HOW DO THE DEMANDS OF REPRODUCTION AND LONG DISTANCE MIGRATION ALTER IMMUNITY IN MONARCH BUTTERFLIES?

by

ALEXANDRA FRITZSCHE MCKAY

(Under the Direction of Sonia Altizer and Vanessa Ezenwa)

ABSTRACT

Migratory species, in addition to accomplishing some of the most impressive physical feats in the animal kingdom, can be exposed to a large number and diversity of parasites or pathogens and have the potential to transport those natural enemies long distances. Thus, migratory animals are important to consider in the context of infectious disease ecology. An essential step in understanding the role migratory animals play in disease transmission is to characterize how their susceptibility to pathogens—by way of changes in immune defenses—varies across the annual migratory cycle. Both strenuous movement and energetically-costly reproduction vary seasonally in migratory species and are each predicted to come at the cost of immunological parasite resistance, especially when food resources are limited during migration. My dissertation explored how an iconic migratory insect, the monarch butterfly (*Danaus plexippus*), balances the competing demands of immune defense, movement, and reproduction. I used the combination of a correlational field study and three laboratory experiments to understand how monarch immune measures varied with (i) food limitation across life stages; (ii) migratory distances travelled and the extent of lipid storage during migration; (iii)

multiple aspects of reproductive activity; and (iv) short-duration forced flight activity. Although trade-offs between immunity and several physical or life history traits were predicted, in many cases I found immune defenses to be fairly resilient to the potential costs of flight-related energy expenditure. This echoes recent findings by other researchers—with both monarchs and other migratory species—that migrants are physiologically adapted to minimize costs of movement. My results also showed that immune defenses were limited by food availability and reduced by several aspects of reproduction, highlighting the potentially increased susceptibility of wild monarchs during the summer breeding season when parasite pressure is high and food is often limited. This work contributes to the disciplines of animal behavior, disease ecology, and ecological immunology and the results inform predictions about when during their annual cycle migratory species may be most vulnerable to infection.

INDEX WORDS: Ecological immunology, disease ecology, migration, trade-off, reproduction, movement, immune defense, insect, *Lepidoptera*

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Overview

Long-distance migration is one of the most physiologically demanding activities in the animal world, entailing repeated cycles of strenuous physical exertion and high metabolic rates interspersed with periods of refueling and physical recovery (Brower et al. 2006, Weber and Stilianakis 2007, Piersma and van Gils 2011, Jachowski and Singh 2015). These intense physiological demands have been found to reduce immune responses (Owen and Moore 2006, Owen and Moore 2008b, Dolan et al. 2016), either directly, or via resource redistribution prior to the onset of migration (reviewed by Altizer et al. 2011 and Buehler et al. 2010). Such changes to host resistance could make migrating animals highly susceptible to pathogen infection, and indeed migrations are suspected to have facilitated the spread of neotropical ticks (Cohen et al. 2015), Phocine Distemper Virus (Harding et al. 2002), and *Mycoplasma gallisepticum* (Hochachka and Dhondt 2000), as well as zoonotic pathogens including Ebola virus (Ogawa et al. 2015), avian influenza viruses (Takekawa et al. 2010, Prosser et al. 2013, Verhagen et al. 2015), and West Nile virus (Dusek et al. 2009).

Complex behavioral traits can affect both exposure to parasites and susceptibility to infection, generating variation in disease among individuals, populations and species (Zuk and Stoehr 2002, Hawley et al. 2011). Because long-distance seasonal migration causes substantial physiological, life history, and behavioral changes, studying migratory

species presents an ideal framework for testing the effects of multiple behaviors on immunity (Buehler et al. 2010, Jachowski and Singh 2015). My dissertation research used a well-studied and experimentally tractable migratory insect, the monarch butterfly (*Danaus plexippus*), to investigate the relationship between migratory behavior and immune defenses. At different points in their annual cycle, monarchs are either migrating but not reproducing, reproducing but not migrating, or engaging in both behaviors simultaneously. This system allows me to examine how behavioral state drives heterogeneity in immune function across different points of the annual cycle, and to identify the stages at which migratory animals are most vulnerable to infection.

Overall, my dissertation asked: how does a migrating animal balance the challenges of investing in movement, reproduction, and immune defense, and what are the consequences of this resource allocation for fitness? Specifically, I used a combination of field observational studies, laboratory assays and experiments to investigate how immune defense depends on: (i) Resource limitation at larval and adult life stages (Chapter 2); (ii) Migratory distances travelled and the extent of lipid storage during migration (Chapter 3); (iii) Multiple aspects of reproductive activity (Chapter 4); and (iv) Experimentally-manipulated reproduction and flight (Chapter 5).

Background

Study system

Monarchs are iconic insects best known for undertaking long-distance migrations in eastern and western North America. Most scientific interest in monarchs has focused on the population that breeds east of the Rocky Mountains and migrates to Central Mexico (Oberhauser and Solensky 2004, Oberhauser et al. 2015). The migratory monarchs'

annual cycle consists of four phases (fall migration, overwintering, spring re-colonization, and summer breeding); my work capitalizes on differences among these stages in both the degree of reproductive investment and the amount of flight activity.

Reproduction and migratory activity are highly seasonal in monarchs. In the late summer and fall, individuals that experience cool night temperatures and decreasing daylength as larvae have diminished levels of juvenile hormone (JH), which causes a physiological state called reproductive diapause (Herman 1981, Herman and Tatar 2001). Rather than reproducing shortly after eclosion, these adults have immature reproductive organs and delay reproduction for up to 8 months, during which time they migrate up to 4000km to overwinter in central Mexico. Thus, monarchs in the fall migratory generation are non-reproductive, long-lived, and experience extreme energetic demands of flight. In the spring, the same individuals regain reproductive capability and fly north to the southern United States to lay eggs and die. Monarchs that develop from these eggs re-colonize the northern U.S. and Canada in two to three successive generations (Wassenaar and Hobson 1998, Miller et al. 2012, Flockhart et al. 2013). The spring re-colonizing generations are reproductive and fly intermediate distances. Monarchs that emerge during mid-summer reproduce immediately, are thought to remain close to their natal grounds, and generally live only a few weeks as adults. Monarchs in the summer breeding generation are reproductive and experience low flight demands.

Monarch natural enemies and immune defenses

A variety of parasitoids, parasites, and pathogens are natural enemies of monarchs in the wild (Altizer and de Roode 2015). Parasitoid wasps and flies infect larvae causing pupal mortality; insects deploy a variety of immunological (Strand and Pech 1995) and non-

immunological (Castelo and Crespo 2012) defenses against parasitoid infection, but little is known about monarchs' defenses against these enemies. Viral, microsporidia, and bacterial pathogens, such as nuclear polyhedrosis virus (Arnott et al. 1968) and *Nosema* spp (Keddie et al. 1989), have been documented in wild monarchs populations and are thought to be exacerbated in insects under captive breeding conditions (Bartel 2012). The most well-studied parasite of monarchs is *Ophyrocystis elektroscirrha* (hereafter OE), a neogregarine protozoan (Altizer and Oberhauser 1999). This parasite is debilitating, reducing reproductive output and lifespan and impairing flight performance (Altizer and Oberhauser 1999, Bradley and Altizer 2005, de Roode et al. 2007). Although genetic variation in susceptibility has been documented (Lefevre et al. 2011), mechanistic determinants of monarch resistance and tolerance to OE remain unknown. The prevalence of OE within eastern migratory monarchs has more than tripled in the last decade (Altizer et al. 2000, Bartel et al. 2011); an important question is whether monarchs might respond to higher OE prevalence by evolving greater resistance to infection, or whether migratory physiology constrains immune defense and might actually limit resistance evolution.

Monarchs, like other insects, maintain multiple lines of immune defenses, which vary between life stages and appear to offer some protection against OE (Lindsey and Altizer 2009, Altizer and de Roode 2015). Insect immune cells (hemocytes) aid in the recognition, phagocytosis, and encapsulation of microbial pathogens (Lavine and Strand 2002, Strand 2008). A second key insect defense is melanization, in which the enzyme phenoloxidase (PO) produces melanin pigment to deposit a dark layer around foreign material to suffocate pathogens or render them inactive (Schmid-Hempel 2005, Rolff and

Reynolds 2009). PO activity can be assessed by measuring the kinetics (absorbance over time) of the PO enzyme in hemolymph mixed with a dopamine substrate and microbial elicitor (Lindsey and Altizer 2009). Insects also produce antimicrobial (lysozyme-like) peptides that can lyse cell membranes and suppress bacterial growth (Dunn 1990, Bulet et al. 2003).

Relationships between immunity, reproduction, and migration

Despite strong selection pressure to minimize infection, most animals have imperfect immunity. If immune defenses are costly (Sheldon and Verhulst 1996, Lochmiller and Deerenberg 2000), then consequently immune defenses are expected to divert resources from other functions, such as competitive ability (Kraaijeveld et al. 2001), growth (van der Most et al. 2011), or reproduction (Lawniczak et al. 2007, Knowles et al. 2009), all of which contribute significantly to fitness. Across a wide diversity of taxa, when immune defense is experimentally up-regulated, reproduction and growth often decrease (Lawniczak et al. 2007, Reaney and Knell 2010, McNamara et al. 2013). Reciprocally, immunity is typically lowest when reproductive investment is high, such as during pregnancy in mammals (Weetman 2010), after copulation in damselflies (Siva-Jothy et al. 1998), during the production of nuptial gifts by crickets (Kerr et al. 2010), or while incubating eggs and rearing offspring in birds (Berzins et al. 2011).

Long distance migration could affect immunity through changes in resource acquisition, stress, and life history traits. First, migration affects access to and allocation of food resources. Even before migration, many animals enter a state known as the 'migratory syndrome', during which they forage excessively, develop flight musculature and rapidly accumulate fat reserves. When animals allocate resources towards muscle or

lipids, immune defense may be automatically down-regulated (Owen and Moore 2008a). Migrating animals often move long distances between stopover sites which limits their opportunities to forage (Wikelski et al. 2003). Migratory mormon crickets travel in pursuit of salt and protein, and the degree to which they encounter essential resources influences their immunity (Srygley and Lorch 2013). Several migratory songbird species have reduced innate immune defense during migration compared to the breeding season (Owen and Moore 2006), possibly owing to energy restriction during migration. Energy expenditure and increased metabolism during strenuous movement further diminishes the pool of resources available for functions like immune defense (Weber and Stilianakis 2007). Forced flight in a laboratory setting has been found to reduce immunity in crickets (Adamo and Parsons 2006), European starlings (Nebel et al. 2012) and pigeons (Matson et al. 2012). In this sense, physical exertion during migration could alter physiological homeostasis and an animal's ability to maintain effective defense against pathogens.

Migration also interacts with the timing of reproduction: some species retain reproductive viability, but many species delay the development of reproductive structures until the migration has been completed (Rankin and Burchsted 1992, Ramenofsky and Wingfield 2006). During breeding periods, hormones related to mating (such as testosterone in vertebrates and juvenile hormone in invertebrates) directly affect metabolism and energy expenditure (Klein 2004), with the expression of secondary sexual characteristics, mate defense, and copulation becoming priority outlets for resource allocation. Animals that remain reproductive during migration may incur simultaneous costs of both reproduction and flight demands resulting in strong reductions

in immunity. Conversely, animals that are non-reproductive during migration might better maintain high or moderate immune defenses.

Because both reproduction and movement can be immunosuppressive, it is challenging to predict whether immunity should be lowest during breeding or migratory seasons. My ongoing research addresses these aspects of migratory trade-offs in wild and captive populations of monarch butterflies**.**

Summary of dissertation chapters

In Chapter 2, I manipulated food availability at larval and adult life stages and measured immune defense and host fitness in order to test how underlying resources affect immune trade-offs. Both theoretical (Houston et al. 2007) and empirical (Boots 2011) work has shown that access to food influences an animal's optimal immune response. Also, physiological trade-offs are more likely to be observed in resource-limited conditions (French et al. 2007b, Adamo et al. 2016), because animals face a tighter constraint on allocation between traits (Van Noordwijk and De Jong 1986, Metcalf 2016). Most studies that investigate costs of food restriction for immunity and immune-fitness relationships only manipulate food at one life stage, but colleagues and I tested for synergistic effects of both larval and adult food restriction. In addition to measuring consequences of food restriction for immunity and fitness, I also tested whether food restriction affected the outcome of infection by the monarch's common protozoan parasite, OE.

In Chapter 3, I explored immune trade-offs in wild rather than in captive monarchs. Specifically, I sampled monarchs at their wintering sites in central Mexico and conducted lab work to characterize relationships between fat storage and two components of immunity. Because favoring the allocation of energy reserves for flight fuel (fat)

should take precedence over immune defense in migrating monarchs, we expected a negative relationship between these two traits. I also tested whether the relationship between fat and immunity was modified by estimated flight distance, measured using stable isotopes of hydrogen. I verified that the hydrogen isotope values estimated flight distance adequately by correlating the values with other physical measurements (i.e. monarch wing traits) known to be related to monarch flight capability or flight distance.

In Chapter 4, I aimed to identify the specific aspects of reproductive activity that generate reductions in immunity. Many aspects of reproduction can be costly for immune defense (Harshman and Zera 2007, Lawniczak et al. 2007, Schwenke et al. 2016), but few studies have asked how the reproductive development (i.e. development of reproductive tissues) versus actively engaging in reproduction (i.e. mating and laying eggs) affect immunity. This distinction is important because the defenses of fall migratory monarchs could be higher because of atrophied reproductive organs, or because adults are not actively investing materials and energy towards breeding (Oberhauser and Hampton 1995, Brower et al. 2007). The goal of this experiment was to compare immunity in monarchs in reproductive diapause (generally representing adults in the fall migratory generation) to reproductively active adults either kept as virgins or allowed to mate and develop eggs (generally representing adults in the summer breeding generation).

In Chapter 5, I examined immune consequences of experimentally-induced powered flight in monarchs. There is currently little empirical evidence for direct physiological links between energetic flight requirements and immunosuppression in wild animals (Matson et al. 2012, Nebel et al. 2012), and insect systems offer a powerful

opportunity to explore the dimensions of immune costs of flight (Chapman et al. 2015). The primary goal of this experiment was to compare immune defense in non-reproductive monarchs that underwent strenuous daily flight (for 4 days) versus those that remained inactive. Additionally, a subset of reproductively active monarchs were flown to determine if reproductive investment compounds the cost of strenuous activity for immune defense; this subset mimicked the dual demands of reproduction and flight experienced by spring re-colonizing monarchs. I quantified immune measures as a function of categorical flight treatment (flown versus control groups) and continuous measures of flight effort (e.g. flight distance, duration, and other measures of efficiency).

Altogether, this interdisciplinary project draws on concepts and methods from animal behavior, physiology and immunology. The physiological and behavioral mechanisms of migration remain under active study owing in part to recent advances in tracking mobile species over long distances. Because of their long journeys and coverage of diverse habitats, migratory animals have far-reaching implications for the spread of infectious diseases (Altizer et al. 2011). This project helps provide a framework for determining when and why migratory species might be most vulnerable to infections and thus enhance infectious disease spread. Using an experimentally tractable insect species, this work advances general understanding of how immune defense can change throughout the annual migratory cycle and how this depends on competing demands of movement and reproduction.

CHAPTER 2

CONSEQUENCES OF FOOD RESTRICTION FOR IMMUNE DEFENSE, PARASITE INFECTION, AND FITNESS IN MONARCH BUTTERFLIES[1]

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[1]Alexa Fritzsche McKay, Vanessa O. Ezenwa, Sonia Altizer.

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Abstract

Organisms have a finite pool of resources to allocate towards multiple competing needs such as development, reproduction, and enemy defense. Abundant resources can support investment in multiple traits simultaneously, but limited resources might promote trade-offs between fitness-related traits and immune defenses. We asked how food restriction at both larval and adult life stages of the monarch butterfly (*Danaus plexippus*) affected measures of immunity, fitness, and immune-fitness interactions. We experimentally infected a subset of monarchs with a specialist protozoan parasite to ask whether parasitism further affected these relationships, and whether food restriction influenced the outcome of infection. Larval food restriction reduced monarch fitness measures both within the same life stage (e.g. pupal mass) as well as later in life (e.g. adult lifespan); adult food restriction further reduced adult lifespan. Larval food restriction lowered both hemocyte concentration and phenoloxidase activity at the larval stage, and the effects of larval food restriction on phenoloxidase activity persisted when immunity was sampled at the adult stage. Adult food restriction reduced only adult phenoloxidase activity but not hemocyte concentration. Parasite spore load decreased with one measure of larval immunity, but food restriction did not increase the probability of parasite infection. Across monarchs we found a negative relationship between larval hemocyte concentration and pupal mass, and a trade-off between adult hemocyte concentration and adult lifespan was evident in parasitized female monarchs. Adult lifespan increased with phenoloxidase activity in some subsets of monarchs. Our results emphasize that food restriction can alter fitness and immunity across multiple life stages. Understanding the consequences of resource limitation for immune defense is therefore

important for predicting how increasing constraints on wildlife resources will affect fitness and resistance to natural enemies.

Introduction

Growth, reproduction, and survival require energy and nutrients. Immune defenses that confer resistance to parasites and pathogens can also be energetically costly, and their expression depends on both available calories and micro- and macro-nutrients derived from food (Sheldon and Verhulst 1996, Lochmiller and Deerenberg 2000, Cotter et al. 2011, Povey et al. 2014). Experimentally food-restricted animals, for example, tend to have lower immune defenses than well-fed animals (Moret and Schmid-Hempel 2000, Butler and McGraw 2012, Laurentz et al. 2012, Simmons 2012), suggesting that sufficient nutrition is necessary to mount costly aspects of an immune response. Because multiple physiological pathways draw from the same pool of resources, phenotypic trade-offs often occur between immunity and other fitness-related traits such as competitive ability (Kraaijeveld et al. 2001), growth (van der Most et al. 2011), or reproduction (Lawniczak et al. 2007, Simmons 2012). The extent or severity of these trade-offs can further depend on diet quality and composition.

Experiments addressing the effects of food limitation on immunity and immune-fitness trade-offs typically restrict diet quantity, quality, timing, or the percentages of specific macronutrients (reviewed in Ponton et al. 2013). Substantial evidence indicates that fitness components and physiological traits such as growth rate, body maintenance, and reproduction can trade-off with immunity in food-limited contexts across systems ranging from crickets (Fedorka et al. 2004) to lizards (French et al. 2007b), poultry (van der Most et al. 2011), and mammals (Canale and Henry 2011). Certain invertebrate

immune defenses (e.g. phenoloxidase and pro-phenoloxidase, encapsulation) have been found to be lower in resource poor environments (Siva-Jothy and Thompson 2002, Triggs and Knell 2012), and can trade-off with fitness measurements such as growth, development rate (Cotter et al. 2011), and reproduction (Karl et al. 2007, Kelly and Tawes 2013, Kelly et al. 2014).

Importantly, the majority of past work focuses on food limitation at a single life stage and examines effects occurring within that same stage, even though early life nutrition can profoundly affect adult fitness and life history (Boggs and Freeman 2005, Bauerfeind et al. 2009, Boggs 2009, Dmitriew and Rowe 2011, Stoks and Córdoba-Aguilar 2012). Further, there may be additive or synergistic effects of early- and late-life food limitation on immunity in both vertebrates (Butler et al. 2011, Butler and McGraw 2012) and invertebrates (Dmitriew et al. 2007, DeBlock and Stoks 2008, Karl et al. 2011, Jiménez-Cortés et al. 2012, Jiménez-Cortés and Córdoba-Aguilar 2013). Emerging trends from these studies in insects are that effects of early-life food restriction on fitness and immunity can persist across metamorphic boundaries and that sexes often differ in the effects of food restriction on immune and fitness traits. Further work is needed to characterize which fitness traits are expected to trade-off with which immune measures, and how these relationships unfold in the contexts of food stress and parasitism.

The monarch butterfly (*Danaus plexippus*) is a well-studied insect whose natural history in temperate environments includes extreme seasonal shifts in energetic demand for reproduction and flight. Monarchs in eastern North America undergo a southward fall migration, overwinter in a non-reproductive state in Mexico for several months, and then migrate north to re-colonize their breeding range during the spring and summer (Urquhart

and Urquhart 1978, Malcolm et al. 1991). As larvae, monarchs feed obligately on a subset of plants in the milkweed subfamily (Asclepiadoideae) from which they sequester cardenolide toxins to use in enemy defense (Malcolm and Brower 1989, de Roode et al. 2008a). As adults, monarchs obtain nectar resources from flowers and convert these to lipids to fuel the fall migration and overwintering period (Alonso-Mejía et al. 1997, Brower et al. 2006). Monarchs can encounter resource limitation as larvae and adults in several ways. Caterpillar densities are generally low during the spring and summer across North America (Pleasants and Oberhauser 2012), but per plant larval densities have been shown to increase from early to late in the summer breeding season (Bartel et al. 2011) and are also high in locations where mild winter climates and the planting of exotic milkweeds allow monarchs to forego migration and breed year-round (Haeger et al. 2015, Satterfield et al. 2015). Moreover, the loss of milkweed habitat throughout the monarchs' breeding range could increase resource competition by crowding monarchs into remaining habitats (Pleasants and Oberhauser 2012). Experimental studies show that high larval density decreases monarch body size, larval survival and adult reproductive output, and increases monarch susceptibility to parasite infection (Lindsey et al. 2009, Flockhart et al. 2012). Modeling studies suggest that current monarch population declines are driven primarily by larval food limitation during the breeding season (Flockhart et al. 2015), a phenomenon exacerbated by habitat loss. Flockhart and colleagues further found that monarchs reared in high densities (with less larval food per animal) had reduced fecundity as adults (Flockhart et al. 2012). Thus, resource acquisition at early life stages has implications for both individual adult fitness and longer term population viability. It has also been suggested that nectar-providing flowering plants are decreasing in

availability across the US (Goulson et al. 2015), creating resource limitation among adults (Brower et al. 2015). Adult monarchs also experience nectar resource limitation during their overwintering period, when they primarily drink water and rely on stored lipids (Alonso-Mejía et al. 1997).

Monarchs can be infected by a variety of parasitoids, parasites, and pathogens (Altizer and de Roode 2015, Oberhauser et al. 2015), and resource limitation at both larval and adult stages might lower immunity and parasite resistance. The most widespread and best-studied monarch parasite is the specialist protozoan *Ophyrocystis elektroscirrha*, hereafter OE (McLaughlin and Myers 1970). This debilitating parasite can reach high prevalence in some monarch populations (Altizer et al. 2000, Satterfield et al. 2015) and reduces monarch body size, adult lifespan and flight performance (Bradley and Altizer 2005, de Roode et al. 2007). OE is primarily transmitted vertically from infected adults to larvae when dormant parasite spores scattered on milkweed leaves are consumed by larvae (Altizer et al. 2004). Parasites replicate internally during larval and pupal stages, and infected monarchs emerge as adults covered with millions of dormant parasite spores. Because OE does not replicate on adults, adult parasite burdens are likely determined by larval and pupal defenses, and could be influenced by larval resources.

Here, we experimentally restricted food at both juvenile and adult stages of captive monarchs to examine the relationships between resources, immune defense, and fitness. Our goals were to ask how food restriction affects different immune components, and to test whether effects of food restriction at both juvenile and adult stages are additive or synergistic. We also examined whether food restriction revealed negative relationships (i.e., trade-offs) between immune defense and fitness. We predicted that

food restriction within a life stage would reduce immune defenses within that life stage, and that larval food restriction would also have consequences for adult immunity. Because resource limitation often increases differential trait allocation, we predicted that trade-offs between immunity and fitness would be primarily evident among resource-limited monarchs. Finally, we experimentally infected a subset of monarchs with OE parasites to ask whether food restriction influenced the outcome of infection in a direction consistent with immune defense responses.

Methods

Host and parasite sources

Monarchs used for this experiment were the grand-progeny of adults captured from two overwintering sites of eastern North American migratory monarchs (Sierra Chincua and Cerro Pelón, Michoacán, Mexico, February 2013). We generated five distinct outcrossed family lines. Monarchs were reared in a naturally-lit room with ambient light from approximately 0630 to 2030 and temperatures from 24 C (average nighttime low) to 29 C (average daytime high) during May – Jun 2013 in Athens, GA, USA. After eclosion, adults were kept in individual glassine envelopes in a 25 C (daytime) to 17 C (nighttime) incubator on a 14:10 hr light:dark cycle.

We used two clones of the OE parasite originally derived from wild monarchs in eastern North America (E3, isolated from a monarch collected in July 2008 from Minnesota; E10, isolated from a monarch sampled in October 2001 from New Jersey). Clones were known to express low (E3) and high (E10) virulence from prior experiments (de Roode et al. 2008b, de Roode and Altizer 2010). Parasite clones had been propagated

in the laboratory for multiple generations before this experiment, including a revival generation completed 6 weeks prior to the start of the study.

