THE UNIVERSITY OF CHICAGO

# MAINTENANCE AND LOSS OF MIGRATION AND MIGRATION-ASSOCIATED PHENOTYPES IN MONARCH BUTTERFLIES

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BY

AYŞE CATHERINE TENGER-TROLANDER

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# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

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# ABSTRACT

North American monarch butterflies maintain alternative migratory phenotypes that develop when individuals experience environmental cues that indicate summer is transitioning into autumn. While the summer generations live relatively short lives in which they reproduce quickly, the migratory generation delays reproduction to migrate thousands of miles to overwinter in Mexico or southern California. As monarchs are freeze susceptible insects, migration is an adaptation to survive the cold of winter. However, not all monarchs need to migrate. In fact, several populations of monarchs live in habitats that never experience extreme cold. They are freed from the selection imposed by the necessity and difficulty of surviving the migration and are no longer exposed to the environmental triggers that lead to the development of the alternative migration phenotypes. The lack of exposure to the relevant environmental cues could result in the dormancy/non-expression of the migration traits like in summer generation North American monarchs. Alternatively, the change in selection pressure could result in loss of migration due to relaxed selection and drift or adaptation to a novel environment. Using common garden experiments, I investigated under what genetic and environmental conditions migration associated traits are lost and maintained in monarchs. I measured migration associated phenotypes including, directional orientation behavior, body mass, forewing size, and forewing shape in several populations reared under different environmental conditions. I found that maintenance of directional orientation and forewing size is very sensitive to the relaxation of selection; without the consistent seasonal pressure of winter, monarchs can lose the genetic mechanisms responsible for plasticity. In addition, the development of the alternative migratory phenotypes in the known migratory population is very sensitive to environmental conditions. When compared with North American monarchs reared completely outdoors, North American

monarchs reared in a variety of autumn-simulating indoor conditions did not develop the alternative migration phenotype.

# CHAPTER ONE: INTRODUCTION

## Plasticity as an Adaptation

Environments determine the evolutionary outcome of the species that live within them. Populations either adapt or go extinct. Adaptive traits often match organisms to their environment in both form and function – walking sticks look astoundingly similar to the branches they climb to avoid predation and cacti have thick waxy cuticles to prevent dehydration in the desert. We often think of environments as moving traits in a particular direction over time. For instance, if an environment becomes more arid, those individuals with traits that prevent dehydration will have greater fitness and persist. Over generations, the trait mean will shift, making the organism more suited to its environment. However, environments can vary episodically, sometimes exerting opposing environmental pressures on the same population. Seasonal differences can be particularly incongruent - freezing temperatures in winter and extreme heat in summer or flooding in wet season and drought in dry season. Organisms cope with this type of environmental heterogeneity without see-sawing between different genotype frequencies generation after generation. And instead of stabilizing selection removing both extremes, natural selection will favor the development of phenotypic plasticity – the ability of a single genotype to produce multiple phenotypes depending upon environment of development or exposure (Lande 2009; Levins 1968; Moran 1992; Sultan and Spencer 2002; Via and Lande 1985; Via 1993; West-Eberhard 2003).

Natural selection favors plasticity in phenotype when there is a correlation between developmental and selective environments which allows for the adaptive matching of the phenotype and environment. Whether or not plasticity will be adaptive for a species is predicted

to depend upon the fitness of each phenotype in its respective environment, the frequency with which an environment is encountered, and how costly plasticity is to maintain (Moran 1992).

Monarch butterflies (*Danaus plexippus*) are an excellent organism in which to study how alternative phenotypes evolved and are maintained. North American migratory populations have distinct phenotypes that develop in response to seasonal shifts in environment (Barker and Herman 1976; Goehring and Oberhauser 2002; Kanz 1977; Taylor et al. 2019; Zhu et al. 2009) as well as non-migratory populations descended from the migratory North American lineage that live in seasonally homogenous environments in the tropics and in captivity (Hilburn 1989; Nail, Drizd, and Voorhies 2019; Neves et al. 2001; Pierce et al. 2014; Zhan et al. 2014). Using these populations, we ask what happens when seasonality disappears? Is plasticity maintained or lost? How do we distinguish between plasticity and genetic change in populations experiencing different environments? To answer these questions, I used common garden experiments and trait measurements to determine how migration phenotypes respond to different environmental conditions and whether non-North American and captive populations have maintained or lost migration-associated ancestral plasticity.

**North American Monarch Butterfly**

North American monarch butterflies maintain alternative phenotypes within a genetically homogenous population by responding plastically to their environment (Goehring and Oberhauser 2002; Reppert, Gegear, and Merlin 2010; Reppert and de Roode 2018; Taylor et al. 2019; Zhu et al. 2009). In summer, monarchs develop into reproductive adults that mate quickly and live for a short time. However, monarchs that develop in the transition between summer and autumn become what is known as the ‘super’ generation, migrating thousands of kilometers from the U.S. and southern Canada to central Mexico where they overwinter before returning north in

the spring (Guerra and Reppert 2013; Urquhart and Urquhart 1978). Only monarchs that survive the migration and hibernation will mate and re-colonize, creating an annual sieve for only the very best migrators to contribute to the next generation. As monarchs are freeze susceptible insects, migration is a critical adaptation to survive the temporal variation present in the northern latitudes of their range (Larsen and Lee 1994; Lee 1989). The offspring of the migratory generation then re-colonize the northern parts of their range in summer (Davis and Howard 2005; Miller et al. 2012)

**Migration Induction**

Migration is often described as a syndrome because it is composed of many different 'symptoms.' These symptoms are plastic phenotypes that include changes in physiology, behavior, and morphology. Migratory generation monarchs suspend their reproductive development, increase longevity, increase fat storage, and change their flight behavior, all in response to autumn (Beall 1948; Herman and Tatar 2001; Zhu et al. 2009). We suspect monarchs are responding to cooling temperatures, shortening photoperiod, host plant quality, and/or the declination of the sun during their development (Goehring and Oberhauser 2002; Reppert and de Roode 2018; Taylor et al. 2019). In particular, shortening day length and cool temperatures in autumn cause a decrease in the synthesis of Juvenile Hormone (JH) which causes reproductive arrest seen in the migratory generation (Barker and Herman 1976; Goehring and Oberhauser 2002; Herman and Brower 1989). These various environmental cues have been discussed as potential signals for other migration associated traits to develop, however, we do not know exactly which are critical or sufficient or if there are critical windows during development. And while we do know that monarchs use a time-compensated sun compass located in their antennae to navigate (Merlin et al., 2009; Reppert et al., 2010), we know relatively little about the

environmental induction of orientation (inclination to fly south) or migration associated morphological traits.

**Resident Monarch Populations**

Though migration is often thought of as a defining characteristic of monarch butterflies, many non-migratory populations live across the globe as well. Genetic evidence suggests that these non-migratory populations are the descendants of North American monarchs that dispersed recently on three independent occasions west across the Pacific, east across the Atlantic, and south into the Caribbean, Central, and South America (Pierce et al. 2014; Zhan et al. 2014). These resident populations no longer experience the temporal selective pressures of their North American ancestors, and do not travel vast distances or enter non-reproductive, overwintering periods (Hilburn 1989; Knight and Brower 2009; Neves et al. 2001; Zalucki et al. 1993). While there are documented morphological differences between the migratory and resident populations (Altizer and Davis 2010; Beall and Williams 1945; Dockx 2007; Li, Pierce, and de Roode 2016), the degree to which resident populations have lost or retained responsiveness to their ancestrally temporally heterogenous environment was relatively unknown, though one study has found some evidence that Pacific lineage monarchs maintain the ability to enter reproductive diapause upon exposure to shortening day lengths (Freedman et al. 2018).

**Captive and Commercial Breeding of Monarchs**

The popularity of monarch butterflies has created a commercial demand for them. Butterfly breeders breed and sell monarchs to school teachers for educational purposes or for release at events like weddings and fairs. In general, butterfly breeding is common, and breeders exist all over the world. Without breeders, we would not have museum exhibits, indoor butterfly gardens or in some cases research specimens. However, selling monarchs for release into the wild

presents a serious potential problem for the migratory population. By keeping colonies of monarchs over years, breeders are effectively eliminating natural selection for migration phenotypes. Individuals that would have perished during the migration because they were poor migrators are instead free to reproduce in captivity. Over many generations, the population could become less responsive to ancestral environment factors or plasticity lost entirely, and in the case where migration associated phenotypes are costly to maintain, we would expect that shift to happen quickly.

Concerned monarch enthusiasts, hoping to increase the monarch population, rear them in their homes for release. Unlike commercial breeders, home rearers usually do not keep colonies of individuals over years, thus maintaining annual natural selection pressures. However, captive rearing has its own potential pitfalls as indoor environments may not provide the full spectrum of environmental cues responsible for migration development.

**Overview of Dissertation**

Chapters 2 through 4 are studies that investigate the plasticity of various migration associated phenotypes in different populations of monarch butterflies reared in varying environmental conditions.

In chapter 2, I assess the directional orientation phenotype in wild-derived and commercially-bred monarchs reared outdoors in summer and autumn. This study was principally concerned with whether 1) the southern directional orientation phenotype seen in autumn is dependent on specific environmental conditions, 2) I could replicate those conditions indoors, and 3) commercially sourced populations have maintained plasticity in directional orientation behavior. I used a flight simulator to assess directional orientation of wild-derived and commercial monarchs reared outdoors in summer and autumn as well as wild-derived monarchs

reared in summer and autumn-like controlled environmental chambers. The outdoor common garden revealed that the commercially bred population was no longer as responsive to autumn environmental cues as the wild-derived population. Further genome sequencing revealed significant genetic divergence between the wild and commercial populations. The wild-derived monarchs reared in the autumn-like environmental chamber did not develop into migratory adults suggesting unknown environmental cues are critical to directional orientation development.

In chapter 3, I furthered our understanding of loss of directional orientation due to both genetic and environmental factors. My main motivation was to understand whether 1) the apparent loss of directional orientation in commercial monarchs as a group is due to a reduction in sensitivity to autumnal cues or complete loss of the trait in the population and 2) natural sunlight (shifting photoperiod and declination) experienced in either a glass top greenhouse or by a window was sufficient to induce directional orientation. The commercial population was a mixture of individuals that repeatedly showed southern orientation and those that did not, suggesting that only consistent seasonal selective pressures will maintain directional orientation. The wild-derived monarchs reared in the greenhouse and near a south-facing window in autumn did not show directional orientation suggesting perhaps a combination of environmental cues is responsible for directional orientation development.

In chapter 4, I investigated the response of migration associated morphological traits, body mass and forewing size and shape, to summer and autumn outdoor conditions in North America in both North American and Costa Rican monarch populations. While morphological plasticity is assumed to be present in North American monarchs, no previous work has established whether observed differences between summer and autumn monarchs are due to plasticity in development

or differential mortality during migration. And though previous comparisons of naturally occurring populations of North American and Costa Rican monarchs found differences in morphology, those are assumed to be due to genetic differences rather than an effect of developmental environment. Here, I investigate whether season of development or population are predictive of morphological trait variation and to what degree. I found that consistent with the seasonal pressures in North America, North American monarchs are highly plastic whereas Costa Rican monarchs are mixed in response to autumn environmental cues.

# CHAPTER TWO: CONTEMPORARY LOSS OF MIGRATION IN MONARCH BUTTERFLIES

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**Abstract**

The annual migration of the monarch butterfly, *Danaus plexippus*, is in peril. In an effort to aid population recovery, monarch enthusiasts across North America participate in a variety of conservation efforts, including captive rearing and release of monarch butterflies throughout the summer and autumn. However, the impact of captive breeding on monarchs remains an open question. Here we show that captive breeding, both commercially and by summertime hobbyists, causes migratory behavior to be lost. Monarchs acquired commercially failed to orient south when reared outdoors in the autumn, unlike wild-caught North American monarchs, yet they did enter reproductive diapause. The commercial population was genetically highly divergent from wild-caught North American monarchs and had rounder forewings, similar to monarchs from non-migratory populations. Furthermore, rearing wild-caught monarchs in an indoor environment mimicking natural migration-inducing conditions failed to elicit southward flight orientation. In fact, merely eclosing indoors after an otherwise complete lifecycle outdoors was enough to disrupt southern orientation. Our results provide a window into the complexity, and remarkable fragility, of migration.

**Significance Statement**

Captive rearing and release of monarch butterflies is a cultural phenomenon in the U.S., where commercial breeders sell monarchs for release by school children and hobbyists raise wild

monarchs in an effort to boost dwindling numbers. Our research shows that the captive breeding of monarchs disrupts critical aspects of their migratory behavior. The results are important because they reveal that different components of the migratory syndrome are easily decoupled and that migratory behavior is remarkably sensitive to genetic and environmental change. These results are relevant to conservation efforts, especially as the US Fish and Wildlife Service considers whether to list the North American monarch as a threatened species under the U.S. Endangered Species Act.

## Introduction

The monarch butterfly, *Danaus plexippus*, is famous for its annual mass migration across North America (Reppert, Gegear, and Merlin 2010; Reppert and de Roode 2018). Unfortunately, the number of overwintering monarchs in Mexico has declined drastically over the past 25 years (Brower et al. 2012; Vidal and Rendón-Salinas 2014). Out of concern that the monarch migration may go extinct in the foreseeable future (Semmens et al. 2016), the United States Fish and Wildlife Service is currently considering whether to list the monarch butterfly as a threatened species under the U.S. Endangered Species Act (The Center for Biological Diversity 2014). While there is some disagreement about primary drivers of monarch population decline (Agrawal and Inamine 2018; Badgett and Davis 2015; Boyle, Dalgleish, and Puzey 2019; Davis 2012; Inamine et al. 2016; Pleasants et al. 2016; Ries, Taron, and Rendón-Salinas 2015; Saunders et al. 2018; Stenoien, Nail, and Oberhauser 2015; Thogmartin et al. 2017), the public maintains a keen interest in monarch conservation and undertakes a variety of activities every year to aid them, including reporting sightings online, planting milkweed, creating migratory waystations, and even raising monarchs for release.

However, captive rearing of monarchs is a contentious practice. Summertime hobbyists raise monarchs in their homes throughout the summer and autumn and then release them, hoping that they or their offspring will fly south to Mexico and ultimately contribute to population recovery. Conservation groups and scientists have expressed concern that captive rearing may result in higher parasite loads and even adaptation to captive conditions (Davis 2018; Journey North 2015; Malcolm 2018; Monarch Joint Venture 2018; Pelton 2018). Formal captive breeding programs, which are sometimes implemented to aid recovery of threatened or endangered species, do not exist for the monarch butterfly, but there are multiple commercial companies that breed monarchs year-round and sell them for release. These commercial monarchs are raised and released by school children across the U.S., again with the belief that they will fly to the overwintering ground. These monarchs are also released at special events like weddings and monarch-themed fall festivals. However, the impact of captive breeding on monarch migration biology has not been investigated. In this study, we explored whether monarch breeding by commercial facilities and hobbyists affects migration phenotypes and genetics of captive reared monarchs.

**Results and Discussion**

To investigate the migratory status of commercially bred monarchs, we reared both commercially sourced and wild-caught North American (NA) monarchs in a common garden experiment. We ordered adult monarchs from a commercial breeder and caught adult wild NA monarchs in July of 2016. We raised the offspring of both groups over two successive generations, summer and autumn, in outdoor insectaries in Chicago, Illinois. Our experiment focused on comparing the descendants of commercial and wild-caught NA monarchs, and crosses between the two groups, raised at the same time in the same, outdoor conditions. The

monarch migratory syndrome is a multifaceted phenotype, encompassing behavioral, physiological, and anatomical traits. We assessed all three of these components by measuring flight orientation, reproductive status, and wing shape.

To measure orientation behavior, we tested monarchs in a monarch flight simulator (Mouritsen and Frost 2002; Fig. 2.1A). Previous work using the simulator has shown that summer generation monarchs do not have a group direction, while autumn generation monarchs fly south (Froy, 2003; Guerra et al., 2013; Merlin et al., 2009; Mouritsen et al., 2002; Zhu et al., 2009). We calculated the group mean vector (0-359°), weighted by the strength of each individual's vector (0-1), as well as the group vector strength. We then used the Rayleigh test to determine whether each group was directional. NA monarchs behaved as expected. NA monarchs that emerged in October flew directionally south (Fig. 2.1B; $\boldsymbol{\sigma}$ = 181°, n = 25, r = 0.65, Rayleigh test, z-score = 10.65, $p < 0.001$), and those that emerged in August flew weakly south (Fig. 2.1B; $\boldsymbol{\sigma}$ = 161°, n = 19, r = 0.37, Rayleigh test, z-score = 2.6, $0.05 < p < 0.1$). Surprisingly, commercial monarchs that were raised side-by-side with NA monarchs did not have a mean direction in either late summer or autumn (Fig. 2.1B; late-summer $\boldsymbol{\sigma}$ = 158°, n = 14, r = 0.32, Rayleigh test, z-score = 1.43, $p > 0.2$ and Fig 2.1B; late-autumn $\boldsymbol{\sigma}$ =183°, n = 14, r = 0.16, Rayleigh test, z-score = 0.36, $p > 0.5$). Consistent with these results, we found that the distribution of directions between commercial and NA did not differ in the summer (Wallraff test, Kruskal-Wallis $\chi_2 = 0.0212$, $p = 0.884$), but did in autumn (Wallraff test, Kruskal-Wallis $\chi_2 = 5.763$, $p = 0.016$). Similarly, the distributions of group vector strengths of commercial and NA groups overlapped in the summer, but not in autumn (Supplementary Information, Fig. 2.4A). Additionally, these results suggest that the genetic basis of directional orientation is dominant to non-orientation because our breeding design involved comparing autumn generation local

monarchs that were actually female NA x commercial hybrids backcrossed to pure NA male monarchs (75% NA, 25% commercial genome). All the hybrid NA x commercial females had NA mothers giving them and their offspring a NA mitochondrial genome.

We next looked at whether commercial monarchs enter diapause, which keeps NA monarchs in reproductive arrest during their migration, by counting the number of mature oocytes in each female that flew in the flight orientation assay. We found that commercial monarchs did enter reproductive arrest like NA monarchs. Commercial monarchs averaged 70.4 $^{20}$ ±14.66 SE oocytes (n = 8) in mid-late August which decreased to just 16.8 ± 5 SE (n =12) in October (Fig. 2.1C). The NA individuals averaged 53 ± 5.72 SE oocytes (n = 15) in mid-late August and decreased to 24.3 ± 7.45 SE (n = 16) in October (Fig. 2.1C). The number of mature oocytes decreased in both populations from August to October (Mann-Whitney U, commercial $p = 0.003$ and NA $p = 0.013$).

There was no difference between commercial and NA oocyte counts in either season (Mann-Whitney U, $p > 0.5$). In contrast to previous results showing that all outdoor-reared females emerging in September had no mature oocytes (Goehring and Oberhauser 2002), only 38% of females had no mature oocytes in the autumn. As a whole, these results demonstrate that components of the migratory syndrome are easily decoupled and that reproductive diapause cannot be used as a proxy for migratory behavior.

Using geometric morphometrics, we compared wing shape and size which are known to differ between migratory NA monarchs and non-migratory populations from other locations (*28*). We found that commercial monarchs had rounder forewings compared to NA monarchs (Mann-Whitney U, $p < 0.001$, Fig. 2.1D, Supplementary Information, Fig. S2.5A)—differences similar to those between natural migratory and non-migratory populations (Altizer and Davis 2010). We

also found that forewing shape is sexually dimorphic in both commercial and NA monarchs with males having rounder forewings than females (Supplementary Information, Fig. 2.6). Additionally, commercial monarchs may have smaller forewings than NA (Mann Whitney U, p = 0.054, Supplementary Information, Fig 2.7), again mirroring differences between natural migratory and non-migratory populations (Altizer and Davis 2010).

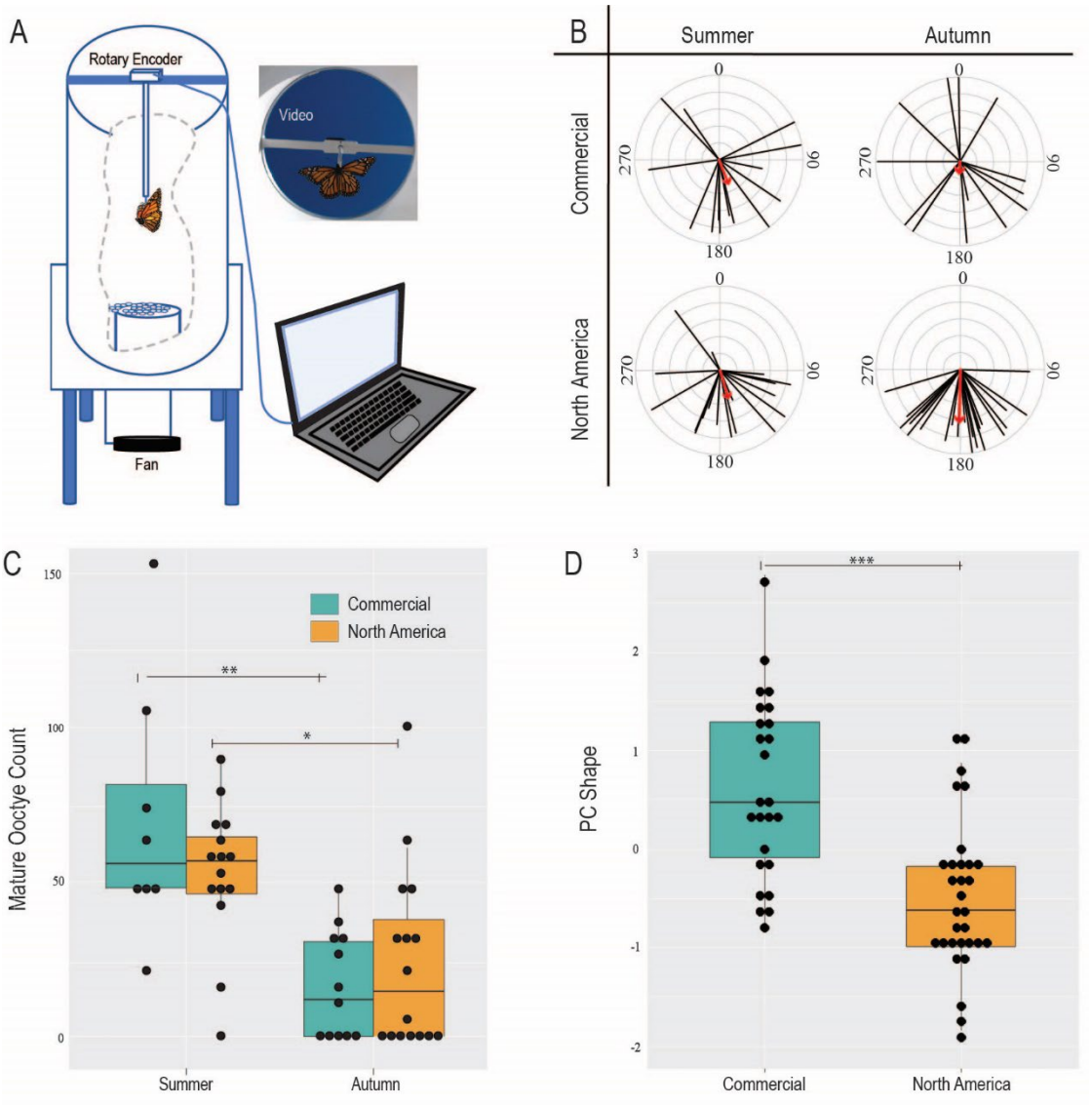


Figure 2.1. Commercial lineage monarchs do not orient south, but enter reproductive arrest. A) A representation of the flight simulator. The rotary encoder captures the orientation data while video is recorded. Laminar air flow is generated by the fan and vertical drinking straws. B) Orientation plots of commercial versus North American (NA) monarchs raised in the summer and autumn. Black lines indicate the mean direction (0-359°) of an individual butterfly and the length represents the strength of that direction (0-1). The red arrow indicates the mean direction

Figure 2.1 (continued) and strength of the group direction. 0° is North. C) The number of mature oocytes of each female who completed a flight test. Both commercial (teal) and NA (orange) females enter reproductive arrest in the autumn as evidenced by their significantly lower oocyte counts. D) Comparison of commercial (teal) and NA (orange) forewing shape. PC Shape is the first principle component of a principle component analysis (PCA) and explains 55.53% of the variation in shape based on geometric morphometric analysis of 13 landmarks on the wing.

