ABSTRACT

JEAN CHI
Wing Morphology, Flight Ability and Immune Measures in Monarch Butterflies
(Under the Direction of Dr. Sonia Altizer)

Monarch butterflies (*Danaus plexippus*) are well-known for their spectacular migrations from eastern Canada and the United States to over-wintering sites in Mexico. In response to wounds and infections, they have a complex immune system that includes the production of hemocytes (insect immune cells) and phenoloxidase (PO) activity, which releases melanin, an important compound in immune defense. Migratory populations of monarchs undergo a high energetic cost associated with flight as well as resistance to infection, but potential trade-offs among flight ability, immunity, and wing morphology are unknown. Hemocyte samples were obtained from the same individuals during larval fifth instar and adult stages, and measured for PO activity. Prior to flight trials, adult monarchs were scanned digitally to obtain wing morphology data such as wing area, loading, aspect ratio, as well as color characteristics. Monarchs were tested for flight ability using a nearly friction-less tethered flight mill; total flight time, distance, initial and final velocity were measured. Results showed no relationships between flight performance and immunity. There were significant relationships between average flight speed and wing coloration, and between adult hemocyte concentrations and almost all measures of wing color. This suggests that wing coloration may be an overall indicator of monarch health in terms of flight performance and immunity.

INDEX WORDS: *Danaus plexippus*, Insect Immunity, Insect Flight, Trade-offs, Flight Mill, Flight Performance

WING MORPHOLOGY, FLIGHT ABILITY AND IMMUNE MEASURES IN MONARCH BUTTERFLIES

by

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CHAPTER ONE
INTRODUCTION

Each fall, monarch butterflies in eastern North America undergo a spectacular journey, flying from areas as far north as Canada to the mountains of central Mexico. There they spend up to 5 months in a non-reproductive state (Urquhart and Urquhart 1978, Brower and Malcolm 1991), In the spring, the same butterflies that flew south then mate and fly north again to recolonize their breeding habitats. This type of seasonal, long-distance migration occurs in many animal systems, and has profound ecological and evolutionary consequences (Dingle 1996). My research examines a little-studied consequence of animal migration, and asks whether the level of investment in potentially costly immune defenses affects flight performance in a migratory insect species. Specifically, this project focuses on the energetic demands of flight in monarch butterflies by investigating relationship between flight performance, innate immune defenses, and adult wing coloration.

Monarch Migration and Infection by Parasites

Monarch butterflies are perhaps best known for their annual migrations during the fall and spring seasons in North America (Brower 1995). The largest population, found east of the Rocky Mountains, undergoes the longest-distance migration of any insect species in the world, traveling distances of over 5200 km round-trip (Urquhart and Urquhart 1978, Brower 1995). A smaller population, found west of the Rocky Mountains, inhabits a smaller breeding range and undergoes a shorter-distance migration to wintering sites on the California coast (Nagano et al. 1993). The energetic demands of migration are associated with a hormonally-mediated delay in

reproductive function (Herman 1985). Specifically, monarchs that emerge between mid-August and early October (at the end of the breeding season) are in a state of reproductive diapause to re-allocate energetic sources for migration (Goehring and Oberhauser 2002). Rather than mating, these adults have undeveloped reproductive organs and instead store lipids to fuel the fall migration and wintering period (Brower 1996). Aside from physiological tradeoffs between reproduction and migratory flight, however, other costs of migration in these butterflies, such as between immune defense and flight performance, remain unknown.

Wild monarchs are infected with a variety of pathogens such as the protozoan parasite *Ophryocystis elektroscirrha* (McLaughlin and Myers 1970), parasitoid flies (Oberhauser et al. 2007), and various bacteria and viruses (Arnott et al. 1968, Oberhauser and Rivers 2003). Best studied of these is *O. elektroscirrha*, which is known to occur in all monarch populations examined to date (Leong et al. 1997); Altizer et al. 2000). Past work has shown that migratory distances are inversely related to parasite prevalence (Altizer et al. 2000), and it appears that monarch populations that migrate long distances experience very low prevalence of infection, whereas populations that undergo short-distance migrations or breed year round without migrating have much higher prevalence of infection. One explanation for this pattern is that infected monarchs are less able to migrate successfully, and thus long-distance migration culls infected butterflies from the population each year. In support of this idea, studies of captive monarchs showed that butterflies infected with parasites exhibit lower flight performance (shorter distances and lower flight speeds) than healthy butterflies (Bradley and Altizer 2005); this result was assumed to be caused by a direct effect of infection, but could also relate to costs of mounting an immune response.

Insect Immunity and Costs of Defenses

Insect immunity consists of an innate immune system that is further broken down into cellular and humoral defense actions (Ling et al. 2005). Cellular immunity is mediated through the action of hemocytes, or insect immune cells, which respond to invading pathogens through phagocytosis, cell aggregation, nodule formation, and encapsulation (Hoffmann 1995, Ling et al. 2005). Humoral immunity is mediated through the release of a prophenoloxidase (proPO) cascade, where the active form phenoloxidase (PO) oxidizes various phenols to form melanin (Hoffmann 1995, Marmaras et al. 1996). Phenoloxidase is predominantly produced by oenocytoid cells, a type of hemocyte, and is an important component of insect immunity, as the melanin products act to encapsulate foreign pathogens and to protect surrounding host tissue (Hoffmann 1995, Gillespie et al. 1997).

Maintaining effective immune defenses and the ability to mount an immune response to infection requires substantial energy expenditures (Rolff and Siva-Jothy 2003, Pomfret and Knell 2006), in some cases to the extent of self-harm (Sadd and Siva-Jothy 2006). Indeed, a persistent theme in studies of ecological immunity is that host defenses can be costly in terms of reductions in other fitness components (Rolff and 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 and Begon 1993, Schmid-Hempel and Ebert 2003).

In some insect species, differences in immunity also relate to variation in color patterns. Specifically, melanin contributes dark pigmentation, and this cuticular melanism (darker body pigmentation) has been associated with greater measures of PO activity and melanin production in beetles and Lepidoptera (Barnes and Siva-Jothy 2000, Wilson et al. 2001, Cotter et al. 2004).

