, $p = 0.4931$).

Tradeoff c): As with the other trade-offs tested above, we found no evidence for a relationship between chemical resistance before damage and regrowth tolerance after damage (initial cardenolides: $F_{1, 195} = 1.17$, $p = 0.2800$, Figure 1c), and no effect of elevated CO_2 on that relationship (CO_2* initial cardenolides: $F_{1, 195} = 0.5856$, $p = 0.4451$, Figure 1c). As with regrowth chemistry (above) regrowth tolerance was determined in large part by milkweed identity (milkweed species: $F_{1, 195} = 0.5856$, $p = 0.4451$, Figure 2c). There were no interactive effects among initial foliar cardenolides, CO_2 treatment, and milkweed species on regrowth tolerance (CO_2* milkweed species* initial cardenolides: $F_{3,199} = 1.82$, $p = 0.1450$).

Tradeoff d): In contrast to the first three potential tradeoffs, we observed a significant tradeoff between regrowth tolerance and the chemical resistance of regrowth foliage (Regrowth rate $F_{1,209} = 20.85$, $P<0.0001$, Figure 1d). However, the tradeoff was determined mainly by two of the four milkweed species (*A. incarnata* and *A. speciosa*) (regrowth rate*milkweed species: $F_{3,209} = 5.53$, $p= 0.0011$, Figure 1d). As above, there was no impact of CO_2 on the tradeoff (regrowth*CO_2: $F_{1,209}= 1.49$, $p = 0.2230$), nor was there an interaction among CO_2, milkweed species and regrowth rate on regrowth cardenolides (CO_2* milkweed species* regrowth: $F_{3,209}= 1.58$, $p = 0.1951$).

Beyond testing for tradeoffs, and any effects of elevated CO_2 in their mitigation, we also explored the effects of CO_2 on growth rates, regrowth tolerance, and chemical resistance. We present these results below.

Elevated CO_2 increased initial plant growth rate but had no effect on regrowth tolerance

The initial growth rates of plants before damage increased in *A. curassavica*, *A. syriaca* and *A. incarnata* under elevated CO_2 by 12%, 42% and 31% respectively, and remained unchanged in *A. speciosa* (CO_2*species: $F_{3,414} = 3.07$, $p = 0.0278$, Figure 3, Table 5.2). Across all milkweed species, elevated CO_2 induced an average 24% increase in growth rate (CO_2: $F_{1,64} = 13.97$, $p = 0.0004$) illustrating the classic pattern of CO_2 fertilization on plant growth (Kimball 1983; Leadley *et al.* 1999). We observed the fastest growth rates in *A. curassavica* and *A. incarnata* (99.60 ± 2.15 mg/day and 98.01± 2.36 mg/day, respectively), while *A. speciosa* grew half as quickly (51.91 ± 2.72 mg/day) (species: $F_{3,414} = 77.98$, $p < 0.0001$).

Following mechanical damage, only 278 of the 442 plants (63%) regrew any above ground tissue. The probability of regrowth varied among milkweed species ($\chi^2 = 55.88$, $p < 0.0001$, Figure 4). Nearly 92% of *A. curassavica*, 86% of *A. syriaca,* 76% of *A. speciosa* and only 32% of *A. incarnata* regrew following damage. CO_2 treatment did not affect the probability of regrowth ($\chi^2 = 1.07$, $p = 0.3009$), nor was there an interaction between milkweed species and CO_2 treatment on regrowth probability ($\chi^2 = 2.58$, $p = 0.4609$).

As mentioned above, milkweed regrowth rate following damage was highest in *A. curassavica* (11.03 ± 0.41 mg/day) and lowest in *A. syriaca* (2.49 ± 0.42 mg/day) ($F_{3,263} = 69.01$, $p < 0.0001$, Figure 2c, Table 5.2). Interestingly, CO_2 treatment had no effect on tolerance across milkweed species ($F_{3,57} = 0.01$, $p = 0.9699$, Table 5.2), nor was there an interaction between species and CO_2 treatment on milkweed tolerance ($F_{3,263} = 0.548$, $p = 0.6502$, Table 5.2). This result contradicted our original predictions that increased carbon (C) availability and reduced water loss through elevated CO_2 would favor faster rates of regrowth in damaged plants. Intriguingly, elevated CO_2 eliminated the positive relationship between initial plant growth rate and regrowth rate following damage ($F_{1,271} = 4.251$, $p = 0.04018$, Figure 5). In other words, future atmospheric

concentrations of CO_2 uncoupled the relationship between regrowth rate following damage and initial growth rate before damage.

Asclepias incarnata plants grew more than twice the dry mass of root tissue (7.297± 0.17 g) than did *A. curassavica* (2.93 ± 0.08 g) ($F_{3,282}$= 213.77, p < 0.0001, Figure 6a, Table 5.2). Across all four species of milkweed, elevated CO_2 induced a 9% increase in belowground dry mass accumulation ($F_{1,45}$= 12.53, p = 0.0001, Figure 6b, Table 5.2).

Elevated CO_2 influenced the resistance of milkweed in a species-specific manner

Similar to the patterns observed in foliar cardenolides, *A. curassavica* produced the highest concentration of root cardenolides, but *A. speciosa* instead had the second highest average root cardenolide concentration, followed by *A. incarnata* and *A. syriaca* (species: $F_{3,291}$= 89.16, p < 0.0001, Figure 7, Table 5.2). In those plants that did regrow following damage, *A. curassavica* again produced the highest concentrations of foliar cardenolides, followed by *A. speciosa, A. syriaca* and *A. incarnata* (species: $F_{3,154}$= 121.32, p < 0.0001, Figure 2b, Table 5.2). Interestingly, elevated CO_2 influenced the concentration of foliar cardenolides of regrowth in a species-specific manner (species*CO_2: $F_{3,154}$= 3.50, p = 0.0170, Figure 2b, Table 5.2). Elevated CO_2 induced a 17% increase in the foliar cardenolide concentrations of regrowth tissue in *A. curassavica*, and a 171% increase in cardenolides in *A. incarnata* regrowth foliage. In contrast, elevated CO_2 reduced the concentrations of regrowth foliar cardenolides in both *A. syriaca* and *A. speciosa* by 59% and 57% respectively.

Of the 380 milkweed plants for which we had initial foliar cardenolide samples before damage, only 332 of those plants produced measurable cardenolides (87%). Exactly 99% of all *A. curassavica*, 95% of *A. speciosa*, and 83% of *A. syriaca* plants produced measurable foliar cardenolides before damage, while only 77% of *A. incarnata* plants produced cardenolides (species: χ^2 = 18.07, p = 0.0004). CO_2 treatment did not affect the probability that milkweeds would produce measurable foliar cardenolides before damage (χ^2 = 2.01, p = 0.1560), nor was there an interaction between milkweed species and CO_2 treatment (χ^2 = 0.31, p = 0.9572). Nearly 96% (343/356) of all root tissues produced measurable cardenolides in our study. Elevated CO_2 induced an ecologically trivial, and marginally non-significant 2% reduction in the probability of

milkweeds producing root cardenolides ($\chi^2 = 3.15$, $p = 0.0761$), but there was no interaction between milkweed species and CO_2 treatment ($\chi^2 = 3.61$, $p = 0.1646$). Within the 271 milkweed plants that regrew following damage, 84% (227) of those plants produced measurable cardenolides. All *A. curassavica* plants, 88% of *A. speciosa* plants, 86% of *A. syriaca* plants and only 40% of *A. incarnata* plants produced foliar cardenolides in their regrowth tissue (species: $\chi^2 = 14.70$, $p = 0.0006$). CO_2 treatment did not affect the probability that milkweeds would produce cardenolides in regrowth tissues ($\chi^2 = 01.38$, $p = 0.2399$), nor was there an interaction between milkweed species and CO_2 treatment ($\chi^2 = 1.28$, $p = 0.5272$).

