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Ecological determinants of host resistance to parasite infection in monarch butterflies

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Ecological determinants of host resistance to parasite infection in monarch butterflies

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ABSTRACT

Ecological determinants of host resistance to parasite infection in monarch butterflies

By Elizabeth A. Lindsey

Heterogeneity in host resistance to infection is common in wild populations despite the obvious fitness benefits of resistance. This variation can be maintained by evolutionary processes and should also be crucially affected by ecological factors. The central goal of my dissertation is to examine how ecological factors influence observed variation in host resistance, focusing specifically on monarch butterflies *Danaus plexippus* and an obligate protozoan parasite *Ophryocystis elektroscirrha*. All populations of monarch butterflies examined to date are infected with this parasite, and parasite prevalence varies with host migratory status. Resistance here was defined broadly to include the ability of an individual to either counter infection or survive and reproduce in the presence of infection. Ecological variables examined included host sex, rearing density and environmental temperature. Initial results showed that host resistance and immunity differed between male and female monarchs in a direction consistent with sex-based tradeoffs between the demands of reproduction and costly immune defenses. Effects of larval rearing density on monarch-parasite interactions included an increase in infection probability and stronger negative effects of parasite infection with increasing density, consistent with predictions that crowding induces greater physiological stress and susceptibility among hosts. Global changes in temperature have been predicted to increase the intensity and prevalence of infection in many host-parasite systems. Effects of temperature reported here showed that infected monarchs in warmer temperatures suffered greater levels of wing deformity and incurred lower parasite loads. Thus, hot temperatures appear to have detrimental consequences for the host and parasite, an effect that could effectively lower parasite prevalence owing to reduced transmission at the population level. Across all studies, unparasitized butterflies showed greater (darker) wing melanism than parasitized monarchs, an observation that could be caused by condition-dependent melanin synthesis or by trade-offs in melanin produced for parasite defense versus wing coloration. Overall, results reported here demonstrate a difference in immune defenses between male and female monarchs, and significant effects of larval density and temperature on host resistance and parasite replication and potential transmission.

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TABLE OF CONTENTS

INTRODUCTION 1

REFERENCES 10

CHAPTER 1

SEX DIFFERENCES IN IMMUNE DEFENSES AND RESPONSE TO PARASITISM IN MONARCH BUTTERFLIES 18

ABSTRACT 19

INTRODUCTION 21

METHODS AND MATERIALS 25

RESULTS 30

DISCUSSION 34

ACKNOWLEDGEMENTS 38

REFERENCES 39

CHAPTER 2

CROWDING AND DISEASE: EFFECTS OF HOST DENSITY ON RESPONSE TO INFECTION IN A BUTTERFLY-PARASITE INTERACTION 53

ABSTRACT 54

INTRODUCTION 56

METHODS AND MATERIALS 59

RESULTS 67

Discussion....71
Acknowledgements....77
References....78

CHAPTER 3

Warmer temperatures limit host and parasite fitness: experimental evidence from a butterfly-parasite system....98
Abstract....99
Introduction....101
Methods and Materials....104
Results....111
Discussion....116
Acknowledgements....122
References....123

TABLES AND FIGURES

CHAPTER 1

TABLE 1. LINDSEY AND ALTIZER. TABLE 1. ANALYSIS OF VARIANCE SHOWING EFFECTS OF PARASITE TREATMENT, SEX, AND HEMOCYTE CONCENTRATION OF PARASITE LOAD, ADULT LONGEVITY, AND MORPHOLOGICAL CHARACTERISTICS....................................49

FIGURE LEGENDS. LINDSEY AND ALTIZER 50

FIGURE 1. LINDSEY AND ALTIZER. FIGURE 1. EFFECTS OF PARASITE TREATMENT AND SEX ON (A) HEMOCYTE CONCENTRATION OF LARVAE (CELLS PER µL OF HEMOLYMPH, SQUARE-ROOT TRANSFORMED), (B) ADULT BODY LENGTH (IN MM), (C) ADULT FOREWING AREA (IN MM^2), AND (D) ADULT LONGEVITY (IN DAYS)............................ 51

FIGURE 2. LINDSEY AND ALTIZER. FIGURE 2. RELATIONSHIP BETWEEN HEMOCYTE CONCENTRATION OF LARVAE (SQUARE-ROOT TRANSFORMED) AND ADULT BODY LENGTH FOR (A) FEMALES AND (B) MALES 52

CHAPTER 2

TABLE 1. LINDSEY, MEHTA ,ET AL. TABLE 1. NUMBER OF MONARCHS USED TO INITIATE THE EXPERIMENT, AND PERCENT SURVIVING TO ADULT ECLOSION, SHOWN SEPARATELY FOR EACH PARASITE TREATMENT AND LARVAL REARING DENSITY..........88

TABLE 2. LINDSEY, MEHTA, ET AL. TABLE 2. ANALYSIS OF VARIANCE SHOWING EFFECTS OF PARASITE TREATMENT, LARVAL REARING DENSITY, AND SEX ON PUPAL MASS AND ADULT FOREWING MORPHOLOGY 89

FIGURE LEGENDS. LINDSEY, MEHTA ET AL. 90

FIGURE 1. LINDSEY, MEHTA ET AL. FIGURE 1. FIFTH INSTAR MONARCH LARVAE FEEDING ON *ASCLEPIAS SYRIACA* (COMMON MILKWEED) IN A FIELD NEAR FORESTPORT, NEW YORK, USA, DURING THE SUMMER OF 2007 (PHOTOGRAPH: MAUREEN CLARK) 93

FIGURE 2. LINDSEY, MEHTA ET AL. FIGURE 2. RELATIONSHIP BETWEEN THE AVERAGE DENSITIES OF MONARCH BUTTERFLY LARVAE BY REGION AND PHASE..........94

FIGURE 3. LINDSEY, MEHTA ET AL. FIGURE 3. EFFECTS OF LARVAL REARING DENSITY AND PARASITE TREATMENT ON A) PUPAL MASS (GRAMS) AND B) DEVELOPMENT TIME (DAYS)...... 95

FIGURE 4. LINDSEY, MEHTA ET AL. FIGURE 4. EFFECTS OF LARVAL REARING DENSITY AND PARASITE TREATMENT ON ADULT LONGEVITY (IN DAYS)......96

FIGURE 5. LINDSEY, MEHTA ET AL. FIGURE 4. EFFECTS OF LARVAL REARING DENSITY AND PARASITE TREATMENT ON A) ADULT FOREWING AREA (MM) AND B) PROPORTION OF BLACK PIGMENTATION ON ADULT FOREWINGS...... 97

CHAPTER 3

TABLE 1. LINDSEY, VALIN ,ET AL. TABLE 1. TARGETED TEMPERATURE SETTINGS, REALIZED AVERAGE MAXIMUM AND MINIMUM TEMPERATURES (IN °C), AND AVERAGE MAXIMUM AND MINIMUM RELATIVE HUMIDITY VALUES FOR COLD, MODERATE, AND HOT TEMPERATURES 132

TABLE 2. LINDSEY, VALIN, ET AL. TABLE 2. ANALYSIS OF VARIANCE SHOWING EFFECTS OF PARASITE TREATMENT, TEMPERATURE, AND SEX ON DEVELOPMENT TIME, PARASITE SPORE LOAD, ADULT MASS, FOREWING AREA, AND MELANISM...... 133

FIGURE LEGENDS. LINDSEY, VALIN, ET AL 134

FIGURE 1. LINDSEY, VALIN ET AL. FIGURE 1. PROPORTION OF MONARCHS SURVIVING FROM INOCULATION TO THE ADULT STAGE UNDER COLD, MODERATE, AND HOT TEMPERATURES 136

FIGURE 2. LINDSEY, VALIN ET AL. FIGURE 2. EFFECT OF TEMPERATURE AND PARASITE TREATMENT ON DEVELOPMENT TIME (DAYS LOG-TRANSFORMED) 137

FIGURE 3. LINDSEY, VALIN ET AL. FIGURE 3. EFFECT OF TEMPERATURE AND PARASITE TREATMENT ON PARASITE SPORE LOAD... 138

FIGURE 4. LINDSEY, VALIN ET AL. FIGURE 4. EFFECTS OF TEMPERATURE AND PARASITE TREATMENT ON A) PROPORTION OF DEFORMED ADULT MONARCHS AND B) ADULT LONGEVITY (IN DAYS) 139

FIGURE 5. LINDSEY, VALIN ET AL. FIGURE 5. EFFECTS OF TEMPERATURE AND PARASITE TREATMENT ON THE PROPORTION OF BLACK ON MONARCH FOREWINGS FOR A) MALES AND B) FEMALES 140

INTRODUCTION

A major question in the field of evolutionary ecology and ecological immunity is 'what factors maintain variation in host resistance to pathogens in the wild?' – especially in light of the strong selective pressures parasites can exert on their hosts. In other words, given the benefits to hosts that harbor resistance traits in the face of debilitating or lethal infections, it might seem that all hosts should become resistant to common infectious agents. Evolutionary explanations based on simple genetic interactions show that polymorphisms in host resistance can be maintained by at least three key mechanisms – frequency-dependent selection, balancing selection, and fitness costs of resistance traits (Schmid-Hempel & Ebert 2003). Indeed, for many years, researchers have studied the evolutionary dynamics of host-parasite relationships, including the evolution of host resistance (Antonovics & Thrall 1994), parasite virulence (Ebert 1994, 1998; Thrall & Burdon 2003), and the co-evolutionary responses of both hosts and parasites (Kraaijeveld & Godfray 1999; Gandon & Michalakis 2002; Altizer *et al.* 2003). One take-home message from this work is that even small costs of resistance, or genotype-specific interactions, between hosts and parasites can allow for the persistence of host genotypes that vary in their susceptibility to infection.

Insects and other arthropods have become popular organisms to study in the growing field of ecological immunity, broadly defined to include studies that examine the evolutionary ecology of immune defenses and factors that determine variation in host resistance to parasites in the wild (Boots & Begon 1993; Rolff & Siva-Jothy 2003; Schmid-Hempel & Ebert 2003). This is probably because many

arthropod hosts are ubiquitous and easy to rear in large numbers for experimental studies and many aspects of arthropod biology depend crucially on environmental variables. During the past two decades, studies of insect-pathogen interactions have focused on how ecological variables (such as host population density, temperature, nutrition, and habitat quality) generate heterogeneity in host resistance (Cotter & Wilson 2002; Schmid-Hempel & Ebert 2003; Wilson *et al.* 2003). Continued understanding concerning causes of heterogeneity in host resistance requires further exploration into the effects of these environmental factors and host traits on host innate resistance and response to parasitism. Studies have suggested that climatic change will facilitate the emergence and spread of wildlife diseases, through selection on host life-history traits affecting parasite virulence and transmission (Marcogliese 2001; Pounds *et al.* 2006; Harvell *et al.* 2007) and through alterations in host population density, geographical distribution, animal migration, and immunity, also affecting parasite virulence and transmission (Harvell *et al.* 2002; Poulin 2006; Harvell *et al.* 2008).

The central goal of my dissertation work is to determine the degree to which ecological factors influence observed variation in host resistance, focusing specifically on monarch butterflies, *Danaus plexippus,* and an obligate protozoan parasite, *Ophryocystis elektroscirrha.* The main focus for each of the three chapters that follow is to examine how host traits and ecologically-relevant environmental variation influence measures of parasite replication and host response to infection. Collectively, this work sheds light on how host sex influences the costs of resistance and response to infection (Chapter 1), effects of

monarch rearing density on host resistance (Chapter 2), and the influence of environmental temperature on monarch-parasite interactions (Chapter 3).

Throughout each of these chapters, I define resistance broadly to include the ability of an individual to either counter infection or survive and reproduce in the presence of infection. In terms of infection parameters, I measured both the presence of infection after challenging hosts with parasites and final parasite load, as an indication into the ability of the host to limit parasite replication. I also quantified both lethal and sub-lethal effects of infection, including survival to the adult life-stage (where reproduction occurs), host development time, pupal and adult mass, body and wing size, wing deformity, and adult longevity. These variables are viewed as important predictors of host fitness in the wild – for example, female body size correlates positively with lifetime fecundity (Oberhauser 1997), and the presence of wing deformities indicate that a monarch is highly unlikely to mate and reproduce, irrespective of body size, adult longevity, or parasite load. Moreover, in each study I examined key links between host body coloration, parasite infection, and design variables for each study, as previous work has shown that insects with greater cuticular melanism can harbor greater resistance to infectious agents (Barnes & Siva-Jothy 2000; Wilson *et al.* 2001; Cotter *et al.* 2004).

Monarch butterflies represent ideal organisms to examine how ecological variables affect resistance to parasite infection. Monarchs are distributed worldwide and inhabit eastern and western N. America, the neo-tropics, Hawaii, Central America, Caribbean, Australia, and New Zealand. All populations of monarchs studied to date are parasitized to some degree by the neogregarine

protozoan *O. elektroscirrha* (Leong *et al.* 1992; Altizer *et al.* 2000). The life history of the parasite is closely correlated with host development. Transmission occurs from adults to larvae only via maternal, paternal, or horizontal routes, depending on the relationship between adults and infected larvae (Altizer et al. 2004). Larvae ingest spores scattered by adults on egg shells and foliage. These spores lyse in the larval gut, sporozoites penetrate the intestinal wall, pass into the mid-gut, and undergo two phases of vegetative schizogony during the pupal stage. Parasites then undergo a brief sexual phase and form spores in and around the scales of developing butterflies, so that infected adults emerge covered with dormant parasite spores on the outsides of their bodies. Severity of infection increases with increased parasite dose and with decreased age at exposure (Altizer & Oberhauser 1999; de Roode *et al.* 2007). Parasite infection results in pre-adult mortality, greater adult mortality, increased deformities, decreased adult longevity, and lower flight ability (Leong *et al.* 1992; Leong *et al.* 1997; Altizer & Oberhauser 1999).

The prevalence of *O. elektroscirrha* varies dramatically among wild monarch populations and in a direction that is inversely correlated with host migration distances (Altizer et al. 2000). Migratory monarch populations over-wintering in California have shown prevalence levels of roughly 30% over the past 2 decades, whereas the eastern migratory population, which travels the farthest distance to overwinter in Mexico, shows the lowest infection prevalence (less than 8% prevalence over the past 30 years). Non-migratory monarchs in S. Florida have the highest prevalence of infection ranging from 70-100% (Altizer et al. 2000). These differences among populations have persisted for many years

and could be caused by differences in monarch migratory behavior (Altizer *et al.* 2000; Bradley & Altizer 2005), local population densities, environmental variation among sites, and/or genetic (heritable resistance) factors. For example, migratory monarchs breed in a temperate climate with relatively low larval host density during the breeding season when larvae are developing on host plants. On the other hand, non-migratory populations residing in tropical climates (such as S. Florida, Hawaii, and Central America) will likely experience greater host density in their breeding habitats (described in more detail in Chapter 2). Moreover, environmental variation in climate could alter the ecological dynamics of natural populations, such as parasite development and infection within individual hosts, and thus significantly influence the host-parasite relationship by altering either host resistance or parasite virulence.

Chapter one explores variation of immune effector traits within monarch butterflies in relation to sex and host infection status. In some insect species, differences in immunity between males and females have been shown to occur, and these can relate to sexual dimorphism in body pigmentation and sexually selected traits. For example, in several arthropods, females are known to harbor greater immunity or resistance to infection (Gray 1998; Radhika *et al.* 1998; Kurtz *et al.* 2000; Kurtz & Sauer 2001; Rolff 2001; Yourth *et al.* 2002; Adamo 2004; Fedorka *et al.* 2004), a pattern that could be explained by females benefitting more from greater immunity because their fitness tends to be limited by longevity and the number of offspring they can rear, whereas males are more often limited by the number of mates they can inseminate (Zuk 1990; Andersson 1994; Zuk & McKean 1996; Moore & Wilson 2002; Yourth *et al.* 2002; Zuk *et al.*

2004). Moreover, in insects such as moths and beetles, cuticular melanism (darker body pigmentation) has been associated with greater activity of phenoloxidase, an enzyme also important for immune defense (Cotter *et al.*, 2004). In damselflies, for example, darker wing pigmentation, larger wingspots and more symmetrical wings of males correlated with increased immunocompetence (Rantala et al. 2000) and parasite resistance (Siva-Jothy 2000; Yourth et al. 2002).

In this first experiment, I quantified two measures of immunity in both healthy and parasitized late instar larvae: hemocyte concentration and mid-gut phenoloxidase activity, and also quantified final parasite loads, adult longevity, and morphological characteristics. The expectation was that parasitized monarchs would recognize and respond to the presence of infection by increasing immune effector traits. Results showed that the concentration of circulating hemocytes increased in parasitized males, but not females. Females, on the other hand, had greater average hemocyte counts in the absence of infection. However, neither hemocyte concentrations nor phenoloxidase activity were significantly correlated with adult parasite loads nor with each other. This work also revealed a cost of immunity for females in the absence of infection, and a benefit for females in the presence of infection. Additionally, across all monarchs, unparasitized butterflies had darker forewings (greater melanism) than parasitized monarchs. This study provides support for differential costs and consequences of immune defense in males and female monarch butterflies following infection by *O. elektroscirrha*.

Chapter two focuses on the effects of host rearing density on the response of monarch butterflies to parasite infection using an experimental approach. This issue is important because some authors have argued that high rearing densities generate crowded environments that enhance host susceptibility to infection (Steinhaus 1958; Goulson & Cory 1995; Reilly & Hajek 2008), whereas others have suggested that hosts can up-regulate immune defenses in response to high densities, thus becoming more resistant to infection (Reeson *et al.* 1998; Barnes & Siva-Jothy 2000; Reeson *et al.* 2000; Cotter *et al.* 2004). Chapter two also includes an analysis of data from a large-scale citizen science monitoring project, the Monarch Larva Monitoring Project (MLMP) (Prysby & Oberhauser 2004; Oberhauser & Prysby 2008) designed to estimate the spatial and temporal variation in monarch larval densities. Analysis of MLMP data showed that larval densities throughout the monarchs' breeding range in eastern N. America varied substantially among regions and throughout the course of the breeding season, thus supporting the ecological relevance of the rearing density for this host-parasite interaction. Larvae in our experiment were divided into three rearing densities and we measured parasite infection and host resistance parameters. We expected that monarchs reared under the highest densities would develop fastest, be the smallest, and show greater susceptibility to parasitism. The study indicates that while highly crowed conditions can increase susceptibility to parasitism, being in a small group has beneficial impacts on some monarch fitness traits.

Chapter three examines the effect of thermal variation on the response of monarch butterflies to parasitism using an experimental approach. Small changes in temperature can alter insect immunity and parasite replication

(Adamo 1998; Blanford & Thomas 1999; Blanford *et al.* 2003), and climate warming is anticipated to assist the emergence and increase of wildlife diseases (Pounds *et al.* 2006; Harvell *et al.* 2007), with warmer temperatures impacting both host resistance (Robb & Forbes 2005) and parasite virulence (Carruthers *et al.* 1992; Watson *et al.* 1993; Elliot *et al.* 2002). In this experiment, larvae were assigned to infection and temperature treatments, and we quantified pre-adult mortality, development rate, final parasite loads, adult longevity, adult deformity, and morphological characteristics. We expected that larvae reared in hot temperatures would develop faster, have higher survival, and lower parasite growth. Results showed that larvae did indeed develop faster (in days) in the hot temperatures across all parasite treatments. While parasite replication was lower in hot temperatures, host survival did not increase. Overall, the study indicates that temperature does impact the host-parasite relationship and that warmer temperatures may be detrimental for both monarchs and their parasites.

Despite a growing interest in factors determining patterns of parasite infection, more information is needed on factors determining host resistance in natural systems – particularly among insects and other invertebrates. Understanding factors affecting levels of resistance in wild animal populations is also vital to conservation efforts, especially for declining or isolated host populations where genetic variation has been reduced (Thorne & Williams 1988; Funk *et al.* 2001), or in species susceptibility to climate warming or habitat changes that lead to crowding or host stress. Environmental changes are occurring with increased frequency and include changes in climate (global warming), changes in habitat (through human intervention and development), and changes in

pathogen exposure (introductions of exotic species and habitat expansions). These fluctuations can alter the ecological dynamics of natural populations, such as migration patterns, and result in alterations of host population densities. Understanding consequences of these changes for host-parasite relationships adds to our understanding of how ecological variation influences population dynamics, and also indicates the degree to which hosts can respond to future environmental changes.

REFERENCES

Adamo S.A. (1998). The specificity of behavioral fever in the cricket *Acheta domesticus*. *Journal of Parasitology*, 84, 529-533.

Adamo S.A. (2004). Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. *Journal of Insect Physiology*, 50, 209-216.

Altizer S.M., Harvell C.D. & Lindsey E. (2003). Rapid evolutionary dynamics and disease threats to biodiversity. *Trends in Ecology & Evolution*, 18, 589-596.

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

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

Altizer S.M., Oberhauser K.S. & Geurts K.A. (2004). Transmission of the protozoan parasite, *Ophryocystis elektroscirrha*, in monarch butterfly populations: implications for prevalence and population-level impacts. In: *The Monarch Butterfly: Biology and Conservation* (eds. Oberhauser KS & Solensky MJ). Cornell University Press Ithaca, NY.

Andersson M. (1994). *Sexual Selection*. Princeton Univeristy Press, Princeton, N.J.

Antonovics J. & Thrall P.H. (1994). Cost of resistance and the maintenance of genetic-polymorphism in host-pathogen systems. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 257, 105-110.

Barnes A.I. & Siva-Jothy M.T. (2000). Density-dependent prophylaxis in the mealworm beetle *Tenebrio molitor L*-(Coleoptera : Tenebrionidae): cuticular melanization is an indicator of investment in immunity. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 267, 177-182.

Blanford S. & Thomas M.B. (1999). Host thermal biology: the key to understanding host-pathogen interactions. *Agricultural and Forest Entomology*, 1, 195-202.

Blanford S., Thomas M.B., Pugh C. & Pell J.K. (2003). Temperature checks the red queen? Resistance and virulence in a fluctuating environment. *Ecology Letters*, 6, 2-5.

Boots M. & Begon M. (1993). Trade-offs with resistance to a granulosis-virus in the Indian meal moth, examined by a laboratory evolution experiment. *Functional Ecology*, 7, 528-534.

Bradley C.A. & Altizer S. (2005). Parasites hinder monarch butterfly flight: implications for disease spread in migratory hosts. *Ecology Letters*, 8, 290-300.

Carruthers R.I., Larkin T.S., Firstencel H. & Feng Z. (1992). Influence of thermal ecology on the mycosis of a rangeland grasshopper. *Ecology*, 73, 190-204.

Cotter S.C., Hails R.S., Cory J.S. & Wilson K. (2004). Density-dependent prophylaxis and condition-dependent immune function in Lepidopteran larvae: a multivariate approach. *Journal of Animal Ecology*, 73, 283-293.

Cotter S.C. & Wilson K. (2002). Heritability of immune function in the caterpillar *Spodoptera littoralis*. *Heredity*, 88, 229-234.

de Roode J.C., Gold L.R. & Altizer S. (2007). Virulence determinants in a natural butterfly-parasite system. *Parasitology*, 134, 657-668.

Ebert D. (1994). Virulence and local adaptation of a horizontally transmitted parasite. *Science*, 265, 1084-1086.

Ebert D. (1998). Experimental evolution of parasites. *Science*, 282, 1432-1435.

Elliot S.L., Blanford S. & Thomas M.B. (2002). Host-pathogen interactions in a varying environment: temperature, behavioural fever and fitness. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 269, 1599-1607.

Fedorka K.M., Zuk M. & Mousseau T.A. (2004). Immune suppression and the cost of reproduction in the ground cricket, *Allonemobius socius*. *Evolution*, 58, 2478-2485.

Funk S.M., Fiorella C.V., Cleaveland S. & Gompper M.E. (2001). The role of disease in carnivore ecology and conservation. In: *Carnivore Conservation* (eds. Gittleman J, Funk S, D. M & Wayne R). Cambridge University Press Cambridge, pp. 241-281.

Gandon S. & Michalakis Y. (2002). Local adaptation, evolutionary potential and host-parasite coevolution: interactions between migration, mutation,

population size, and generation time. *Journal of Evolutionary Biology*, 15, 451-462.

Goulson D. & Cory J.S. (1995). Responses of *Mamestra-brassicae* (Lepidoptera, Noctuidae) to crowding -interactions with disease resistance, color phase and growth. *Oecologia*, 104, 416-423.

Gray D.A. (1998). Sex differences in susceptibility of house crickets, *Acheta domesticus*, to experimental infection with *Serratia liquefaciens*. *Journal of Invertebrate Pathology*, 71, 288-289.

Harvell C.D., Altizer S., Cattadori I., Harrington L. & Weil E. (2008). Climate change and wildlife diseases: when does the host matter the most? *Ecology*. (in review).

Harvell C.D., Jordan-Dahlgren E., Merkel S., Raymundo L., Rosenberg E., G. S., Weil E. & Willis B. (2007). Coral disease, environmental drivers and the balance between coral and microbial associates. *Oceanography*, 20, 58-81.

Harvell C.D., Mitchell C.E., Ward J.R., Altizer S.M., Dobson A.P., Ostfeld R.S. & Samuel M.D. (2002). Ecology - climate warming and disease risks for terrestrial and marine biota. *Science*, 296, 2158-2162.

Kraaijeveld A.R. & Godfray H.C.J. (1999). Geographic patterns in the evolution of resistance and virulence in Drosophila and its parasitoids. *American Naturalist*, 153, S61-S74.

Kurtz J. & Sauer K.P. (2001). Gender differences in phenoloxidase activity of *Panorpa vulgaris* hemocytes. *Journal of Invertebrate Pathology*, 78, 53-55.

Kurtz J., Wiesner A., Gotz P. & Sauer K.P. (2000). Gender differences and individual variation in the immune system of the scorpionfly *Panorpa vulgaris* (Insecta : Mecoptera). *Developmental and Comparative Immunology*, 24, 1-12.

Leong K.L.H., Kaya H.K., Yoshimura M.A. & Frey D.F. (1992). The occurrence and effect of a protozoan parasite, *Ophryocystis elektroscirrha* (Neogregarinida, Ophryocystidae) on overwintering monarch butterflies, *Danaus plexippus* (Lepidoptera, Danaidae) from two California winter sites. *Ecological Entomology*, 17, 338-342.

Leong K.L.H., Yoshimura M.A., Kaya H.K. & Williams H. (1997). Instar susceptibility of the monarch butterfly (*Danaus plexippus*) to the neogregarine parasite, *Ophryocystis elektroscirrha*. *Journal of Invertebrate Pathology*, 69, 79-83.