In damselflies, darker wings correlated with parasite resistance and hence could signal quality to potential mates (Siva-Jothy 2000, Yourth et al. 2002). If melanin is involved in both immunity and wing pigmentation (as part of the overall color display), and there are possible trade-offs between the two, wing coloration might serve as a predictor for overall health and immunity. It is important to note that sexual dimorphism in wing coloration exists among adult monarch butterflies, with females having thicker black veins on their wings than males (Davis et al. 2005). Therefore, it is possible that differences in immune response between males and females might correlate with darker pigments in females. Studies on the cabbage white butterfly (*Pieris rapae*) suggest that there may be a melanin-based trade-off between wing pattern and encapsulation because of sexual dimorphism (Stoehr 2007), but this is an untested hypothesis in monarch butterflies.

Research Questions and Goals

In this study, I investigated the relationship between immune defenses, wing color and morphology, and flight performance in captive monarch butterflies. Specifically, uninfected monarch butterflies were reared to the adult stage, sampled twice for immune defenses (once each as larvae and adults), and flown on a tethered flight mill to quantify their flight distances and speeds. The first prediction I tested is whether adult butterflies showed evidence of a trade-off between immunity and flight performance. In other words, do monarchs with larger measures of hemocyte concentration and PO activity show poorer flight performance, due to the energetic demands of maintaining high defenses? I also hypothesized that monarchs would show negative correlations between measures of immune defense and wing melanism, if a finite pool of phenoloxidase must generate both wing pigmentation and PO for immune defenses. Finally, I

examined the relationship between wing coloration and flight performance, specifically asking whether wing melanism and color hue were correlated with flight speeds and distances flown. Although less work has been done on this question, I predicted that monarchs with brighter wing coloration or darker black pigmentation might show stronger flight measures, given that wing color patterns might provide a signal of overall adult quality.

CHAPTER TWO
METHODS

Butterfly Sources and Rearing

Between Jul-Oct 2008, I examined larval and adult immune defenses and adult flight performance using laboratory-raised monarch butterflies. I initiated this experiment using three genetic lineages consisting of full and half-siblings from third generation progeny of wild-caught monarchs from Mexico (original adults were captured in February 2008 at the monarch wintering sites in Michoacan, Mexico). After obtaining eggs from adult female monarchs in the laboratory, I selected twenty first instar individuals from each lineage; these individuals were reared together by lineage in plastic storage containers (15 cm x 15 cm x 4.5 cm) for approximately 4 days, until the early third instar stage.

Upon reaching third instar, each larva was placed in a plastic pint container and reared singly until adult eclosion. Containers were supplied with a moist paper towel and cut milkweed stalks (greenhouse-raised *Asclepias incarnata*), and were placed in a temperature-controlled room (range: 23.6°C to 28.4°C, average=26.2°C) with ambient light during the larval and pupal stages. Every 1-2 days containers were cleaned to remove frass, paper towels were re-moistened, and fresh milkweed was added. Pupation occurred between August 1 and 3, and adult eclosion occurred between August 8 and 11. Approximately two days prior to adult eclosion, I transferred pupae from the larval rearing area to the main laboratory (average temperature=24°C) to minimize possible infection from the protozoan parasite *Ophryocystis elektroscirrha* (OE). Newly-eclosed adult monarchs were given 6 hours post-eclosion (for their wings to fully expand and harden) before being placed in glassine envelopes and held at 12°C for further processing.

Larval Hemolymph Collection

Larval hemolymph was collected from 48 randomly-chosen individuals (16 per lineage) during the fifth instar stage. A total of 12 individuals (four from each lineage) were selected as un-bled controls. To obtain hemolymph samples, the front tubercle was cut near the base (Fig. 1) and I extracted approximately 20 µL of raw hemolymph. Samples were obtained at 8 to 10 days after hatching, and the average larval age at bleeding was 8.9 days. Larvae were immediately returned to their individual containers after bleeding procedures and were reared to adulthood.

For hemocyte counts, 3 µL of hemolymph was mixed with 27 µL of Pringle's saline solution (1.0 L dD water, 9.0 g NaCl, 0.2 g KCl, 0.2 g CaCl, 4.0 g dextrose) and loaded into two replicate chambers of a pre-labeled Kova® hemocytometer slide. Hemocyte concentrations per µL were obtained under a light microscope by counting the total number of hemocyte cells in the four corner grids (1 mm x 1 mm x 0.1 mm) of each chamber for a total of 8 replicate counts per individual. Cells were counted within one hour of hemolymph collection and before melanization could occur. For the phenoloxidase (PO) assays, 15 µL of hemolymph was mixed with 15 µL Pringle's solution and immediately stored at -80°C until further processing. I retrieved frozen samples and thawed them on ice before testing for PO activity. Samples were loaded into a 96-count well plate at a volume of10 µL per well, and 2 replicate wells were loaded per individual. 190 µL of a pre-mixed assay buffer consisting of 50 mM $NaPO_4$, 2mM dopamine, and *Micrococcus luteus* elicitor, following Hall et al. (1995), was mixed into each well. The plate was held on ice prior to being loaded onto a BioTek® EL808 microplate reader set at 30°C; absorbance at 490 nm was read every 24 seconds for 2 hours. Final absorbance (after 2 hr) was used as the primary measure of PO activity.

Adult Hemolymph Collection

Adult monarchs were examined for possible infection by the protozoan *Ophryocystis elektroscirrha*, and un-infected individuals were held at 12°C in glassine envelopes and fed every 10 days with a 20% honey solution. The age range at adult bleeding was 22 to 24 days after hatching (average = 22.8 days), and 3 to 5 days after eclosion (average= 3.58 days). I fed adults again immediately prior to collecting hemolymph to replenish any fluids that were removed. Adult hemolymph samples were obtained by puncturing the intersegmental membrane of the abdomen along the dorsal vein with a 25-gauge needle. Whenever possible, a total of 10 μL adult hemolymph was collected. Procedures for hemocyte counts and PO assays were the same as described earlier for larval monarchs except that 2 μL hemolymph was used for counts (mixed with 18 μL Pringle's solution), and 6 μL was used for PO assays (mixed with 6 μL Pringle's solution and using only a single well per individual).

