Distribution Agreement

Ali Ebada April 10th, 2023

An analysis of monarch butterfly flight performance and navigational orientation

by

Ali Ebada

Jacobus C. de Roode

Advisor

Biology

Jacobus C. de Roode

Advisor

Levi Morran

Committee Member

Ali J. Zarrabi

Committee Member

2023

An analysis of monarch butterfly flight performance and navigational orientation

by

Ali Ebada

Jacobus C. de Roode

Advisor

An abstract of

a thesis submitted to the Faculty of Emory College of Arts and Science

of Emory University in partial fulfillment

of the requirements of the degree of Bachelor of Science with Honors

Biology

2023

Abstract

An analysis of monarch butterfly flight performance and navigational orientation

by Ali Ebada

Successful, efficient long-distance flight is integral to the fitness of migratory monarch butterfly (*Danaus plexippus*) populations. The behavior allows the insect to accomplish its world-renowned migratory journey that encompasses up to a 4,500-kilometer distance from areas in the United States and Canada into overwintering sites in Central Mexico. Despite how well-regarded monarch migration is among the public, there continues to be a lack of understanding of the factors that affect successful flight performance. Monarch overwintering populations in Mexico have declined over the last few decades, while the incidence of non-migratory, sedentary populations has increased. Despite this, it remains unknown how sedentary populations drive migration loss in terms of flight behavior and efficiency.

To address these two areas, in two sequential studies, we investigated the impact of monarch larval diet on the mechanisms of flight and comparatively analyzed the flight performance displayed by a migratory and non-migratory population of monarchs. The first study displayed that the milkweed source that monarchs are reared on has important implications for flight performance, while the second study showed that migratory monarchs did not display differential flight performance when compared alongside non-migratory monarchs. Thus, overall, these two studies displayed the significant role that environment plays in the successful flight function of the monarch butterfly.

Additionally, while the genetic, physiological, and environmental components of monarch migratory navigation have been thoroughly investigated in prior studies, the ability of the monarch to display such precise navigational ability amid their long journey remains elusive. Thus, in a third study, we investigated the efficacy of two methodologies meant to understand the navigational orientation of the monarch. In this study, we studied eastern North American monarchs in the migratory stop-over site in St. Marks, Florida, from 2019 to 2022. Overall, the purpose of these sequential studies was to confer an increased understanding of the mechanisms that allow the monarch butterfly to orient, fly, and, as a result, thrive in its environment.

Keywords: Lepidoptera, migration, milkweed, flight performance, metabolism, Puerto Rico, *Danaus plexippus*, flight orientation, directionality, Savannah

An analysis of monarch butterfly flight performance and navigational orientation

by

Ali Ebada

Jacobus C. de Roode

Advisor

A thesis submitted to the Faculty of Emory College of Arts and Science

of Emory University in partial fulfillment

of the requirements of the degree of Bachelor of Science with Honors

Biology

2023

Acknowledgements

I thank Dr. Jacobus de Roode and Dr. Ania Majewska for their continued mentorship throughout my undergraduate research career, including aiding in the development of the research design of the studies that encompass this thesis, monarch sourcing and capture, and, overall, teaching me the multitude of skills that define a scientist. I also want to thank Mitchell Kendzel for his help with data analysis and compilation. This work was supported by NSF grant DEB-1754431.

Many thanks to my family and closest friends for their support and encouragement throughout my undergraduate career.

Table of Contents

1. Chapter 1: Larval diet impacts flight performance in monarch butterflies from two populations.

2. Chapter 2: Monarch butterflies from two populations display similar flight performance.

3. Chapter 3: Gone with the Wind: Testing the efficacy of two experimental orientation procedures in the eastern North American monarch butterfly.

4. References

List of Figures

1. Chapter 1: Larval diet impacts flight performance in monarch butterflies from two populations.

2. Chapter 2: Monarch butterflies from two populations display similar flight performance.

3. Chapter 3: Gone with the Wind: Testing the efficacy of two experimental orientation procedures in the eastern North American monarch butterfly.