Marcogliese D.J. (2001). Implications of climate change for parasitism of animals in the aquatic environment. *Canadian Journal of Zoology-Revue Canadienne de Zoologie*, 79, 1331-1352.

Moore S.L. & Wilson K. (2002). Parasites as a viability cost of sexual selection in natural populations of mammals. *Science*, 297, 2015-2018.

Oberhauser K. & Prysby M.D. (2008). Citizen science: creating a research army for conservation. *American Entomologist*, 54, 97-99.

Oberhauser K.S. (1997). Fecundity, lifespan and egg mass in butterflies: effects of male-derived nutrients and female size. *Functional Ecology*, 11, 166-175.

Poulin R. (2006). Global warming and temperature-mediated increases in cercarial emergence in trematode parasites. *Parasitology*, 132, 143-151.

Pounds J.A., Bustamante M.R., Coloma L.A., Consuegra J.A., Fogden M.P., Foster P.N., La Marca E., Masters K.L., Merino-Viteri A., Puschendorf R., Ron S.R., Sanchez-Azofeifa G.A., Still C.J. & Young B.E. (2006). Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature*, 439, 161-167.

Prysby, M.D. & Oberhauser, K., eds. (2004). Temperal and geographic variation in monarch densities: citizen scientists document monarch population patterns. In *The Monarch Butterfly: Biology and Conservation* (eds K.S. Oberhauser & M.J. Solensky). Cornell University Press, Ithaca, NY.

Radhika M., Nazar A.K.A., Munuswamy N. & Nellaiappan K. (1998). Sex-linked differences in phenol oxidase in the fairy shrimp *Streptocephalus dichotomus Baird* and their possible role (Crustacea : Anostraca). *Hydrobiologia*, 377, 161-164.

Rantala M.J., Koskimaki J., Taskinen J., Tynkkynen K. & Suhonen J. (2000). Immunocompetence, developmental stability and wingspot size in the damselfly *Calopteryx splendens L. Proceedings of the Royal Society of London Series B-Biological Sciences*, 267, 2453-2457.

Reeson A.F., Wilson K., Cory J.S., Hankard P., Weeks J.M., Goulson D. & Hails R.S. (2000). Effects of phenotypic plasticity on pathogen transmission in the field in a Lepidoptera-NPV system. *Oecologia*, 124, 373-380.

Reeson A.F., Wilson K., Gunn A., Hails R.S. & Goulson D. (1998). Baculovirus resistance in the noctuid *Spodoptera exempta* is phenotypically plastic and responds to population density. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 265, 1787-1791.

Reilly J.R. & Hajek A.E. (2008). Density-dependent resistance of the gypsy moth, *Lymantria dispar,* to its nucleopolyhedrovirus and the consequences for population dynamics. *Oecologia*, 154, 691-701.

Robb T. & Forbes M.R. (2005). On understanding seasonal increases in damselfly defence and resistance against ectoparasitic mites. *Ecological Entomology*, 30, 334-341.

Rolff J. (2001). Effects of age and gender on immune function of dragonflies (Odonata, Lestidae) from a wild population. *Canadian Journal of Zoology-Revue Canadienne de Zoologie*, 79, 2176-2180.

Rolff J. & Siva-Jothy M.T. (2003). Invertebrate ecological immunology. *Science*, 301, 472-475.

Schmid-Hempel P. & Ebert D. (2003). On the evolutionary ecology of specific immune defence. *Trends in Ecology & Evolution*, 18, 27-32.

Siva-Jothy M.T. (2000). A mechanistic link between parasite resistance and expression of a sexually selected trait in a damselfly. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 267, 2523-2527.

Steinhaus E.A. (1958). Crowding as a possible stress factor in insect disease. *Ecology*, 39, 503-514.

Thorne E.T. & Williams E.S. (1988). Disease and endangered species: the black-footed ferret as a recent example. *Conservation Biology*, 2, 66-74.

Thrall P.H. & Burdon J.J. (2003). Evolution of virulence in a plant host-pathogen metapopulation. *Science*, 209, 1735-1737.

Watson D.W., Mullens B.A. & Petersen J.J. (1993). Behavioral fever response of *Musca domestica* (Diptera: Muscidae) to infection by Entomophthora

muscae (Zygomycetes: Entomophthorales). *Journal of Invertebrate Pathology*, 61, 10-16.

Wilson K., Cotter S.C., Reeson A.F. & Pell J.K. (2001). Melanism and disease resistance in insects. *Ecology Letters*, 4, 637-649.

Wilson K., Knell R., Boots M. & Koch-Osborne J. (2003). Group living and investment in immune defence: an interspecific analysis. *Journal of Animal Ecology*, 72, 133-143.

Yourth C.P., Forbes M.R. & Baker R.L. (2002). Sex differences in melanotic encapsulation responses (immunocompetence) in the damselfly *Lestes forcipatus Rambur*. *Canadian Journal of Zoology-Revue Canadienne de Zoologie*, 80, 1578-1583.

Zuk M. (1990). Reproductive strategies and disease susceptibility - an evolutionary viewpoint. *Parasitology Today*, 6, 231-233.

Zuk M. & McKean K.A. (1996). Sex differences in parasite infections: patterns and processes. *International Journal for Parasitology*, 26, 1009-1023.

Zuk M., Simmons L.W., Rotenberry J.T. & Stoehr A.M. (2004). Sex differences in immunity in two species of field crickets. *Canadian Journal of Zoology-Revue Canadienne de Zoologie*, 82, 627-634.

CHAPTER 1

Sex differences in immune defenses and response to parasitism in monarch butterflies

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Sex differences in immune defenses and response to parasitism in monarch butterflies

Elizabeth Lindsey and Sonia Altizer

ABSTRACT

Host susceptibility and patterns of infection are predicted to differ between males and females due to sex-based tradeoffs between the demands of reproduction and costly immune defenses. In this study, we examined immune defenses and the response to experimental infection by a protozoan parasite, *Ophryocystis elektroscirrha*, in male and female monarch butterflies, *Danaus plexippus.* We quantified two measures of immunity in late instar larvae: the concentration of circulating hemocytes and mid-gut phenoloxidase activity, and also quantified final parasite loads, body size, longevity, and wing melanism of adult butterflies. Results showed that females had greater average hemocyte counts than males in the absence of infection; males, but not females, showed an increased concentration of hemocytes in the presence of infection. However, higher hemocyte concentrations in larvae were not significantly correlated with lower adult parasite loads, and mid-gut phenoloxidase activity was not significantly associated with hemocyte counts or parasite treatments. Among unparasitized females, greater hemocyte concentrations were costly in terms of reduced body size, but for parasite-treated females, hemocyte concentrations and body size were positively associated. Across all monarchs, unparasitized butterflies showed greater wing melanism (darker forewings) than parasitized monarchs. Overall, this study provides support for differential costs of immune defenses in male and

female monarch butterflies, and a negative association between parasite infection and monarch wing melanism.

Key words: hemocytes, insect immunity, phenoloxidase, *Danaus plexippus*, *Ophryocystis elektroscirrha*, trade-offs

INTRODUCTION

A persistent theme in studies of ecological immunity is that host defenses can be costly in terms of reductions in other fitness components (Norris & Evans 2000; Rolff & Siva-Jothy 2003; Viney *et al.* 2005) and that tradeoffs arising from these fitness costs can maintain variation in host susceptibility in natural populations despite the obvious benefits of resistance (Boots & Begon 1993; Schmid-Hempel & Ebert 2003). The costs and benefits of host resistance could differ greatly between males and females due to their differential reproductive strategies (Zuk 1990; Zuk & McKean 1996; Yourth *et al.* 2002; Zuk *et al.* 2004). Specifically, females may benefit more from greater immunity because their fitness tends to be limited by longevity and the number of offspring they can rear, whereas males are more often limited by the number of mates they can inseminate (Andersson 1994; Moore & Wilson 2002). Thus, conventional wisdom predicts that males should show lower measures of immune defenses, in part due to a tradeoff between investing in longer-term survival versus competition for increased mating opportunities (McKean & Nunney 2001; Zuk & Stoehr 2002). On the other hand, when longevity is equally important for males and females, or if the impact of parasites on host condition is greater for males, sexual selection could favor greater male investment in immunity (Stoehr 2006; Stoehr & Kokko 2006; Stoehr 2007).

While the majority of support for sex differences in immunity is found in mammals, insects are well-suited for studies of the costs and benefits of immune function and their correlation with other host traits (Rolff & Siva-Jothy 2003; Schmid-Hempel 2005). Many ecological and evolutionary studies of insect

resistance have focused on two generalized immune effector traits: hemocyte concentration and melanic encapsulation. The concentration of circulating hemocytes reflects the number of cells available to recognize and phagocytize foreign particles. Hemocytes also participate in cellular encapsulation, in which hemocytes adhere to parasites in layers, and melanic encapsulation, which involves the production and deposition of melanin onto foreign particles (Gillespie *et al.* 1997; Silva *et al.* 2002). Melanic encapsulation (hereafter called melanization) occurs through the activation of the phenoloxidase (PO) enzymatic cascade, and can be measured through the enzyme kinetics of PO using hemolymph or tissue (Siva-Jothy 2000; Schmid-Hempel 2005). A number of studies have quantified hemocyte concentration, melanization and cellular encapsulation to infer levels of parasite defense by insect hosts (Paskewitz *et al.* 1989; Dunphy 1991; Eslin & Prevost 1996; Nigam *et al.* 1997; Cotter & Wilson 2002; Rolff & Siva-Jothy 2004) and to examine costs of insect immune defenses via tradeoffs with other fitness components (Moret & Schmid-Hempel 2000; Adamo *et al.* 2001; McKean & Nunney 2001). However, although a few correlational analyses indicate that greater measures of immune effector traits (Paskewitz *et al.* 1989; Adamo 2004) and external melanism (Wilson *et al.* 2001) are associated with resistance to some pathogens, the effectiveness of these mechanisms against a range of naturally-occurring parasites in insect hosts remains relatively unknown.

Sex differences in host defenses, where females show greater immunity or resistance to infection, have been demonstrated for several arthropods including scorpionflies, crickets, shrimp, dragonflies, and damselflies (Gray 1998; Radhika

et al. 1998; Kurtz *et al.* 2000; Kurtz & Sauer 2001; Rolff 2001; Yourth *et al.* 2002; Adamo 2004; Fedorka *et al.* 2004). In many cases, females show greater levels of hemocytes (da Silva *et al.* 2000; Kurtz *et al.* 2000) or PO activity (Gray 1998; Kurtz *et al.* 2000; Adamo *et al.* 2001; Rolff 2001). However, other studies showed greater measures of immune effector in males (Stoehr 2007), including when food resources were limited (Zuk *et al.* 2004; McKean & Nunney 2005), suggesting that immune effector traits are phenotypically plastic and vary under resource limitations.

In some insect species, differences in immunity also relate to sexual dimorphism in body pigmentation and sexually selected traits. Specifically, cuticular melanism (darker body pigmentation) has been associated with greater measures of PO activity and melanin production in beetles and Lepidoptera (Barnes & Siva-Jothy 2000; Wilson *et al.* 2001; Cotter *et al.* 2004a). In damselflies, larger wingspots and more symmetrical wings of males correlated with increased immunocompetence (Rantala *et al.* 2000); other work on damselflies showed that darker wing pigmentation correlated with parasite resistance and hence could signal higher male quality to potential mates (Siva-Jothy 2000; Yourth *et al.* 2002). Variations in melanism are particularly interesting because of the direct role melanin plays in insect immunity. Sexual dimorphism in wing pigmentation has been observed in monarch butterflies (Davis *et al.* 2005), such that females have darker wings than males. Importantly, this is the first study to explore the relationship between immune effector traits, parasite infection and wing pigmentation in monarchs.

In this study, we quantified two immune effector traits (hemocyte concentration and phenoloxidase activity) in male and female monarch butterflies (*Danaus plexippus*) following experimental infection with the naturally occurring protozoan parasite *Ophryocystis elektroscirrha*. We predicted that monarchs exposed to parasites would show greater measures of immunity, provided that the immune defenses we examined are inducible. We also predicted that innate host resistance could be evolutionarily beneficial for both male and female monarch butterflies because this parasite causes wing deformities, decreased body size, reduced longevity, poorer flight ability and decreased mating success (Leong *et al.* 1992; Leong *et al.* 1997; Altizer & Oberhauser 1999). This is particularly true for monarchs in eastern N. America that undergo extreme long-distance migrations and endure a long wintering period prior to the onset of breeding activity (Brower & Malcolm 1991; Brower *et al.* 1995). Hence, even small reductions in survival or flight ability caused by infection could preclude reproductive success the following spring. We further predicted that the costs of parasite infection, and hence the benefits of immunity, might be greater for female monarchs than for males. This is because overwintering males that are in poor condition can mate and transfer spermatophores to females rather than re-migrate north in the spring (Van Hook 1993, 1996; Oberhauser & Frey 1999); females in poor condition do not have this option, and must remigrate > 500km to oviposit on host plants. Previous work has also shown that female body size at eclosion is positively related to lifetime fecundity and egg size (Oberhauser 1997, 2004); thus, parasite-induced reductions in host body size could have additional negative effects on female

reproduction, beyond reduced longevity and mating success. Finally, we examined the relationship between host defenses and adult fitness components in the presence and absence of infection to investigate their potential costs and benefits to both males and females, and we quantified dark pigmentation on the wings of adult butterflies to test the prediction that external melanism correlates positively with measures of parasite resistance.

METHODS AND MATERIALS

The study system. Monarch butterflies (*Danaus plexippus)* are geographically widespread (Ackery & Vane-Wright 1984); all populations examined to date are infected by the neogregarine protozoan *Ophryocystis elektroscirrha,* and prevalence varies dramatically among populations with different migratory strategies (Altizer *et al.* 2000). The life cycle of *O. elektroscirrha* is closely correlated with host development (McLaughlin & Myers 1970). Transmission occurs when larvae ingest spores scattered by infected adults onto eggs and foliage. Parasite spores lyse in the larval gut, and emerging sporozoites penetrate and pass through the gut wall, migrate to the hypoderm, and undergo vegetative schizogony during the host's late larval and early pupal stages. Several days prior to adult butterfly eclosion, parasites undergo sexual reproduction and haploid spore formation. Infected butterflies emerge covered with dormant parasite spores on the outside of their bodies (McLaughlin & Myers 1970; Leong *et al.* 1992).

Monarch sources and mating design. Monarchs used in this experiment were the offspring of 11 wild-caught females mated with 4 wild-caught males

collected from Giles County (Virginia, USA) during June 2005. All monarchs were examined for the presence of *O. elektroscirrha* (Altizer *et al.* 2000) and only uninfected individuals were used to obtain progeny. After mating, female monarchs were placed in 2 separate 0.6m^3 cages supplied with potted greenhouse-reared *Asclepias incarnata*. Plants were transferred to a laboratory and maintained at 24°C after 60 or more eggs were laid on a single plant.

Inoculation and host rearing. Larvae were randomly divided into two groups: parasite treated (N=107) and control (N=110). Parasite spores were obtained from an eastern N. America monarch infected with an eastern strain of *O. elektroscirrha* (collected in Clarkston, GA, USA). We inoculated second instar larvae individually by feeding them 1-cm^2 *A. incarnata* pieces to which 10-15 parasite spores had been manually transferred. Control larvae were fed pieces of *A. incarnata* free of parasite spores. Larvae were maintained singly in 10 cm diameter Petri dishes until they consumed all of the plant material.

After inoculation, we transferred larvae to individual 0.47 L plastic containers with mesh screen lids. We fed fresh cuttings of greenhouse-raised *A. incarnata* to larvae daily, removed frass, changed the lining of the container, and kept the lining moist. *A. incarnata* was sterilized by soaking it in a 20% bleach solution for 20 minutes and rinsing it in tap water prior to use. All rearing containers were kept on laboratory benches (24°C) and their positions were rotated daily. After an individual pupated, the container was moved to a separate laboratory (26°C). We recorded the dates of pupation and eclosion and placed each adult butterfly into a glassine envelope 6-12 hrs post-eclosion. Following eclosion, we recorded sex and time to death in days based on holding monarchs

inactive in the laboratory at 26°C in envelopes without access to food. The measure of longevity reflects both the duration of adult life as well as the amount of stored energy reserves.

Immune parameters. We measured two immune effector traits in late instar larvae: concentration of circulating hemocytes and phenoloxidase activity of the mid-gut tissue. Samples were collected from 5th (final) instar larvae. Hemolymph was extracted from the second to last proleg of a random subset of parasite-treated and control group larvae (*N*=149); 10 μl of hemolymph was placed into 50 μl of an anti-coagulant and anti-melanization PBS 1X [NaCl 1.37 mM, KCl 0.268 mM, Na_2HPO_4 1.08 mM, KH_2PO_4 0.147 mM, EDTA 10mM, Citric Acid 10 mM, PTC 0.0197 mM, pH 7.4 stored at 4°C] solution on ice. Hemocytes were counted on the day of collection using a 6.6 μl chamber hemocytometer under 400X. We performed counts from each of two separate hemocytometer chambers and calculated the average number of cells per μl.

For a subset of larvae sampled for hemocyte concentration (65 out of 149), we extracted the mid-gut immediately after hemolymph collection. The mid-gut section of the larva was removed, rinsed in 4°C anti-coagulant PBS 1X [NaCl 1.37 mM, KCl 0.268 mM, Na_2HPO_4 1.08 mM, KH_2PO_4 0.147 mM, EDTA 10mM, Citric Acid 10mM, pH 7.4], and placed in a 1.5 ml microcentrifuge tube with a 1 ml solution of anti-coagulant PBS and 20 μl of 3% paraformaldehyde. Samples were homogenized in the tube and placed at -20°C. The mid-gut sample was thawed to 4°C, vortexed, and 20 μl of 3% paraformaldehyde was added. We homogenized and vortexed samples a second time and centrifuged at 9000 rpm for one minute. 20 μl of sample was added to 140 μl of distilled water, 20 μl of PBS (1X) ph 7.4,

and 20 µl L Dopa (4mg/mL). We measured the absorbance at 490 nm every 27 seconds at 30°C for 300 measures (02:14:33) using a Biotek microplate reader and KC Junior software. We calculated both the slope of the kinetic curve (absorbance per hour) and the final absorbance during linear phase (verified visually for each sample) to estimate the rate of production of the precursor to melanin, Dopachrome (Barnes & Siva-Jothy 2000; Siva-Jothy 2000; Cerenius & Soderhall 2004). Each sample was assayed twice and the average of the two samples was used for analysis.

Parasite assessment. For all monarchs that survived to adulthood (and not sacrificed for measures of mid-gut phenoloxidase activity N=65), we quantified parasite reproduction based on the number of *O. elektroscirrha* spores on adult butterflies (N=83). Upon death, the abdomen of each monarch was removed and placed into a vial containing 5 ml of deionized water. We vortexed each sample for 5 minutes at maximum speed (Vortex Genie II) and counted the number of dislodged *O. elektroscirrha* spores in two replicate hemocytometer chambers at a magnification of 400X. Average counts per monarch were multiplied by 5×10^5 to estimate the total number of spores per individual.

Body size and melanism. Adult body length (from tip of head to end of abdomen, in mm) was measured using a digital caliper (N=83). We used digital image analysis to measure adult wing size and the external melanism (N=79). Upon death we removed and scanned monarch forewings using a flatbed HP scanner set to 300 dpi using the same exposure for each scan. Measurements were made using Adobe Photoshop software with the Image Processing Tool Kit plugin (Reindeer Graphics, Inc.). Total forewing area (in mm^2) and two measures

of wing melanism were obtained for both forewings of each adult butterfly following Davis *et al.* (2005). We quantified the proportion of area on each forewing encompassed by black pigmentation. We also estimated the density of black pigmentation as an indicator of the overall intensity or opacity of black, with the units of density set so that lower values corresponded to an increased intensity of black. Average measures per individual were based on results for L and R forewings.

Statistical Analysis. Count variables were log transformed (number of spores, development time, and adult longevity) or square root transformed (hemocyte concentration) prior to analysis to normalize the error variance. We tested for equal variances between parasite treatment groups using Fisher's F-test, for normally distributed data, and Levene's Test for non-normally distributed but continuous data, at significance levels of $\alpha=0.05$. Unequal variances based on Levene's Test at $\alpha=0.05$ were observed for adult longevity. We used two-tailed Pearson's correlations to explore the relationships between immune effector traits and morphological measures within and between parasitized and control monarchs. Directed t-tests were used to test the specific prediction that immune parameters were greater in parasite-treated versus control monarchs (Rice & Gaines 1994). Directed tests allocate a greater probability to the tail of the distribution in the predicted direction (γ), while retaining a smaller probability in the opposite tail to detect unexpected deviations opposite to predictions ($\delta<\gamma$; where $\delta + \gamma=\alpha$). We followed the guidelines in Rice and Gaines (1994) by setting γ/α to 0.8, giving values of $\gamma=0.04$ and $\delta=0.01$.

We ran ANCOVA using GLM in SPSS 13.0 to examine the effects of hemocyte concentration, sex and parasite treatment on monarch longevity, parasite load, body length, wing area, and wing melanism (Full model: Dependent variable = parasite + sex + hemocyte concentration + all two- and three-way interactions). We followed Crawley (2002) in performing model simplification and used Akaike's information criterion adjusted for small sample sizes (AICC) for model comparison. Predictor variables with p-values greater than 0.10 were sequentially deleted from the full model starting with the variable with the highest p-value, and then model fit was evaluated at each step. In cases where missing data affected sample sizes amongst statistical models, we used adjusted-R^2 over AICC for model comparison. If a factor with $p>0.10$ affected the significance ($\alpha=0.05$) of another variable it was retained in the final model. We tested for normal distributions of the residuals and all of the minimum adequate models showed normally distributed residuals.

RESULTS

General results. A total of 36 parasitized (48.6%) and 47 control (62.7%) monarchs survived to adulthood (excluding those sacrificed to measure PO activity). Differences in pre-adult mortality of parasitized and control monarchs were not significant (Mann-Whitney $Z=-0.534$, $p=0.593$). Of the monarchs surviving to adulthood, 21 parasitized and 23 control monarchs were sampled for circulating hemocyte concentration as larvae. Although there was greater mortality among individuals sampled for hemocyte concentration the difference was not significant (Mann-Whitney $Z=-0.532$, $p=0.595$). All monarchs in the

parasite-treated group were infected with *O. elektroscirrha*, but no monarchs in the control treatment were infected. Average parasite loads removed from the abdomens of inoculated monarchs were 1.29 x 10^6 spores (range: 0.32 x 10^6 – 5.13 x 10^6). Average parasite loads did not differ between male and female butterflies (t_{80}=0.291, $P_{2\text{-tailed}}$=0.772).

Immune parameters. Across all monarchs sampled for hemolymph, hemocyte concentrations were higher for parasite-treated (29 ± 12 S.D.; N=74) versus control monarchs (23 ± 11 S.D.; N=75) and this difference was significant (t_{147}=-2.991, $P_{Directed}$=0.002), supporting the notion that hemocyte levels increased upon recognition of the parasite. Similar but non-significant differences were observed among the subset of monarchs that survived to adulthood (t_{42}=-1.40, $P_{Directed}$=0.106). No significant difference was found between parasitized monarchs sampled for hemocytes that survived to adulthood and those that did not survive to adulthood (t_{40}=0.512 P=0.612). Females had similar hemocyte concentrations across control and parasite treatments, whereas control males had lower hemocyte concentrations than parasitized males (Figure 1a). A two-way ANOVA did not provide support for effects of treatment or sex on hemocyte concentrations (treatment: $F_{1, 78}$=1.378, p=0.248; sex: $F_{1, 78}$=0.054, p=0.818; treatment*sex: $F_{1, 78}$=1.192, p=0.282). However, directed t-tests demonstrated that hemocyte counts were significantly lower in control versus parasitized males (t_{23}=-1.79, $P_{Directed}$=0.044), whereas concentrations in parasitized and control females were similar (t_{16}=-0.052, $P_{Directed}$=0.480).

Within infected females, parasite loads decreased with increased hemocyte concentrations, and the opposite pattern was observed for males. However,

statistical analysis indicated that parasite load was not significantly associated with sex or hemocyte concentration, and no interactions involving parasite treatment, sex and hemocyte concentration were retained in the final model (Table 1).

Two measures of larval mid-gut PO activity -- final absorbance and the slope of increasing product over time -- were highly correlated with one another (Pearson correlation: R=0.684; N=65; P=0.000). However, neither measure of mid-gut PO activity was correlated with hemocyte concentration (e.g., for reaction slope, Pearson correlation: R=-0.152; N=65; p=0.227). Mid-gut PO activity measures were similar for parasitized and control larvae (Parasitized: mean slope =0.0639 ± 0.0058 S.D.; Control: mean slope =0.0648 ± 0.0079 S.D.) and there was no significant effect of parasite treatment on PO activity (t_{63}=0.534, p=0.595). Mid-gut PO activity could not be compared between males and females because sex was not recorded for dissected larvae.

Adult body size and longevity. Adult longevity, forewing area and body length were lower among parasite-treated versus control monarchs and males incurred a greater proportional decrease in each of these measures relative to females (Figures 1b-d). However, for adult longevity neither sex nor the three way interaction between sex, parasite infection and hemocyte concentration were statistically significant (Table 1). Among control females, hemocyte concentrations were negatively associated with both body length and wing area, whereas parasitized females showed the opposite trend (Figure 2a, only body length shown). Among males, both control and parasite-treated monarchs showed a positive association between hemocyte concentration and measures of

body size (Figure 2b). These patterns were supported by statistical analyses showing a significant 3-way interaction between sex, parasite infection and hemocyte concentration for analyses involving both body length and forewing area (Table 1).

Wing melanism. Females had a greater proportion of black pigmentation on their forewings (65.86 ± 2.60 S.D.; N=35) than males (56.20 ± 1.96 S.D.; N=44) and also showed a higher intensity (i.e., density) of black pigmentation (Females: 133.80 ± 7.80 S.D.; N=36; Males: 147.02 ± 8.29 S.D.; N=46; lower density scores are associated with more intense black). Control females had the greatest proportion of black pigmentation on their forewings (66.71 ± 1.98 S.D.; N=21), followed by parasitized females (64.59 ± 2.95 S.D.; N=14), control males (56.80 ± 2.13 S.D.; N=26), and parasitized males (55.33 ± 1.29 S.D.; N=18). The main effects of parasite treatment and sex on the proportion of black pigmentation were significant, but not the two-way interaction (Table 1), consistent with the observation that greater wing melanism among uninfected monarchs was consistent for both males and females. Within the subset of parasitized females, final spore loads were negatively associated with the proportion of black on adult forewings, although this relationship was not significant (Pearson's correlation: R=-0.260, N=14, P=0.370). No relationship was observed between wing melanism and parasite loads for males, and we also observed no relationship between hemocyte concentration and measures of wing melanism (Table 1).