Root Mass was influenced by Tolerance and Resistance

Our data suggest that most milkweeds used significant root resources to regrow after clipping. In *A. speciosa, A. syriaca,* and *A. incarnata,* root biomasses were 9%, 16%, and 13% lower in those plants that regrew following damage than in those plants that failed to regrow ($F_{3,282} = 7.01$, $p = 0.0001$, Figure 8a). However, root biomass was 39% higher in the *A. curassavica* plants that regrew than in those plants that failed to regrow. As noted earlier, we have no measures of root biomass from plants prior to damage and cannot conclude with certainty that regrowth occurred at the cost of root material, or that those plants with less root material regrew more frequently following damage. Within the subset of milkweed plants that did regrow following damage, regrowth rate was correlated negatively to root mass ($F_{1,179}=6.18$, $p=0.0138$, Figure 8b). This negative correlation further supports the argument that regrowth comes at the cost of root biomass. There was no relationship between root mass and foliar cardenolide concentrations of regrowth tissue ($F_{3,126}=0.88$, $p=0.3503$), nor was there an interaction among foliar cardenolide concentrations, milkweed species, and CO_2 treatment ($F_{3,132}=0.60$, $p=0.6134$).

Root cardenolide concentrations of plants previously grown under elevated CO_2 were 119% higher in plants that regrew following damage, and 84% higher in plants previously grown under ambient CO_2 that regrew following damage (regrowth binary* CO_2: $F_{1,302}=4.97$, $p=0.02651$, Figure 9a). In other words, there was a positive relationship between regrowth and root cardenolides that became stronger when plants were previously grown under elevated CO_2. Within the plants that regrew following damage, there was no relationship between the rate of regrowth and the concentration of root cardenolides ($F_{1, 326} = 0.01$, $p = 0.9373$), nor was there an

interaction between regrowth rate, milkweed species and CO_2 treatment ($F_{3,321} = 0.3691$, $p = 0.7754$). Finally, in *A. curassavica, A. syriaca* and *A. incarnata* plants previously grown under elevated CO_2, root cardenolides were positively correlated with regrowth cardenolide concentrations (CO_2*species*regrowth cardenolides: $F_{3, 156} = 2.86$, $p = 0.03810$). Conversely, the root cardenolides of *A. speciosa* plants previously grown under elevated CO_2 were negatively correlated with regrowth foliar cardenolides (Figure 9b). Intriguingly, the relationship between root cardenolides and regrowth foliar cardenolides switched signs between ambient and elevated CO_2 treatments in *A. speciosa* (from positive to negative) and *A. incarnata* (from negative to positive, Figure 9b).

Pre-defoliation growth rates above ground were correlated positively with root mass ($F_{1,286}=81.29$, $p < 0.0001$) but were unrelated to root cardenolide concentrations ($F_{1,144}=1.71$, $p = 0.19348$). Additionally, there were no relationships between initial foliar cardenolides and root biomass ($F_{1,172}=0.28$, $=0.5988$) or root cardenolide concentrations ($F_{1,283}=0.01$, $p = 0.9290$).

5.5 Discussion

Our study examined the potential effects of elevated CO_2 on four hypothesized trade-offs between aspects of plant growth and resistance traits. We found support for only one of the four potential trade-offs described: within regrown plants, there was a trade-off between plant tolerance (regrowth rate) and resistance traits (foliar cardenolide concentrations) (Figure 1d). This trade-off varied substantially among milkweed species but was unaffected by previous exposure to elevated CO_2. In all tests of potential trade-offs, milkweed species was by far the most important factor determining plant growth and resistance. Our data add to growing body of work that demonstrates the complex nature of plant growth and resistance relationships and the need to test these energetic decisions in the context of changing resource availability on ecological time scales as well as in evolutionary contexts.

Among plants that regrew following clipping, we found evidence of a trade-off between foliar cardenolide concentrations and regrowth rate in two of four milkweed species (Figure 2). This finding supports previous studies that have demonstrated negative relationships between milkweed growth and cardenolide production (Hochwender *et al.* 2000; Züst *et al.* 2015; Tao *et*

al. 2016a). Multiple mechanisms may govern the magnitude of this tradeoff including limitation by other nutrients, allocation costs within the plant, genetic linkage of defense traits, ontogeny, and other ecological costs such as negative effects on critical mutualists (Simms & Rausher 1987; Strauss *et al.* 1999; Fine *et al.* 2006; Boege *et al.* 2007; Wise & Abrahamson 2007; Tucker & Avila-Sakar 2010; Tao *et al.* 2016a; Züst & Agrawal 2017). Ours is the first study within the milkweed system to show differences in tolerance-resistance relationships among milkweed species. However, this could be an artifact of our use of clipping as a defoliation treatment. Plants can modulate tolerance and resistance in response to specific herbivore species (Fornoni 2011; Carmona & Fornoni 2013), and therefore alter the strength and magnitude of the relationship between resistance and tolerance in response to herbivory. It would be interesting to repeat our experiment with a range of defoliation treatments by a range of herbivores to assess effects of CO_2 and herbivore identity on resistance-tolerance tradeoffs.

Within the plants that did regrow following mechanical damage, *A. syriaca* and *A. speciosa* individuals grown under elevated CO_2 had cardenolide concentrations in regrowth foliage (our measure of resistance) nearly 60% lower than individuals grown under ambient CO_2 (Figure 2b). Conversely, both *A. curassavica* and *A. incarnata* produced higher cardenolide concentrations in regrowth foliage when plants were grown previously under elevated CO_2 (Figure 2b). A change in the chemical resistance traits of regrowth foliage in response to elevated CO_2 is perhaps surprising, given that there were no effects of CO_2 treatment on the concentration of cardenolides in either the foliage prior to damage or in the roots (Figure 2a & 7, Table 5.2). Phylogenetic relatedness may play a role in the direction and magnitude of response to elevated CO_2 exhibited by the milkweed species we tested. Within the American *Asclepias* genus, *A. syriaca* and *A. speciosa* are more closely related to each other than they are to *A. curassavica* and *A. incarnata* (Fishbein *et al.* 2011), and vice versa. These data support previous work suggesting that closely related species have similar underlying mechanisms governing plant chemical defense (Agrawal & Fishbein 2008), such as resource allocation patterns and phytohormone concentrations. It also suggests that there may be useful phylogenetic signals to predict how plant species may respond to global environmental change (DeLucia *et al.* 2012; Guo *et al.* 2012; Zavala *et al.* 2013, 2017), although we would need a much more comprehensive phylogenetic comparison to be sure.

Surprisingly, the probability of regrowing and the rate of that regrowth following damage were unaffected by past atmospheric carbon enrichment. In our study, the expected pattern of CO_2 fertilization manifested in both higher original growth rates (Figure 5.3) and belowground biomass (Figure 5.6) under elevated CO_2. However, post-clipping effects of elevated CO_2 were limited to chemical resistance (Figure 5.2b), not regrowth tolerance. Generally, elevated CO_2 reduces plant tolerance and resistance (Wilsey 2001; Marshall *et al.* 2008; Lau & Tiffin 2009) likely through altered phytohormonal signaling pathways (Guo *et al.* 2012). We did not fertilize plants following mechanical damage, so it is possible that the plants were limited by soil nutrients such as nitrogen or phosphorous, which are known to alter the tolerance-resistance relationship in milkweed (Tao *et al.* 2016a). The lack of an effect of CO_2 treatment on regrowth may therefore have arisen from simultaneous nutrient limitation and altered plant signaling that suppressed regrowth. Further, elevated CO_2 uncoupled the relationship between initial plant growth rate and regrowth rate following damage (Figure 5.5). Those plants that were fast growers initially and had grown previously under ambient CO_2 were also fast growers following damage (had higher tolerance). However, those plants that were fast growers and had grown under elevated CO_2 were much slower to regrow following damage. The elimination of a relationship between initial growth rate and regrowth rate lends further support to the argument that elevated CO_2 suppresses regrowth mechanisms.