List of Tables

1. Chapter 1: Larval diet impacts flight performance in monarch butterflies from two populations.

3. Chapter 3: Gone with the Wind: Testing the efficacy of two experimental orientation procedures in the eastern North American monarch butterfly.

Chapter 1: Larval diet impacts flight performance in monarch butterflies from two populations.

Ali Ebada[1], Jacobus C. de Roode[1], Ania A. Majewska[2]

Institutional affiliations:

[1] Department of Biology, Emory University, Atlanta, GA 30322, USA

[2] Department of Physiology and Pharmacology, College of Veterinary Medicine, University of Georgia, Athens, GA 30602, USA

Introduction

Flight plays a critical role in dispersal, predator avoidance, acquisition of food, mate finding, and fitness and is impacted by numerous environmental factors (Goldsworthy & Wheeler, 1989). In flying insects, the environmental conditions experienced early in life are particularly important because their development is dictated by food availability, nutrition quality, and temperature fluctuations (Damos & Savopoulou-Soultani, 2012; Scriber, 1981). Prior studies show that poor nutrition during larval stages is detrimental for flight ability and wing morphology in butterflies, fruit flies, and moths (Portman et al., 2015; Reim et al., 2019; Yama et al., 2019; Zahran et al., 2018). Larval diet can therefore have long-lasting effects on insect dispersal.

The plants that insects use for larval rearing vary in quality as a food source and can have strong impacts on performance, largely is due to the variation in nutrients (e.g., protein) as well as the chemical defenses, which can reduce consumption, digestion, and growth (Chen, 2008; Couture et al., 2016). For instance, cabbage looper larvae (*Trichoplusia ni*) experience decreased survival, development, and size with higher toxin concentrations in their diet (Paul et al., 2021). With smaller body size and wing area, plant defenses can thereby indirectly affect flight. The effects on wing area and shape might be particularly critical for migratory insects, which rely on wing morphologies that increase energy efficiency and survival to successfully complete the journey (Wootton, 1992). For instance, in the monarch butterfly (*Danaus plexippus*), migratory individuals have elongated wings and utilize high-performance gliding, while non-migratory adults tend to have shorter, more rounded wings and use powered flight (Altizer & Davis, 2010; Flockhart et al., 2017). Besides wing morphology, plant toxins can also impact flight via effects on muscle composition and metabolism (Portman et al., 2020; Portman et al., 2015).

To contend with plant defenses, insects evolved strategies to minimize the negative impacts, with many herbivores specializing on particular plant species (Ali & Agrawal, 2012). For instance, monarch caterpillars evolved to feed on cardenolide-containing milkweed plants (*Asclepias* spp) and even to benefit by sequestering the toxins for anti-predatory defense (Agrawal et al., 2015). Thus, insect herbivores raised on a plant that they share co-evolutionary history with are expected to experience little to no impact on flight performance due to the adaptations to avoid plant defenses and maximize nutritional value. Conversely, insect herbivores raised on an introduced plant may experience deleterious effects on their flight performance if the plant presents novel toxins and/or higher toxin levels. At the same time, more beneficial effects on flight performance might be observed if an introduced plant lacks detrimental toxins, and/or yields higher nutrition.

Here we asked whether milkweed food plant species, which differ in their origin and toxin concentrations, impact flight metrics and metabolism in two monarch butterfly populations (Puerto Rican and eastern North American). The eastern North American population breeds during the spring and summer season and migrates each fall to overwinter in central Mexico, while the Puerto Rican population breeds year round due to the tropical climate and availability of food plants (Zhan et al., 2014). We selected two milkweed species: *Asclepias incarnata* (native to eastern North America but not Puerto Rico) and *A. curassavica* (native to Puerto Rico but not eastern North America). While phosphorus and nitrogen levels are comparable in the two species, *A. curassavica* has a much higher concentration of cardenolide toxins, which are detrimental to caterpillar growth rate (Tao et al., 2014). Thus, we hypothesized that monarchs would exhibit better flight performance when fed a less toxic diet as caterpillars. At the same time, diet that is native relative to the population might provide superior performance due to the co-evolutionary history. Therefore, we anticipated lowered flight performance in eastern North American monarchs raised on non-native and more toxic *A. curassavica* compared to the less toxic native *A. incarnata* and vice versa in Puerto Rican monarchs.