Flight Trial Protocol

Flight trials were conducted following the protocols of Bradley and Altizer (2005) using a tethered flight mill in a room controlled for light and temperature. At least 24 hours prior to flight trials, a light steel wire 9 cm long was glued onto the dorsal side of the thorax with rubber cement (average wire weight= 0.117 g, range wire weight= 0.0485 to 0.162 g). Monarchs were immediately placed in a mesh holding cage (0.6 m x 0.6 m x 0.6m) provided with sponges soaked in 20% honey water to allow for ad libidum feeding. Adult monarchs were flown at an average age of 19 days post eclosion (range = 10-30 days).

The flight mill was constructed of a 66.4 cm long graphite rod that pivoted around a metal stand (Fig. 2). One end of the rod was attached to the wire glued onto the monarch's thorax

and the other end held three moveable weights to counterbalance the weight of the butterfly. This design provided a nearly frictionless flight with a circumference of 4.27 m around the metal stand. A small piece of flagging tape was tied to the end of the rod opposite the monarch; each rotation of the rod allowed the flag to pass through an infrared beam emitted by the photogate sensor (PasPort Xplorer PS-2000, Pasco Scientific, Roseville, CA, USA) that automatically recorded time in seconds of each rotation. Additionally, I placed four silk flower bouquets uniformly around the perimeter of the flight mill to serve as visual landmarks for the butterflies. (Fig. 2).

I recorded the mass (to nearest thousandth gram) of each butterfly immediately before and after flight on an analytical balance. The temperature of the room during each flight trial averaged 24.8°C (range= 23.3°C to 27.0°C). Individuals that paused more than 10 times during the first 10 rotations (N = 5) were removed from the flight mill apparatus and excluded from the dataset. Flights were terminated when wing movement and flight mill rotations completely ceased for more than 10 seconds. To measure flight performance, I used raw data on the total number of rotations flown and time required for each rotation to calculate the following summary flight parameters: total distance flown (km), total time in flight (hr), average speed of flight (km/hr), and total loss of body mass during flight, both as a proportion (relative to initial body weight) and as a proportion relative to the total km flown (i.e., % weight lost per km).

Morphological Variables

Digital image analysis was used to quantify wing size, adult body size and wing coloration. One day post-eclosion, adult monarchs were placed on a Microtek® ScanMaker i320 flatbed scanner set at 300 dpi to obtain a digital image of their entire dorsal side that included

wings and body. Monarchs were placed in standard pinning position and immobilized by placing 1.0 g metal nuts on each wing. Wing and body size were digitally measured following Davis et al. (2005) using Adobe® Photoshop® image processing software. Using the Image Processing Tool Kit plugin (IPTK; Reindeer Graphics, Inc.), average forewing area and body area (in cm^2) were computed, as well as wing aspect ratio (length of forewing divided by width). Wing loading, a measure of the weight of the animal relative to its total wing area, was measured using both body area (wing $load_{area}$= body area/ total wing area) and weight in mg (wing $load_{mass}$= weight before flight/ total wing area).

Wing coloration (black and orange) was also quantified using IPTK. First, a thresholding step produced a pure black and white image, and the total area (cm^2) of black versus white patches was determined. The proportion of black pigmentation was measured by dividing the area of black by total forewing area. The density of black (overall intensity or opacity of black) was quantified on a 0-255 scale, with 0 being completely black or having the greatest color density. To measure orange color, the discal cell of the left and right forewings was isolated (Davis et al. 2007). The average density, hue, saturation, luminosity, and brightness of each pixel in the cell were computed using Fovea Pro color measurements routine. Orange hue was measured in degrees up to 360, and indicated the distinction between red, orange, or yellow pigmentation. Saturation was the intensity of color, or the difference between orange and gray, on a scale of 1 to 255. Brightness was the lightness or darkness of color on a scale of 1 to 255. Final values for wing morphology and color for each monarch were determined using the average values of the left and right forewings.

Data Analysis

I performed all statistical analyses using SPSS 16.0 software (2008). First, I tested the effects of monarch sex, age (days post-eclosion), lineage, wing loading (by area and by mass), body area, and wire weight on each of the summary flight measures (total distance, total time, average speed, and proportion of weight lost after flight) to determine whether any of these variables should be included in further analyses. Second, univariate analysis of variance was used to compare (i) measures of flight performance with measures of larval and adult immunity (adult hemocyte counts, larval hemocyte counts larval PO activity), and (ii) measures flight performance with measures of wing color (proportion of black, density of black, density of orange, and orange hue). In addition, I performed a bivariate correlation analysis to compare different measures of wing coloration, different measures of adult and larval immunity, and the associations between color and immune measures.

Figure 1: Larval hemolymph was extracted after tubercles of fifth instar larvae were clipped near the head.