Larval and adult food restriction regimes

We used a factorial design to restrict food at the larval stage, adult stage, both stages, or neither stage (N=199-201 monarchs per food treatment). Larvae were reared singly in 0.5L plastic containers with mesh screen lids, and fed daily with fresh cuttings of greenhouse-raised swamp milkweed (*Asclepias incarnata*). Monarch development includes five larval instars, each separated by a molt, with the fourth and fifth instars constituting the stage of the greatest absolute weight gain (Lavoie and Oberhauser 2004). Monarchs in the "Larva unrestricted" treatment group were fed milkweed *ad libitum*. Monarchs in the "Larva restricted" treatment group were deprived of milkweed for 6 hrs between 0900 and 1600 each day during the 4^{th} and 5^{th} larval instars. This resulted in between 3 and 8 (mean ± SEM: 5.2 ± 0.04) total days of food restriction during larval development, depending on the time to progress from fifth instar to pupation. In the wild, this form of food restriction may occur when larvae consume an entire milkweed plant and must seek additional plants in the same or neighboring milkweed patches. Because monarchs undergo approximately 12-hour molting cycles between larval instars, during which time they do not feed (A. Fritzsche McKay, personal observation), we confined the food restriction interval to 6 hours so that all monarchs had the opportunity for several hours of foraging in a 24 hour period.

As adults, food restriction was implemented by halving the caloric concentration in the diet of experimental subjects compared to controls. "Adult unrestricted" monarchs were fed with a 20% concentration (1:4 honey:water) and "Adult restricted" monarchs

were fed with a 10% concentration. Both food-restricted and control adults were fed to satiation every second day until ten days post-eclosion. We standardized the feeding protocol by manually unrolling each adult's proboscis into the honey water solution initially, and again after feeding cessation, up to three times before terminating the feeding bout. We note, however, that this protocol did not control precisely for the volume of honey ingested by each monarch; thus our results must be interpreted in light of the fact that monarchs could have adjusted honey intake rate in response to sugar concentration, as has been shown in other Lepidoptera (Boggs 1988). By feeding all monarchs to satiation but with different nutrient concentration, we mimicked a naturally occurring source of food restriction. Adult monarchs, prone to dessication, will drink water from dewdrops or other sources in the absence of nectar resources (Masters et al. 1988, Brower 1999) which could dilute the ingested concentration of sugars. Also the abundance of nectar flowers is increasingly patchy in the landscape, and drought can reduce nectar volume and sugar concentration (Carroll et al. 2001, Nabhan 2004).

Parasite infection

We inoculated half of the monarchs in each food restriction treatment with the protozoan parasite OE. Second instar larvae were fed a small square of milkweed dosed with 10 parasite spores following de Roode et al. (2007). Control animals consumed milkweed squares with no spores. We monitored the progression of OE by visually assessing spore development in pupae following de Roode et al. (2008b). No control animals showed signs of infection, and 95.3% of inoculated monarchs were found to be infected as emerging adults. To quantify adult spore load as an index of within-host parasite replication, after an infected monarch died we vortexed the abdomen in 5mL of distilled

water on high speed for 5 min to dislodge spores. A 10µL aliquot was loaded into a hemocytometer and spores were counted at 400X magnification. Average spore counts per 0.1ul (from 5 replicate grids) were multiplied by 5 x 10^4 to estimate the total number of spores per monarch abdomen. Because not all inoculated monarchs became infected, we use the predictor variable "infection status" in all statistical analyses except the analysis of survival to adulthood, in which case we used "inoculation treatment" because true infection status was not assessed until adulthood.

Immune assays

We collected hemolymph to measure immunity at two time points, towards the end of the larval fifth instar and nine days after adult eclosion. Hemolymph was collected from larvae by clipping a front tubercle at the base and from adults by puncturing the cuticle of an intersegmental vein on the dorsal side of the abdomen. All larvae were weighed to the nearest 0.001g at the time of hemolymph collection to account for potential relationships between body size and immune measures.

We quantified two immune measures using well-described assays. First, we conducted hemocyte counts to quantify the concentration of immune cells in the blood. Hemocytes have various functions including phagocytosis, encapsulation, and production of humoral immune effector molecules such as antimicrobial peptides (Lavine and Strand 2002, Strand 2008). The total concentration of hemocytes at the larval stage has been found to be mildly protective against OE in adult monarchs (S. Altizer, unpublished data). Immediately after collection, 2µl hemolymph was rapidly diluted 1:10 in sterile Pringle's Saline [1x in 1L dD H_20: 9.0gNaCl, 0.2g KCl, 0.2g CaCl, 4.0g dextrose] and loaded onto Kova ® glasstic hemocytometer slides. We counted hemocytes under phase

contrast microscopy at 400x in two replicate chambers per sample and calculated the average number of hemocytes per µl.

Second, we measured the propensity of monarch blood to melanize in response to a bacterial elicitor, through the activity of the enzyme phenoloxidase (hereafter PO). PO activity involves the production of melanin pigment which is deposited onto foreign bodies to suppress growth (Söderhäll and Cerenius 1998). A 6µl sample of hemolymph was mixed 1:1 with ice cold Pringle's saline in an eppendorf tube. A total of 10µl of diluted sample was loaded into a well of a 96-well plate with 190ul assay buffer [in dD H_20: 50mM Na_2PO_4 monobasic monohydrate adjusted to 6.5pH, 2mM dopamine, and heat-killed *Micrococcus luteus* elicitor at 3% total volume]. We measured absorbance at 490nm every 24 seconds at 30°C for 300 measures (total time: 01:59:36) using a Biotek microplate reader. We calculated the slope of the kinetic curve (absorbance per hr) during the linear phase of the reaction to estimate the rate of melanization (Hall et al. 1995, Barnes and Siva-Jothy 2000).

All bled larvae yielded enough hemolymph for both the hemocyte and PO assays, but in many adult monarchs the volume of hemolymph collected was insufficient for both assays to be implemented. In these cases, we prioritized quantifying hemocyte concentration over PO activity. Of the 195 adults selected for hemolymph sampling, 105 bled a sufficient volume for both immune assays, 77 bled enough for hemocyte counts only, 3 bled enough for PO activity only, and 10 adults bled an insufficient volume for either immune measure. Finally, because prior work showed that wounding monarchs to draw hemolymph affected OE infection outcome (S. Altizer, unpublished data), we examined the effect of bleeding on infection status and other response variables using a

factorial design. Some monarchs were bled at both of the time points (N=86), some only as larvae (N=112), some only as adults (N=109), and a subset remained unbled (N=94).

Fitness metrics

To examine effects of food restriction on monarch development and fitness proxies, we recorded body mass (to the nearest mg) on day 5 post-pupation and on day 10 following adult eclosion. Pupal mass reflects the total resources that each individual amassed during the larval stage and thus reflects the effect of larval food restriction, while adult mass (measured after 10 days of feeding) reflects the cumulative effects of both larval and adult food restriction. We calculated monarch development as either larval development rate (1/days from hatching to pupation) or total development rate (1/days from hatching to adult eclosion). On day 10 after eclosion, half of the adults were placed into a 14°C refrigerator (following deRoode et al 2007) where they remained unfed and were checked daily for mortality to quantify lifespan. The remaining adults were frozen. During the course of the study, adult females were held in conditions that promoted egg development, although they remained unmated; logistical constraints prevented us from quantifying the number of mature eggs in females.

Data analyses

We compared survival to adulthood across larval food restriction and OE inoculation treatments using binary logistic regression. We used two-way ANOVAs to test the main and interactive effects of food restriction and parasite infection status (fixed factors) on continuous fitness variables including body size (pupal and adult mass), larval and total development rates, and adult lifespan. A series of focused tests examined the influence of drawing hemolymph on fitness variables. In cases where a fitness measure

was sensitive to hemolymph collection, we retained bleed treatment in the final model for the two-way ANOVAs. The only variable determined to be sensitive to larval bleed was pupal mass, and the only variable determined to be sensitive to adult bleed was lifespan.

To explore how food restriction influenced immunity and parasite infection, we tested effects of larval food restriction on larval immune measures (one-way ANOVA), and of both larval and adult food restriction on adult immune measures (two-way ANOVA). As another measure of susceptibility to disease, we also used a logistic regression to examine the effects of larval food restriction on the outcome of infection (infected or not infected) among larvae that were inoculated with OE. Body mass could be an important predictor of immune defenses and was strongly influenced by food restriction in our experiment (Effect of larval food restriction on larval body mass at the time of immune sampling, ANOVA: $F_{1,196}=37.51$, $P<0.005$; Effect of adult food restriction on adult body mass one day after emergence: $F_{1,369}=48.00$, $P<0.005$). As such, we incorporated mass into our preliminary analyses in two ways. First, we tested the effects of food treatment on immune measures uncorrected for body size. Next, we re-ran the models including size as a covariate (ANCOVA) to explore the degree to which the effects of food were mediated by size.

We ran analyses of variance on linear models ("anova(lm())" in R) to test if relationships between immunity and fitness were affected by our design variables. Two larval fitness metrics, development rate and pupal mass, were used as response variables against predictors of larval immunity (either hemocyte concentration or PO activity), larval food treatment, larval mass, sex, and two-way interactions between immune measures and design variables (food treatment and sex). Because only a subset of the

animals sampled for hemocytes could be sampled for PO, we included the two immune measures in separate models to maximize statistical power. Infection status, parasite clone, and monarch family line were included in initial models but were not significant in models of either larval fitness measure and were removed from final reported models. For adult lifespan we tested effects of adult immunity (hemocyte concentration or PO activity in separate models), OE infection status, larval food treatment, adult food treatment, adult mass, sex, and select two-way and three-way interactions between immune measures and design variables (Table 2). Because OE infection had a pronounced negative effect on adult lifespan, generating a bimodal distribution in the data, we conducted model diagnostic procedures following Venables and Ripley (2002) and we verified that model residuals were normally distributed.

Response variables for all immune measures (larval and adult hemocyte concentrations and PO activity) were $\log_{10}$-transformed to normalize the error variance. Adult lifespan data distributions were improved ($W=0.979$, $P=0.003$) by a power transformation using a lambda of 0.3 as determined by Box-Cox procedure in R; model residuals were also approximately normal for development rate (Larval: $W=0.957$, $P<0.005$; Total: $W=0.975$, $P<0.005$). Prior to analyses, we removed five outliers (greater than ± 2 SD from mean) for pupal mass, one outlier for adult body mass, and one outlier for PO activity that were biologically unrealistic and deemed to be the result of mechanical or observer error. All statistical models used Type 1 (sequential) Sum of Squares. One-way and two-way ANOVAS were implemented in SPSS version 22.0 (2013) while linear models were implemented in R (R Core Team 2015).

Results

Food restriction and infection effects on survival and fitness measures

Larval food restriction did not affect the likelihood of survival to adulthood (94% for restricted (N= 199) and 95.5% for unrestricted (N= 201); $\chi^2_1 = 0.81$, P=0.37). Likewise, survival to adulthood did not depend on OE inoculation (94.5% and 95% for control (N=201) and inoculated (N=199) monarchs, respectively, $\chi^2_1 = 0.001$, P=0.98).

Larval and total development rates responded similarly to design variables, so we report results only for larval development rate (1/days from hatch to pupation). Food-restricted larvae developed more slowly than those fed ad libitum (Figure 2.1A; $F_{1,378}$=41.92, P<0.005), but the rate was unaffected by parasite infection ($F_{1,378}$=0.86, P=0.355). Development rate varied among monarch lineages ($F_{4,376}$=7.28, P=0.002), but lineages responded in the same way to food restriction (no interaction between food treatment and lineage; $F_{4,376}$=0.43, P=0.789). Because pupal mass was lower in monarchs sampled for hemolymph as larvae ($F_{1,379}$=6.34, P=0.012), the effect of hemolymph sampling was included as a covariate in models investigating the effects of larval food restriction and infection. Pupal mass was significantly lower in food-restricted larvae (Figure 2.1B; $F_{1,371}$=93.56, P<0.005) but was unaffected by parasite infection ($F_{1,371}$=0.88, P=0.417) and the interaction between food treatment and infection ($F_{1,371}$=0.48, P=0.487).

Adult mass was significantly reduced by both larval and adult food restriction (Figure 2.2A; larval restriction: $F_{1,364}$=69.04, P<0.005; adult restriction: $F_{1,364}$=55.77, P<0.005), but there was no interactive effect of the two food treatments ($F_{1,364}$=0.21, P=0.644), indicating that the effect of food restriction at multiple life stages is additive

but not synergistic. Additionally, adult mass was lower in infected than uninfected monarchs ($F_{1,364}$=4.26, P=0.040). Because adult lifespan was lower in adults sampled for hemolymph ($F_{1,204}$=4.92, P=0.028), the effect of hemolymph sampling was included in subsequent models for this response variable. Adult lifespan was significantly shorter in monarchs that were restricted as larvae (Figure 2.2B, $F_{1,195}$=6.76, P=0.01) and as adults ($F_{1, 195}$=93.15, P<0.005), but there was no significant interaction between the two food restriction treatments ($F_{1, 195}$=0.05, P=0.817). Infected adults had shorter lifespans ($F_{1, 195}$=174.22, P<0.005), and we found an interaction between adult food restriction and infection ($F_{1, 195}$=10.23, P=0.002), such that monarchs that were both restricted as adults and infected had shorter lifespans than expected by additive effects of these variables alone (Figure 2.2B).

Food restriction effects on immune defense and infection

Larval food restriction decreased larval immune defenses. Uncorrected for body size, both PO activity ($F_{1,103}$=5.97, P=0.016) and hemocyte concentration ($F_{1,160}$=4.35, P=0.039) were lower in larvae fed restricted diets (Figure 2.3A). When larval mass was included as a covariate in the models of immune measures as a function of larval food restriction the effect of the food restriction disappeared (hemocyte concentration: $F_{1,155}$=0.39, P=0.534; PO activity: $F_{1,101}$=1.47, P=0.299), suggesting that the effect of larval food restriction on immunity was mediated by changes in body size.

Adult PO activity was reduced by both larval and adult food restriction (Figure 2.3; larval food: $F_{1,110}$=5.03, P=0.027; adult food: $F_{1,110}$=7.25, P=0.008), but not by the interaction between the two treatments ($F_{1,110}$=1.39, P=0.241). Adult PO activity increased with adult body mass (r^2=0.05, P=0.013), and when we included adult mass as

a covariate in the above model the effect of both larval and adult food restriction disappeared (larval food: $F_{1,105}$=0.17, P=0.682; adult food: $F_{1,110}$=0.06, P=0.808). Adult hemocyte concentration was unaffected by food restriction at either stage, or the interaction between the two restriction regimes (Figure 2.3, larval food: $F_{1,186}$=0.32, P=0.574; adult food: $F_{1,186}$=0.27, P=0.61; interaction: $F_{1,186}$=0.01, P=0.934).

There was no effect of food restriction on infection probability by the OE parasite among inoculated monarchs (χ^2_1=0.08, P=0.78). Among a subset of infected monarchs, the final spore load differed by OE clone ($F_{1,175}$=13.14, P<0.005) and was higher for clone E10 (known to be more virulent based on prior work) than clone E3. In the initial model of OE spore load (as a function of parasite clone and larval food restriction), spore load was higher in monarchs fed ad libitum as larvae ($F_{1,175}$=4.37, P=0.038); however, when pupal mass was included as a covariate in this model, the effect of larval food restriction disappeared, suggesting that OE growth is limited by host resources amassed as larvae. Finally, we found a weak, but significant negative relationship between larval hemocyte concentration and OE spore load (Appendix A).

Across all monarchs, larval hemocyte concentration was positively correlated with larval PO activity (r^2= 0.15, p<0.005). The two immune measures were not correlated in adults (r^2= 0.01, p=0.19), and neither larval hemocytes nor larval PO activity predicted levels of hemocytes or PO activity in adults (hemocyte concentration: r^2= -0.001, p=0.35; PO activity: r^2= r^2= -0.03, p=0.94).

Food restriction effects on immunity-fitness relationships

Larvae that developed faster to the pupal stage had greater larval hemocyte concentration and greater larval PO activity, counter to our expectations (Table 2.1). Larval

development rate was reduced by larval food restriction, but was not affected by sex or the two-way interaction between any pair of variables (Table 2.1). Food-restricted and female monarchs formed smaller pupae than unrestricted and male monarchs; across all monarchs, having higher hemocyte concentration was associated with forming smaller pupae (Table 2.1). Pupal mass was not associated with PO activity or interactions between PO activity and design variables.

Our models of adult lifespan showed significant three-way interactions between OE infection status, sex, and each measure of immunity, which we interpret in lieu of significant two-way interactive or main effects including these terms (Table 2.2). When monarchs were not infected by OE, lifespan increased with hemocyte concentration in both sexes (Figure 2.1A); when monarchs were infected by OE, lifespan decreased with hemocyte concentration among female monarchs only (Figure 2.1B). In other words, infected females with higher adult hemocyte concentrations died more quickly, whereas there was no effect of hemocyte concentration on lifespan in infected males. Adult lifespan was also affected by a three-way interaction between OE infection status, sex, and PO activity. In males, lifespan increased with PO activity equally as strongly in OE-infected and uninfected monarchs; in females, lifespan increased with PO activity more strongly in OE-infected than in uninfected monarchs (Figs. 2.4C and 2.4D).

Discussion

Our study showed that food restriction lowered fitness measures and components of immunity on short timescales, and that food restriction early in life affected a subset of traits at later life stages. Food restriction in larval monarchs lowered both measures of immunity and reduced larval growth. Larval food restriction also lowered adult

immunity and fitness (mass and lifespan), and these effects were additive, not interactive, with adult food restriction. Adult food restriction reduced adult mass, lifespan, and phenoloxidase activity, but not hemocyte concentration. We also found evidence for trade-offs between immune defense and fitness measures for a subset of monarchs. Across all monarchs there was a negative relationship between larval hemocytes and pupal mass, and in parasite-infected females there was a negative relationship between adult hemocytes and lifespan. These immune trade-offs only occurred with hemocyte concentration, as phenoloxidase activity was actually associated with longer adult lifespan, especially in infected females.

Individuals acquiring fewer resources might sacrifice immune defense in favor of other fitness traits, and abundant resources might eliminate such trade-offs and lead to higher investment in immunity. Although substantial past work has shown that food limitation can reveal trade-offs (Moret and Schmid-Hempel 2000, Alonso-Alvarez and Tella 2001, French et al. 2007b, Boots 2011, Simmons 2012, Kelly et al. 2014), most experiments explore resource-dependent trade-offs only within the same life stage in which the food restriction occurred. Our study also asked how food restriction at both juvenile and adult stages affect immune traits and fitness both within the life-stage of food restriction as well as later in life. These ontogenetic effects are particularly important in holometabolous insects, whose acquisition and allocation of resources as larvae constrain the resources available as adults (Boggs 2009). We found that larval food restriction influenced adult immune and fitness measures, and had some limited consequences for the relationships between immune and fitness traits.

Like food restriction, parasite infection could reveal trade-offs between traits by depleting the hosts' available resources. In this study, we found a negative relationship between adult lifespan and adult hemocyte concentration, which was only significant in female monarchs infected with the OE parasite. Monarchs with high concentrations of hemocytes as larvae had lower OE spore loads as adults (Figure S1), suggesting that investing more in larval hemocytes suppresses parasite growth. Because the OE parasite replicates internally prior to the host's adult lifestage, defenses earlier in life are more likely to limit parasite development. Although average larval and adult hemocyte concentrations did not differ between parasitized and unparasitized monarchs, OE infection lowered adult body size and lifespan substantially, suggesting that the parasite depleted host resources or damaged hosts in a way that limited their survival. We also see evidence that having higher phenoloxidase activity as an adult corresponded to increased longevity, an effect which was stronger in infected than in uninfected monarchs. This result suggests a protective quality of phenoloxidase activity against OE pathology, yet previous work has shown that larval phenoloxidase does not protect OE during its critical establishment period during the hosts' larval stage (S. Altizer unpublished data).

The trade-off we observed between adult hemocyte concentration and adult lifespan was most evident in infected female monarchs. Sex differences in immunity, with males typically displaying reduced immune function relative to females, are well-documented in vertebrates (Nunn et al. 2009), but remain less well-characterized in invertebrates. In particular, the direction of sex biases in immunity is inconsistent in invertebrate studies: in some cases females have been found to have higher

phenoloxidase activity than males, especially at reproductively mature stages (Adamo et al. 2001), but in other cases females were more poorly defended against pathogens than males (Rantala and Roff 2007, Stoehr 2007). In monarchs, past work showed that females tend to have higher hemocyte concentrations than males in the absence of OE infection, but fewer than males in the presence of infection (Lindsey and Altizer 2009). Further, there is little evidence and theory predicting why the sexes may differ in the relationships between immunity and life history traits, and the response of these traits to food stress (Rolff 2002). Females, whose fitness is linked to lifetime egg production, should invest more strongly in immunity to survive pathogen damage for a longer time, but they might sacrifice immunity under food limitation because resources limit egg production (McKean and Nunney 2005, Kelly and Jennions 2009, Kelly 2011). An experiment by McKean and colleagues (2011) with *Drosophila* showed that females, but not males, exhibited immunosuppression under food-limited conditions, and males only experienced immunosuppression under high mating demand. Experiments similar to ours by Karl and colleagues (2011) in tropical butterflies and by Kelly and Tawes (2013) in field crickets have shown that relationships between food restriction and immune defenses depended on sex. For example, female tropical butterflies showed a greater reduction in phenoloxidase activity (but not hemocyte concentration) than males under food stress (Karl et al. 2011). The differences between males and females in the severity of immune trade-offs under food restriction may derive from difference in the costs of reproductive tissue (Kelly 2011). In our study, both females and males remained unmated virgins, but females did most likely develop mature eggs. This egg development could represent another outlet for resource allocation faced by females, but not males. Our study provides

further evidence that the cost of immunity for fitness traits is context-dependent, with food availability, reproductive activity, and parasitism each potentially driving sex differences in the optimal investment in immunity.

This study explored immune defenses over time and across life stages, with repeated measures for individuals. There are relatively few examples of the ontogeny of insect immune profiles (but see Doums et al. 2002, Schmid et al. 2008, Wilson-Rich et al. 2008, Laughton et al. 2011, Urbański et al. 2014), and our experiment further explores the degree to which lepidopteran insects shift their investment in different defenses across life stages, and the extent to which these changes depend on resources. We found that investment in two immune defenses changed across monarch ontogeny, with larvae investing more strongly in immune cell (hemocyte) production and adults investing more strongly in melanization (PO activity). Further, the two immune measures responded differently to food restriction at both life stages. Larval food restriction lowered both hemocyte concentration and PO activity at the larval stage, and the effects of larval food restriction on PO activity persisted when immunity was sampled at the adult stage. Adult food restriction reduced only adult phenoloxidase activity but not hemocyte concentration. Karl and colleagues (2011), working with tropical butterflies, also found that PO activity but not hemocyte concentration was reduced by larval food restriction, but they found that both measures were reduced by adult food restriction. The similarity in results suggests a degree of generality in the responses of these invertebrate immune measures to stress and life history changes. Although in our study hemocytes were relatively insensitive to food restriction, the trade-offs we observed with fitness traits (both at larval and adult stages) involved hemocytes, not PO activity. On one hand, this

could be driven by our lower sample size for PO activity and poorer statistical power to detect effects. On the other hand, hemocytes and PO activity represent different components of defense that might respond differently to resource limitation.

Although lab experiments cannot perfectly replicate natural conditions, our selected modes of food restriction paralleled natural sources of resource limitation in wild monarchs. Monarch larval densities in the wild are typically low, but during phases of high larval density (peak summer breeding season and winter breeding in mild climates), larvae can deplete entire plants or patches of milkweed. In such cases, larvae have been observed wandering on the ground in search of new milkweed plants (Satterfield, Fritzsche McKay, Altizer personal observation). Milkweed densities vary tremendously across habitat types, with the lowest density now in herbicide-tolerant corn and soy monoculture plots, in the areas that once represented a crucial component of the breeding range (Hartzler and Buhler 2000, Oberhauser et al. 2001, Pleasants and Oberhauser 2012). In our study, we also reduced the caloric content of food provided to adults (mimicking reduced nectar availability while ensuring adequate hydration) and found significant negative effects on monarch mass, lifespan, and the phenoloxidase immune defense. Throughout their migration, monarchs forage on nectar and convert these resources to stored lipids, which fuel the butterflies through the remainder of their migration and overwintering period (Alonso-Mejía et al. 1997, Brower et al. 2006). After extensive studies of the nectaring behavior of overwintering monarch butterflies in Mexico, Brower (1999) found that in some years monarchs forage from flowers nearly devoid of nectar, and may actually expend more energy and lose more water than they gain by foraging. Of growing importance under climate change, drought stress can reduce

flower nectar volume and sugar concentration (Halpern et al. 2010, Brower et al. 2015), reducing the nutritional benefit to foraging butterflies during their migration. While the potential drivers of monarch decline are numerous (Brower et al. 2012, Pleasants and Oberhauser 2012, Flockhart et al. 2015), our work supports other suggestions that conserving food resources is of prime importance for monarchs and identifies potential physiological and fitness consequences of resource loss.