Materials and Methods

Monarch rearing

We used F2 inbred progeny of F1 outbred lineages established prior to the experiment. The F1 lineages were the offspring of adults captured in St. Marks, Florida (eastern North American population) in October 2020 and in Puerto Rico (Puerto Rican population) in June 2021. The eastern North American monarchs were collected during peak of fall migration at St.

Marks, Florida, a major flyway and stopover site for migrating monarchs originating from across eastern North America. Butterflies collected in St. Marks, Florida in the midst of migration are representative of the entire eastern North American population and were not part of the breeding population in Florida.

We screened all monarchs for the parasite *Ophryocystis elektroscirrha* to ensure the insects were free of infection (following (Altizer et al. 2000)). To create the F2 progeny lineages, we placed adults with potential mates in mesh cages in a large temperature-controlled growth chamber (Conviron Ltd., Winnipeg, Canada) held at 26°C and 16:8 light:dark cycle. We checked mating cages twice daily (morning and evening) and we recorded all mating pairs. We collected eggs from mated females on greenhouse-grown *A. incarnata* and *A. curassavica*, reared under similar conditions. We acquired eggs from five eastern North American and five Puerto Rican lineages.

At the second instar stage, we transferred four randomly selected Puerto Rican caterpillars to *A. incarnata.* This transferring was repeated for 5 separate Puerto Rican lineages for a total of 20 Puerto Rican caterpillars reared on *A. incarnata*. To *A. curassavica*, we transferred six randomly selected Puerto Rican caterpillars. Again, this process was repeated for 5 separate Puerto Rican lineages for a total of 30 Puerto Rican caterpillars reared on *A. curassavica.* However, due to error, two extra Puerto Rican caterpillars were transferred to an *A. incarnata* plant, and another two extra Puerto Rican caterpillars were transferred to an *A. curassavica.* We transferred two randomly selected caterpillars from each lineage and population to four *A. incarnata* (n=8 per lineage) and six *A. curassavica* (n=12 per lineage) plants (for sample sizes see Table S1). The milkweeds, grown from seed in a greenhouse with natural light and controlled temperature, were two months old and approximately two feet (61 cm) tall. We reared the caterpillars in a greenhouse with an *ad libitum* supply of their respective milkweed diet. Feeding the monarchs *ad libitum* prevented food limitation, which provided a standardized means of feeding the monarchs: caterpillars were allowed to consume as much food as needed to meet their physiological needs. Following emergence, we placed the adults in mesh cages for 24 hours to allow their wings to fully dry before handling them for wire attachment for flight trials.

Flight trials and metrics

Flight trials and metabolic rates acquisition were performed following (Schroeder et al., 2020). For description of wire attachment, flight mill and equipment see Supporting Information. Following eclosion, we attached a steel wire to the dorsal thorax and acclimated monarchs to the wire for 48-72 hours in a mesh cage with 20% honey water *ad libitum*. We performed trials indoors during Sept-Nov 2021 using two flight mills in a windowless room between 0900 and 1800 h. We used two flight mills with identical assemblies to maximize the number of monarchs that could be flown per day. The flight mill selection for each monarch was randomized. Prior to a trial, we weighed the monarch with the wire using a digital scale and measured resting oxygen consumption rate for 5 min with an O_2 Sensor (The Vernier O_2 Gas Sensor).

To initiate a flight trial, we attached a monarch to the carbon rod of the flight mill. Data was collected via the PASCO® Capstone software connected to a wireless photogate which detected passage of a flag on the rod, opposite to the monarch. Monarchs flew to exhaustion but no more than 60 minutes due to logistical constraints. The 60-minute time cut-off did not limit the sample size and permitted us to fly all monarchs within one week of emergence. We considered a successful flight to be at least 15 min. Immediately after flight, we measured oxygen consumption for 5 min to attain post-flight rate and weighed monarchs with and without the wire.