Our results indicate that either: a) long-term captive breeding of this commercial monarch population resulted in the loss of migratory behavior and a change in wing morphology, or b) the commercial population in our study was originally founded by or supplemented with monarchs from a non-migratory population. Over the past hundreds or thousands of years, NA monarchs dispersed out of North America at least three times, once south into Central and South America and the Caribbean, once west across the Pacific Islands and into Australia, and once east into southern Europe and North Africa (Zhan et al. 2014). Each of these dispersal events produced populations that reproduce year-round and do not migrate. It remains unknown whether these populations do not migrate because they have lost the ability or because they do not experience the relevant environmental cues. To determine the ancestry of the commercial population, we generated whole genome sequencing (WGS) data from 15 commercial specimens, 14 of which successfully completed an autumn flight test, and compared them to a worldwide sample of monarch genomes (Zhan et al. 2014). After filtering, our analysis was based on 4,593,379 single nucleotide polymorphisms (SNPs) with an overall genotyping rate of 0.995. Principal Component Analysis (PCA) showed that the commercial lineage did not cluster with any other known monarch population, including North America (Zhan et al. 2014, Fig. 2.2A). Using a pruned dataset of 1 million variants, we inferred population subdivision and admixture using Frappe, v1.1 (Tang et al. 2005). Consistent with previous work, we found that samples collected from around the world represent at least four distinct populations: North America, Central/South America, Pacific, and Atlantic (Zhan et al. 2014). The commercial individuals represent a

distinct and previously unknown population of monarchs (Fig. 2.2B). We also found evidence that the commercial breeder does introduce NA genetic variation into their captive population as two of the fifteen commercial samples shared ancestry with NA (Fig. 2.2B). However, this supplementing of genetic variation does not appear to have a lasting impact on the commercial population.

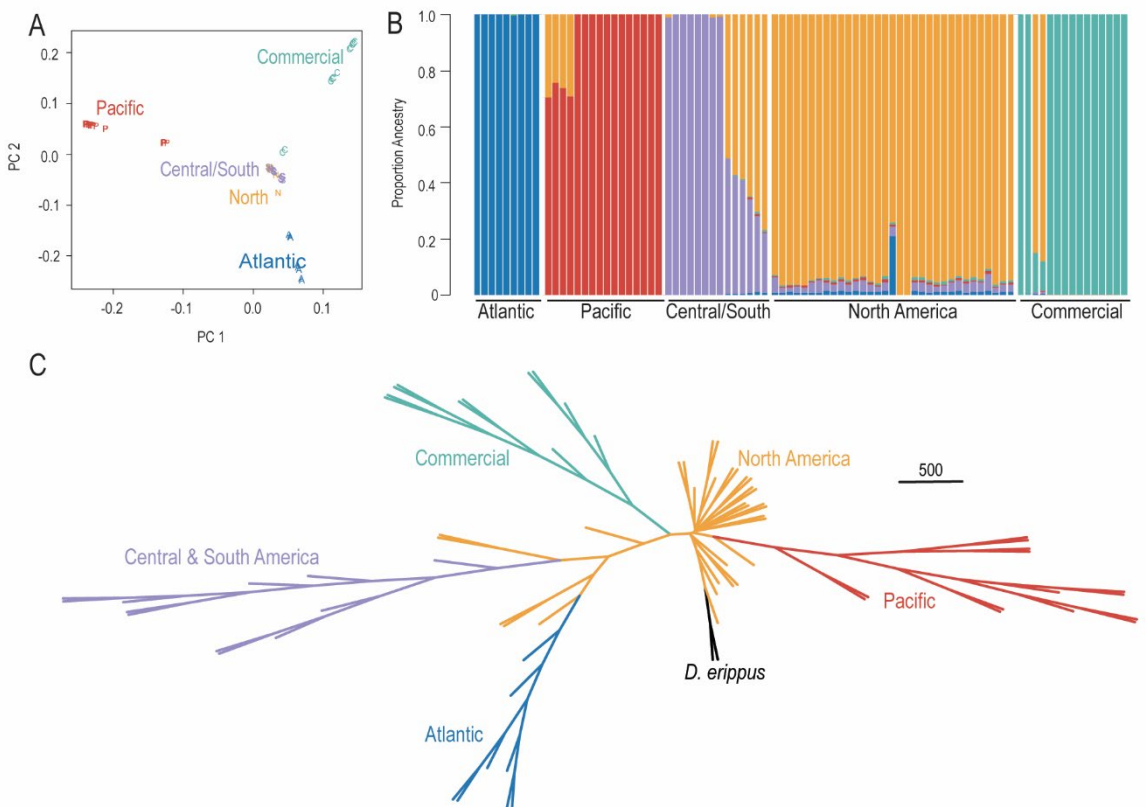


Figure 2.2. Commercial monarchs represent a distinct population of monarch butterflies derived from North American monarchs. A) Principle component analysis of single nucleotide polymorphisms (SNPs). Principle component 1 (PC1) accounts for 12.3% of the variation in the data and principle component 2 (PC2) for 10.7%. B) Analysis of SNP structure using Frappe. Each bar represents a single individual and the colors the proportion of ancestry for five populations. C) Neighbor-joining consensus tree based on SNP data. Scale bar is equivalent to 500 bootstraps.

Subsequent phylogenetic analyses indicate that the commercial monarch population was originally derived from North America and there has been no appreciable gene flow into the commercial population from non-migratory populations. For instance, our phylogenetic analysis

20 recovered the signatures of independent dispersal events out of North America in the founding of worldwide monarch populations, with the addition of a fourth independent event leading to the origin of the commercial population (Fig. 2.2C). Furthermore, analysis with TreeMix (Pickrell and Pritchard 2012) found no evidence of gene flow between the commercial population and any non-migratory population (Supplementary Information, Fig. 2.8). Consistent with the inferred NA ancestry of the commercial population, we found that the commercial samples that we sequenced were fixed for the NA haplotype at a migration-associated collagen gene, suggesting there is more than one way to become non-migratory (Zhan et al. 2014). The population genetic consequences of commercial rearing appear to mirror natural dispersal; the commercial lineage, similar to the Atlantic and Pacific populations, was genetically differentiated from NA and had reduced nucleotide diversity ($\pi$) (Supplementary Information, Table 2.1).

We do not know what effect the introduction of non-orienting monarchs might have on the wild NA population or whether these results apply more generally to commercial monarch breeding. However, our results indicate that at least one group of commercially bred monarchs are much less likely to migrate than wild NA monarchs. Non-orienting monarchs released in the autumn are unlikely to migrate successfully and will not contribute to monarch population recovery or to the gene pool. However, non-orienting monarchs released in the summer could mate with wild NA individuals leading to the introduction of non-migratory variation that may not be purged. We suspect that without the strong annual selective pressure of migration, migration associated traits can be lost in captivity.

Unlike commercial breeders, hobbyist breeders tend to collect wild eggs throughout the spring and summer and rear them for immediate release or for a few generations during the

summer and autumn. When released, autumn generation butterflies are expected to fly south and experience the same selection pressures as wild individuals. However, we do not know whether 20 rearing a monarch butterfly indoors, where natural environmental cues (temperature, light, etc) may be absent, affects the induction of migratory behavior. To determine whether indoor captive rearing affects migration, we reared NA monarchs indoors in both an autumn-like (18°C with a 14-hour day) and summer-like (25°C with a 16-hour day) environmental chamber in 2018. We also reared a summer and autumn generation outdoors and caught wild autumn generation monarchs as they migrated south through Chicago in mid-September to act as controls. As expected, outdoor summer and chamber summer groups did not orient in a specific direction (Fig. 2.3A; outdoor: $\boldsymbol{\sigma}$ = 348°, r = 0.12, n = 16, Rayleigh test, z-score = 0.23, p > 0.5, Fig 2.3A; chamber: $\boldsymbol{\sigma}$ = 124°, r = 0.295, n = 19, Rayleigh test, z-score = 1.65, p > 0.1). In contrast, the outdoor reared autumn generation and the wild-caught autumn generation monarchs showed southern group orientation (Fig. 2.3A; outdoor $\boldsymbol{\sigma}$ = 185°, r = 0.4, n = 9, Rayleigh test, z-score = 1.44, p > 0.2 and Fig. 2.3B wild $\boldsymbol{\sigma}$ = 164°, r = 0.39, n = 14, Rayleigh test, z-score = 2.13, p > 0.1). Our sample sizes for these groups were limited but they did have a southward direction when combined (autumn positive controls, $\boldsymbol{\sigma}$ = 172°, n = 23, r = 0.39, Rayleigh test, z-score = 3.50, p < 0.05). Unexpectedly, monarchs reared in the autumn-like chamber did not orient south (Fig. 2.3A; $\boldsymbol{\sigma}$ = 295°, r = 0.21, n = 17, Rayleigh test, z-score = 0.75, p > 0.2). The distributions of individual directions and group vector strengths differed between autumn chamber monarchs and autumn positive controls (Wallraff test, Kruskal-Wallis $\chi_2 = 5.970$, p = 0.015, Supplementary Information, Fig. 2.4B).

Since reproductive diapause is also an important component of the migratory syndrome, we counted mature oocytes in our chamber and outdoor reared monarchs. As expected, the autumn

females reared outdoors averaged 35.6 ± 11.7 (SE) mature oocytes (n = 7), a marked decrease from those reared in the summer which averaged 77.2 ± 7 (SE) (n = 9) (Supplementary Information, Fig. 2.9). Unlike the outdoor reared group, the autumn-like chamber females did not

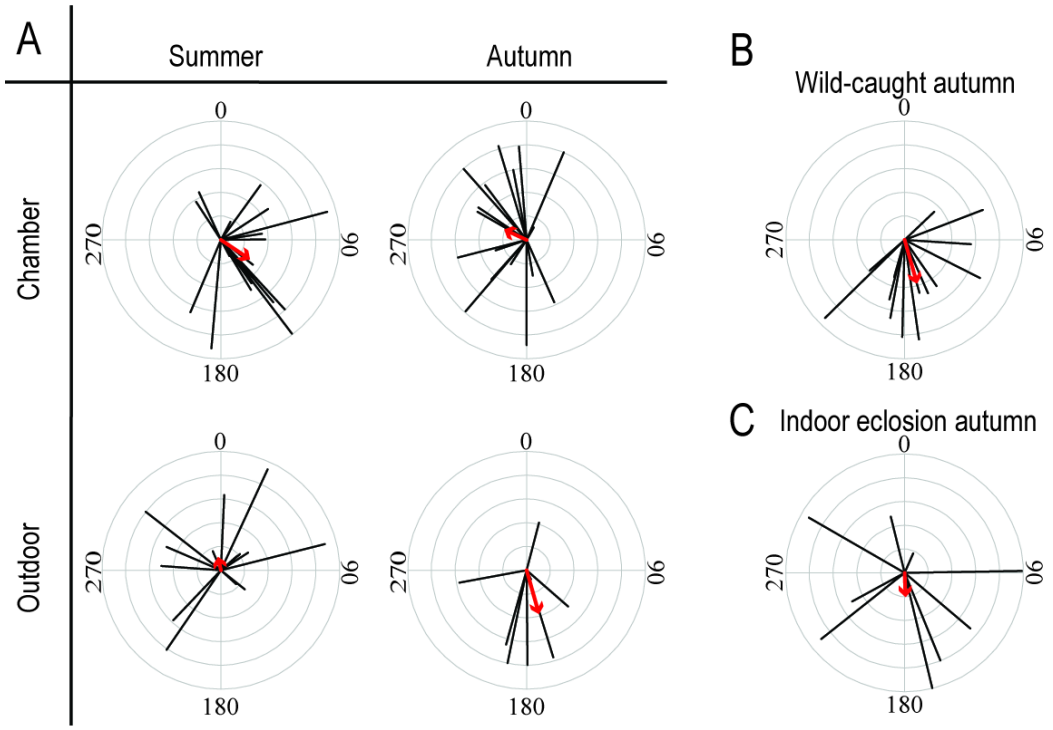


Figure 2.3. North American (NA) monarchs reared in environmental chambers do not orient south. A) Orientation plots of NA monarchs raised in a summer-like and autumn-like chamber and NA monarchs raised outdoors in both a summer and autumn generation. Each black line indicates the mean direction (0-359°) of an individual butterfly and the length of the line represents the strength of that direction (0-1). The red arrow indicates the mean direction of the group and the length of the arrow, the strength of the group direction. 0° is North. B) Orientation plot of wild NA monarchs caught along their migration route through Chicago, IL. C) Orientation plot of NA monarchs raised outdoors, but brought indoors for eclosion.

have lower egg counts when compared to the summer-like chamber females, averaging 60.4 ± 16.5 (SE) (n=8) oocytes and 69.9 ± 10.8 (SE) (n=13) respectively (Supplementary Information, Fig. 2.9). Although some autumn chamber reared individuals entered diapause, the cool temperature and early autumn day length conditions were not sufficient to induce diapause in the entire group suggesting a missing environmental cue.

We do not know what specifically about the indoor environment prevents the development of migration behavior. Perhaps there are critical developmental periods or environmental conditions that prime monarchs to develop as migratory individuals. We do, however, have one additional observation that illustrates the fragility of migratory orientation behavior. On October 24th, 2016, we moved a number of outdoor-reared NA pupae indoors to an autumn-like chamber kept at 21°C and with an 11-hour day (07:00-18:00) to mimic the outdoor environment. The 9 individuals who flew in the simulator emerged on either day 3 or 4 after being brought indoors. Even though these individuals spent the vast majority of their development outdoors during the autumn, they did not all orient towards the south like their completely outdoor-reared siblings (Fig. 2.3C; indoor eclosion NA $\boldsymbol{\sigma}$ = 177°, r = 0.21, n = 9, Rayleigh test, z-score = 0.40, p > 0.5). The indoor eclosion and the autumn positive control groups differed in the distribution of their individual directions (Wallraff test, Kruskal-Wallis $\chi_2$ = 7.574, p = 0.006) and group vector strengths (Supplementary Information, Fig. 2.4C). These results suggest that brief exposure to unnatural conditions—and even late in development—may be enough to disrupt flight orientation behavior in some monarchs. However, given the small sample size, this result merits further attention in the future.

Our results have a number of practical implications. First, captive bred monarchs that are reared year-round could potentially lose flight orientation behavior which would seriously impact their ability to migrate. That being said we assessed only one commercially bred lineage, and there is evidence that other commercially bred monarchs do migrate. Recently 720 monarchs that were raised by a different commercial breeder were tagged and released in San Antonio, Texas, of which five were recovered at overwintering sites in Mexico (Maeckle 2018). We do not know if different husbandry practices affect whether a captive population is likely to lose

migration behavior or if some proportion of all commercial monarchs have the potential to orient and migrate successfully. Additional flight testing may reveal that a percentage of the commercially bred monarchs orient correctly; however, as a group, these commercial monarchs are not directional.

In terms of seasonal rearing by summer hobbyists and school groups, we would argue that the practice of raising monarchs in this setting is a net positive, especially in the link it creates between people and their natural environment. The fact that so many school-age children raise monarchs is probably one of the reasons the insect is so popular and why the public directly participates in conservation efforts on behalf of the species. This practice should absolutely continue with the added caveats that the butterflies should be locally sourced, and then subsequently reared outdoors where they will be exposed to the full spectrum of natural environmental conditions, ensuring that reared monarchs will have the best chance of migrating successfully.

Our results also have important implications for the larger issue of monarch conservation, especially as it relates to potentially listing the monarch as a threatened species. Even though NA monarchs have dispersed and colonized many parts of the world, these populations do not migrate. While globally the species may survive, the spectacular annual migration of monarchs in NA may be nearing an end. This reality has inspired scientists and conservationists to ask about the nature of migration loss in other environments—are non-migratory populations simply never exposed to the environmental cues that induce migration or have they lost the trait? A recent study demonstrated that a Pacific monarch lineage enters diapause when reared under autumn-like conditions (Freedman et al., 2018) suggesting non-migratory monarchs may retain migration associated adaptations. However, that study did not assess flight orientation. Our

results suggest that recurring selective pressure in the form of annual migration is necessary to maintain the entire suite of migration associated adaptations.

## Materials and Methods

### *Animal Husbandry*

For outdoor reared monarchs in 2016, we captured summer generation adult North American (NA) monarchs in Chicago and ordered adult monarchs from a commercial breeder in July. However, the number of wild monarchs in the Chicago area was very low in 2016, and so we mated some of our NA wild caught adults to commercially sourced as well as wild caught monarchs to produce our late summer generation. The summer generation monarchs tested in the flight simulator and dissected for mature oocyte counts emerged between August 11th and August 22nd. Since we had very few pure bred NA monarchs in our summer generation, we crossed the few remaining 100% NA monarchs to the F1 hybrids to create our NA autumn generation (roughly 75% NA to 25% commercial). We mated pure commercial individuals to each other to produce the commercial autumn generation. We reared the autumn generation outdoors, and they emerged between October 10th and 24th, 2016.

In 2018, we captured summer generation adult North American (NA) monarchs in Chicago in June. We collected eggs from adults housed outdoors in early July and reared the offspring in a summer or fall like chamber as well as keeping a group outdoors. We kept the summer-like chamber kept at 25°C with a 16-hour day and the autumn-like chamber at 18°C with a 14-hour day. Summer chamber adults emerged between July 31st and August 3rd, 2018.

Autumn chamber adults emerged between August 26th and 30th, 2018. The outdoor 2018 summer generation emerged between August 1st and 3rd, 2018 while the outdoor autumn generation emerged between September 7th and 19th, 2018. Additionally, we caught 24 wild

adult monarchs as they migrated through Chicago on September 14th, 2018. Details of rearing and care are described in SI Appendix, Materials and Methods.

*Flight Testing*

We performed flight testing in a monarch flight simulator adapted from Mouritsen and Frost (Mouritsen and Frost 2002, Fig. 2.1A), and used methods consistent with previous flight experiments (Froy 2003; Guerra and Reppert 2013; Merlin, Gegear, and Reppert 2009; Mouritsen and Frost 2002; Zhu et al. 2009) when testing. We performed all tests under sunny skies, and a successful test required that an individual fly continuously for 10 minutes. After each successful test, we froze the sample for future dissection and potential genetic analysis. We calculated the mean vector ($\boldsymbol{\sigma}$ = 0 - 359°) and vector strength (r = 0 - 1) for each individual. We then calculated a weighted group mean vector and vector strength. We used the Rayleigh test to determine whether the group mean was significantly directional and the Wallraff test to determine whether the distribution of individual directions differed. To determine whether our groups had significantly different distributions of their group vector strengths, we applied a bootstrapping analysis (Supplementary Information, Fig. 2.4). For detailed descriptions see Supplementary Information, Materials and Methods.

*Mature Oocyte Counts*

We dissected females who completed a flight test by making a longitudinal cut down the abdomen to remove ovaries and eggs and then counted the number of mature oocytes (see Supplementary Information, Materials and Methods).

*Geometric Morphometrics*

To examine the shape and size of monarch forewings, we performed geometric morphometric analyses using 13 landmarks (Supplementary Information, Fig. 2.5B). To examine shape alone,

we applied a generalized Procrustes analysis to exclude effects unrelated to shape including reflection, position, scale, and orientation. We examined variation in size and shape with principal component analysis (PCA) (see Supplementary Information, Materials and Methods).

*Population Genetics and Phylogenetics*

We extracted DNA from 15 commercial monarchs, generated paired-end 75bp libraries, and sequenced the libraries on the NextSeq500 Illumina platform. We downloaded whole genome sequencing data from 72 monarch and 9 outgroup samples collected from around the world (Zhan et al. 2014) from NCBI SRA (Supplementary Information, Table 2.2). We mapped sequences to the North American monarch reference genome (version 3, repeat masked) (Zhan et al. 2011). We then assigned sample genotypes and called single nucleotide polymorphisms (SNPs/variants) using Genome Analysis Toolkit's (GATK) (McKenna et al. 2010). After filtering, we performed a principal component analysis (PCA) using the remaining 4,593,379 variants. We estimated population identity from 2 to 8 distinct populations (K=2 to K=8) using Frappe, version 1.1 (Tang et al. 2005, Fig. 2.12). We built a neighbor-joining tree with 500 rapid bootstrap replicates using FastMe (Lefort, Desper, and Gascuel 2015, Fig. 2.2C) and used TreeMix to investigate historical relationships and gene flow among monarch populations (Pickrell and Pritchard 2012, Fig. 2.8). Details of these analyses are available in Supplementary Information, Materials and Methods.

**Data availability**

Whole genomes sequences are available at the National Center for Biotechnology Information's website under BioProjectID: PRJNA509269.

**Acknowledgments**

We thank Dr. Andre Green for significant help in raising the butterflies used in the flight simulator experiments. We thank Dr. Patrick Guerra for his instruction on using the monarch flight simulator. This work was funded by NSF Graduate Research Fellowship Program, NIH Genetics and Regulation Training Grant T32 GM07197, US Fish and Wildlife award F17AC01222, NSF IOS-1452648, and NIH grant GM108626.

**Supplementary Information**

*Supplemental Materials and Methods*

*Animal Husbandry*

For outdoor reared monarchs in 2016, we captured summer generation adult North American (NA) monarchs in Chicago and ordered adult monarchs from Commercial breeder (commercial) in July. However, the number of wild monarchs in the Chicago area was very low in 2016, and so we mated some of our NA wild caught adults to commercially sourced as well as wild caught monarchs to produce our late summer generation. Our local late summer group contains 12 pure NA and 8 F1 hybrids (Fig. 2.1B). We also mated commercial monarchs to each other to produce our commercial late summer group (Fig. 2.1B). The summer generations emerged between August 9th and 31st, 2016; however, summer monarchs tested in the flight simulator and dissected for mature oocyte counts emerged between August 11$^{th}$ and August 22$^{nd}$.

Since we had very few pure-bred NA monarchs in our summer generation, we crossed the few remaining 100% NA monarchs to the F1 hybrids to create our NA autumn generation (roughly 75% NA to 25% commercial). We mated pure commercial individuals to each other to produce the commercial autumn generation. We reared the autumn generation outdoors, and they emerged between October 10th and 24th, 2016 (Fig. 2.1B). We then brought all remaining pupae

indoors on Oct. 24th as the temperature was expected to drop below freezing that night. The remaining pupae emerged over the course of 3 to 4 days in an environmental chamber kept at 21°C with an 11-hour day (07:00-18:00). These monarchs are the indoor eclosion autumn group and siblings of the autumn NA monarchs (Fig. 2.3C).

We reared all monarchs in 1.83$m^3$ outdoor cages with steel frames covered in screens with 0.63mm openings purchased from Bioquip. The eggs and larvae were further contained inside 30.5$cm^3$ mesh popup cages. The adult butterflies were also double contained in 91.5cm x 30.5$cm^2$ mesh popup cages. All larvae ate fresh *Ascelpias syriaca*, the common milkweed, which is native to Illinois. Adults ate Birds Choice Butterfly nectar, replenished daily. We labeled each adult monarch with a unique ID number in permanent marker on the hindwing and we recorded the ID, population of origin, emergence date, and sex of each individual.

We followed the same protocol in 2018 for the outdoor rearing of our NA summer and autumn generations; however, we did not cross NA monarchs to the commercial line in 2018. The 2018 summer generation emerged between August 1st and 3rd, 2018 while the autumn generation emerged between September 7th and 19th, 2018. Additionally, we caught 24 wild adult monarchs as they migrated through Chicago on September 14th, 2018.

For monarchs reared in environmental chambers in 2018, we captured NA adults in Chicago in June 2018, mated the adults and housed them outdoors. We collected eggs from the adults in early July and then reared half of the eggs in a summer-like chamber kept at 25°C with a 16-hour day and half in an autumn-like chamber kept at 18°C with a 14-hour day. The environmental chambers were fitted with Zilla tropical 25 UVB fluorescent T8 bulbs, which emit low levels of UVA and UVB light, and Philips bright white light mercury Alto II grow lights. Larvae ate fresh *Ascelpias syriaca* while adults ate Birds Choice Butterfly nectar. The summer

chamber adults emerged between July 31st and August 3rd, 2018. The autumn chamber adults emerged between August 26th and 30th, 2018. We labeled each adult monarch with a unique ID number in permanent marker on the hindwing as well as recorded the ID, population of origin, emergence date, and sex of each individual.

*Flight Simulator Design*

We performed all flight testing in a monarch flight simulator adapted from Mouritsen and Frost (Mouritsen and Frost 2002). The simulator consists of four main components: a large tube, a rotary encoder embedded in a metal ring at the top of the tube, a metal base which the tube sits on top of and a fan. We purchased a white semi-translucent acrylic tube open on both ends with a diameter of 30.5cm and height of 50.8cm. The base is constructed from steel and consists of four 22.9cm tall legs holding up a 30.5cm square platform with a 15.24cm wide hole in the center for the fan. Air flows through parallel drinking straws contained by an aluminum tube. The straws sit just above a computer fan (Comair Rotron 19028254A, Digikey, Part number: CR136-ND) which is connected to a variable transformer to create laminar airflow (*1*). We record all flight tests with a Logitech B910 webcam mounted under the platform and a rotary optical encoder suspended from a steel ring sitting on the tube. We purchased the optical encoder (Catalog # E5-360-250-NE-S-D-D-B) and the cable to connect the encoder to a USB port (Catalog # CA-FC5-SH-FC5-20) from US Digital.