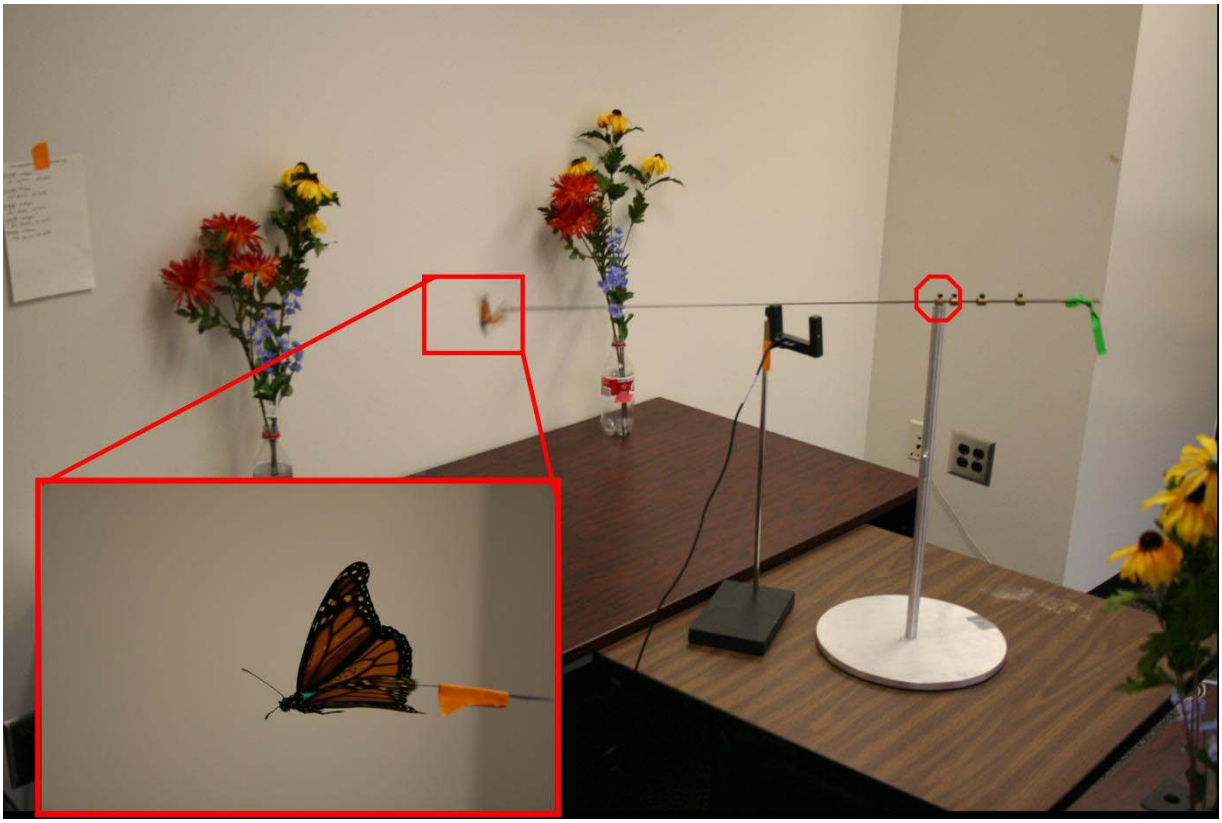

Figure 2: Adult monarchs were attached to one end of the flight mill arm (shown enlarged) as they flew around a frictionless pivot (circled).

CHAPTER THREE
RESULTS

General Results

Of the 60 monarchs initially reared as larvae, a total 48 larvae provided hemolymph for immune measures, and 12 more were unbled. Eight individuals died prior to adult eclosions. Fifty-three adults successfully eclosed, and 52 provided wing scans for morphometric measurements (one individual had a deformed wing upon eclosion and was excluded from the rest of the study). There were 42 adults that provided hemolymph samples plus 11 unbled individuals. Finally, a total of 43 monarchs (34 experimental monarchs and 9 controls) provided flight mill data, and represented the core set of individuals included in the final comprehensive data analyses (Table 1).

Average values for immune measures, wing morphology, and flight performance are summarized in Table 2. Different measures of adult flight performance were significantly positively correlated with each other: for example, total distance (km, log-transformed) and total time in flight were highly correlated (hr, log-transformed, $r=0.856$, $p<0.001$), and both of these measures were positively correlated with average flight speed. There were no significant correlations between adult and larval immune measures (as might be expected if animals better-defended early in life also mounted stronger defenses later in life), and there was no relationship between hemocyte concentrations (both larval and adult) and PO activity. However, monarch larvae had higher average hemocyte concentrations than adults (Table 2a).

Measures of wing morphology and color are shown in Table 2b. Importantly, many of these variables differed between male and female monarchs, indicative of sexual wing

dimorphism. For example, female monarchs had darker wings (based on percent black and black density) and greater hue values (indicative of yellower wings, as compared to redder wings in the males). All measures of orange (hue, saturation, luminosity, and brightness) were highly correlated with each other (e.g. correlation between orange hue and saturation, $r= -0.79$, $p<0.001$; correlation between orange luminosity and hue, $r= -0.55$, $p<0.001$). Therefore, I selected orange hue as the primary measure for color for subsequent analyses. Orange hue and measures of black pigmentation were also highly correlated with each other (Table 3). This correlation might have been caused, at least in part, by sexual differences, as females had both a higher proportion of black than males and higher values of orange hue.

Flight Performance and General Measures

Analysis of variance showed no significant effects of monarch sex, lineage, age (days post-eclosion), body area (cm^2), wire weight (g), and wing $loading_{area}$ (g/cm^2) on any of the following measures of flight performance: total distance (km, log-transformed), total time in flight (hr, log-transformed), and average speed (km/hr, log-transformed). I found a significant positive relationship between the proportion of body mass lost (mass lost during flight/initial mass in grams) and wing $loading_{mass}$ ($F_{1,39}= 13.17$, $df= 43$, $p=0.001$; Fig. 3). There was also a significant positive relationship between proportionate mass lost per km flown ($F_{1, 38}= 4.86$, $df= 43$, $p= 0.03$) and wing $loading_{mass}$. As a result, I retained wing $loading_{mass}$ as a covariate in subsequent analyses of flight performance.

Flight Performance and Immunity

I found no significant relationships between measures of flight performance (total distance, total time, average speed, and proportion of mass lost) and immune measures (larval hemocyte counts, adult hemocyte counts, and larval PO activity). For these analyses, the ANOVA model was as follows: Flight performance measure = larval hemocytes + adult hemocytes + larval PO + wing $loading_{mass}$. Model simplification was performed to determine whether removal of the least significant terms increased the explanatory power of other variables; however, other variables remained non-significant after model simplifications.