DISCUSSION

Our results showed that average hemocyte concentrations, but not measures of mid-gut phenoloxidase (PO) activity, were greater among larvae experimentally challenged with the neogregarine protozoan *O. elektroscirrha*. Among monarchs surviving to adulthood, only males, showed evidence of greater hemocyte production following parasite infection. Together, these findings suggest that 1) production of hemocytes increased in the presence of a debilitating parasite, and 2) males and females may differ in baseline hemocyte production and response to infection. Previous work has demonstrated that insects can show increased expression of immune defenses, including hemocyte concentrations, following acute challenges by foreign materials (Freitak *et al.* 2003; Robb & Forbes 2006) although patterns vary with insect species and the specific immune effector traits assayed (Siva-Jothy *et al.* 2001; Yourth *et al.* 2002; Adamo 2004; Chernysh *et al.* 2004; Armitage & Siva-Jothy 2005). The increased hemocyte production observed here indicates that this immune effector trait might be an inducible (rather than constitutive) defense under some circumstances.

Although we predicted that mid-gut defenses could be important during the initial phase of parasite infection when *O. elektroscirrha* sporozoites penetrate the gut wall (McLaughlin & Myers 1970), there was no association between mid-gut PO activity and parasite treatment. However, it is possible that mid-gut PO activity responds to infection at an earlier life stage than we examined here. Because larvae were sampled destructively for the mid-gut assay, we were unable to examine how mid-gut PO activity affects the final outcome of infection, or how this measure differed between males and females. Moreover,

our results provided no evidence for a correlation between hemocyte concentrations and mid-gut PO activity, which appears to be consistent with a general phenomenon that associations between different immune effort traits vary among insect species, with some studies showing positive associations between multiple measures of immune defenses e.g., in desert locusts, crickets, scorpionflies and Egyptian cotton leafworms (Gillespie *et al.* 2000; Kurtz *et al.* 2000; Adamo 2004; Cotter *et al.* 2004b), and others showing no association between measures of immune defenses e.g., in crickets and damselflies (Adamo *et al.* 2001; Rolff 2001).

The finding that parasitized males had greater average hemocyte counts than control males, but that females showed similar concentration of hemocytes following infection, was supported by directed t-tests of hemocyte concentrations in relation to parasite treatment. One explanation for this finding is that females could benefit more from maintaining higher baseline immune defenses than males (Zuk 1990; Zuk & McKean 1996; Yourth *et al.* 2002; Zuk *et al.* 2004). Greater investment or benefits from immunity by females have been demonstrated for other insect species including milkweed beetles (Abbot & Dill 2001), wolf spiders (Ahtiainen *et al.* 2005), scorpionflies (Kurtz *et al.* 2000; Kurtz & Sauer 2001), field crickets (Adamo *et al.* 2001), house crickets (Gray 1998), and damselflies (Rolff 2001; Yourth *et al.* 2002). Alternatively, counterpoints to this pattern have been demonstrated in *Drosophila* (McKean & Nunney 2005), crickets (Zuk *et al.* 2004), earwigs (Rantala *et al.* 2007), butterflies (Stoehr 2007), and in a meta-analysis of studies from arthropod hosts (Sheridan *et al.* 2000). For example, Stoehr (2007) demonstrated that sex

differences in the encapsulation response of adult cabbage butterflies changed with age, such that younger males, but older females had higher rates of encapsulation.

If host defenses are costly, then greater investment in immune defenses should correlate negatively with host longevity and body size in the absence of infection. Among control females, greater hemocyte concentrations were indeed associated with reduced body length and forewing area. The opposite trend was observed for parasite-treated females; monarchs with greater hemocyte concentrations suffered less from the negative effects of *O. elektroscirrha*. These trends are consistent with the idea that female monarchs benefit from maintaining a costly defense if the risk of infection is sufficiently high. This finding is important in light of previous work that demonstrated negative effects of *O. elektroscirrha* on adult monarch body size (Altizer & Oberhauser 1999); indeed, de Roode *et al.* (2007) showed that although male and female butterflies showed similar longevity reductions in response to parasite infection, the negative effect of parasitism on adult body size was much greater for females. Because female body size in monarchs is known to correlate positively with lifetime fecundity and egg size (Oberhauser 1997, 2004), the consequences of infection could be more substantial for females than for males. Moreover, in monarch populations that migrate long distances, such as in eastern N. America, selection pressures on female longevity and greater body size could be even more intense. This is because males in poor condition can mate before the wintering season ends (Van Hook 1996; Oberhauser & Frey 1999), but females must fly hundreds of kilometers to oviposit on milkweed host plants in the southeastern

United States. Heavily parasitized females are likely to have an extremely low probability of reproducing under such demanding conditions, leading to greater benefits for potentially costly immune defenses in females as compared to males.

In monarchs, females have darker forewings than males, in terms of the proportion of wing area covered by scales with black pigmentation (Davis *et al.* 2005). Our study confirmed this difference in wing melanism between males and females, and further showed that wing melanism correlates with monarch butterfly infection status. Specifically, control monarchs had darker forewings than parasitized monarchs, and this difference was observed among both males and females. It is therefore possible, as demonstrated for other insect systems (Barnes & Siva-Jothy 2000; Wilson *et al.* 2001; Cotter *et al.* 2004a), that resistance to parasitism is correlated with external melanism on monarch forewings, and that wing pigmentation could be an indicator of the infection status of potential mates. Two further explanations for darker wings among unparasitized butterflies are that 1) resources for melanin production are limited and may be allocated to only one function (i.e. wing melanism or parasite defense; Hooper *et al.* 1999; Talloen *et al.* 2004; Freitak *et al.* 2005), or that 2) coloration/melanism is influenced by the general overall "health" of an individual (Stoehr 2006).

In summary, this study provides support for differential costs and consequences of immune defenses in male and female monarch butterflies following infection by *O. elektroscirrha*. Further work incorporating a range of parasite doses and larger samples sizes is needed to evaluate the degree to which males and females differ in their baseline and induced defenses, and the degree

to which different immune effector traits influence parasite replication. To our knowledge, this study is the first to quantify immune defenses of a butterfly species in response to a naturally-occurring protozoan parasite, and further emphasizes the need to understand how insect species balance investment in potentially costly immune defenses with other life history demands in the face of uncertain infection risk.

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REFERENCES

Abbot P. & Dill L.M. (2001). Sexually transmitted parasites and sexual selection in the milkweed leaf beetle, *Labidomera clivicollis*. *Oikos*, 92, 91-100.

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

Adamo S.A. (2004). Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. *Journal of Insect Physiology*, 50, 209-216.

Adamo S.A., Jensen M. & Younger M. (2001). Changes in lifetime immunocompetence in male and female *Gryllus texensis* (formerly *G-integer*): trade-offs between immunity and reproduction. *Animal Behaviour*, 62, 417-425.

Ahtiainen J.J., Alatalo R.V., Kortet R. & Rantala M.J. (2005). A trade-off between sexual signalling and immune function in a natural population of the drumming wolf spider *Hygrolycosa rubrofasciata*. *Journal of Evolutionary Biology*, 18, 985-991.

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

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

Andersson M. (1994). *Sexual Selection.* Princeton Univeristy Press, Princeton, N.J.

Armitage S.A.O. & Siva-Jothy M.T. (2005). Immune function responds to selection for cuticular colour in *Tenebrio molitor*. *Heredity*, 94, 650-656.

Barnes A.I. & Siva-Jothy M.T. (2000). Density-dependent prophylaxis in the mealworm beetle *Tenebrio molitor L*-(Coleoptera : Tenebrionidae): cuticular melanization is an indicator of investment in immunity. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 267, 177-182.

Boots M. & Begon M. (1993). Trade-offs with resistance to a granulosis-virus in the Indian meal moth, examined by a laboratory evolution experiment. *Functional Ecology*, 7, 528-534.

Brower L.P., Fink L.S., Brower A.V., Leong K., Oberhauser K., Altizer S., Taylor O., Vickerman D., Calvert W.H., Vanhook T., Alonsomejia A., Malcolm S.B., Owen D.F. & Zalucki M.P. (1995). On the dangers of interpopulational transfers of monarch butterflies - discussion. *Bioscience*, 45, 540-544.

Brower L.P. & Malcolm S.B. (1991). Animal migrations: endangered phenomena. *American Zoologist*, 31, 265-276.

Cerenius L. & Soderhall K. (2004). The prophenoloxidase-activating system in invertebrates. *Immunological Reviews*, 198, 116-126.

Chernysh S.I., Filatova N.A., Chernysh N.S. & Nesin A.P. (2004). Cytotoxic activity of blowfly *Calliphora vicina* hemocytes. *Journal of Insect Physiology*, 50, 777-781.

Cotter S.C., Hails R.S., Cory J.S. & Wilson K. (2004a). Density-dependent prophylaxis and condition-dependent immune function in Lepidopteran larvae: a multivariate approach. *Journal of Animal Ecology*, 73, 283-293.

Cotter S.C., Kruuk L.E.B. & Wilson K. (2004b). Costs of resistance: genetic correlations and potential trade-offs in an insect immune system. *Journal of Evolutionary Biology*, 17, 421-429.

Cotter S.C. & Wilson K. (2002). Heritability of immune function in the caterpillar *Spodoptera littoralis*. *Heredity*, 88, 229-234.

Crawley M.J. (2002). *Statistical Computing: An Introduction to Data Analysis using S-Plus*. John Wiley and Sons, West Sussex, England.

da Silva C., Dunphy G.B. & Rau M.E. (2000). Interaction of hemocytes and prophenoloxidase system of fifth instar nymphs of *Acheta domesticus* with bacteria. *Developmental and Comparative Immunology*, 24, 367-379.

Davis A.K., Farrey B.D. & Altizer S. (2005). Variation in thermally induced melanism in monarch butterflies (Lepidoptera : Nymphalidae) from three North American populations. *Journal of Thermal Biology*, 30, 410-421.

de Roode J.C., Gold L.R. & Altizer S. (2007). Virulence determinants in a natural butterfly-parasite system. *Parasitology*, 134, 657-668.

Dunphy G.B. (1991). Phenoloxidase Activity In The Serum Of 2 Species Of Insects, The Gypsy-Moth, *Lymantria-Dispar* (Lymantriidae) and the greater wax moth, *Galleria-Mellonella* (Pyralidae). *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, 98, 535-538.

Eslin P. & Prevost G. (1996). Variation in *Drosophila* concentration of haemocytes associated with different ability to encapsulate *Asobara tabida* larval parasitoid. *Journal of Insect Physiology*, 42, 549-555.

Fedorka K.M., Zuk M. & Mousseau T.A. (2004). Immune suppression and the cost of reproduction in the ground cricket, *Allonemobius socius*. *Evolution*, 58, 2478-2485.

Freitak D., Ots I., Vanatoa A. & Horak P. (2003). Immune response is energetically costly in white cabbage butterfly pupae. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 270, S220-S222.

Freitak D., Vanatoa A., Ots I. & Rantala M.J. (2005). Formation of melanin-based wing patterns is influenced by condition and immune challenge in *Pieris brassicae*. *Entomologia Experimentalis et Applicata*, 116, 237-243.

Gillespie J.P., Burnett C. & Charnley A.K. (2000). The immune response of the desert locust *Schistocerca gregaria* during mycosis of the entomopathogenic fungus, *Metarhizium anisopliae var acridum*. *Journal of Insect Physiology*, 46, 429-437.

Gillespie J.P., Kanost M.R. & Trenczek T. (1997). Biological mediators of insect immunity. *Annual Review of Entomology*, 42, 611-643.

Gray D.A. (1998). Sex differences in susceptibility of house crickets, *Acheta domesticus*, to experimental infection with *Serratia liquefaciens*. *Journal of Invertebrate Pathology*, 71, 288-289.

Hooper R.E., Tsubaki Y. & Siva-Jothy M.T. (1999). Expression of a costly, plastic secondary sexual trait is correlated with age and condition in a damselfly with two male morphs. *Physiological Entomology*, 24, 364-369.

Kurtz J. & Sauer K.P. (2001). Gender differences in phenoloxidase activity of *Panorpa vulgaris* hemocytes. *Journal of Invertebrate Pathology*, 78, 53-55.

Kurtz J., Wiesner A., Gotz P. & Sauer K.P. (2000). Gender differences and individual variation in the immune system of the scorpionfly *Panorpa vulgaris* (Insecta : Mecoptera). *Developmental and Comparative Immunology*, 24, 1-12.

Leong K.L.H., Kaya H.K., Yoshimura M.A. & Frey D.F. (1992). The occurrence and effect of a protozoan parasite *Ophryocystis elektroscirrha* (Negregarinida: Ophryocystidae) on overwintering monarch butterflies *Danaus plexippus* (Lepidoptera: Danaidae) from two California winter sites. *Ecological Entomology*, 17, 338-342.

Leong K.L.H., Yoshimura M.A., Kaya H.K. & Williams H. (1997). Instar susceptibility of the monarch butterfly (*Danaus plexippus*) to the neogregarine parasite, *Ophryocystis elektroscirrha*. *Journal of Invertebrate Pathology*, 69, 79-83.

McKean K.A. & Nunney L. (2001). Increased sexual activity reduces male immune function in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, 98, 7904-7909.

McKean K.A. & Nunney L. (2005). Bateman's principle and immunity: phenotypically plastic reproductive strategies predict changes in immunological sex differences. *Evolution*, 59, 1510-1517.

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

Moore S.L. & Wilson K. (2002). Parasites as a viability cost of sexual selection in natural populations of mammals. *Science*, 297, 2015-2018.

Moret Y. & Schmid-Hempel P. (2000). Survival for immunity: the price of immune system activation for bumblebee workers. *Science*, 290, 1166-1168.

Nigam Y., Maudlin I., Welburn S. & Ratcliffe N.A. (1997). Detection of phenoloxidase activity in the hemolymph of tsetse flies, refractory and susceptible to infection with *Trypanosoma brucei rhodesiense*. *Journal of Invertebrate Pathology*, 69, 279-281.

Norris K. & Evans M.R. (2000). Ecological immunology: life history trade-offs and immune defense in birds. *Behavioral Ecology*, 11, 19-26.

Oberhauser K.S. (1997). Fecundity, lifespan and egg mass in butterflies: effects of male-derived nutrients and female size. *Functional Ecology*, 11, 166-175.

Oberhauser K.S. (2004). Effects of female age, female mass and nutrients from males on monarch egg mass. In: *The Monarch Butterfly: Biology and Conservation* (eds. Oberhauser KS & Solensky MJ). University Press Cornell, pp. 21-26.

Oberhauser K.S. & Frey D.F. (1999). Coercive mating by overwintering male monarch butterflies. In: *The 1997 North American Conference on the Monarch Butterfly* (eds. Hoth J, Merino L, Oberhauser KS, Pisanty I, Price

S & Wilkson T). Commission for Environmental Cooperation Montreal, pp. 67-68.

Paskewitz S.M., Brown M.R., Collins F.H. & Lea A.O. (1989). Ultrastructural-localization of phenoloxidase in the midgut of refractory *Anopheles-gambiae* and association of the enzyme with encapsulated *Plasmodium-cynomolgi*. *Journal of Parasitology*, 75, 594-600.

Radhika M., Nazar A.K.A., Munuswamy N. & Nellaiappan K. (1998). Sex-linked differences in phenol oxidase in the fairy shrimp *Streptocephalus dichotomus baird* and their possible role (Crustacea : Anostraca). *Hydrobiologia*, 377, 161-164.

Rantala M.J., Koskimaki J., Taskinen J., Tynkkynen K. & Suhonen J. (2000). Immunocompetence, developmental stability and wingspot size in the damselfly *Calopteryx splendens L. Proceedings of the Royal Society of London Series B-Biological Sciences*, 267, 2453-2457.

Rantala M.J., Roff D.A. & Rantala L.M. (2007). Forceps size and immune function in the earwig *Forficula auricularia L. Biological Journal of the Linnean Society*, 90, 509-516.

Rice W.R. & Gaines S.D. (1994). Heads I win, tails you lose - testing directional alternative hypotheses in ecological and evolutionary research. *Trends in Ecology & Evolution*, 9, 235-237.

Robb T. & Forbes M.R. (2006). Sex biases in parasitism of newly emerged damselflies. *Ecoscience*, 13, 1-4.

Rolff J. (2001). Effects of age and gender on immune function of dragonflies (*Odonata, Lestidae*) from a wild population. *Canadian Journal of Zoology-Revue Canadienne de Zoologie*, 79, 2176-2180.

Rolff J. & Siva-Jothy M.T. (2003). Invertebrate ecological immunology. *Science*, 301, 472-475.

Rolff J. & Siva-Jothy M.T. (2004). Selection on insect immunity in the wild. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 271, 2157-2160.

Schmid-Hempel P. (2005). Evolutionary ecology of insect immune defenses. *Annual Review of Entomology*, 50, 529-551.

Schmid-Hempel P. & Ebert D. (2003). On the evolutionary ecology of specific immune defence. *Trends in Ecology & Evolution*, 18, 27-32.

Sheridan L.A.D., Poulin R., Ward D.F. & Zuk M. (2000). Sex differences in parasitic infections among arthropod hosts: is there a male bias? *Oikos*, 88, 327-334.

Silva J.E.B., Boleli I.C. & Simoes Z.L.P. (2002). Hemocyte Types and Total and Differential Counts in Unparasitized and Parasitized *Anastrepha obliqua* (Diptera, Tephritidae) Larvae. *Brazilian Journal of Biology*, 62, 689-699.

Siva-Jothy M.T. (2000). A mechanistic link between parasite resistance and expression of a sexually selected trait in a damselfly. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 267, 2523-2527.

Siva-Jothy M.T., Tsubaki Y., Hooper R.E. & Plaistow S.J. (2001). Investment in immune function under chronic and acute immune challenge in an insect. *Physiological Entomology*, 26, 1-5.

Stoehr A.M. (2006). Costly melanin ornaments: the importance of taxon? *Functional Ecology*, 20, 276-281.

Stoehr A.M. (2007). Inter- and intra-sexual variation in immune defense in the cabbage white butterfly, *Pieris rapae L.* (Lepidoptera : Pieridae). *Ecological Entomology* 32, 188-193.

Stoehr A.M. & Kokko H. (2006). Sexual dimorphism in immunocompetence: what does life-history theory predict? *Behavioral Ecology*, 17, 751-756.

Talloen W., Van Dyck H. & Lens L. (2004). The cost of melanization: butterfly wing coloration under environmental stress. *Evolution*, 58, 360-366.

Van Hook T. (1993). Non-random mating behavior in monarch butterflies overwintering in Mexico. Biology and Conservation of the Monarch Butterfly In: *Biology and Conservation of the Monarch Butterfly* (eds. Malcolm SB & Zalucki MP). History Museum of Los Angeles County Los Angeles, California, pp. 49-60.

Van Hook T. (1996). *Monarch butterfly mating ecology at a Mexican overwintering site: proximate causes of non-random mating*. University of Florida, Gaineville, FL.

Viney M.E., Riley E.M. & Buchanan K.L. (2005). Optimal immune responses: immunocompetence revisited. *Trends in Ecology & Evolution*, 20, 665-669.

Wilson K., Cotter S.C., Reeson A.F. & Pell J.K. (2001). Melanism and disease resistance in insects. *Ecology Letters*, 4, 637-649.

Yourth C.P., Forbes M.R. & Baker R.L. (2002). Sex differences in melanotic encapsulation responses (immunocompetence) in the damselfly *Lestes*

forcipatus Rambur. Canadian Journal of Zoology-Revue Canadienne de Zoologie, 80, 1578-1583.

Zuk M. (1990). Reproductive strategies and disease susceptibility - an evolutionary viewpoint. *Parasitology Today*, 6, 231-233.

Zuk M. & McKean K.A. (1996). Sex differences in parasite infections: Patterns and processes. *International Journal for Parasitology*, 26, 1009-1023.

Zuk M., Simmons L.W., Rotenberry J.T. & Stoehr A.M. (2004). Sex differences in immunity in two species of field crickets. *Canadian Journal of Zoology-Revue Canadienne de Zoologie*, 82, 627-634.

Zuk M. & Stoehr A.M. (2002). Immune defense and host life history. *American Naturalist*, 160, S9-S22.

Table 1. Analysis of variance of dependent variables (column headings) and independent variables included in the final minimum adequate models. Analyses began with the full model: Response variable = parasite treatment + sex + hemocyte concentration + treatment*sex + hemocyte*treatment + hemocyte*sex + treatment*sex*hemocyte + error term. Model simplification was performed as described in Methods text. F- and P-values are shown only for explanatory variables retained in the final model; adjusted R^2 is shown for the final reduced models. The direction of the effect of independent variables on dependent variables is indicated as follows: ↑ increase/positive relationship and ↓decrease/positive relationship, and ↑♀ greater in females. For the independent variable "Parasite Treatment" the direction indicated is for the parasitized monarchs.

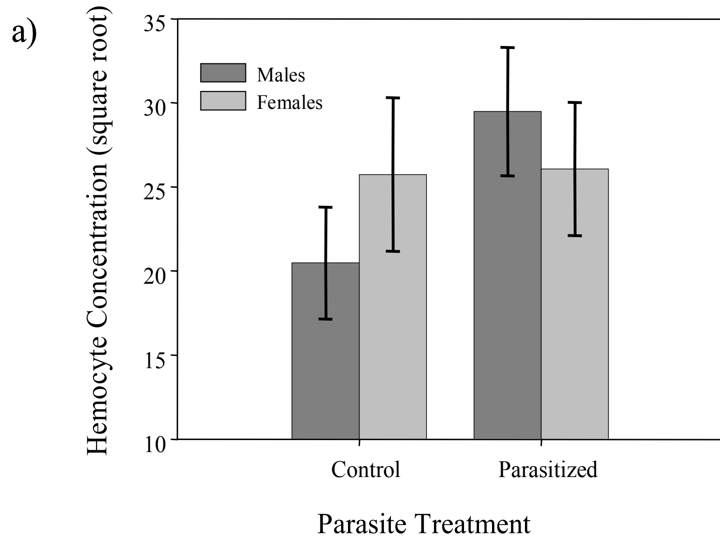
Source	Parasite Load	Adult Longevity	Body length	Forewing area	Proportion black	Density black
Parasite Treatment	$F_{1,80}$=4786.107, **P=0.000** ↑	$F_{1,80}$=292.319, **P=0.000** ↓	$F_{1,35}$=6.204, **P=0.018** ↓	$F_{1,35}$=2.180, P=0.149	$F_{1,76}$=13.367, **P=0.000** ↓	$F_{1,79}$ = 1.961, P = 0.165
Sex			$F_{1,35}$=0.741, P=0.395	$F_{1,35}$=0.510, P=0.480	$F_{1,76}$=410.714, **P=0.000** ↑♀	$F_{1,79}$ = 54.361, **P = 0.000** ↑♀
Hemocytes			$F_{1,35}$=3.956, **P=0.055** ↑	$F_{1,35}$=3.897, **P=0.056** ↑		
Sex* Parasite Treatment			$F_{1,35}$=2.398, P=0.130	$F_{1,35}$=3.483, P=0.070		
Sex* Hemocytes			$F_{1,35}$=0.108, P=0.744	$F_{1,35}$=0.010, P=0.922		
Parasite Treatment* Hemocytes			$F_{1,35}$=2.612, P=0.115	$F_{1,35}$=0.531, P=0.471		
Sex* Parasite Treatment*Hemocytes			$F_{1,35}$=4.155, **P=0.049**	$F_{1,35}$=4.845, **P=0.034**		
Adjusted R^2	**.983**	**.782**	**.233**	**.085**	**.844**	**.406**

FIGURE LEGENDS

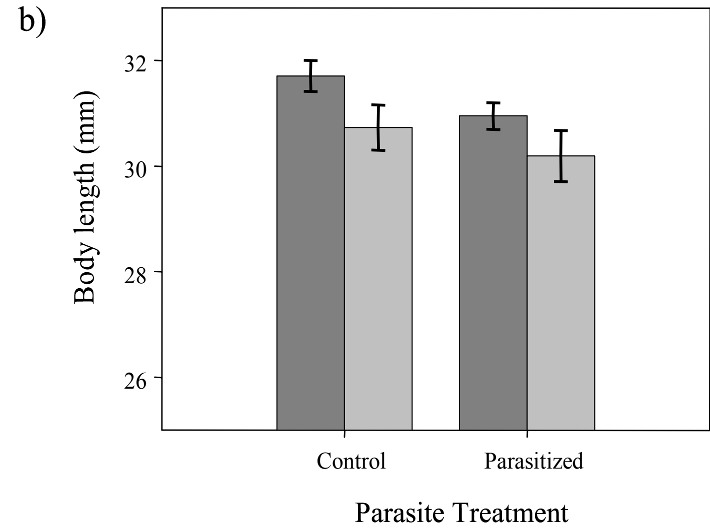
Figure 1. Effects of parasite treatment and sex on (a) hemocyte concentration of larvae (cells per μl of hemolymph, square-root transformed), (b) adult body length (in mm), (c) adult forewing area (in mm^2), and (d) adult longevity (in days). In each figure, data for males are shown in black and females are shown in grey. Error bars represent standard error.

Figure 2. Relationship between hemocyte concentration of larvae (square-root transformed) and adult body length for (a) females and (b) males. Separate trend lines are shown for unparasitized (dashed line, open circles) and parasitized (solid line, closed circles) treatment groups. Linear regression slopes and adjusted R^2 values based on simple regression models are as follows: for control females, slope = -0.303, t_9 = -0.899, P = 0.395; R^2 = 0.092; for parasitized females, slope = 0.615, t_7 = 1.913, P = 0.104; R^2 = 0.379; for control males, slope = 0.441, t_{12} = 1.630, P = 0.131; R^2 = 0.195; for parasitized males, slope = 0.411, t_{11} = 1.428, P = 0.184; R^2 = 0.169.