Aspects of our design differ slightly from Mouritsen and Frost (Mouritsen and Frost 2002). Instead of using a plastic axle for our shaft and inserting it into Teflon cylinders, we milled an aluminum axle and inserted it into two stainless steel ball bearings (Ultra-Precision mini stainless-steel ball bearings, McMaster-Carr, Catalog #3826T15, Dimensions: 1/4" Shaft Diameter, 3/8" OD, 1/8" W) to minimize friction and make the encoder easy for butterflies to

turn. We disassembled the encoder and attached the 16.5cm long hollow aluminum shaft using a small screw. The two ball bearings sit inside the metal ring and hold the axle steady making the shaft's rotation smooth. The bottom 2.5cm of the shaft extending down into the tube is milled to a narrower diameter so that flexible plastic tubing can be slid over the shaft. The butterflies are attached at this junction via a tungsten tether (A-M Systems, Dimensions: 0.020 x 6 inches). The tether is inserted into their thorax, and the small wound is sealed with beeswax. The end of the tether inserted into the butterfly has a small hook that holds the tether in place under the dorsal cuticle of the thorax. The tether should be the approximate length of the butterfly's forewing and have the top 0.5 cm of a P20 pipette tip super glued to the top. The tether's pipette tip "hat" ensures quick transition between samples in the simulator. The hat fits into the flexible plastic tubing junction of the simulator shaft. Butterflies can rotate 360 degrees while flying in place.

*Flight Simulator Testing*

We used methods consistent with previous experiments utilizing a monarch flight simulator (Froy 2003; Guerra and Reppert 2015; Merlin, Gegear, and Reppert 2009b; Mouritsen and Frost 2002; Zhu et al. 2009). Outdoor reared monarchs remained outdoors for no less than 3 full days after emergence. Environmental chamber reared monarchs also remained in their respective environmental chambers for at least 3 days post eclosion. Once tethered, all butterflies spent at least 4 days resting in a glassine envelope in an environmental chamber before flight testing. In both 2016 and 2018, our tethered outdoor and environmental chamber reared autumn generation monarchs were stored in chambers at 21°C with a short 12-hour photoperiod. In the summer of 2016, we stored outdoor reared individuals at 26.7°C with a 16-hour day; however, we lowered the temperature to 25°C in 2018 which resulted in lower morality. We performed all tests under sunny skies, and a successful test required that an individual fly continuously for 10 minutes.

After each successful test, we froze the sample for future dissection and potential genetic analysis. Each test was video recorded to ensure continuous flight. The rotary encoder records every change of position (0-359°) along with the time difference in milliseconds between the previous and current angle.

*Circular Data and Statistics*

Using custom scripts and the packages "Circular" and "Plotrix" written in R, we converted degree counts into cartesian coordinates, X and Y (Agostinelli and Lund 2017; Lemon 2006; R Core Team 2013). We then calculated the mean vector ($\boldsymbol{\sigma}$ = 0 - 359°) and vector strength (r = 0 - 1) for each individual. Next, we calculated a weighted group mean vector ($\boldsymbol{\sigma}$) and vector strength (r) by multiplying each individual's mean Cartesian coordinates (X,Y) by their individual vector strength. We used the Rayleigh test (sample size* vector strength$^2$) to determine whether the group mean was significantly directional. Given the strength of the NA autumn generation's group direction (r = 0.65), a sample size of n = 7 is required for significance (Supplementary Information, Fig. 2.10). We used the Wallraff test to determine whether the distribution of individual directions differed significantly from each other. The Wallraff test computes the angular distances from a reference angle, in each group, which are then compared with a rank sum test.

To determine whether our groups had significantly different distributions of their group vector strengths we applied a bootstrap analysis. We randomly sampled, with replacement, each dataset with a sample size set to the smaller of the two groups, 1000 times. We then took the 1000 subsampled datasets and calculated a group mean and vector strength. We calculated 95% confidence intervals around each mean (Supplementary Information, Fig. 2.4).

*Mature Oocyte Counts*

We dissected females who completed a flight test by making a longitudinal cut down the abdomen to remove ovaries and eggs. We then counted each mature oocyte. Mature oocytes have ridges that form prior to oviposition and run from the top to the bottom of the egg. We used the Mann-Whitney U test to determine whether mature oocyte counts differed significantly between groups. Age and mating status of the female affects the numbers of mature oocytes (Oberhauser and Hampton 1995). Our females were never mated, but the females were not dissected at the same age. On average, females were 15 days old and varied between 7 and 27 days old at time of dissection. We noticed a positive trend between age of female and number of mature oocytes in both summer and autumn generations (Summer's $R^2 = 0.599$ and Autumn's $R^2 = 0.368$).

*Geometric Morphometrics*

Forewings of dead monarchs were detached from the thorax using forceps. We placed the forewings on grid paper (0.05 × 0.05 cm squares) and took photos of the ventral side with a Canon EOS 70d camera. Using tpsDig232, a software designed for digitizing landmarks and outlines for geometric morphometric analyses (Rohlf 2006), we put 13 landmarks on each forewing (Supplementary Information, Fig. 2.5B). Each landmark, either at vein intersections or margins, was an anatomically homologous point between individuals. We loaded landmarks coordinates into R and performed principal component analysis to examine variation in wing size. To examine variation in shape, we used Generalized Procrustes Analysis (GPA) in the R "Shapes" package to exclude effects unrelated to shape including reflection, position, scale, and orientation (Dryden, 2018.). After the Procrustes analysis, we performed principal component analysis. We included 14 commercial and 15 NA individuals raised in a summer-like environmental chamber, and 11 commercial and 16 NA individuals raised outdoors in the

summer in the forewing shape analysis (commercial n = 29 and NA n = 31). We found no difference in forewing shape by rearing condition (Supplementary Information, Fig. 2.11). These commercial and NA samples were pure bred. We tested for differences in group means using the Mann-Whitney U test.

*Population Genetics and Phylogenetics*

We extracted DNA from the thorax of fifteen autumn generation commercial monarchs raised outdoors during autumn 2016, 14 of which successfully completed flight testing. We used the modified VDRC Drosophila genomic extraction, a chloroform/phenol method (Veinna Biocenter Core Facilities). After extraction, we checked the quality of the extracted DNA with an Agilent bioanalyzer. We used a KAPA Hyper Prep kit from KAPA Biosystems along with 15 custom Illumina barcodes to generate paired-end 75bp libraries. We checked the quality of the libraries with an Agilent bioanalyzer and then pooled them for sequencing on the NextSeq500 Illumina platform. Raw reads for each individual were trimmed using Trimmomatic (Bolger, Lohse, and Usadel 2014) to remove barcode adapters, and quality checked using FastQC (Andrews 2010). To establish the ancestry and degree of genetic change in the commercial monarchs, we downloaded whole genome sequencing data from 72 monarch and 9 outgroup samples from NCBI SRA (Supplementary Information, Table 2.2). These genome data originate from monarchs collected from around the world (Zhan et al. 2014). The previously sequenced samples were collected from North American (California, North Florida, South Florida, Massachusetts, Mexico, New Jersey, Texas), Central/South American (Aruba, Belize, Bermuda, Costa Rica, Ecuador), Pacific (Australia, Hawaii, Fiji, New Caledonia, New Zealand, American Samoa), and Atlantic (Morocco, Portugal, and Spain) populations. Coverage for all 96 genomes, including the commercial and wild populations, averaged 13.74X. We mapped all sequences using Burrows-

Wheeler aligner (H. Li and Durbin 2010) to the North American monarch reference genome (version 3, repeat masked) (Stensmyr and Hansson 2011) and converted file formats using SAMtools (H. Li et al. 2009). The average percent of reads mapped for all samples was 81.7%. We marked duplicates with Picard and assigned sample genotypes using Genome Analysis Toolkit's (GATK) HaplotypeCaller command with heterozygosity set to sample pi, 0.0127, and all other settings at default values (McKenna et al. 2010). We then jointly called single nucleotide polymorphisms (SNPs/variants) in all samples using GATK's GenotypeGVCFs command with default settings, with the exception that heterozygosity which was set at sample pi, 0.0127 (McKenna et al. 2010). We removed all insertions, deletions, and all variants with a quality score lower than 30 and kept only biallelic sites leaving 23,058,661 variants with a genotyping rate of 0.9612. We then filtered each site by a genotyping rate of at least 90% and removed variants not in Hardy-Weinberg equilibrium. With all samples merged, we then removed variants with minor allele frequencies less than 0.01. Finally, we pruned variants in high linkage disequilibrium (LD); such that any pair of SNPs in a 1kb window with a correlation of 0.95 or greater were removed until no such pairs remained. These further filtering steps left 4,593,379 variants with an overall genotyping rate of 0.9954 for analysis. We filtered using a combination of the programs Plink version 1.90 and VCFtools version 0.1.14 (Danecek et al. 2011; Purcell et al. 2007). We performed Principal Component Analysis (PCA) using Plink version 1.90 on the 4,593,379 variants left after filtering and pruning. We then randomly pruned the 4,593,379 variants to 1 million, and estimated population identity from 2 to 8 distinct populations (K=2 to K=8) using Frappe, version 1.1 (Tang et al. 2005, Supplementary Information, Fig. 2.12). We averaged nucleotide diversity across 10kb windows 20 generated by VCFtools (Supplementary Information, Table 2.1). We used FastMe to build a neighbor-joining

tree based on the 4,593,379 SNPs used for the population genetic analyses (Lefort, Desper, and Gascuel 2015). We used the BioNJ method with subtree pruning and regrafting (SPR) tree refinement and performed 500 rapid bootstrap replicates (Lefort, Desper, and Gascuel 2015). We then used Phylip's consense program to draw a tree with branches proportional to the number of bootstraps supporting each branch (Felsenstein, 2005, Fig. 2.2C). Using the same SNP set, we ran TreeMix, which infers a maximum likelihood tree, to investigate historical relationships and gene flow among monarch butterfly populations (Pickrell and Pritchard 2012, Supplementary Information, Fig. 2.8).

*Supplemental Figures and Tables*

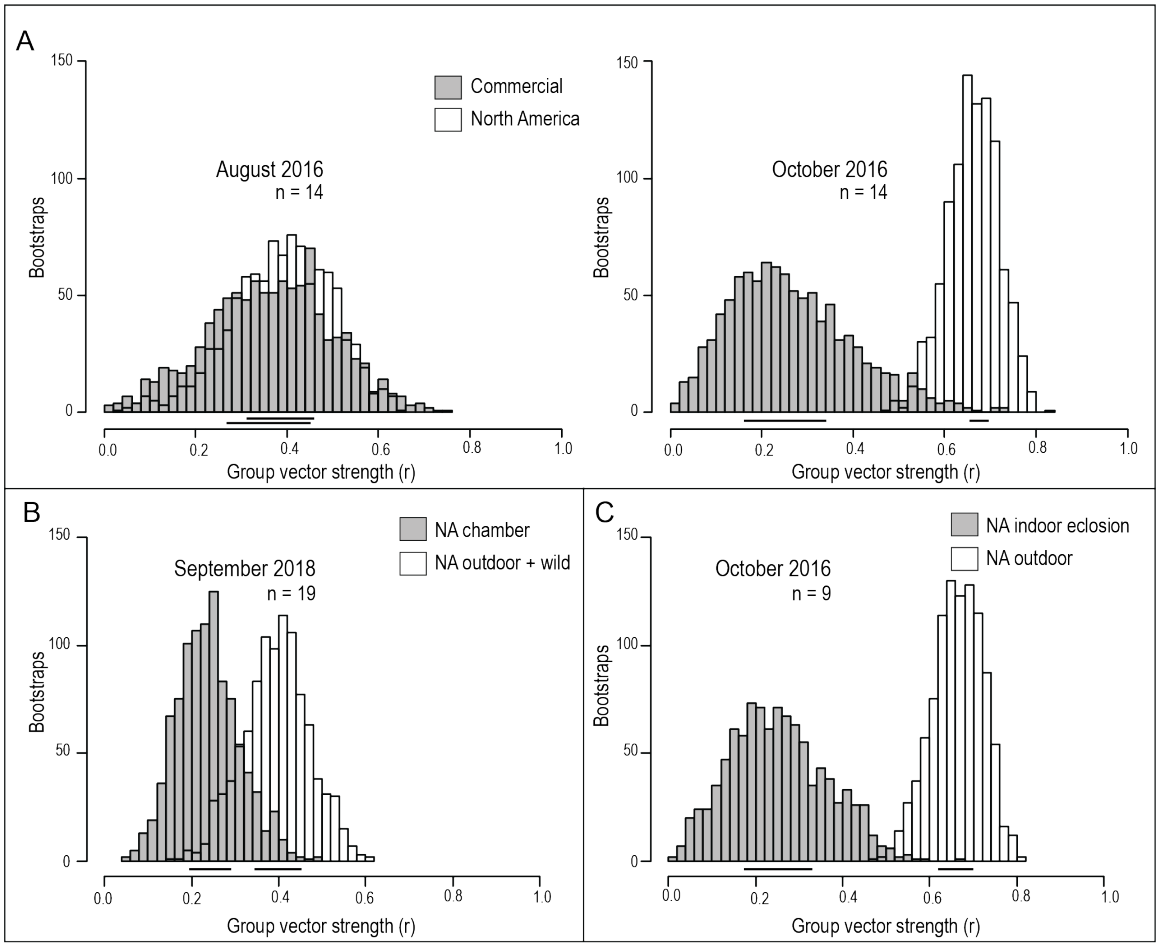


Figure 2.4. Distribution of group vector strengths (r) generated from subsampled orientation data. Black bars under histograms are 95% confidence intervals. A) In August 2016, NA: 95% CI [0.316, 0.460] and commercial: 95% CI [0.274,0.452]. In October NA: 95% CI [0.659, 0.700] and commercial: 95% CI [0.177, 0.352]. B) In September 2018, NA autumn chamber: 95% CI [0.190, 0.284] and combined NA outdoor + wild: 95% CI [0.345,0.445]. C) In October 2016, indoor eclosion: 95% CI [0.180,0.325] and outdoor eclosion: 95% CI [0.625,0.701].

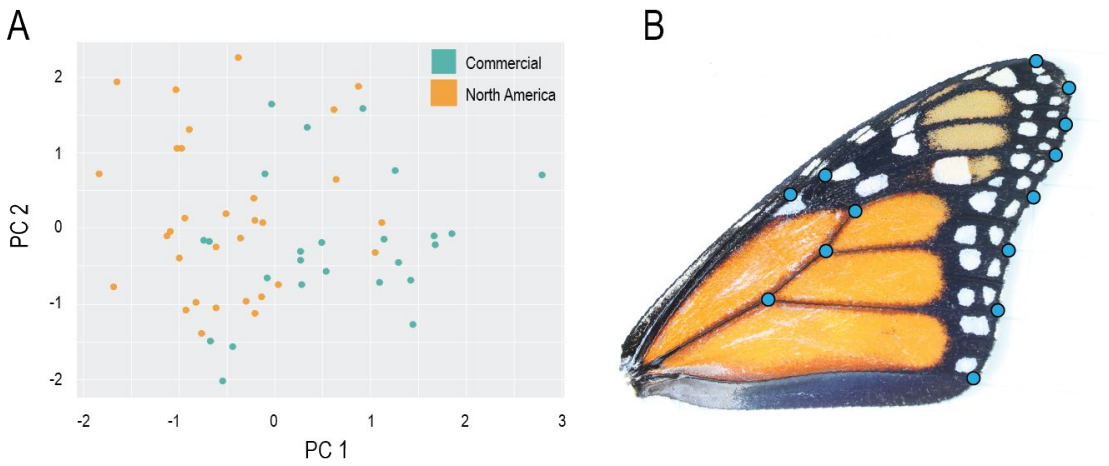


Figure 2.5. Commercial monarchs and North American (NA) monarchs have differently shaped forewings. A) PC1 and PC2 represent the top two vectors of a principle component analysis (PCA) explaining 55.53% and 10.53% of the variation in shape respectively. B) A representative monarch forewing, blue dots indicate the placement of landmarks.

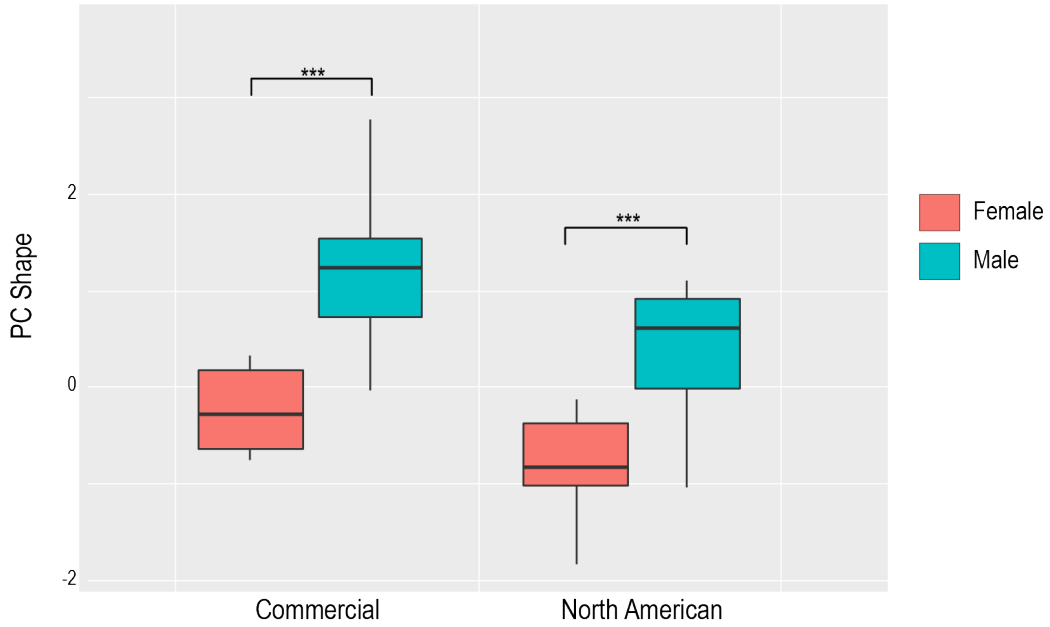


Figure 2.6. There is significant sexual dimorphism in the shape of monarch forewings in both commercial and North American (NA) populations. Males (blue) have significantly rounder forewings than the females (red) in both populations. PC Shape refers to the first vector of the principle component analysis (PCA).

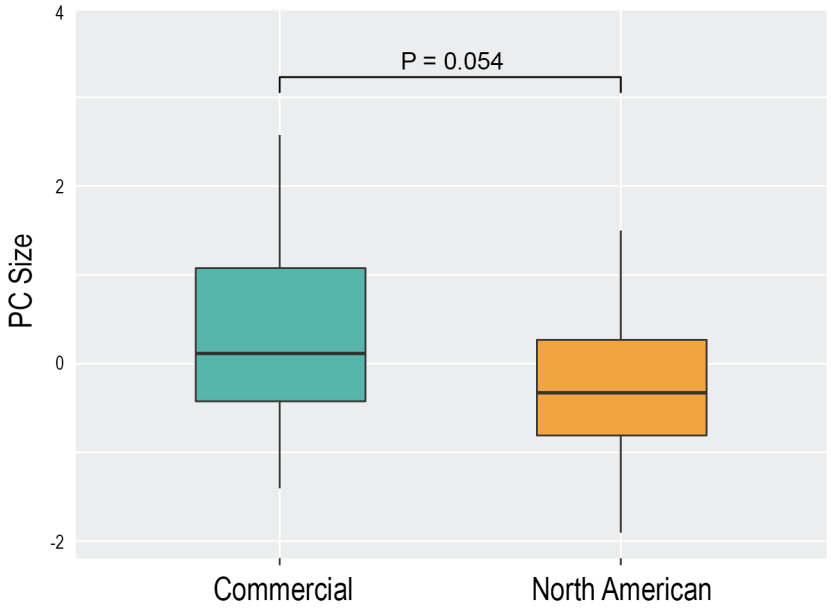


Figure 2.7. Commercial monarch forewings tend to be smaller than North American (NA) monarchs though the difference is not significant. PC Size refers to the first vector of the principle component analysis (PCA) and explains 61.73% of the variation. Higher PC values correspond to a smaller forewing.

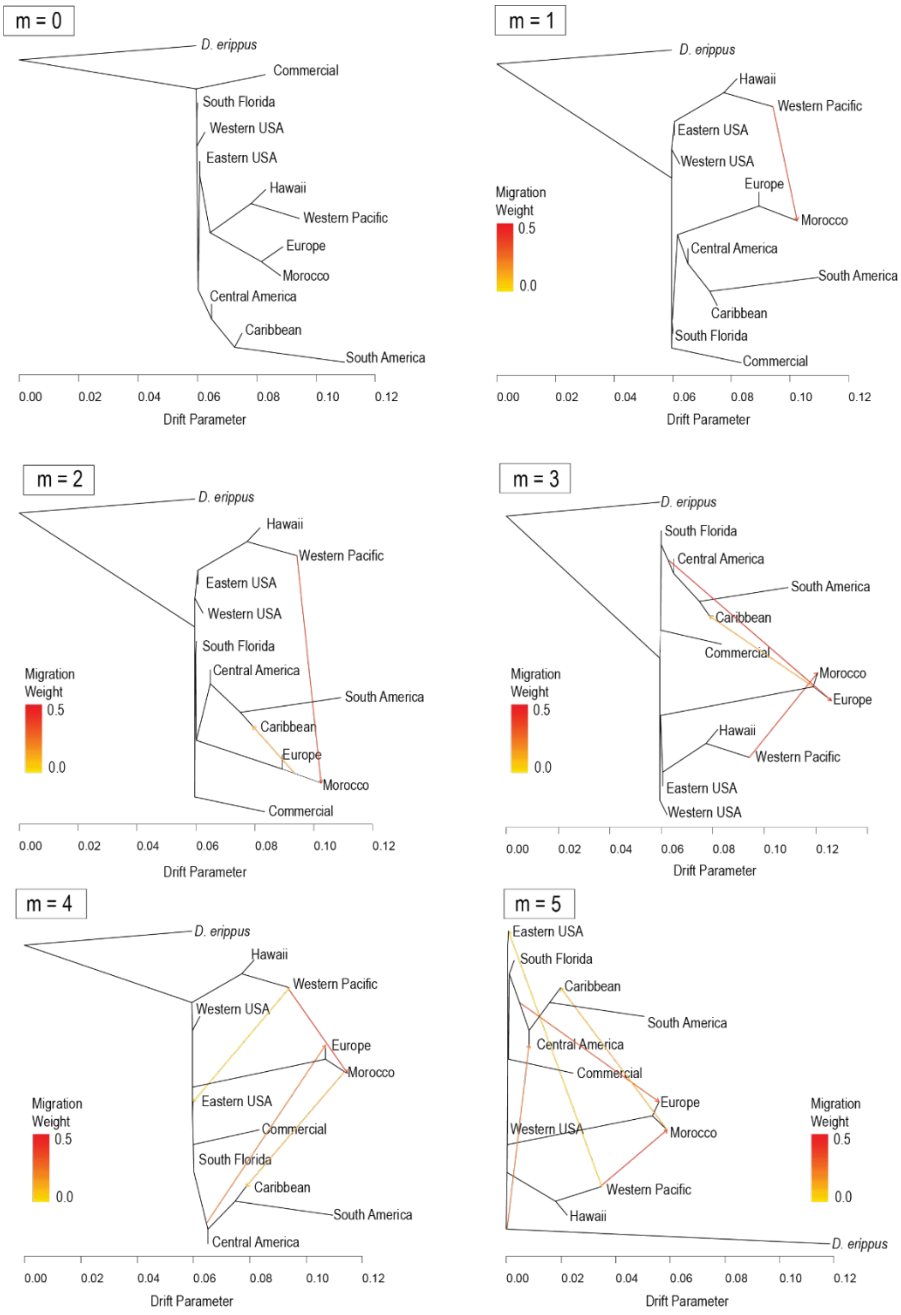


Figure 2.8. Results of TreeMix analysis for commercial, North American (NA), and non-migratory monarch populations reveal no evidence of migration between commercial and other monarch butterfly populations. *Danaus erippus* is the outgroup. Arrows indicate direction of migration given a migration event (m= 0, 1, 2, 3, 4, 5). Colors of the arrows represent migration weights which are correlated to admixture proportions.

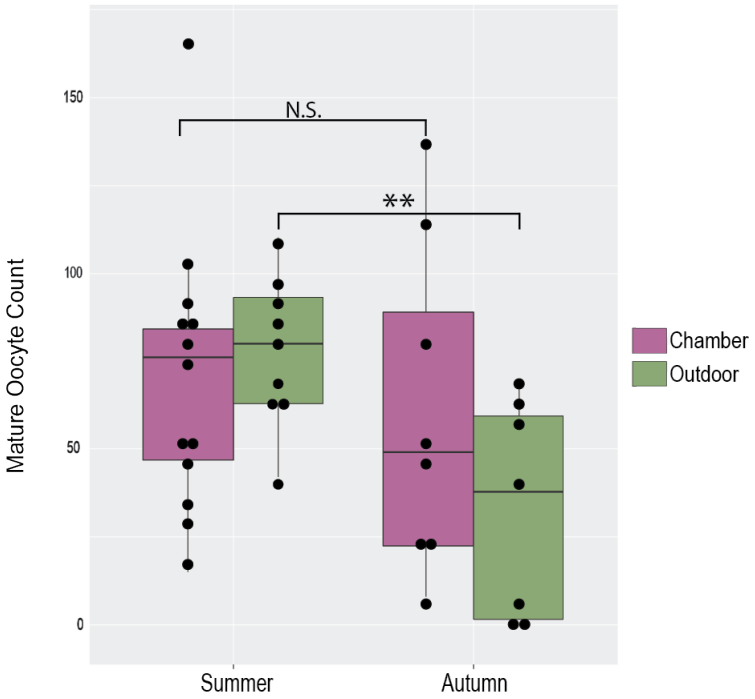


Figure 2.9. Mature oocyte counts for environmental chamber reared monarchs. The number of mature oocytes significantly decreases between the summer and autumn generations of outdoor reared North American monarchs (green), but there is no significant difference in oocyte counts between the autumn and summer-like environmental chamber treatments (purple).