Natural environments are not benign, but stressful, and predictions about individual and population health should be made assuming such limits to fitness and defense against natural enemies. In this study, we showed that food restriction at multiple life stages affects monarch fitness and immunity, and that both food stress and parasite infection can reveal trade-offs whereby organisms sacrifice immune defense in favor of other fitness traits. Resource limitation is an escalating concern for monarchs, as milkweed and nectar flowers are diminishing in availability across a human dominated landscape. Understanding to what extent these migratory insects suffer reduced performance as larvae and adults due to resource limitation is important to both the basic field of ecoimmunology and to the long-term persistence of this iconic butterfly.

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Table 2.1. The response of juvenile fitness measures (development rate and pupal mass) to immune measures, food restriction, and sex. The order of explanatory terms in the table reflects the order they appeared in the models; terms were sequentially assessed by Type 1 sum of squares. We indicate the direction of effects for continuous mass and immunity covariates with + or –; for larval food restriction with R for restricted and U for unrestricted; and for sex with ♀ for females and ♂ for males.

	Immunity covariate					
	Larval log(hemocyte concentration)			**Larval log(PO activity)**		
	F_{df}	p	Direction	F_{df}	p	Direction
Larval development rate						
Immunity covariate	36.84_{1}	***	+	9.49_{1}	***	+
Larval food restriction	4.65_{1}	*	R<U	0.00_{1}		
Larval mass	14.60_{1}	***	+	6.36_{1}	*	+
Sex	2.37_{1}			0.40_{1}		
Immunity covariate * Larval food	0.48_{1}			0.29_{1}		
Immunity covariate * Sex	3.65_{1}			2.15_{1}		
Error	$_{151}$			$_{98}$		
Pupal mass						
Immunity covariate	7.62_{1}	**	–	0.15_{1}		
Larval food restriction	72.64_{1}	***	R<U	62.31_{1}	***	R<U
Larval mass	0.18_{1}			0.40_{1}		
Sex	40.21_{1}	***	♀<♂	24.26_{1}	***	♀<♂
Immunity covariate * Larval food	0.00_{1}			0.54_{1}		
Immunity covariate * Sex	2.20_{1}			2.77_{1}		
Error	$_{150}$			$_{97}$		

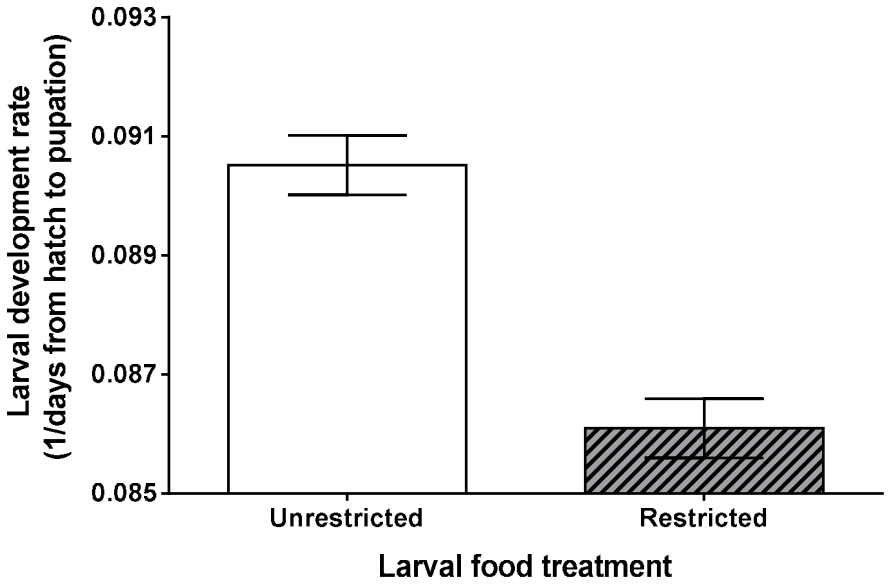
* = $0.01 < p < 0.05$.
** = $0.005 < p < 0.01$.
*** = $p < 0.005$.

Table 2.2. The response of adult lifespan to immune measures, food restriction, adult body mass, sex, and several two-way and three-way interactions. The order of explanatory terms in the table reflects the order they appeared in the models; terms were sequentially assessed by Type 1 sum of squares.

	Immunity covariate			
	Adult log(hemocyte concentration)		**Adult log(PO activity)**	
Explanatory variables	F_{df}	p	F_{df}	p
OE infection status	78.57_{1}	***	48.05_{1}	***
Immunity covariate	0.65_{1}		17.10_{1}	***
Larval food restriction	9.10_{1}	**	0.02_{1}	
Adult food restriction	73.14_{1}	***	27.24_{1}	***
Adult mass	6.66_{1}	*	2.07_{1}	
Sex	25.76_{1}	***	14.96_{1}	***
OE infection status * *Immunity covariate*	0.10_{1}		9.48_{1}	**
OE infection status * Larval food restriction	0.31_{1}		0.70_{1}	
OE infection status * Adult food restriction	4.47_{1}	*	3.43_{1}	
OE infection status * Sex	1.20_{1}		3.46_{1}	
Immunity covariate * Larval food	2.68_{1}		0.01_{1}	
Immunity covariate * Adult food	0.93_{1}		1.79_{1}	
Immunity covariate * Sex	3.15_{1}		0.20_{1}	
Larval food restriction * Adult food restriction	0.14_{1}		2.32_{1}	
OE infection status * *Immunity covariate* * Sex	$\mathbf{4.18_{1}}$	*	$\mathbf{4.12_{1}}$	*
OE infection status * *Immunity covariate* * Larval food	0.22_{1}		0.37_{1}	
OE infection status * *Immunity covariate* * Adult food	0.61_{1}		0.52_{1}	
Error degrees of freedom	77		40	

* = $0.01 < p < 0.05$.
** = $0.005 < p < 0.01$.
*** = $p < 0.005$.

A)



B)

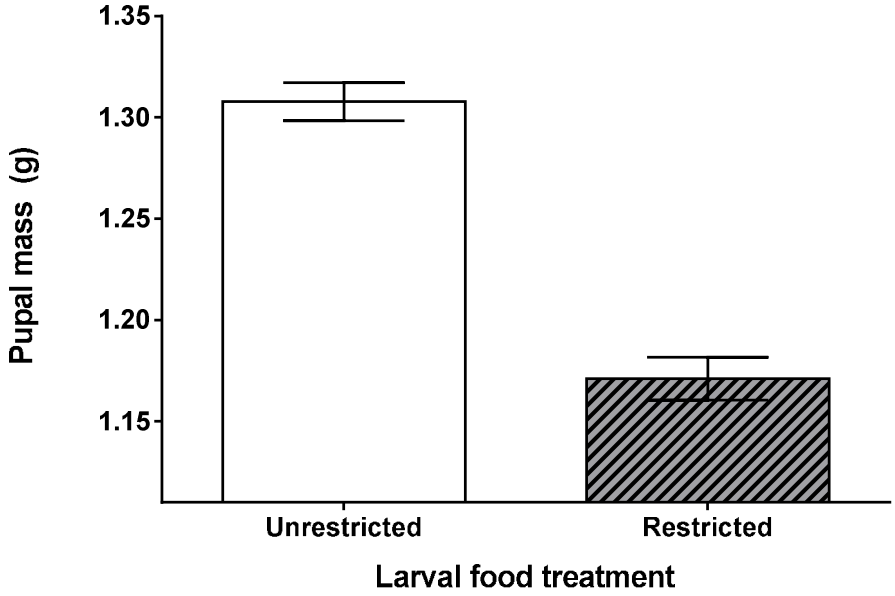
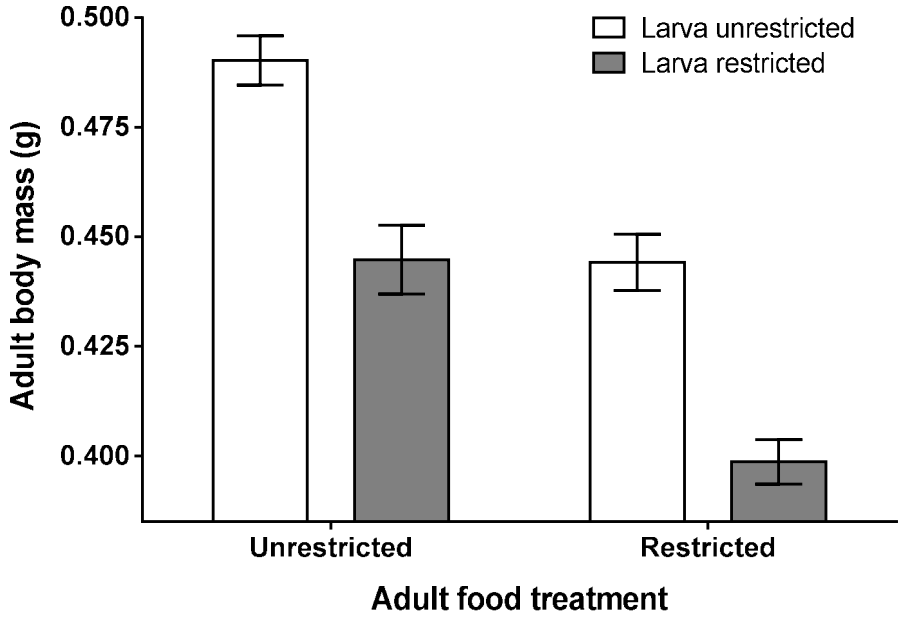


Figure 2.1. Larval food restriction reduces two measures of juvenile fitness: A) larval development rate (time from hatch to pupation in day^{-1}) and B) pupal mass (in g). Infection by a protozoan parasite did not influence these two variables. Data show means ± 1 SE.

A)



B)

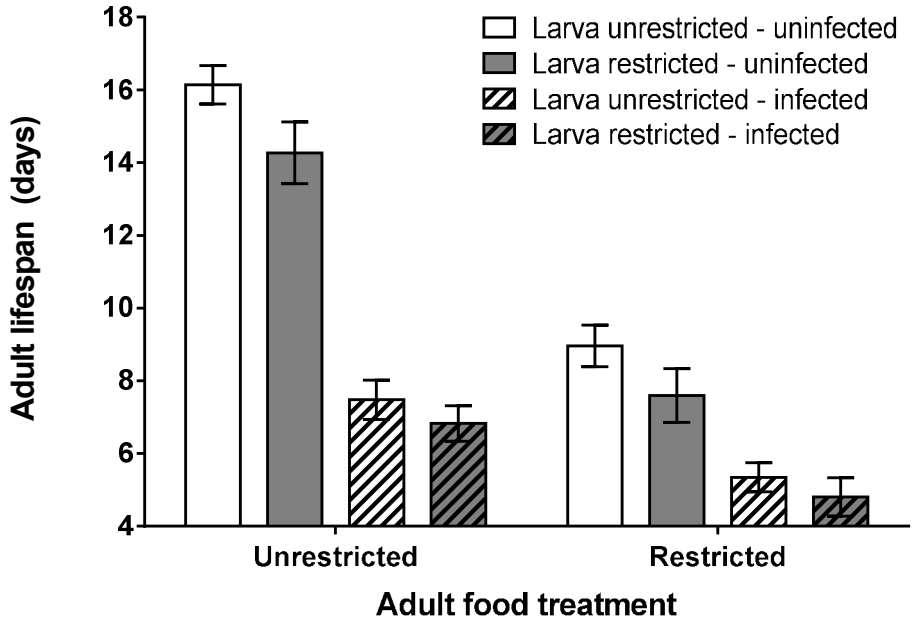
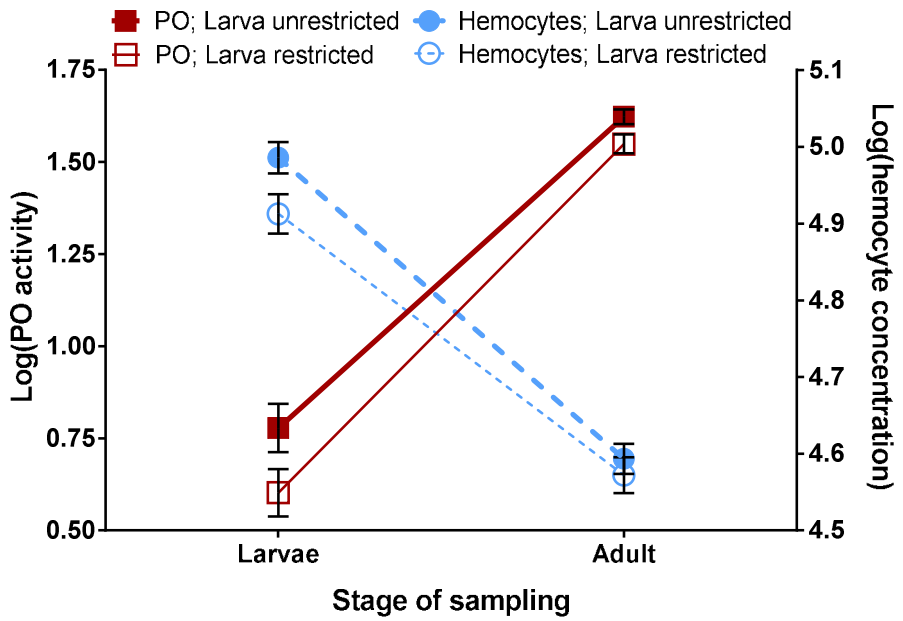


Figure 2.2. Larval and adult food restriction reduces two measures of adult fitness: A) body mass (in g) and B) adult lifespan (in days). Adult lifespan and mass were significantly reduced by food restriction at both life stages. Lifespan was also significantly reduced by protozoan parasite infection and the interaction between adult food restriction and parasite infection. Data show means ± 1 SE.

A)



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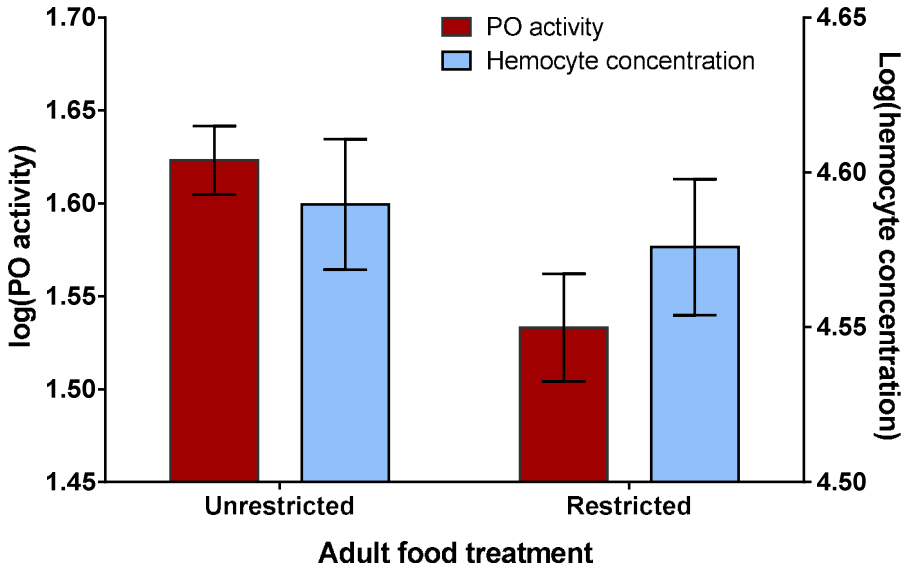

Figure 2.3. **A) Effects of larval food restriction on larval and adult immune measures.** Monarchs unrestricted as larvae had higher average PO activity (red lines) than food restricted larvae when assayed at both the larval and adult stages. Hemocyte concentration (blue lines) was higher in unrestricted larvae when measured at the larval stage, but there was no difference in adult hemocyte concentration. **B) Effects of adult food restriction on adult immune measures.** PO activity, but not hemocyte concentration, was significantly lower in food-restricted relative to unrestricted adult monarchs. Data show means ± 1 SE.

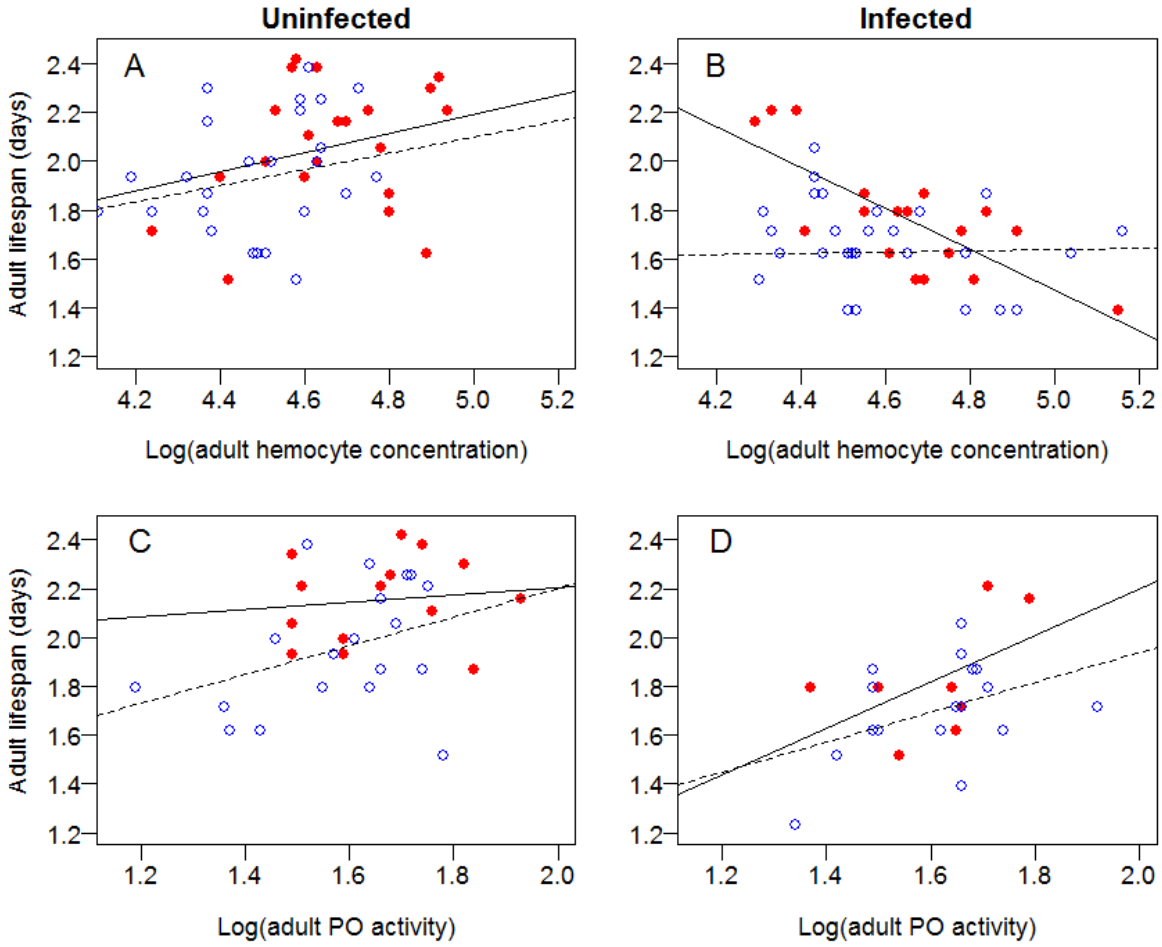

Figure 2.4. **Relationships between adult lifespan and immune measures differ by OE infection status and sex.** In all panels, females are denoted by red filled circles and solid lines while males are denoted by blue hollow circles and dashed lines. Top-row panels show hemocyte concentration and bottom-row panels show PO activity in uninfected (A,C) and infected (B,D) monarchs. Lifespan decreased with hemocyte concentration in uninfected monarchs (A) but decreased with hemocyte concentration in infected female monarchs (B). Lifespan generally increased with PO activity, especially in infected monarchs (D).

CHAPTER 3

WHAT DOESN'T KILL THEM MAKES THEM STRONGER: SUCCESSFUL MONARCH MIGRANTS DO NOT TRADE-OFF IMMUNE DEFENSE FOR FAT STORAGE

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Abstract

Migrating animals undergo drastic physical and physiological changes which alter the allocation of resources to optimize movement. One common alteration is to store fat as fuel for flight, which could come at the cost of immune defenses against parasites. This may be especially the case for insects, in which both movement and immunity compete for lipid reserves. Whether or not migrating animals sacrifice immune defenses in favor of flight-related tissue or energy expenditure has implications for the animal's susceptibility to infection. We examined trade-offs between fat storage and two components of immunity in migratory monarch butterflies sampled at their wintering sites in central Mexico. We also tested whether the relationship between the two traits was modified by estimated flight distance, measured using stable isotopes of hydrogen. Monarchs did not trade-off immunity for fat storage. Conversely, both measures of immunity (hemocyte concentration and phenoloxidase activity) were positively related to the proportion of body mass containing lipids. We did find evidence of a mild cost of migration for immunity, as hemocyte concentration decreased with estimated flight distance. Our results suggest that monarchs in the best physical condition were most likely to survive the complete migration to Mexico, and the findings underscore the critical importance of nectar flowers at stopover sites to provide monarchs with the lipid reserves that fuel flight and help maintain defenses against parasites.

Introduction

In preparation for and throughout migration, animals that move long distances undergo sweeping physiological changes to facilitate the energetically demanding journey (Dingle 2006, Ramenofsky and Wingfield 2006, Jachowski and Singh 2015).

One major physiological change in migrating animals is the accumulation and storage of fat as energy to fuel flight. Migrating bats (McGuire et al. 2012), whales (Silva et al. 2013), birds (Ramenofsky 1990), and butterflies (Brower et al. 2006) use stopover sites to forage excessively and to accumulate fat, but then rapidly expend these stores during bouts of energetically costly movement. While these physiological changes are essential to migratory success, they could draw resources away from immunological defenses against pathogens. In Swainson's thrushes, the pre-migratory state of fattening and prioritizing the development of flight muscle and energy storage reduced several components of immunity (Owen and Moore 2008a). In insects, lipid resources are mobilized for immune responses, stress responses, and physical exertion (Adamo and Parsons 2006, Adamo 2010, Adamo 2014), making a trade-off between flight-related lipid storage and immunity likely.

Given the role of some migrating animals in spreading pathogens of human health concern (Altizer et al. 2011, Prosser et al. 2013) considerable attention has been given to understanding whether migration induces immunosuppression and increases pathogen susceptibility. The prioritization of movement-related tissue and rapid expenditure of energy reserves during migration are assumed to be costly to immunity, but evidence of the immunological costs of migration is relatively rare. Arguably the most direct evidence for migration-related immunosuppression comes from work by Owen and colleagues with Swainson's thrushes. Owen and Moore showed that immune defense were lower in actively migrating birds compared to during the breeding season (2006) and that birds arriving at stopover sites were in poor condition and had very low immune defenses (2008b).

While much of the work on the immune costs of migration has come from bird species, long-distance migratory insects offer more tractable opportunities to assess the existence of trade-offs between immune defenses and flight (Chapman et al. 2015). Monarch butterflies (*Danaus plexippus*) in eastern North America travel up to 3500 km each fall in an annual migration from Canada and the United States to wintering sites in Central Mexico. During the migration, monarchs stop frequently to forage from nectar plants, and they convert these resources into lipids to fuel the remaining flight and the four-month overwintering period (Gibo and McCurdy 1993, Alonso-Mejía et al. 1997, Brower et al. 2006). Because lipids are depleted both by physical exertion (Rankin and Burchsted 1992) and by mounting an immune response (Adamo et al. 2008), we anticipate that monarchs storing and mobilizing lipids for migratory flight would face a trade-off (negative phenotypic correlation) between immune measures and lipid storage.

The extent to which the physical exertion of migration becomes costly to the maintenance of immune defenses could depend on the duration or distance of the journey. Monarchs that successfully migrate to Mexico vary greatly in their migratory distance because the breeding range is incredibly large—an estimated 12 million km^2 (Flockhart et al. 2013)—extending from the 30^{th} to 50^{th} north latitude parallels (Journey North, 2016). Insights about the monarch breeding range extent and occupation, and migratory distances during the fall migration and spring recolonization have been yielded by the use of hydrogen isotopic analysis (Hobson et al. 1999, Miller et al. 2012, Altizer et al. 2015, Yang et al. 2015). Stable signatures of the deuterium hydrogen isotope (δ_2H) in precipitation become more depleted with increasing latitude; these environmental isotopic signatures are integrated into the milkweed host plants of monarch larvae and

subsequently fixed in adult wing chitin tissue (Wassenaar and Hobson 1998, Hobson et al. 1999). Analysis of the degree of δ_2H depletion in tissue can approximate the geographical natal origins of migratory animals (Wassenaar and Hobson 1998); for monarchs captured at their migratory terminus in central Mexico, hydrogen isotope values can estimate migratory distance.