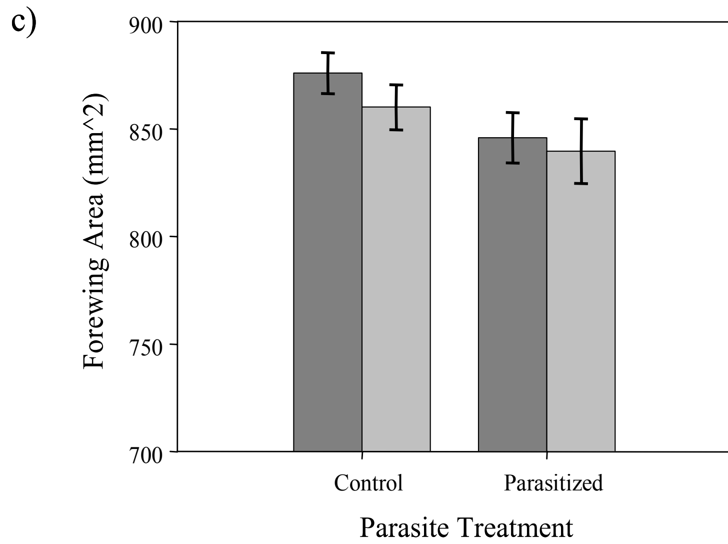
a)



b)



c)



d)

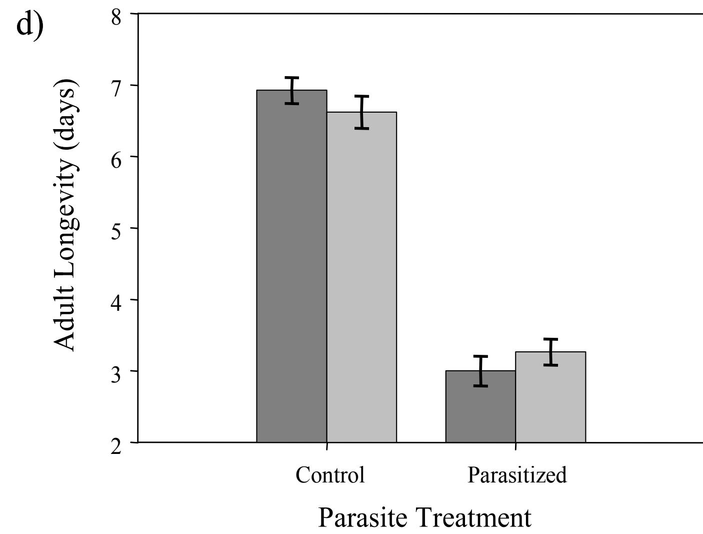
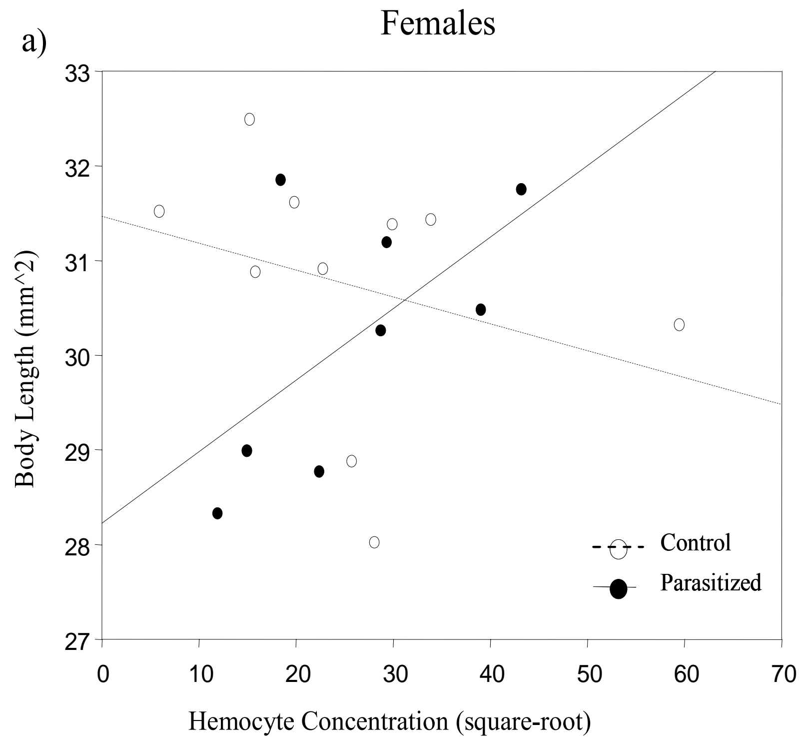


Figure 1



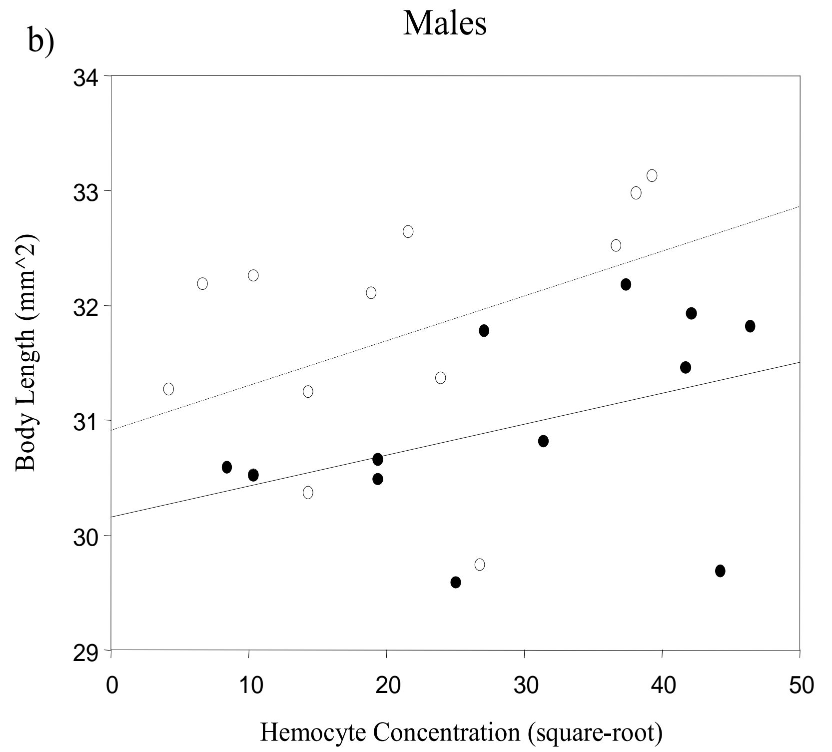


Figure 2

CHAPTER 2

Crowding and disease: effects of host density on response to infection in a butterfly-parasite interaction

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Crowding and disease: effects of host density on response to infection in a butterfly-parasite interaction

Elizabeth Lindsey, Mudresh Mehta, Varun Dhulipala, Karen Oberhauser, and Sonia Altizer

ABSTRACT

1. Hosts experiencing frequent variation in density are thought to benefit from allocating more resources to parasite defense when density is high ('density-dependent prophylaxis'). However, high density conditions can increase intraspecific competition and induce physiological stress, hence increasing host susceptibility to infection ('crowding-stress hypothesis').
2. We studied monarch butterflies (*Danaus plexippus*) and quantified the effects of larval rearing density on susceptibility to the protozoan parasite *Ophryocystis elektroscirrha*. Larvae were inoculated with parasite spores and reared at three density treatments: low, moderate, and high. We examined the effects of larval density on parasite loads, host survival, development rates, body size, and wing melanism.
3. Results showed an increase in infection probability with greater larval density. Monarchs in the moderate and high density treatments also suffered the greatest negative effects of parasite infection on body size, development rate, and adult longevity.
4. We observed greater body sizes and shorter development times for monarchs reared at moderate densities, and this was true for both unparasitized and parasite-treated monarchs. We hypothesize that this

effect could result from greater larval feeding rates at moderate densities, combined with greater physiological stress at the highest densities.

5. Although monarch larvae are assumed to occur at very low densities in the wild, an analysis of continent-wide monarch larval abundance data showed that larval densities can reach high levels in year-round resident populations and during the late phase of the breeding season. Treatment levels used in our experiment captured ecologically-relevant variation in larval density observed in the wild.

Key words: density-dependent prophylaxis, rearing density, melanism, host-parasite interaction, monarch butterfly, *Danaus plexippus*, neogregarine protozoan, *Ophryocystis elektroscirrha*

INTRODUCTION

It is often assumed that animals living in larger groups or at higher population densities should experience a greater risk of acquiring infectious diseases (Alexander, 1974; Altizer *et al.*, 2003; Freeland, 1976; Krause & Ruxton, 2002; Møller *et al.*, 1993; Moore, 2002). This is mainly because host contact rates and the transmission of parasites spread by close proximity among individuals are predicted to increase with host population density (Anderson & May, 1979, 1981; Lloyd-Smith *et al.*, 2005; McCallum *et al.*, 2001). Several field and experimental studies support this assumption; mammals (Freeland, 1979; Hoogland, 1979, 1995; Wilkinson, 1985), birds (Brown & Brown, 1986; Shields & Crook, 1987), and insects (Dwyer & Elkinton, 1993; Knell *et al.*, 1996; Ryder *et al.*, 2005) exhibit positive relationships between measures of parasite prevalence or intensity and host population density or group size. For example, in the African army worm (*Spodoptera exempta*), high host density results in increased host activity (Reeson *et al.*, 2000), which directly affects contact rates between susceptible hosts and pathogens.

As a result of increased parasite risk, animals living at higher population densities are predicted to invest more resources in resistance to infection, including behavioral and immune defenses (Møller & Erritzoe, 1996; Møller *et al.*, 2001). Within a species, increased host resistance in response to crowding or higher host density has been termed 'density-dependent prophylaxis' (Reeson *et al.*, 1998); under this scenario, greater investment in parasite resistance is presumed to counter the risk of increased transmission (Barnes & Siva-Jothy, 2000; Wilson *et al.*, 2001). There is substantial evidence that animals

experiencing higher densities do indeed show greater resistance to infectious diseases (Goulson & Cory, 1995; Kunimi & Yamada, 1990; Reeson *et al.*, 1998; Wilson *et al.*, 2002). For example, one study showed that in a phase-polymorphic moth species, dark-colored larvae (the high density phenotype) exhibited greater immune defenses than pale-colored larvae (based on higher hemolymph and cuticular phenoloxidase activity and a stronger encapsulation response; Cotter *et al.*, 2004). Other studies have shown that larvae reared under higher densities tend to develop darker cuticular melanism (Hagen *et al.*, 2003; Lee & Wilson, 2006; Simmonds & Blaney, 1986). This is important because for some species, an increase in external melanism correlates with an increase in immune effector traits (Barnes & Siva-Jothy, 2000; Cotter *et al.*, 2004; Reeson *et al.*, 1998; Wilson *et al.*, 2001), although this trend does not hold for all insect species (Hagen *et al.*, 2006; Pie *et al.*, 2005; Robb *et al.*, 2003). It is also important to note that some studies have found no effect of host density on measures of immunity, including for field crickets (Adamo, 2006) and termites (Pie *et al.*, 2005).

Animals living in high density populations might also experience more intense competition for resources. Thus, a second key hypothesis is that high density leads to physiological or nutritional stress and that animals in crowded conditions will be more susceptible to infectious diseases relative to less crowded hosts. In insects, this hypothesis was initially examined by Steinhaus (1958) in studies of caterpillars and their natural pathogens. More recent experimental studies on lepidopteran hosts have shown that animals reared at higher densities experience reduced disease resistance and/or decreased time to death (Goulson &

Cory, 1995; Reilly & Hajek, 2008). However, Brown *et al.* (2003) found no effect of host-resource stress on infection or immunity in bumblebees.

Here we ask how host rearing density affects the outcome of infection by a common protozoan parasite, *Ophryocystis elektroscirrha*, in monarch butterflies, *Danaus plexippus*. Parasitism by *O. elektroscirrha* occurs in all monarch populations examined to date, but prevalence varies widely both within and among populations (Altizer *et al.*, 2000; Leong *et al.*, 1997). Monarch densities in the wild vary over space and time (Ackery & Vane-Wright, 1984; Prysby & Oberhauser, 2004); hence, it is possible that effects of host density on susceptibility to infection will affect patterns of infection in the wild. We reared monarch larval stages under low (single larva), moderate, and high densities and compared infection probability, development, and survival among treatments. We expected that monarchs reared under the highest density treatment would develop the fastest (based on time to pupation and adulthood), and show smaller body sizes than monarchs reared at lower densities. Monarchs reared under the highest densities could show greater susceptibility to infection, in support of the stress-disease hypothesis, and would thus experience greater lethal and sub-lethal effects of parasitism on host fitness. Alternatively, monarchs reared under high densities could invest more in disease resistance, in support of the density-dependent prophylaxis hypothesis. This could be manifested by darker wing coloration, which may correlate with resistance to infection (Barnes & Siva-Jothy, 2000).

MATERIALS AND METHODS

Host-parasite system. Monarch butterflies inhabit islands and continents worldwide (Ackery & Vane-Wright, 1984), migrate annually in temperate North America and Australia (Brower, 1995; James, 1993; Urquhart & Urquhart, 1978), and form resident populations that breed year-round in tropical locations such as South Florida and Hawaii (Knight, 1998; Stimson & Berman, 1990). Although monarchs generally lay eggs singly on host plants (Farrey & Davis, 2004; Prysby & Oberhauser, 2004; Zalucki & Kitching, 1982), multiple larvae can occupy the same plant, especially in areas where host plants are patchily distributed or rare. In support of this, observations of monarchs breeding year-round in South Florida indicate that it is not unusual to find plants with several larvae feeding on them (e.g., Brower, 1964; Farrey & Davis, 2004). By comparison, across the large breeding range of monarchs in North America, host plants (including common milkweed, *Asclepias syriaca*) are common and widespread, and larval densities per plant can be exceedingly low, with a single larva occurring on roughly one out of every 30-50 host plants examined (Prysby & Oberhauser, 2004). Other studies have shown that per-plant larval densities can vary over time and space in response to weather-related abiotic factors (Zalucki & Rochester, 1999; Figure 1) and as monarch numbers increase over the course of a breeding season (Prysby & Oberhauser, 2004).

The neogregarine protozoan parasite *Ophryocystis elektroscirrha* occurs naturally in wild monarch populations (Altizer *et al.*, 2000; Leong *et al.*, 1997) and is transmitted when adult butterflies scatter parasite spores on eggs and milkweed leaves. After spores are ingested by larvae, emerging sporozoites

penetrate the gut wall, migrate to the larval hypoderm, and undergo vegetative schizogony (McLaughlin & Myers, 1970). During the host pupal stage, the parasite undergoes sexual reproduction and haploid spores are formed 2-3 days before adult butterflies eclose from their pupal cases. Infected butterflies emerge covered with dormant parasite spores on the outside of their bodies, concentrated primarily on the abdomen (Leong *et al.*, 1992; McLaughlin & Myers, 1970). Negative effects of *O. elektroscirrha* depend on initial dose and the stage at which hosts are infected (de Roode *et al.*, 2007), and include pre-adult mortality, shorter adult longevity, smaller adult body sizes, smaller forewings, and lower flight ability (Altizer & Oberhauser, 1999; Bradley & Altizer, 2005; de Roode *et al.*, 2007; Lindsey & Altizer, 2008).

All monarch populations examined to date have been parasitized by *O. elektroscirrha,* and prevalence is highly variable across different regions (Altizer *et al.*, 2000; Leong *et al.*, 1997). Monarchs in resident populations that breed year round (i.e., in southern Florida and Hawaii) bear the highest parasite loads (over 70% heavily infected). Approximately 30% of monarchs from a migratory population in western North America are heavily infected (Altizer *et al.*, 2000; Leong *et al.*, 1992). Less than 8% of monarchs from the eastern migratory population (longest-distance migrants) are heavily infected (Altizer *et al.*, 2000). These differences among populations have persisted for many years and could be caused by differences in monarch migratory behavior (Altizer *et al.*, 2000; Bradley & Altizer, 2005), local population densities, or environmental variation among sites. In support of a role for monarch density in affecting parasite prevalence, prevalence of *O. elektroscirrha* in eastern N. America increases from

early spring to late summer, as might occur with increases in adult and larval abundance during the summer months (S. Altizer, unpublished data, www.monarchparasites.org).

Monarch sources and mating design. Monarchs used in this experiment were the great-grand progeny of monarchs collected as larvae and adult butterflies from three sites in eastern N. America during Aug-Oct 2004: Virginia (Giles County), Georgia (Dekalb County), and New York (Tompkins County). All monarchs were examined for the presence of *O. elektroscirrha* following Altizer *et al.* (2000) and only uninfected individuals were used to obtain progeny ($N_{initial}$ = 33 adults). Captive monarchs were reared from egg to adult using a breeding design that eliminated the possibility of full-sib mating and maximized the contribution of initial founders to each generation (with N per generation > 200). Eggs for this experiment were obtained from 15 females that oviposited onto potted greenhouse-reared *Asclepias incarnata*. Plants were transferred to a laboratory and maintained at 24°C, and larvae remained on their natal plants until they reached the second instar.

Inoculation and host rearing. We used a fully factorial design where infection treatment (parasitized and control) and larval rearing density (low, moderate and high) were experimental factors (Table 1). Parasite inoculum was derived from the abdomen of a monarch captured in Atlanta, GA, USA. Following Altizer and Oberhauser (1999), we vortexed the abdomen for 5 minutes in 10 ml of distilled water and calibrated inoculum to a dose of 300 spores per larva using a hemocytometer. Control inoculum was prepared by vortexing the abdomen of an uninfected eastern adult monarch. We inoculated second instar larvae

individually by pipetting 10 µl drops of inoculum onto 1-cm^2 milkweed pieces placed on dampened filter paper inside sterile 8.5-cm Petri dishes. Larvae were maintained singly in the Petri dish until they consumed all of the plant material, which occurred within 48 hrs.

Following inoculation, larvae were transferred to plastic 3.8 L containers with mesh-screen lids and reared to adulthood in a laboratory exposed to ambient light (~14h:10h L:D) and maintained at 26°C. A total of 420 larvae were randomly assigned to density treatment groups as follows: 1 larva/container (low density), 5 larvae/container (moderate density) and 10 larvae/container (high density; Table 1). We checked containers twice daily, and at least once per day added fresh cuttings of greenhouse-raised milkweed (*A. incarnata*) to each container, removed frass, and maintained a clean, moist paper lining. We adjusted the total food supply so that the number of leaves per larva remained relatively constant by provisioning approximately 5 leaves per larva to each container per feeding. Milkweed cuttings were held in florist tubes and sterilized by soaking in a 20% bleach solution for 20 minutes and rinsing thoroughly in tap water prior to use.

After all monarchs in a container had pupated, containers were transferred to an adjacent laboratory maintained at 26°C to avoid contaminating the larval rearing area with parasite spores. Pupal mass was measured on an analytic balance to the nearest 0.0001g. Pupae were transferred to single 0.5 L plastic containers to avoid transfer of parasite spores among individual butterflies. We recorded the development time of monarchs based on the number of days from oviposition to pupation and eclosion. After adults emerged, we recorded the sex

of each butterfly and placed adults individually into glassine envelopes 6-12 hrs post-eclosion. Monarchs were held at 24ºC without feeding, and mortality counts were taken daily to record adult longevity (in days). Adult longevity may also be referred to as starvation resistance and provides a measure of the ability on an individual monarch to survive on lipid reserves alone, which is similar to the survival needs of monarchs at over-wintering sites. We used latex gloves to handle milkweed, monarchs, and inoculum; gloves were frequently changed and laboratory surfaces and utensils were sterilized with 20% bleach solution to prevent unintentional transmission of parasite spores.

Quantifying infection and monarch wing parameters. We assessed the infection status of each adult by estimating the total number of spores on the insects' abdomens. Upon death, the abdomen of each monarch was removed and placed into a vial containing 5 ml of deionized water. After vortexing at high speed for 15 minutes, a hemocytometer counting chamber was used to estimate the number of spores per butterfly based on replicate counts for each sample.

We used digital image analysis to quantify adult monarch wing size and the degree of melanism (dark coloration). We removed left and right forewings from preserved adults and scanned them using a flatbed HP scanner set to 300 dpi using the same exposure settings for each scan. Measurements were made using Adobe Photoshop software with the Image Processing Tool Kit plugin (Reindeer Graphics, Inc.). Total forewing area (mm^2) and two measures of wing melanism were obtained for both forewings of each adult butterfly, following Davis *et al.* (2005). First, we quantified the proportion of forewing area encompassed by black pigmentation. Second, we estimated the density of black

pigmentation, an indicator of the intensity or level of opacity of black. The scoring measurement of density is on a 0-255 scale, with 0 being completely black (greatest color density). Average measures per individual were based on results for L and R forewings.

Regional and temporal variation in monarch density. We used data from the Monarch Larva Monitoring Project (MLMP; Oberhauser & Prysby, 2008; Prysby & Oberhauser, 2004), a citizen science program, to further indicate the degree to which monarch butterfly larval densities vary over time and space in eastern N. America. Volunteer observers for the MLMP began collecting weekly abundance data during the monarch's breeding season in 1997, with per plant densities of monarch egg and larval (reported to individual stadia) stages on milkweed plants available for 32 states and provinces across North America. Data included the total number of larvae and milkweed plants observed at a specific time and location. Because a high proportion of monarchs die as eggs and early instar larvae, we calculated average larval density per site based on count data for the final three instars (3, 4 and 5) only. All sites used in the analysis had been monitored for more than 1 year with a minimum of 4 weekly observations per year.

We divided observations from 1997 through 2006 in the eastern U.S. and Canada into three geographic regions: Midwest (MN, WI, MI, IA, IL, IN, MO, OH, NE), Northeast (VT, MA, NY, NJ, PA, MD, ON, and DC), and South (TX, GA, NC, VA, and TN), and three temporal breeding phases: early (before June 1st), middle (June 1st-July 31st), and late (after July 31st) to examine changes in larva abundance. Geographic regions were selected based on previously-described

patterns of monarch spring re-colonization, whereby adults returning from Mexico lay eggs in the southern-most states during April-May (here represented by the region denoted 'South'), and a second generation continues the journey north followed by a brief time lag (Malcolm *et al.*, 1993; Howard & Davis, 2004; Davis & Howard 2005). In addition, northeastern and mid-western states were examined separately because these areas are associated with two major fall migratory flyways at the end of the breeding season (Davis and Howard in press). We analyzed the average larval density per site based on the total number of larvae divided by the number of milkweed plants examined each week, and averaged the weekly density values for each site within a given phase. We then excluded zero density reports (where sites were monitored but no larvae were reported for a given phase), and log-transformed the remaining density estimates prior to analysis.

Statistical Analysis. Analyses to examine the effects of parasite treatment and rearing density were conducted using average values for all monarchs reared in a container as the unit of observation. Dependent variables included pupal mass, adult forewing area, development time from inoculation to adulthood, adult spore load, adult longevity and two measures of wing melanism (proportion of black and density of black on forewings). Count data were log-transformed prior to calculating container means. We tested for equal variances between density treatment groups using Fisher's F-test, for normally distributed data, and Levene's Test, for non-normally distributed data, at significance levels of α = 0.05. Variances between density treatments were equal for spore load data and all continuous response variables.

Analysis of variance was used to examine effects of design variables on one count variable, final spore load, and all continuous variables: pupal mass, adult forewing area, and two measures of wing melanism (proportion of black and density of black on forewings). The nonparametric Kaplan- Meier analysis was used to examine treatment effects on development time and adult longevity, and multinomial logistic regression to examine treatment effects on the proportion of monarchs that survived to adulthood, the proportion of adults infected with *O. elektroscirrha* and the proportion of adults with deformed wings. For analyses of adult measures, monarch sex (M/F) was included as fixed factor, and the final density of larvae (based on the actual number of monarchs that survived to pupation per container) was included as a continuous covariate (full model: dependent variable = parasite treatment + density treatment + final density + sex + parasite*density + infection*sex + density*sex).

Analyses were performed in SPSS (ver. 15.0; SPSS, Inc., Chicago, IL) and we used comparisons of Akaike's Information Criterion (AIC) and Hurvich and Tsai's Criterion (AICC) for model simplification following Crawley (2002). In Table 2, we report significance values only for variables included in the final minimum adequate model. Bonferroni pairwise comparisons of means were used to further examine differences between the three density treatments in cases where rearing density was significant, and results are reported in the figure legends. We examined the distribution of residuals for each minimum adequate model and in most cases found that these approximated a normal distribution.

RESULTS

Regional and temporal variation in monarch density. Analysis of MLMP data indicated that the average number of larvae per plant differed among breeding phases (early, middle, and late) and between regions in eastern N. America (Midwest, Northeast, and South). The final data set included a total of 641 density estimates by sampling location and breeding phase, as recorded by 78 observers over all 10 years. The number of density values (calculated for individual sites within a given breeding phase) used for each region by phase combination ranged from 14 (Northeast, early phase) to 307 (Midwest, middle phase), after reports of zero density were removed from the data set. Average densities per location were highest in the South during the early phase of the breeding season (Figure 2). During the middle of the breeding season, monarch density was very low in the South, and increased again late in the breeding season. In the Northeast and Midwest, average larval density increased from early to late in the breeding season (Figure 2). Effects of phase of breeding season ($F_{2, 627}$ =3.709 p=0.025) and the two-way interaction between breeding phase and region ($F_{4, 627}$ =2.820 p=0.024) were highly significant, but the main effect of region was not significant ($F_{2, 627}$ =0.556 p=0.574).

It is important to note that average larval densities reported in Figure 2 underestimate the actual numbers of larvae per host plant. This is because plants with larvae and those without were included equally in the count of plants examined. Because the number of larvae on a single plant for sites with monarchs present must be at least 1.0, averages shown in Figure 2 could be higher if only those plants with larvae had been counted. Moreover, within each region, the

maximum larval density for any given site was greater than 1.0 in several cases, leaving no doubt that some plants carried > 1 larva (e.g., Figure 1). Finally, an observer in Delray Beach, FL (excluded from the analysis here due to its close proximity to the S. Florida resident monarch population) reported average numbers of 7.0 and 6.5 larvae per plant examined during the middle phase of the breeding season in both 2005 and 2006, respectively. This observation indicates that the numbers of larvae per plant could reach higher levels in year-round resident populations as compared to the relatively low larval densities experienced by the eastern migratory population.

Survival, infection status, and parasite load. Monarchs in the density-infection experiment experienced high larval and pupal mortality across all treatments; only 49% of all monarchs survived from third instar to the adult stage (Table 1). On average, the probability of survival to adulthood did not differ between control and parasite-treated monarchs (χ^2=7.012, df=6, p=0.320) or across density treatments (χ^2=17.426, df=12, p=0.134), and the interaction between rearing density and parasite treatment was also not significant (χ^2=27.026, df=30 p=0.622). However, the survival of parasite-treated monarchs was greatest in the moderate density treatment and lowest in the high density treatment (Table 1) and a separate analysis focused on survival within parasite-treated monarchs alone showed that this effect of rearing density was highly significant (χ^2=165.770, df=12, p=0.000).