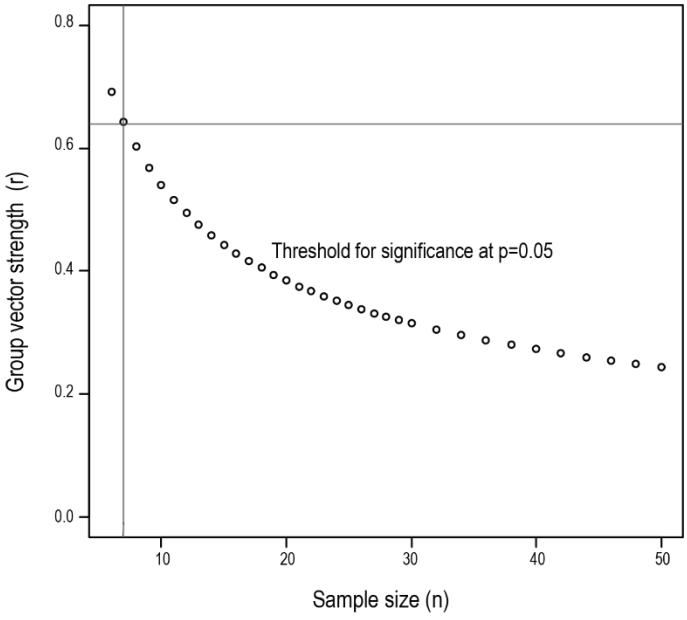


Figure 2.10. Relationship between group vector strength (r) and sample size. Circles are the sample size required for significance (p = 0.05) at a given vector strength. The vertical and horizontal grey lines show that an r = 0.64 requires a sample size of n = 7 to detect significant directionality.

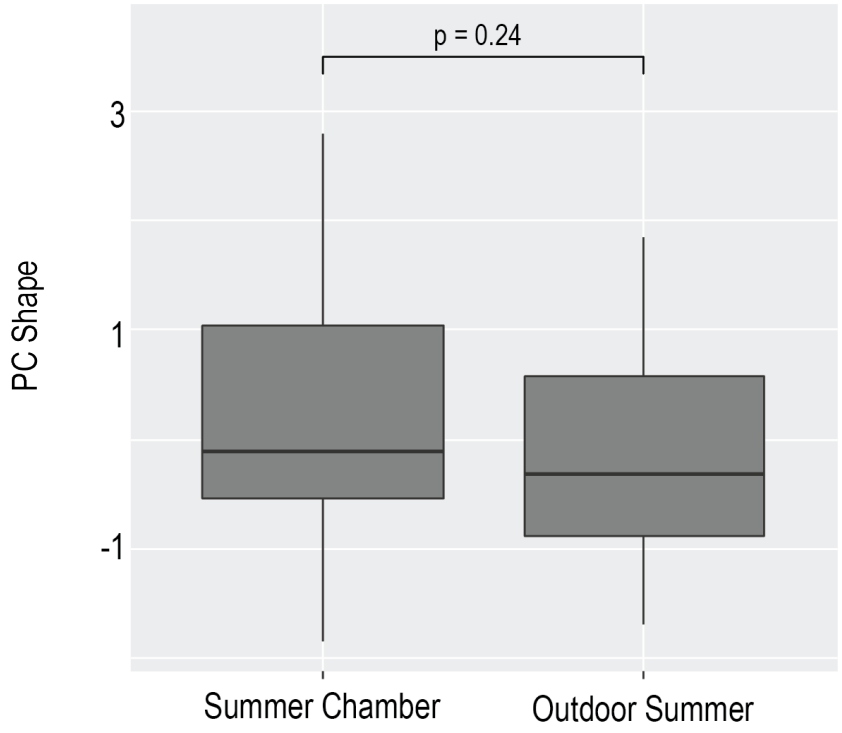


Figure 2.11. There is no significant difference in wing shape between monarchs raised outdoors in the summer versus in a summer-like environmental chamber. Of the total 25 commercial monarchs used in the geometric morphometric analyses, 11 were reared outdoors in the summer while 14 were reared in an environmental chamber. Of the total 31 North American monarchs, 16 were raised outdoors and 15 in a summer-like environmental chamber. PC Shape refers to the first vector of the principle component analysis (PCA).

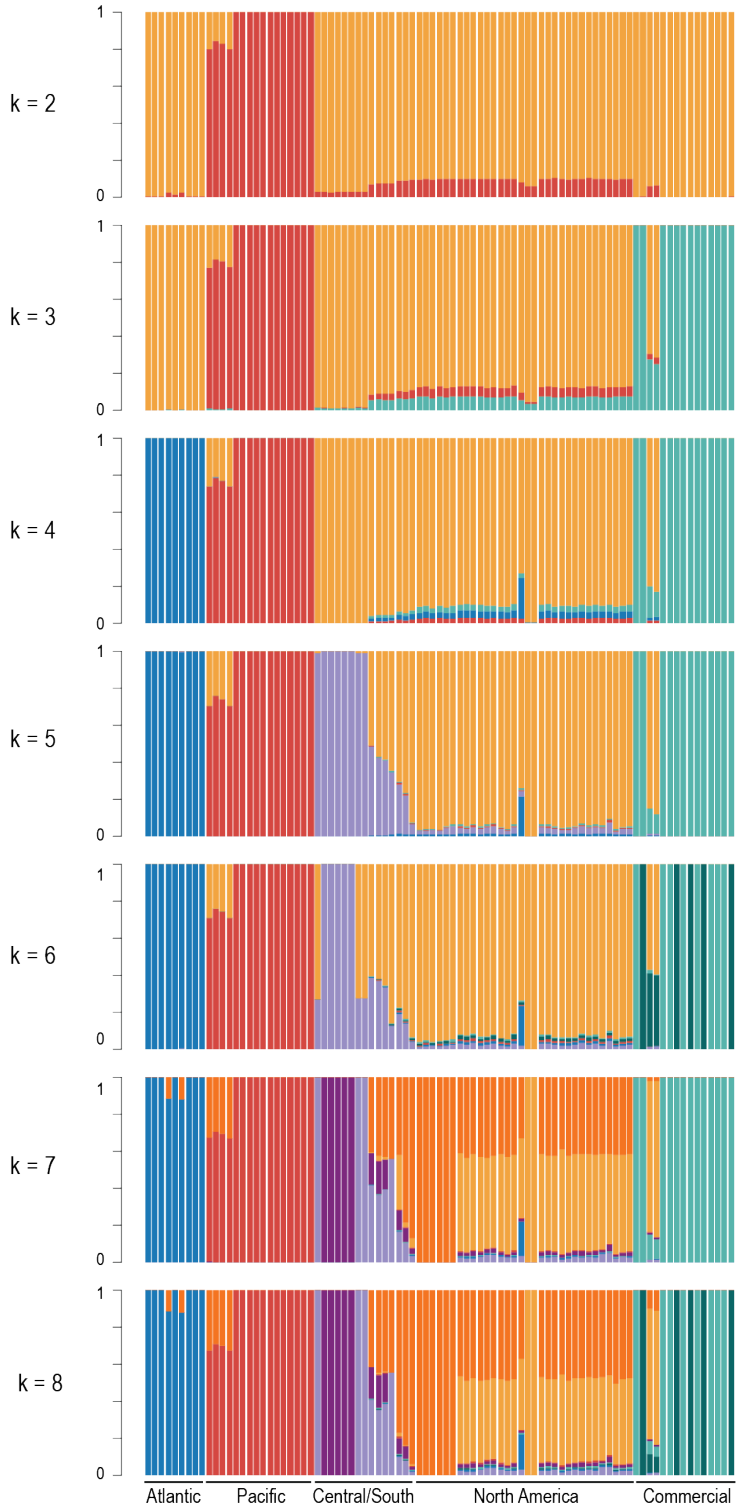


Figure 2.12 Analysis of single nucleotide polymorphisms (SNPs) using Frappe reveals the commercial lineage is a distinct population with recent North American (NA) admixture. Each column represents an individual sample and colors in each column the proportion of ancestry over population sizes ranging from K=2 to K=8, where K refers to the distinct number of subpopulations that make up the total monarch population.

Table 2.1. Nucleotide diversity ($\pi$) in the commercial lineage is less than North American (NA) wild populations and comparable to non-migratory populations of monarchs world-wide. SD stands for standard deviation.

| **Populations** | **$\pi$** | **SD** |
|---|---|---|
| Atlantic | 0.00805 | 0.00487 |
| Pacific | 0.00812 | 0.00490 |
| Commercial | 0.00892 | 0.00525 |
| Central/South American | 0.00990 | 0.00577 |
| North American | 0.01019 | 0.00596 |
| South Florida | 0.01050 | 0.00609 |
| All Samples | 0.01273 | 0.00730 |

Table 2.2. Monarch and outgroup species sample information and statistics.

| **Accession #** | **Collection Location** | **Source** | **Raw (Mb)** | **Post QC (Mb)** | **Depth** | **Mapped %** |
|---|---|---|---|---|---|---|
| SAMN10576418 | Commercial breeder, USA | This study | 2029 | 1628 | 10.02 | 79.23 |
| SAMN10576419 | Commercial breeder, USA | This study | 3781 | 3714 | 22.04 | 80.40 |
| SAMN10576420 | Commercial breeder, USA | This study | 4378 | 4095 | 21.99 | 88.66 |
| SAMN10576421 | Commercial breeder, USA | This study | 2336 | 2058 | 18.32 | 88.23 |
| SAMN10576422 | Commercial breeder, USA | This study | 3350 | 2767 | 18.50 | 79.40 |
| SAMN10576423 | Commercial breeder, USA | This study | 4533 | 4237 | 23.53 | 75.90 |
| SAMN10576424 | Commercial breeder, USA | This study | 3632 | 3451 | 18.57 | 79.44 |
| SAMN10576425 | Commercial breeder, USA | This study | 3081 | 2654 | 17.97 | 80.32 |
| SAMN10576426 | Commercial breeder, USA | This study | 3031 | 2295 | 21.52 | 77.93 |
| SAMN10576427 | Commercial breeder, USA | This study | 4265 | 3318 | 11.42 | 78.76 |
| SAMN10576428 | Commercial breeder, USA | This study | 3889 | 3383 | 17.06 | 80.23 |
| SAMN10576429 | Commercial breeder, USA | This study | 3264 | 2865 | 23.02 | 79.99 |
| SAMN10576430 | Commercial breeder, USA | This study | 3734 | 3614 | 17.73 | 78.31 |
| SAMN10576431 | Commercial breeder, USA | This study | 4871 | 4438 | 15.39 | 80.13 |
| SAMN10576432 | Commercial breeder, USA | This study | 3704 | 3290 | 15.12 | 79.55 |

Table 2.2 continued.

| | | | | |
|---|---|---|---|---|
| SRR1549530 | Spain | Zhan et al. 2014 | 8.43 | 88.59 |
| SRR1549531 | Spain | Zhan et al. 2014 | 14.12 | 82.04 |
| SRR1549532 | Spain | Zhan et al. 2014 | 16.72 | 89.68 |
| SRR1551990 | Portugal | Zhan et al. 2014 | 12.44 | 76.14 |
| SRR1551991 | Portugal | Zhan et al. 2014 | 5.99 | 71.42 |
| SRR1551992 | Portugal | Zhan et al. 2014 | 24.02 | 89.30 |
| SRR1552005 | Morocco | Zhan et al. 2014 | 10.05 | 83.92 |
| SRR1552006 | Morocco | Zhan et al. 2014 | 7.68 | 88.67 |
| SRR1552007 | Morocco | Zhan et al. 2014 | 7.28 | 88.30 |
| SRR1551994 | Belize | Zhan et al. 2014 | 5.86 | 70.47 |
| SRR1551996 | Costa Rica | Zhan et al. 2014 | 10.83 | 90.80 |
| SRR1552000 | Puerto Rico, USA | Zhan et al. 2014 | 10.02 | 91.40 |
| SRR1552102 | Aruba | Zhan et al. 2014 | 20.55 | 78.98 |
| SRR1552103 | Aruba | Zhan et al. 2014 | 21.04 | 89.11 |
| SRR1552107 | Bermuda | Zhan et al. 2014 | 9.74 | 86.59 |
| SRR1552228 | Belize | Zhan et al. 2014 | 6.69 | 87.96 |
| SRR1552229 | Belize | Zhan et al. 2014 | 8.40 | 85.80 |
| SRR1552230 | Costa Rica | Zhan et al. 2014 | 18.53 | 91.32 |
| SRR1552231 | Costa Rica | Zhan et al. 2014 | 10.41 | 91.22 |
| SRR1552310 | Ecuador | Zhan et al. 2014 | 16.76 | 90.89 |
| SRR1552311 | Ecuador | Zhan et al. 2014 | 15.77 | 90.15 |
| SRR1552312 | Ecuador | Zhan et al. 2014 | 8.69 | 87.72 |
| SRR1552313 | Puerto Rico, USA | Zhan et al. 2014 | 14.04 | 91.90 |
| SRR1552314 | Puerto Rico, USA | Zhan et al. 2014 | 8.61 | 91.52 |
| SRR1548504 | Massachusetts, USA | Zhan et al. 2014 | 22.74 | 88.39 |
| SRR1548506 | Massachusetts, USA | Zhan et al. 2014 | 18.29 | 85.56 |
| SRR1548571 | Massachusetts, USA | Zhan et al. 2014 | 11.46 | 92.20 |
| SRR1548572 | New Jersey, USA | Zhan et al. 2014 | 9.42 | 85.38 |
| SRR1548573 | New Jersey, USA | Zhan et al. 2014 | 12.17 | 77.49 |
| SRR1548574 | New Jersey, USA | Zhan et al. 2014 | 9.95 | 82.47 |
| SRR1548575 | Massachusetts, USA | Zhan et al. 2014 | 7.66 | 80.86 |
| SRR1548576 | Massachusetts, USA | Zhan et al. 2014 | 6.83 | 88.69 |
| SRR1548578 | Massachusetts, USA | Zhan et al. 2014 | 23.35 | 68.64 |
| SRR1549524 | Saint Marks, Florida, USA | Zhan et al. 2014 | 13.75 | 84.60 |
| SRR1549525 | Saint Marks, Florida, USA | Zhan et al. 2014 | 13.20 | 88.40 |
| SRR1549526 | Saint Marks, Florida, USA | Zhan et al. 2014 | 21.10 | 83.34 |
| SRR1549527 | Texas, USA | Zhan et al. 2014 | 10.67 | 87.72 |
| SRR1549528 | Texas, USA | Zhan et al. 2014 | 9.54 | 87.72 |
| SRR1549529 | Texas, USA | Zhan et al. 2014 | 7.68 | 79.87 |

Table 2.2 continued.

| | | | | |
|---|---|---|---|---|
| SRR1551995 | California, USA | Zhan et al. 2014 | 9.22 | 87.00 |
| SRR1552204 | Mexico | Zhan et al. 2014 | 7.05 | 88.35 |
| SRR1552205 | Mexico | Zhan et al. 2014 | 13.03 | 81.09 |
| SRR1552206 | Mexico | Zhan et al. 2014 | 9.39 | 89.20 |
| SRR1552207 | Mexico | Zhan et al. 2014 | 9.41 | 80.45 |
| SRR1552208 | Mexico | Zhan et al. 2014 | 10.73 | 89.70 |
| SRR1552209 | Mexico | Zhan et al. 2014 | 9.31 | 88.73 |
| SRR1552222 | Saint Marks, Florida, USA | Zhan et al. 2014 | 21.51 | 89.03 |
| SRR1552223 | Saint Marks, Florida, USA | Zhan et al. 2014 | 6.47 | 65.12 |
| SRR1552224 | California, USA | Zhan et al. 2014 | 9.19 | 50.50 |
| SRR1552225 | California, USA | Zhan et al. 2014 | 12.24 | 89.85 |
| SRR1548393 | Hawaii, USA | Zhan et al. 2014 | 20.46 | 79.17 |
| SRR1549537 | Western Samoa | Zhan et al. 2014 | 38.95 | 86.12 |
| SRR1551989 | Western Samoa | Zhan et al. 2014 | 23.59 | 54.28 |
| SRR1551993 | Australia | Zhan et al. 2014 | 7.24 | 87.74 |
| SRR1551997 | Hawaii, USA | Zhan et al. 2014 | 6.78 | 90.62 |
| SRR1551999 | New Zealand | Zhan et al. 2014 | 8.99 | 90.11 |
| SRR1552002 | New Caldonia | Zhan et al. 2014 | 9.98 | 69.63 |
| SRR1552004 | New Caldonia | Zhan et al. 2014 | 6.58 | 50.39 |
| SRR1552111 | Fiji | Zhan et al. 2014 | 16.08 | 89.55 |
| SRR1552113 | Fiji | Zhan et al. 2014 | 13.13 | 65.72 |
| SRR1552232 | Hawaii, USA | Zhan et al. 2014 | 10.44 | 89.37 |
| SRR1552233 | Hawaii, USA | Zhan et al. 2014 | 18.92 | 88.32 |
| SRR1552234 | Australia | Zhan et al. 2014 | 11.98 | 90.48 |
| SRR1552235 | Australia | Zhan et al. 2014 | 16.70 | 90.92 |
| SRR1552236 | New Zealand | Zhan et al. 2014 | 15.29 | 93.22 |
| SRR1552237 | New Zealand | Zhan et al. 2014 | 18.39 | 84.88 |
| SRR1551998 | Miami, Florida, USA | Zhan et al. 2014 | 11.41 | 88.58 |
| SRR1552211 | Miami, Florida, USA | Zhan et al. 2014 | 12.32 | 86.52 |
| SRR1552213 | Miami, Florida, USA | Zhan et al. 2014 | 7.86 | 70.07 |
| SRR1552214 | Miami, Florida, USA | Zhan et al. 2014 | 14.07 | 63.74 |
| SRR1552216 | Miami, Florida, USA | Zhan et al. 2014 | 13.96 | 87.92 |
| SRR1552226 | Miami, Florida, USA | Zhan et al. 2014 | 14.38 | 91.27 |
| SRR1552518 | Brazil | Zhan et al. 2014 | 11.01 | 58.61 |
| SRR1552519 | Brazil | Zhan et al. 2014 | 6.17 | 55.19 |
| SRR1552522 | Costa Rica | Zhan et al. 2014 | 15.47 | 65.60 |
| SRR1552523 | Florida, USA | Zhan et al. 2014 | 13.84 | 60.53 |
| SRR1552520 | Australia | Zhan et al. 2014 | 16.73 | 88.77 |
| SRR1552521 | Australia | Zhan et al. 2014 | 24.28 | 92.58 |
| SRR1552524 | Costa Rica | Zhan et al. 2014 | 14.44 | 60.71 |

Table 2.2 continued.

| | | | | |
|---|---|---|---|---|
| SRR1552525 | Florida, USA | Zhan et al. 2014 | 5.47 | 89.85 |
| SRR1980588 | Texas, USA | Zhan et al. 2014 | 13.41 | 42.24 |

# CHAPTER THREE: MIGRATION BEHAVIOUR OF COMMERCIAL MONARCHS REARED OUTDOORS AND WILD-DERIVED MONARCHS REARED INDOORS

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**Abstract**

Captive rearing of monarch butterflies is a commercial and personal pursuit enjoyed by many different groups and individuals. However, the practice remains controversial especially after new evidence showed that both a group of commercially-derived monarchs reared outdoors and a group of wild-derived but indoor-reared monarchs failed to orient south, unlike wild-derived monarchs reared outdoors. To more fully characterize the mechanisms responsible for the loss of orientation in both commercial and indoor-reared monarchs, we performed flight simulator experiments to determine: 1) whether any fraction of commercial monarchs maintains a southern heading over multiple tests, and 2) whether indoor conditions with the addition of sunlight can induce southern flight in wild-derived monarchs. Commercial monarchs changed their flight direction more often over the course of multiple tests than wild-derived monarchs. While as a group the commercial monarchs did not fly south on average, a subset of individuals did orient south over multiple tests, potentially explaining the discordance between flight simulator assays and the recovery of tagged commercial monarchs at overwintering locations. We also show that even when raised indoors with sunlight, wild-derived monarchs did not consistently orient south in the flight simulator, though wild-derived monarchs reared outdoors did orient south.

## Introduction

Captive-reared monarch butterflies are reared and released at schools, weddings, conservation events, fairs, and by individual enthusiasts. However, the term 'captive reared' represents a spectrum of practices, including 1) raising wild-collected eggs and caterpillars in non-natural environments for eventual release 2) breeding wild-collected individuals for a few generations and releasing them into the wild and 3) raising eggs and caterpillars bought from a commercial source for release. Captive breeding can affect reared individuals' behavior, morphology, and physiology in two distinct ways: changes to the genetic background of the population through inbreeding and adaptation to captive environments and exposure to and development in non-natural conditions (Frankham 2008; Jonsson and Jonsson 2006).

Long-term breeding in captivity is known to alter behaviour in fishes, mice, drosophila, and toads (Gilligan and Frankham 2003; Jonsson, Jonsson, and Jonsson 2019; Kraaijeveld-Smit et al. 2006; McPhee 2003). In monarchs, we have previously identified a population of commercially bred individuals that are genetically divergent from North American wild-type monarchs that no longer orient south as a group even when reared in conditions known to induce directional orientation in wild-derived individuals (Tenger-Trolander et al. 2019). While the orientation of the commercial monarchs was non-directional as a group (Tenger-Trolander et al. 2019), other tagged commercial monarchs have been found at Mexican overwintering sites, prompting the question of whether some fraction of the commercial individuals can, in fact, migrate. To assess the individual directionality of commercial monarchs, we assessed directional orientation of individuals from a known 'non-directional' North American commercial population (Tenger-Trolander et al. 2019) and North American wild-derived population (from here on referred to as commercial and wild type respectively) multiple times to establish whether an individual would

repeatedly fly south. Previous work, including our own, concluded testing after a single, successful orientation flight trial per individual (Froy 2003; Merlin, Gegear, and Reppert 2009; Mouritsen and Frost 2002; Tenger-Trolander et al. 2019). By testing an individual repeatedly, we aim to determine whether specific individuals within the larger population exhibit directional orientation.

While rearing wild-collected monarch eggs will not change the genetic background of the individuals, artificial captive environments induce fitness differences in numerous fish species and monarch butterflies reared captively for a single generation (Carr, Whoriskey, and O'reilly 2004; Davis, Smith, and Ballew 2020; Metcalfe, Valdimarsson, and Morgan 2003; Milot et al. 2013; Rosengren et al. 2016; Putman, Meinke, and Noakes 2014; Schwinn et al. 2017). In general, artificial rearing environments produce individuals that fare worse than wild individuals when released (Jonsson and Jonsson 2006). In both migratory fish and monarchs, changes to rearing environment affect migratory behavior (Putman, Meinke, and Noakes 2014; Tenger-Trolander et al. 2019). Specifically, rearing migratory wild-type North American monarchs in an autumn-like environmental chamber (short day length and cool temperature) resulted in a group that oriented in random directions, while rearing wild types outdoors resulted in a group that oriented south (Tenger-Trolander et al. 2019).

Since the environmental chamber does not replicate natural sunlight, we reared wild-type monarchs indoors with access to sunlight as filtered through glass windows during autumn and tested their directional orientation. Changes in photoperiod and declination of the sun during the transition between summer and autumn are hypothesized to be important environmental cues to induce migratory monarch development (Goehring and Oberhauser 2002; Oberhauser 2019; Taylor et al. 2019). Monarchs are known to use a time-compensated sun compass to navigate; in

fact, shifting their circadian clock with different light entrainment shifts orientation in migrating individuals (Froy 2003; Merlin, Gegear, and Reppert 2009; Mouritsen and Frost 2002). While the position of the sun throughout the day plus light entrainment is critical for navigation, we do not know how important natural sunlight is for the development and triggering of directional orientation.