Flight Performance and Wing Color

There was no relationship between two measures of flight performance (total distance flown and total time in flight) and measures of wing color (proportion of black, black density, orange density, and orange hue). Sex was also included as a fixed factor in the statistical model due to the morphological differences between males and females; however, this variable had no effect on flight distance or time. I found a significant positive relationship between average speed and the proportion of black on monarch forewings ($F_{1, 36} = 5.45$, df= 43, p= 0.025; Fig. 4). This analysis also included the two-way interaction between sex and proportion of black. However, there was no difference in average flight speeds between males and females even though females have a higher proportion of black on their forewings (Fig. 4).

Immunity and Wing Color

A bivariate correlation analysis revealed a significant relationship between adult hemocyte counts (Table 3) and most measures of adult wing color including the proportion of

black pigmentation (positive correlation; Fig. 5), orange hue (positive correlation; Fig. 6) and black density (negative correlation, where higher measures of density refer to reduced opacity). There were no significant correlations between larval hemocyte counts, larval PO activity, and any measures of adult wing color.

Table 1: Sample sizes for each stage of assessment nested within overall experimental design.

	Total individuals (N)	
Stage of assessment	**Bled (immune assay treatment)**	**Controls (unbled)**
Initial sample size	48	12
Larval immune measures	48	12
Adult wing scans	42	11
Adult immune measures	42	11
Adult flight performance	34	9

Table 2a: Average values for immune measures and flight performance measures for 43 monarchs used in the final analyses.

		Average	**Minimum**	**Maximum**
Immune measures:	*Larval hemocyte counts (per μL)*	1600	55	3420
	Adult hemocyte counts (per μL)	546	125	1624
	Larval PO activity (final absorbance)	2.18	1.13	3.59
	Adult PO activity (final absorbance)	2.58	1.32	3.36
Flight performance measures:	*Total distance (km)*	3.87	0.26	9.53
	Total time (hr)	1.20	0.15	2.39
	Average speed (km/hr)	3.11	1.44	4.67
	%Mass lost	5.46	2.03	16.7
	%Mass lost per km	2.40	0.511	12.1

Table 2b: Average measures of adult wing variables for 43 monarchs used in the final analyses.

Variable	**Males: Average (min-max)**	**Females: Average (min-max)**
Wing area (cm^2)	8.84 (7.98-9.91)	8.79 (7.35-9.54)
Wing aspect ratio	1.93 (1.86-1.98)	1.92 (1.87-1.99)
Wing loading$_{mass}$	0.06 (0.05-0.07)	0.06 (0.05-0.09)
Wing loading$_{area}$	5.30 (4.81-5.19)	5.87 (5.23-6.46)
Proportion of black	61.07 (57.72-64.67)	71.81 (69.02-76.30)
Black density	131.94 (119.06-140.67)	120.80 (115.50-131.08)
Orange hue	38.37 (35.92-40.35)	41.96 (37.93-45.71)
Orange saturation	245.86 (234.74-249.58)	222.59 (202.44-237.46)
Orange luminosity	136.28 (128.11-146.58)	113.39 (101.24-130.34)
Orange density	76.36 (69.95-82.11)	70.02 (63.08-82.30)

Table 3: Bivariate correlation matrix shows significant relationships between adult hemocyte concentration (log-transformed) and measures of adult wing color.

		Adult hemocytes	**Propn Black**	**Orange Hue**	**Orange Density**	**Black Density**
Adult hemocytes:	*Pearson correlation:*	1	0.506	0.416	-0.422	-0.403
	Significance:		0.003	0.016	0.014	0.020
	N:	33	33	33	33	33
Propn Black	*Pearson correlation:*	0.506	1	0.627	-0.827	-0.748
	Significance:	0.003		<0.001	<0.001	<0.001
	N:	33	43	43	43	43
Orange Hue	*Pearson correlation:*	0.416	0.627	1	-0.790	-0.317
	Significance:	0.016	<0.001		<0.001	0.038
	N:	33	43	43	43	43
Orange Density	*Pearson correlation:*	-0.422	-0.827	-0.790	1	0.734
	Significance:	0.014	<0.001	<0.001		<0.001
	N:	33	43	43	43	43
Black Density	*Pearson correlation:*	-0.403	-0.748	-0.317	0.734	1
	Significance:	0.020	<0.001	0.038	<0.001	
	N:	33	43	43	43	43

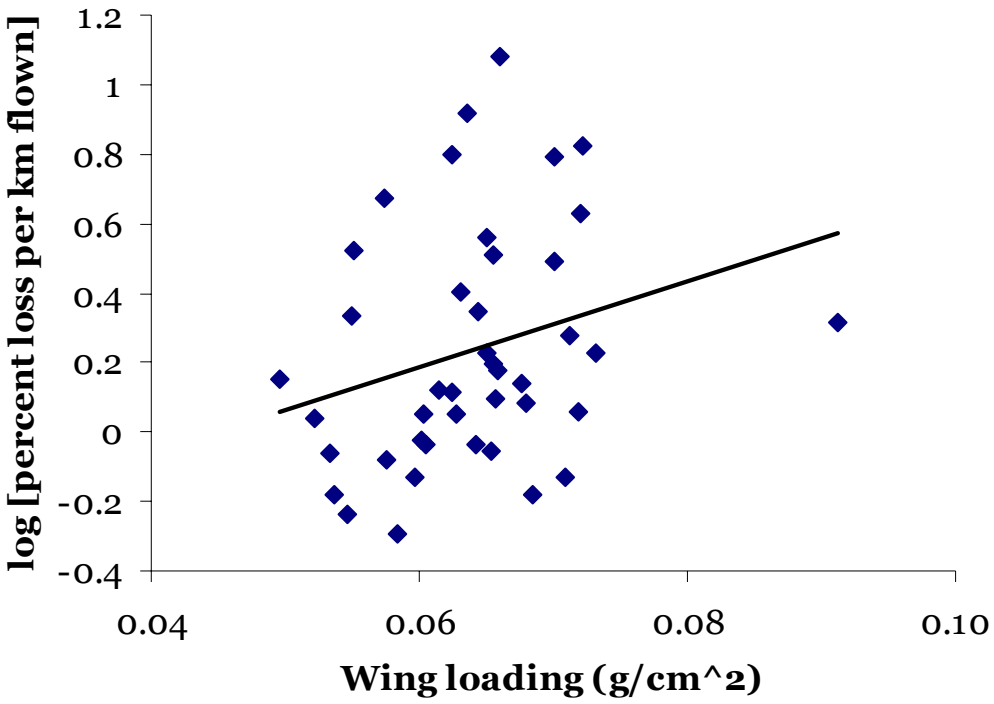

Figure 3: Relationship between wing loading$_{mass}$ and the percent of mass lost per km flown, log-transformed ($F_{1, 38}$= 4.86, df= 43, p= 0.03). The equation that describes this relationship based on least-squares regression is: $y = 12.39 – 0.56x$; $R^2 = 0.0735$.