All surviving control monarchs were parasite-free (N=97), whereas 85% of the parasite treated monarchs (N=91) were infected with *O. elektroscirrha.* The proportion of infected monarchs within the parasite-treated class increased with

larval rearing density (Table 1). This effect of density on infection status was significant (χ^2=23.324, df=10, p=0.010). The average parasite load per infected monarch was 3.08 x 10^5 spores (range: 5.56 x 10^3 – 1.16 x 10^6), and we noted a trend of increasing spore load with increased rearing density (average spore load by rearing density: Low 2.46 x 10^5; Moderate 2.91 x 10^5; High 3.39 x 10^5). However, analysis within the subset of parasitized monarchs showed that average spore load was not significantly affected by either rearing density ($F_{2, 47}$ = 0.725, p=0.490) or sex ($F_{1, 47}$=0.322, p=0.573).

Pupal mass and development time. Pupal mass was greatest in the moderate density treatment (Figure 3a) and this effect was highly significant (Table 2). Average pupal mass was lower among parasite-treated monarchs in both the moderate and high density treatments, but parasite-treated and control monarchs had similar pupal mass in the low density treatment (Figure 3a). Statistical analysis showed a significant main effect of parasite treatment and a significant two-way interaction between parasite treatment and density (Table 2). Pupal mass was also greater in males than females across all treatment categories, and this effect was highly significant (Table 2).

Parasite-treated monarchs developed more slowly (based on time from hatching to adult eclosion) than monarchs in the control treatment (Log Rank: χ^2=6.891, df=1, p=0.09), and this effect was strongest in the low and high density treatments (Figure 3b). Moreover, larvae in the moderate density treatment developed faster than those in the low and high density treatments, and this effect of rearing density on development time was highly significant (Log Rank: χ^2=8.773, df=2, p=0.013). There was also a significant interaction between

parasite treatment and density (Log Rank: χ^2=15.108, df=5, p=0.013), such that the slower development time for infected monarchs was observed in the low and high density treatments only. In addition, female monarchs developed more quickly than males across all parasite and density treatments (Log Rank: χ^2=8.447, df=1, p=0.004).

Adult longevity and wing traits. Parasite-treated monarchs experienced a 23% reduction in adult longevity (starvation resistance) as compared to control monarchs, and this effect of parasite treatment was highly significant (Figure 4; Log Rank: χ^2=48.056, df=1, p=0.000). Adult longevity was not affected by larval rearing density alone (Log Rank: χ^2=3.951, df=2, p=0.139), however there was a significant interaction between parasite treatment and density (Log Rank: χ^2=58.310, df=5, p=0.000; Figure 4), such that adult longevity decreased with increasing larval density within parasite treated monarchs only.

Only two unparasitized monarchs (N=105) emerged with wing deformities, whereas five parasitized adults (N=77) had deformed wings. Four of the five parasitized monarchs with wing deformities were in the high density treatment. However, neither parasite treatment nor rearing density was significantly associated with wing deformities (parasite treatment: χ^2=5.031, df=3, p=0.170; rearing density: χ^2= 9.934, df=6, p=0.127).

Adult forewing area was significantly influenced by rearing density, parasite treatment, and sex (Table 2). Males were larger than females, and monarchs in the moderate density treatment had the largest forewings, whereas those in the low density treatment had the smallest forewings (Figure 5a). Forewing area was also affected by the density by parasite treatment interaction

(Table 2). Specifically, control monarchs had larger forewings than parasite-treated monarchs in both the moderate and high density treatments, whereas parasite-treated monarchs had larger forewings in the low density treatment (Figure 5a).

The proportion of black coloration on monarch forewings was marginally greater for control monarchs than for parasite-treated butterflies ($p = 0.053$), and females were darker than males across all treatment groups (Figure 5b, Table 2). The density of wing pigmentation, a measure of the opacity or intensity of black, was greater for female than for male monarchs, but this variable was not significantly affected by parasite treatment (Table 2). Finally, neither measure of wing coloration was significantly affected by rearing density based on main or interaction effects (Table 2).

DISCUSSION

Both larval rearing density and infection by the protozoan *O. elektroscirrha* affected measures of size and development in monarch butterflies. First, we observed significant negative effects of parasite treatment on monarch fitness, with infection resulting in decreased monarch pupal mass, slower development rate, reduced wing area and shorter adult longevity (starvation resistance). This is consistent with previous studies demonstrating that high replication of *O. elektroscirrha* within monarch hosts results in substantial negative consequences for adult lifespan, body size, wing size, mating success and flight ability (Altizer & Oberhauser, 1999; Bradley & Altizer, 2005; de Roode *et al.*, 2007; Lindsey & Altizer, 2008).

While monarch infection probability increased significantly with increasing larval densities, average spore loads following exposure to *O. elektroscirrha* increased only slightly with larval densities. The effect of density treatment on host infection status weakly suggests increased susceptibility to infection with increasing larval density. Thus, in the case of rearing density, results here do not support the 'density-dependent prophylaxis' hypothesis, which predicts that increased resistance to parasitism can result from increased rearing density (Barnes & Siva-Jothy, 2000; Reeson *et al.*, 1998; Wilson *et al.*, 2003). On the one hand, because monarchs appear to experience ecologically relevant variation in density in the wild, they should, in theory, benefit from tailoring levels of immunity or resistance to variation in host density. This is because infection patterns by *O. elektroscirrha* in wild populations show that prevalence increases from early to late summer within migratory populations (S. Altizer, unpublished data) and is higher in year-round breeding populations as compared with migratory populations (Altizer *et al.*, 2000; Altizer *et al.*, 2004; Leong *et al.*, 1997). Because both of these situations also correlate with higher numbers of larvae on plants (e.g., Figure 2), it seems likely that monarchs in high-density populations could also experience higher risks of infection. On the other hand, monarch immune defenses can be costly (Lindsey & Altizer, 2008); hence, although greater measures of immunity correlate with higher host survival following infection with *O. elektroscirrha*, these defenses might not be readily mobilized under high-risk conditions.

An alternative hypothesis to density-dependent prophylaxis is the 'crowding and stress' hypothesis, which predicts that individuals reared at lower

population densities will be in better physical condition, and hence will be better able to resist the negative effects of parasitism (Adamo, 2006; Goulson & Cory, 1995; Reilly & Hajek, 2008). This idea is based on the assumption that increased stress and intraspecific competition will make hosts more susceptible to infection, or less able to tolerate the negative consequences of parasitism. In support of this hypothesis, monarchs in the moderate and high density treatments generally experienced slightly higher infection rates and suffered the greatest negative effects of infection (based on differences between the parasite-treated and untreated groups). Moreover, monarchs in the moderate and high density rearing treatments also suffered the greatest negative effects of infection on development and body size (based on relative differences between the parasite-treated and untreated class). By comparison, parasitized monarchs in the low density treatment had mean values of pupal mass, development rate and adult wing size that were similar to or slightly greater than uninfected monarchs. Collectively, these effects of rearing density on host infection and fitness measures suggest that monarchs under high-density conditions are more susceptible to parasite infection and its costly effects.

Counter to our initial expectations, we observed a nonlinear relationship between larval density and measures of development and body size for both parasite-treated and untreated larvae. Specifically, each of these variables was greatest for monarchs reared under moderate density, and averages were 10-20% lower for monarchs reared singly and at the highest density. Previous studies of Lepidoptera and other insect species have shown that immature stages reared under high density conditions experience decreased survivorship, slower

development rates, and/or achieve smaller adult body sizes (Gibbs *et al.*, 2004; Mercer, 1999; Tammaru *et al.*, 2000). On the other hand, cabbage moth *Mamestra brassicae* larvae reared both singly and at the highest densities weighed less than larvae reared at intermediate densities (Goulson & Cory 1995). Our findings are consistent with those of Goulson and Cory (1995), and suggest that for both healthy and infected monarchs, the ideal rearing conditions are to be neither solitary nor in a large group, but to occur at moderately low densities, provided that food resources are not limiting.

Observations of greater body size and faster development rate for intermediate density conditions, irrespective of host infection status, might be best explained by two different mechanisms. Given that high densities could cause greater intraspecific competition under natural conditions, larvae might feed more rapidly to attain a large body size before food supplies are depleted. Because food supplies, ultimately, were not limited in this experiment, this could have resulted in both more rapid development *and* greater size at pupation when compared with solitary larvae. Consistent with this explanation, Atterholt & Solensky (2008) reported greater body size for monarch larvae grown under higher density conditions (5 larvae/container) as compared to larvae reared singly. Monarch larvae reared under the highest density conditions in our experiment (10/container) could have suffered from physical or developmental stress associated with overcrowding or interference competition. For example, Gibbs *et al.* (2004) observed aggressive encounters, including head and tail biting and head butting, among speckled wood butterfly larvae (*Pararge aegeria)* reared at high densities. Although we did not quantify feeding behaviors in this

study, monarchs engage in similar aggressive interactions in captivity, and larvae can also bear integument scars and missing tubercules from previous injuries (S. Altizer, personal observation). Finally, we note that in experiments described here, a greater number of host plant leaves provisioned to containers with more larvae could have altered the micro-environmental conditions in ways that enhanced monarch survival and development in the moderate density treatment.

External melanism (i.e., dark body coloration) is often thought to correlate positively with resistance to infection (Barnes & Siva-Jothy, 2000), but not in all insect species (Robb *et al.*, 2003). Moreover, previous work has shown that body or wing melanism can increase under crowded host conditions (Wilson *et al.*, 2001). For example, polyphenism in Lepidoptera can occur such that the high density phenotype is darker than the low density phenotype (e.g., as demonstrated for *Spodoptera littoralis*; Cotter *et al.*, 2004). Within monarchs, we found that unparasitized adults had darker forewings (based on the proportion of black pigmentation) than parasite-treated butterflies, indicating that parasite infection might lower the ability of monarchs to produce dark body coloration, or that monarchs experience tradeoffs between melanin production for parasite defense versus wing coloration. However, our results did not support an effect of rearing density (or a density by parasite treatment interaction) on measures of dark coloration in adult butterflies.

Field data from the MLMP do not allow us to determine per plant larval densities for occupied plants alone, and thus it is impossible to determine how often the experimental larval densities examined here occur in the wild. However, MLMP data do clearly demonstrate that per-plant monarch densities vary up to

five-fold across space and time in North America, and we have anecdotal reports of densities of late-instar larvae similar to those used in our study (Figure 1). It is therefore possible that the faster development observed here for monarchs, in the moderate density treatment, is an evolved response to crowding that occurs in nature.

Ultimately, hosts that live in variable environments can experience changes in population structure and extrinsic forces that influence variation in disease risk. These risks are particularly relevant to monarch butterflies that have been threatened at their overwintering sites and breeding habitats in recent years (Brower *et al.*, 2002; Brower & Malcom, 1991; Oberhauser & Peterson, 2003; Zalucki & Rochester, 1999). Specifically, destruction of over-wintering sites, climate warming and planting of tropical milkweed host plant species in non-native regions can alter the ecological dynamics of migratory populations, potentially resulting in the replacement of the large migratory populations with smaller remnant populations that breed year-round. These non-migratory populations could experience higher local population densities, and are also likely to become heavily parasitized (e.g., Altizer *et al.*, 2000; Altizer *et al.*, 2004).

As indicated by this study, both parasite infection and larval density can affect monarch butterfly fitness, although results reported here were not always consistent with predictions from previous work. Specifically, we found that contact with other larvae at moderate (but not high) local densities could increase pre-adult survival, stimulate larval growth, and increase pupal and adult body size, irrespective of host infection status. However, it is important to note that this finding probably relies on non-limiting food resources given modest

increases in density. Moreover, our study indicates that highly crowded conditions can increase the potential for monarchs to suffer from the negative consequences of disease, probably owing to increased stress and intra-specific competition. In summary, being in a group may have beneficial impacts on a number of fitness traits, even for species like monarchs that do not have gregarious larval stages. However, high density conditions appear to have negative effects on monarch resistance and tolerance to infection and results such as those reported here can help reveal the mechanisms that underlie the positive and negative consequences of host density for individual performance.

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REFERENCES

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

Adamo, S.A. (2006). The emergency life-history stage and immunity in the cricket, *Gryllus texensis*. *Animal Behaviour*, 72, 235-244.

Alexander, R.D. (1974) The evolution of social behavior. *Annual Review of Ecology and Systematics*, 5, 325-383.

Altizer, S., Nunn, C.L., Thrall, P.H., Gittleman, J.L., Antonovics, J., Cunningham, A.A., Dobson, A.P., Ezenwa, V., Pedersen, A.B., Poss, M., & Pulliam, J.R.C. (2003). Social organization and parasite risk in mammals: integrating theory and empirical studies. *Annual Review of Ecology, Evolution and Systematics*, 34, 517–47.

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

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

Altizer, S.M., Oberhauser, K.S., & Geurts, K.A. (2004). Transmission of the protozoan parasite, *Ophryocystis elektroscirrha*, in monarch butterfly populations: implications for prevalence and population-level impacts. Pp. 203-218. In *The Monarch Butterfly: Biology and Conservation* (eds K.S. Oberhauser & M.J. Solensky). Cornell University Press, Ithaca, NY.

Anderson, R.M. & May, R.M. (1979). Population biology of infectious diseases: Part 1. *Nature*, 280, 361-367.

Anderson, R.M. & May, R.M. (1981). The population-dynamics of micro-parasites and their invertebrate hosts. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, 291, 451-524.

Atterholt, A.L. & Solensky, M.J. (2008). Effects of larval rearing density and food availability on adult size and coloration in monarch butterflies, *Danaus plexippus*. *Entomologia Experimentalis et Applicata*. In press.

Barnes, A.I. & Siva-Jothy, M.T. (2000). Density-dependent prophylaxis in the mealworm beetle *Tenebrio molitor* L (Coleoptera : Tenebrionidae): cuticular melanization is an indicator of investment in immunity. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 267, 177-182.

Bradley, C.A. & Altizer, S. (2005). Parasites hinder monarch butterfly flight: implications for disease spread in migratory hosts. *Ecology Letters*, 8, 290-300.

Brower, L.P. (1964). Experimental analyses of egg cannibalism in the monarch and queen butterflies, *Danaus plexippus* and *D. gilippus berenice*. *Physiological Zoology*, 287-296.

Brower, L.P. (1995). Understanding and misunderstanding the migration of the monarch butterfly (Nymphalidae) in North America: 1857-1995. *Journal of the Lepidopterists' Society*, 49, 304-385.

Brower, L.P., Castilleja, F., Peralta, A., Lopez-Garcia, J., Bojorquez-Tapia, L., Diaz, S., Melgarejo, D., & Missrie, M. (2002). Quantitative changes in forest

quality in a principal overwintering area of the monarch butterfly in Mexico, 1971-1999. *Conservation Biology*, 16, 346-359.

Brower, L.P. & Malcom, S.B. (1991). Animal migrations: endangered phenomena. *American Zoologist*, 31, 265-276.

Brown, C.R. & Brown, M.B. (1986). Ecotoparasitism as a cost of coloniality in cliff swallows (*Hirundo pyrrhonota*). *Ecology*, 67, 1206-1218.

Brown, M.J.F., Moret, Y., & Schmid-Hempel, P. (2003). Activation of host constitutive immune defence by an intestinal trypanosome parasite of bumble bees. *Parasitology*, 126, 253-260.

Cotter, S.C., Hails, R.S., Cory, J.S., & Wilson, K. (2004). Density-dependent prophylaxis and condition-dependent immune function in Lepidopteran larvae: a multivariate approach. *Journal of Animal Ecology*, 73, 283-293.

Crawley, M.J. (2002). *Statistical Computing: An Introduction to Data Analysis using S-Plus*. John Wiley and Sons, West Sussex, England.

Davis, A.K., Farrey, B.D., & Altizer, S. (2005). Variation in thermally induced melanism in monarch butterflies (Lepidoptera : Nymphalidae) from three North American populations. *Journal of Thermal Biology*, 30, 410-421.

Davis, A.K. & Howard, E. (2005). Spring recolonization rate of monarch butterflies in eastern North America: new estimates from citizen-science data. *Journal of the Lepidopterists' Society,* 59, 1-5.

de Roode, J.C., Gold, L.R., & Altizer, S. (2007). Virulence determinants in a natural butterfly-parasite system. *Parasitology*, 134, 657-668.

de Roode, J.C., Yates, A.J., & Altizer, S. (2008). Virulence-transmission trade-offs and population divergence in virulence in a naturally occurring butterfly

parasite. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 7489-7494.

Dwyer, G. & Elkinton, J.S. (1993). Using simple-models to predicts virus epizootics in gypsy-moth populations. *Journal of Animal Ecology*, 62, 1-11.

Farrey, B. & Davis, A.K. (2004). Monarch butterfly larvae (Lepidoptera: Nymphalidae) with 3 tubercle pairs in South Florida. *Florida Entomologist*, 87, 408-411.

Freeland, W.J. (1976). Pathogens and the evolution of primate sociality. *Biotropica*, 8, 12-24.

Freeland, W.J. (1979). Primate social groups as biological islands. *Ecology*, 60, 719-728.

Gibbs, M., Lace, L.A., Jones, M.J., & Moore, A.J. (2004). Intraspecific competition in the speckled wood butterfly *Pararge aegeria*: effect of rearing density and gender on larval life history. *Journal of Insect Science*, 4, 16.

Goulson, D. & Cory, J.S. (1995). Responses of *Mamestra brassicae* (Lepidoptera, Noctuidae) to crowding - interactions with disease resistance, color phase and growth. *Oecologia*, 104, 416-423.

Hagen, S.B., Ims, R.A., & Yoccoz, N.G. (2003). Density-dependent melanism in sub-arctic populations of winter moth larvae (*Operophtera brumata*). *Ecological Entomology*, 28, 659-665.

Hagen, S.B., Sorlibraten, O., Ims, R.A., & Yoccoz, N.G. (2006). Density-dependent melanism in winter moth larvae (Lepidoptera : Geometridae): A

countermeasure against parasitoids? *Environmental Entomology*, 35, 1249-1253.

Hoogland, J.L. (1979). Aggression, ectoparasitism, and other possible costs of prairie dog (Sciuridae, *Cynomys* spp) coloniality. *Behaviour*, 69, 1-35.

Hoogland, J.L. (1995). *The Black-Tailed Prairie Dog*. University of Chicago, Chicago, IL.

Howard, E. & Davis, A.K. (2004). Documenting the spring movements of monarch butterflies with Journey North, a citizen science program. Pp. 105-114 (In: Oberhauser, K. and Solensky, M. Eds.) *The Monarch Butterfly. Biology and Conservation*. Cornell University Press. Ithaca, NY.

Howard, E. & Davis, A.K. (2008). The fall migration flyways of monarch butterflies in eastern North America revealed by citizen scientists. *Journal of Insect Conservation*. In press.

James, D.G., ed. (1993). *Migration biology of the monarch butterfly in Australia*, pp 189-200. Natural History Museum of Los Angeles County, Los Angeles, CA.

Knell, R.J., Begon, M., & Thompson, D.J. (1996). Transmission dynamics of *Bacillus thuringiensis* infecting *Plodia interpunctella*: A test of the mass action assumption with an insect pathogen. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 263, 75-81.

Knight, A. (1998). *A Population Study of Monarch Butterflies in North-Central and South Florida*. University of Florida, Gainesville, FL.

Krause, J. & Ruxton, G.D. (2002). *Living in Groups*. Oxford University Press, Oxford.

Kunimi, Y. & Yamada, E. (1990). Relationship of larval phase and suseptibility of the armyworm, *Pseudaletia separata* Walker (Lepidoptera, Noctuida) to a nuclear polyhedrosis-virus and a granulosis-virus. *Applied Entomology and Zoology*, 25, 289-297.

Lee, K.P. & Wilson, K. (2006). Melanism in a larval Lepidoptera: repeatability and heritability of a dynamic trait. *Ecological Entomology,* 31, 196-205.

Leong, K.L.H., Kaya, H.K., Yoshimura, M.A., & Frey, D.F. (1992). The occurrence and effect of a protozoan parasite, *Ophryocystis elektroscirrha* (Neogregarinida, Ophryocystidae) on overwintering monarch butterflies, *Danaus plexippus* (Lepidoptera, Danaidae) from two California winter sites. *Ecological Entomology*, 17, 338-342.

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

Lindsey, E. & Altizer, S. (2008). Sex differences in immune defenses and response to parasitism in monarch butterflies. *Evolutionary Ecology*. In press.

Lloyd-Smith, J., Cross, P., Briggs, C., Daugherty, M., Getz, W., Latto, J., & Sanchez, M. (2005). Should we expect population thresholds for wildlife disease? *Trends in Ecology & Evolution*, 20, 511-519.

Malcolm, S.B., Cockrell, B.J., & Brower, L.P. (1993). Spring recolonization of eastern North America by the monarch butterfly: successive brood or single sweep migration? In Biology and Conservation of the Monarch Butterfly

(eds. S.B. Malcolm & M.P. Zalucki), Science Series Vol. No. 38, pp. 253-267. Natural History Museum of Los Angeles County, Los Angeles.

McCallum, H., Barlow, N., & Hone, J. (2001). How should pathogen transmission be modeled? *Trends in Ecology & Evolution*, 16, 295-300.

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

Mercer, D.R. (1999). Effects of larval density on the size of *Aedes polynesiensis* adults (Diptera : Culicidae). *Journal of Medical Entomology*, 36, 702-708.

Møller, A.P., Dufva, R., & Allander, K. (1993). Parasites and the evolution of host social behavior. *Advances in the Study of Behavior*, 22, 65-102.

Møller, A.P. & Erritzoe, J. (1996). Parasite virulence and host immune defense: Host immune response is related to nest reuse in birds. *Evolution*, 50, 2066-2072.

Møller, A.P., Merino, S., Brown, C.R., & Robertson, R.J. (2001). Immune defense and host sociality: a comparative study of swallows and martins. *American Naturalist*, 158, 136-145.

Moore, J. (2002). *Parasites and the Behavior of Animals.* Oxford University Press, Oxford.

Oberhauser, K. & Prysby, M.D. (2008). Citizen science: Creating a research army for conservation. *American Entomologist*, 54, 97-99.

Oberhauser, K.S. & Peterson, A.T. (2003). Modeling current and future potential wintering distributions of eastern North American monarch butterflies.

Proceedings of the National Academy of Sciences U.S.A., 100, 14063-14068.

Pie, M.R., Rosengaus, R.B., Calleri, D.V., & Traniello, J.F.A. (2005). Density and disease resistance in group-living insects: do eusocial species exhibit density-dependent prophylaxis? *Ethology Ecology & Evolution*, 17, 41-50.

Prysby, M.D. & Oberhauser, K. (2004). Temperal and geographic variation in monarch densities: citizen scientists document monarch population patterns. pp. 9-20. In *The Monarch Butterfly: Biology and Conservation* (eds K.S. Oberhauser & M.J. Solensky). Cornell University Press, Ithaca, NY.

Reeson, A.F., Wilson, K., Cory, J.S., Hankard, P., Weeks, J.M., Goulson, D., & Hails, R.S. (2000). Effects of phenotypic plasticity on pathogen transmission in the field in a Lepidoptera-NPV system. *Oecologia*, 124, 373-380.

Reeson, A.F., Wilson, K., Gunn, A., Hails, R.S., & Goulson, D. (1998). Baculovirus resistance in the noctuid *Spodoptera exempta* is phenotypically plastic and responds to population density. *Proceedings of the Royal Society of London Series B-Biological Sciences,* 265, 1787-1791.

Reilly, J.R. & Hajek, A.E. (2008). Density-dependent resistance of the gypsy moth *Lymantria dispar* to its nucleopolyhedrovirus, and the consequences for population dynamics. *Oecologia*, 154, 691-701.

Robb, T., Forbes, M.R., & Jamieson, I.G. (2003). Greater cuticular melanism is not associated with greater immunogenic response in adults of the polymorphic mountain stone weta, *Hemideina maori*. *Ecological Entomology*, 28, 738-746.

Ryder, J.J., Webberley, K.M., Boots, M., & Knell, R.J. (2005). Measuring the transmission dynamics of a sexually transmitted disease. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 15140-15143.

Shields, W.M. & Crook, J.R. (1987). Barn swallow coloniality - a net cost for group breeding in the Adirondacks. *Ecology*, 68, 1373-1386.

Simmonds, M.S.J. & Blaney, W.M. (1986). Effects of rearing density on development and feeding behavior in larvae of *Spodoptera exempta*. *Journal of Insect Physiology*, 32, 1043-1053.

Steinhaus, E.A. (1958). Crowding as a possible stress factor in insect disease. *Ecology*, 39, 503-514.

Stimson, J. & Berman, M. (1990). Predator induced color polymorphism in *Danaus plexippus* L (Lepidoptera, Nymphalidae) In Hawaii. *Heredity*, 65, 401-406.

Tammaru, T., Ruohomaki, K., & Montola, M. (2000). Crowding-induced plasticity in *Epirrita autumnata* (Lepidoptera : Geometridae): weak evidence of specific modifications in reaction norms. *Oikos*, 90, 171-181.

Urquhart, F.A. & Urquhart, N.R. (1978). Autumnal migration routes of Eastern population of monarch butterfly (*Danaus plexippus* L-Danaidae-Lepidoptera) in North America to overwintering site in Neovolcanic Plateau of Mexico. *Canadian Journal of Zoology*, 56, 1759-1764.

Wilkinson, G.S. (1985). The social-organization of the common vampire bat. 1. Pattern and cause of association. *Behavioral Ecology and Sociobiology*, 17, 111-121.

Wilson, K., Cotter, S.C., Reeson, A.F., & Pell, J.K. (2001). Melanism and disease resistance in insects. *Ecology Letters*, 4, 637-649.

Wilson, K., Knell, R., Boots, M., & Koch-Osborne, J. (2003). Group living and investment in immune defence: an interspecific analysis. *Journal of Animal Ecology*, 72, 133-143.

Wilson, K., Thomas, M.B., Blanford, S., Doggett, M., Simpson, S.J., & Moore, S.L. (2002). Coping with crowds: Density-dependent disease resistance in desert locusts. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 5471-5475.

Zalucki, M.P. & Kitching, R.L. (1982). Temperal and spatial variation of mortality in field populations of *Danaus plexippus* L. and *D. chrysippus* L. larvae (Lepidoptera: Nymphalidae). *Oecologia*, 53, 201-207.

Zalucki, M.P. & Rochester, W.A. (1999). Estimating the effect of climate on the distribution and abundance of the monarch butterfly, *Danaus plexippus* (L): a tale of two continents. In *1997 North American Conference on the Monarch Butterfly* (eds J. Hoth, L. Merino, K. Oberhauser, I. Pisanty, S. Price & T. Wilkson), pp. 150-163. The Commission for Environmental Cooperation, Montreal, Canada, Morelia, Mexico.