Our data also reveal a complicated relationship between the biomass and chemistry of roots and plant tolerance. Because we measured root mass and chemistry at the end of the experiment (after roots are removed, the plant is completely unable to grow), we are unable to distinguish the exact factor driving the relationships among root mass, root chemistry, and regrowth and resistance traits. Storage in root tissues is known to improve milkweed tolerance (Strauss & Agrawal 1999; Tiffin & Rausher 1999; Stowe *et al.* 2000), in a context-dependent fashion (Hochwender *et al.* 2000). For instance, *A. syriaca* plants with more root biomass are more tolerant under nutrient poor conditions, but this trend disappears when soil nutrients are abundant (Hochwender *et al.* 2000). Given that the regrowth period of 21 days is sufficient time for plants to utilize root energy stores, we think it likely that differences in root mass related to tolerance are the result of regrowth costs, rather than the determinant of regrowth ability. Regrowth in the three perennial species of milkweed native to North America in our study was associated with

low root mass, suggesting a strategy whereby milkweeds invest stored energy from below ground into new above ground structures for one last period of photosynthesis before winter. The opposite strategy is exhibited by *A. curassavica*, the one tropical species used in our study that has no life-history strategy to prepare for dormancy. Less clear is the positive relationship between root cardenolides and regrowth that is strengthened by previous exposure to elevated CO_2 (Figure 5.9). Because root tissues are well-known sites of secondary metabolite synthesis (Hartmann *et al.* 1989; Erb *et al.* 2009), factors that alter the amount of root material produced (such as elevated CO_2) or the allocation of resources to roots may influence the production of secondary metabolites in the plant. Our data indicate that plants which produce low to moderate foliar and root cardenolides (*A. syriaca*, *A. speciosa*, and *A. incarnata*), employ different resource allocation strategies into chemical resistance when grown under different CO_2 treatments: *A. speciosa* and *A. incarnata* exhibit opposite relationships between root and regrowth cardenolides between CO_2 treatments. To improve our understanding of these defense strategies, we urge further studies testing the relationship between below ground and above ground chemical resistance traits under future environmental conditions.

Monarch caterpillars are one of the most iconic herbivores found on milkweed and are suffering massive declines, due in part to changing environmental conditions (Stenoien *et al.* 2016; Malcolm 2017). Roadside milkweed habitats are important for monarchs (Mueller & Baum 2014; Kasten *et al.* 2016), and appropriately timed mowing treatments can increase monarch fecundity within milkweed patches by increasing the availability of regrowth foliage (Borkin 1982; Fischer *et al.* 2015). Our study reveals that foliar cardenolide concentrations in *A. syriaca*, the most common species of milkweed available to monarchs in eastern N. America, decline by by 59% following clipping treatments (Figure 5.7c). Reductions in foliar cardenolide concentrations may initially prove beneficial to monarchs because toxin catabolism is energetically costly (Tao *et al.* 2016b). However, these reductions in cardenolide concentrations could increase the energetic cost of toxin sequestration by monarchs to defend against predators (Reichstein *et al.* 1968; Malcolm & Brower 1989), or lower the antimicrobial properties of milkweed for protection against parasite infection (de Roode *et al.* 2008). Given the conservation importance of roadside milkweed patches that are regularly mowed throughout N. America, changes in regrowth tissue chemical quality could have implications for monarch populations.

However, attempts to predict how migratory monarchs that depend on roadside milkweed corridors will fare under global environmental change remain challenging (Zipkin *et al.* 2012).

Many theories of plant defense assume the existence of a trade-off between tolerance and resistance (Strauss & Agrawal 1999), however detecting this trade-off has been difficult (Tiffin & Rausher 1999; Leimu & Koricheva 2006; Agrawal 2011; Carmona & Fornoni 2013). In our study, the identity of the milkweed influenced any trade-off between tolerance and resistance following mechanical damage, and the potential costs of regrowth to plant energy stores in root tissues. Effects of CO_2 on plant defenses are highly context dependent (Hunter 2001, Vannette & Hunter 2011) and our study lends further support to this claim. Although our data provide another step towards understanding mixed defense strategies under future environmental conditions, further studies exploring the ultimate fitness costs of tolerance and resistance, and the responses of herbivore populations to these changes, are greatly needed. This knowledge can be used to inform policy decisions which reduce the use of pesticides (Strauss & Murch 2004) and inform weed control programs (Williams *et al.* 2004).

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5.7 Tables & Figures

Table 5.1. Sample sizes of 442 milkweed plants grown under either ambient (400 ppm) or elevated (760 ppm) CO_2 grouped (a) by species and (b) by their distribution in 40 open-top chambers. Species codes are: CUR = *A. curassavica*, SYR = *A. syriaca*, SPE = *A. speciosa*, INC= *A. incarnata*.

a)

CO_2 Treatment	species	N
ambient	*A. curassavica*	84
	A. incarnata	105
	A. speciosa	22
	A. syriaca	25
elevated	*A. curassavica*	81
	A. incarnata	91
	A. speciosa	23
	A. syriaca	11

b)

CO_2 Treatment	chamber	CUR	INC	SPE	SYR	chamber	CUR	INC	SPE	SYR
elevated	**1**	4	3	0	2	**21**	4	4	2	1
ambient	**2**	6	6	1	2	**22**	5	6	2	0
elevated	**3**	6	7	1	0	**23**	2	5	3	0
ambient	**4**	3	6	2	3	**24**	5	5	0	1
elevated	**5**	4	4	3	1	**25**	6	6	2	1
ambient	**6**	4	4	0	1	**26**	4	6	1	0
elevated	**7**	5	6	2	0	**27**	2	4	0	1
ambient	**8**	4	6	1	1	**28**	4	6	0	1
elevated	**9**	1	6	0	0	**29**	5	5	2	0
ambient	**10**	4	5	3	1	**30**	3	5	0	2
elevated	**11**	4	5	1	0	**31**	5	2	0	1
ambient	**12**	4	6	2	1	**32**	4	3	0	0
elevated	**13**	3	1	1	0	**33**	4	6	3	0
ambient	**14**	2	5	2	3	**34**	4	5	0	1
elevated	**15**	6	3	1	1	**35**	2	3	1	2
ambient	**16**	5	5	0	3	**36**	6	6	3	2
elevated	**17**	3	4	1	0	**37**	4	6	1	1
ambient	**18**	5	4	0	0	**38**	5	6	2	1
elevated	**19**	5	6	0	0	**39**	6	5	0	0
ambient	**20**	5	6	2	1	**40**	2	4	0	1

Table 5.2. ANOVA Table detailing the effects of CO_2 treatment, milkweed species, and their interaction on plant growth rate before damage, regrowth rate after damage, final root biomass, foliar cardenolide concentration before damage, root cardenolide concentration at harvest, and cardenolide concentrations of regrowth foliage.

	Plant Species		**CO_2 treatment**		**Plant species* CO_2**		**N**
	F	P	F	P	F	P	
sqrt(Growth rate)	**$F_{3,414}$ =77.98**	**< 0.0001*** **	**$F_{1,64}$ =13.97**	**0.0004*** **	**$F_{3,414}$ = 3.07**	**0.0278***	442
sqrt(Regrowth rate)	**$F_{3,263}$ = 69.01**	**< 0.0001*** **	$F_{1,56.95}$ = 0.01	0.9699	$F_{3,263}$ = 0.548	0.6502	278
log(Root biomass)	**$F_{3,270}$ = 210.31**	**< 0.0001*** **	**$F_{1,270}$ =14.89**	**0.0001*** **	$F_{3,270}$ = 1.45	0.2283	278
log(Initial Foliar Cardenolide Concentration)	**$F_{3,288}$ = 217.19**	**< 0.0001*** **	$F_{1,288}$= 2.49	0.1185	*$F_{3,57}$ = 2.49*	*0.0817*	299
log(Root Cardenolide Concentration)	**$F_{3,291}$ = 89.16**	**< 0.0001*** **	$F_{1,291}$ = 0.08	0.7817	$F_{3,291}$ = 0.88	0.4528	299
log(Regrowth Cardenolide Concentration)	**$F_{3,154}$ = 121.32**	**< 0.0001*** **	$F_{1,154}$ = 0.58	0.4492	**$F_{3,154}$ = 3.50**	**0.0170***	162