This study tested for a trade-off between immune defenses and fat content among monarchs that successfully migrated to Mexico. We predicted that monarchs storing more energy as fat would have fewer resources dedicated to immune defense, resulting in lower levels of two common invertebrate immune measures. We also investigated whether migratory distance (as estimated by hydrogen isotope values) altered the relationship between fat storage and immunity. We predicted that monarchs that flew the longest distances would face a steeper physiological trade-off (i.e.a stronger negative relationship between lipids and immunity) than monarchs that few shorter distances.

Methods

Animal source and maintenance

We collected monarchs at two overwintering colonies, Sierra Chincua and Cerro Pelón, near Michoacán, Mexico in February 2013. We sampled monarchs from different limbs of trees using nets with expandable poles. In the field, we assessed the status of infection by a common protozoan parasite, *Ophryocystis elektroscirrha* (OE), by pressing a clear sticker on the abdomen and counting OE spores under 20x microscopy. Samples containing over 100 spores indicate that the monarch had been infected as a larva and became heavily infected, whereas spore loads under 100 typically indicate that the monarch obtained spores transmitted horizontally as an adult but did not likely

experience a true infection (de Roode et al. 2007). For this study, we used only uninfected monarchs (having fewer than 100 spores) to avoid confounding immune measures with variation in parasitism.

Monarchs were stored in individual glassine envelopes and kept chilled to be returned to our laboratory at the University of Georgia. We sampled blood (hemolymph) for assessment of immune measures (N=135 for hemocyte concentration and N=153 for phenoloxidase activity) and froze monarchs at -12C for subsequent analyses of wing morphometrics (N=158), lipid content (N=152), and stable isotopes of hydrogen (N=110) described below. We aimed to acquire all lines of data for every individual, but this was not feasible owing to mortality between sampling timepoints.

Assessment of cellular and humoral immune defenses

We collected hemolymph by puncturing the cuticle of an intersegmental vein on the dorsal side of the abdomen and subsequently measured two important components of insect immunity: hemocyte concentration and phenoloxidase activity. Hemocytes are immune cells that have several functions including phagocytosis, encapsulation, and production of humoral immune effector molecules such as antimicrobial peptides (Lavine and Strand 2002, Strand 2008). To quantify hemocyte concentration, 2µl hemolymph was diluted 1:10 in sterile Pringle's Saline [1x in 1L dD H20: 9.0gNaCl, 0.2g KCl, 0.2g Cacl, 4.0g dextrose] immediately after collection and loaded onto Kova ® glasstic hemocytometer slides. We counted hemocytes under phase contrast microscopy at 400x in two replicate chambers and calculated the average number of hemocytes per µl.

Second, we quantified melanization, the process through which the invertebrate enzyme phenoloxidase produces melanin, a toxic compound, in response to a bacterial

pathogen or elicitor (Söderhäll and Cerenius 1998). A 6µl sample of hemolymph was mixed 1:1 with ice cold Pringle's saline in an eppendorf tube. A total of 10µl of diluted sample was loaded into a well of a 96-well plate with 190ul assay buffer [in dD H_20: 50mM Na_2PO_4 monobasic monohydrate adjusted to 6.5pH, 2mM dopamine, and heat-killed *Micrococcus luteus* elicitor at 3% total volume]. We measured absorbance at 490nm every 24 seconds at 30°C for 300 measures (total time: 01:59:36) using a Biotek microplate reader. We calculated the slope of the kinetic curve (absorbance per hr) during the linear phase of the reaction to estimate the rate of melanization (Hall et al. 1995, Barnes and Siva-Jothy 2000).

Measurement of wing physical properties

Characteristics of monarch wings, such as wing length, area, and aspect ratio have been identified as key predictors of migratory status or capability (Altizer and Davis 2010, Satterfield and Davis 2014). To digitally assess these wing traits, we scanned the dorsal sides of left and right monarch forewings on a flatbed scanner. We then measured the area of each wing in mm^2 and the aspect ratio with the FoveaPro plugin (Reindeer Graphics, Inc.) for Adobe Photoshop ® (Davis et al. 2007, Altizer and Davis 2010). Wing length (mm) was assessed at capture using digital calipers.

Estimation of natal origins by hydrogen isotopes

We used a protocol modified from Wassenaar and Hobson (1998) to estimate the hydrogen isotopic signatures of the keratin in monarch wing tissue. We prepared tissue from the monarch right hindwing, which had been previously removed from the body with ethanol-sterilized scissors. Following approximately 48 h of freeze-drying at -50C, the hindwing was finely ground to homogenize isotope values that might vary across the

wing. Initially our protocol was to grind the tissue with a glass rod in a 10mL scintillation vial on a high-speed vortex. This method, used for N=54 samples, was time- and labor-intensive (est. 45 minutes of grinding per sample) so we changed the grinding protocol for the remaining 55 samples. Owing to several weeks' passing between grinding methods, we re-dried remaining samples for 48 hours in the freeze-drier before using a Precellys®24 Dual Tissue Homogenizer to pulverize wing tissue for 30 seconds in specialized 7mL tubes with 2.8mm stainless steel beads. Hydrogen isotope values (δ^2H) did not differ between the two preparation methods (ANOVA: $F_{1,107}=1.74$, $p=0.19$).

Following grinding, small aliquots of ground wing tissue were packed into silver capsules and sent to the UC Davis Stable Isotope Laboratory for elemental analysis. Wing samples were compared against keratin working standards and international reference materials. The final raw hydrogen isotope values (δ^2H) values are expressed relative to the V-SMOW (Vienna Standard Mean Ocean Water) international standard.

Measurement of fat content by lipid extraction

We used a lipid extraction protocol modified from methods initially described by Alonso-Mejía et al. (1997) and modified by Satterfield et al. (2013). Because we used the right hindwing for stable isotope analyses, our measurement of lipid concentration is of the remaining tissue (i.e. whole body except right hindwing). Thus our lipid values cannot be compared to other studies, which used whole bodies in the measurement (Alonso-Mejía et al. 1997, Brower et al. 2006, Satterfield et al. 2013), but they are internally consistent and comparable within this study.

We obtained an initial dry weight (in mg) after monarchs were dried whole for approximately 48h at -50C in a freeze-drier. We finely homogenized monarch tissue by

grinding the tissue with a glass rod in a 10mL scintillation vial on a high-speed vortex for three minutes. We added three mL of petroleum ether and homogenized the mixture for an additional three minutes. After transferring to a 25mL vial and vortexing with an additional 7mL of petroleum ether, we heated the mixture for 30 minutes in a 35C water bath. The tube was vortexed for 10 sec every 10 minutes, and was then centrifuged at 1000 rpm for 10 min. We pipetted the supernatant into a pre-weighed aluminum pan. We repeated the extraction process after adding an additional 20mL of petroleum ether and adding additional supernatant to the pan. The contents of each pan was air-dried in a fume hood for two days while petroleum ether evaporated, and the lipid mass remaining was weighed to the nearest mg. To account for variations in whole-monarch body mass, we calculated lipid proportion as the mass of lipids divided by the whole-monarch dry mass. Lipid mass and lipid proportion were tightly correlated (r^2=0.86, $p<0.001$), and results of analyses with the two variables were qualitatively similar. Here, we present results only for analyses using lipid proportion.

Statistical analyses

We first tested for relationships between continuous δ^2H values and monarch physical measurements (wing morphometrics and fat storage) to validate whether δ^2H values could serve as estimators of flight distance. We predicted that larger wing size (i.e. length or area) would enable monarchs to fly longer distances, based on previous work showing that larger wings confer the ability for increased dispersal and flight capability and tend to be associated with migratory phenotypes (Hill et al. 1999, Dudley and Srygley 2008, DeVries et al. 2010). We conducted univariate linear models of δ^2H values as a function of each monarch physical measurement: wing area (in mm^2), wing

length (in mm), wing aspect ratio, and lipid proportion. An exploratory analysis showed that continuous δ^2H values were significantly influenced by monarch collection site (either Sierra Chincua or Cerro Pelón wintering colony) so we included collection site as a fixed factor in these models.

We next tested whether immune measures (either hemocyte concentration or PO activity in separate models) were influenced by fat storage (lipid proportion), estimated migration distance (δ^2H values) and sex. We tested for potential differences in immune measures by collection site but found no effect and excluded site from further analyses. We used general linear models to model each immune measure as a function of lipid proportion, continuous δ^2H value, sex, and all two-way interactions. Models were simplified by progressively removing least-significant terms, beginning with the interaction terms and following with main effect terms, until all terms in the model were significant or only the intercept was remaining (Crawley 2002).

Hemocyte concentration (cells/μL) was log-transformed to normalize error variance. All continuous covariates were standardized to the consistent unit of standard deviations ($y= (x – mean(x))/(2*SD(x))$) prior to inclusion as predictor variables in linear models to facilitate comparisons of estimates and effect sizes. We used R version 3.2.2 (R Core Team, 2015) for all analyses.

Results

δ^2H values approximate flight distance

Consistent with our expectations, monarchs with larger wing area (β: -0.28±0.09, $p=0.003$, $R^2=0.09$) and wing length (β: -0.25±0.09, $p=0.008$, $R^2=0.08$) had lower δ^2H values and thus likely flew longer distances (Figure 3.1). Wing aspect ratio was unrelated

to δ^2H values (β: 0.04±0.10, p=0.64, R^2=0.01). Further, lipid proportion decreased with δ^2H value (β: -0.21±0.09, p=0.02, R^2=0.07), indicating that monarchs that flew the furthest had established the greatest fat stores during migration.

Fat storage and migration distance influence monarch immune measures

After model simplification, PO activity was predicted only by lipid proportion (Table 3.1); monarchs with more body mass stored as fat (higher lipid proportion) had higher PO activity (β: 0.27±0.08, p=0.0009, R^2=0.07). Further, hemocyte concentration was significantly predicted by both lipid proportion and δ^2H value (Table 3.1). Monarchs with more body mass stored as fat (higher lipid proportion) had higher hemocyte concentration (β: 0.20±0.09, p=0.04); however, monarchs that flew longer distances had lower hemocyte concentration (β: 0.24±0.10, p=0.02), suggesting a mild cost of flight (Figure 3.2).

Discussion

A frequent assumption about migratory animals is that the development of tissues required for migratory flight comes at a cost to immunological resistance to infection. We investigated whether there was such a trade-off between lipids (the substance that fuels monarch flight) and two immune measures among a subset of uninfected monarchs that successfully migrated to wintering sites in central Mexico. Contrary to our expectations, we found a positive association between both immune measures and the proportion of body mass containing lipids. This result indicates that the largest and best-fueled monarchs were also in the best immunological condition. Our results also showed that monarchs that flew further distances had larger wings and stored more fat. Migration

distance was negatively associated with one measure of immunity (hemocyte concentration), indicating that reduction in immune cells may be a cost of migration.

We anticipated a trade-off (negative relationship) between fat storage and immunity, because in monarchs and other insects, lipids are shared between energetic expenditure for movement, stress responses, and immune responses (Adamo et al. 2008, Adamo 2014) and storing lipids for fuel should be prioritized during migratory phases (Rankin and Burchsted 1992). However, migrating animals may be able to maintain immune responses despite the costs of migration. First, animals undergoing migratory flight have evolved efficient flight modes and metabolic performance (Weber 2009). For example, long-distance migratory birds have highly adjustable "metabolic machinery" (Piersma et al. 1996) and efficient utilization of fatty acids in flight muscle (Jenni and Jenni-Eiermann 1998) to mitigate the costs of migration. Recent investigations of the monarch butterfly genome have discovered that migratory populations of monarchs have unique genes involved in flight metabolism and have lower flight metabolic rate than non-migratory populations (Zhan et al. 2014). As further evidence for traits that maximize flight efficiency, morphological traits such as elongated wings with lower wing loading have been documented in birds (Bowlin and Wikelski 2008) and insects (Angelo and Slansky 1984, Dudley and Srygley 2008, Satterfield and Davis 2014). Monarchs also conserve energy by taking advantage of thermal vents and using gliding/soaring flight rather than powered/flapping flight as much as possible to reduce flight costs (Gibo and Pallett 1979, Gibo 1986). During migration or other strenuous activity, animals might mitigate costs by reconfiguring rather than universally suppressing the immune system. This adaptive reconfiguration of the immune system to less energetically-costly

components has been shown in captive birds (Matson et al. 2012, Nebel et al. 2012) and crickets (Adamo et al. 2008, Adamo 2014) forced to fly moderate distances.

Flight distance often varies for individuals within migratory populations and could affect the severity of immunosuppression and physiological trade-offs. Some migrating monarchs fly from as far north as southern Canada (~4000km from overwintering sites) while others undergo a much shorter migration from the southern United States (~1500km from overwintering sites).We investigated to what extent migration distance affected immune defenses in overwintering monarchs and the relationships between immunity and fat storage. Although we did not find an interactive effect between migration distance and fat storage on immunity, we did find evidence of a mild cost of migration distance for hemocyte concentration. To our knowledge, no studies have documented immune costs of long-distance migration in free-living insects. Although flying further distances did induce a cost to cellular immune defenses in monarchs, it also allowed monarchs to acquire larger stores of fat (based on the negative relationship between lipid proportion and δ^2H values). Monarchs accrue fat during migration by foraging on nectar flowers (Brower et al. 2006, Brower et al. 2015), and although longer distance migrations are assumed to deplete energy, monarchs flying from more northerly latitudes may gain additional foraging opportunities.

Although our results did not support a trade-off between fat storage and immunity, a previous study in monarchs by Satterfield and colleagues (2013) did find a negative relationship between lipid proportion and PO activity among monarchs sampled in Georgia while actively migrating to Mexico. The difference in results between Satterfield et al (2013) and our study suggests that migrants with low fat and high

immunity or high fat and low immunity may have existed but were unsuccessful migrants (i.e. they did not survive the full journey to Mexico). Satterfield et al (2013) also took into account infection by a protozoan parasite, *Ophryocystis elektroscirrha*, and found that infection by this parasite did not modify the relationship between lipids and immunity. An experiment with captive monarchs showed that monarchs infected by the *O. elektroscirrha* parasite performed more poorly in tethered flight, suggesting a cost of parasitism for flight capability (Bradley and Altizer 2005). In the present study, we used only monarchs that were not infected by the OE parasite; consequently we cannot determine whether monarchs infected by OE or other pathogens would have been more likely to experience trade-offs between fat storage and immunity.

Like other studies (Hobson et al. 1999, Miller et al. 2011, 2012, Altizer et al. 2015, Yang et al. 2015), we drew inferences about monarch natal origins from signatures of stable hydrogen isotopes. Our use of isotopes is coarse relative to other studies, but our goals in using these data differ from previous efforts. We aimed to estimate roughly the latitude of origin as a proxy for flight distance, and our questions did not necessitate assigning each monarch to a precise region of origin. We are confident that our raw hydrogen isotope values sufficiently estimate migratory flight distance because they co-varied with monarch wing area in a way consistent with prior work showing that physically larger monarchs are capable of flying longer distances (Yang et al. 2015).

Ultimately, we conclude that monarchs that were in the best physical condition (larger bodied, greater fat storage) flew longer distances in their migration to Mexico. The subset of monarchs we sampled had successfully migrated and survived the majority of the overwintering population; this sample may represent those that were able to amass

large enough lipid stores during migration so as to avoid energy constraints and immune costs. Indeed, rather than the trade-off we expected, we observed that having more fat storage was associated with higher immune measures. This conclusion highlights the importance of maintaining ample patches of nectar flower resources throughout the migratory corridor (Alonso-Mejía et al. 1997, Brower et al. 2006, Brower et al. 2015); monarchs that obtain substantial lipid reserves could avoid immunosuppression during migration and, crucially, survive the overwintering period.

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Table 3.1: Effects of fat storage, flight distance, and sex on immune measures. Full linear models were initially structured as: immune measure ~ lipid proportion + δ^2H value + sex + lipid proportion*δ^2H value + lipid proportion*sex + δ^2H value*Sex. We report the model formula retained following model simplification by Crawley (2002), in which non-significant terms were removed until all terms in model were significant.

Retained model structure and predictor variables		Estimate ± S.E.	p-value
(A) PO activity ~ Lipid proportion			
(p=0.0009, adjusted R^2=0.07)			
	Lipid proportion	0.27 ± 0.09	0.0009
(B) Hemocyte concentration ~ Lipid proportion + δ^2H value			
(p=0.02, adjusted R^2=0.06)			
	Lipid proportion	0.20 ± 0.09	0.04
	δ^2H value	0.24 ± 0.10	0.02

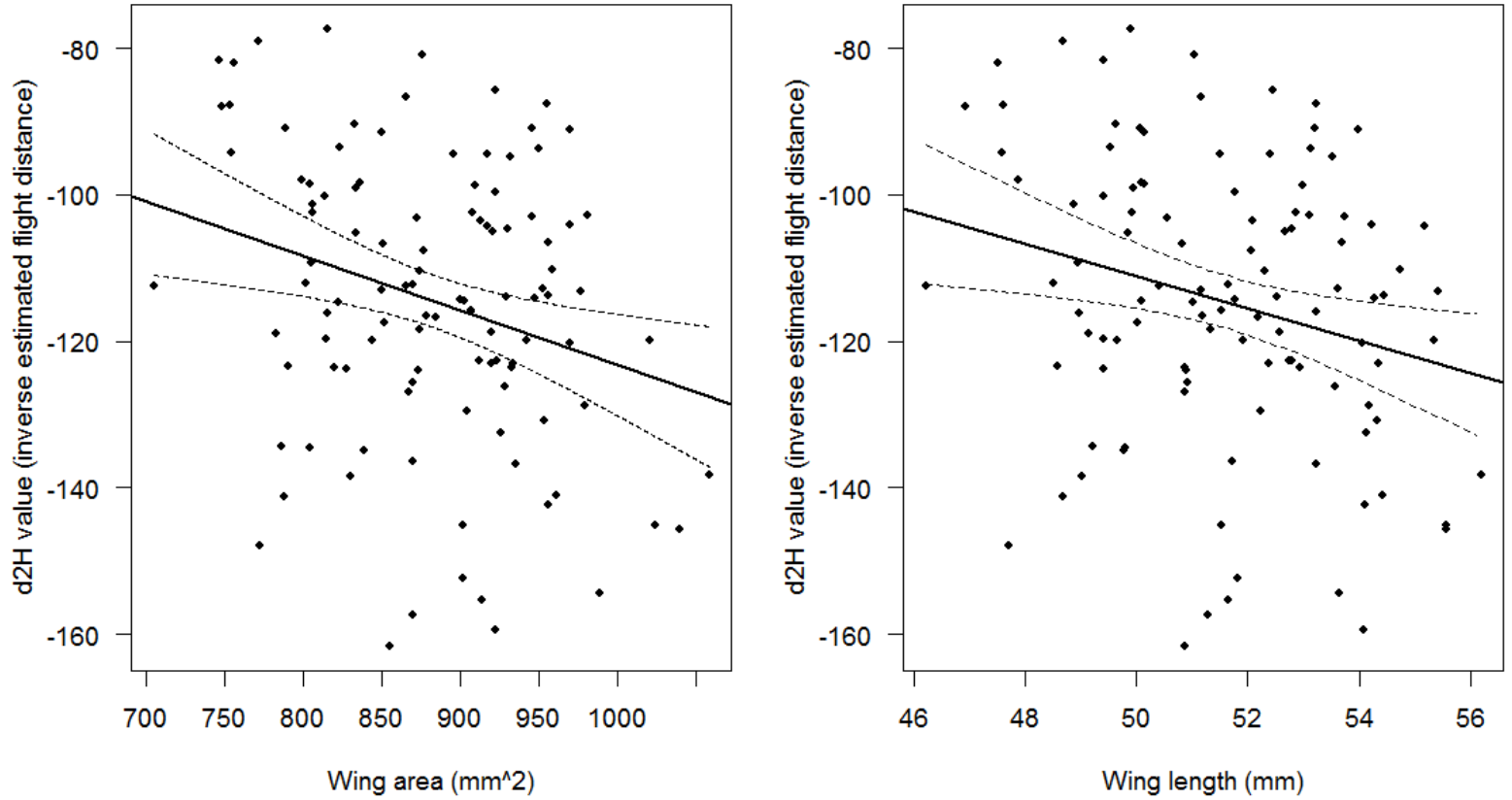

Figure 3.1. Two monarch physical measurements (wing area and wing length) are significant predictors of δ^2H values, an index of migratory flight distance. More negative δ^2H values correspond to monarchs that flew to Mexico from more northerly latitudes. Monarchs with larger wing area (β: -0.28±0.09, p=0.003; Panel A) and wing length (β: -0.25±0.09, p=0.008; Panel B) flew further distances. Lines show predictions from the univariate linear regression models and bands represent 95% confidence intervals.

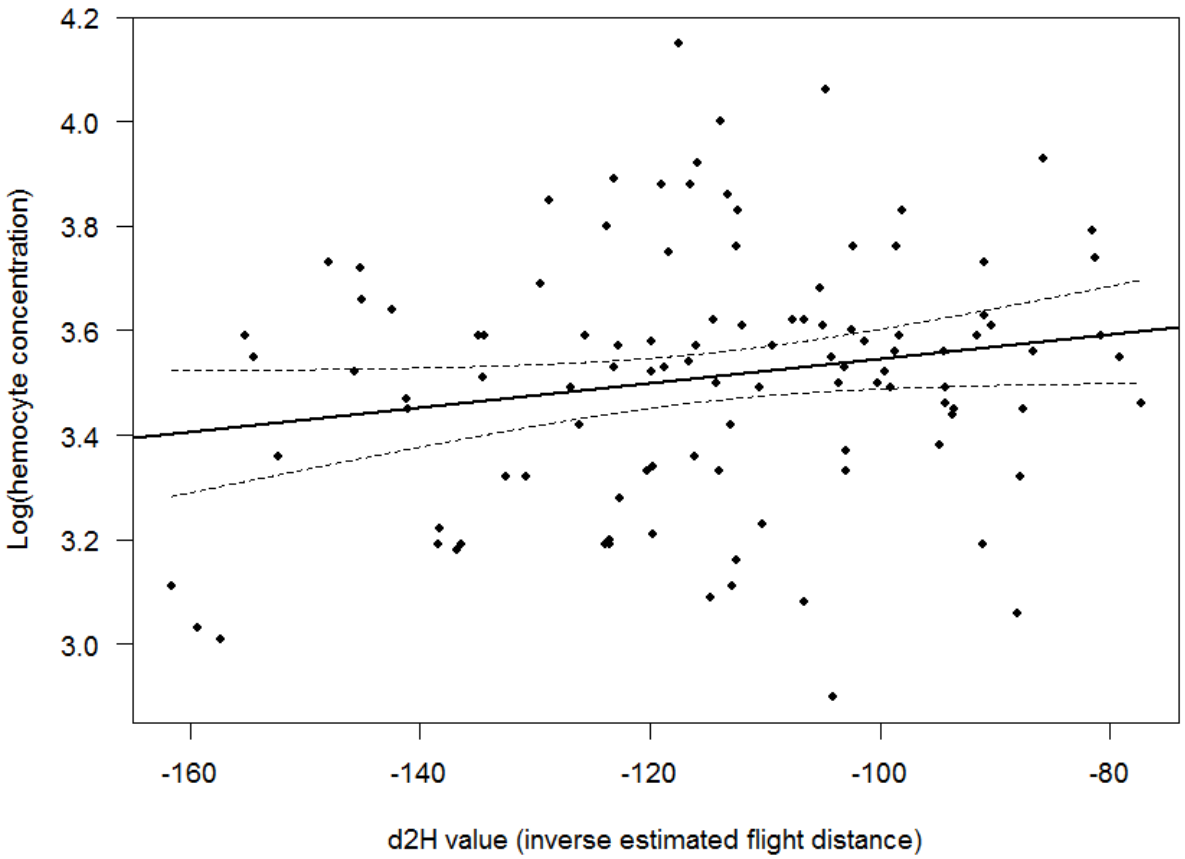

Figure 3.2. **Longer migration distance (more negative δ^2H value) is associated with lower concentrations of hemocytes (β: 0.24±0.10, p=0.02).** The line shows the prediction from the linear regression model (hemocyte concentration ~ lipid proportion + δ^2H value) and band represents 95% confidence interval.

CHAPTER 4

BOTH REPRODUCTION AND MATING ACTIVITY SUPPRESS IMMUNITY IN MONARCH BUTTERFLIES

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Abstract

Because reproduction and immune defense are both energetically costly activities, trade-offs between the two are common. While the trade-off between reproduction and investment in immune defense has been well documented, few studies have tested immune costs at different stages of reproduction, e.g., pre- versus post-copulatory periods. Using the experimentally-tractable monarch butterfly (*Danaus plexippus*) system, we manipulated reproductive tissue development (via larval rearing conditions and adult hormonal manipulation with juvenile hormone) and subsequent mating activity (via treatment groups varying in access to mating opportunities) to identify whether reproductive maturity alone results in immunosuppression, or whether animals must actively reproduce to experience immune costs. We found that monarchs treated with a juvenile hormone analog (methoprene) which initiates reproductive development had reduced measures of hemocyte concentration and phenoloxidase activity (from pre- to post-treatment sampling time points) relative to control-treated monarchs. Further, phenoloxidase was lowest in male monarchs that—when provided access to females—attempted mating but did not actually mate, suggesting a cost of strenuous courtship contests even without actual copulation. In females, a higher frequency of mating corresponded to a greater loss in hemocytes (immune cells) between sampling time points, but this loss of immunity was unrelated to the number of mature eggs developed, counter to expectations. Collectively, our results suggest an immunosuppressive effect both of reproductive tissue development and aspects of courtship and mating. In the context of monarch natural history, these costs of reproduction for immunity could increase susceptibility of summer breeding or spring re-colonizing monarchs to parasites.