Table 1. Number of monarchs used to initiate the experiment, and percent surviving to adult eclosion, shown separately for each parasite treatment and larval rearing density.

		Density Treatment			
Parasite Treatment		*Low (1 larva per container)*	*Moderate (5 larvae per container)*	*High (10 larvae per container)*	*Total*
Control	Initial number	40	60	110	210
	Third Instar	40	55	88	183
	Adult	22	28	47	97
	Egg to adult survival[1]	55%	47%	43%	46%
	Third instar to adult survival[1]	55%	51%	53%	53%
	Parasitized	0	0	0	0
Parasitized	Initial number	40	60	110	210
	Third Instar	39	56	105	200
	Adult	18	40	33	91
	Egg to adult survival[1]	45%	67%	30%	43%
	Third instar to adult survival[1]	46%	71%	31%	46%
	Parasitized	14 (78%)	34 (85%)	29 (88%)	77 (85%)

[1] Survival estimates used in the statistical analysis were based on the number of adult monarchs divided by the number of third instar larvae, because deaths of larvae from earlier instars were difficult to observe (larvae were frequently missing but no carcass was found). Overall survival (egg to adult) is shown for comparison.

Table 2. Analysis of pupal mass and adult forewing characteristics as a function of experimental design variables (density and infection treatment) and sex (full model: Response variable = rearing density + parasite treatment + sex + rearing density*treatment + final larval density + rearing density*sex + treatment*sex + error term). Model simplification was performed as described in Methods text. F- and P-values are shown only for those explanatory variables remained in the final model; adjusted R^2 is shown for the final reduced models. All analyses use container means as the unit of observation.

Independent variable	Pupal mass	Wing area	Wing proportion black	Wing black density
Rearing Density	$F_{2,104}$=10.38 **p=0.000**	$F_{2,102}$=12.71 **p=0.000**		
Parasite Treatment	$F_{1,104}$=4.80 **p=0.032**	$F_{1,102}$=2.13 p=0.148	$F_{1,107}$=3.81 p=0.053	
Sex	$F_{1,104}$=39.07 **p=0.000**	$F_{1,102}$=4.97 **p=0.028**	$F_{1,107}$=388.99 **p=0.000**	$F_{1,108}$=209.94 **p=0.000**
Final Density		$F_{1,102}$=3.88 p=0.052		
Rearing Density * Parasite Treatment	$F_{2,104}$=4.14 **p=0.019**	$F_{2,102}$=3.94 **p=0.022**		
Adjusted R-square	0.389	0.254	0.788	0.657

FIGURE LEGENDS

Figure 1. Fifth instar monarch larvae feeding on *Asclepias syriaca* (common milkweed) in a field near Forestport, New York, USA, during summer 2007. Wild monarch larvae at this location are typically scattered at low density (a single larva per plant; Maureen Clark, MLMP personal observation). However, in 2007, up to 8 eggs were laid on single plants, and multiple late instar larvae were seen feeding on some milkweeds (as shown here), probably owing to low rainfall and scarcity of milkweeds during this year. (Photograph: Maureen Clark)

Figure 2. Relationship between the average densities of monarch butterfly larvae reported by Monarch Larva Monitoring Project observers (measured as the total number of larvae divided by the number of milkweed plants examined per site) for three regions in eastern North America: Northeast (white bars), Midwest (gray bars), and South (black bars). Phases are early (before Jun 1st), middle (Jun 1st-July 31st),), and late (after July 31st). Error bars represent standard errors. We included data only for sites where observers recorded data for > 1 year, and conducted 4 or more weekly observations per year, and for phase-site combinations where monarch larvae were reported (i.e., we excluded zero density observations).

Figure 3. Effects of larval rearing density and parasite treatment on a) pupal mass (g) and b) development time (days). Data are shown for unparasitized (white bars) and parasitized (gray bars) treatment groups within each larval rearing density (low: 1 larva per container; moderate: 5 larvae per container;

high: 10 larvae per container). Error bars represent standard errors. Bonferroni pairwise comparisons focused on rearing density treatments showed that mean pupal mass for monarchs reared under moderate densities were significantly different than means for monarchs reared at low and high densities ($p < 0.05$), whereas means for high and low density treatments were not statistically distinguishable ($p > 0.50$). Comparison of means for development time (here estimated using Bonferonni pairwise comparisons assuming normally-distributed data) showed that mean development time for monarchs reared at moderate densities were significantly different than means for monarchs reared at high densities ($p<0.03$), whereas means for all other combinations were not statistically distinguishable ($p>0.30$).

Figure 4. Effects of larval rearing density and parasite treatment on adult longevity (in days). Data are shown for unparasitized (white bars) and parasitized (gray bars) treatment groups within each larval rearing density (low: 1 larva per container; moderate: 5 larvae per container; high: 10 larvae per container). Error bars represent standard errors.

Figure 5. Effects of larval rearing density and parasite treatment on a) adult forewing area (mm) and b) proportion of black pigmentation on adult forewings. Data are shown for unparasitized (white bars) and parasitized (gray bars) treatment groups within each larval rearing density. Error bars represent standard errors. Bonferroni pairwise comparisons showed that for forewing area, mean values for monarchs reared under moderate density were significantly

greater than means for monarchs reared at low and high densities ($0.000 < p < 0.002$), whereas means for high and low density treatments were not statistically distinguishable ($p = 0.098$). Rearing density was not significantly associated with the proportion of black pigmentation on monarch forewings.

Figure 1

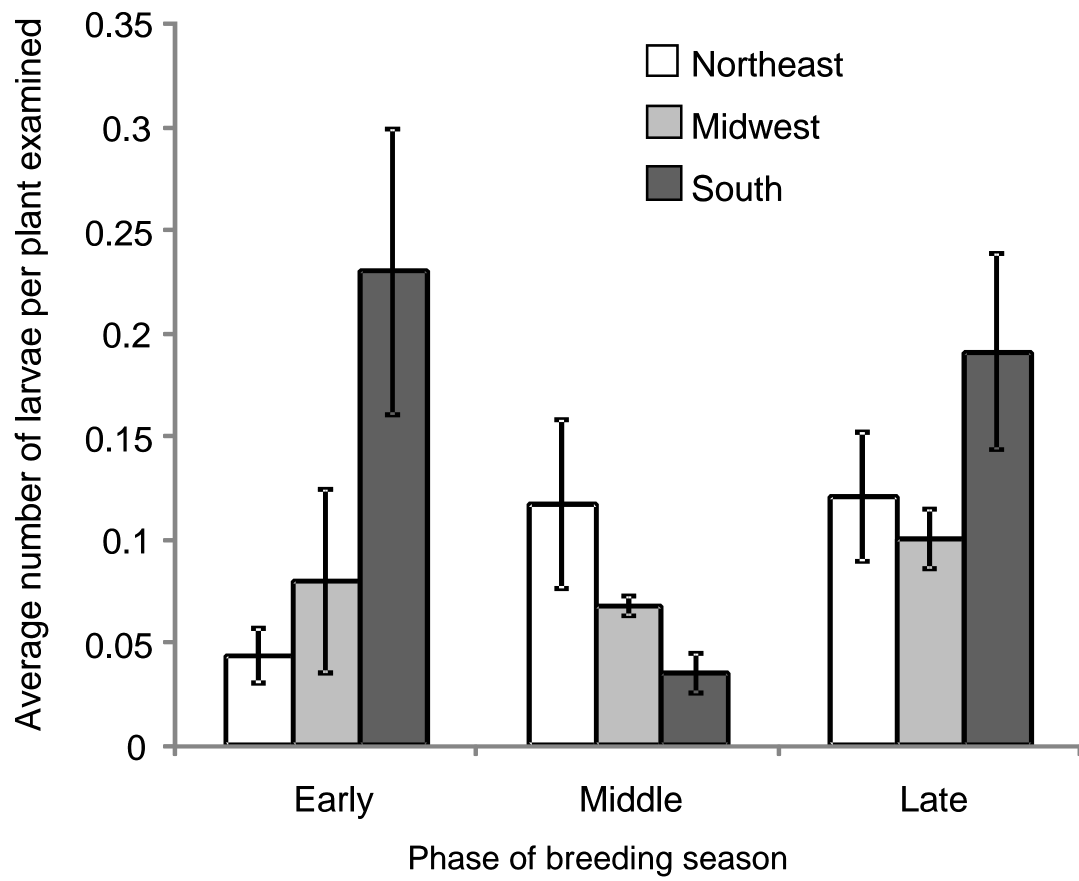

Figure 2

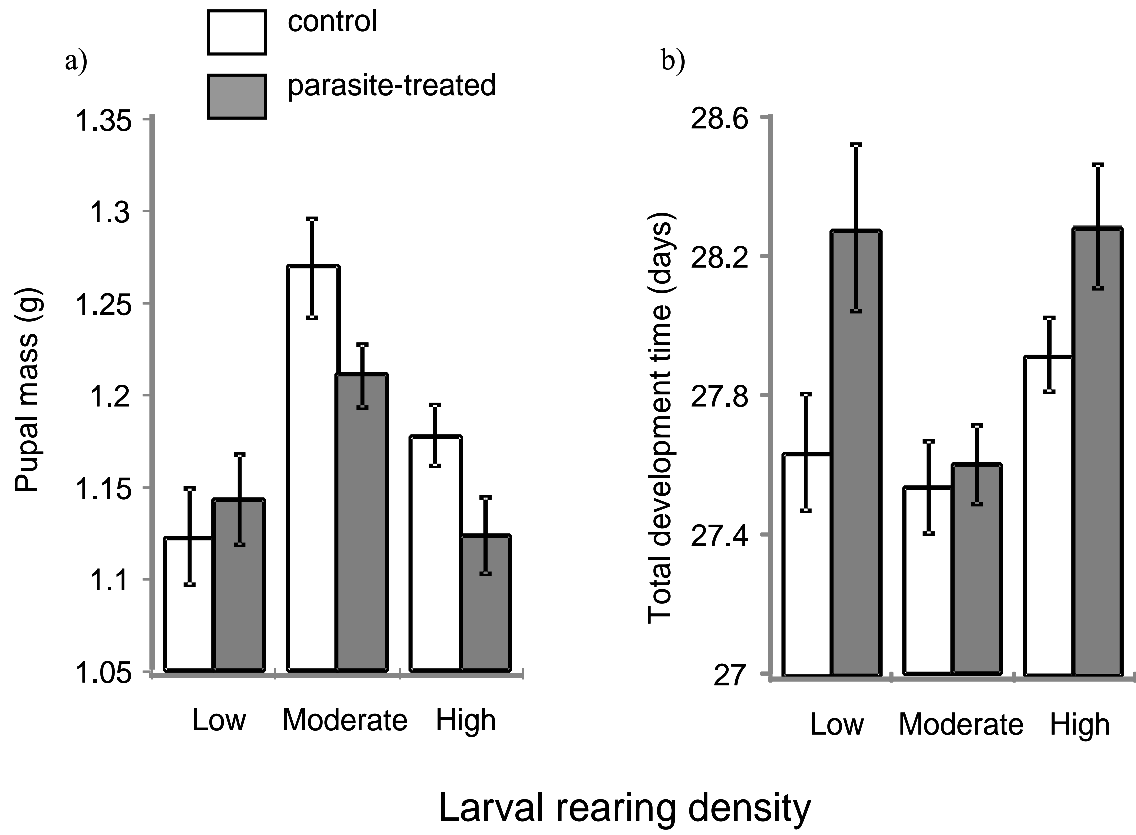

Figure 3

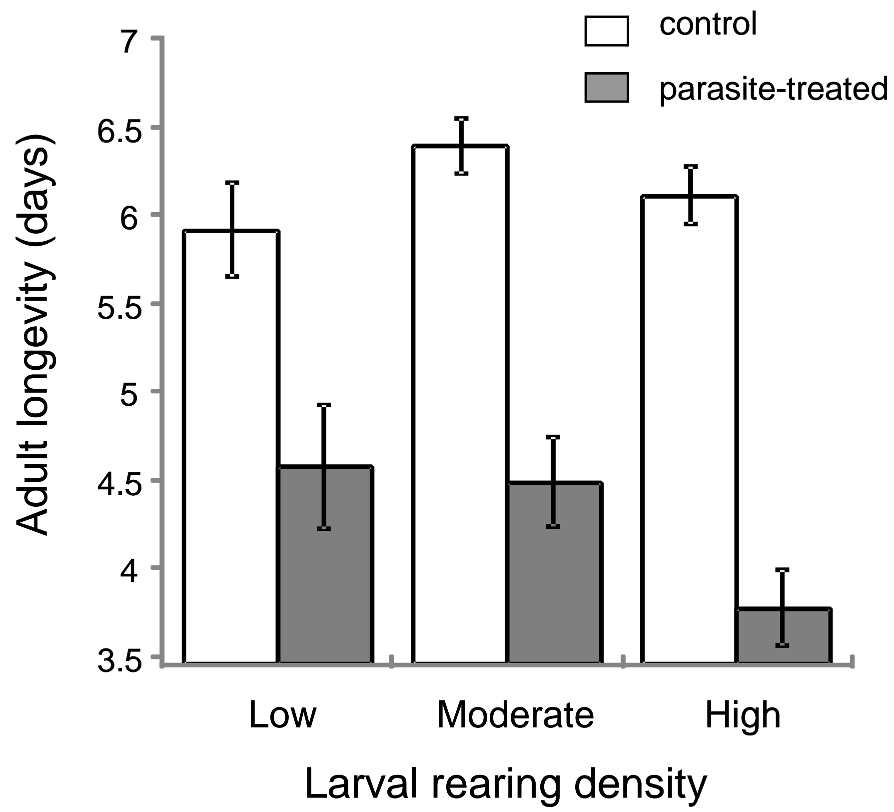

Figure 4

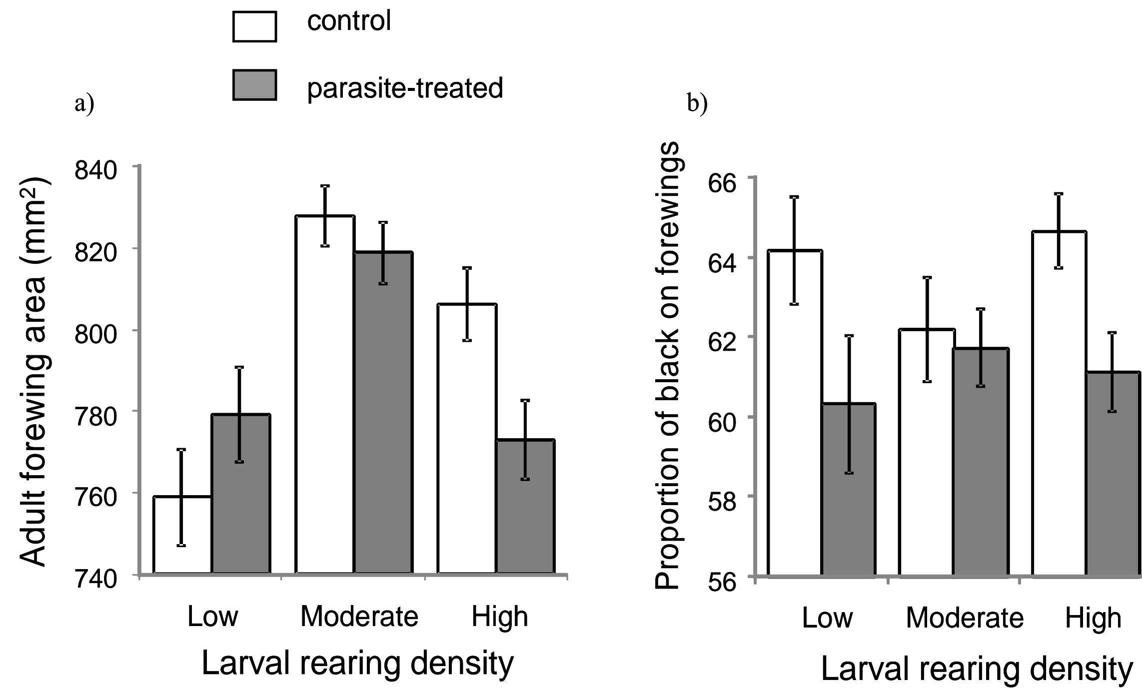

Figure 5

CHAPTER 3

Warmer temperatures limit host and parasite fitness: experimental evidence from a butterfly-parasite system

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Warmer temperatures limit host and parasite fitness: experimental evidence from a butterfly-parasite system

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ABSTRACT

Previous studies have shown that interactions between insect hosts and their pathogens can depend on environmental temperature. Global changes in temperature have been predicted to cause an increase in the intensity and prevalence of infection in many host-parasite systems. We examined the effect of temperature on the response to experimental infection by an obligate protozoan parasite, *Ophryocystis elektroscirrha*, in monarch butterflies, *Danaus plexippus.* We infected monarch larvae with one of three parasite doses and reared individuals to adulthood under one of three temperature treatments: hot (31˚C), moderate (26˚C), and cold (21˚C). We quantified monarch development and pre-adult survival, parasite loads, adult body size and longevity, and larval and adult wing melanism. Results showed that infected monarchs exposed to warmer temperatures emerged with greater levels of wing deformities and emerged with lower parasite loads when compared with monarchs reared under cooler temperatures. We also noted strong effects of rearing temperature on cuticular melanism of larvae and effects of temperature and infection on wing melanism in adult butterflies. Although monarch survival to adulthood, adult body size, and longevity were not significantly affected by temperature in this experiment, results reported here suggest that monarchs exposed to higher temperatures

suffered greater costs of infection, and their parasites experience reduced replication and transmission potential, as compared to monarchs reared under cooler conditions. Warmer temperature neither explains the increased prevalence or intensity of parasites in non-migratory monarch populations nor is likely to cause in an increase in parasite prevalence or intensity. However, warmer temperatures appear to be detrimental to both monarchs and *O. elektroscirrha*. Our results are important for understanding patterns of infection in wild monarch populations and for predicting how climate change might influence the distribution of disease in monarchs and other insect hosts.

Key words: temperature, host-parasite interaction, cuticular melanism, monarch butterfly, *Danaus plexippus*, neogregarine protozoan, *Ophryocystis elektroscirrha*

INTRODUCTION

As ectothermic organisms, insects are sensitive to environmental temperatures throughout all life stages (Huey & Kingsolver 1989), and the effects of temperature extend to interactions between insects and their parasites (Elliot *et al.* 2002; Thomas & Blanford 2003). Specifically, host immune defenses and parasite replication and virulence can all respond to small changes in temperature (Adamo 1998; Blanford & Thomas 1999; Blanford *et al.* 2003). In many cases, warmer temperatures can improve host fitness following infection. For example, in both *Drosophila* and damselflies affected by parasitoids and parasitic mites, high temperatures resulted in greater host resistance (Robb & Forbes 2005) and reduced parasite survival and development (Fellowes et al. 1999). Warmer temperatures can also increase the longevity of infected hosts, as demonstrated for fungal pathogens affecting crickets, grasshoppers, locusts and flies (Carruthers *et al.* 1992; Watson *et al.* 1993; Elliot *et al.* 2002). Indeed, many insects behaviorally raise their body temperature by basking or moving to warmer areas following infection (Adamo 1998; Karban 1998; Elliot *et al.* 2002). On the other hand, for mosquitoes, high temperatures were shown to lower host immunity based on melanic encapsulation (Suwanchaichinda & Paskewitz 1998), thus emphasizing that the direction and strength of temperature effects can differ among host and parasite species.

In addition to affecting infection parameters, environmental temperature can alter the body pigmentation of many insects, with cooler temperatures leading to the production of darker morphs in larval and adult stages (e.g., (Wicklund & Tullberg 2004; Hazel 2007). This is important ecologically because

darker pigmentation under cooler conditions can improve thermoregulation or enhance predator avoidance, as has been shown for some Lepidoptera (Kingsolver & Wiernasz 1991; Nice & Fordyce 2006). A separate body of work has demonstrated links between body pigmentation and the strength of an insect's immune response (Wilson et al. 2001); but see (Robb et al. 2003). In the moth *Spodoptera littoralis*, for example, Cotter et al. (2004) showed that darker larvae harbored a higher density of hemocytes and were more resistant to parasite infection. Moreover, in beetles, moths, and other insect species, cuticular melanism is known to be positively associated with the activity of phenoloxidase, an enzyme that is also important for parasite defense via melanic encapsulation (Reeson et al. 1998; Wilson et al. 2001). However, despite the known two-way relationships between temperature, insect body coloration, and immune defenses, few studies have examined how these three factors interact simultaneously.

Here, we examine the effects of temperature on the interaction between monarch butterflies, *Danaus plexippus*, and an obligate protozoan parasite, *Ophryocystis elektroscirrha*. Owing to their broad geographic range, monarchs are commonly exposed to a range of temperatures throughout all life stages, with migratory monarchs in temperate regions generally exposed to cooler temperatures and greater thermal variation than monarchs that inhabit tropical locations. Moreover, previous studies have shown that monarch survival and development rate are highly sensitive to temperature variation (Zalucki 1982; York & Oberhauser 2002; Solensky & Larkin 2003). For example, in eastern North America, monarch larvae that develop in the cooler spring and early

summer months experience a twofold increase in development time when compared to larvae developing in the warmer mid-summer months (Malcolm et al. 1987; Prysby 2001). In addition, monarch body pigmentation depends on temperature, with colder temperatures leading to darker coloration of both larval and adult monarchs (Smithers 1972; Solensky & Larkin 2003). Thus, the variation in temperatures experienced by monarchs in the wild, together with known effects of temperature on monarch development and survival, emphasize the ecological relevance of temperature variation for monarch-parasite interactions.

Our specific goals were to determine the effect of environmental temperature on parasite replication, and to quantify both lethal and sub-lethal effects of infection on the host. We predicted that monarch larvae reared in hot temperatures would develop faster and have higher survival in the absence of infection, and would also experience lower parasite replication and suffer less severe fitness consequences in the presence of infection. In addition, we predicted that monarch larvae reared in cooler temperatures would develop slower and experience greater rates of parasitism, owing to either greater parasite replication or lower host resistance. Based on previous work, we predicted that monarchs reared under cooler temperatures would appear darker (Solensky and Larkin 2003; Davis et al. 2005), and that parasite infection may limit the ability of monarchs to produce dark body pigmentation (Lindsey and Altizer 2008). Finally, it is important to note that monarchs have been identified as a species vulnerable to global change, with habitat loss and climate warming cited as threats to the monarchs' annual migration (Brower *et al.* 2002; Oberhauser &

Peterson 2003). Because climate warming has been linked to pathogen-mediated declines in several host species (Harvell *et al.* 2002; Harvell *et al.* 2008), examining how temperature affects monarch-parasite interactions is very relevant for predicting how climate change might influence the distribution of disease in monarchs and other insect hosts.

METHODS AND MATERIALS

Host-parasite system. Native and introduced monarch butterfly populations inhabit islands and continents worldwide (Ackery & Vane-Wright 1984) and form both migratory and non-migratory populations. Specifically, monarchs inhabiting tropical areas such as Hawaii, S. Florida, and the Caribbean Islands experience warm temperatures and can breed year-round. On the other hand, monarchs that inhabit seasonal environments in N. America and Australia experience more variable temperatures, despite the annual migration of adults to milder regions during the winter months (Urquhart & Urquhart 1978; James 1993; Brower *et al.* 1995). For example, during the monarchs' spring northward migration in eastern N. America, larvae from eggs laid in the gulf coast region as early as March (Howard & Davis 2004) can experience temperatures as cool as 7-20°C (Climate Diagnostics Center 2005). Once in their northern range (across much of eastern US and Canada), eastern monarchs undergo several summer generations, where temperatures often exceed 27°C in midsummer (Climate Diagnostics Center 2005). Moreover, previous studies have shown that both monarch survival and development rate are highest when larvae are reared at 26˚C, and that constant exposure to temperatures above 32°C and below 19°C leads to high pre-adult

mortality (e.g., Zalucki 1982; Oberhauser and York 2002; Solensky and Larkin 2003).

The life cycle of the neogregarine protozoan parasite *Ophryocystis elektroscirrha* closely follows monarch development. Transmission occurs when larvae ingest spores scattered by adult butterflies onto eggs and host plant leaves (McLaughlin & Myers 1970; Altizer *et al.* 2004; de Roode *et al.* 2008). Spores lyse within the larval gut and emerging sporozoites migrate to the hypoderm and undergo vegetative replication. During the hosts' late pupal stage, the parasite undergoes spore formation and infected butterflies emerge covered with dormant parasite spores on the outside of their bodies, primarily concentrated on the abdomen (McLaughlin & Myers 1970; Leong *et al.* 1992). Heavily infected monarchs experience negative effects on fitness including slower development, smaller adult forewings, shorter adult lifespans, and reduced flight ability (Leong *et al.* 1997; Altizer & Oberhauser 1999; Bradley & Altizer 2005; de Roode *et al.* 2007; Lindsey & Altizer 2008).

All monarch populations examined to date are affected by *O. elektroscirrha*, and prevalence varies dramatically among wild populations. Specifically, prevalence is highest (70-100%) in non-migratory populations in S. Florida and Hawaii (Leong et al. 1997; Altizer et al. 2000); intermediate (30-50%) in populations that migrate shorter distances in California and Australia, and lowest (2-8%) in the eastern N. American population that undergoes the longest distances (Altizer et al. 2000). These differences in prevalence have persisted for many years and could be caused by differences in monarch migratory behavior (Altizer *et al.* 2000; Bradley & Altizer 2005) or other

ecological variation, including host population density (Lindsey et al. in review) or host-parasite responses to environmental temperatures. For example, prevalence might be highest in populations from tropical regions if parasite development is favored by warmer temperatures, or if hosts are less likely to die from parasitism (and hence survive longer to transmit infections) when reared under warm conditions.

Host and parasite sources. Monarchs used in this experiment were the progeny of adults derived from wild-captured eggs and early instar larvae from Dade County, FL, USA (April 2004) and laboratory-raised monarchs derived from adults captured in Dekalb County, GA, USA (Fall 2003). All monarchs were screened for parasite infection as described in Altizer and Oberhauser (1999) and only uninfected monarchs were used to obtain progeny. Eggs were obtained from a total of 8 females mated to 3 males using a breeding design that eliminated the possibility of full-sib mating. Each mated female oviposited in a separate mosquito-net cage (0.6-m^3) onto potted greenhouse-reared *Asclepias incarnata* and eggs remained in the greenhouse at 26˚C until hatching. Larvae remained on their natal host plants until they reached late second instar, and were then randomly assigned to infection and temperature treatments described below.