Figures

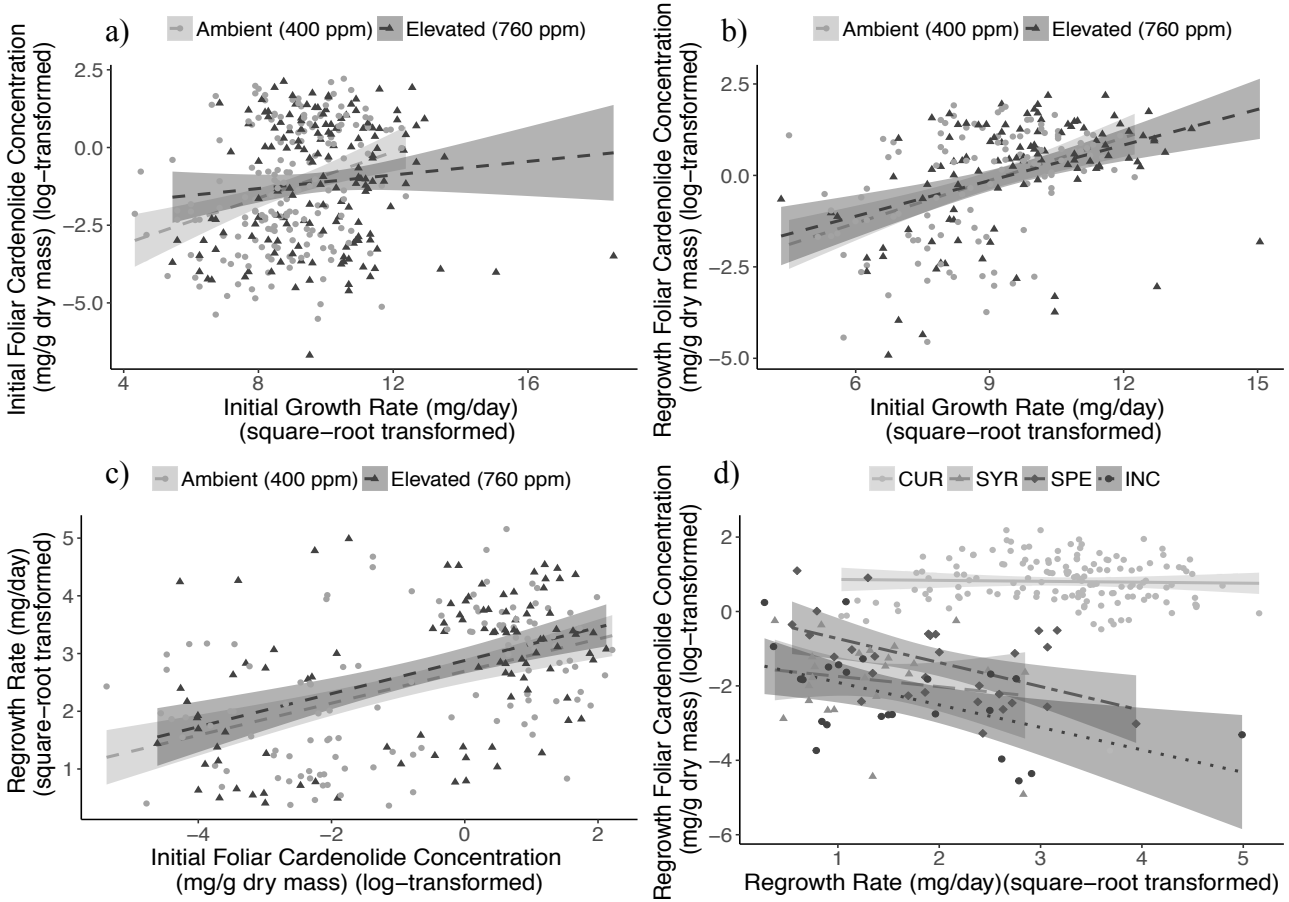

Figure 5.1. a) Nonsignificant effects of CO_2 treatment and initial growth rate on milkweed foliar cardenolide concentrations (mg/g dry mass) before mechanical damage. b) Nonsignificant effects of CO_2 treatment and initial growth rate on milkweed regrowth foliar cardenolide concentrations (mg/g dry mass) after mechanical damage. c) Nonsignificant effects of CO_2 treatment and milkweed foliar cardenolide concentrations before mechanical damage on regrowth rate after damage. d) The relationship between log-transformed foliar cardenolide concentrations (mg/g dry mass) after damage and square-root-transformed regrowth rate (mg/day) within those milkweed plants that regrew. Regressions are represented with 95% confidence intervals and milkweed species codes: CUR = *A. curassavica*, SPE = *A. speciosa*, SYR = *A. syriaca*, and INC= *A. incarnata.*

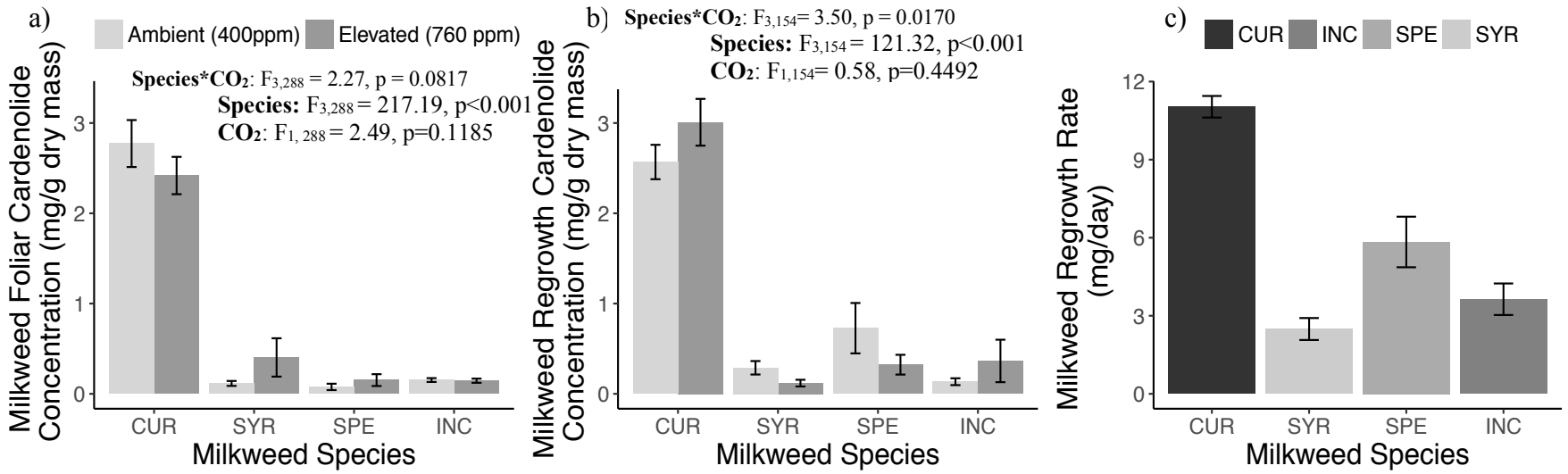

Figure 5.2. The effects of CO_2 treatment and milkweed species on a) initial foliar cardenolide concentrations (mg/g dry mass), and b) regrowth foliar cardenolide concentrations (mg/g dry mass) after damage. Interaction statistics from the accompanying ANOVAs are presented with each graph; although some interactions were not significant, we present the data in this fashion for ease of comparison. The main effect of Species was significant in every case (Table 2). The effect of milkweed species on c) the regrowth rate of plants (mg dry mass of above ground tissue per day over 21 days). Error bars represent ±1 SEM from the mean values and milkweed species codes are the same as in Figure 1.