For each monarch, we calculated the average velocity (m per sec), duration (min), distance traveled (m), and power (Watts). For velocity we disregarded the first five minutes of flight as monarchs appeared to fly quickly to escape the handler. Distance traveled was quantified as the number of revolutions times the circumference of the circle flown. We calculated power of each revolution by dividing the estimated kinetic energy (½ x mass x velocity^2) by time and calculated an average for the flight.

Finally, we acquired wing area and aspect ratio (wing length divided by the width) of the forewing by scanning the right and left wings with a flatbed scanner and processed the images with ImageJ 1.52k (Alaidrous et al., 2022).

Statistical analyses

Statistical analyses were performed using R computing language (R Core Team, 2022). We asked whether eastern North American and Puerto Rican monarchs differed in mass, forewing area, and forewing aspect ratio. We ran separate models because preliminary analyses revealed that mass, forewing area and aspect ratio were highly correlated (Pearson's correlation >0.7). Individual plants were not included as random effects as we did not keep track of which caterpillars came from which plant. However, milkweed provision to individual caterpillars was random and plants were highly homogeneous: because monarch caterpillars are voracious feeders, each rearing tube with caterpillars received multiple plants throughout the experiment, and these plants derived from the same batch of plants, propagated from the same source of seeds, and grown under the same conditions.

We examined whether eastern and Puerto Rican monarchs and plant species diet impacted flight metrics. First, we used the Rosner outlier test (package *EnvStats*) to test for outliers in the response variables (velocity, distance, duration, power, and oxygen consumption), which identified no significant outliers ($p>0.05$). Next, we used linear mixed-effects model (LMM) with gaussian errors, where fixed effects included population, plant species, the interaction between population and plant species, and wing aspect ratio. Models that included wing area or mass instead of aspect ratio showed qualitatively similar results (see Supporting Information). Lineage was included as a random effect. We began with a full model and performed model selection with the function *dredge* (MuMIn package) to identify variables that best explain the response variables. We retained population and plant species (diet) factors given our a priori hypotheses. The model with lowest AICc was selected as the most parsimonious model regardless of diet and population (Table 1). We present results for the single best model selected in the main text.

Results

The proportion of monarchs that survived to adulthood was higher among Puerto Rican monarchs ($t=3.88$, $df = 111$, $p <0.001$), but did not differ between plant species diets ($t =1.57$, $df = 111$, $p = 0.12$). Puerto Rican monarchs weighed less ($t= -3.76$, $df = 6.19$, $p < 0.01$), had smaller wing area ($t= -4.92$, $df = 8.32$, $p = 0.001$), and had lower wing aspect ratio ($t =-4.21$, $df = 8.60$, $p = 0.002$) than eastern North American monarchs.

Population and plant species diet were retained for model selection and therefore are present in most-parsimonious models (Table 1). The most-parsimonious model of velocity included population and plant species diet only and showed that monarchs from both Puerto Rico and Eastern North America raised on *A. incarnata* flew more slowly than those raised on *A. curassavica* (t = -4.21, df = 52.03, p <0.001; Fig. 1A). Eastern North American and Puerto Rican monarchs flew at similar velocities (t = 1.60, df = 6.20, p = 0.16; Fig. 1A). Best models for flight distance and duration included all factors, although none showed strong influence (Table 1). Flight distance was marginally shorter for monarchs raised on *A. incarnata* (t =-1.68, df = 52.27, p = 0.10; Fig. 1B) and duration was positively related, marginally, to wing aspect ratio (t=1.89, df = 50.98, p = 0.06). The best model for power included population and plant species diet and showed that monarchs raised on *A. incarnata* had less powerful flight than those raised on *A. curassavica* (t = -2.16, df = 54.88, p = 0.04, Fig. 1C). The best model for oxygen consumption rate did not include the interaction between population and plant species diet. Only mass showed a significant negative relationship with oxygen consumption rate (t=-2.21, df = 55.94, p=0.03).