Parasite inoculum was prepared from the abdomens of two infected wild-caught monarchs (Dade County, FL, USA) following the protocols of Altizer and Oberhauser (1999). Abdomens were vortexed in a 10 mL solution of distilled water for 3 minutes, and parasite spore concentration was calibrated using a hemocytometer to generate the following three doses in units of spores per larva: 50 (low dose), 500 (medium dose) and 1000 (high dose). Control inoculum (0

spores/larva) was prepared separately by vortexing the abdomen of an uninfected adult monarch. We inoculated late second instar larvae individually by pipetting 10 µl of inoculum onto to pieces of 1-cm^2 milkweed placed on dampened filter paper inside sterile 8.5-cm diameter plastic Petri dishes. Larvae were maintained singly in dishes until they consumed more than 80% of the plant material.

Experimental design. The experiment was fully factorial, with a total of 347 larvae divided among four experimental infection treatments and three rearing temperatures. Upon reaching third instar, larvae were transferred to individual 1.0 L plastic containers with mesh screen lids and reared singly to adulthood. Containers were placed in one of three temperature treatments set to a photoperiod of 12hr light: 12hr dark. Each treatment imposed a different day-night temperature regime as follows: cold (19-23˚C), moderate (24-28˚C) and hot (29-33˚C). These temperatures were selected because previous studies have shown that both monarch survival and development rate are highest when larvae are reared at 26˚C, and that constant exposure to temperatures above 32°C and below 19°C leads to high pre-adult mortality (e.g., Zalucki 1982; Oberhauser and York 2002; Solensky and Larkin 2003) and our primary focus was to evaluate the effects of varying temperature on the response to parasitism. Additionally, monarchs experience these ranges of temperatures (Climate Diagnostics Center 2005). Controlled environment chambers (Conviron Model E15) set to 1-hr ‘ramp’ periods for each temperature transition were used for hot and cold treatments; containers for the moderate temperature treatment were placed on metal shelving in the same experimental room with a thermostat setting that was closed to outside light and temperature sources. The lighting was standardized

across all treatments by using a photometric cell and set to 5000 Lux with cool white fluorescent tubs. Twice each day for the duration of the experiment, we recorded the current, maximum and minimum temperature and humidity, with averages shown in Table 1. Twice daily we added fresh cuttings of greenhouse-raised milkweed (*A. incarnata*) to each container, removed frass, and maintained a clean, moist paper lining until monarchs reached pupation. Milkweed stalks were held in florist tubes and sterilized by soaking in a 10% bleach solution for 20 minutes and rinsing thoroughly in tap water prior to use. Latex gloves were used to handle milkweed, monarchs, and inoculum. Gloves were frequently changed and laboratory surfaces and utensils were sterilized with 20% bleach solution.

Quantifying development, cuticular melanism, size, and infection status. We recorded the development time of monarchs from inoculation to pupation and eclosion in days. When larvae reached late fifth instar, we took a digital image of the larvae using an Olympus C-3000 digital camera set to macro focus mode and mounted on a tripod 20 cm above each individual. Larvae were placed on a bright green background and photographed from above, without a flash, using maximum aperture and ambient light to avoid any shadow. The images were imported into Adobe Photoshop and processed with the Image Processing Tool Kit (IPTK) plugin (Reindeer Graphics, Inc.). We digitally cropped tubercles from each larva, removed the background, and thresholded the image to produce a pure black and white image (so that all non-black colors became white; (Davis et al. 2004; Davis et al. 2005). We measured cuticular melanism as the percentage of black pigmentation (i.e., area of solid black as determined by the thresholding step divided by the total body area measured).

Both pupal and adult mass were measured on an analytic balance to the nearest 0.0001g, with adult mass recorded 24 hours after emergence. We recorded the proportion of monarchs that successfully eclosed as adults, and the presence and severity of wing deformities as follow: 0 - not deformed, 1 - wings folded or not completely expanded, and 2 - wings severely crumpled and/or adults not able to completely detach from the pupal case. We recorded the sex of each butterfly and placed individuals into a glassine envelope 6-12 hrs post-eclosion. Envelopes with monarchs were held at 12°C without feeding and mortality counts were taken daily to record adult longevity (in days). Adult longevity may also be referred to as starvation resistance and provides a measure of the ability on an individual monarch to survive on lipid reserves alone, which is similar to the survival requirements of monarchs at over-wintering sites.

We quantified the proportion of adults that became infected and the parasite load of each individual by estimating the total number of spores on the insects' abdomens. Upon death, the abdomen of each monarch was removed and placed into a vial containing 5 ml of water. This was vortexed at high speed for 5 min, and a hemocytometer counting chamber was used to estimate the number of spores per butterfly based on 20 replicate counts for each sample.

For adults that did not show visible wing deformity, we removed the L and R forewings following death, and used an HP flatbed scanner set to 300 dpi, using the same exposure for each scan, to scan the dorsal sides of both forewings. As with measures of larval pigmentation, we performed a thresholding step to produce a pure black and white image, and ran the IPTK measurements function to quantify the total area (in mm^2) of all black versus white patches on the

resulting image. We measured the proportion of black pigmentation on adult forewings based on the area of black divided by the total wing area.

Statistical Analysis. We used binary logistic regression to examine the effect of design variables (infection treatment and rearing temperature) on three binomial response variables (recorded as 0/1 for each individual): survival to eclosion, adult infection status (yes/no), and the presence of wing deformities. For logistic regression, significance tests are reported as Wald χ^2 statistics and associated P-values. The nonparametric Kaplan- Meier analysis was used to examine treatment effects on variables measuring the length of time: development time from oviposition to eclosion (in days) and adult longevity (in days). Analysis of variance was used to examine experimental effects on final spore load. Count variables were log-transformed prior to analysis to normalize the error variance. We further examined five continuous traits including the proportion of black pigmentation on larvae, pupal mass, adult mass, adult forewing area and the proportion of black pigmentation on adult forewings. All analyses were performed in SPSS (ver. 15.0; SPSS, Inc., Chicago, IL). We used comparisons of Akaike's Information Criterion (AIC) and Hurvich and Tsai's Criterion (AICC) for model simplification (Crawley 2002) and report test statistics and P-values only for variables included in the final simplified model (Full Model: Dependent variable = Temperature Treatment + Parasite Treatment + Sex + all two-way and three-way interactions). Bonferroni pairwise comparisons of means were used to examine differences between the three temperature treatments and the four parasite treatments. We examined the distribution of the residuals of each minimum adequate model and found each to

approximate a normal distribution. For logistic regression analyses, model simplification was performed using likelihood ratio tests based on comparison of nested models.

RESULTS

Pre-adult mortality and development. A total of 298 monarchs survived from inoculation to the adult stage, representing 86% of the larvae used to initiate the experiment. Pre-adult survival was lowest for monarchs in the high dose treatment (73%) as compared with monarchs in the control (93%), low (92%) and medium (85%) dose treatments (Figure 1). This main effect of parasite treatment on pre-adult survival was highly significant (χ^2=16.8, df=3, p=0.001). Average monarch survival was similar across temperature treatments (cold: 88%; moderate: 82%; hot: 87%). Monarchs infected with the highest parasite dose appeared to survive better in the cold temperature treatment (Figure 1), but neither the main effect of temperature nor the temperature by dose interaction effect were included in the final model of pre-adult survival.

We observed differences in development time from inoculation to eclosion arising from both temperature and infection treatments. Development time decreased sharply with increasing temperature and increased moderately with increasing parasite dose (Figure 2). The main effect of both temperature and parasite dose and the interaction of dose and temperature treatment on development time were highly significant (temperature: Kaplan-Meier Log Rank δ=411.713 df=2 p=0.000; parasite: Kaplan-Meier Log Rank δ=9.940 df=3

p=0.019; parasite treatment and temperature: Kaplan-Meier Log Rank δ=466.355 df=11 p=0.000).

Infection status and parasite load. None of the control monarchs were infected, while all of the monarchs in the medium and high dose treatments and 97% of the monarchs in the low dose treatment emerged as infected. There was no effect of temperature on the proportion of adults that became infected (χ^2=0.908, df=2, p=0.635). Among the subset of monarchs that emerged with parasites, the average parasite load across all treatments was 5.83 x 10^6 spores (± 3.28 x 10^5 SE). Counter to our predictions, parasite load was greatest in the low dose (50 spores/larva) treatment and decreased with increasing parasite dose (Figure 3; Table 2). Moreover, for all infection treatments, parasite load was lowest in the hot temperature treatment (Figure 3) and this effect was highly significant (Table 2).

Pupal and adult mass. Average pupal mass was similar for all experimental treatments (Avg. mass=1.32 g ± 0.008 SE) and we observed no significant effects of infection or temperature on this trait. However, the pupal mass of males was significantly greater than that of females ($F_{1,287}$=56.280, p=0.000). Among newly emerged adults, average body mass was greatest in the control treatment group (0.576 g ± 0.007 SE), second greatest in the low dose parasite dose treatment (0.527 g ± 0.008 SE), and lowest for both medium (0.490 g ± 0.011 SE) and high dose (0.490 g ± 0.017 SE) parasite treatments. This effect of parasite treatment on adult mass was highly significant, and Bonferroni pairwise comparisons provided support for similar means between medium and high dose parasite treatments, but significant differences between

all other treatment combinations ($0.000 < p < 0.035$). Across all treatments, males weighed more than females, and this effect of sex was highly significant (Table 2). There was no significant effect of temperature on adult mass.

Adult wing deformity and size. A total of 39% of monarchs across all treatments (N=298) emerged with mild to severe wing deformities. The proportion of monarchs with any wing deformity was lowest for the control and low dose treatments and was greatest for monarchs in the medium and high dose treatments (Figure 4a). The main effect of infection treatment on wing deformities was highly significant (χ^2=39.7, df=3, p=0.000). Although, wing deformities among infected monarchs were more common in the moderate and hot temperature treatments (Figure 4a), the main effect of temperature (χ^2=1.27, df=2, p=0.531) and the interaction effect between temperature and infection treatment were not significant (χ^2=5.47, df=6, p=0.485).

Across all monarchs with wing deformities (N=116), 66% did not completely eclose from their pupal cases and had severely deformed wings (i.e., Deformity Index 2). As with overall deformities, the proportion of monarchs with severe deformities increased significantly with increasing parasite dose (χ^2=12.7, df=3, p=0.005), such that 0 (N=82), 1.3% (N=79), 44% (N=75), and 71.2% (N=59), respectively, of the monarchs in the control, low, medium, and high dose treatments were severely deformed and did not completely eclose. As with overall deformity, this proportion was lowest in the cold temperature treatment (17%; N=105), and higher in the moderate (33%; N=96) and hot (28%; N=94) temperature treatments. The main effect of temperature on severe wing deformities was significant (χ^2=6.70, df=2, p=0.035).

We observed a strong negative association between the level of deformities and estimated spore loads, focusing on the subset of infected monarchs. Specifically, average spore loads were highest among monarchs with no wing deformities (N=97; $7.36 \times 10^6 \pm 4.81 \times 10^5$ SE), intermediate for monarchs with mildly deformed wings (Deformity Index 1; N=37; $6.15 \times 10^6 \pm 8.17 \times 10^5$ SE), and lowest for monarchs that did not completely eclose from their pupal cases (Deformity Index 2; N=75; $3.69 \times 10^6 \pm 4.39 \times 10^5$ SE). This negative association between deformity level and spore load was highly significant ($F_{1, 110}=8.534$, $p=0.004$).

Individual monarchs with deformed wings were excluded from analyses of wing size (measured here as forewing area). Out of the 191 monarchs with non-deformed forewings, 79% were in the control and low parasite dose treatment groups combined; we therefore grouped monarchs from both medium and high dose treatments into a single level prior to analysis of this variable. Monarch forewing area decreased with increasing parasite dose (means for control: 902 ± 6 SE; low dose: 894 ± 8 SE; moderate and high dose: 842 ± 11 SE), and males were significantly larger than females across all treatments. The main effects of parasite treatment and sex on forewing area were highly significant (Table 2). We also observed a significant interaction between sex and dose treatment, (Table 2), in that females experienced a sharper decrease in wing area with increasing dose as compared to males. There was no significant effect of temperature on adult wing area.

Adult longevity (starvation resistance). Parasite treatment had a strong negative effect on adult longevity (starvation resistance) (Figure 4b); most adults

from the control treatment (N = 58) lived for up to 14 days, whereas 89% of adults from the medium and high dose treatments (N = 130) died within 3 days post-eclosion. We also observed a significant main effect of parasite dose and an interaction between temperature and dose on adult longevity (starvation resistance) (parasite: Kaplan-Meier Log Rank δ=302.971 df=3 p=0.019; parasite and temperature: Kaplan-Meier Log Rank δ=322.603 df=11 p=0.000). Specifically, control (uninfected) monarchs had the shortest adult lifespan when reared in the hot treatment (Figure 4b), whereas monarchs in the low dose treatment lived longest when reared under hot temperatures. Monarchs in the medium and high dose treatments had similarly short lifespans across all temperatures (Figure 4b).

Larval and adult melanism. The proportion of black pigmentation on monarch larvae increased with decreasing temperature. Larvae in the cold treatment had the greatest proportional area of black on their bodies (60% ± 0.6 SE), and larvae from the moderate (49% ± 0.5 SEM) and hot (34% ± 0.6 SE) treatments had lower proportions of black pigmentation. This main effect of temperature on larval melanism was highly significant (Table 2), but we detected no main effect or interaction involving parasite treatment.

Across all temperature treatments, the proportion of black pigmentation on adult forewings decreased with increasing parasite dose, and this main effect of parasite treatment on adult forewing melanism was highly significant (Table 2). As with analyses of forewing area, we excluded data from adults with wing deformities and grouped monarchs from medium and high dose treatments into a single level prior to analysis. Females were significantly darker than males

(Figure 5) and both the main effect of sex and the two-way interaction involving parasite treatment and sex were highly significant (Table 2). Specifically, females showed a sharper reduction in wing melanism with increasing dose as compared to males (Figure 5). Among females, forewing melanism increased with temperature, but among males, forewing melanism decreased with temperature for all but the highest dose treatments (Figure 5). The interaction between temperature and sex was highly significant, but there was no main effect of temperature on wing melanism (Table 2; Figure 5). Finally, there was no relationship between larval melanism and adult wing melanism, either when the two variables were examined alone (Pearson's R=0.01, N=335, p=0.894) or when larval melanism was included as a covariate in the final model shown in Table 2.

DISCUSSION

Our results showed that the temperature to which monarchs were exposed during their larval and pupal stages affected the outcome of infection by *O. elektroscirrha*. Specifically, infected monarchs exposed to warmer temperatures emerged with greater levels of wing deformities and emerged with lower parasite loads when compared with monarchs reared under cooler temperatures. It is important to note that with the exception of two variables (development time and larval melanism), the relative effects of parasite dose on response variables were generally stronger than the effects of rearing temperature. Nevertheless, results reported here suggest that monarchs exposed to higher temperatures suffered greater costs of infection – and their parasites experience reduced replication and

transmission potential – as compared to monarchs reared under cooler conditions.

Consistent with previous studies, we found that *O. elektroscirrha* caused significant fitness reductions for monarch hosts, with higher parasite doses leading to increased infection probability and lower pre-adult survival, smaller adult body mass and forewing area and shorter adult longevity (Altizer & Oberhauser 1999; Bradley & Altizer 2005; de Roode *et al.* 2007; Lindsey & Altizer 2008). Some of these variables, particularly adult longevity (starvation resistance), pupal and adult mass, and adult wing area, did not change significantly across the range of temperatures examined. However, warm temperatures strongly increased the probability that monarchs infected with high parasite doses emerged with wing deformities or failed to eclose properly. Because monarchs with substantial wing deformities would not survive to reproduce or transmit infections, the interactive effects of high temperature and parasite infection on host fitness would certainly limit the parasites' own reproductive potential under natural conditions.

Parasite spore loads were also dramatically reduced for monarch hosts reared under high temperatures. As noted by studies of other insect-pathogen systems (Fellowes et al. 1999), this could be caused by hot temperatures impeding parasite development directly (Elliott et al. 1995) or enhancing host immune defenses (Robb & Forbes 2005). Alternatively, because monarchs developed faster (in terms of days from inoculation to adulthood) at high temperatures, it is possible that *O. elektroscirrha* did not have enough time to fully compete its replication cycle within monarchs reared in the hot treatment.

Thus, the decrease in parasite load at high temperatures could be caused by the parallel decrease in monarch development time.

In addition to cooler temperatures resulting in a longer time for monarchs to reach the adult stage, cold temperatures were also associated with greater cuticular melanism of late-instar larvae. Both of these results are consistent with previous studies of temperature effects on monarch development (e.g., Goehring and Oberhauser 2002; Solensky and Larkin 2003; Farrey and Davis 2004; Davis et al. 2005). However, unlike some past work (e.g., Zalucki 1982; Solensky and Larkin 2003), we found no effect of temperature on pre-adult survival. This could be due to the fact that we did not consider survival of eggs or early instar larvae, as was done in previous studies, both of which can experience low survival under extreme temperatures. Moreover, temperatures used in this study were relatively close to the 27-29°C at which monarch survival is maximized (Zalucki 1982), thus dampening any effects of temperature on host survival.

Importantly, the development time of monarchs increased with increasing parasite dose. This change in development time could be due to parasites consuming resources previously allocated to host growth and development. Another possibility is that heavily infected hosts re-allocated resources to immune defense or to repair host tissue damage caused by replicating parasites.

Consistent with previous studies of monarchs, females had significantly darker forewings than males (e.g., Davis et al. 2005), and wing melanism decreased significantly with greater parasite dose (Lindsey & Altizer 2008; Lindsey *et al.* 2008). One explanation for lighter forewings among parasitized monarchs could be that, because melanin is costly to synthesize (e.g., Talloen et

al. 2004), external melanism is influenced by the overall "health" of an individual (Stoehr 2006), so that only unparasitized monarchs can produce the darkest wings. A related explanation is that if the ability to produce melanin is constrained by finite resources, then insects might face trade-offs between investments in immune defense versus body coloration (Hooper *et al.* 1999; Talloen *et al.* 2004; Freitak *et al.* 2005). Our results were also consistent with an earlier study noting an interesting interaction between temperature, wing melanism and sex (Davis et al. 2005). Specifically, males were darkest when reared under cold temperatures and lightest in the hot temperature treatment, whereas females showed the opposite trend. Davis et al. (2005) suggested that this result could represent a non-adaptive response to thermal stress among female monarchs; another possibility is that the female monarch immune system functions better at hotter temperatures. Therefore, melanin production may be increased at hotter temperatures as a by-product of greater phenoloxidase activity (Robb and Forbes 2005).

We were surprised to find a negative relationship between infection dose and final parasite loads (an index of within-host replication and spore formation) across all temperature treatments, as previous studies have shown positive effects of dose on final parasite loads of *O. elektroscirrha* (e.g., de Roode et al. 2007). One possible explanation for the relationship observed here is that the two highest doses used in this experiment (500 and 1000 spores per larva) were greater than the maximum doses used in previous studies. Thus, the strong density-dependent effects reported by de Roode et al. (2007), whereby per capita replication declined with increasing dose, could lead to negative effects of dose on

final parasite loads when the initial dose is exceptionally high. Moreover, we noted that the majority of monarchs infected with the highest parasite doses not only suffered from wing deformities, but were also missing entire sections of abdominal scales – and, presumably, parasite spores that would have developed around those scales. Thus, a second explanation is that at high doses, spore formation by the parasites was either greatly impaired, or the majority of spores remained in the pupal cases and were not present on the abdomens of badly deformed monarchs.

Recent reviews on climate change and wildlife disease emphasize the importance of understanding the threat of climate warming on host population declines, animal migrations, and host immunity (Harvell *et al.* 2002; Harvell *et al.* 2008). While some studies have suggested that climate warming will facilitate the emergence and spread of wildlife diseases (such as has been reported for corals and frogs; (Pounds *et al.* 2006; Harvell *et al.* 2007), our results suggest that for some insect pathogens, the opposite effect could occur, in that direct effects of temperature on insects and their parasites may lead to reduced transmission and lower prevalence. While the precise mechanisms behind this observation remain unclear, studies that examine the influence of temperature on hosts and parasites could inform predictions regarding effects of global warming on insect-parasite systems (Poulin 2006). Of equal importance to host-parasite dynamics is the effect of climate change on animal migrations and breeding ecology. For monarch butterflies, combined effects of climate warming and habitat loss at wintering sites could lead to the gradual replacement of the large migratory population in eastern N. America with resident populations that breed

year-round in parts of their current summer range (Brower & Malcolm 1991; Brower *et al.* 2002; Oberhauser & Peterson 2003). Based on current parasite data from different populations, these non-migratory populations are expected to harbor high parasite burdens (Altizer et al. 2000) and could favor more virulent parasite strains (de Roode et al. 2008). Therefore, while results from this study indicate that warmer temperatures *per se* do not favor increased prevalence of *O. elektroscirrha*, in part because lower parasite loads and greater host mortality under high temperatures would reduce parasite transmission, changes in temperature could increase parasite prevalence and intensity through other mechanisms, for example, through effects on the monarchs' annual migratory behavior.

In summary, our study demonstrated effects of temperature on both host and parasite fitness, with results showing that increased temperatures amplified the negative effects of parasitism on monarch fitness, and decreased the production of parasite transmission stages on emerging adult butterflies. In other words, hotter temperatures appear to be detrimental to both the host and parasite. These results have key implications for parasite infection rates in wild populations; specifically, they suggest that, all else being equal, the prevalence of *O. elektroscirrha* should be lower in hotter climates owing to decreased parasite transmission and more rapid removal of infected butterflies. However, observed differences in parasite prevalence among populations show the opposite pattern, such that parasite infections are known to be lowest in the eastern U.S. migratory population that experiences more variable temperatures, and cooler temperatures on average, than monarchs inhabiting tropical locations (Leong et

al. 1997; Altizer et al. 2000). Thus, findings from our study indicate that observed differences in prevalence between migratory versus resident monarch populations are unlikely to result from temperature effects operating on infection processes and virulence within individual hosts.

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REFERENCES

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

Adamo S.A. (1998). The specificity of behavioral fever in the cricket *Acheta domesticus*. *Journal of Parasitology*, 84, 529-533.

Altizer S., Oberhauser K.S. & Geurts K.A. (2004). Transmission of the protozoan parasite, *Ophryocystis elektroscirrha,* in monarch butterfly populations. In: *The monarch butterfly. Biology and Conservation* (eds. Oberhauser KS & Solensky MJ). Cornell University Press Ithaca, NY.

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

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

Blanford S. & Thomas M.B. (1999). Host thermal biology: the key to understanding host-pathogen interactions. *Agricultural and Forest Entomology*, 1, 195-202.

Blanford S., Thomas M.B., Pugh C. & Pell J.K. (2003). Temperature checks the red queen? Resistance and virulence in a fluctuating environment. *Ecology Letters*, 6, 2-5.

Bradley C.A. & Altizer S. (2005). Parasites hinder monarch butterfly flight: implications for disease spread in migratory hosts. *Ecology Letters*, 8, 290-300.

Brower L.P., Castilleja G., Peralta A., Lopez-Garcia J., Bojorquez-Tapia L., Diaz S., Melgarejo D. & Missrie M. (2002). Quantitative changes in forest quality in a principal overwintering area of the monarch butterfly in Mexico, 1971-1999. *Conservation Biology*, 16, 346-359.

Brower L.P., Fink L.S., Brower A.V., Leong K., Oberhauser K., Altizer S., Taylor O., Vickerman D., Calvert W.H., Vanhook T., Alonsomejia A., Malcolm S.B., Owen D.F. & Zalucki M.P. (1995). On the dangers of interpopulational transfers of monarch butterflies - discussion. *Bioscience*, 45, 540-544.

Brower L.P. & Malcolm S.B. (1991). Animal migrations: endangered phenomena. *American Zoologist*, 31, 265-276.

Carruthers R.I., Larkin T.S., Firstencel H. & Feng Z. (1992). Influence of thermal ecology on the mycosis of a rangeland grasshopper. *Ecology*, 73, 190-204.

Climate Diagnostics Center (2005). National Oceanic and Atmospheric Administration and Cooperative Institute for Research in Environmental Science, Climate Diagnostics Center Website - http://www.cdc.noaa.gov/index.html.

Cotter S.C., Hails R.S., Cory J.S. & Wilson K. (2004). Density-dependent prophylaxis and condition-dependent immune function in Lepidopteran larvae: a multivariate approach. *Journal of Animal Ecology*, 73, 283-293.

Crawley M.J. (2002). *Statistical Computing: An Introduction to Data Analysis using S-Plus*. John Wiley and Sons, West Sussex, England.

Davis A.K., Farrey B. & Altizer S. (2004). Quantifying monarch butterfly larval pigmentation using digital image analysis. *Entomologia Experimentalis et Applicata*, 113, 145-147.

Davis A.K., Farrey B.D. & Altizer S. (2005). Variation in thermally induced melanism in monarch butterflies (Lepidoptera : Nymphalidae) from three North American populations. *Journal of Thermal Biology*, 30, 410-421.

de Roode J.C., Gold L.R. & Altizer S. (2007). Virulence determinants in a natural butterfly-parasite system. *Parasitology*, 134, 657-668.

de Roode J.C., Yates A.J. & Altizer S. (2008). Virulence-transmission trade-offs and population divergence in virulence in a naturally occurring butterfly parasite. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 7489-7494.

Elliot S.L., Blanford S. & Thomas M.B. (2002). Host-pathogen interactions in a varying environment: temperature, behavioural fever and fitness. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 269, 1599-1607.

Elliott N.C., Burd J.D., Kindler S.D. & Lee J.H. (1995). Temperature effects on development of three cereal aphid parasitoids (Hymenoptera: Aphidiidae). *Great Lakes Entomologist*, 28, 199-204.

Fellowes M.D.E., Kraaijeveld A.R. & Godfray H.C.J. (1999). The relative fitness of *Drosophila melanogaster* (Diptera, Drosophilidae) that have successfully defended themselves against the parasitoid *Asobara tabida*

(Hymenoptera, Braconidae). *Journal of Evolutionary Biology*, 12, 123-128.

Freitak D., Vanatoa A., Ots I. & Rantala M.J. (2005). Formation of melanin-based wing patterns is influenced by condition and immune challenge in *Pieris brassicae*. *Entomologia Experimentalis Et Applicata*, 116, 237-243.

Harvell C.D., Altizer S., Cattadori I., Harrington L. & Weil E. (2008). Climate change and wildlife diseases: when does the host matter the most? *Ecology*.