**Methods**

*Animal Husbandry*

In late July 2019, we caught approximately 20 wild monarchs in Hyde Park, Chicago, Illinois and ordered 20 commercial monarchs from the same source of commercial monarchs documented in Tenger-Trolander *et al* 2019. We then checked the abdomens of each monarch for signs of *Ophryocystis elektroscirrha* (OE) spores and froze individuals with apparent infection. We housed the uninfected male and female monarchs from their respective populations in medium size (91.5cm x 30.5cm$^2$) mesh pop-up cages outdoors with access to their host plant, *Asclepias syriaca*. Once females laid eggs, we washed and transferred the eggs to small (30.5cm$^3$) mesh pop-up cages. We reared caterpillars in groups rather than individually. We fed caterpillars a diet of wild-collected *Asclepias syriaca* that we replenished daily. We washed the milkweeds in a 1% bleach solution and then in water before offering them to the larvae. Upon emergence, we labeled each adult with a unique identification number in permanent marker on the hindwing. Adults were housed in medium size (91.5cm x 30.5cm$^2$) mesh pop-up cages before directional orientation testing and fed a diet of Birds Choice butterfly nectar.

*Treatments*

We housed the developing wild-type monarchs in one of three treatment groups: (1) outdoors, (2) indoors in a glass-top greenhouse, and (3) indoors in our laboratory (lab) next to a south-

facing window. The commercial monarchs developed outdoors and had no other treatments. For the outdoor treatment, pop-up cages were contained within a large outdoor 1.83cm$^3$ mesh cage. The greenhouse received only natural light, and we kept the temperature at 23ºC during the day and 18ºC at night. Temperatures in the lab remained fairly consistent between 22-23ºC, 24 hours a day. Both indoor groups emerged between September 24$^{th}$ and October 28$^{th}$ of 2019. Commercial monarchs reared outdoors emerged between September 12$^{th}$ and October 1$^{st}$ 2019, and wild-type monarchs reared outdoors emerged between September 8$^{th}$ and September 16$^{th}$ 2019.

Unfortunately, we experienced a suspected outbreak of nuclear polyhedrosis virus (NPV) which reduced expected sample sizes and pushed back the dates of emergence of our wild types reared indoors as we attempted to control spread of the virus. We found the wild-type population was particularly susceptible; however, final sample sizes were sufficient to determine directional orientation.

*Flight Simulator and Testing*

After four days in their respective rearing conditions (outdoor, indoor greenhouse, or indoor lab), we tethered the monarch adults following the protocol outlined in Tenger-Trolander *et al* 2019. All tethered monarchs then spent five days recovering in glassine envelopes stored in a 12:12 hour light-dark cycle, 21ºC environmental chamber before testing. Directly before testing, all monarchs spent at least a full hour in an outdoor cage free to move.

We tested all individuals in the monarch flight simulator developed by Mouritsen and Frost (Mouritsen and Frost 2002, Fig. 3.1A, see Tenger-Trolander *et al.* 2019 for description of modified flight simulator). All testing occurred outdoors in sunny conditions between the hours of 10am and 2:30pm. We counted the orientation test as successful if the individual flew

continuously for 10 minutes as confirmed by video recording. We only tested individuals once per day whether the test was successful or not. Due to changing weather conditions, time restrictions on testing, and variability in emergence dates, every tethered monarch could not be tested each day of testing. We focused testing on the outdoor wild type and commercial individuals to determine individual preferences in directional orientation in these groups. Table 3.2 in 'Supplementary Information' details the number of orientation tests and successful tests of each individual by treatment and population.

In total, we tethered 83 monarchs. 74 survived long enough to be tested, including 15 wild types reared outdoors, 18 wild types reared in the greenhouse, 4 wild types reared in the lab, and 37 commercials reared outdoors. Of these 74 tested, 65% (N = 48) flew at least once, including 8 wild types reared outdoors, 12 wild types reared in the greenhouse, 3 wild types reared in the lab, and 25 commercials reared outdoors. Of the 48 individuals that flew at least once, 56% (N = 27) completed at least one additional test, including 6 wild types reared outdoors, 4 wild types reared in the greenhouse, 1 wild type reared in the lab, and 16 commercials reared outdoors. The number of repeated tests in the indoor-reared group was small (N = 5); however, we were not attempting to determine whether a portion of these individuals was migratory, but rather if the group as a whole (N=15) headed south on average. The number of successful tests per individual ranged between one and seven (Supplementary Information, Table 3.2). Flight headings were recorded using a US Digital optical rotary encoder and captured on video (Fig. 3.1A). Since orientation data and video were recorded autonomously, testing was not conducted blind.

*Data Analysis and Circular Statistics*

In our flight simulator assays, tethered monarchs were attached to a rotary encoder and placed inside the simulator. As the monarch changed position, the rotary encoder recorded the new

position (in degrees, 0 - 359°) and the amount of time elapsed (in milliseconds) between each change. We used circular statistics packages, Circular and Plotrix in R, to analyze and plot flight simulator data (Agostinelli and Lund 2017; Lemon 2006; R Core Team 2013). After converting degrees to cartesian coordinates, we found the mean vector direction ($\boldsymbol{\sigma}$ = 0 - 359°) and mean resultant vector magnitude (r = 0 - 1) of each test. The mean vector direction is the average heading and the vector magnitude is a measure of consistency of the heading (r = 1 - variance). We also calculated a weighted group mean vector and magnitude and used the Rayleigh test to determine whether the group mean was directional.

Additionally, we calculated overall vector mean direction and vector magnitude for each monarch with between two and seven independent orientation tests. For example, an individual monarch with three tests with strong vector magnitudes (r>0.5) could still have a weak overall vector magnitude if it headed in vastly different directions (e.g. $0^0$, $90^o$, $180^o$) for each test. The weak overall vector magnitude indicates the monarch chose a different direction for each of the three tests. We then took each individual's overall heading and subtracted that from the individual's first heading. We compared the difference in degrees between commercial and wild type with a Welch's t-test. We also calculated each individual's vector magnitude variance for all tests and compared commercial and wild type means with a Welch's t-test.

*Random Re-sampling of Migratory Flight Data*

To determine whether any commercial monarchs with multiple orientation tests were likely migrators, we assessed the probability of finding each individual's multiple flight headings within a distribution of known migrators using a random re-sampling approach. Including data from the autumns of 2016, 2018, and 2019, we have orientation data for 55 wild-type North American monarchs raised outdoors. Directional orientation data from the outdoor-reared wild

types tested in 2016 and 2018 are available in Dataset_S01.xlsx file of Tenger-Trolander *et al*. 2019. Data from 2019 are available in the supplementary file TengerTrolander_Data_S1.xlsx of this paper. Monarchs from 2016, 2018, and 2019 were all reared outdoors in the same conditions, but with variability in eclosion dates. Monarchs reared in 2016 eclosed between October 7$^{th}$ – 20$^{th}$, those reared in 2018 eclosed between Sept 7$^{th}$-18$^{th}$, and those reared in 2019 eclosed between September 8$^{th}$ – 16$^{th}$.

We binned the 55 orientation tests into either north (270-89º) or south (90-269º) bins, resulting in 51 southern binned and four northern binned tests. From those 55 binned wild-type migratory tests, we randomly sampled, with replacement, the number of tests an individual completed (between 2-7), 5,000 times. Each random sample had several possible orientation patterns going north and south. For instance, in the case of 5,000 random samples of three tests, the possible patterns encountered are SSS, SSN, SNN, or NNN (where S was south and N was north and order was not considered). We then counted how many of those 5,000 random patterns were SSS, SSN, SNN, and NNN. In the case of SSN, we found it appeared 350 times out of 5000 trials or 7% of the time in the known migratory group. An individual with 3 orientation tests that oriented south twice and north once has a 7% probability of being a migrator.

The number of bins and degree cutoffs for each bin was arbitrary and could be changed. We also analyzed the data in 90º bins with the following degree cutoffs: northeast (0-89º), southeast (90-179º), southwest (180-269º), and northwest (270-359º). While the specific probabilities changed, which individuals are least and most likely to be migratory did not; exceptions are highlighted in white in Table 3.1. In our dataset, degree cut-offs affected the probabilities of two individuals (E101 and E103) described in detail in the results.

*Outdoor Exposure and Southern Orientation*

After the conclusion of our study, new work suggested that indoor-reared monarchs could re-orient when released outdoors (Wilcox et al. 2020). We were interested in whether increasing outdoor exposure would potentially correlate with southern orientation. Since tethered monarchs were brought outdoors during each testing session and remained outdoors for the full testing period, which lasts several hours, most individuals spent many hours outside over the course of days. Using flight records from each test day, we calculated the minimum time spent outdoors by each of the indoor-reared monarchs. We tested whether there was any correlation with directional orientation south using a non-parametric Kruskal-Wallis H test.

**Results**

*Multiple Directional Orientation Tests in Wild-type and Commercial Monarchs Reared Outdoors*

For the orientation tests comparing wild type and commercial, we reared monarchs outdoors during autumn. We tested eight wild-type and 27 commercial monarchs. Six of the wild types and 16 of the commercial yielded multiple orientation tests (Fig. 3.1B wild type: 24 total flights = 8 first flights + 16 additional flights from the six individuals with multiple tests, commercial: 61 total flights = 27 first flights + 34 additional flights from the 16 individuals with multiple tests). Wild-type monarchs flew with an average heading south ($\boldsymbol{\sigma}$ =143º) and a vector magnitude of r = 0.35 (Fig. 3.1B, Rayleigh test, z-score = 2.88, $0.05 < p < 0.10$). Commercial monarchs' average heading was also south ($\boldsymbol{\sigma}$ =155º), but with a much weaker magnitude, r = 0.11 (Fig. 1B, Rayleigh test, z-score = 0.68, $p > 0.50$).

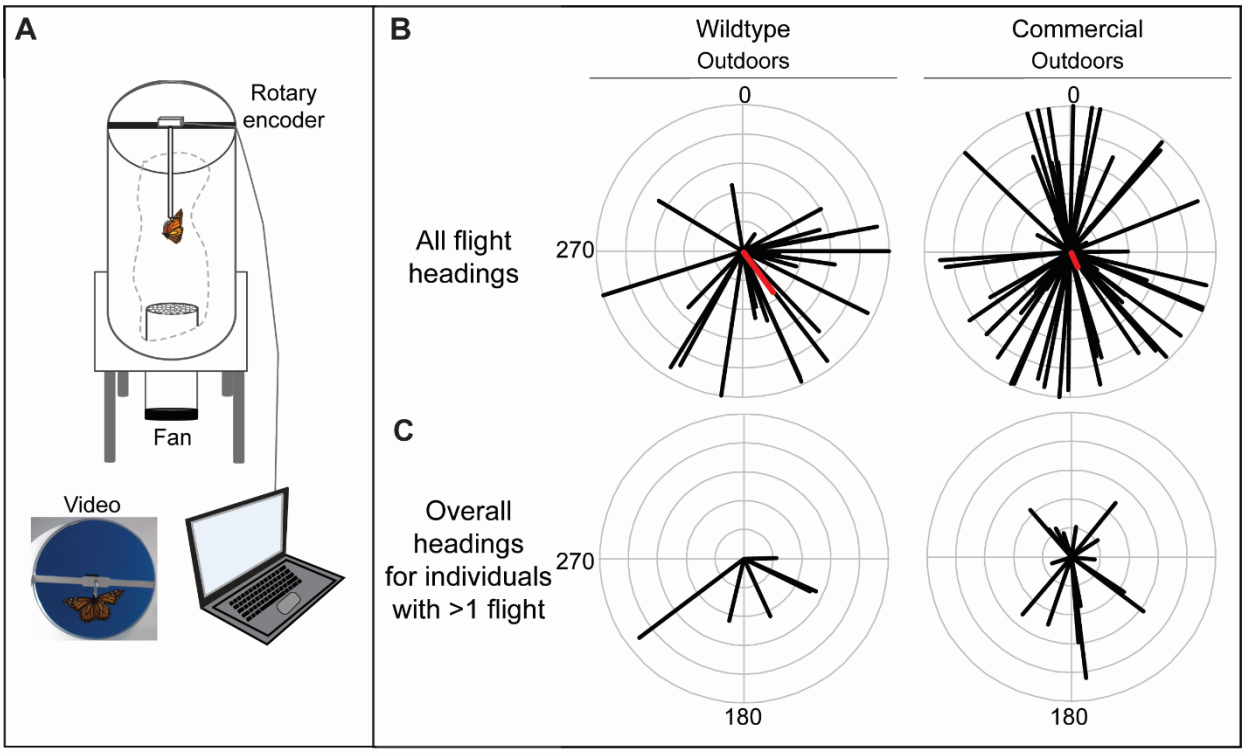


Figure 3.1. A) Flight simulator schematic. B) Orientation plots of wild-type and commercial monarch butterflies reared outdoors in autumn in Chicago, IL. Black lines indicate the vector direction (0-359º) and the length of that line is the vector magnitude, indicating consistency of flight (0 to 1). 0º is North. All flight tests for eight wild-type monarchs with 24 total flights and 27 commercial monarchs with 61 total flights. Group mean direction and magnitude highlighted in red. C) Overall mean directions for six wild-type and 16 commercial monarchs with at least two flight tests.

We then determined overall orientation headings for each of the monarchs with multiple orientation tests (Fig. 3.1C). Five of the six (83.33%) wild types had overall vector magnitudes > 0.4 with overall headings south (90-270º), while the 6$^{th}$ individual's overall direction was 89º with a relatively weak vector magnitude, 0.22 (Fig 3.1C and Table 3.1, wild type). Six of the 16 (37.5%) commercial individuals had overall headings south with vector magnitudes > 0.4 while the remaining 10 individuals' overall headings were north and/or with magnitudes < 0.4 (Fig. 3.1C and Table 3.1, commercial). The difference in degrees between an individual's first flight and the mean of all their flights showed wild-type monarchs chose more similar headings over multiple tests than commercial (t-test, t = 1.64, df = 18.88, p-value = 0.058, Fig. 3.2A).

Additionally, we compared the variance of vector strengths in each individual's multiple tests between the two groups. Commercial monarch vector magnitudes varied significantly more around an individual's mean than wild type (t-test, t = 2.29, df = 19.33, p-value = 0.016, Fig. 3.2B), indicating that commercial monarchs were sometimes very directional during a test and then much less directional for the subsequent test whereas the wild types maintained similar vector strengths over multiple tests.

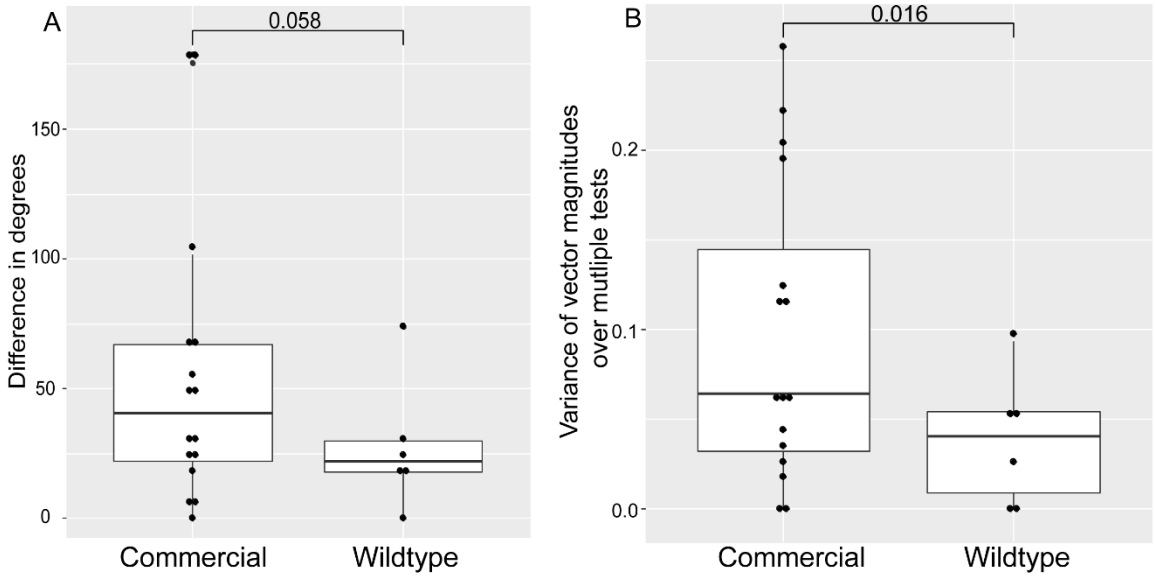


Figure 3.2. Wild-type monarchs are more directional over multiple tests than commercial monarchs. A) The difference (0-180º) between an individual's first vector heading and overall vector heading is nearly significant between commercial and wild-type monarchs. B) Individual wild-type monarchs' vector magnitudes vary less around the individual's overall mean than commercial.

We next determined whether commercial monarchs with multiple orientation tests were possible migrators, by assessing the probability of finding each individual's multiple flight headings within a distribution of known migrators. 37.5% (six of 16) of commercial and 83.33% (five of six) of wild-type monarchs had orientation test patterns consistent with the known migratory distribution of orientations (Table 3.1). Panels A and B of figure 3.3 are examples of

four individuals whose test patterns suggest a strong probability of southern orientation. Panel C of figure 3.3 shows the patterns of two individuals with low probability.

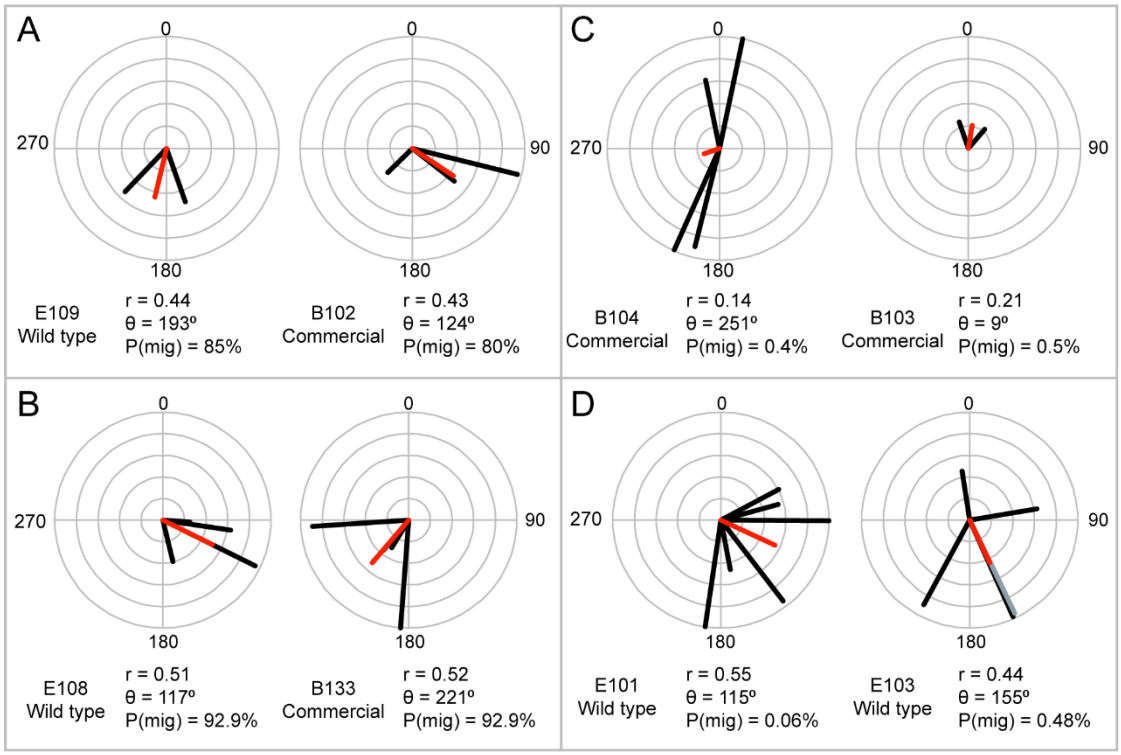


Figure 3.3. Orientation plots of 8 monarchs reared outdoors. The overall mean of orientation tests in red. Line direction indicates the vector heading (θ = 0-359º) and the length of that line is vector magnitude (r = 0 to 1). 0º is North. P(mig) is the probability the individual's pattern of orientation tests is migratory given 180º bins. A) E109 and B102 are wild-type and commercial monarchs respectively with all tests heading south, strong overall vector magnitudes, and strong probabilities of being migratory. B) E108 and B133 are wild-type and commercial monarchs respectively with all tests heading south and strong probabilities of being migratory when binned by 180º, but significantly lower when binned by 90º. C) B104 and B103 are both commercial monarchs with low probabilities of being migratory. D) E101 and E103, wild types reared outdoors, have a lower than expected probabilities of being migratory due to the constraints imposed by strict binning cutoffs (i.e. all flights must be exactly between 90º-269º to count as part of the migratory distribution in 180º bins). For E103, note two flights are overlapping one shown in black and the other in grey.

We noted that though E103 and E101 (wild types reared outdoors) had low probabilities of being part of the migratory distribution in the 180º binning procedure, both have overall southern headings with strong vector magnitudes (Fig. 3.3D, Table 3.1). E103 headed north on two out of five tests, but one of those flights was within 10º of being binned as south (Fig. 3.3D). This is in contrast to the low probability commercial individuals, which all had northern mean headings or

weak southern vector magnitudes (Table 3.1). In total, only 6 of 16 commercial monarchs showed signs of directional orientation south (Table 3.1).

Table 3.1 Data for all individuals with multiple flight tests from wild-type and commercial groups. Identification number (ID), mean vector strength (R), overall mean vector (Direction Flown), the probability that individual's flight pattern is part of the migratory distribution using 180º binning (180º Bin), the probability that individual's flight pattern is part of the migratory distribution using 90º binning (90º Bin), and the total number of successful flight tests per individual (Flights) are reported. Data is organized from lowest to highest probability using 180º bin. Shading indicates the probability of the individual being part of the migratory flight distribution. Dark grey denotes those monarchs with very clear migratory results, light grey those monarchs with unclear results, and white highlights either discrepancies between 90º and 180º binning probabilities (E101, E108, B133) or a low probability of being part of the migratory distribution.

| | ID | R | Direction Flown | 180º Bin | 90º Bin | Flights |
|---|---|---|---|---|---|---|
| Wild type | E101 | 0.55 | 114.76 | 0.06% | 24.82% | 7 |
| | E103 | 0.44 | 155.29 | 0.48% | 1.33% | 5 |
| | E111 | 0.22 | 89.07 | 14.84% | 5.22% | 2 |
| | E104 | 0.91 | 232.83 | 85.16% | 17.31% | 4 |
| | E109 | 0.44 | 193.37 | 85.16% | 41.73% | 2 |
| | E108 | 0.51 | 116.65 | 92.9% | 5.67% | 2 |
| Commercial | B105 | 0.48 | 39.39 | 0.00% | 0.80% | 2 |
| | B104 | 0.14 | 250.89 | 0.40% | 0.50% | 3 |
| | B142 | 0.43 | 318.36 | 0.40% | 0.60% | 2 |
| | B103 | 0.21 | 8.82 | 0.50% | 5.20% | 4 |
| | B117 | 0.25 | 320.60 | 0.50% | 0.20% | 5 |
| | B111 | 0.16 | 96.76 | 6.70% | 12.80% | 3 |
| | B106 | 0.59 | 175.49 | 7.00% | 0.90% | 3 |
| | B109 | 0.21 | 58.22 | 7.00% | 4.60% | 3 |
| | B146 | 0.22 | 331.64 | 7.00% | 4.60% | 4 |
| | B115 | 0.17 | 342.15 | 14.30% | 3.40% | 2 |
| | B102 | 0.44 | 124.15 | 79.90% | 55.40% | 2 |
| | B100 | 0.50 | 198.65 | 85.20% | 41.70% | 3 |
| | B144 | 0.84 | 173.09 | 85.20% | 41.70% | 4 |
| | B110 | 0.34 | 169.28 | 92.60% | 55.40% | 4 |
| | B124 | 0.63 | 127.36 | 92.60% | 12.20% | 2 |
| | B133 | 0.52 | 220.48 | 92.90% | 3.10% | 3 |

*Directional Orientation in Wild-type Monarchs Reared Indoors*

We reared wild-type monarchs with natural light (as filtered through glass windows) during autumn in both a glass-top greenhouse and near a south-facing window in our laboratory and compared them to the outdoor reared group. We tested 15 indoor-reared monarchs, five of which produced multiple orientation tests (Fig. 3.4, indoor wild type: 26 total flights = 15 first flights +

11 additional flights from five individuals with multiple tests). The mean heading for those reared indoors was west, $\boldsymbol{\sigma} = 259^{o}$ with a weak vector magnitude, r = 0.12 (Fig. 3.4A, Rayleigh test, z-score = 0.40, p > 0.50). 11 of the 26 flights (42.3%) (from nine distinct individuals) had northern headings (Fig. 3.4A) compared to six (from three distinct individuals) of 24 (25%) flights in wild type reared outdoors (Fig. 3.1B). The five wild types reared indoors with multiple tests had overall means both south and north with strong and weak vector magnitudes (Fig. 3.4B). Even with the addition of autumnal sunlight through windows, we found outdoor wild-type flight behaviour was not completely recapitulated in the indoor-reared group.