Introduction

Associations between male secondary sexual traits, parasites, and immune responsiveness have provided a strong foundation for investigating trade-offs between reproduction and immunity, and resulting measures of individual fitness (Hamilton and Zuk 1982, Folstad and Karter 1992). Males with more elaborate sexual ornaments typically have lower burdens of parasites (either because they have acquired substantial resources and/or mounted large immune defenses), a tendency often attributed to an underlying physiological trade-off between immune defenses and reproduction-related energy allocation (Zera and Harshman 2001, Harshman and Zera 2007). A large body of subsequent work in diverse taxa has shown that when immune defense is experimentally up-regulated, reproduction often decreases (Reaney and Knell 2010, McNamara et al. 2013, Reavey et al. 2014). Reciprocally, immunity is typically lowest when reproductive investment is high, such as during courtship in a number of species (Kerr et al. 2010), after copulation in damselflies (Siva-Jothy et al. 1998), during pregnancy in mammals (Weetman 2010), or while incubating eggs and rearing offspring in birds (Berzins et al. 2011). While several aspects of reproduction can be costly for immune defense, few studies have addressed the relative immune costs of reproductive development versus actively engaging in reproduction (i.e. mating.) by manipulating multiple aspects of reproduction within a study.

Sex hormones are considered to be key mediators of trade-offs between reproductive development and immunity (Folstad and Karter 1992, Klein 2004). Most of the work on this topic, to date, has come from vertebrate systems. For example, experimental manipulations have shown that increasing testosterone levels suppresses

immune functions like wound repair, and the effects are exacerbated when animals are nutritionally limited (French et al. 2007a). A meta-analysis across multiple vertebrate species showed relatively consistent support for the negative effects of sex hormone manipulation, specifically testosterone, on immune function in males (Foo et al. 2016). However, less is known about the hormonal underpinnings of changes in immunity in other taxa. In insects, but there is evidence that increases in juvenile hormone (JH) associated with reproductive maturity can suppress immunity (Lawniczak et al. 2007, Schwenke et al. 2016). Rolff and Siva-Jothy (2002) demonstrated in flour beetles that mating behavior increased JH and subsequently reduced a key invertebrate immune response, phenoloxidase activity. Further, increased JH in mealworm beetles resulted in heightened male attractiveness to females but reduced phenoloxidase activity and encapsulation of foreign particles (Rantala et al. 2003). While some of these studies manipulated JH by removing the brain region (the corpora allata) that synthesizes the hormone, an alternative approach of using methoprene, a synthetic JH analog, has been useful in many systems. Treatment of damselflies with methoprene has been found to mediate the trade-off between phenoloxidase activity and male aggression (Contreras-Garduño et al. 2009), increase sexual traits at the expense of somatic maintenance (Contreras-Garduño et al. 2010), and reduce survival of bacterial infection (González-Tokman et al. 2012). These studies suggest a central role of the insect juvenile hormone in mediating a physiological trade-off between reproduction and immunity in insects analogous to the role that testosterone plays in vertebrates.

After reproductive maturity has been reached, many aspects of the breeding process—from courting mates to copulating, laying eggs, and caring for young—are

potentially energetically costly and likely to reduce immune defenses. In females, these costs could result from the allocation of limited energy (or specific micro- or macro-nutrients) away from immune defense in favor of synthesizing eggs (Lochmiller and Deerenberg 2000, Lee 2006, Boggs 2009, Stahlschmidt et al. 2013). In males, the physical challenge (and energy expenditure) of courtship and copulation have been found to elevate metabolic rates (van Dijk and Matson, in review; and references therein), with downstream consequences for immune investment. The costs to immunity are potentially even higher in systems in which mating entails significant aggression—either when males compete actively for mates or when females resist males' advances (Leman et al. 2009). Finally, an energetic cost unique to males in some insect species is the generation of nitrogen-rich bundles of sperm called spermatophores that males transfer to the female during copulation (Barbosa et al. 2016).

In a recent review of reproduction-immunity trade-offs in insects, Schwenke and colleagues (2016) highlight that the field is still lacking a synthetic understanding of which specific components of immunity are suppressed by reproduction and which aspects of reproduction (i.e., pre- or post-copulatory phases) are more likely to suppress immunity. The monarch butterfly (*Danaus plexippus*) is an experimentally tractable system amenable to testing the distinct effects of reproductive development versus mating activity on immune defenses. In the well-studied North American migratory population, the monarchs' annual cycle consists of four phases (fall migration, overwintering, spring re-colonization, and summer breeding). Monarchs that experience cool night temperatures and decreasing day lengths as larvae (in late summer and fall) have diminished levels of juvenile hormone III, which causes a physiological state called

reproductive diapause (Herman and Tatar 2001). Rather than reproducing shortly after eclosion, these adults have severely reduced, immature reproductive organs and delay reproduction for up to 8 months, during which time they migrate up to 4000km to overwinter in central Mexico. In contrast, monarchs that emerge during mid-summer reproduce immediately, are thought to breed in a narrow geographic area, and generally live only a few weeks as adults. Evidence from differential gene expression studies comparing fall migratory monarchs either control-treated or treated with methoprene – a JH analog – showed that JH treatment was associated with down-regulation of genes related to innate immunity (Zhu et al. 2009). This work suggests that wild monarchs' immune defenses might be lowest during summer when JH levels (and reproductive investment) are high.

The two hallmark aspects of monarch reproductive biology—a hormonally-driven reproductive development dichotomy (diapause) and a competitive mating system—are both amenable to experimental manipulation. This allows us to uniquely test the relative contributions of hormonal reproductive development versus active mating to changes in immune function. We compared two measures of immunity in monarchs in reproductive diapause to reproductively active adults either kept as virgins or allowed to mate and develop eggs. We were able to disentangle effects of larval rearing environment, hormonal underpinnings of reproduction, and several nuanced aspects of mating behavior on immune measures, with implications for understanding of seasonal changes in parasite resistance.

Methods

Larval rearing conditions

Our experiments used individuals derived from several generations of lab rearing, with original monarchs captured from Savannah, Georgia and Saint Marks, Florida. We generated three distinct outcrossed family lines, and reared larvae (initial N=320) singly in 0.5L plastic containers with mesh screen lids. We fed larvae daily with fresh cuttings of greenhouse-raised swamp milkweed (*Asclepias incarnata*).

We used several manipulations to cross reproductive development with mating activity (Appendix B, Figure B1). We raised approximately half (N=140) of the monarch larvae in summer-like conditions to promote reproductive development, and half (N=180) in fall-like conditions to promote reproductive diapause. The summer conditions were 28°C daytime and 26°C nighttime temperature with a 16h daylight period from 0500 to 2100h. The fall conditions were 24°C daytime and 18°C nighttime temperature; the photoperiod began with 14h daylight (from 0630 to 2030h) but was reduced by 5 min per day to a final day length of 10h. Because monarch development depends strongly on temperature, monarchs in cooler temperatures (fall conditions) require more days to reach certain developmental stages (Zalucki 1982). In an effort to sample immunity, body mass, oocyte counts, etc. at similar developmental stages in summer- and fall-reared monarchs, we adjusted all experimental time points to occur at equivalent degree days for summer and fall monarchs (Appendix B, Figure B1).

Experimental manipulation of reproductive development and mating

Upon adult emergence, we treated half of the fall-reared monarchs with topically-applied methoprene (a JH analog) to initiate reproductive development. To do so, we

diluted methoprene in acetone to a concentration of 40µg/1µL, and applied 5µL of this solution to the monarchs' abdomen where it quickly absorbed into the cuticle. We treated the remaining half of individuals with a control acetone vehicle to remain non-reproductive.

We subsequently divided each of the three hormone treatment groups (summer naturally reproductive, fall artificially reproductive, and fall non-reproductive) into adult mating treatment groups (Appendix B, Figure B1). Monarchs in the "unmated and immobilized" treatment group were housed in a glassine envelope in the incubator of the original larval rearing conditions, and thus were not provided an opportunity to mate. Monarchs in the "access to mates" treatment group were housed in mesh cages (located inside the incubators they were reared as larvae) containing members of the opposite sex and allowed freely to mate. A subset of females, in the "access to mates and milkweed" treatment group, were housed in a cage with males and also with cut stalks of the milkweed hostplant (*Asclepias incarnata*), which also provided opportunity to oviposit following mating. For all monarchs in mating cages, we observed mating pairs nightly (at approximately 1900 h) following peak daily mating activity; once paired, monarchs can engage in prolonged copulation for up to a full day (Svard and Wiklund 1988). Importantly, not all monarchs were observed to mate during their time in the mating cage; thus, we considered a fourth category— "access to mates but unmated" —in the mating treatment variable.

Monarchs were "exposed" to the mating treatments for 5 days in summer conditions and 9 days in fall conditions (accounting for developmental degree days). Because individuals can only mate approximately once per day, we calculated mating

frequency (number of nights mated divided by number of days in mating cage) as a measure of continuous mating effort. In both mated and unmated groups, we dissected females at the end of the study (at 11 days in summer conditions and 19 days in fall conditions) to quantify oocytes (eggs). A small subset of females were dissected at an early time point (after JH treatments but before mating treatments) to assess the success of diapause induction in the fall-like rearing conditions.

Immune defense sampling regime

To assess the effects of reproductive development and mating activity on immunity, we assayed two components of immune defenses at two time points for each individual. The first measurement was obtained one day (in summer monarchs) or two days (in fall monarchs) post eclosion; this baseline measure reflects differences in immunity based only on reproductive development caused naturally by environmental conditions. However, this measure may be confounded by the effect of temperature on immune defenses (Karl et al. 2011, Murdock et al. 2012), so a second immune sample was taken following hormonal manipulation of reproductive development. The second sample was taken for most monarchs following both the JH (or control) treatment and mating treatments (at either 10 days post-eclosion in summer conditions or 17 days post-eclosion in fall conditions). This sample should cumulatively reflect differences in immunity based on both reproductive development and mating activity.

A small subset of females were sampled for the second measure of immunity (and subsequently dissected) at an earlier time point—following JH treatment but prior to mating treatments (Appendix B, Figure B1). The comparison of immune defenses between fall-reared monarchs that received JH or control treatments should reflect

differences in hormonal reproductive development without the confound of temperature that arises when comparing the baseline immune measures between summer and fall-reared monarchs.

We collected hemolymph non-lethally from adults by puncturing the cuticle of an intersegmental vein on the dorsal side of the abdomen. We quantified two immune pathways using two well-described assays. First, we conducted hemocyte counts to quantify the concentration of immune cells in the blood. Hemocytes have various functions including phagocytosis, encapsulation, and production of humoral immune effector molecules such as antimicrobial peptides (Lavine and Strand 2002, Strand 2008). Second, we measured the propensity of monarch blood to melanize in response to a bacterial elicitor, an immune response called phenoloxidase activity (hereafter PO activity). PO activity involves the production of melanin pigment which is deposited onto foreign bodies to suppress growth (Söderhäll and Cerenius 1998). Details on the protocols for measurement of hemocyte concentration and PO activity and sample sizes obtained for each measure are given in supplementary material (Appendix B). In some cases time constraints prevented us from completing immune assays for some individuals; in these cases, we pricked monarchs to consistently apply the stress of bleeding and loss of hemolymph across all monarchs.

Statistical analyses & predictions

We removed from analyses 18 monarchs that were reared in diapause-inducing (fall) conditions but were found to be reproductive after mating in the mating cage or possessing mature eggs upon dissection. This fraction of unexpectedly reproductive individuals is consistent with prior studies showing that diapause induction is rarely

100% successful (Goehring and Oberhauser 2002). We also removed 4 female monarchs from analyses that were reared in summer conditions but upon dissection had zero mature or immature eggs.

We sampled blood for immune assays at two time points for most individuals; in the majority of cases, both hemocyte concentration and phenoloxidase activity were lower at the second time point. The reduction in immune measure could be attributed to loss of hemolymph between time points, but it also importantly reflects resilience of immunity to the onset of reproductive development or mating activity and is thus a critical variable for this study (Boughton et al. 2011). For PO activity, this calculated response variable (called change in PO activity) is the PO activity at the second assay timepoint minus the PO activity at the first assay timepoint; for hemocyte concentration, this variable (called change in hemocyte concentration) is the average number of hemocytes at the second assay timepoint minus the average number of hemocytes at the second assay timepoint.

We used a series of general linear models to explore the effects of reproductive development, mating activity, and continuous measures of reproductive investment on immune defenses. Initial exploratory analyses did not reveal that any interaction terms were important in explaining variation in immune measures, so final models included only main effects of the primary reproduction-related predictor variables (JH treatment, mating treatment, or continuous measures of mating effort) and additional fixed effects described below. All models were implemented in R version 3.2.2 (R Core Development Team 2015).

Does development of reproductive tissue affect immunity?

First, we tested whether rearing conditions—summer or fall—affected baseline immune measures by modeling either hemocyte concentration or PO activity at the first sampling time point as a function of rearing conditions, genetic lineage, sex, and body mass (at 1 day post-eclosion). We predicted that baseline immune measures would be lower in Summer than in Fall monarchs.

Second, we tested if JH treatment (artificial reproductive development) affected immune measures. We restricted this analysis to only the subset of monarchs: i) that were reared in fall-like conditions and ii) that were assigned to the "unmated and immobilized" mating treatment group that did not have access to mating opportunities. These linear models included JH-treatment, genetic lineage, sex, and body mass (at 1 day post-eclosion) as predictors of either the change in hemocyte concentration or change in PO activity. We predicted that reductions in immune measures would be greater in JH-treated than control (untreated) monarchs.

Does mating activity affect immunity?

We next explored whether mating activity affected immune measures using the categorical mating treatment predictor variable which included either three or four levels depending on sex: unmated and immobilized (both sexes), access to mates but unmated (both sexes), access to mates (both sexes), and access to mates and milkweed (females only). Because this predictor variable had different levels for males and females, we conducted separate models for each sex. The change in either hemocyte concentration or PO activity was modeled as a function of mating group, genetic lineage, sex, and body mass (at 1 day post-eclosion). Tukey's HSD post-hoc analysis was used to identify

differences among the treatment levels. We predicted that the change in immune measures would be greatest in monarchs that mated, or for females, mated and laid eggs than those that did not mate while in the mating cage or were not put in the mating cage.

Finally, we determined if changes in immune measures were predicted by continuous measures of mating effort. For both males and females we modeled the change in either hemocyte concentration or PO activity as a function of mating frequency (number of nights mated/number of nights in mating cage), and for females only we also tested for an effect of the number of mature eggs on immune measures. In the analyses of the effect of oocyte number on immune measures, we included an interaction with mating treatment group because egg number is known to be higher in mated females relative to virgins (Oberhauser and Hampton 1995). We predicted that higher measures of mating effort (mating frequency or number of oocytes) would correspond to greater negative change in immune measures.

Results

Does development of reproductive tissue affect immunity?

241 (75%) the monarchs initially used in the study survived to adulthood. The surviving set of adults included 109 females and 132 males, with 62 monarchs from lineage F-MI, 98 from lineage J, and 81 from lineage M. Baseline hemocyte concentration was influenced only by genetic lineage (Table 4.1) and was unaffected by larval rearing conditions (Figure 4.1A). The baseline measurement of PO activity was lower in female than male monarchs and varied across monarch genetic lineages (Table 4.2). Baseline PO activity was also significantly higher in monarchs reared in summer conditions than monarchs reared in fall conditions (Table 4.1, Figure 4.1B). Among fall-reared monarchs

that differed in their reproductive development manipulated hormonally instead of environmentally, monarchs treated with JH had a significantly greater loss in both hemocyte concentration and PO activity than untreated controls (Table 4.2, Figure 4.1C and 4.1D).

Does mating activity affect immunity?

The proportion of monarchs that mated when provided access to the opposite sex varied depending on larval rearing conditions and JH treatment. Of the 57 summer-reared monarchs that were provided access to mates in a mating cage, 46 (15 females and 21 males) mated at least one time over 5 days (Appendix B, Figure B1). Of the 41 fall-reared and JH-treated monarchs that were provided access to mates, 16 (9 females and 7 males) mated at least once. Of the 22 fall-reared and control-treated (non-reproductive) monarchs that were provided access to mates, zero mated while in the mating cage (Appendix B, Figure B1).

In male monarchs, mating treatment group significantly affected PO activity but not hemocyte concentration (Table 4.2). Post-hoc group comparisons showed that males that were provided access to mates but that did not mate had significantly greater reductions in PO activity than males that were immobilized in glassine envelopes (Tukey HSD: $p=0.02$; Figure 4.2). In females however, there was no difference across mating treatment groups for either hemocyte concentration or PO activity (Table 4.2).

Female monarchs that mated more frequently (higher number of mates per number of days in the mating cage) showed a greater reduction in hemocytes (Table 4.2, Figure 4.3). There was no relationship between mating frequency and the change in PO activity in females or between mating frequency and either immune measure in males

(Table 4.2). Further, there was no relationship between the number of mature eggs and either immune measure in females; this relationship was not modified by whether or not the females were mated or virgins (Table 4.2).

Discussion

This experiment accomplished what is rarely attempted in investigations of immune costs of reproduction: manipulating multiple aspects of reproduction to disentangle the source of immune costs. Our results suggest that both aspects of reproduction reduced two measures of immune defense in monarchs. Evidence that reproductive development carried immune costs came from the result that monarchs reared in fall conditions (emerging as adults in a non-reproductive state) showed strong reductions in both measures of immune defenses when they were treated with a juvenile hormone analog (methoprene) that restored reproductive tissue development. Mating activity also reduced immune defenses, but in more complex ways. In male monarchs, being given access to females but not actively mating resulted in greater losses of the melanization immune response (PO activity) than both mating and remaining immobilized without access to mates. In female monarchs, mating at a higher frequency (more nights mated while in mating cage) corresponded to a greater loss of cellular immune defenses (hemocytes concentration), but changes in immunity were not associated with the amount of eggs developed. As a whole, this experiment shows immune costs of multiple aspects of reproduction in the monarch butterfly.

One aspect of our results that did not strongly support a cost of reproductive development for immunity was that the baseline measure of PO activity was higher in reproductively mature monarchs reared in summer conditions than in fall-reared

monarchs. However, we interpret this finding cautiously because invertebrate immune responses can depend on temperature (Karl et al. 2011, Catalán et al. 2012, Murdock et al. 2012). Although we aimed to avoid the temperature effects by further manipulating hormonal reproductive development in fall-reared monarchs, there may be insight in considering whether free-living monarchs during summer generations do mount higher levels of immunity compared to free-living monarchs in the fall. If so, this temperature-related increase in immunity would fortunately coincide with the time of heightened parasitoid and parasite risk for monarchs (Altizer and de Roode 2015). Field sampling of monarchs across their seasonal generations (summer breeding, fall migrating, and spring re-colonizing) could elucidate broad patterns of immunity in wild monarchs.

A secondary goal of this study was to understand when monarchs should be most vulnerable to infections across their annual migratory cycle. Making the distinction between reproductive development and active mating is important because the defenses of fall migratory monarchs could be higher because of atrophied reproductive organs, or because adults are not actively investing materials and energy towards breeding (Oberhauser and Hampton 1995, Brower et al. 2007). In adult females, JH initiates the production of vitellogenin, a yolk protein in eggs (Pan and Wyatt 1971, Pamminger et al. 2016); in our study, exposure to methoprene initiated egg development and came at a cost to two measures of immunity. Interestingly, we did not find that the number of mature eggs was associated with immune defenses, contrary to our predictions of a trade-off. In essence, this suggests that the negative effect of egg development is binary rather than continuous. Interpreting this result in the light of monarch natural history suggests that females could be equally vulnerable to immunosuppression in the spring re-colonizing

and summer breeding generations, as both are intervals of reproductive maturity. Spring re-colonizing monarchs are reproductively mature (having emerged from diapause before the onset of the return journey from Mexico) but likely mate less frequently as they undergo substantial movement to recolonize the breeding range; conversely, summer breeding monarchs mate and lay eggs almost continuously during short breeding lifespans. Our results suggest that male monarchs may have especially low immune defenses when they undergo active mating (even when matings are attempted but unsuccessful), suggesting elevated parasite susceptibility in the summer breeding generation relative to fall migratory and spring re-colonizing generations.

The majority of studies investigating immune costs of reproduction measure or manipulate a single – usually a post-copulatory – component of reproduction (Honkavaara et al. 2009, Kerr et al. 2010, Nava-Sánchez et al. 2015, Barbosa et al. 2016). Yet critical differences in reproductive investment occur prior to the onset of mating activity, when hormones mediate the development of reproductive tissue (Klein 2004, Lawniczak et al. 2007). The effects of sex hormones on immunity prior to the onset of mating have been thoroughly explored in vertebrates (Roberts et al. 2004, Martin et al. 2008, Foo et al. 2016), but far fewer studies have investigated these effects in insects. A notable study by Cotter and colleagues (2008) in dung beetles investigated changes in phenoloxidase activity throughout the critical larval period in which changes in JH titers lead to either major or minor male morphs; this pre-copulatory (JH-mediated) difference in sexual development corresponded to differences in levels of phenoloxidase activity, with higher immunity in higher-quality (major) males (Cotter et al. 2008). Typically, effects of vertebrate sex hormones on immunity have been more clearly shown in males

than in females (Foo et al. 2016), potentially because testosterone has a more direct antagonistic effect on immune molecules, whereas reproductive development in females results in more general shifts in allocation of resources. With just one hormone (JH) driving reproductive development in monarchs and other insects, it remains unclear whether sex differences in the costs of reproductive development are to be expected (Nunn et al. 2009). A recent review paper by Schwenke and colleagues (2015) described the effects of mating activity on immunosuppression reported across female insects, and suggests that the effects of JH on immunity should, if anything, be stronger in females because of its clear physiological link to oogenesis.

Our results suggest that mating attempts during courtship are a nuanced source of immune costs warranting further investigation, especially in systems with aggressive mating behavior (Leman et al. 2009). When reproductively mature, monarchs have a coercive mating system wherein males force females into copulation following lengthy—and likely taxing—physical contests (Oberhauser and Frey 1999, Brower et al. 2007). In this study, males that were provided access to females but did not successfully mate had the lowest levels melanization (PO activity). These males likely undertook energetically-costly mating attempts but if their attempts were not permitted by females, they may have been poor quality, immunologically or otherwise. The males in our study that successfully mated may have achieved success in fewer mating attempts (potentially because they were perceived by females as higher-quality), enabling them to expend less energy in conquests. We were unable to observe mating attempts separately from mating successes, but this behavior is measurable in monarchs (as well as other animals) and would be a beneficial component of future studies.

Among the central predictions set in place by Folstad and Karter (1992) is that more ornamented males should have lower immune defenses—and consequently higher parasite burdens—owing to the immunosuppressive effects of testosterone. However, the link between testosterone and immune is not universally found, and in some cases higher testosterone has corresponded to higher immunity, which (Peters 2000) suggested could occur when both traits are linked to higher quality (access to more resources). In tree frogs, males with the largest body mass did not experience immune costs of testosterone supplementation, and in fact showed immunoenhancement (Desprat et al. 2015). In wolf spiders, a trade-off between parasite encapsulation and courtship rate was apparent only in unsuccessful males presumably of low quality and unable to bear the cost of mating effort (Ahtiainen et al. 2006). A recent meta-analysis (Habig and Archie 2015) showed a weak association between male social rank and immunity, but instead a strong trend that higher-ranked males had more parasites; the authors argue that this result indicates that high-quality males can "afford" the immunosuppression of testosterone, or in other words be more tolerant of parasite burdens (Habig and Archie 2015). Our results add to this accumulating evidence that costs of reproduction or mating for immunity must be considered in light of variation in individual quality.

A goal of this study was to identify whether reproductive development, active mating or both result in immunosuppression. Our findings have implications for understanding when monarchs should be most vulnerable to parasitism owing to trade-offs between reproduction and immunity. This work also demonstrates that immune costs of mating activity may occur only for low-quality males. This result is important because it shows that rather than a simple, energetic trade-off, wherein greater investment in

mating (i.e. transferring more energetically-costly spermatophores) results in greater immunosuppression, instead changes in immunity are related to a more integrated aspect of quality, the ability to compete for mates.

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Table 4.1. Results of linear models investigating effects of reproductive development on two measures of monarch immunity.