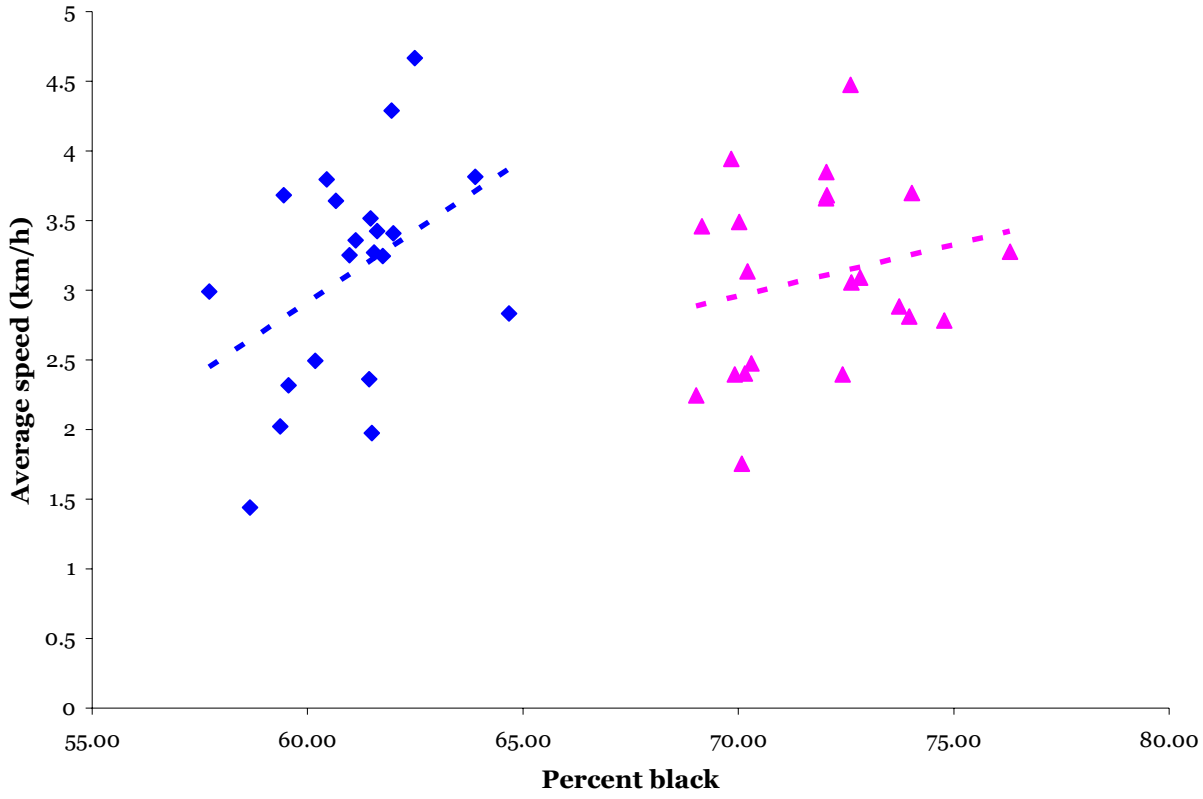

Figure 4: Relationship between average flight speed and the percent of black on monarch forewings ($F_{1, 36} = 5.45$, df= 43, p= 0.025). Blue diamonds denote male monarchs ($R^2 = 0.171$), and pink triangles are female monarchs ($R^2 = 0.048$). Note that females are darker than males, but within each sex a positive relationship exists between black pigmentation and average flight speed.