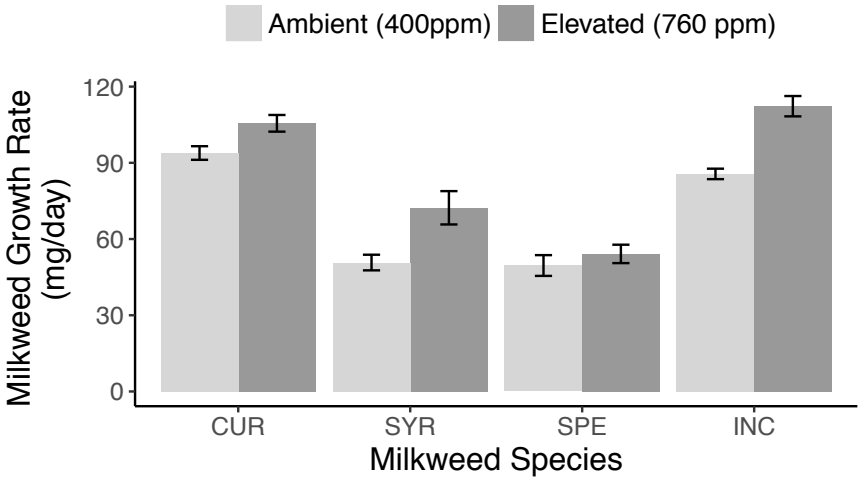

Figure 5.4. Effects of elevated CO_2 and milkweed species on the initial growth rate of milkweeds prior to mechanical damage (mg dry mass of aboveground tissue accumulated per day for 64 days). Dark gray bars represent plants grown under elevated CO_2 and light gray bars are those grown under ambient CO_2. Error bars represent ±1 SEM from the mean values. Innate growth rate was transformed for analyses but we present raw data here for ease of interpretation. Milkweed species codes: CUR = *A. curassavica*, SYR = *A. syriaca*, SPE = *A. speciosa*, INC= *A. incarnata.*

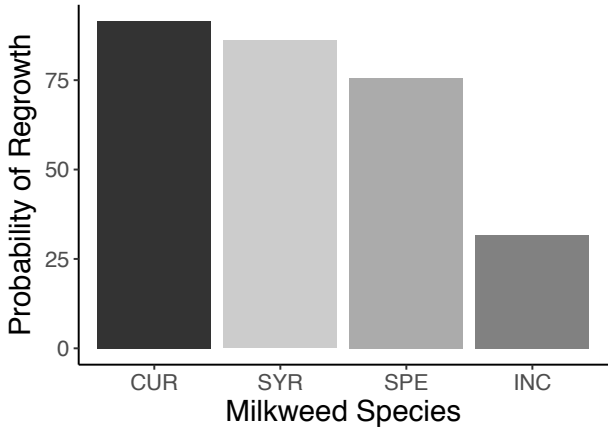

Figure 5.3. Variation among milkweed species in the probability of regrowth after mechanical damage. Regrowth growth rate was transformed for analyses but we present raw data here for ease of interpretation. Milkweed species codes are the same as in Figure 1.

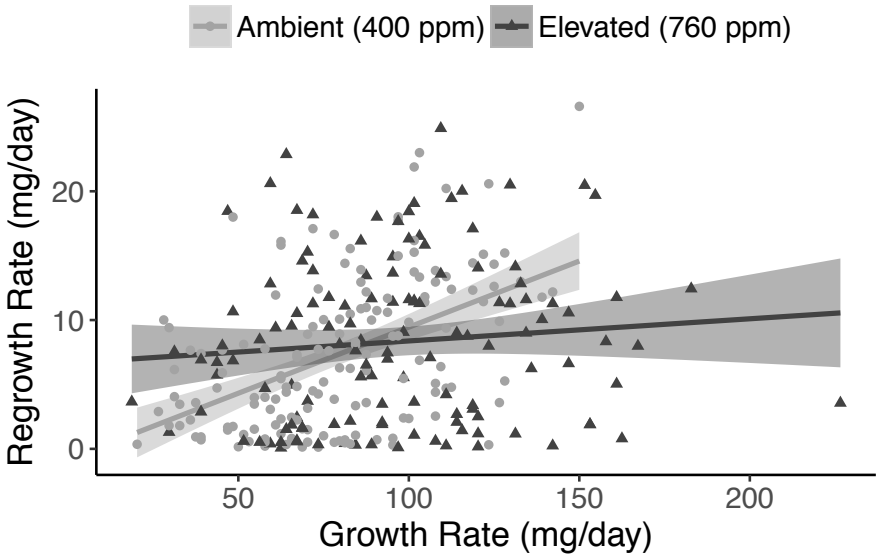

Figure 5.5. Effects of elevated CO_2 and initial growth rate on milkweed regrowth rate following mechanical damage. Light gray points and lines represent plants grown under ambient CO_2 and dark gray points and lines are those grown under elevated CO_2. Regressions are represented with 95% confidence intervals. Variables were transformed for analyses but we present raw data here for ease of interpretation.

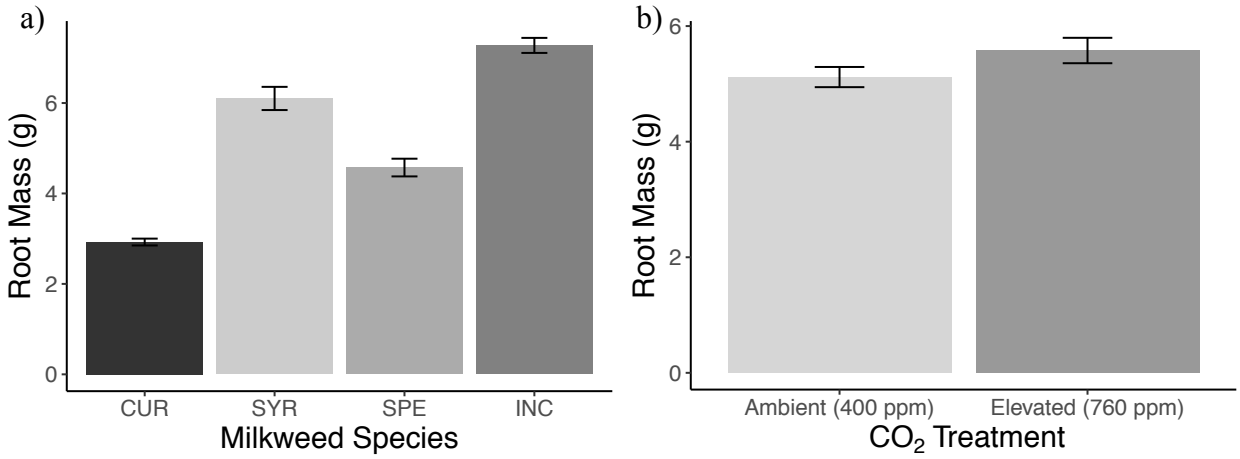

Figure 5.6. Effects of a) milkweed species and b) CO_2 treatment on the root mass of milkweeds. Error bars represent ±1 SEM from the mean values. Root mass was transformed for analyses but we present raw data here for ease of interpretation. Milkweed species codes are the same as in Figure 1. Dark gray bars represent plants grown under elevated CO_2 and light gray bars are those grown under ambient CO_2.

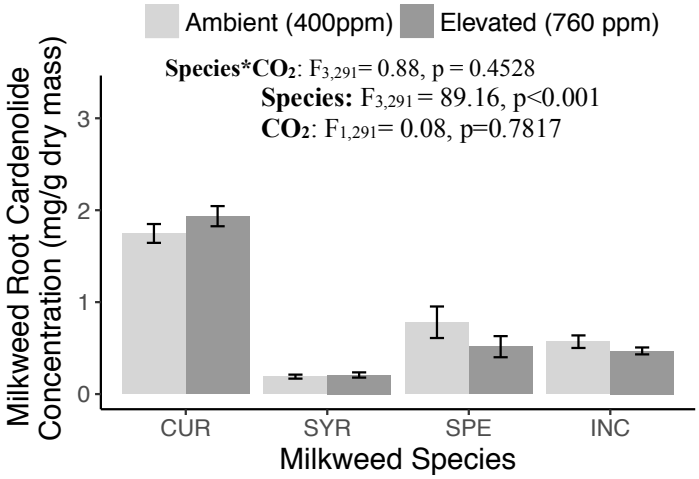

Figure 5.7. The effects of CO_2 treatment and milkweed species on root cardenolide concentrations (mg/g dry mass). Interaction statistics from the accompanying ANOVAs are presented; although some interactions were not significant, we present the data in this fashion for ease of comparison. Error bars represent ±1 SEM from the mean values and milkweed species codes are the same as in Figure 1. Dark gray bars represent plants grown under elevated CO_2 and light gray bars are those grown under ambient CO_2.