New work has suggested that monarchs reared indoors, but then released are capable of re-orienting outdoors (Wilcox et al. 2020). In light of this work, we used our testing records to calculate the total amount of time that each indoor-reared monarch spent outdoors prior to their test and found no correlation with directional orientation – more outdoor time did not increase the likelihood of southern orientation (Kruskal-Wallis chi-squared = 25, df = 25, p-value = 0.4624, Supplementary Information, Table 3.3).

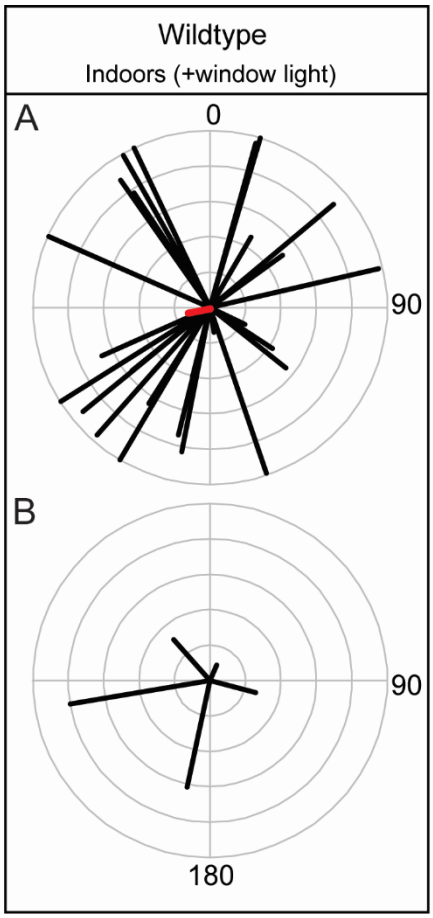


Figure 3.4 Wild-type monarchs raised indoors with window light. A) all flight tests, for 15 individuals reared indoors with a total of 26 flights. Group mean direction and magnitude highlighted in red. B) The overall mean directions for five indoor reared individuals with at least two flight tests.

## Discussion

While a great deal is known about inducing diapause (Green and Kronforst 2019; Goehring and Oberhauser 2002; Barker and Herman 1976) as well as how the monarch utilizes its circadian clock to navigate (Froy 2003; Merlin, Gegear, and Reppert 2009; Mouritsen and Frost 2002), how monarchs develop and maintain directional orientation is less clear. The southern directional orientation phenotype requires a yet unknown combination of environmental conditions and genetics. Our earlier work suggested changes in long-term selection pressures and short-term

developmental conditions can affect whether monarchs orient south in a flight simulator (Tenger-Trolander et al. 2019). Here, we looked more closely at the behaviour of individual commercially sourced monarchs and investigated the effects of indoor rearing conditions with sunlight exposure on directional orientation.

We found that the commercial monarchs are a mix of southern-orienting and non-southern orienting individuals, suggesting that the directional orientation phenotype is not fixed in this population. Migration imposes a strong selective pressure on migratory monarchs as only successful migrators will pass on their genes in the coming spring. In commercial facilities, the difficulties of flying thousands of kilometers, finding the overwintering ground, and surviving till spring are no longer barriers to successful breeding. Add to that small population sizes inherent to commercial breeding and long-term captivity could lead to stochastic increase in the frequency of non-migratory alleles that do not respond to the correct environmental cues or alter the reaction norm of the population making responses to the environment more variable. While this study is limited to a single population of commercial monarchs, the mechanism of loss may be relevant to all long-term captive breeding populations.

While the effect of commercial releases on the North American monarch population is currently unknown, it may be ultimately inconsequential if natural selection purges the wild population of non-migratory individuals. After all, any non-migratory individuals would simply die in winter, their alleles never passed on to the next generation. However, this argument ignores two things, 1) the presence of new resident populations in the southern U.S. that can offer refuge to poor migrators and 2) the likely recessive (Tenger-Trolander et al. 2019) and polygenic nature of migration genetics. In fact, crosses of the commercial and wild-type monarchs resulted in offspring that oriented south in autumn (Tenger-Trolander et al. 2019).

Non-migratory alleles could persist in the genetic background of a migratory individual. Releasing these commercial individuals may result in more monarchs in Mexican overwintering grounds in the short term, but have unintended consequences on their genetics in the long term. Additionally, the introduction of non-migratory alleles into the wild population may actually increase the number of individuals that breed year-round in the southern U.S.(Howard, Aschen, and Davis 2010; Knight and Brower 2009; Satterfield et al. 2018) which has implications for the increased transmission of the monarch parasite OE. Resident populations have higher rates of OE infections (Satterfield, Maerz, and Altizer 2015), and having more resident populations could lead to increased infection in the migratory population as it travels between the overwintering grounds and summer habitat (Brower, 1996). Beneficial, neutral, or detrimental, the release of non-migratory alleles into a wild migratory population is worth discussing critically.

The effect of rearing environment should also be considered. Wild-type monarchs reared indoors with full exposure to natural autumn sun did not consistently orient south, though their genetic background is identical to the wild types reared outdoors. That being said, our results do not fully answer the question of what degree of "naturalness" is required to rear a directional adult. As we have only 5 indoor-reared individuals with multiple tests, we do not know if some proportion of the indoor-reared individuals are directional. However, placing captive-reared monarch larvae/pupae near a window does not result in as many directionally oriented monarchs as full outdoor exposure. Scientists have long speculated about the potential environmental variables that "turn on" the migration developmental program including photoperiod changes, temperature variation, sun declination, and host plant quality (Goehring and Oberhauser 2002; Oberhauser 2019; Perez and Taylor 2004; Merlin, Gegear, and Reppert 2009; Taylor et al. 2019). While we do not know which cue or combination of factors is responsible or the critical

development times, we do know that the following conditions did not result in adults with consistent southern orientation: 1) rearing in an autumn-like environmental chamber 2) rearing in a room with sunlight and autumn-like temperatures during autumn and 3) eclosing in an environmental chamber after almost complete juvenile development outdoors. So far, in our flight simulator experiments, only wild adult monarchs caught in autumn and wild-type monarchs reared outdoors in autumn fly consistently south. And once oriented south, storing monarchs in an environmental chamber does not affect their southern orientation (Guerra and Reppert 2013) unless the temperature is dropped. Exposure to very cool temperatures in an environmental chamber causes re-orientation north(James et al. 2018) in preparation for the spring re-migration.

New work from Wilcox *et al.* suggests that monarchs reared indoors may recover southern orientation after release. In their study, Wilcox *et al.* used a flight simulator to find the headings of a group of indoor-reared monarchs and found they did not orient south, consistent with our flight results of monarchs reared indoors (Wilcox et al. 2020). They also released groups of radio transmitter tagged monarchs reared indoors and found that the individuals flew an average of 37.4 km south (Wilcox et al. 2020). These results imply that regardless of rearing conditions experienced during development, adults given sufficient time outdoors in autumn would eventually fly south, suggesting monarchs are capable of re-orienting.

Currently, we cannot directly compare the flight simulator or radio-tracking data from Wilcox *et al.* to wild-caught or wild-type monarchs reared outdoors, which are known to fly south in autumn, because Wilcox *et al.* did not employ positive or negative controls (Wilcox et al. 2020). While our results and those of Wilcox *et al.* do not give us a completely clear understanding of the development of southern orientation in autumn in monarchs, together they

suggest southern directional flight behaviour could be engaged in adulthood. In light of this possibility, we calculated the amount of time each indoor-reared monarch spent outdoors prior to each test but found no correlation between increased time spent outdoors and propensity to fly south.

In addition to radio-tracking data, mark-recapture studies of indoor-reared monarchs do recover a number of individuals at overwintering sites (Steffy 2015). However, a study that tagged and released groups of both wild-caught and captive-reared eastern monarchs showed the recovery rate of captive-reared monarchs was significantly lower than that of the wild monarchs. 56 of 11,333 wild-caught monarchs were recovered in Mexico whereas only 2 of 3,056 captive-reared monarchs were recovered ($\chi^2 = 10.96$, $p = 0.00093$) (Steffy 2015). The same study also re-captured monarchs as they traveled south. While only 3 reared and 5 wild recoveries are reported in the paper (Steffy 2015), a total of 10 indoor-reared and 6 wild-caught individuals were eventually recovered (Steffy, 2020). The captive-reared traveled an average distance of 120km and the wild-caught an average of 560 km (Mann-Whitney U, p-value = 0.002997). Even in the case that monarchs reared indoors re-orient upon release, captive-reared monarchs were less successful in reaching Mexico than wild monarchs (Steffy 2015).

While many people hope that captive rearing is helping a declining population, the cumulative data available suggest that captive breeding of monarchs has negative consequences for migration behaviour and that monarchs reared indoors are not as well equipped to survive migration as those left in the wild (Davis, Smith, and Ballew 2020; Steffy 2015; Tenger-Trolander et al. 2019). We also know that rearing monarchs at home and in educational settings inspires new generations of conservationists, nature-lovers, and scientists. For those who love rearing monarchs, we advise the following: rear caterpillars individually in clean enclosures, rear

outdoors when possible (especially in late summer and autumn), limit the total number reared, avoid purchasing, and participate in citizen science projects. The non-profit, Monarch Joint Venture (monarchjointventure.org/get-involved/study-monarchs-citizen-science-opportunities), lists links to many on-going studies which have contributed vastly to our understanding of monarch biology. Finally, if we want to ensure the future of migratory monarch populations, we must promote longer-term solutions, like protecting and restoring habitat and addressing climate change.

**Acknowledgments**

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**Supplementary Information**

*Supplemental Tables*

Table 3.2. Number of total flight trials and successful flight tests for all tested monarchs (N=74) by population of origin (wild type or commercial) and treatment (outdoor or indoor-reared). Greyed areas indicate individuals with successful flight tests and white those that never flew. Laboratory reared individual are S123, S124, S122, and S126.

| Wild type Outdoor | | | Wild type Indoor | | | Commercial Outdoor | | |
|---|---|---|---|---|---|---|---|---|
| ID | Trials | Successful flights | ID | Trials | Successful flights | ID | Trials | Successful flights |
| E104 | 12 | 2 | S101 | 8 | 6 | B102 | 9 | 3 |
| E108 | 11 | 4 | S123 | 4 | 3 | B103 | 9 | 2 |
| E110 | 10 | 1 | S113 | 3 | 3 | B100 | 8 | 2 |
| E101 | 9 | 7 | S111 | 3 | 2 | B108 | 8 | 1 |
| E100 | 9 | 0 | S115 | 3 | 1 | B105 | 7 | 5 |
| E107 | 9 | 0 | S118 | 3 | 1 | B142 | 7 | 5 |
| E103 | 8 | 5 | S104 | 3 | 0 | B146 | 7 | 3 |
| E111 | 8 | 2 | S124 | 3 | 0 | B144 | 7 | 2 |
| E106 | 8 | 1 | S117 | 2 | 2 | B133 | 6 | 4 |
| E105 | 8 | 0 | S016 | 2 | 1 | B139 | 6 | 0 |
| E113 | 8 | 0 | S110 | 2 | 1 | B143 | 6 | 0 |
| E109 | 7 | 2 | S112 | 2 | 1 | B111 | 5 | 3 |
| E102 | 6 | 0 | S119 | 2 | 1 | B124 | 5 | 3 |
| E112 | 4 | 0 | S122 | 2 | 1 | B134 | 5 | 1 |
| E114 | 2 | 0 | S107 | 2 | 0 | B138 | 5 | 1 |
| | | | S108 | 2 | 0 | B140 | 5 | 1 |
| | | | S109 | 2 | 0 | B141 | 5 | 1 |
| | | | S105 | 1 | 1 | B145 | 5 | 0 |
| | | | S114 | 1 | 1 | B104 | 4 | 4 |
| | | | S126 | 1 | 1 | B110 | 4 | 4 |
| | | | S103 | 1 | 0 | B106 | 4 | 3 |
| | | | S116 | 1 | 0 | B115 | 4 | 2 |
| | | | | | | B117 | 4 | 2 |
| | | | | | | B128 | 4 | 1 |
| | | | | | | B132 | 4 | 1 |
| | | | | | | B136 | 4 | 1 |
| | | | | | | B109 | 3 | 3 |
| | | | | | | B101 | 3 | 0 |
| | | | | | | B130 | 3 | 0 |
| | | | | | | B147 | 3 | 0 |
| | | | | | | B126 | 2 | 1 |
| | | | | | | B129 | 2 | 1 |
| | | | | | | B127 | 2 | 0 |
| | | | | | | B122 | 1 | 1 |
| | | | | | | B125 | 1 | 0 |
| | | | | | | B131 | 1 | 0 |
| | | | | | | B137 | 1 | 0 |

Table 3.3. Flight test data for all individuals from wild-type group reared indoors. Individual (ID), mean vector magnitude (R), mean vector (Direction Flown), minimum time in hours spent outdoors post tethering and rest period (Time outdoors), age in days, and the date of the flight test (Flight date). Data is organized by increasing amount of time spent outdoors. Data are color coded by their direction flown, S stands for south and N for north.

| ID | R | Direction Flown | | Time outdoors (hrs) | Age (days) | Flight Date |
|---|---|---|---|---|---|---|
| S111 | 0.83 | 191.11 | S | 1.03 | 11 | 10/27/2019 |
| S115 | 0.96 | 15.61 | N | 1.08 | 10 | 10/27/2019 |
| S113 | 0.99 | 161.2 | S | 1.37 | 10 | 10/27/2019 |
| S114 | 1 | 210.5 | S | 1.38 | 10 | 10/27/2019 |
| S110 | 0.98 | 77.06 | N | 1.67 | 11 | 10/27/2019 |
| S117 | 0.74 | 193.86 | S | 1.82 | 10 | 10/27/2019 |
| S105 | 0.64 | 212.53 | S | 2.07 | 12 | 10/27/2019 |
| S123 | 0.67 | 245.92 | S | 2.37 | 12 | 10/27/2019 |
| S101 | 0.42 | 123.5 | S | 3.95 | 13 | 10/7/2019 |
| S123 | 0.99 | 237.85 | S | 5.28 | 13 | 10/28/2019 |
| S111 | 0.4 | 194.32 | S | 6.82 | 19 | 11/4/2019 |
| S113 | 0.88 | 325.12 | N | 6.98 | 18 | 11/4/2019 |
| S117 | 0.91 | 50.12 | N | 7.1 | 18 | 11/4/2019 |
| S118 | 0.22 | 115.32 | S | 7.3 | 17 | 11/4/2019 |
| S123 | 0.99 | 293.7 | N | 7.33 | 20 | 11/4/2019 |
| S112 | 0.93 | 230.58 | S | 7.53 | 18 | 11/4/2019 |
| S106 | 0.5 | 54.2 | N | 7.68 | 20 | 11/4/2019 |
| S126 | 0.46 | 30.18 | N | 7.87 | 7 | 11/4/2019 |
| S119 | 0.96 | 221.65 | S | 7.95 | 17 | 11/4/2019 |
| S122 | 0.99 | 330.5 | N | 8.7 | 20 | 11/4/2019 |
| S113 | 1 | 334.61 | N | 10 | 20 | 11/6/2019 |
| S101 | 1 | 16.47 | N | 11.53 | 15 | 10/9/2019 |
| S101 | 0.77 | 326.5 | N | 14.7 | 20 | 10/14/2019 |
| S101 | 0.14 | 171.01 | S | 21.52 | 33 | 10/27/2019 |
| S101 | 0.55 | 128.5 | S | 22.98 | 34 | 10/28/2019 |
| S101 | 0.57 | 231.01 | S | 26.13 | 41 | 11/4/2019 |

# CHAPTER FOUR: SEASONAL PLASTICITY IN MIGRATORY NORTH AMERICAN AND RESIDENT COSTA RICAN MONARCH BUTTERFLY MORPHOLOGIES

**Abstract**

The migratory generation of the North American (N.A.) monarch butterflies lead distinctly different lives from their summer generation parents as well as their tropical descendants living in Costa Rica (C.R). Migratory N.A. monarchs postpone reproduction, travel thousands of kilometers south to hibernate in Mexico, and subsist on little food for months, before returning north to reproduce. To be successful in North America, monarchs have seasonally plastic development which changes their behavior and physiology; however, we do not know to what whether there is seasonal plasticity in morphology. And though C.R. monarchs are descended from the N.A. population, they are no longer subject to selection imposed by migration, and whether they retain ancestral seasonal plasticity is unclear. To investigate the effect of environment and genetic background on plasticity in morphology in monarchs, we reared both N.A. migratory and C.R. resident monarchs outdoors in summer and autumn in Chicago, Illinois, USA. We found seasonal plasticity in morphology of N.A. monarchs; they increased the area of and decreased the circularity of the forewing and increased thorax investment in females in response to autumn. C.R. monarchs had mixed responses to their ancestral environment. While they did not enlarge the forewing, they did decrease circularity and females increased thorax investment in autumn. Landmark based geometric morphometric analysis of the forewing in both populations revealed that shape, as predicted by wing vein morphology, is not different between populations and not responsible for the seasonal plasticity seen in shape (circularity) in autumn in either population.

**Keywords**

Seasonal plasticity, migration phenotypes, wing morphology, *Danaus plexippus*, monarch butterfly, Costa Rican monarch

## Introduction

Unlike many North American (N.A.) insects, monarch butterflies (*Danaus plexippus*) do not overwinter in their summer habitat (Larsen and Lee 1994; Lee 1989). While several generations reproduce through summer (Flockhart et al. 2013), the final generation of adults flies thousands of kilometers south to Mexico to overwinter before returning north in the spring. N.A. monarchs deal with these different environmental pressures through plasticity in their developmental responses. Monarch larvae developing in the transition between summer and autumn respond to the changing environment by entering reproductive diapause, altering their directional orientation from random to southern, and increasing fat storage (Brower, Fink, and Walford 2006; Goehring and Oberhauser 2002; Herman and Tatar 2001; Tenger-Trolander et al. 2019; Zhu, Casselman, and Reppert 2008; Zhu et al. 2009). These physiological and behavioral changes extend monarch adult lives and are required for the long journey south toward their overwintering grounds. Here we investigate the extent to which monarch wing and body morphology are seasonally plastic as well. Though previous studies have found summer and migratory generations to have morphological differences, they measured only wild-caught individuals, making it difficult to determine whether those differences are due to plasticity in development or differential mortality during migration (Freedman and Dingle 2018; Flockhart et al. 2017; Li, Pierce, and de Roode 2016). To address whether monarch morphology is seasonally plastic, we reared N.A. monarchs in a common garden outdoors in both summer and autumn in Chicago, Illinois.

Morphological differences also appear when comparing N.A. and tropical or sub-tropical resident populations (Altizer and Davis 2010; Dockx 2007). These resident populations are all descended from the migratory N.A. population (Zhan et al. 2014). They are genetically divergent and no longer migratory, with the possible exception of an Australian population (Hilburn 1989; Knight and Brower 2009; Neves et al. 2001; Zalucki et al. 1993). Differences in their morphology are attributed to their non-migratory status; resident populations are no longer subject to annual selection to maintain migration traits (Altizer and Davis 2010), resulting in selection favoring investment in other traits. However, many resident populations also lack migration relevant environmental cues (declining day lengths, cool night temperatures, senescing milkweed) in their new equatorial homes. Without those cues, migration-associated traits could be retained and simply lying dormant. In fact, recent work on a Pacific lineage of monarchs showed that the resident population entered reproductive diapause when exposed to ancestral autumn-like conditions (Freedman et al., 2018) suggesting some resident populations do retain ancestrally plastic traits. Given the staggering decline in the number of western migratory monarchs as a result of habitat loss in recent wildfires as well as clear declines in Eastern migratory population (Agrawal and Inamine 2018; Lincoln P. Brower et al. 2012; Inamine et al. 2016; Pelton et al. 2019; Schultz et al. 2017; Vidal and Rendón-Salinas 2014), there is interest in whether resident populations have the adaptive potential to recolonize North American in the event of extinction of migratory populations (Nail, Drizd, and Voorhies 2019). To investigate, we reared resident C.R. monarchs, in the common garden alongside the N.A. monarchs, outdoors in summer and autumn. We compared body mass, forewing size, and forewing morphologies between seasons and populations.

## Methods

*Animal husbandry*

We captured 31 adult N.A. monarch butterflies in Chicago, IL and ordered 20 C.R. monarch pupae from a butterfly breeder in Costa Rica. We housed the monarchs from their respective populations in medium size (91.5cm x 30.5cm$^2$) mesh pop-up cages outdoors with access to host plant, *Asclepias syriaca*. After females laid eggs, we washed and transferred the eggs to small (30.5cm$^3$) outdoor mesh pop-up cages and fed them exclusively on a diet of wild-collected *Asclepias syriaca* cuttings. All pop-up cages were contained inside two large outdoor 1.83cm$^3$ mesh cages separated by population of origin. Adults from both populations emerged between July 1$^{st}$ and July 12$^{th}$.

We used a small number of these individuals to produce the autumn generation which emerged between Aug 31$^{st}$ and Sept 26$^{th}$. We did not measure forewing traits of parents or mass of these individuals as their wings were damaged over time by the constant contact with the cages, and all other individuals used in the study were virgin males and females.

*Body mass measurements*

We dissected off the wings, antennae, head, and legs from the body. We separated the thorax and abdomen and dried them at 60ºC in an incubator with a silica crystal desiccant for 72 hours. After drying, we weighed the thorax and abdomen separately and together on an analytical balance (N = 185, Supplementary Information, Table 4.2).

*Wing size and shape measurements*

We removed both forewings from the thorax of each individual (N = 247, Supplementary Information, Table 4.2), placed them on a sheet of gridded paper with 0.635 cm squares, and photographed them individually using a DSLR Canon EOS 70d camera with an 18-55mm lens.

We scaled photos relative to the 0.635 cm squares in each photo and then measured the area (in $cm^2$), aspect ratio (length/width), and circularity ($4 \pi$ *area/perimeter$^2$) of each forewing in ImageJ (Rueden et al. 2017). To measure aspect ratio, ImageJ finds the longest length (major axis) and width (minor axis) of the object while maintaining the perpendicular intersection of both lines and divides the major axis length by the minor. Higher circularity scores indicate a more circular wing shape whereas lower scores more polygonal or angular shapes. Circularity should not be confused with the measure of roundness ($4*area/(\pi*major\_axis^2)$) though these terms have been used interchangeably. For example, a hexagon has high circularity and low roundness where as an oval has low circularity and high roundness.

We also used 2D landmark-based geometric morphometrics to assess shape differences. Using the software tpsDIG2ws, we placed 16 landmarks at homologous points (vein intersections and margins) on each forewing (Rohlf 2006, Supplementary Information, Fig. 4.5). We analyzed the resulting landmark data in R using the package 'Geomorph' (Adams and Collyer 2020). We performed a general Procrustes analysis that removed differences in orientation and size, allowing us to focus exclusively on shape differences (Supplementary Information, Fig. 4.6). We then calculated mean shape which is the average landmark coordinates for a set of aligned wings. For each of the 16 landmarks, we calculated the distance between the individual's coordinates and the group mean coordinates and summed those distances to find each specimen's total distance from the mean shape.

*Statistical analyses*

Thorax mass (grams), forewing area ($cm^2$), and aspect ratio were normally distributed (Supplementary Information, Fig. 4.7). We used the R package 'glmulti' to automatically select the best fit general linear model (glm) for each and determine which of the three independent

variables (sex, population, and season) were significant predictors of the measurements (Calcagno and de Mazancourt, Claire 2010) (Supplementary Information, Table 4.3). To quantify the association between each independent variable and dependent variable, we calculated the odds ratio (OR) with the package 'oddsratio' (Schratz 2017).

Abdomen mass, circularity scores, and mean shape distances were not normally distributed, and various transformations of the measurement did not yield normal distributions (Supplementary Information, Fig. 4.7). For these three dependent variables we did not fit a glm. Instead, to assess significance, we employed the Kruskal-Wallis H test, a rank-based nonparametric test. We then used Dunn's test that employs a Bonferroni correction to account for multiple testing.

**Results**

*Abdomen and thorax mass combined*

The combined mass of the thorax and abdomen did not differ by population of origin, season of development, or sex ($\mu = 0.0845$ grams, Kruskal-Wallis chi-squared = 11.4, df = 7, p-value = 0.12).

*Abdomen mass*

Considering the abdomen alone, season of development and sex both affect mass (Kruskal-Wallis chi-squared = 16.8, df = 7, p-value = 0.019). Males reared in summer had significantly lighter abdomens than females reared in summer ($\mu = 0.0352$g vs $\mu = 0.043$g, Dunn test with Bonferroni correction, df = 7, p = 0.0035) and males increased abdomen mass in response to fall ($\mu = .0454$g vs $\mu = 0.0352$g, Dunn test with Bonferroni correction, df = 7, p = 0.0043).

*Thorax mass*

Thorax mass differed across seasons, population, and sex with little evidence for an interaction (Fig. 4.1 and Supplementary Information, Table 4.3). However, each independent variable explained only a small amount of the variation in thorax mass as indicated by the low r-squared for the model ($r^2 = 0.19$) and small odds ratios. We found that the odds a N.A. thorax was heavier than a C.R. monarch's thorax is 1.005 (95% confidence interval (CI): 1.002–1.007), that a male thorax was heavier than a female's is 1.005 (95% CI: 1.002-1.008), and that an autumn thorax was heavier than a summer generation thorax is 1.005 (95% CI: 1.003-1.008). Summer-reared C.R. females had the lightest thoraxes and autumn-reared N.A. males the heaviest, with all other groups falling in between (Fig. 4.1).