Discussion

Our results confirm findings of previous work indicating that insect herbivore performance is impacted by food plant species: milkweed species used for larval rearing influenced the monarch butterfly's flight velocity, distance, and power. Yet, we did not detect the expected differences in performance based on plant species origin (native vs. non-native) relative to the two different monarch populations (eastern North America and Puerto Rico) and differences in levels of plant toxicity. Instead, regardless of population, we observed slower, shorter, and less powerful flights in monarchs fed on *A. incarnata* than those fed on the more toxic *A. curassavica*, suggesting *A. incarnata* might provide fewer nutrients for monarchs and be less favorable for development of muscles necessary for fast flight. We found no effect of wing size on flight metrics, nor did the data suggest a relationship between food type and wing size. Our results conflict with the findings of previous studies, which have found a positive correlation between wing size and flight metric performance in both lab-reared and wild-caught monarchs (Büyükyilmaz & Tseng, 2022).

Past studies have found that host plant nutritional quality influences flight muscle development, energetics, and maintenance in monarchs, whereby the plant impacts the fuel requirements for sustained flight (Pocius et al., 2022). However, Pocius et al., 2022 found no significant difference in the flight muscle development between monarchs raised on *A. curassavica* versus *A. incarnata*. Given that we found differences in flight metrics between monarchs raised on these respective milkweeds, it is possible that the milkweed nutritional profile affects flight through a mechanism different from flight muscle development. In particular, the difference in flight patterns could be explained by changes in wing morphology induced by the milkweed plant species. However, our analysis of flight metrics which included wing aspect ratio, indicated no significant differences on the basis of rearing plant. We did find a marginal increase in flight duration in monarchs with higher aspect ratios (more elongated wings), a result supported by previous studies. Specifically, migratory monarchs, which utilize energetically efficient gliding behavior to sustain long flight durations have more elongated wings (Altizer & Davis, 2010). On the other hand, non-migratory monarchs, including those from Puerto Rico, tend to have smaller aspect ratios and more flapping-dependent flight (Altizer & Davis, 2010). One possible explanation for our finding of no significant differences in flight metrics in relation to plant species might be a behavioral effect induced by the plant species.

Namely, *A. incarnata* may result in adults that use more gliding versus active, flapping flight, yet these behavioral differences are difficult to measure and were not investigated in this study.

Previous work showed that eastern North American monarchs reared on *A. incarnata* had longer and more elongated wings compared to monarchs reared on *A. curassavica* (Soule et al., 2020), which we did not find. While the researchers in that study fed the milkweed to monarch larvae in the form of cuttings, our work involved rearing the caterpillars on intact plants grown in pots. The difference in feeding methods may explain the conflicting experimental results, as feeding cuttings of milkweed may alter the associated phytochemistry of the tissue. For example, cutting the leaves of milkweed can induce cardenolide defenses in the intact portions of the plant, and severing the leaves of the plant has been found to reduce latex flow (Malcolm & Zalucki, 1996; Zalucki et al., 2001). Consumption of plant cuttings versus an intact plant may also have implications for the insect's ability to acquire nutrients as cuttings likely experience water loss. Further, Soule *et al.* 2020 found weak effects of milkweed diet on maximum flight velocity, yet the flight trials lasted less than 10 minutes, making direct comprisons between study findings difficult.

Contrary to our expectations, eastern North American and Puerto Rican monarchs did not differ in flight performance or metabolism, despite showing differences in morphology. A possible explanation is that the flight differences are only detectable between monarchs reared to show active migratory state and those in non-migratory state, as previously observed (Schroeder et al., 2020). Indeed, we reared monarchs in summer-like conditions with the intent of avoiding physiological changes associated with fall conditions (e.g., reproductive diapause). We suspect that flight demands in the two populations during the breeding season are similar, which might explain why we did not detect differences in flight metrics between the populations. The similarity in flight demands between migratory monarchs out of diapause of non-migratory monarchs has been supported in previous work. For example, one study has found that a vast majority of recolonization of North America by monarchs in the spring is done through successive broods, or a series of short term distance flights completed by sequential generations in order to progressively repopulate regions of eastern North America (Miller et al., 2012). It is important to note that our study investigated food plant effects after only one generation and population-level differences might emerge after multiple generations of rearing, a possibility that should be explored in future studies.