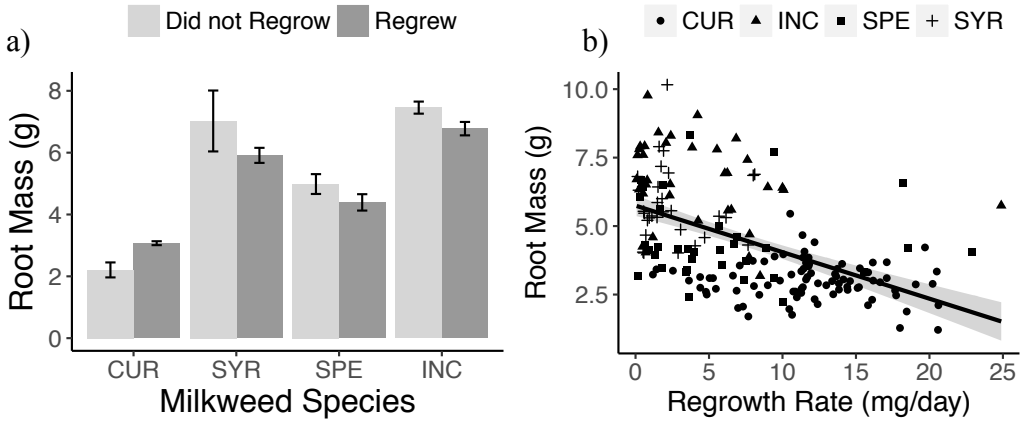

Figure 5.8. The effects of a) milkweed species on the relationship between milkweed regrowth following mechanical damage and final root mass. Within those plants that regrew following damage, b) the rate of regrowth was related negatively to final root mass. Error bars represent ±1 SEM from the mean values, regressions are represented with 95% confidence intervals, and milkweed species codes are the same in Figure 1

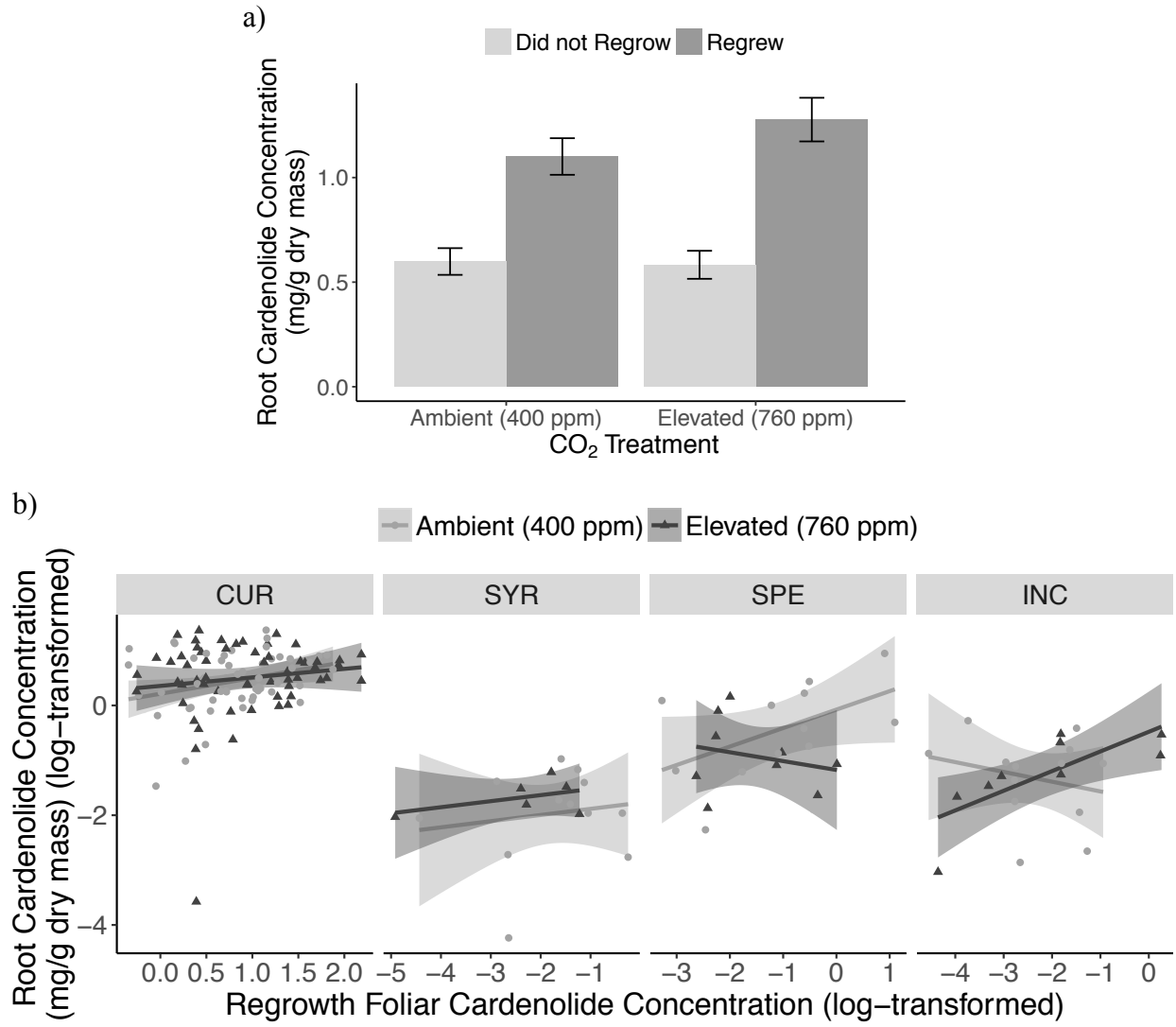

Figure 5.9. The effects of CO_2 on a) the relationship between regrowth probability on root cardenolide concentrations. b) The effects of CO_2 and milkweed species on the relationship between root cardenolide concentrations and regrowth foliar cardenolide concentrations. Dark gray points and lines represent plants grown under elevated CO_2 and light gray points and lines are those grown under ambient CO_2. Milkweed species codes are the same as in Figure 1, and regressions are represented with 95% confidence intervals. Note the different x-axes.

Chapter 6 : Conclusion

6.1 Summary of Findings

Anthropogenic drivers of global change influence the biology of organisms, accelerating extinction events, altering the abundance and distribution of organisms, and shifting community composition (Sala *et al.* 2000; Pearson & Dawson 2003). Simultaneously, changing climate and biogeochemical cycles may cause less obvious, but equally important, alterations to the networks of ecological interactions among species (Tylianakis *et al.* 2008; Gunderson *et al.* 2017).

In this dissertation, I combine a series of manipulative experiments to assess the effects of a critical global environmental change variable, increasing CO_2 concentration, on a plant-phytophagous host-parasite system. To understand how global change will alter disease dynamics, I investigate the effects of elevated CO_2 on the medicinal and nutritional chemistry of plants. I assess the subsequent impacts of those phytochemical changes on host (herbivore) tolerance to parasites, and parasite virulence. I then examine how those phytochemical changes induced by elevated CO_2 may also affect host defenses against predation, including the sequestration of chemical defense and mobility. To better understand one potential mechanism mediating the effects of diet quality on host-parasite interactions, I examine effects of elevated CO_2 on the host immune response mediated by diet chemistry. Finally, I explore how elevated CO_2 alters how plants allocate resources to defense, potentially shifting the relationships between plant resistance traits and regrowth tolerance. This matters to herbivore disease dynamics because any reduction in allocation to resistance traits may also reduce the pharmaceutical nature of plant foliage. I summarize the results of these four manipulative experiments below.

I use the monarch butterfly, *Danaus plexippus*, and its protozoan parasite, *Ophryocystis elektroscirrha*, as the central host-parasite interaction of my dissertation due to the strong influence of the monarch's obligate host plant, *Asclepias*, on the interaction (de Roode *et al.*

2008a, 2011; Sternberg *et al.* 2012). Plants of the genus *Asclepias,* milkweed, produce a group of toxic steroids known as cardenolides, which are medicinally active against *O. elektroscirrha* (Gowler *et al.* 2015). Given the strong dependence of monarch-enemy interactions on phytochemistry, any changes in the quality of milkweed should have important implications for monarch populations.