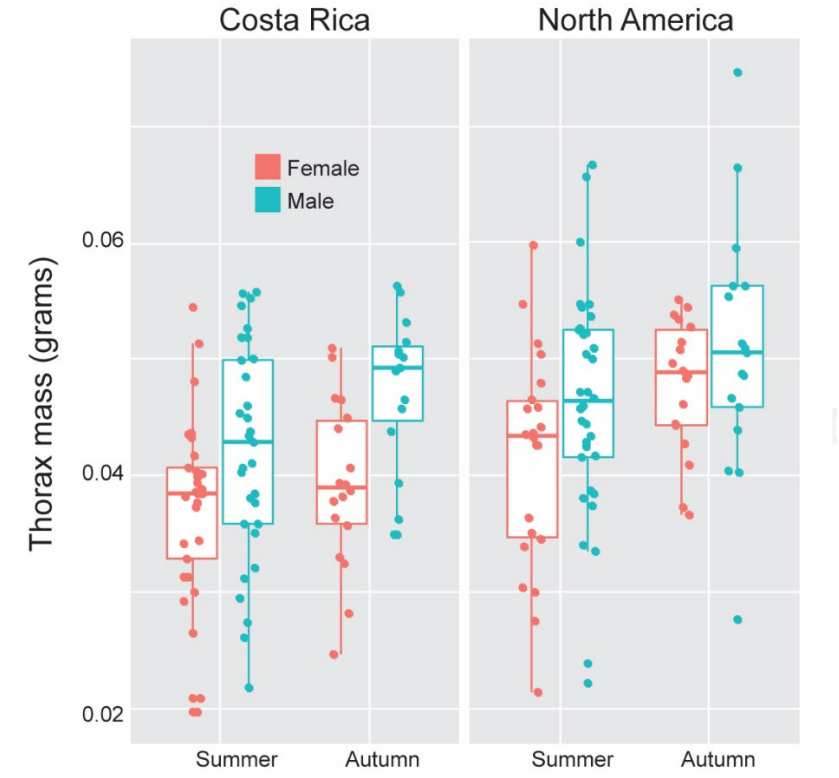


Figure 4.1. Boxplot of thorax mass of Costa Rican and North American monarchs separated by season of development and sex.

*Thorax to body mass ratio*

While there was no interaction effect for thorax mass, the ratio of thorax mass to total body mass is explained by population, sex, season, and interactions between population and sex as well as sex and season. These variables together explained only a small amount of the variation in thorax:body ratio as indicated by the low r-squared for the model ($r^2 = 0.27$) and odds ratios. We found that the odds a N.A. monarch invested more mass in the thorax than a C.R. monarch is 1.055 (95% confidence interval (CI): 1.029–1.082), that a male invested more than a female is 1.023 (95% CI: 0.989-1.057). Interestingly, the odds a N.A. female invests more in the thorax is 1.047 (95% CI: 1.012-1.084) and the odds an autumn generation female invests more in the thorax is 1.077 (95% CI: 1.039-1.116). In summer, males invest more of their total body mass in the thorax than females (Kruskal-Wallis chi-squared = 52.97, df = 7, P = 0.0, Dunn test with Bonferroni correction, P = 0 and P = 0.0047 for C.R and N.A groups respectively), but in autumn, females and males are no longer significantly different in thorax to body mass ratio (Dunn test with Bonferroni correction, P = 1 and P = 0.9970 for C.R and N.A groups respectively). Though Costa Rican females do increase investment in thorax tissue in autumn, they still fall significantly behind N.A. autumn generation females (Dunn test with Bonferroni correction, P = 0.0125).

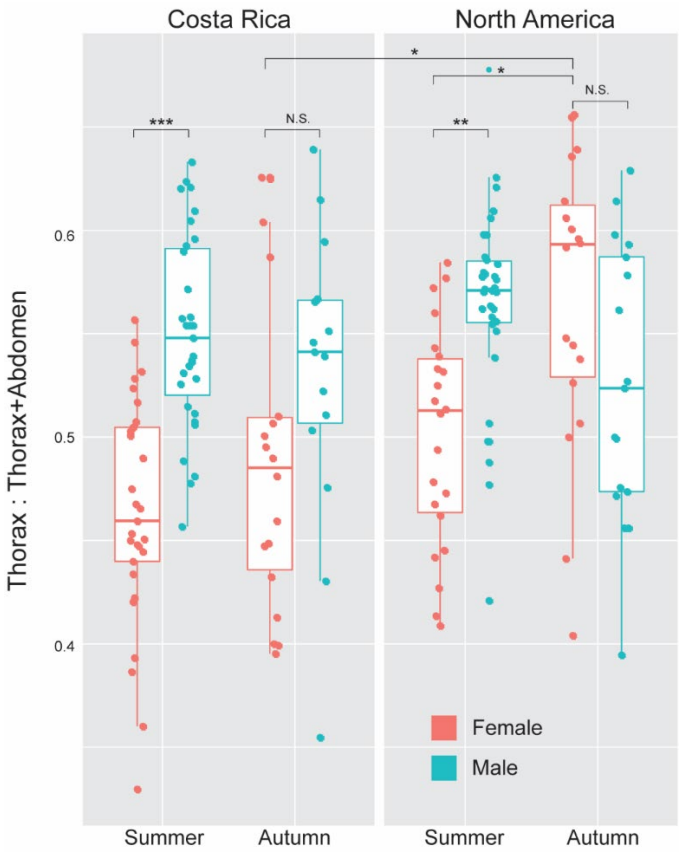


Figure 4.2. Boxplot of thorax:body mass ratio of Costa Rican and North American monarchs separated by season of development and sex.

Table 4.1. Best fit general linear models for the dependent variables, thorax mass, thorax/body mass ratio, and forewing area.

| *Thorax mass ~ Population + Sex + Season* | | | | |
|---|---|---|---|---|
| Independent Variable | Estimate | Std. Error | t value | P value |
| Population | 0.004810 | 0.001277 | 3.767 | 0.000223 |
| Season | -0.004546 | 0.001325 | -3.432 | 0.000742 |
| Sex | 0.004999 | 0.001283 | 3.897 | 0.000137 |
| *Thorax mass/Body Mass ~ Population + Sex + Season + Population:Sex + Season:Sex* | | | | |
| Independent Variable | Estimate | Std. Error | t value | P value |
| Population | 0.05343 | 0.01277 | 4.185 | 4.47e-05 |
| Season | -0.04460 | 0.01292 | -3.452 | 0.000694 |
| Sex | 0.02244 | 0.01699 | 1.321 | 0.188297 |
| Population:Sex | -0.04591 | 0.01755 | -2.616 | 0.009658 |
| Sex:Season | 0.07416 | 0.01818 | 4.080 | 6.79e-05 |
| *Forewing Area ~ Population + Season + Population:Season* | | | | |
| Independent Variable | Estimate | Std. Error | t value | P value |
| Population | 0.99408 | 0.11582 | 8.583 | 1.11e-15 |
| Season | -0.02497 | 0.12305 | -0.203 | 0.839370 |
| Population:Season | -0.58212 | 0.16380 | -3.554 | 0.000456 |

*Forewing area*

Variation in wing area was best explained with the glm forewing area ~ population + season + population:season with an $R^2 = 0.28$ (Supplementary Information, Table 4.3). In this case, only population and the interaction of population and season were significant (Table 4.1 and Supplementary Information, Fig. 4.8). We found that the odds a N.A. forewing was larger than a C.R. monarch's forewing is 1.51 (95% confidence interval (CI): 1.203–1.895), that an autumn N.A. monarch's forewing was larger than the summer generation was 1.79 (95% CI: 1.298-2.467).

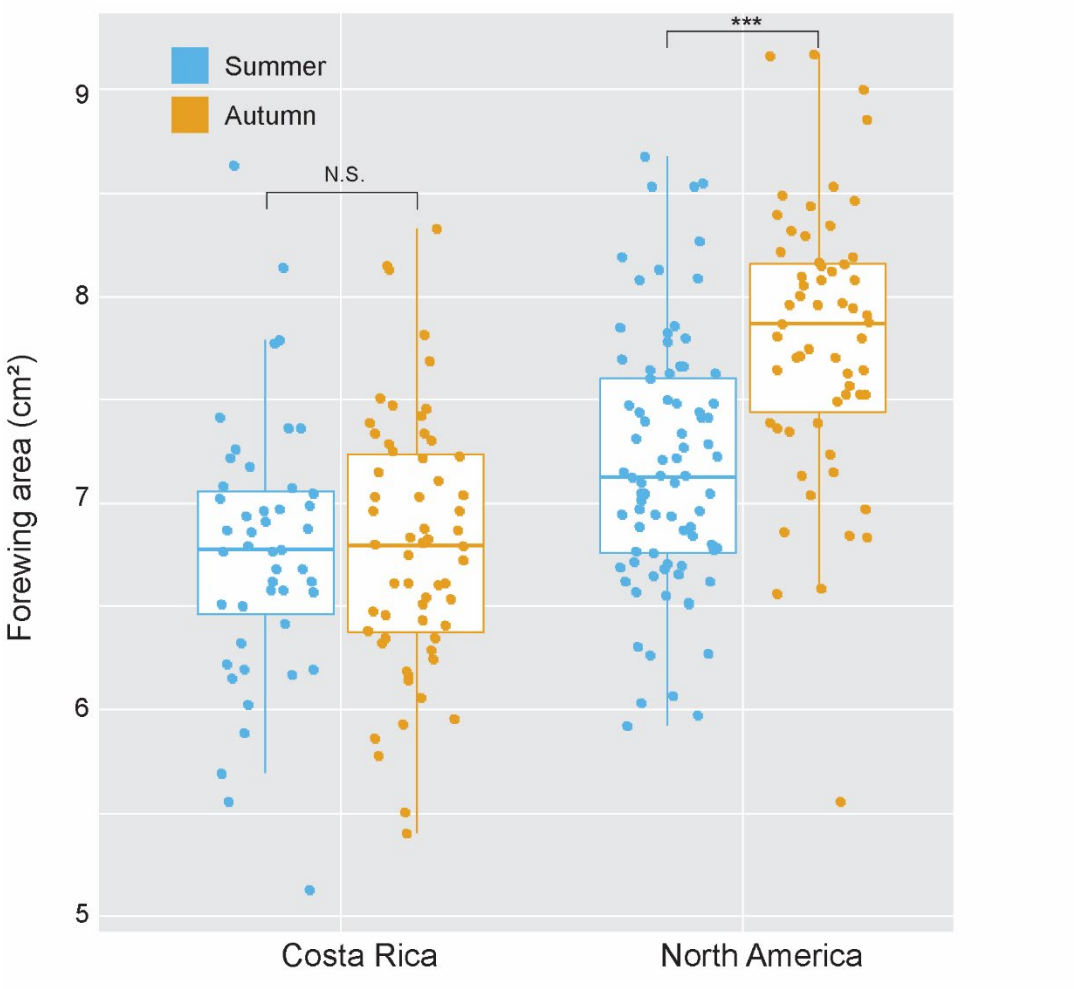


Figure 4.3. Boxplot of forewing are separated by population or origin and season of development. N.S. indicates that the difference between groups is not significant and three asterisks correspond to the p value < 0.001.

C.R. monarchs were not seasonally plastic in forewing area and had smaller wings than N.A. monarchs reared in either summer or autumn. N.A. monarchs' forewings measured 7.82 $cm^2$ on average when reared in autumn versus 7.21 $cm^2$ when reared in summer (Fig. 4.2, Kruskal-Wallis chi-squared = 71.6611, df = 3, p-value = 0, Dunn test with Bonferroni correction, p-value = 0.0). Meanwhile, C.R. monarchs reared in autumn and summer maintained nearly identical forewing areas with averages of 6.77$cm^2$ in summer and 6.76$cm^2$ in autumn (Fig. 4.2,

Kruskal-Wallis chi-squared = 71.6611, df = 3, p-value = 0, Dunn test with Bonferroni correction, p-value = 1.0).

*Forewing shape (aspect ratio, circularity, and geometric morphometrics)*

Variation in forewing aspect ratio was not significantly explained by sex, season, population or their interactions (Supplementary Information, Table 4.7D). These results are consistent with previous work which found no significant difference between N.A. and C.R. monarch forewing aspect ratios (Altizer and Davis 2010).

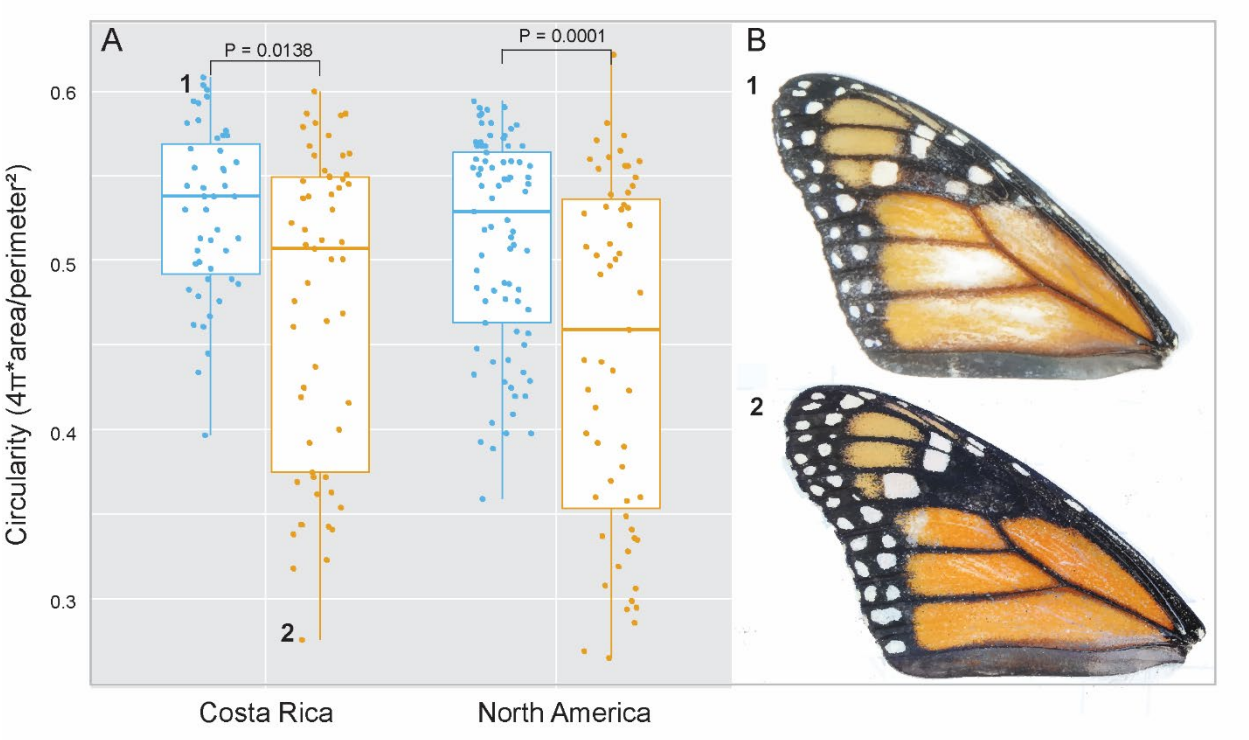


Figure 4.4. A) Boxplot of forewing circularity scores separated by population of origin and season of development. P values are indicated for those comparisons that are significantly different. B) Examples of differently shaped Costa Rican forewings, (1) high circularity score and (2) low circularity score. The numbers 1 and 2 label the location of these two individual wings on the boxplots.

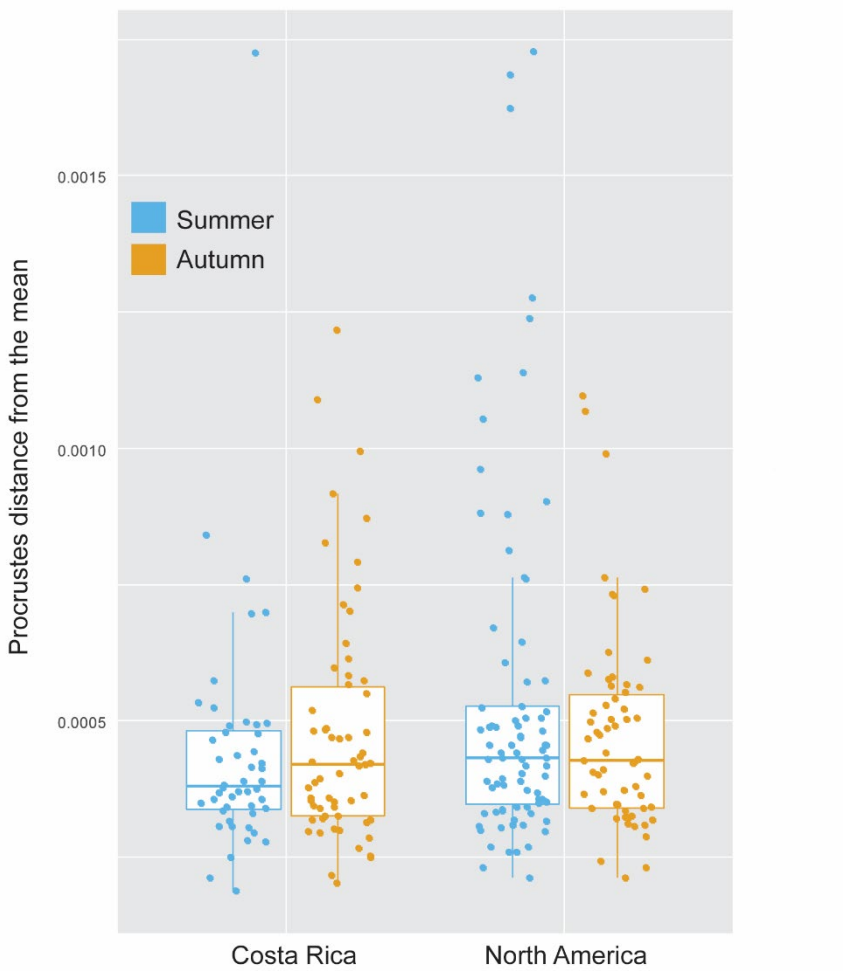


Figure 4.5. A boxplot of Procrustes distances from the mean consensus forewing shape. Each dot represents the summed distance of 16 coordinates (landmarks) from their respective mean shape coordinate. There are no significant differences between groups, Kruskal-Wallis chi-squared = 3.579, df = 3, p-value = 0.31.

In addition to aspect ratio scores, we measured circularity of the forewing ($4\pi$*area/perimeter$^2$). A value of 1 is a perfect circle; decreasing scores indicate more polygonal (angular) forewings. Both N.A. and C.R. monarchs decrease the circularity of the forewing in response to autumn conditions (Fig. 4.3, Kruskal-Wallis chi-squared = 24.2523, df = 3, p-value = 0, Dunn test with Bonferroni correction, p = 0.003 and p = 0.0138).

We then used geometric morphometrics to determine the mean shape of C.R. and N.A. forewings using 16 homologous landmarks (veins intersections and margins). Surprisingly, mean

shape did not differ between the two populations (Supplementary Information, Fig. 4.9) and did not exhibit seasonal plasticity in either group (Supplementary Information, Fig. 4.10). Comparing autumn reared C.R. and N.A. populations, where we might expect to see the largest difference given their differing ecologies, mean shape is the same (Supplementary Information, Fig. 4.9). Since the mean shapes were so similar, we wondered whether any group might show more variation around the mean shape than another. To investigate, we measured the distance of each individual specimen's landmark to the respective consensus mean landmark and summed those distances. The total distance from the mean shape did not vary by population or season (Fig. 4.4). Wing shape, as understood from vein placement, has not changed appreciably in C.R. monarchs suggesting the elongation of the wing in autumn occurs by changing the amounts of wing tissue within existing vein boundaries.

**Discussion**

Consistent with the differing demands on summer and autumn (migratory) generations, N.A. monarchs are seasonally plastic in both body mass and wing morphology. N.A. migratory generation monarchs have heavier thoraxes and larger, more angular wings, that are, at least in part, due to differences between the summer and autumn generation development. The comparison of summer and autumn reared groups is important in controlling for potential differences observed between summer and autumn wild-caught adults due to the possibility of differential mortality during migration. C.R. monarchs, perhaps unsurprisingly, have mixed responses to autumn in North America. They retain some phenotypic plasticity; both male and females' forewings become as angular as N.A. monarchs in autumn and females increase investment in the thorax tissue much like N.A. females; however, neither males nor females increase forewing size.

As the location of flight muscles and the point of attachment for the wings, thorax mass is suspected to play a critical role in migration performance. While population is a significant predictor of thorax mass, there is no interaction. However, when comparing the ratio of thorax mass to total body mass, we found an interaction between sex and population and sex and season. Females invest more of their total mass in thorax tissue in autumn versus summer in both populations indicating that some amount of seasonal plasticity is maintained in Costa Rican population. One other paper that measured thorax mass in reared N.A. monarchs found significant differences between thorax mass in males and females, but did not explicitly compare different rearing conditions (Davis and Holden 2015). And though we did not find significant sexual dimorphism in thorax mass of N.A. monarchs in either season, our sample sizes for these particular comparisons were smaller (Supplementary Information, Table 4.2) and trends in our data also suggest there is some degree of sexual dimorphism especially when reared in summer conditions. Additionally, a study that reared N.A. monarchs indoors and compared them to wild autumn migrators caught in Georgia found increased grip strength in the wild group (Davis, Smith, and Ballew 2020). As monarchs caught in Georgia have potentially come from further north, the difference suggests increased strength is critical to improved survival during migration in monarchs. C.R. monarchs appeared to retain some ability to respond to autumn conditions by altering investment in thorax tissue, though not to the same degree as the N.A. population.

Forewing size is the most divergent trait when comparing N.A. and C.R. monarchs. And while population and season are both predictors of forewing size in monarchs, there is also an interaction between season and population in that only N.A. monarchs increase forewing size in autumn. Consistent with other work, we found forewing size is smaller in Costa Rican monarchs (Altizer and Davis 2010; Beall and Williams 1945; Dockx 2007; Li, Pierce, and de Roode 2016).

The smaller forewing size of C.R. and other resident monarch populations plus the C.R. monarchs' lack of plasticity suggests that adaptation to the local environment has either directionally selected for smaller wing size because the larger migratory monarch forewing is costly to maintain and unnecessary. In N.A. monarchs, wing sizes are smaller in summer than autumn, consistent with a study of museum specimens collected in North America between 1878-2017 in which the authors found that autumn individuals had larger wings than summer (Freedman and Dingle 2018).

For forewing shape, the previously available data are variable in conclusions. Some studies find differences in shape between resident and migratory monarchs (S. Altizer and Davis 2010; Dockx 2007) while others do not (Li, Pierce, and de Roode 2016). However, part of the confusion stems from the term 'elongation' which can be used to describe many different aspects of forewing shape including, wing length, aspect ratio (measured as major axis/minor axis or length from attachment to the thorax/perpendicular width), and circularity ($4\pi$ area/perimeter$^2$).

In forewing shape, we found no differences in shape using geometric morphometric analyses which rely on the placement of homologous landmarks on vein intersections and margins or in aspect ratio. Previous work comparing N.A. wild-caught individuals from early and late in the migration found that the earlier migrators had higher aspect ratios (Satterfield and Davis 2014). The lack of a difference in aspect ratio between the summer and autumn reared N.A. monarchs in our experiment suggests the differences observed in the wild may arise due to differential mortality.

However, when we measured circularity ($4\pi$ area/perimeter$^2$) of the forewings, we found that both N.A. and C.R. monarchs increase the angularity of their forewings in response to autumn. Our results suggest that little has changed in wing shape between C.R. and N.A.

monarchs in terms of vein placement, but angularity of the wings does change in response to autumn conditions. These results together with previous measurements (Altizer and Davis 2010) suggest that the differences seen in circularity when comparing wild caught C. R. monarchs to N.A. monarchs are largely driven by differences in rearing environment rather than genetics – as C.R. monarchs clearly maintain the plasticity associated with forewing circularity.

Our results provide evidence for the seasonal plasticity of migration associated morphological traits in N.A. migratory monarch butterflies and show that wing size in the Costa Rican resident population is smaller due to genetic rather than environmental differences. Larger wing size is clearly costly to maintain and under constant directional selection in the migratory population.

**Acknowledgments**

Thank you to Dr. Andre Green, Michelle Noyes, and Elena Sparrow for collaboratively rearing the monarch specimens used in this study. Thank you especially to Dr. Green and Dr. Micah Freedman for our many conversations on monarch butterflies and their morphology. This work was funded by the Graduate Research Fellowship Program, National Institute of Health Genetics and Regulation Training Grant T32 GM07197, US Fish and Wildlife Service Award F17AC01222, National Science Foundation Grant IOS-1452648, and National Institute of Health Grant GM108626.