Table 1. Summary of the most parsimonious models after global selection for population, plant species, interaction between population and plant species, and wing aspect ratio describing flight metrics (velocity, distance, duration, power, and oxygen consumption). Each row represents a distinct model. Population and plant species were retained in model selection. Wing aspect ratio was not included in the power and oxygen consumption models. Oxygen consumption model included mass. "//" denotes that a factor was not included in the best model. "ns" indicates the included factor was not significant. "↓" represents a negative relationship between response and the factor, while "↑" represents a positive relationship. P-values are denoted with symbols: $^{\cdot}p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$. For full model results, see Supporting Information.

	Population	**Plant species**	**Population × Plant species**	**Wing aspect ratio**
Velocity	*ns*	↓*incarnata* ***	//	//
Distance	*ns*	↓*incarnata* •	*ns*	*ns*
Duration	*ns*	*ns*	*ns*	↑ •
Power	*ns*	↓*incarnata* *	//	//
Oxygen consumption	*ns*	ns	//	//

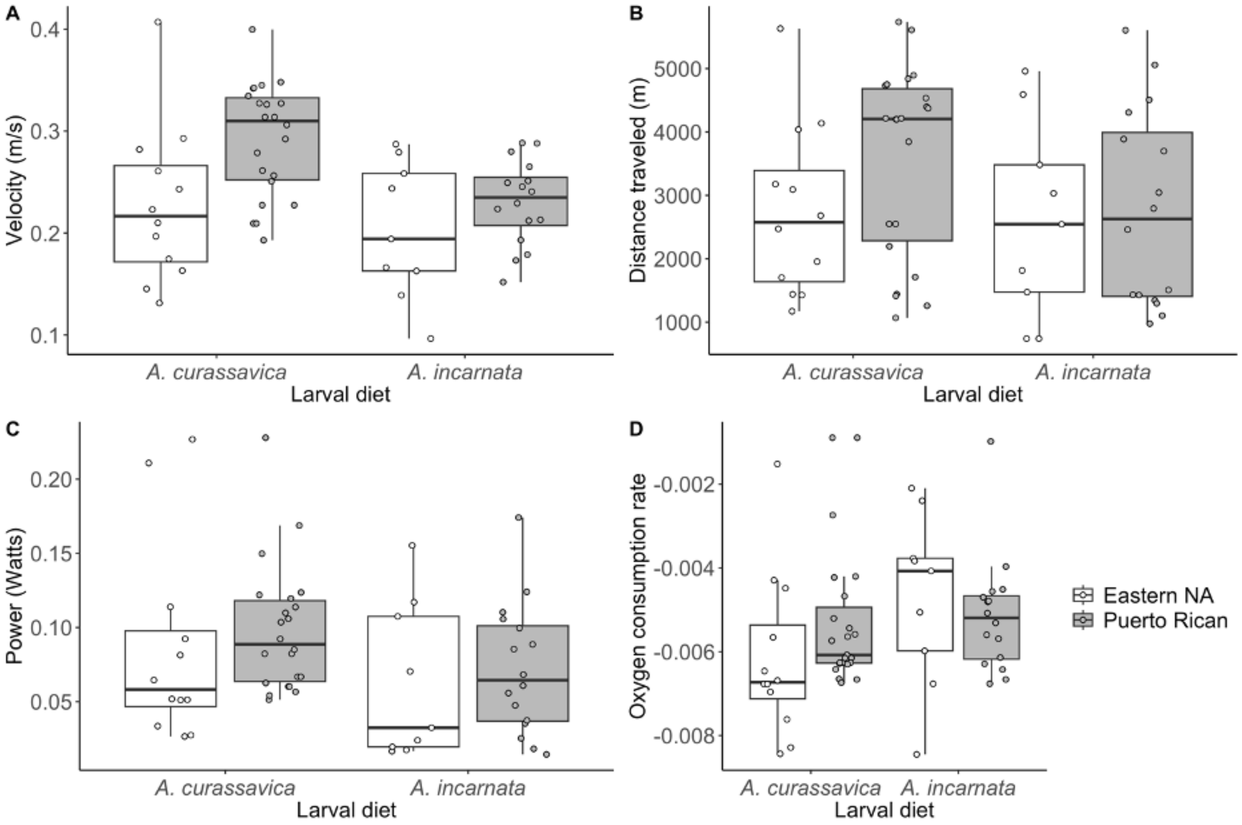


Figure 1. Flight metrics of monarchs from eastern North America (white fill boxplots and symbols) and Puerto Rico (gray fill boxplot and symbols) raised on diet of *A. curassavica* or *A. incarnata:* (A) velocity, (B) total distance traveled, (C) power, and (D) post-flight oxygen consumption rate. The top horizontal bar represents the third quartile, followed by the median and the first quartile. Each whisker indicates the full data range.

Chapter 1 Supporting Information

Methods

Lineages

We used F2 inbred progeny of F1 outbred lineages established prior to the experiment. The F1 lineages were the offspring of adults captured in St. Marks, Florida (eastern North American population) in October 2020 and in Puerto Rico (Puerto Rican population) in June 2021. We screened all monarchs for *Ophryocystis elektroscirrha* to ensure the insects were free of infection (following (Altizer et al. 2000)). To create the F2 progeny lineages, we placed adults with potential mates in mesh cages in a large temperature-controlled growth chamber (Conviron Ltd., Winnipeg, Canada) held at 26°C and 16:8 light:dark cycle. We checked mating cages twice daily (morning and evening) and we recorded all mating pairs. We collected eggs from mated females on two greenhouse-grown milkweed species: *Asclepias incarnata* and *Asclepias curassavica.*