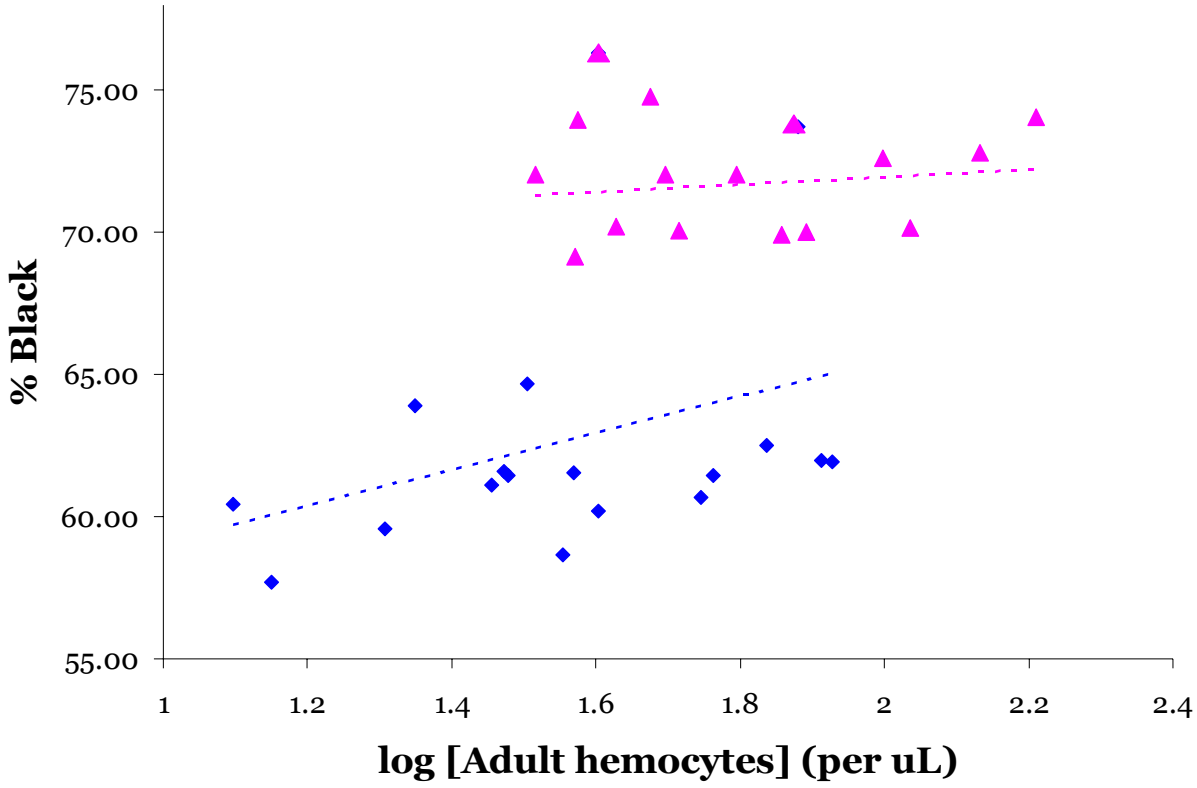

Figure 5: Relationship between adult hemocyte concentrations (log-transformed) and the percentage of black on wings (r= 0.506, p=0.003). Blue diamonds denote male monarchs (R^2 = 0.111), and pink triangles are female monarchs (R^2 = 0.0003).

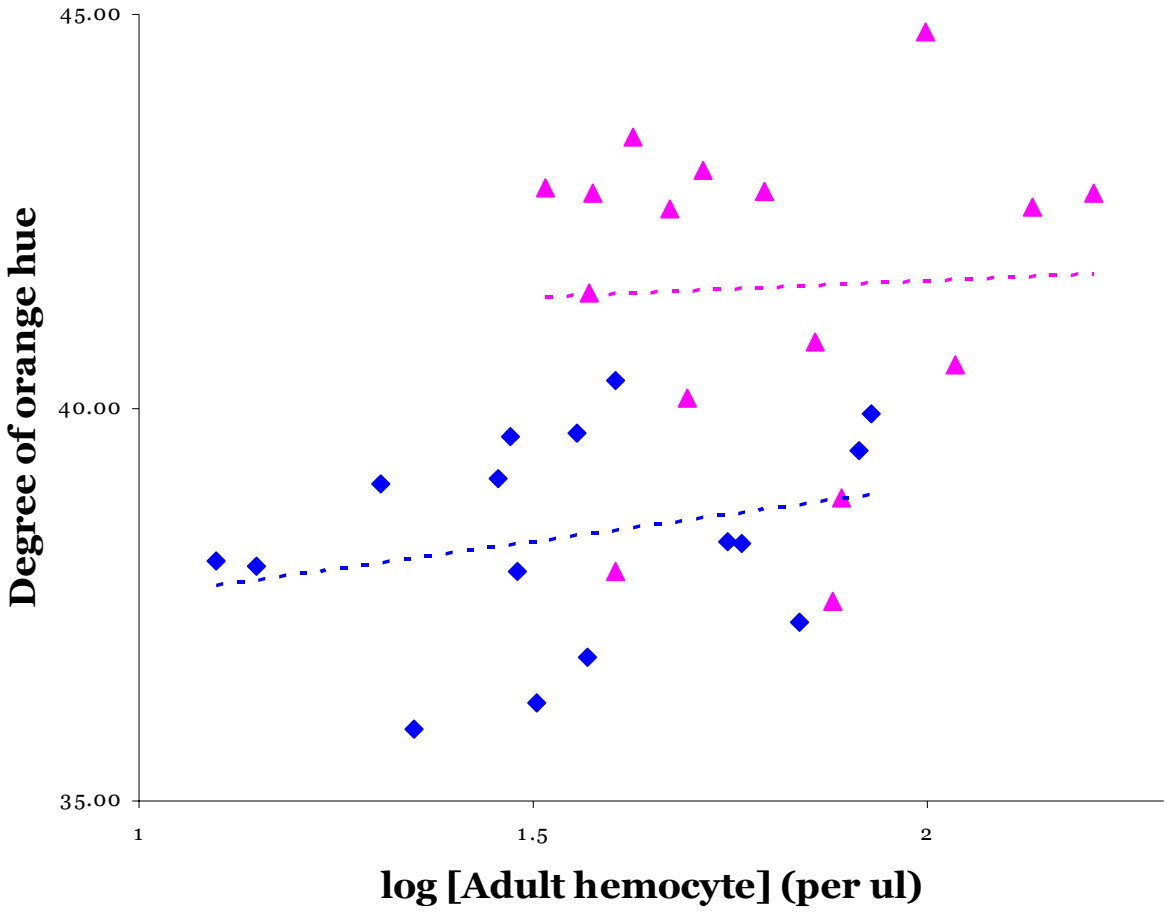

Figure 6: Relationship between adult hemocyte concentration (log-transformed) and orange hue on monarch forewings. Lower values indicate redder hues and higher values indicate more yellow hues. Blue diamonds denote male monarchs ($R^2 = 0.069$), and pink triangles are female monarchs ($R^2 = 0.002$).

CHAPTER FOUR
DISCUSSION

Despite the fact that parasites and pathogens can generate strong selective pressures on host species to evolve resistance traits, a great deal of variation in resistance and immunity exists in natural populations. An important question therefore is why aren't all hosts highly resistant to infectious diseases, and what factors maintain variation in immunity between individuals? During the past two decades, research in the field of ecological immunity has examined the ecological causes and consequences of variation in immune function (Norris and Evans 2000, McDade 2003). A central idea has suggested that immune defenses may be costly, such that not all hosts can invest equally in immunity to all pathogens (Cotter et al. 2004). In addition, hosts that invest in greater immunity could suffer in other ways, such as reduced competitive ability or lower reproduction (Lochmiller and Deerenberg 2000). Such costs of resistance could affect other traits associated with animal fitness (Schmid-Hempel and Ebert 2003), including energetically demanding flights required for long-distance migrations. Therefore, the main idea I initially sought to test with this project was whether immune defenses in monarch butterflies are negatively correlated with flight performance, as might be expected if animals that invest finite resources in one activity then lose the ability to invest in other important functions.