Chapter II: Elevated Atmospheric Concentrations of Carbon Dioxide Reduce Monarch Tolerance and Increase Parasite Virulence by Altering the Medicinal Properties of Milkweeds.
Environmental change imposes both direct and indirect effects on host resistance, host tolerance, and parasite virulence. In this chapter, I explored how elevated CO_2 alters the medicinal quality of milkweeds and, in turn, influences monarch host tolerance and parasite virulence. A high-cardenolide (medicinal) milkweed species lost its medicinal properties when grown under elevated CO_2. In response, monarch tolerance to *O. elektroscirrha* decreased, and *O. elektroscirrha* virulence increased. We related declines in medicinal quality with declines in the production of lipophilic foliar cardenolides. No such effects were observed on low cardenolide (non-medicinal) milkweeds under elevated CO_2. Our results emphasize that key parameters of host-parasite dynamics are susceptible to environmental change. Notably, the alterations in host tolerance and parasite virulence described here could modify selective pressures on parasites, which may favor the evolution of less virulent pathogens under future environmental conditions.

Chapter III: Managing Migration and Defense in a Changing World.
Higher trophic levels are affected by CO_2-induced shifts in plant quality (Hentley *et al.* 2014; Ode *et al.* 2014) as these changes may render herbivores more vulnerable to predation. In this chapter, I examined the indirect effects of elevated CO_2 on toxin sequestration and flight morphology of the monarch butterfly mediated by plant quality. Our results demonstrate that 1) monarchs compensate for lower plant toxin concentrations under elevated CO_2 by increasing toxin sequestration rate. Namely, monarchs maintain the same composition and concentrations of cardenolides in their wings under the two CO_2 treatments despite declines in foliar cardenolide concentrations under elevated CO_2. 2) Flight morphology, including wing shape, wing loading,

and wing density vary by elevated CO_2, milkweed species, infection status, and sex. 3) Feeding on high cardenolide milkweed is associated with the formation of rounder, thinner wings, which are less efficient at gliding flight and more prone to tearing.

We suggest that changes in the rate of sequestration under elevated CO_2 are a byproduct of compensatory feeding aimed at maintaining a nutritional target in response to declining dietary quality. Ingesting larger amounts of foliage from milkweed high in cardenolides may come at a cost to the monarch. Such costs may manifest as lower quality flight phenotypes: rounder, thinner wings with lower wing loading values. Small changes in wing morphology may have important consequences for flight ability and migration success. Energetic costs due to alterations in sequestration and morphology may, therefore, have important consequences for monarch defense in a changing world.

Chapter IV: Effects of CO_2 on Environmentally-Mediated Immunity in a Specialist Herbivore

The mechanisms underlying the impacts of global change on host-parasite interactions, such as those found in Chapter II, often remain unresolved. In this chapter, I investigated the plant-mediated effects of elevated CO_2 on monarch cellular (hemocyte concentrations) and humoral (immune enzyme activity) immunity in response to infection by *O. elektroscirrha* and to challenge by simulated parasitoid attack. I found that the immune enzyme activity of early-instar monarchs declined under parasite infection but was "rescued" by consuming foliage grown under elevated CO_2. Additionally, infection and a diet of foliage from elevated CO_2 increased the hemocyte concentrations of early-instar monarchs. However, in late-instar monarchs, the immune response against parasitoids declined on "medicinal" milkweed, suggesting a potential tradeoff between resistance against parasitoids and resistance against agents of disease. An improved understanding of immune mechanisms underlying host-enemy interactions may enhance our ability to make predictions about alterations in trophic cascades and emerging infectious diseases.

Chapter V: Variation Among Individual Milkweed Species, Not Elevated CO_2, Influences the Relationship Between Plant Resistance and Tolerance.

Changing environmental conditions alter resource availability and influence the defensive strategies of plants. Milkweed plants must employ two major avenues of defense against attacking herbivores: chemical resistance and regrowth tolerance. In this study, we investigated the effects of elevated CO_2 on the resistance and tolerance traits of the four milkweed species used in Chapters II and III. We demonstrate that a trade-off between plant tolerance and resistance traits varies in strength among milkweed species. Previous exposure to elevated CO_2 did not affect the strength of the trade-off between tolerance and resistance observed in our study despite the species-specific effects of elevated CO_2 on the chemical resistance of regrowth tissue. Our data add to a growing body of work that demonstrates the complex nature of plant growth and resistance relationships. Given the conservation importance to monarchs of roadside milkweed patches that are regularly mowed throughout N. America, changes in the chemical quality of regrowth tissue could have implications for future monarch populations.

6.2 Synthesis & Future Directions

Anthropogenic environmental change regularly influences both abiotic and biotic factors that operate in combination and at multiple scales to alter host-parasite interactions (Tylianakis *et al.* 2008; Altizer *et al.* 2013; Gunderson *et al.* 2017). My dissertation provides strong evidence that interactions between monarch butterflies and their protozoan parasites will be affected by ongoing environmental change. Notably, elevated concentrations of atmospheric CO_2 had important effects on milkweed chemistry, monarch tolerance, and parasite virulence. However, my dissertation spans a relatively short period. Long-term evolution experiments are very difficult to perform in this system but would provide much needed empirical data illustrating the evolution of both parasite virulence and host tolerance under future environmental conditions. There is strong support for the evolution of optimal virulence within *O. elektroscirrha* populations (de Roode *et al.* 2008b). During my three field seasons I observed substantial variation in parasite virulence, upon which selection may act. In the absence of external stressors, theory predicts that virulence should decline under elevated CO_2 back to a level that best optimizes parasite lifetime fitness (Best *et al.* 2008; Schneider & Ayres 2008). Given the accelerating rise in atmospheric CO_2 however, future empirical and theoretical studies should

investigate the relationship between the timescale of virulence evolution, rapid environmental change, and ultimate host-parasite dynamics. These studies will be crucial for evaluating policy decisions that recommend intervention in ecological processes to manipulate virulence evolution in human and animal pathogens in our changing world (Galvani 2003).

Rising concentrations of atmospheric CO_2 are also accompanied by increasing ambient temperatures, variability in precipitation regimes, and other aspects of environmental change (IPCC 2013). Reductionist studies focused on the effects of single environmental change drivers on multitrophic interactions, like the chapters presented here, are needed to infer causal relationships and population level effects. However, more studies combing the effects of multiple global change drivers, such as elevated CO_2 and temperature, on host-parasite interactions over varying temporal scales are sorely needed to improve our predictive abilities. Higher order interactions between environmental change drivers have already been illustrated in other systems (*reviewed* in Tylianakis *et al.* 2008), and may help to explain the considerable variation in the strength and magnitude of ecological responses to environmental change. Observational studies across gradients of environmental stress (for environmental change drivers such as drought) will also add to our understanding of disease under future conditions and better inform the design of further empirical work.

Much like a growing number of other chemical ecology studies, my work suggests that total concentrations of secondary metabolites are not indicative of ecological function, rather synergies amongst different compounds and chemical structures determine biological activity (Hay *et al.* 1994; Nelson & Kursar 1999; Dyer *et al.* 2003). In all of my studies where herbivore performance was influenced by phytochemistry, it was the diversity and polarity of cardenolides that was biologically relevant. This highlights the importance of measuring and categorizing the diversity of molecular structures in future studies of chemical ecology (Ayres *et al.* 1997; Dyer *et al.* 2014; Richards *et al.* 2015). Further analyses of our H^1-NMR foliar metabolomics data are currently underway to correlate important molecular features with the immune responses of monarchs. With these methods, we may better detect synergies between plant compounds critical to monarch defense, better describe the phytochemical changes induced by elevated CO_2, and perhaps aid in future drug discovery.

Additionally, my dissertation reveals an alarming reduction in phytochemical diversity under elevated CO_2. Given the extensive variation that exists on the phytochemical landscape, and its importance to multitrophic interactions and nutrient cycling (Hunter 2016), it would be interesting to explore the ubiquity of phytochemical homogenization by global environmental change and its effects on trophic dynamics. Additionally, we know very little about how environmental patchiness and resource instability (Hite & Cressler 2018), or its disappearance, influences the evolution of virulence across landscapes. Therefore, I urge future observational and empirical studies to examine the phytochemical dependence of host-parasite interactions at larger spatial scales of varying resource heterogeneity.