Question 1.1. Do baseline immune measures depend on rearing conditions and other predictors?
Data subset: All surviving monarchs with an initial baseline immune sample

Response variable	Terms	F_{df}	p
Baseline hemocyte concentration ~	Rearing conditions	0.65_{1}	0.43
	Lineage	9.83_{2}	<0.005
	Sex	0.44_{1}	0.51
	Mass	0.19_{1}	0.66
	Residual	$_{204}$	
Baseline PO activity ~	**Rearing conditions**	$\mathbf{5.99_{1}}$	**0.02**
	Lineage	$\mathbf{6.65_{2}}$	**0.002**
	Sex	$\mathbf{8.90_{1}}$	**0.003**
	Mass	3.43_{1}	0.07
	Residual	$_{164}$	

Question 1.1. Does the change in immune measures depend on treatment with juvenile hormone and other predictors?
Data subset: Monarchs reared in fall conditions, and either dissected at the early 2nd timepoint OR in the "mated and immobilized" mating treatment group

Response variable	Terms	F_{df}	p
Change in hemocyte concentration ~	**JH treatment**	$\mathbf{4.04_{1}}$	**0.05**
	Lineage	1.01_{2}	0.37
	Sex	0.17_{1}	0.68
	Mass	0.22_{1}	0.64
	Residual d.f.	$_{42}$	
Change in PO activity ~	**JH treatment**	$\mathbf{9.55_{1}}$	**0.007**
	Lineage	$\mathbf{4.96_{2}}$	**0.02**
	Sex	3.42_{1}	0.09
	Mass	2.26_{1}	0.15
	Residual d.f.	$_{14}$	

Table 4.2. Results of linear models investigating effects of mating activity on two measures of monarch immunity.

Question 2.1. Does the change in immune measures depend on mating treatment group and other predictors?
Data subset: All surviving monarchs with initial and final immune samples.

		♀		♂	
Response variable	**Predictors**	**F_{df}**	**p**	**F_{df}**	**p**
Change in hemocyte concentration ~	Mating group	0.81_{3}	0.49	2.45_{2}	0.09
	Lineage	$\mathbf{4.81_{2}}$	**0.01**	0.38_{2}	0.69
	Mass	0.04_{1}	0.84	0.27_{1}	0.60
	Residual d.f.	60		88	
Change in PO activity ~	Mating group	0.44_{3}	0.73	$\mathbf{4.31_{2}}$	**0.02**
	Lineage	0.46_{2}	0.64	1.06_{2}	0.36
	Mass	0.28_{1}	0.60	1.95_{1}	0.17
	Residual d.f.	39		34	

Question 2.2. Does the change in immune measures depend on continuous measures of mating?
Data subset: All monarchs that either mated while in mating cage, and/or were dissected for quantification of oocyte number.

		♀		♂	
Response variable	**Predictors**	**F_{df}**	**p**	**F_{df}**	**p**
Change in hemocyte concentration ~	Mating frequency	$\mathbf{4.35_{1,45}}$	**0.04**	$0.27_{1,52}$	0.60
Change in PO activity ~	Mating frequency	$0.08_{1,30}$	0.77	$0.26_{1,24}$	0.61
Change in hemocyte concentration ~	Oocyte number	$0.94_{1,60}$	0.34		
	Mating treatment group	$0.37_{3,60}$	0.77		
	Interaction	$1.12_{3,60}$	0.35		
Change in PO activity ~	Oocyte number	$0.17_{1,39}$	0.68		
	Mating treatment group	$0.55_{3,39}$	0.65		
	Interaction	$0.97_{3,39}$	0.42		

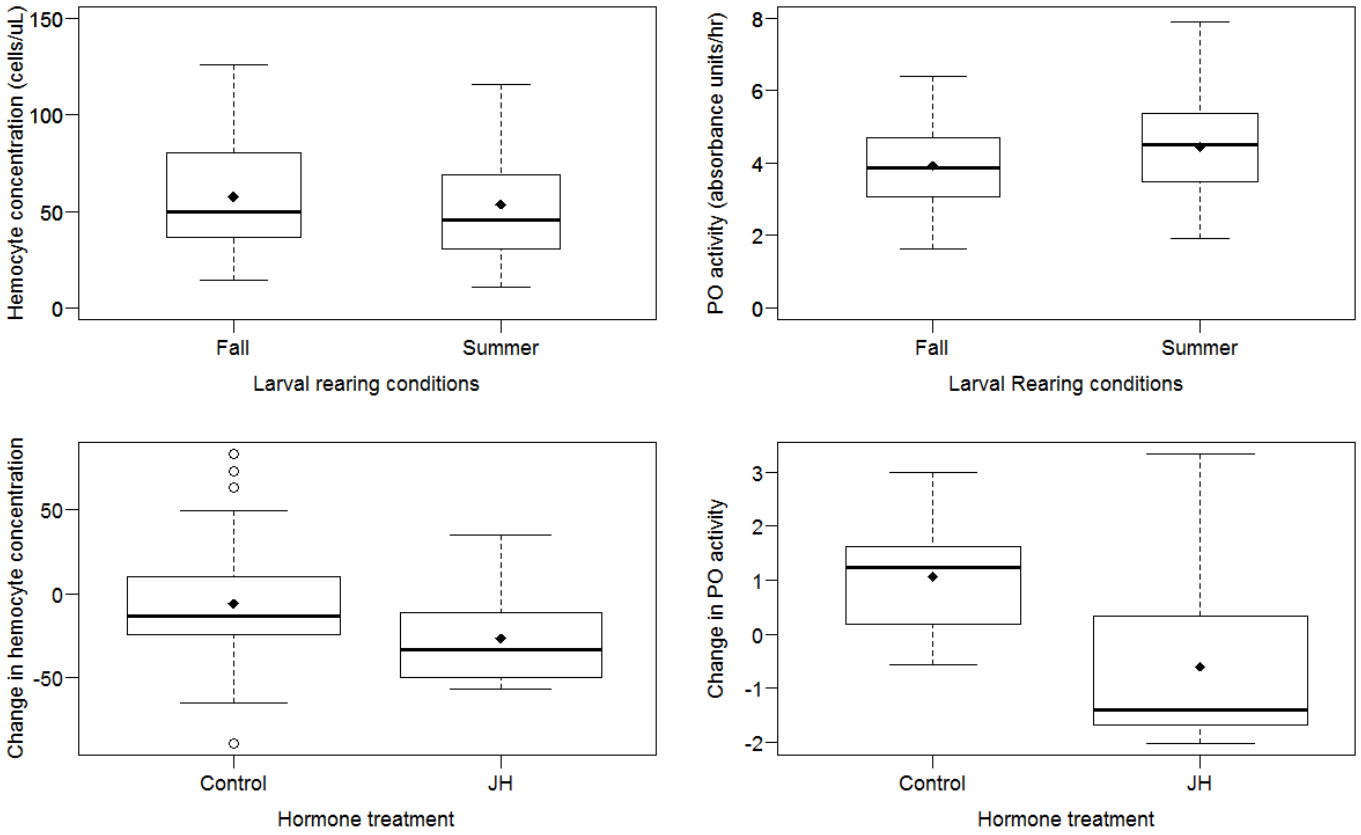

Figure 4.1. Baseline levels of PO activity (B) were higher in summer-reared than in fall-reared monarchs, but hemocyte concentration (A) did not differ by larval rearing conditions. Monarchs reared in fall conditions and subsequently treated with methoprene (a JH analog) have significantly lower measures of (C) hemocyte concentration and (D) phenoloxidase activity compared to monarchs that were control-treated and remained in reproductive diapause. Center lines show medians and black diamonds show means; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range, and outliers are represented by dots.

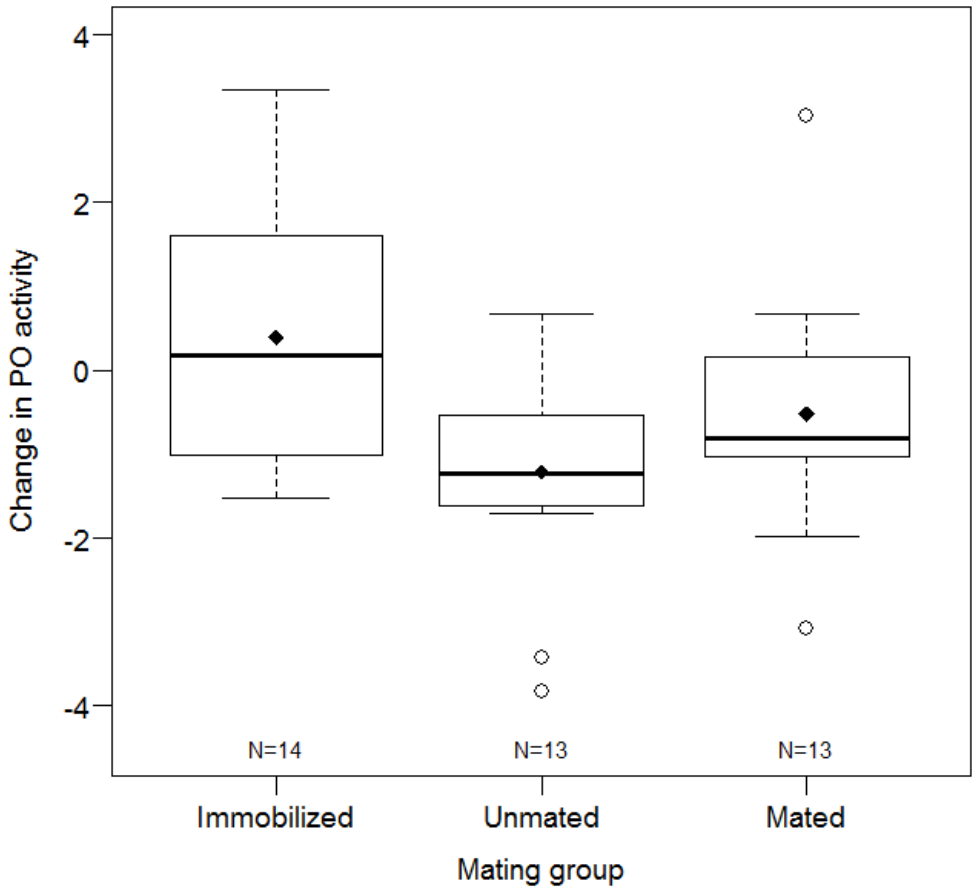

Figure 4.2. The degree to which male monarchs engaged in mating activity affected the change in PO activity from the first to the second timepoint. Males that were in the mating cage but remained unmated lost significantly greater PO activity than monarchs that remained immobilized in glassine envelopes, and than males that mated when provided access to mates. Center lines show medians and black diamonds show means; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range, and outliers are represented by dots.

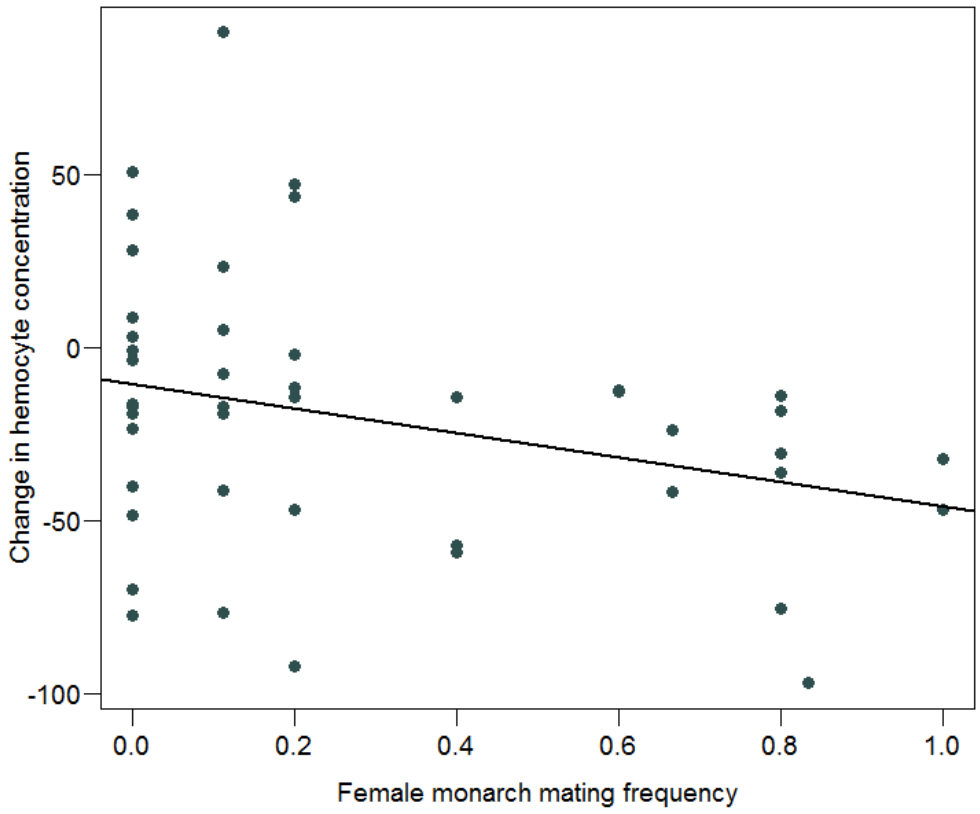

Figure 4.3. Female monarchs that mated more times over the period they were in the mating cage with males (higher mating frequency) had greater reductions in hemocyte concentration. (Linear model: Estimate -35.15 ± 16.8 s.e., $p = 0.04$, $r^2 = 0.09$).

CHAPTER 5

UNRAVELLING THE COSTS OF FLIGHT FOR IMMUNE DEFENSES IN THE MIGRATORY MONARCH BUTTERFLY

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Abstract

Migratory animals undergo extreme physiological changes to prepare for and to sustain energetically costly movements; one potential change is reduced investment in immune defenses. However, because some migrants have evolved to minimize the energetic demands of movement (for example, through the temporary atrophy of non-essential organs such as those involved in reproduction), migratory animals could potentially avoid immunosuppression during long-distance journeys. In this study, we used a tethered flight mill to examine immune consequences of experimentally induced powered flight in eastern North American monarch butterflies. These butterflies undergo an annual two-way long-distance migration each year from as far north as Canada to wintering sites in Central Mexico. We quantified immune measures as a function of categorical flight treatment (flown versus control groups) and continuous measures of flight effort (e.g. flight distance, duration, and other measures of efficiency). We also examined whether relationships between flight and immune measures depended on reproductive investment by experimentally controlling whether monarchs were reproductive or in a pre-migratory state of reproductive diapause (having atrophied reproductive organs) prior to flight. Of the three immune responses we measured, hemocyte concentration was lower in flown monarchs relative to controls but increased with flight distance among flown monarchs; the other two immune measures showed no relationship to monarch flight. We also found that monarchs that were reproductively active were less efficient fliers, as they exerted more power during flight than monarchs in the migratory condition of reproductive diapause. However, reproductive status did not modify relationships between flight and immune responses. Results of this study add to a growing body of work suggesting that

migratory monarchs – like some other animals that travel vast distances – can complete their journeys with efficient use of resources and minimal costs.

Introduction

Animals that walk, fly, or swim long distances can expend massive amounts of energy (reviewed by Bonte et. al. 2012; Matson and VanDijk 2016). In some cases, investment in energetically costly movements can come at the cost of defense against pathogens (Buehler et al. 2010, Altizer et al. 2011). Several observational studies have documented reduced immune defense before or during migratory intervals (Owen and Moore 2006, Owen and Moore 2008a, Owen and Moore 2008b), although it is important to note that other variables such as temperature, age and access to food resources can affect immune defense in wild animals. Experimental approaches have been successful in examining the effects of forced movement while controlling for other variables that further affect immunity in the wild (Matson et al. 2012, Nebel et al. 2012). Although migratory birds have been a primary focus of such work, Chapman and colleagues (2015) recently highlighted the potential for tethered flight studies with experimentally-tractable migratory insects to unravel the link between migratory effort and pathogen defense.

Costs of migratory movement for immune defense might depend on whether or not animals are in reproductive condition during migration (Rankin and Burchsted 1992, Buehler and Piersma 2008). Migrating animals can vary in the extent to which reproduction and migration are synchronous (Dingle 2006, Ramenofsky and Wingfield 2006, Ramenofsky and Wingfield 2007). Some birds, for example, atrophy reproductive organs (and other organ systems) when they enter a pre-migratory state (Bauchinger et al. 2007, Vézina and Salvante 2010, Vézina et al. 2012). Similarly, migratory or dispersing

insects can undergo a phenomenon called the "oogenesis flight syndrome" wherein the reproductive system shuts down at the onset of a flight or movement interval (Lorenz 2007, Guerra 2011). Because reproduction is known to be costly and can trade-off with immune defense (Schwenke et al. 2016), being reproductively mature during migration could intensify the costs of movement for immunity, making the decoupling of reproductive and migratory intervals an adaptive strategy (Rankin and Burchsted 1992, Buehler et al. 2010).

In this study, we examined whether active flight lowers immune defense in a migratory butterfly and further asked whether reproductive maturity compounds the cost of strenuous activity. We focused on monarch butterflies (*Danaus plexippus*) in eastern North America that undergo an annual long-distance migration traveling up to 3500 km each fall from Canada and the US to wintering sites in Central Mexico. In the fall, monarch adults emerge in a pre-migratory state called reproductive diapause, thought to be a necessary precursor to the long-distance migration (Herman 1973, Herman 1981). This state can be experimentally induced by exposing monarch larvae to cooling temperatures and decreasing day length, mimicking fall conditions (Goehring and Oberhauser 2002). Adult monarchs in reproductive diapause have atrophied reproductive organs and excess stores of lipids needed to survive the overwintering period (Brower et al. 2006). In the early spring, monarchs emerge from reproductive diapause and initiate the reverse migration that re-colonizes the southern part of their breeding range. Two to three successive generations of reproductively active monarchs fly substantial distances northward during this re-colonization (Malcolm et al. 1993, Miller et al. 2012), and potentially incur simultaneous costs of reproduction and flight on immune defenses.

Monarchs use both soaring and powered flight during migration; in soaring flight, monarchs gain altitude on thermal vents and coast long distances (Gibo and Pallett 1979, Gibo 1986) while in powered flight monarchs must continually flap their wings. Gibo and McCurdy (1993) estimated that a monarch would deplete a 140mg fat supply in 1060 hours of soaring flight versus only 11 hours of powered flight. Monarchs would be unlikely to survive the migration by relying on powered flight alone, but sustained flapping is a necessary component of migration under low-wind conditions or when facing adverse weather. Thus, powered flight is crucial to monarch migration and is likely to be energetically costly relative to gliding (Gibo and McCurdy 1993).

To test whether migrating monarchs experience lower immune defense as a result of energetically expensive flight, we induced powered flight by flying monarchs on a tethered flight apparatus over consecutive days and measured subsequent changes in immune defenses. We tested the effects of forced flight on immunity in both reproductive and non-reproductive (diapause) monarchs to explore whether reproductive maturity compounds the costs of strenuous activity. We predicted that monarchs that were both reproductively active and forced to fly (compared to immobilized controls) would mount the lowest immune responses, and that immune defense would decrease with continuous measures of flight effort, based on a trade-off between flight-related energy expenditure and immune defense.

Methods

Monarch rearing

Five non-inbred monarch genetic lines were used in experiments; these were the grand-progeny of eastern North American migratory monarchs originally collected at St.

Marks Florida in Oct 2014 and held to overwinter in the laboratory. Mating and egg laying occurred in a naturally-lit room with ambient light from approximately 0630 to 2030 and temperatures from 26°C (average nighttime low) to 29°C (average daytime high) during June 2015 in Athens, GA, USA. Eggs were collected on stalks of greenhouse-reared *Aclepias incarnata* (swamp milkweed) and were laid in daily cohorts staggered across 14 days. At the early 2nd instar stage, monarch larvae were transferred from milkweed stalks to individual 0.5L containers with mesh screen lids and housed in controlled environmental chambers (Model I-36VL, Percival Scientific, Perry IA) for the remainder of the experiment.

Manipulation of reproductive status

In monarchs, adult reproductive diapause is triggered by environmental conditions, especially temperature, photoperiod and host plant quality, experienced at the larval stage (Goehring and Oberhauser 2002). "Fall" conditions to induce reproductive diapause in our experiment were 17°C nighttime, 23°C daytime with a decreasing photoperiod (13:11 l:d reduced by 2 minutes per 24 hours). "Summer" conditions to maintain reproductively active adults were 26°C nighttime, 28°C daytime with a constant long photoperiod (16:8 l:d). Monarchs developed at different rates under these temperature regimes (mean days from hatch to adult eclosion: 19.8 days in Summer and 37.1 days in Fall), so all of the Summer monarchs eclosed prior to the Fall monarchs. Upon emergence as adults (eclosion), males were euthanized by freezing; this study used only female monarchs because their reproductive status is easier to assess by dissection.

Manipulation of flight activity

On the first day after eclosion, monarchs were assigned to one of three flight treatment groups: Flown, Tethered Control and Unhandled Control. Monarchs in the Flown group (N=35 Summer, N=53 Fall) were forced to fly to exhaustion (or to a maximum of 60 minutes, whichever came first) on each of four consecutive days between 0900 and 1800hrs. In all cases monarchs were flown on days 4-7 post-eclosion to control for effects of age on flight and immune responses. The Tethered Control group (N=13 Summer, N=14 Fall) experienced the same handling procedures as the Flown group (application of a wire for tethered flight) but instead of forced flight they were restrained in a glassine envelope for sixty minutes. The Unhandled Control group (N=6 Summer, N=6 Fall) served as a further control for handling stress; they did not have a wire attached and remained unhandled (in glassine envelopes) for the duration of the study, except for approximately 5 minutes every day when they were manually fed 20% honey water.

We applied wires to monarchs in the Flown and Tethered Control groups on the second day after eclosion. An 8cm length of lightweight steel wire was affixed to the dorsal side of the monarch's thorax with a small piece of lab tape and rubber cement. Monarchs were weighed immediately before and after wire attachment, and the average mass of the wire and adhesive was 0.20g (range 0.09 – 0.27g, or approximately 35% of adult monarch body weight). Monarchs acclimated to the wire attachment for 48 hours in a 0.6m^2 mesh cage located inside the environmental incubator set to the same environmental conditions experienced as larvae; monarchs remained in these cages for the duration of the experiment except when flight treatments were applied. Flown and

Tethered monarchs had *ad libitum* access to 20% honey water in petri dishes in their cages.

Flight trials were conducted in two separate interior rooms (to avoid daily variation in intensity of natural light) with one flight mill apparatus per room. The flight trial rooms and tethered flight mill were configured similarly to Bradley and Altizer (2005) and are described in detail in the supplementary material (Appendix C). Briefly, the flight mill consisted of a lightweight carbon rod (120cm in length and 3mm in diameter) attached to a stand on a nearly-frictionless steel pivot. At one end of the rod, a tape "flag" passed through a photogate (interrupting an infrared beam and transmitting information to a datalogger) upon each rotation of the monarch affixed to the opposite end of the carbon rod. The datalogger and associated software (Appendix C) record the timestamp of each rotation and the instantaneous velocity (m/s) of the flag's passage through the photogate. Given the dimensions of the rod, the circumference of the monarch's circular flight path was 4.23m.

To initiate a trial, we taped the wire attached to the monarch to the carbon rod of the flight mill and released the monarch. Throughout the trial, if the monarch ceased flight for 10 consecutive seconds, the observer blew lightly on the monarch from behind (in the direction of flight) to stimulate flight. A trial was terminated when the monarch failed to resume flapping after three consecutive "blows" separated by 10 seconds of gliding. All trials that did not end by this mechanism were terminated at 60 minutes (the maximum flight time permitted by logistical constraints). If a monarch's flight was terminated in five or fewer minutes, the monarch was fed 20% honey water and was re-flown one to three hours later. In all cases of re-flight (N= 27 total trials across 21

individuals), the second trial was longer in duration than the first and was subsequently used in data analyses. One monarch was excluded from the study after having flown fewer than 5 minutes on each of the first two days of flight trials.

Flight metrics and physical covariates

For each flight trial, we calculated the duration of flight (sec), the distance flown (number of rotations * 4.23m circumference, in m), and the average speed (distance flown/flight duration, in m/s). From these data, we calculated four summary flight effort metrics to assess the cumulative impact of four days of flight on immune measures. First, we summed the total flight duration and total distance flown over four flights. Using monarch weights obtained immediately before and after each flight, we calculated the mass lost per distance flown ((pre-flight mass – post-flight mass)/distance flown), then averaged (and log-transformed) this measure across the four flights to index the monarch's ability to retain mass during flight. Finally, we coarsely estimated mechanical power as an index of energy spent over time in flight (Ellington 1991, Hedenström et al. 2001, Hasselquist et al. 2007). To calculate power, we first estimated energy expended as kinetic energy (Joules) using the formula $KE = ½ * mass(kg) * velocity^2 (m/s)$; we then divided KE by flight duration to obtain power (in Watts or J/s). Given the calculation of power $(mass * velocity^2)/(2*time)$, a high power could result from short duration but high-velocity flights or from a heavy monarch traveling at low velocity (large numerator), while a low power could result from a long-duration flight (large denominator). Power was averaged across the four flight trials.