## Supplementary Information

*Supplemental Figures and Tables*

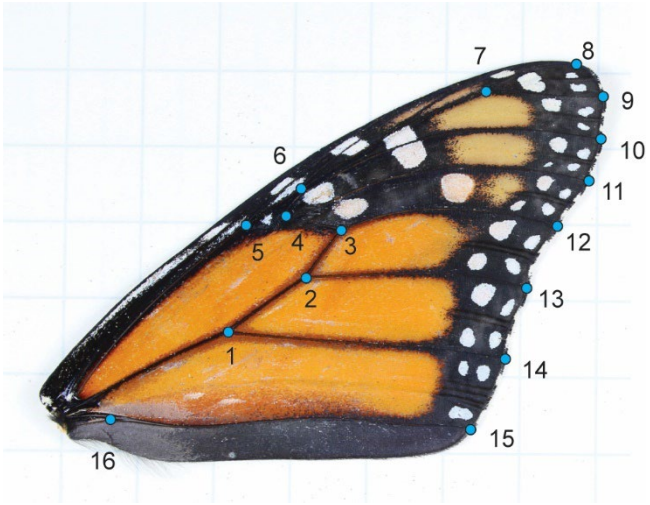


Figure 4.6. Blue dots indicate the positions of 16 landmarks on monarch butterfly forewing.

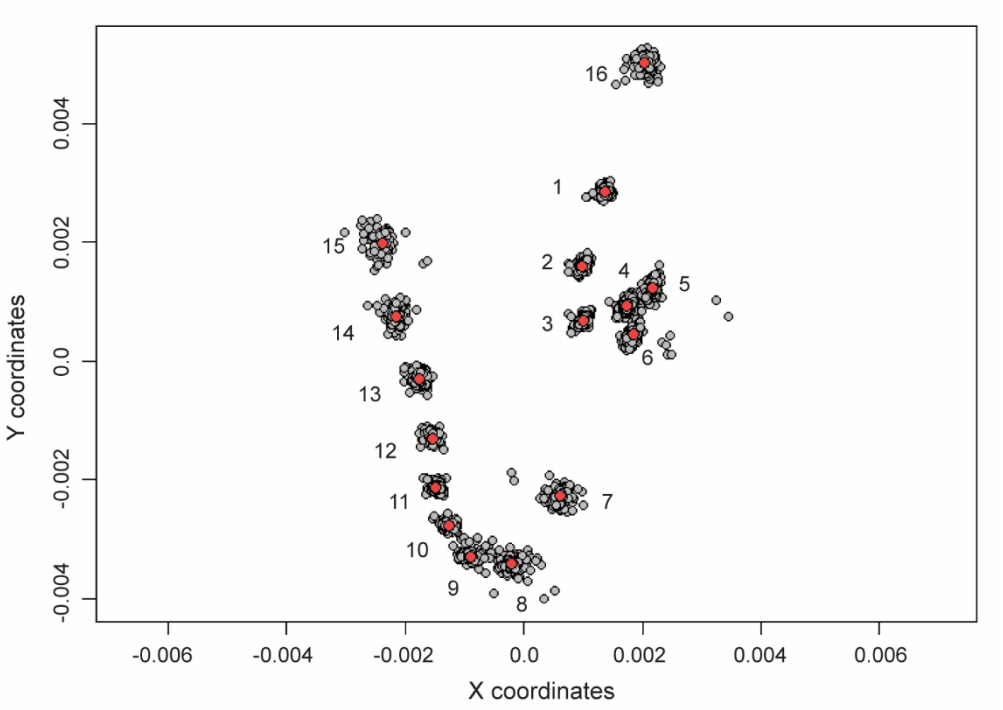


Figure 4.7. Plot of all specimens' landmarks after general Procrustes alignment in grey. Red dots are the consensus mean coordinates for each landmark. Landmarks numbered 1 through 16 correspond to vein intersections and vein margins in figure 4.6.

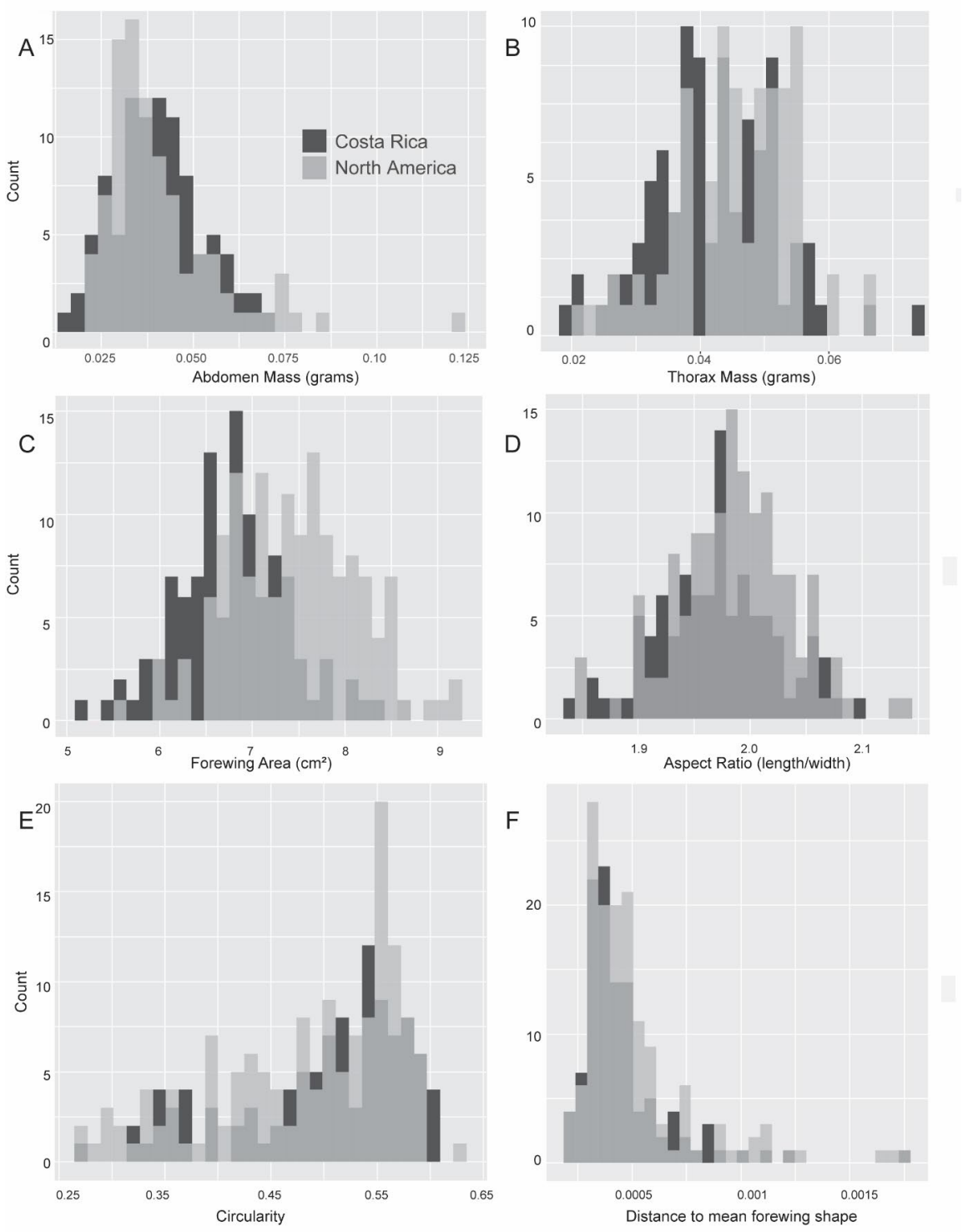


Figure 4.8. Histograms of response variables with North American measurements in light grey and Costa Rican in dark grey. A) Distribution of abdomen masses measured in grams skews right. B) Distribution of thorax masses measured in grams is normal. C) Distribution of forewing area in cm² is normal. D) Distribution of forewing aspect ratios is normal. E) Distribution of forewing circularity scores skews left. F) Distribution of Procrustes distances from consensus mean shape skews right.

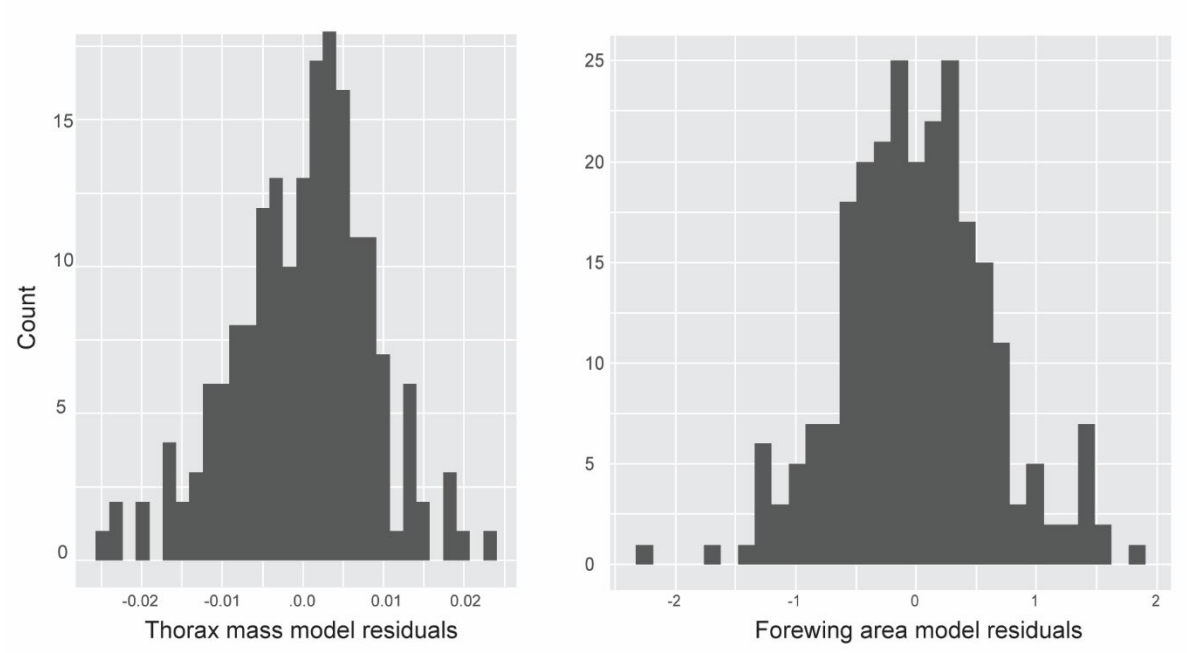


Figure 4.9. Histograms of residuals for thorax mass (left) and forewing area (right) general linear models. Residuals are normally distributed.

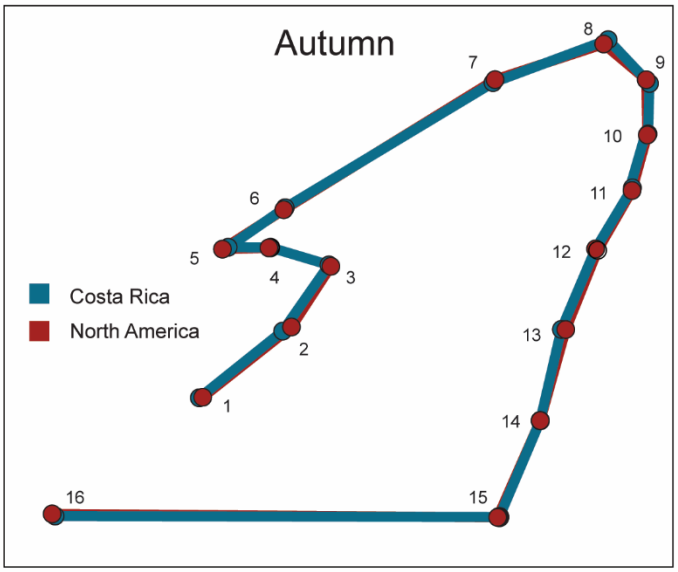


Figure 4.10. Comparison of mean forewing shape of Costa Rican (blue) and North American (red) monarchs reared in autumn. Each dot is the consensus mean coordinate for landmarks 1-16. Straight lines are drawn between landmarks to outline the forewing.

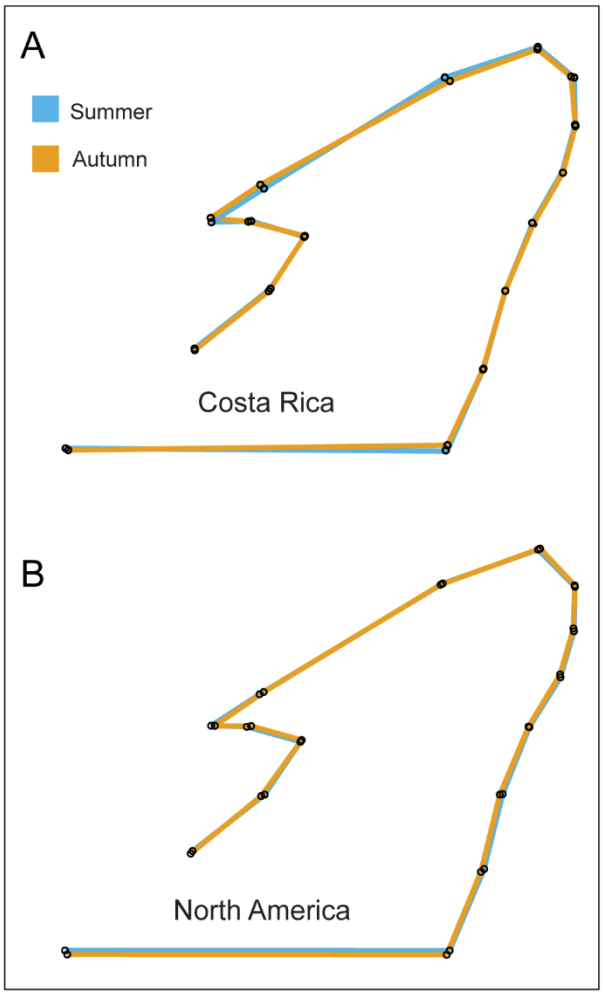


Figure 4.11. Comparison of mean forewing shapes of Costa Rican and North American monarchs reared in summer and autumn. Each dot is the consensus mean coordinate for landmarks 1-16. Straight lines are drawn between landmarks to outline the forewing. A) Mean shape of Costa Rican summer (yellow) plotted on top of mean shape of Costa Rican Autumn (blue). B) Mean shape of North American autumn (yellow) plotted on top of mean shape of North American summer (blue).

Table 4.2. Number of monarch specimens measured. Top table shows the number of forewings photographed and measured by population, season, and sex. Bottom table shows the number of bodies massed by population, season, and sex. There are 87 individuals that appear in both data sets

| Wing Specimens (N = 247) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Population | Costa Rican 107 | | | | North American 140 | | | |
| Season | Summer 47 | | Autumn 60 | | Summer 81 | | Autumn 59 | |
| Sex | Female 39 | Male 8 | Female 36 | Male 24 | Female 45 | Male 36 | Female 34 | Male 25 |

| Body Specimens (N = 185) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Population | Costa Rican 93 | | | | North American 92 | | | |
| Season | Summer 60 | | Autumn 33 | | Summer 56 | | Autumn 36 | |
| Sex | Female 29 | Male 31 | Female 18 | Male 15 | Female 22 | Male 34 | Female 19 | Male 17 |

Table 4.3 Candidate general linear models for thorax mass, thorax/body mass ratio, and forewing area ranked by Bayesian Information Criterion (BIC) score and model weights. The weight scores are the probability that the candidate model is the best among the entire set of models. The best model is highlighted in red.

| Thorax mass candidate models | BIC | Weights |
|---|---|---|
| Thorax ~ Population + Sex + Season | -1204.551 | 0.729999 |
| Thorax ~ Population + Sex + Season + Sex:Population | -1199.945 | 0.072964 |
| Thorax ~ Population + Sex + Season + Season:Population | -1199.904 | 0.071476 |
| Thorax ~ Population + Sex + Season + Season:Sex | -1199.461 | 0.057276 |
| Thorax ~ Population + Sex | -1198.101 | 0.029015 |
| Thorax ~ Sex + Season | -1195.801 | 0.009185 |
| Thorax ~ Population + Sex + Season + Sex:Population + Season:Population | -1195.203 | 0.006812 |
| Thorax ~ Population + Sex + Season + Sex:Population + Season:Sex | -1194.878 | 0.005791 |
| Thorax ~ Population + Season | -1194.865 | 0.005754 |
| Thorax ~ Population + Sex + Season + Season:Population + Season:Sex | -1194.788 | 0.005535 |
| Thorax ~ Population + Sex + Sex:Population | -1193.626 | 0.003096 |
| Thorax ~ Population | -1191.25 | 0.000944 |
| Thorax ~ Sex + Season + Season:Sex | -1190.64 | 0.000696 |
| Thorax ~ Population +Sex + Season + Sex:Population + Season:Population + Season:Sex | -1190.107 | 0.000533 |
| Thorax ~ Population + Season + Season:Population | -1190.027 | 0.000512 |
| Thorax ~ Sex | -1189.298 | 0.000356 |
| Thorax ~ Season | -1185.265 | 4.73E-05 |
| Thorax ~ 1 | -1181.654 | 7.78E-06 |
| **Thorax to body ratio candidate models** | **BIC** | **Weights** |
| Thorax/Body ~ Population + Sex + Season + Sex:Population + Season:Sex | -487.649 | 0.611 |
| Thorax/Body ~ Population + Sex + Season + Season:Sex | -485.922 | 0.258 |
| Thorax/Body ~ Population + Sex + Season + Sex:Population + Season:Population + Season:Sex | -482.772 | 0.0534 |
| Thorax/Body ~ Population + Sex + Season + Season:Population + Season:Sex | -481.361 | 0.0263 |
| Thorax/Body ~ Population + Sex + Sex:Population | -481.047 | 0.0225 |
| Thorax/Body~ Sex + Season + Season:Sex | -480.441 | 0.0166 |
| Thorax/Body ~ Population + Sex | -479.008 | 0.00813 |
| Thorax/Body ~ Population + Sex + Season + Sex:Population | -476.415 | 0.00222 |
| Thorax/Body ~ Population + Sex + Season | -474.484 | 0.000846 |
| Thorax/Body ~ Sex | -473.543 | 0.000529 |
| Thorax/Body ~ Population + Sex + Season + Sex:Population + Season:Population | -471.307 | 0.000173 |
| Thorax/Body ~ Population + Sex + Season + Season:Population | -469.58 | 7.29E-05 |
| Thorax/Body ~ Sex + Season | -469.184 | 5.98E-05 |
| Thorax/Body ~ Population | -461.033 | 1.02E-06 |
| Thorax/Body ~ Population + Season | -455.948 | 7.99E-08 |
| Thorax/Body ~ 1 | -450.882 | 6.34E-09 |
| Thorax/Body ~ Population + Season + Season:Population | -449.842 | 3.77E-09 |
| Thorax/Body ~ Season | | |
| **Forewing area candidate models** | **BIC** | **Weights** |
| Forewing Area ~ Population + Season + Season:Population | 497.55 | 0.8987 |
| Forewing Area ~ Population + Sex + Season + Season:Population | 502.977 | 0.0596 |
| Forewing Area ~ Population + Season | 504.556 | 0.0271 |
| Forewing Area ~ Population + Sex + Season + Sex:Population + Season:Population | 507.506 | 0.0062 |
| Forewing Area ~ Population + Sex + Season + Season:Population + Season:Sex | 507.97 | 0.0049 |
| Forewing Area ~ Population + Sex + Season | 509.797 | 0.002 |
| Forewing Area ~ Population + Sex + Season + Sex:Population + Season:Population + Season:Sex | 510.792 | 0.0012 |
| Forewing Area ~ Population + Sex + Season + Season:Sex | 515.206 | 0.0001 |
| Forewing Area ~ Population + Sex + Season + Sex:Population | 515.283 | 0.0001 |
| Forewing Area ~ Population | 516.701 | 6E-05 |
| Forewing Area ~ Population + Sex + Season + Sex:Population + Season:Sex | 520.619 | 9E-06 |
| Forewing Area ~ Population + Sex | 522.159 | 4E-06 |
| Forewing Area ~ Population + Sex + Sex:Population | 526.743 | 4E-07 |
| Forewing Area ~ Season | 561.61 | 1E-14 |
| Forewing Area ~ 1 | 563.649 | 4E-15 |
| Forewing Area ~ Sex + Season | 565.228 | 2E-15 |
| Forewing Area ~ Sex | 566.205 | 1E-15 |
| Forewing Area ~ Sex + Season + Season:Sex | 568.502 | 4E-16 |

# CHAPTER 5: CONCLUSIONS

I explored the effect of environment on migration and migration associated phenotypes in monarch butterflies and identified conditions under which migration associated phenotypes can be lost in both individuals and entire populations. Monarchs are sensitive to environmental conditions and environmentally induced traits can be lost if a population no longer experiences selective pressure to maintain plasticity. In particular, the loss of directional orientation in a commercial population and both the loss of plasticity and reduction in forewing size of Costa Rican monarchs suggests that migration each autumn is a significant selective episode and critical to maintaining migration behavior even in the short term.

We suspect that in new environments without North American autumnal environmental cues, individuals with either weak migration phenotypes or weak responses to environmental cues, that would have perished on the journey to Mexico, are instead free to pass on their genes. Without selection, the population shifts away from optimal migratory phenotypes. Indeed, the speed with which this occurred in the commercial (<200 generations) population may indicate which traits are costly to maintain in the absence of selection or alternatively, which traits are still adaptive in their new habitat (diapause).

In addition to loss of migration phenotypes due to either human captive breeding or dispersal into warmer climates, I showed North American individual monarchs are at risk of not developing migratory phenotypes when reared indoors. The development of the alternative migratory phenotype, directional orientation, is very sensitive to environmental conditions. When compared with North American monarchs reared completely outdoors, North American monarchs reared in a variety of autumn-simulating indoor conditions did not develop in southern orienting adults.

**Implications**

Beyond the interest of scientists who study them, monarchs have captivated the public as well. They are conspicuous as they fly south, streaming en masse over us each autumn. Monarchs are one of the most well-known insect species in North America, and have garnered significant attention due to their decline (Agrawal and Inamine 2018; Brower et al. 2012; Schultz et al. 2017; Semmens et al. 2016; Vidal and Rendón-Salinas 2014), even being considered for endangered species status by the U.S. government (The Center for Biological Diversity 2014).

Our results have important implications for monarch conservation. Though North American monarchs have dispersed and colonized many parts of the world, these populations largely do not migrate. While globally the species may survive, we worry that the annual migration of monarchs as we now know it now may be nearing an end.

While we do not know if introduction of non-orienting monarchs affects migration in the wild North American population, our results do show that commercial monarchs released in the autumn are significantly less likely to migrate successfully and may adversely affect monarch population recovery. The release of non-orienting monarchs could be insignificant if natural selection purges the wild population of non-migratory individuals. However, when released in summer, non-orienting monarchs may mate with wild North American individuals leading to the introduction of non-migratory variation. These non-migratory alleles may not be purged and instead persist in the genetic background of migratory individuals.

The introduction of non-migratory alleles into the wild population has serious implications for the increased transmission of the monarch parasite, *Ophryocystis elektroscirrha* (OE). Because resident populations have higher rates of OE infections (Satterfield, Maerz, and Altizer 2015), having more resident populations may lead to more infections in the migratory population

as it travels between the overwintering grounds and summer habitat (L. Brower 1996). OE reduces the fitness of migratory individuals and could contribute to further declines of monarch populations overwintering in Mexico (Bradley et al., 2005; Altizer et al., 1999).

While breeding programs are often touted as helping populations recover from declines, our work adds to the mounting evidence that captive breeding can negatively affect trait development in individuals and the population (Araki, Cooper, and Blouin 2007; Courtney Jones, Munn, and Byrne 2017; Davis, Smith, and Ballew 2020; Frankham 2008; Gilligan and Frankham 2003; Jonsson, Jonsson, and Jonsson 2019; Jonsson and Jonsson 2006; Kraaijeveld-Smit et al. 2006; Putman, Meinke, and Noakes 2014). Specifically, there is inherent difficulty in breeding a population that is under selection pressures which cannot be replicated in captive environments (Putman, Meinke, and Noakes 2014). Releasing monarchs may increase the numbers briefly, but could contribute to the decline in migration behavior.

The cumulative data available suggest that captive breeding of monarchs has negative consequences for migration behaviour in monarchs and that monarchs reared indoors are not as well equipped to survive migration as those left in the wild (Tenger-Trolander et al. 2019; Davis, Smith, and Ballew 2020; Steffy 2015). Although I discourage the practice of mass rearing for population recovery, I believe rearing as an educational tool is very important. The practice of raising monarchs for educational purposes fosters a link between people and their natural environment. Raising monarchs in schools is one of the reasons the insect is so popular and why so many people participate in conservation efforts. However, if we want to ensure the future of migratory monarch populations, we must address the clear threats of climate change and habitat loss because without seasonal variation and a place to live, migration is likely to disappear.

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