I was surprised to find no evidence for negative relationships between measures of monarch flight performance and innate immune defenses, indicating no apparent trade-offs between immunity and energetically demanding flight. Specifically, larval and adult hemocyte concentrations and PO activity showed no association with flight distances, time, speed, and weight lost during flight. In addition, I found no relationship between monarch age, sex, genetic

lineage and body size and measures of flight performance using the tethered flight mill. This could be explained by other underlying physiological constraints affecting flight (beyond what was measured here). For example, I did not assess monarchs for reproductive diapause status, a physiological change that coincides with monarch migration in the wild (Herman 1985), which may have accounted for variations in flight performance and immunity. Moreover, the monarchs used for this study were healthy and uninfected; perhaps costs and trade-offs between immunity and flight performance would be more obvious if comparisons were made between infected and uninfected individuals. Indeed, a previous study by Bradley and Altizer (2005) showed that monarchs infected by the protozoan *Ophryocystis elektroscirrha* (but otherwise appearing normal) experienced 10-20% reductions in overall flight performance, based on measures similar to those reported here.

Among the morphometric variables I examined, wing $\text{loading}_{\text{mass}}$ was the only measure correlated with flight performance, and was positively associated with the proportion of body mass lost during flight. Because wing $\text{loading}_{\text{mass}}$ was calculated as initial mass of the monarch divided by total forewing area, monarchs that were heavier relative to their wing size lost more weight during flight. I also noted that monarchs that flew further also lost more weight, and so computed a second measure of weight loss relative to the total distance flown. I again found that monarchs with higher values of wing loading lost more mass per km flown. This may have implications for general body weight restrictions on flight performance. Heavier monarchs (i.e. those with higher wing loading) may have lost more weight because they excreted more water during flight (i.e., dropped ballast), or they may have required more energy for flight, which may have caused their weight to decline more rapidly. A study conducted on migratory Neotropical butterflies indicates that high lipid content is highly correlated with air speed to optimize energy

expenditure (Dudley and Srygley 2008), so assessing lipid content post-flight may have provided insights on flight speed and weight loss.

An unexpected finding of this study involved the association between wing melanism (black pigmentation) and average flight speeds. Specifically, monarchs with a higher proportion of black on their forewings flew faster on average. This effect was not related to sexual dimporphism in wing melanism: females were shown to have darker forewings than males, but there was no difference in average flight speeds between the two sexes. Rather, both males and females showed a similar trend of increasing speed with darker wings. This is the first time that such a result has been shown for monarch butterflies. Some studies of other butterfly species and beetles have shown that animals with darker wings have increased flight times and distances most likely due to solar absorption during flight (Guppy 1986). However, this mainly applies to darkening on parts of the body closest to the thorax, whereas the black coloration on monarch wings is farthest from their bodies. At this time, it is not clear what mechanisms account for the relationship between wing melanism and flight speeds observed here, but it could relate to a biochemical link between flight muscle activity and melanin production. Alternatively, this relationship could be caused by monarchs in better condition simply having a greater ability to fly faster, and the ability to produce more melanin pigments.

A final key result from this study is that adult immune measures were significantly associated with adult wing coloration. Specifically, I observed a positive correlation between adult hemocyte concentration and the proportion of black on monarch forewings, with a significant difference between females and males. Females have darker wing pigmentation and higher adult hemocyte counts than males, and within males, I observed a positive relationship between hemocyte concentration and wing melanism. This relationship could be caused by the

role of hemocytes in melanin production, which is associated with both melanization as an immune defense, and in providing dark pigmentation for body coloration. Higher hemocyte counts in females may suggest that healthy females, in general, maintain an elevated immune response, perhaps to increase success in reproduction and egg laying. For example, it is known that adult longevity has a stronger positive effect on female lifetime reproduction than on males, because females are limited by the time required to search for host plants and produce mature eggs (Oberhauser and Hampton 1995). In addition, wing melanism could be an external indicating for overall health and immunity in monarch butterflies. Similarly, the negative correlation between orange hue and adult hemocyte counts also supports the difference between the sexes. Females had higher values of hue, causing them to have yellower tones in their wings as opposed to the redder tones in males. Hence, although adult males may have fewer hemocytes than females, this may be a desirable trade-off when considering sexual selection for brighter and more vivid hues in male wings (Davis et al. 2007). Importantly, this is the first study to examine and report a significant association between immunity and wing coloration in monarch butterflies.

This study established baseline measures for the relationships between immunity, wing morphology, and flight performance in healthy monarchs. Darker monarchs, that is, monarchs with higher melanin levels and darker wing pigmentation, flew faster. When coupled with the relationship between adult hemocytes and wing color, results suggested an overall visual indicator of immunity and flight instead of expected trade-offs between the two (Rolff and Siva-Jothy 2003). A study performed on migratory red knots produced similar findings where long flights did not influence immune response (Hasselquist et al. 2007); this may indicate that migrants are able to cope with the energetic demands of flight. To the extent of my knowledge,

this has been the first study to look at immunity and flight performance in insects as well as the first study of immunity and color in monarch butterflies. Further inquiries can be made to compare these findings with those of parasitized monarchs. If parasitized monarchs exhibit poorer flight performance than healthy ones (Bradley and Altizer 2005), what are the implications for immunity and color? Additionally, do the different adult hemocyte counts between males and females indicate a difference in allocation of resources given the known costs of female reproduction (Oberhauser and Hampton 1995)? Despite what is already known about monarch biology, this study reveals the need for a more comprehensive picture of the mechanisms behind flight performance, color, and immunity in butterflies.

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