Both flight capability and immune responses in insects can be influenced by physical factors such as body mass, wing size, and relative masses of thorax and

abdomen body segments (Srygley and Kingsolver 2000, Berwaerts et al. 2002, Berwaerts et al. 2006), which we measured and controlled for in statistical analyses. On their first day post-eclosion, we measured the length (in mm) of the monarchs' right forewing, and we weighed monarchs to quantify initial mass (in g). Following the conclusion of the study, monarchs were euthanized in a -20°C freezer for one hour and the abdomens were removed and dissected to assess reproductive status as the presence or absence of mature oocytes (Goehring and Oberhauser 2002). Dissections were performed in pre-weighed aluminum pans so that abdomen tissue could be retained for drying; the pans and their contents were oven-dried at 60°C for 72 hours and re-weighed (subtracting the original weight of the pan) to obtain the dry mass of the abdomen. We also separated, oven-dried (at 60°C for 72 hours), and weighed the thoraxes to subsequently calculate the abdomen:thorax mass ratio; larger relative thorax mass is associated with higher flight capability in butterflies (Berwaerts et al. 2002, Berwaerts et al. 2006, Saastamoinen et al. 2010).

Monarch immune responses

Approximately sixty minutes after the conclusion of the fourth and final flight (or restraint in the case of Tethered Controls), we sampled hemolymph (insect blood) by puncturing an intersegmental vein on the dorsal side of the monarch's abdomen. We measured cellular immunity (hemocyte concentration) with fresh blood and two aspects of humoral immunity (phenoloxidase and lysozyme-like activities) on aliquots of blood frozen at -80°C.

Hemocytes are invertebrate immune cells with functions including phagocytosis, encapsulation, and production of humoral immune effector molecules such as

antimicrobial peptides (Lavine and Strand 2002, Strand 2008). Under phase contrast microscopy at 400x, we counted total hemocytes (and calculated the average number of hemocytes per µl) and differentially counted each of the four cell types – granulocytes, plasmatocytes, oenocytoids, and spheroid cells – scored as a percentage out of 100 hemocytes. Granulocytes, typically the most abundant, are phagocytic; plasmatocytes aggregate to encapsulate pathogens; oenocytoids produce molecular precursors to the melanization response; spheroid cells have an unknown function in monarchs (Lavine and Strand 2002, Strand 2008).

Melanization is an invertebrate immune response through which the enzyme phenoloxidase (PO) produces melanin, a toxic compound, in response to a bacterial pathogen or elicitor (Söderhäll and Cerenius 1998). Procedural details for this immune assay are provided in supplementary material (Appendix C). We define PO activity as the slope of the kinetic curve (absorbance per hr) during the linear phase of the melanization reaction (Hall et al. 1995, Barnes and Siva-Jothy 2000).

Lysozyme-like activity is the capacity of antimicrobial peptides in hemolymph to lyse bacterial cell wall (Adamo 2004). Hemolymph samples were incubated in agar plates containing freeze-dried *M. luteus* bacteria, and we measured the diameter of the clearance zones surrounding sample wells (see Appendix C for procedural details). These diameters were calibrated against a standard curve of known concentrations of chicken egg white lysozyme, so here lysozyme-like activity is in units of estimated concentration (µg/mL).

Statistical analyses

Dissections to determine reproductive status showed that 66% of monarchs reared in Fall conditions (intended to induce reproductive diapause) had zero mature oocytes,

indicative of diapause (Goehring and Oberhauser 2002). 100% of monarchs reared in summer-like conditions had mature oocytes. Thus, in primary analyses concerning the effect of reproductive status on immunity, flight, or flight-immunity relationships, we restricted the dataset to "Fall Diapause" (fall-reared and absent mature eggs) and "Summer Reproductive" monarchs (summer-reared and present mature eggs). Results were qualitatively similar when we used the presence or absence of mature eggs, regardless of rearing conditions, as a categorical variable.

First, we used three separate one-way ANOVAs to test if reproductive status or rearing conditions affected flight effort metrics (total duration, total distance, average power, and log(average mass lost/distance flown)). We next asked if flight treatment group and reproductive status affected immune defenses using separate two-way ANOVA models for each of the three immune measures (hemocyte concentration, PO activity, and lysozyme-like activity). We modeled these immune measures as a function of flight treatment category (Unhandled control, Tethered control, Flown), reproductive status (Fall Diapause and Summer Reproductive), and the interaction between flight treatment and reproductive status. Initially, the Flown flight treatment category included all monarchs that were forced to fly; a second round of analyses restricted this comparison to monarchs that flew a sum total duration of 7200 sec across four days (approximately 30 minutes per day, or half of the maximum flight time). Tukey's HSD post-hoc tests were used to evaluate differences among treatment groups. The relative percentages of the different hemocyte types were modeled with the same predictor variables but using a generalized linear model (glm in base R) with a quasi-binomial error structure.

Our final analytical question was to investigate if, among flown monarchs, immune measures were influenced by physical covariates, continuous flight effort metrics, and interactions between flight effort and reproductive status. Initial general linear models (run separately for hemocyte concentration, PO activity, and lysozyme-like activity) included as predictors all flight effort metrics, all physical covariates (initial monarch mass, wing length, mass of the wire attachment, and abdomen:thorax mass ratio), and reproductive status both as a main effect and in interaction terms with each flight effort metric. We simplified models by progressively removing least-significant terms, beginning with the interaction terms and following with main effect terms, until all terms in the model were significant or only the intercept was remaining (Crawley 2002).

We log-transformed the immune response variables for hemocyte concentration (cells/μL) and lysozyme-like activity (estimated lysozyme concentration in μg/mL) to normalize error variance. All continuous flight metrics and physical covariates were standardized to the consistent unit of standard deviations ($y= (x – mean(x))/(2*SD(x))$) prior to inclusion as predictor variables in linear models to facilitate comparisons of estimates and effect sizes. We investigated correlations among continuous flight effort metrics with general linear models. For all models of immune response variables, we initially used linear mixed effects models in the R package lme4 (Bates, Maechler & Bolker, 2015) to test effects of random intercepts for hatch date (cohorts of eggs laid across 14 days) and monarch genetic lineage; however these random effects explained very low amounts of variance in immune measures so final models contained only fixed effects modeled with general linear models. We used R version 3.1.3 (R Core Team, 2015) for all analyses.

Results

General results

Summer Reproductive monarchs (N=53) emerged at lighter weights than Fall Diapause monarchs (i.e. Fall-reared monarchs lacking mature eggs, N=46; $F_{1,97}$=10.64, p=0.002), but did not differ in wing length ($F_{1,97}$=0.29, p=0.59). Given the presence of oocytes in the abdomen, Summer Reproductive monarchs also had higher ratios of abdomen mass to thorax mass than Fall Diapause monarchs ($F_{1,97}$=21.58, p<0.005).

Our initial analyses revealed relationships among the flight effort metrics. Total flight distance was tightly correlated with total flight duration (r^2=0.86, p<0.005). Monarchs that flew longer duration flights lost less mass per distance flown (r^2=0.16, p<0.005), highlighting that mass lost per distance represents flight efficiency needed for long duration flights. Monarchs that had higher flight power flew significantly shorter total flight distances (r^2= 0.29, p<0.005), and also lost significantly more mass per distance flown (r^2= 0.09, p=0.01). Cumulatively, these two relationships indicate that higher power corresponds to lower flight efficiency, as more energy is expended per time.

Does reproductive status predict flight effort?

Summer Reproductive monarchs flew shorter-duration flights than Fall Diapause monarchs ($F_{1,61}$=5.32, p=0.02), but did not necessarily fly shorter distances ($F_{1,61}$=2.81, p=0.10). Summer Reproductive monarchs exhibited more power during flight than Fall Diapause monarchs ($F_{1,59}$=7.76, p=0.01), even after correcting for mass (power/initial monarch mass: $F_{1,59}$=10.64, p=0.002); in other words, reproductively active monarchs used more energy per unit time than monarchs in diapause (Figure 5.1). However,

Summer Reproductive monarchs did not lose more mass per distance flown than Fall Diapause monarchs ($F_{1,61}$=0.76, p=0.39).

Do forced flight and reproductive status predict immunity?

Our results show limited evidence that flight treatment and reproductive status interacted to influence immunity (Table 5.1). PO activity did not depend on either of the treatment variables, but lysozyme-like activity was significantly lower in Summer Reproductive than Fall Diapause monarchs (Table 5.1). Hemocyte concentration was affected by both flight treatment and reproductive status (Table 5.1). Post-hoc analyses showed that hemocyte concentration was significantly lower in Fall Diapause than Summer Reproductive monarchs and in Flown monarchs compared to both control groups (Figure 5.2); and among Flown monarchs, this measure was lower in Fall Diapause than Summer Reproductive individuals (p = 0.002). There was no difference, however, in the control groups compared across reproductive status (Figure 5.2). These results were qualitatively similar when we restricted this analysis to include only the Flown monarchs that flew a total duration of approximately 30 minutes per day across the four days of flight (Appendix C, Figure C.1). We further investigated whether the reduction in hemocyte concentration in flown monarchs was driven by changes in any particular cell type. The relative percentage of granulocytes (phagocytic cells) was higher in Flown monarchs relative to Unhandled and Tethered controls, regardless of monarch reproductive status (GLM estimate ± standard error for effect of forced flight: 1.07 ± 0.29, p<0.005). The decline in granulocytes was mirrored by an increase in plasmatocytes (cells involved in the encapsulation response) in Flown monarchs relative

to Unhandled and Tethered controls (GLM estimate ± standard error for effect of forced flight: -1.17 ± 0.32, $p<0.005$).

Do flight effort and monarch physical traits predict immunity?

There was no evidence that immune measures traded-off with measures of flight effort (Table 5.2). No flight effort metric or interactions with reproductive status were retained in final models of either PO activity or lysozyme-like activity (Table 5.2). PO activity was only predicted by the mass of the wire attached to the monarch: monarchs showed higher PO activity when they had larger wires attached. Lysozyme-like activity was negatively related to the ratio of abdomen to thorax mass, with larger relative abdomens corresponding to higher lysozyme activity (Table 5.2). On the other hand, a measure of flight was retained in the hemocyte model. Hemocyte concentration was predicted by the total distance flown, reproductive status, and the mass of the wire attachment; but contrary to expectation monarchs that were reproductive and that flew further total distances had higher hemocyte concentrations (Table 5.2).

Discussion

The results of this study show that multiple components of insect immunity do not decline in monarchs forced to use powered flight in the lab environment. Although we found that hemocyte concentration was lower in flown monarchs relative to unflown controls, hemocyte concentration actually increased with the distance flown among monarchs that were forced to fly. This result could reflect a common physiological response to stress in insects, in which hemocytes are mobilized into the hemolymph (Adamo 2010). Our prediction that immunity would be lowest in monarchs both reproductively active and forced to fly was also not supported; hemocyte concentration

was lower in flown Fall Diapause monarchs than flown Summer Reproductive monarchs. Moreover, there were no instances of interactive effects between reproductive status and flight effort, further indicating that reproduction does not modulate the costs of short-term powered flight for immunity in monarchs. Despite the large expenditure of energy during long-distance movement and the assumption that this expenditure should come at the cost of immune defenses, migration-adapted animals could be resilient to the costs of flight. Monarchs indeed are emerging as a "poster child" for migratory efficiency; recent genome analyses and metabolic studies have demonstrated that monarchs from migratory populations maintain lower metabolic rates during flight than monarchs from non-migratory populations and possess unique genes (e.g. additional collagen in wing tissue) thought to streamline their flight (Zhan et al. 2014).

Other studies inducing flight in a captive environment have yielded similar results – that flight does not consistently reduce immunity. In red knots forced to fly in a wind tunnel, flown and unflown birds mounted similar levels of cell-mediated and humoral immune responses, and birds that failed to fly at all mounted the weakest immune responses, a result which the authors say indicates that poor-condition birds choose not to undertake strenuous journeys (Hasselquist et al. 2007). Similarly, western sandpipers flown in a wind tunnel experienced immune costs of flight only when the birds were previously challenged with a non-pathogenic simulated bacterial infection (Nebel et al. 2013). In fact, among healthy birds, bacterial killing ability was positively correlated with flight duration (Nebel et al. 2013), a result comparable to ours showing a positive relationship between hemocyte concentration and flight distance. Among insects, there are additional examples of animals maintaining immune defenses during strenuous

movement. In the Glanville fritillary, individuals forced to fly for 10 minutes while shaken in a jar were better able to encapsulate a foreign body compared to unflown controls, indicating that flight (or stress) may actually mobilize immune cells (Saastamoinen and Rantala 2013). During a "fight or flight" response to tethered flight in crickets, hemocytes were found to rush into the hemolymph (Adamo 2010). In our study, the only immune measure that responded to forced flight was hemocyte concentration. Hemocyte activity was lower in flown monarchs relative to controls, potentially reflecting that forced flight shifts monarchs' molecular resources (e.g. apolipoporin III, a protein utilized by both stress and immune responses in insects) from immunity towards flight-related functions such as lipid transfer (Adamo and Parsons 2006, Adamo et al. 2008). However, hemocyte concentration is known in other insects to increase with stress, as hemocytes are released from the hemopoietic organ into the hemolymph (Adamo 2010); the positive relationship in our study between hemocyte concentration and flight distance could result from this link between increasing flight stress and mobilization of hemocytes. PO activity and lysozyme-like activity are less directly coupled to stress (Adamo 2014) and had the potential to demonstrate immune costs based on energetic trade-offs, however, we found no evidence of such trade-offs in our study.

Wild, actively migrating animals make decisions about flight paths, times of departure, and speed to best conserve resources and maximize distances travelled (Meitner et al. 2004, Srygley and Dudley 2008). While we acknowledge that the short-term flights we experimentally induced in the lab do not represent an entire migratory journey, powered flight is essential to migration and controlled flight effort in captive insects can yield important knowledge of the costs of flight (Chapman et al. 2015). In

this experiment, we were able to compare monarchs in two unique reproductive and physiological conditions – Fall Diapause and Summer Reproductive – that experience very different flight demands in the wild. We believe that our experimental conditions adequately yielded "migration-condition" monarchs, evidenced by the facts that Fall Diapause monarchs were heavier than Summer Reproductive monarchs and had larger proportions of their mass in thorax (flight muscle) tissue than abdomen tissue. Although reproductive status did not affect the relationships between flight effort and immunity as we predicted, we found differences in flight metrics between Summer reproductive and Fall diapause consistent with the idea that fall migrants are adapted for more efficient flight. Summer monarchs had higher power during flight, meaning that they use more (kinetic) energy per flight time than Fall monarchs. Given the calculation of power ((½ mass * velocity^2)/time), a high power could result from short duration but high-velocity flights or from a heavy monarch traveling at low velocity (large numerator), while a low power could result from a long-duration flight (large denominator). Summer breeding monarchs are laden with eggs, have less flight muscle, and should be able to move quickly in short bouts within and among resource patches (i.e. nectar-flower gardens); whereas fall migrating monarchs should use less energy during flight to be capable of flying long distances.

Given the results of this study, we can conclude either that short-term forced flight is not costly to monarch immunity or that the flight demand we imposed was not sufficient to incur costs. Forced flights as short as 5 minutes in lab experiments with other butterflies have been sufficient to induce significant costs to lifespan and fecundity (Saastamoinen et al. 2010). Although tethered flight experiments are confined logistically

to relatively short flights, they contribute crucial information about the link between migration effort and parasite susceptibility. For example, Bradley and Altizer (2005) showed that monarchs infected by a protozoan parasite performed more poorly on the flight apparatus we used in this study. Currently, our study and others in captive birds (Hasselquist et. al. 2007, Nebel et. al. 2013) provide a growing body of evidence that immune responses are resilient to forced flight. Increasing the number and scope of these studies, as well as testing for immunosuppression in actively migrating wild animals, will inform predictions about the challenges migrants face in terms of susceptibility to pathogen infection.

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Table 5.1. Results of two-way ANOVA models investigating main and interactive effects of flight treatment category (Flown, Tethered Control, and Unhandled Control) and reproductive status (Fall Diapause versus Summer Reproductive) category on immune defense measures.

Response variable	Predictors	Mean square	df	F	p-value
(A) Phenoloxidase activity					
	Flight treatment	0.75	2	0.38	0.68
	Reproductive status	0.71	1	0.36	0.55
	Flight treatment * reproductive status	2.03	2	1.04	0.36
	Error	1.96	75		
(B) Lysozyme-like activity					
	Flight treatment	0.05	2	0.23	0.79
	Reproductive status	**2.38**	**1**	**11.10**	**<0.005**
	Flight treatment * reproductive status	0.00	2	0.01	0.99
	Error	0.21	78		
(C) Hemocyte concentration					
	Flight treatment	**0.63**	**2**	**12.86**	**<0.005**
	Reproductive status	**0.64**	**1**	**13.20**	**0.005**
	Flight treatment * reproductive status	0.10	2	1.96	0.15
	Error	0.05	93		

Table 5.2. Effects of continuous flight measures, physical covariates, and reproductive status on immune measures. Full linear models were initially structured as: immune measure ~ Avg. Power + Sum Distance + log(Avg. Mass Lost) + Sum Duration + Reproductive Status + Avg. Power * Reproductive Status + Sum Distance * Reproductive Status + log(Avg. Mass Lost) * Reproductive Status + Sum Duration * Reproductive Status + Day 1 Mass + Wing Length + Abdomen:Thorax Ratio + Wire Mass). We report the model formula retained following model simplification by Crawley (2002), in which non-significant terms were removed until all terms in model were significant.

Retained model structure and predictor variables		Estimate ± S.E.	p-value
(A) Phenoloxidase activity ~ Wire Mass *($p=0.03$, adjusted $R^2=0.05$)*			
	Wire Mass	0.72 ± 0.33	0.03
(B) Lysosyme-like activity ~ Abdomen:Thorax Ratio *($p=0.03$, adjusted $R^2=0.05$)*			
	Abdomen:Thorax Ratio	-0.31 ± 0.11	0.005
(C) Hemocyte concentration ~ Distance + Repro. Status + Wire Mass *($p<0.005$, adjusted $R^2=0.28$)*			
	Sum Distance	0.12 ± 0.06	0.04
	Reproductive Status	0.26 ± 0.06	<0.005
	Wire Mass	-0.15 ± 0.06	0.02

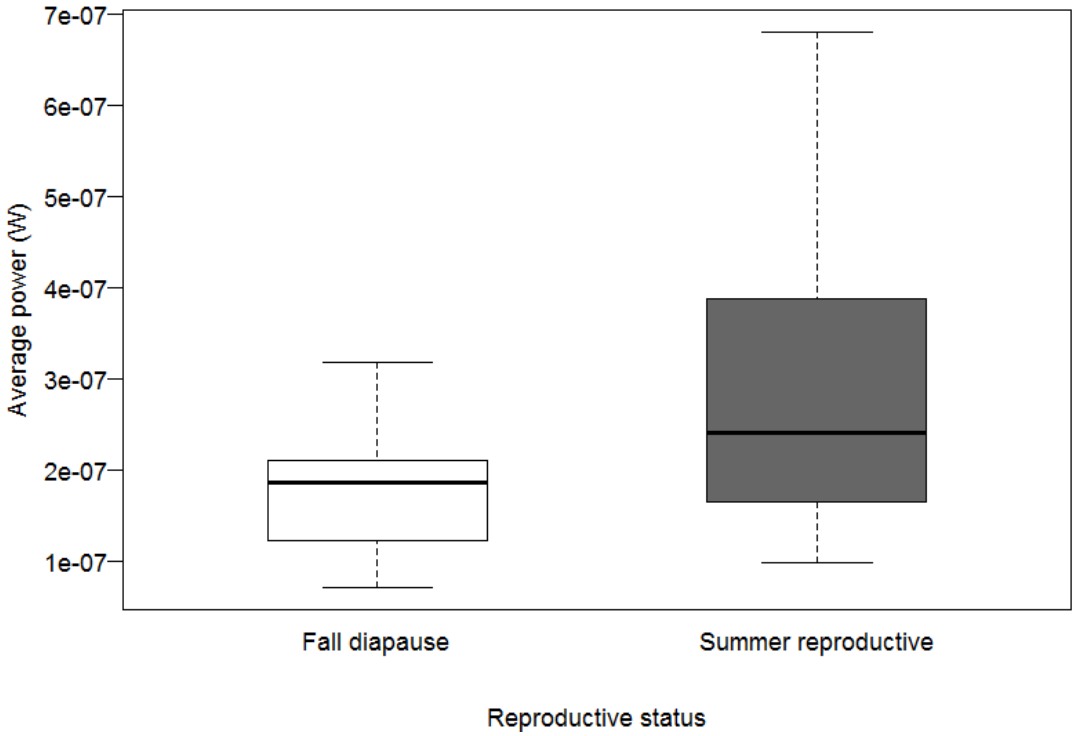

Figure 5.1. Fall Diapause and Summer Reproductive monarchs differ in the power exerted during flight. Mechanical power (in Watts) was estimated as *(mass * velocity^2)/(2*time)* and the average power across four flight trials reflects how much energy the monarch expended per time. Reproductively active Summer monarchs had higher power (used more energy per unit time) than Fall Diapause monarchs ($F_{1,59}$=7.76, p=0.01). Boxes designate the interquartile range divided by the median, and whiskers extend to 1.5 times the interquartile range beyond the box.

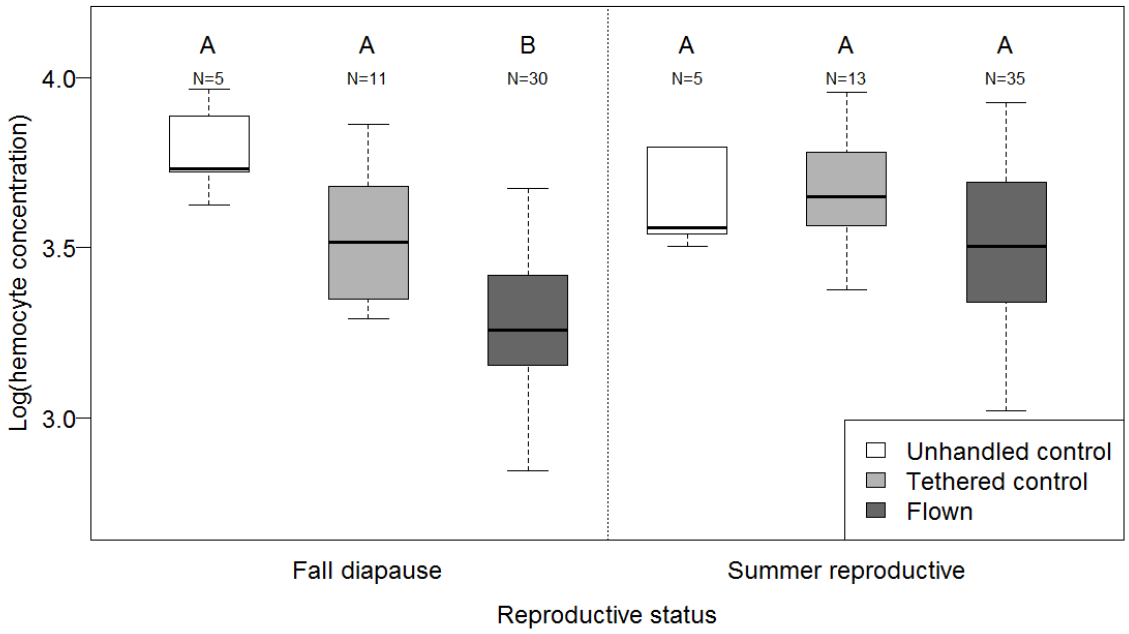

Figure 5.2. Effects of flight treatment and reproductive status on hemocyte concentration. Post-hoc analyses of the significant main effects (in two-way ANOVA) of flight treatment and reproductive status show that hemocyte concentration is lower in Flown monarchs relative to both Unhandled and Tethered controls and in Fall Diapause monarchs relative to Summer Reproductive monarchs. Boxes designate the interquartile range divided by the median, and whiskers extend to 1.5 times the interquartile range beyond the box.

CHAPTER 6

CONCLUSIONS

The overarching goal of my dissertation was to understand how a migrating animal balances the challenges of investing in movement, reproduction, and immune defense. Energetically costly behaviors such as reproduction and movement can play a crucial role in shaping animal defense against infectious diseases if they divert resources away from immunity. Migratory species, such as the monarch butterfly, provide an excellent opportunity to examine how behavioral state drives heterogeneity in immune function across an annual cycle with changing life history requirements. My dissertation used a combination of field observational studies, laboratory assays and experiments to explore immune consequences of food restriction, investment in flight-related tissue, reproduction, and strenuous flight behavior.

In Chapter 2, I asked how food restriction at both larval and adult life stages affected measures of immunity, fitness, and immune-fitness interactions. Immune measures were sensitive to food restriction, but food restriction did not necessarily induce fitness trade-offs as predicted. Across monarchs (regardless of food restriction) we found a negative relationship between larval hemocyte concentration and pupal mass, and a trade-off between adult hemocyte concentration and adult lifespan was evident in parasitized female monarchs. Adult lifespan increased with phenoloxidase activity in some subsets of monarchs. The results emphasize that food restriction can alter fitness

and immunity across multiple life stages, and they suggest that resource limitation could constrain fitness and resistance to natural enemies in the monarch.