Implications for the Monarch

Despite the multitude of negative predictions accumulating about monarch survival in the face of global change (Oberhauser *et al.* 2015; Stenoien *et al.* 2016; Malcolm 2017), my dissertation work reveals possible mechanisms of monarch resilience. Elevated CO_2 improved early-instar monarch immunity and flight phenotype (made wings more elongated) despite inducing reductions in monarch host tolerance of *O. elektroscirrha* (see Chapters, II, III, & IV). Additionally, mowing milkweed at optimal times (Fischer *et al.* 2015) during the breeding season under future concentrations of CO_2, may improve the survival of monarchs depending on the species of milkweed and the prevalence of *O. elektroscirrha* infection in the monarch population (see Chapter V). However, this dissertation warns of the complexity of responses by organisms to global change. Thus, monarchs may be more resilient than previously proposed in the face of elevated CO_2, but how this resilience will fair under combined environmental change drivers remains uncertain.

Overall, the work described here contributes to our knowledge of multitrophic interactions in a changing world. Understanding the factors that govern the strength of host-parasite interactions (i.e. host immunity, resistance and tolerance) and the importance of environmental context (i.e. plant defensive and nutritional chemistry) under future conditions is critical to predicting future disease risk, community composition, and ecosystem function.

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Appendix A Supplementary Figures & Table for Chapter 2

Table A 2. Experimental treatments and the numbers of uninfected (control) and infected larvae used to explore the effects of elevated CO_2 and milkweed species on the interaction between monarch butterflies and a protozoan parasite, *Ophryocystis elektroscirrha*.

Year	Milkweed Species	CO_2 Treatment	Uninfected Initial	Uninfected Surviving	Infected Initial	Infected Surviving
2014	*A. curassavica*	ambient	10	8	20	15
		elevated	10	6	21	13
	A. incarnata	ambient	10	10	20	17
		elevated	10	9	19	16
	A. speciosa	ambient	10	5	14	13
		elevated	10	7	18	11
	A. syriaca	ambient	10	9	18	15
		elevated	11	8	19	12
2015	*A. curassavica*	ambient	19	19	20	13
		elevated	21	20	21	17
	A. incarnata	ambient	21	20	19	16
		elevated	20	18	21	18
	A. speciosa	ambient	21	18	19	13
		elevated	20	19	22	16
	A. syriaca	ambient	22	20	19	13
		elevated	21	20	20	12

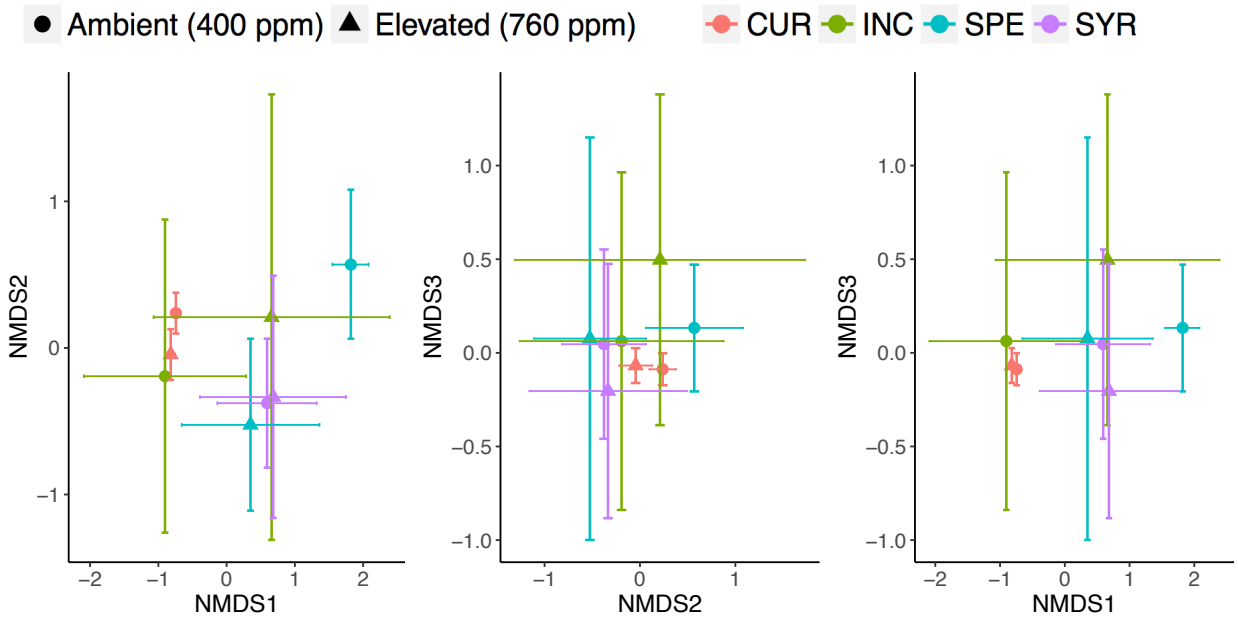

Figure A 2. Effects of milkweed species and CO_2 treatment on foliar cardenolide communities, separated in a 3-dimensional NMDS analysis. Circular points represent plants grown under ambient CO_2 and triangular points represent plants grown under elevated CO_2. The color of the points indicates the species of milkweed: CUR =*A. curassavica* (red), SYR=*A. syriaca* (purple), SPE=*A. speciosa* (blue), INC=*A. incarnata* (green).

Appendix B : Supplementary Figures & Table for Chapter 3

Table B 2. Effects of milkweed species on foliar cardenolide diversity and polarity. Numbers represent mean values ± 1 SE. *A. incarnata* were omitted from the analyses of cardenolide diversity because most individuals plants contained only a single cardenolide.

	A. curassavica	***A. syriaca***	***A. speciosa***	***A. incarnata***
Cardenolide Diversity	0.66 ± 0.02	0.12 ± 0.042	0.10 ± 0.04	-
Cardenolide Polarity	3.12 ± 0.07	4.41 ± 0.22	2.57 ± 0.40	2.07 ± 0.50

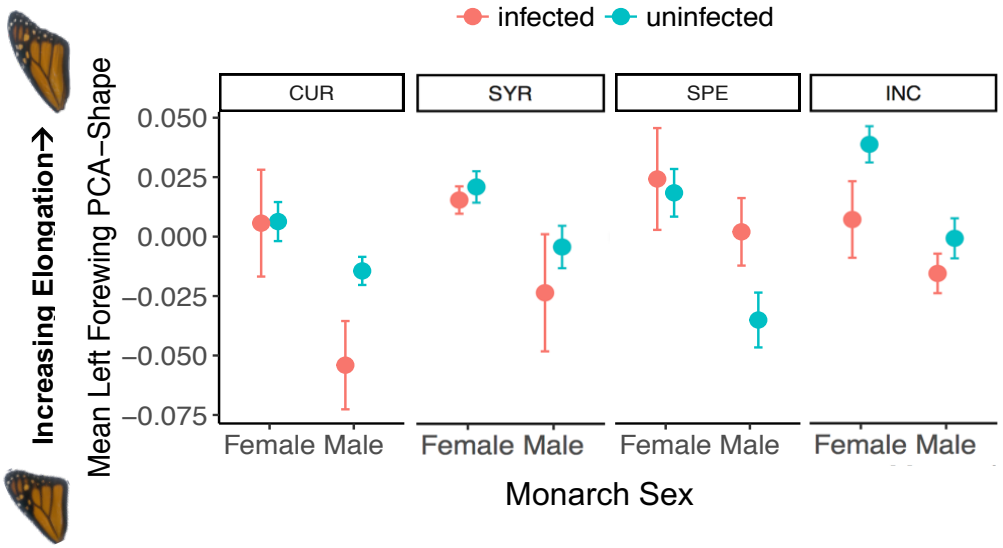

Figure B 2. The interaction between milkweed host-plant species, infection by *Ophryocystis elektroscirrha* and sex on a composite measure of monarch forewing shape. Points represent mean PCA-shape values ±1 SE. Red points indicate mean shape values of infected monarchs while, blue points represent uninfected monarchs. With increasing PCA-shape values wings become more elongated and angular. Milkweed species codes: CUR = *A. curassavica*, SYR = *A. syriaca*, SPE = *A. speciosa*, INC= *A. incarnata*.