Harvell C.D., Jordan-Dahlgren E., Merkel S., Raymundo L., Rosenberg E., G. S., Weil E. & Willis B. (2007). Coral disease, environmental drivers and the balance between coral and microbial associates. *Oceanography*, 20, 58-81.

Harvell C.D., Mitchell C.E., Ward J.R., Altizer S.M., Dobson A.P., Ostfeld R.S. & Samuel M.D. (2002). Ecology - climate warming and disease risks for terrestrial and marine biota. *Science*, 296, 2158-2162.

Hazel W.D. (2007). The environmental and genetic control of seasonal polyphenism in larval color and its adaptive significance in a swallowtail butterfly. *Evolution*, 56, 342-348.

Hooper R.E., Tsubaki Y. & Siva-Jothy M.T. (1999). Expression of a costly, plastic secondary sexual trait is correlated with age and condition in a damselfly with two male morphs. *Physiological Entomology*, 24, 364-369.

Howard E. & Davis A.K. (2004). Documenting the spring movements of monarch butterflies with Journey North, a citizen science program. In: *The Monarch Butterfly. Biology and Conservation* (eds. Oberhauser K & Solensky M). Cornell University Press Ithaca, NY, pp. 105-114.

Huey R.B. & Kingsolver J.G. (1989). Evolution of thermal sensitivity of ectotherm performance. *Trends in Ecology & Evolution*, 4, 131-135.

James, D.G., ed. (1993). *Migration biology of the monarch butterfly in Australia*, pp 189-200. Natural History Museum of Los Angeles County, Los Angeles, CA.

Karban R. (1998). Caterpillar basking behavior and nonlethal parasitism by tachinid flies. *Journal of Insect Behavior*, 11, 712-723.

Kingsolver J.G. & Wiernasz D.C. (1991). Seasonal polyphenism in wing-melanin pattern and thermoregulatory adaptation in *Pieris* butterflies. *American Naturalist*, 137, 816-830.

Leong K.L.H., Kaya H.K., Yoshimura M.A. & Frey D.F. (1992). The occurrence and effect of a protozoan parasite, *Ophryocystis elektroscirrha* (Neogregarinida, Ophryocystidae) on overwintering monarch butterflies, *Danaus plexippus* (Lepidoptera, Danaidae) from two California winter sites. *Ecological Entomology*, 17, 338-342.

Leong K.L.H., Yoshimura M.A., Kaya H.K. & Williams H. (1997). Instar susceptibility of the monarch butterfly (*Danaus plexippus*) to the neogregarine parasite, *Ophryocystis elektroscirrha*. *Journal of Invertebrate Pathology*, 69, 79-83.

Lindsey E. & Altizer S. (2008). Sex differences in immune defenses and response to parasitism in monarch butterflies. *Evolutionary Ecology*.

Lindsey E., Mehta M., Dhulipala V., Oberhauser K. & Altizer S. (2008). Crowding and disease: effects of host density on response to infection in a butterfly-parasite interaction. *Ecological Entomology*. (in review).

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

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

Nice C.C. & Fordyce J.A. (2006). How caterpillars avoid overheating: behavioral and phenotypic plasticity of pipevine swallowtail larvae. *Oecologia*, 146, 541-548.

Oberhauser K. & Peterson A.T. (2003). Modeling current and future potential wintering distributions of eastern North American monarch butterflies. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 14063-14068.

Poulin R. (2006). Global warming and temperature-mediated increases in cercarial emergence in trematode parasites. *Parasitology*, 132, 143-151.

Pounds J.A., Bustamante M.R., Coloma L.A., Consuegra J.A., Fogden M.P., Foster P.N., La Marca E., Masters K.L., Merino-Viteri A., Puschendorf R., Ron S.R., Sanchez-Azofeifa G.A., Still C.J. & Young B.E. (2006). Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature*, 439, 161-167.

Prysby M.P. (2001). Temporal and geographical variation in monarch egg and larval densities (*Danaus plexippus*): an ecological application of citizen science. In. University of Minnesota St. Paul, MN.

Rawlins J.E. & Lederhouse R.C. (1981). Developmental influences of thermal-behavior on monarch caterpillars (*Danaus-Plexippus*) - an adaptation for migration (Lepidoptera, Nymphalidae, Danainae). *Journal of the Kansas Entomological Society*, 54, 387-408.

Reeson A.F., Wilson K., Gunn A., Hails R.S. & Goulson D. (1998). Baculovirus resistance in the noctuid *Spodoptera exempta* is phenotypically plastic and responds to population density. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 265, 1787-1791.

Robb T. & Forbes M.R. (2005). On understanding seasonal increases in damselfly defence and resistance against ectoparasitic mites. *Ecological Entomology*, 30, 334-341.

Robb T., Forbes M.R. & Jamieson I.G. (2003). Greater cuticular melanism is not associated with greater immunogenic response in adults of the polymorphic mountain stone weta, *Hemideina maori*. *Ecological Entomology*, 28, 738-746.

Smithers C.N. (1972). Observations on a breeding population of *Danaus plexippus* (L.) (Lepidoptera: Nymphalidae) at Camden, New South Wales. *Australian Zoologist*, 17, 142-148.

Solensky M.J. & Larkin E. (2003). Temperature-induced variation in larval coloration in *Danaus plexippus* (Lepidoptera : Nymphalidae). *Annals of the Entomological Society of America*, 96, 211-216.

Stoehr A.M. (2006). Costly melanin ornaments: the importance of taxon? *Functional Ecology*, 20, 276-281.

Suwanchaichinda C. & Paskewitz S.M. (1998). Effects of larval nutrition, adult body size, and adult temperature on the ability of *Anopheles gambiae* (Diptera : Culicidae) to melanize Sephadex beads. *Journal of Medical Entomology*, 35, 157-161.

Talloen W., Van Dyck H. & Lens L. (2004). The cost of melanization: butterfly wing coloration under environmental stress. *Evolution*, 58, 360-366.

Thomas M.B. & Blanford S. (2003). Thermal biology in insect-parasite interactions. *Trends in Ecology & Evolution*, 18, 344-350.

Urquhart F.A. & Urquhart N.R. (1978). Autumnal migration routes of Eastern population of monarch butterfly (*Danaus-p-plexippus* L-Danaidae-Lepidoptera) in North-America to overwintering site in Neovolcanic Plateau of Mexico. *Canadian Journal of Zoology-Revue Canadienne de Zoologie*, 56, 1759-1764.

Watson D.W., Mullens B.A. & Petersen J.J. (1993). Behavioral fever response of *Musca domestica* (Diptera: Muscidae) to infection by *Entomophthora muscae* (Zygomycetes: Entomophthorales). *Journal of Invertebrate Pathology*, 61, 10-16.

Wicklund C. & Tullberg B.S. (2004). Seasonal polyphenism and leaf mimicry in the comma butterfly. *Animal Behaviour*, 68, 621-627.

Wilson K., Cotter S.C., Reeson A.F. & Pell J.K. (2001). Melanism and disease resistance in insects. *Ecology Letters*, 4, 637-649.

York H.A. & Oberhauser K.S. (2002). Effects of duration and timing of heat stress on monarch butterfly (*Danaus plexippus*) (Lepidoptera : Nymphalidae) development. *Journal of the Kansas Entomological Society*, 75, 290-298.

Zalucki M.P. (1982). Temperature and rate of development in *Danaus-plexippus L* and *Danaus-chrysippus L* (Lepidoptera, Nymphalidae). *Journal of the Australian Entomological Society*, 21, 241-246.

Table 1. Targeted temperature settings, realized average maximum and minimum temperatures (in °C), and average maximum and minimum relative humidity values for cold, moderate, and hot treatments used in this experiment. Numbers in parentheses represent standard errors.

Treatment	Targeted range (day-night)	Avg. diurnal maximum	Avg. nocturnal minimum	Avg. maximum humidity	Avg. minimum humidity
Cold	23-19	23.1 (±0.08)	18.8 (±0.24)	86.8 (±0.7)	71.4 (±0.8)
Moderate	28-24	29.2 (±0.13)	25.5 (±0.25)	69.6 (±1.1)	50.6 (±1.3)
Hot	33-29	33.2 (±0.03)	30.0 (±0.25)	67.3 (±1.1)	49.7 (±1.1)

Table 2. Analysis of spore load, adult mass, forewing area, larval melanism, and wing melanism as a function of experimental design variables (temperature and infection treatment) and sex (full model: Response variable = temperature + parasite treatment + sex + temperature*parasite trt + temperature*sex + parasite trt*sex + temperature*parasite trt*sex + error term). For spore load, only data from parasite-treated monarchs were included in the analysis. For forewing area and wing melanism, medium and high dose parasite treatment levels were combined into one category owing to small sample sizes. Model simplification was performed as describe in Methods text. F- and P-values are shown only for those explanatory variables remaining in the final model; adjusted R^2 values are shown for the final reduced models.

	Spore Load	Adult Mass	Forewing Area	Larval Melanism	Wing Melanism
Temperature	$F_{2,196}$=85.371, **p=0.000**			$F_{2,276}$=425.094, **p=0.000**	$F_{2,176}$=2.273, p=0.106
Parasite Treatment	$F_{2,196}$=25.027, **p=0.000**	$F_{3,285}$=22.493, **p=0.000**	$F_{2,184}$=15.299, **p=0.000**		$F_{2,176}$=13.192, **p=0.000**
Sex		$F_{1,285}$=36.407, **p=0.000**	$F_{2,184}$=23.846, **p=0.000**	$F_{1,276}$=0.759, p=0.384	$F_{1,176}$=408.773, p=**0.000**
Temperature * Parasite	$F_{4,196}$=4.399, **p=0.002**				$F_{4,176}$=1.765, p=0.138
Temperature*Sex				$F_{2,276}$=3.211, **p=0.042**	$F_{2,176}$=15.788, **p=0.000**
Parasite*Sex			$F_{2,184}$=4.367, **p=0.014**		$F_{2,176}$=6.029, **p=0.003**
Adjusted R^2	0.527	0.255	0.211	0.757	0.758

FIGURE LEGENDS

Figure 1. Proportion of monarchs that survived from inoculation to the adult stage (± SEM) when reared under cold (21˚C), moderate (26˚C), and hot (31˚C) conditions. Colored bars represent inoculation treatments for parasite doses 0 (control), 50 (low), 500 (medium) and 1000 (high) spores per larva.

Figure 2. Effect of temperature and parasite treatment on development time from inoculation to eclosion measured in days (log-transformed) (± SEM). Data are shown for monarchs reared under cold (21˚C), moderate (26˚C), and hot (31˚C) temperatures. Colored bars represent inoculation treatments for parasite doses 0 (control), 50 (low), 500 (medium) and 1000 (high) spores per larva.

Figure 3. Effect of temperature and parasite treatments on parasite spore load (± SEM). Data are shown only for infected monarchs reared under cold (21˚C), moderate (26˚C) and hot (31˚C) temperatures. Colored bars represent inoculation treatments for parasite doses 0 (control), 50 (low), 500 (medium) and 1000 (high) spores per larva. Bonferroni pairwise comparisons showed that mean values for monarchs reared under each of the three parasite treatments were significantly different ($p < 0.015$), and that mean values for monarchs reared under the hot temperature treatment were significantly different than means for monarchs reared at cold and moderate temperature treatments ($p = 0.000$).

Figure 4. Effects of temperature and parasite treatments on measures of adult monarch fitness (± SEM). Data are shown for monarchs reared under cold (21˚C), moderate (26˚C) and hot (31˚C) temperatures. Colored bars represent inoculation treatments for parasite doses 0 (control), 50 (low), 500 (medium) and 1000 (high) spores per larva. (a) Proportion of adults with deformed wings (deformity levels 1 and 2 combined). (b) Adult longevity (starvation resistance) in days.

Figure 5. Effect of temperature and parasite treatments on the proportion of black on monarch forewings, shown separately for (a) males and (b) females (± SEM). Data are for monarchs reared under cold (21˚C), moderate (26˚C), and hot (31˚C) temperatures. Colored bars represent inoculation treatments for parasite doses 0 (control), 50 (low), and 500-1000 (medium and high combined) spores per larva. Bonferroni pairwise comparisons for males provided support for a significant difference between means for control and low dose treatments ($p=0.019$) and similar means for all other dose levels. Comparisons of means across temperature treatments showed that cold treatment males were significantly darker than those from the hot temperature treatment ($p=0.001$). For females, pairwise comparisons showed that all parasite dose treatments were significantly different ($p < 0.002$). Mean values for females reared under the hot temperature treatment were significantly greater than those reared in either the cold or moderate treatments ($p < 0.004$).

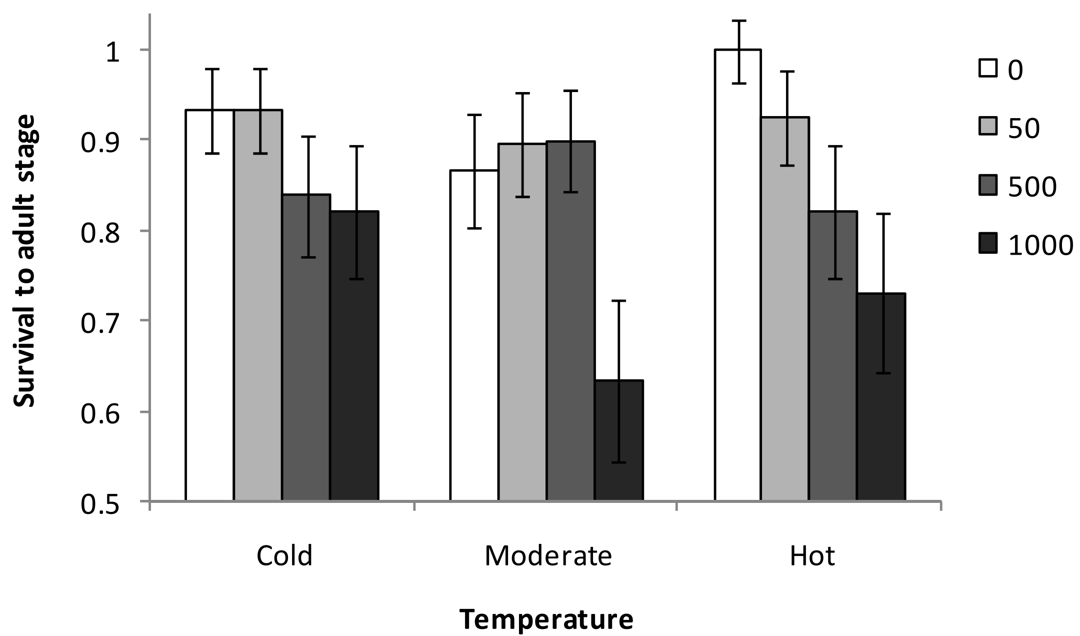

Figure 1

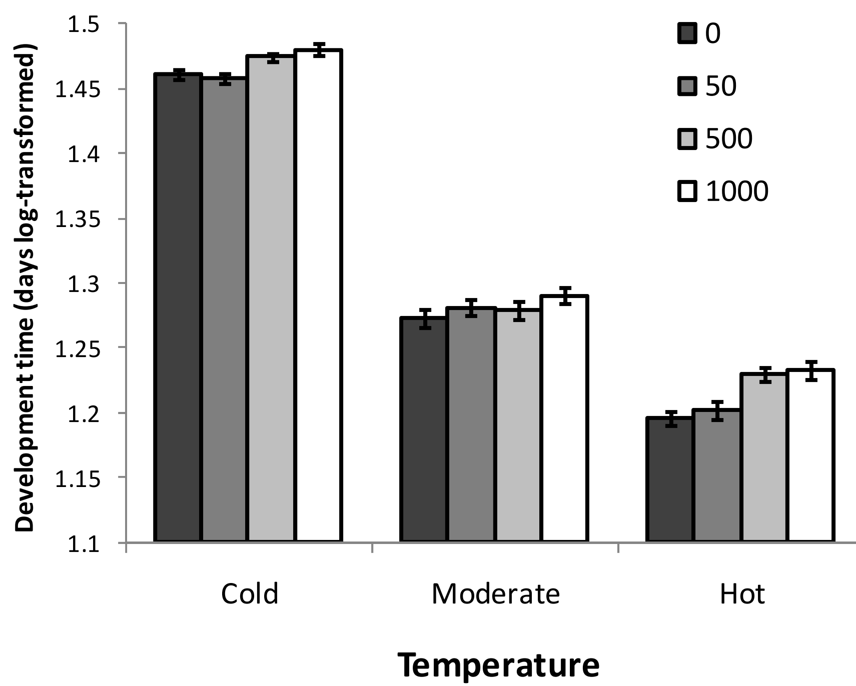

Figure 2

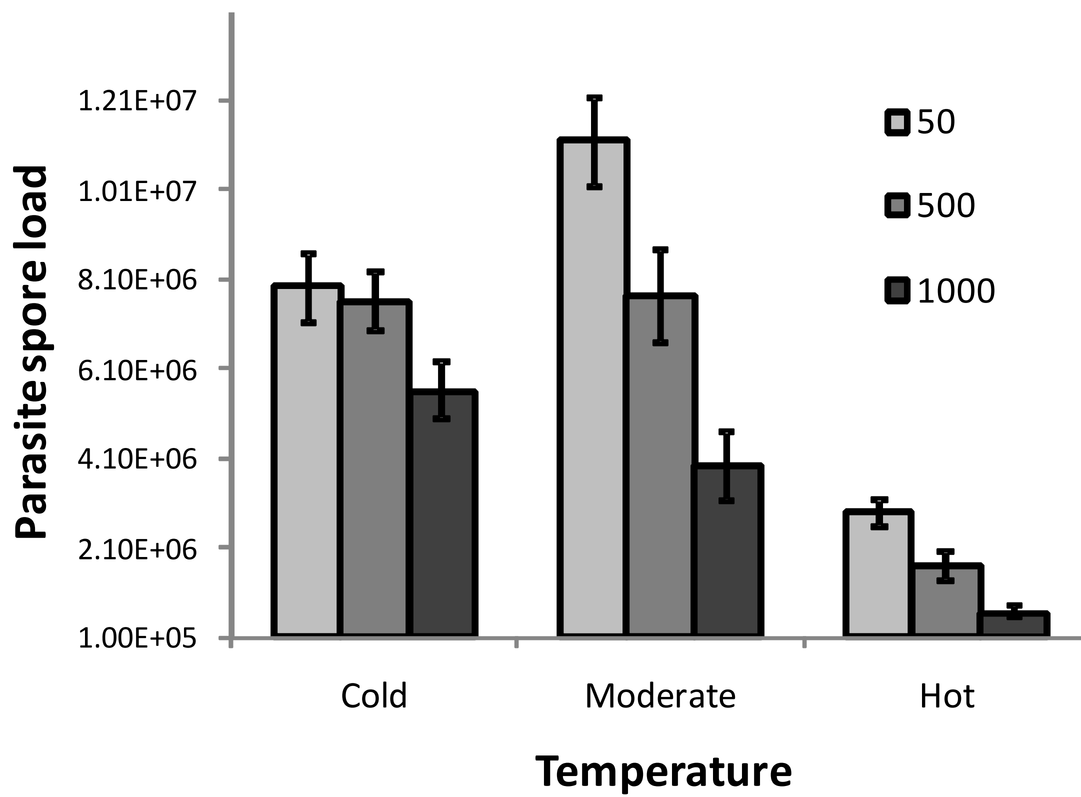

Figure 3

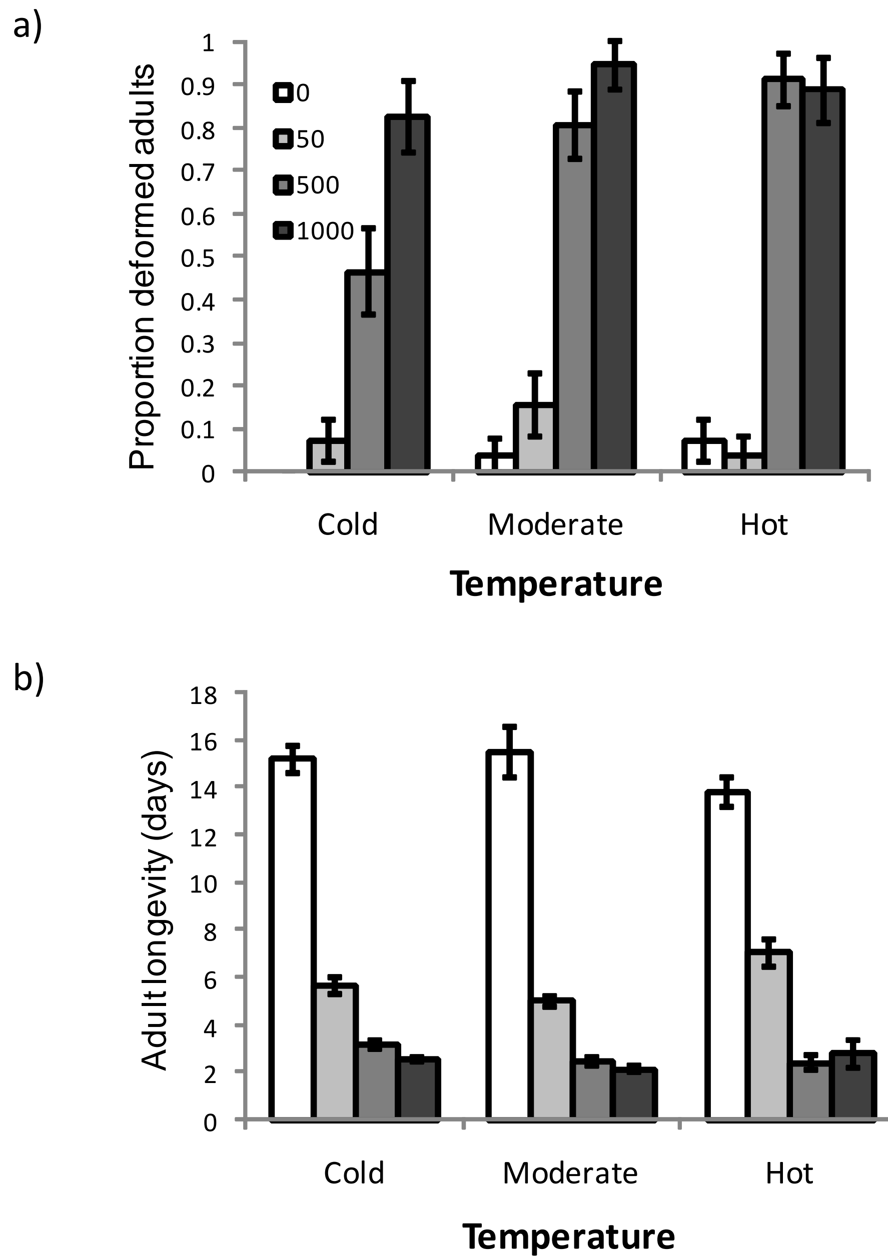

Figure 4

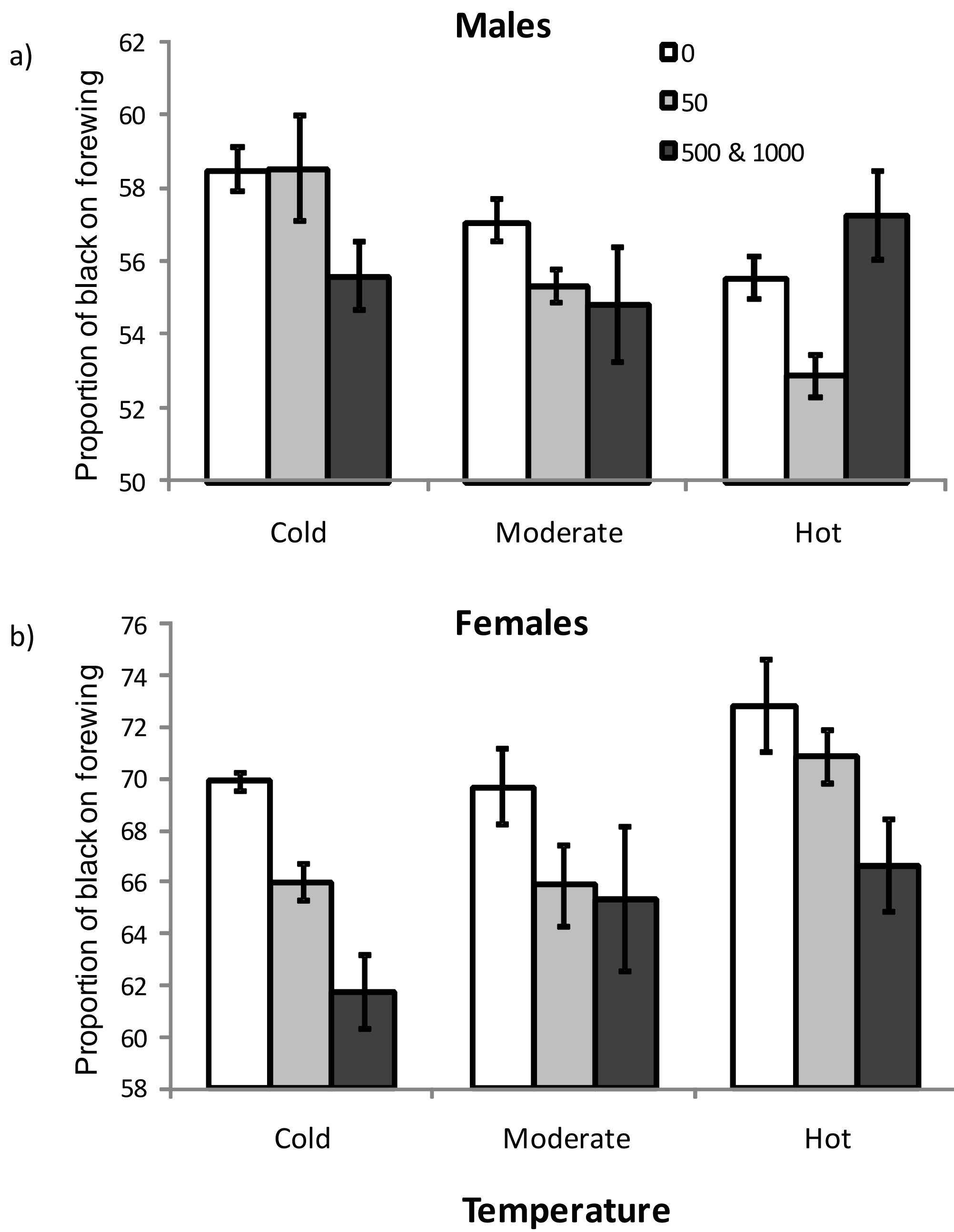

Figure 5