In Chapter 3, I explored trade-offs between immunity and flight-related tissue in wild monarchs sampled at their wintering sites in central Mexico. I anticipated a trade-off between lipid (fat) storage and immunity, but instead found that both measures of immunity were positively related to the proportion of body mass containing lipids. I did find evidence of a mild cost of migration for immunity, as hemocyte concentration decreased with estimated flight distance. The results suggest that monarchs in the best physical condition were most likely to survive the complete migration to Mexico, and the findings underscore the critical importance of nectar flowers at stopover sites to provide monarchs with the lipid reserves that fuel flight and help maintain defenses against parasites.

In Chapter 4, I aimed to identify the specific aspects of reproductive activity (either reproductive tissue development or mating activity) that generate reductions in immunity. The results showed that reproductively mature monarchs (treated with a hormone that initiated development) had more strongly reduced measures of hemocyte concentration and phenoloxidase activity than control monarchs. I also found evidence for a subtle cost of strenuous courtship contests; males that attempted mating but did not actually mate had the lowest measures of phenoloxidase activity. In females, a higher frequency of mating corresponded to a greater loss in hemocytes. Collectively, these results suggest an immunosuppressive effect both of reproductive tissue development and certain aspects of courtship and mating. In the context of monarch natural history, these

immune costs of reproduction could increase susceptibility of summer breeding (or spring re-colonizing) monarchs to parasites.

In Chapter 5, I used a tethered flight mill to examine immune consequences of experimentally induced powered flight in monarchs that varied in their reproductive status. I found that monarchs that were reproductively active were less efficient fliers, as they exerted more power during flight than monarchs in the migratory condition of reproductive diapause. However, reproductive status did not modify relationships between flight and immune responses. Of the three immune responses measured, hemocyte concentration was lower in flown monarchs relative to controls but increased with flight distance among flown monarchs; the other two immune measures showed no relationship to monarch flight. Results of this study add to a growing body of work suggesting that migratory monarchs – like some other animals that travel vast distances – can complete their journeys with efficient use of resources and minimal costs.

In summary, my dissertation established that monarch immune defense is resource-limited, can trade-off against growth and survival, and is reduced by certain aspects of reproduction and flight (Table 6.1). I found that one immune measure—hemocyte concentration—was more frequently involved in trade-offs with other physical or life history traits (Table 6.1). This tendency suggests that hemocytes, as a more constitutive component of immunity, are more costly to produce and maintain than the enzyme phenoloxidase. Phenoloxidase activity was generally responsive to treatments in male monarchs only, calling into question whether this particular immune response (and the molecules it necessitates) are under greater demand for other functions (i.e. the deposition of melanin in wing scale tissue) in males than in females. Future studies could

explore whether phenoloxidase and wing melanization are associated, and if female mate choice exists for darker black wings.

In several cases, my dissertation results did not show evidence for a physiological trade-off despite our predictions. Theoretical work initially provided by de Jong and van Noordwijk (1986, 1992) and recently revisited by Metcalf (2016) offers an explanation regarding why positive (or non-significant) relationships emerge when a trade-off (negative relationship) is predicted. When individuals in a population vary in their acquisition of resources, then the underlying resource availability makes some individuals able to invest strongly in two traits of interest and others to invest weakly in the two traits, leading to a positive relationship. Conversely, if acquisition of resources is constrained (for instance by experimental manipulation) and individuals only vary in their allocation of energy to two traits of interest, a negative relationship indicative of a trade-off is more likely to be observed (Metcalf 2016). When I controlled resource acquisition in Chapter 2, I observed trade-offs between larval hemocyte concentration and larval growth, and between adult hemocyte concentration and lifespan. Yet in Chapter 3, with field-collected monarchs lacking experimental controls on resource intake, I observed positive relationships between immunity and fat storage. Thus, my results must be interpreted in light of the fact that existing trade-offs could have been masked by variation in resource intake.

This work was undertaken, in part, to predict when monarchs could be most vulnerable to parasites. Because both reproduction and movement can be immunosuppressive, it is challenging to predict whether immunity should be lowest during breeding or migratory generations. Altogether, this work indicates that monarchs

in the wild likely have the lowest capability to mount an immune response during the summer breeding season, especially in areas or conditions with limited food resources (i.e. isolated patches of nectar flowers or depleted patches of the milkweed host plant). Despite facing enormous energetic expenditure during migration, the fall migratory generation may consist of monarchs physiologically adapted to utilize energy efficiently. This assertion is based on evidence from Chapter 3 showing that larger and better fueled monarchs had higher immune defenses, and from Chapter 5 showing that forced flight treatments did not consistently reduce immunity. Further, other recent work has shown that migratory monarchs maintain lower metabolic rate during flight than non-migratory monarchs, allowing them to retain energy (Zhan 2014, Majewska unpublished data).

Monarchs, among other migratory animals, are facing population declines owing to habitat loss and other aspects of anthropogenic change (Satterfield et al. 2015, Gilroy et al. 2016). Reduced availability of resources, on top of extreme energetic expenditure during migration, could limit the extent to which these animals invest in immune defenses against pathogens. My dissertation adds to our understanding of how migration influences immunity (and potentially pathogen susceptibility) by way of changes in physiology and behavior.

Table 6.1. **Simplified summary of the significant responses of my two key immune measures—hemocyte concentration and phenoloxidase activity—to the experimental treatments I imposed or covariates I measured in my dissertation chapters**. The direction of the response is indicated by a (+) when the immune measure increased with the covariate or under the treatment imposed, and by a (–) when it decreased. Hemocytes tended to be reduced by treatments I imposed (e.g. reproductive development and mating, flight activity) and to correlate negatively with physical traits or fitness proxies (e.g. pupal mass, migration distance). Phenoloxidase activity typically responded to experimental treatments in male monarchs only.

	Physical, behavioral or life history trait	**Immune measure**	**Direction of response**
Chapter 2	Development rate	Hemocytes	+
		PO activity	+
	Pupal mass	Hemocytes	–
	Lifespan	Hemocytes	– (in infected ♀)
		PO activity	+ (in infected ♀ and ♂)
Chapter 3	Lipid storage	Hemocytes	+
		PO activity	+
	Migration distance	Hemocytes	–
Chapter 4	Reproductive tissue development	Hemocytes	–
		PO activity	–
	Mating activity	Hemocytes	– (in ♀)
		PO activity	– (in ♂)
Chapter 5	Forced flight treatment	Hemocytes	–

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APPENDIX A

CHAPTER 2 SUPPLMENTARY RESULTS

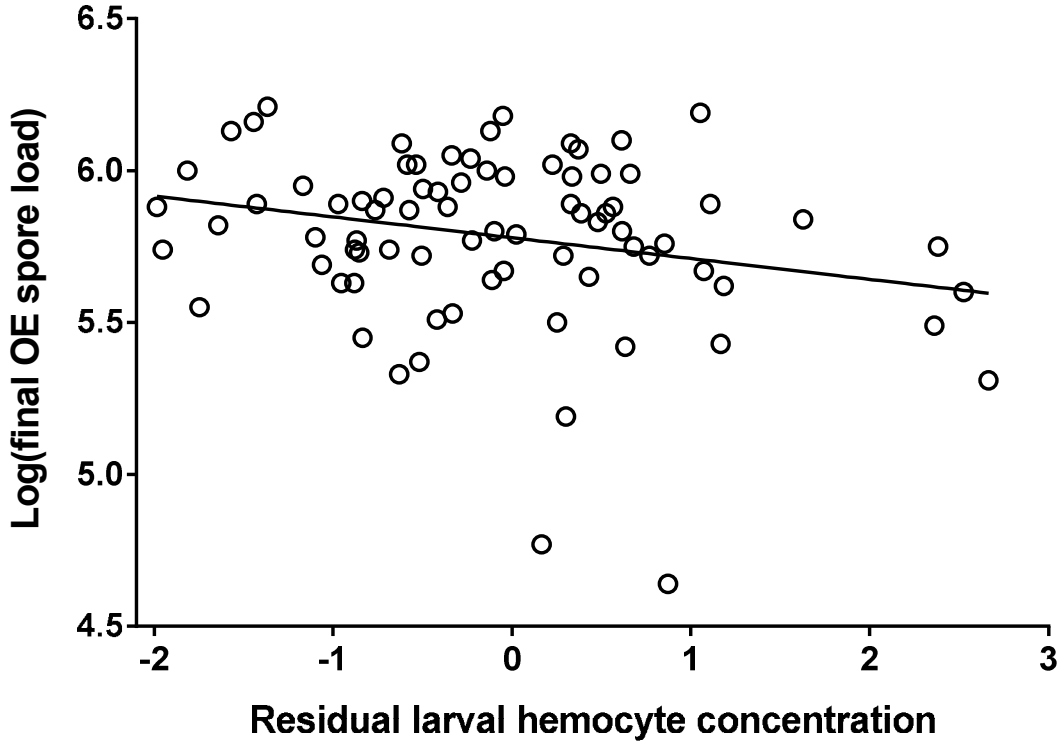

Figure A1. Relationship between residual larval hemocyte concentration and the outcome of OE parasitism (log of the final spore load). This weak but significant relationship ($r^2 = 0.051$, $p=0.029$) suggests some protective value of investing in hemocytes early in life in terms of minimizing parasite growth later in life.

APPENDIX B

CHAPTER 4 SUPPLMENTARY METHODS AND RESULTS

CHAPTER 4 SUPPLMENTARY METHODS AND RESULTS

Methods

Environmental rearing conditions to induce reproduction or diapause

Half of the monarchs were raised as larvae in summer-like conditions to promote reproductive development. We used one Percival incubator set to warm conditions with constant photoperiod (mean daily temperature 27.3ºC, 16 day hours : 12 night hours). We also kept some monarchs in the "Rearing Room", which is a temperature-controlled room naturally-lit by light from a south-facing window (mean daily temperature 27.4 ºC, ambient photoperiod in Athens, GA from 18 August 2014 through 14 October 2014); the Rearing Room group was expected to develop reproductively like the Summer incubator monarchs. The remaining half of the monarchs were raised as larvae in fall-like conditions to trigger reproductive diapause (atrophied reproductive organs and cessation of mating activity). We used three Percival incubators each set to cool temperatures and a progressively decreasing photoperiod (24ºC during daytime, 18ºC during nighttime, 14 day hours reduced to 10 day hours by 5 minutes per day). Temperatures in all five rearing locations were monitored by I button data loggers. Because in pilot experiments methoprene was found to affect unintended control monarchs housed in the same room, we housed methoprene-treated monarchs in one incubator (on fall rearing conditions) and the control monarchs divided across two other incubators (also on fall rearing conditions).

Adjusting experimental time points to account for degree day accumulation

The developmental zero (temperature at which development ceases) in monarchs is estimated at 11.5°C. By dividing the estimated average temperatures of the fall- and summer-rearing conditions by 11.5, we established that monarchs in our study accrued approximately 16 degree days per day in the summer and approximately 9 degree days per day in the fall. Using these figures, we adjusted all experimental timepoints for fall monarchs to match the degree day equivalent of summer monarchs. For example, the first round of dissections (in a small subset of females) occurred at 7 days post-eclosion in summer monarchs, which was estimated to be an equivalent of ~12 days in fall monarchs according to the following calculations: 1) we multiplied the summer time point's days post eclosion by the degree days per day (7 days * 15.83 degree days = 31.66) and 2) we divided this amount of degree days by the degree days accrued by fall monarchs (31.66/9.25 degree days per day = 11.98 days).

Immune assay protocol details

For hemocyte concentration measurement: Immediately after collection, 2µl hemolymph was rapidly diluted 1:10 in sterile Pringle's Saline [1x in 1L dD H20: 9.0gNaCl, 0.2g KCl, 0.2g CaCl, 4.0g dextrose] and loaded onto Kova ® glasstic hemocytometer slides. We counted hemocytes under phase contrast microscopy at 400x in two replicate chambers per sample and calculated the average number of hemocytes per µl.

For phenoloxidase (PO) activity measurement: A 6µl sample of hemolymph was mixed 1:1 with ice cold Pringle's saline in an eppendorf tube. A total of 10µl of diluted sample was loaded into a well of a 96-well plate with 190ul assay buffer [in dD H20:

50mM Na2PO4 monobasic monohydrate adjusted to 6.5pH, 2mM dopamine, and heat-killed *Micrococcus luteus* elicitor at 3% total volume]. We measured absorbance at 490nm every 24 seconds at 30°C for 300 measures (total time: 01:59:36) using a Biotek microplate reader. We calculated the slope of the kinetic curve (absorbance per hr) during the linear phase of the reaction to estimate the rate of melanization (Hall et al. 1995, Barnes and Siva-Jothy 2000).

At the first immune sampling time point (1-2 days post-emergence), we obtained sufficient hemolymph to assess PO activity for 175 monarchs and to measure hemocyte concentration for 218 monarchs. A small subset of 15 summer-reared and 12 fall-reared females (6 JH-treated and 6 control) were sampled for immune measures (an early second timepoint following JH-treatment), euthanized, and dissected to determine effectiveness of JH-treatment in initiating egg development. All other monarchs in the study were sampled for immunity for the second time following the mating treatment (Figure S1). Collectively, we obtained hemolymph at two time points (and were thus able to calculate the change in immune measure) for measurement of PO activity in 49 monarchs and measurement of hemocyte concentration in 102 monarchs.

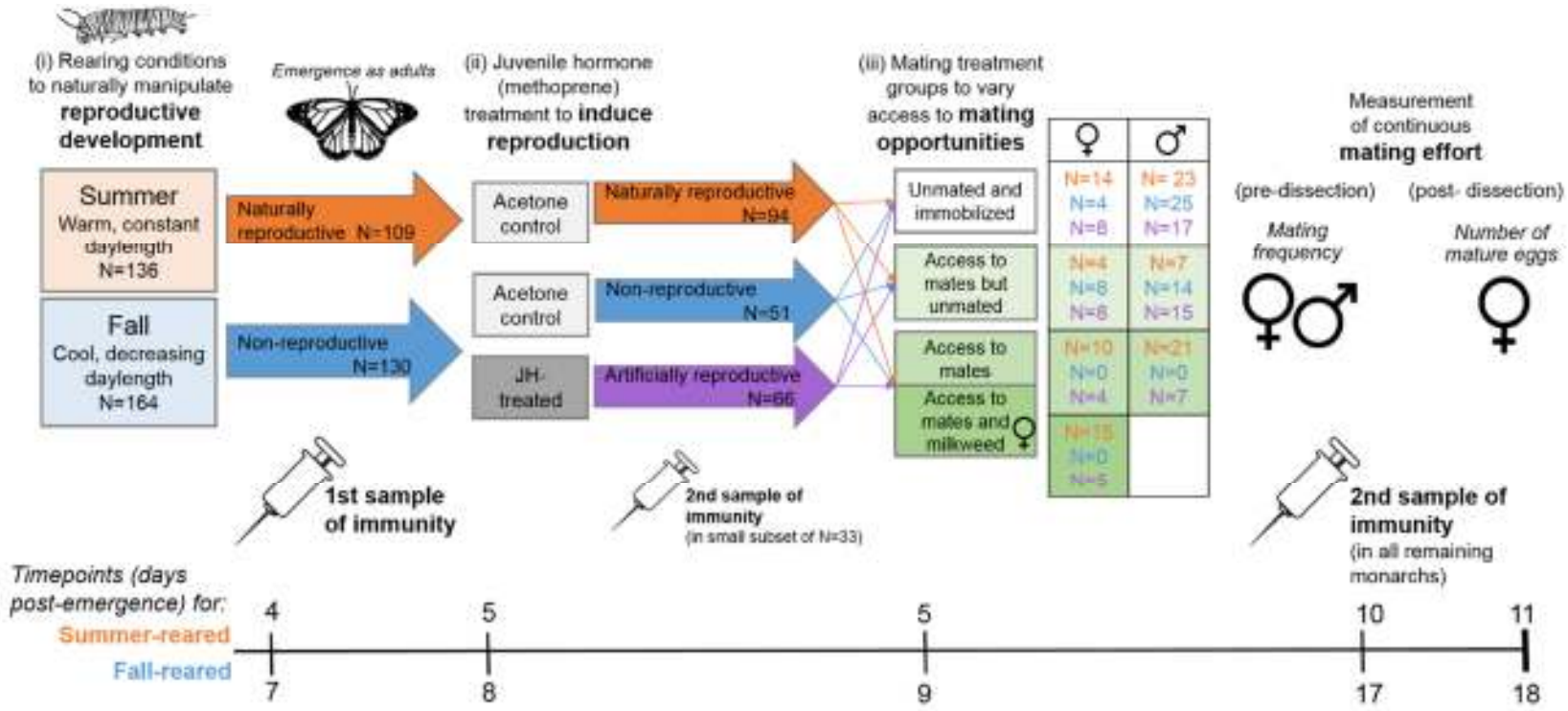

Figure B1. Aspects of experimental design used to manipulate reproductive development (at the monarch larval stage via environmental rearing conditions and/or at the adult stage via hormonal manipulation) and mating activity (at the adult stage by providing varying degrees of mating opportunity.

APPENDIX C

CHAPTER 5 SUPPLMENTARY METHODS AND RESULTS

CHAPTER 5 SUPPLMENTARY METHODS AND RESULTS

Methods

Additional details on flight apparatus

Flight trials were conducted in interior rooms lit with three 60W lamps, heated to approximately 25-26ºC with electric space heaters, and containing vases of artificial flowers surrounding the flight mill to incentivize flying. The flight mill apparatus (Bradley and Altizer 2005) consisted of a lightweight carbon rod (120cm in length and 3mm in diameter) attached to a stand on a nearly-frictionless steel pivot. From one end of the rod we hung a tape "flag" which passed through a photogate (interrupting an infrared beam and transmitting information to a datalogger) upon each rotation of the monarch affixed to the opposite end of the carbon rod. The datalogger (PS-2100A, PASCO Scientific, Roseville, CA, USA) interfaced with associated PASCO Capstone software to record the timestamp of each rotation and the instantaneous velocity (m/s) of the flag's passage through the photogate. Given the dimensions of the rod, the circumference of the monarch's circular flight path was 4.23m.

Additional details on insect immune assays

PO activity – A 6µl sample of hemolymph was mixed 1:1 with ice cold Pringle's saline in an eppendorf tube and frozen at -80°C. Later, a 10µl of thawed sample was loaded into a well of a 96-well plate with 190ul assay buffer [in dD H_20: 50mM Na_2PO_4 monobasic monohydrate adjusted to 6.5pH, 2mM dopamine, and heat-killed *Micrococcus luteus*

elicitor at 3% total volume]. We measured absorbance at 490nm every 24 seconds at 30°C for 300 measures (total time: 01:59:36) using a Biotek microplate reader.

Lysozyme-like activity – We plated 1µL of undiluted sample into wells on a prepared agar plate [in dD H_20: 10mg/mL Agar, 0.1mg/mL, 5mg/mL lyophilized *M. luteus,* and 0.1mL/mL Triton-X]. Plates were incubated at 30°C for 24 hours and clearance zones surrounding sample wells were measured (in mm) with digital calipers, and were compared to a standard curve of known concentrations [32, 20, 16, 10, 8, 5, and 4 µg/µL] of chicken egg white lysozyme (Sigma Aldrich L6876) run at the same time as the samples.

Results

Table C1. Results of two-way ANOVA models investigating main and interactive effects of flight treatment category (Flown, Tethered Control, and Unhandled Control) and reproductive status (Fall Diapause versus Summer Reproductive) category on immune defense measures. In this analysis, the Flown flight category includes only monarchs that flew for at least 7200 total seconds across four days (an average of approximately 30 minutes of flight per day).

Response variable	Predictors	Mean square	df	F	p-value
(A) Phenoloxidase activity					
	Flight treatment	0.36	2	0.20	0.82
	Reproductive status	0.33	1	0.19	0.67
	Flight treatment * reproductive status	1.90	2	1.09	0.34
	Error	1.74	62		
(B) Lysozyme-like activity					
	Flight treatment	0.14	2	0.64	0.53
	Reproductive status	**2.43**	**1**	**11.19**	**<0.005**
	Flight treatment * reproductive status	0.01	2	0.03	0.97
	Error	0.22	64		
(C) Hemocyte concentration					
	Flight treatment	**0.53**	**2**	**10.00**	**<0.005**
	Reproductive status	**0.60**	**1**	**11.27**	**<0.005**
	Flight treatment * reproductive status	0.11	2	2.14	0.12
	Error	0.05	78		

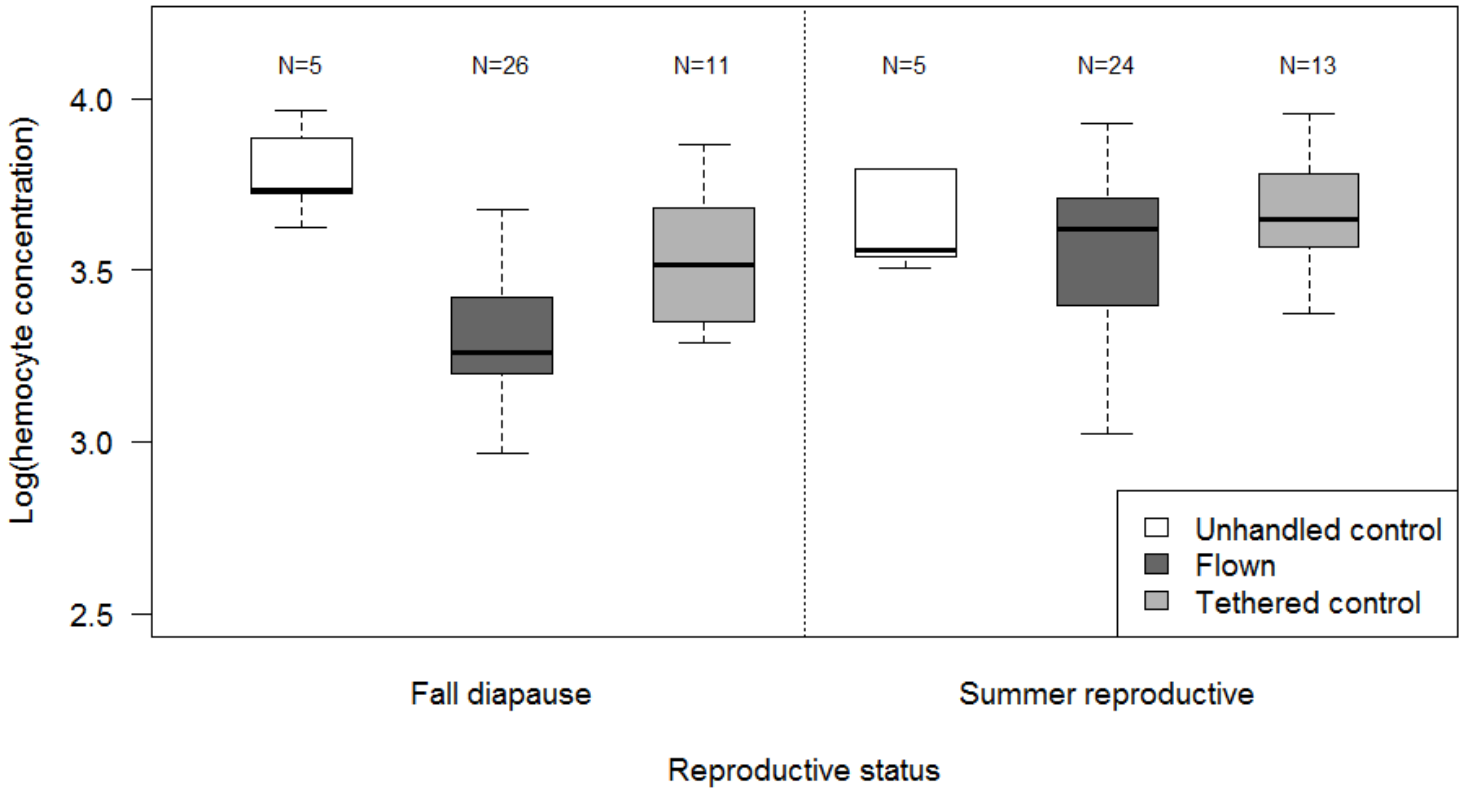

Figure C1. Effects of flight treatment and reproductive status on hemocyte concentration in the subset of monarchs that flew for at least 7200 total seconds across four days (an average of approximately 30 minutes of flight per day). Tukey's HSD Post-hoc analyses show that, in Fall Diapause monarchs only, hemocyte concentration is significantly lower in Flown monarchs relative to Unhandled Controls ($p=0.001$) and marginally lower in Flown monarchs relative to Tethered Controls ($p=0.09$). Flown monarchs in diapause also had significantly lower hemocyte concentration than Flown monarchs that were reproductively active ($p=0.005$). Among Summer Reproductive monarchs there was no difference among any of the flight treatment groups. Boxes designate the interquartile range divided by the median, and whiskers extend to 1.5 times the interquartile range beyond the box.