Wire attachment

We attached a folded piece of lightweight steel wire (AFM Monel Trolling Wire 15lb (7kg) test) to the dorsal thorax of the butterfly. Briefly, we immobilized the insects using an index card (7.62 x 12.7 cm) folded in half, with rectangular openings cut on the center opposite to the fold. We sized the rectangular opening relative to the body length and width of the monarch. To restrain the butterfly, we separated their wings and closed them within the index card with the insect's body and legs placed within the rectangular opening. We used paper clips on each side of the opening to keep the monarch wings fastened inside. We placed a flat, elevated surface underneath each side of the index card to ensure the butterfly and its legs were properly suspended from the working surface. With the dorsal thorax exposed, we applied a thin layer of rubber cement to the dorsal side of the monarch's thorax and spread it evenly using a small paintbrush. The layer of rubber cement provided additional adhesion to the small piece of bandage (Band-aid tough strips; length: 6 mm, width: 4 mm), which we placed on the thorax using fine-tip forceps. Next, we glued the wire to the bandage with a small amount of super glue. We shaped the wire to permit the movement of the butterfly's abdomen without interference. The folded wire weighed 0.125 g on average (range: 0.104, 0.151). Finally, we affixed a small piece of lab tape (~0.02 cm) to the end of the wire to facilitate attachment to the flight mill (see Supporting Information). Following wire attachment, we allowed monarchs to acclimate to the wire for 48-72 hours in a small mesh cage within incubators with the same temperature conditions previously noted. We provided monarchs with 20% honey water *ad libitum*. By allowing monarchs to consume honey water ad libitum for at least 24 hours, individuals consumed until they were satisfied, which we assumed to be proportional to their dietary needs. We chose not to standardize on the basis of volume of honey water, for example, as different monarchs require different amounts of nutrients to reach the same level of need.

Flight trials

We performed all flight trials indoors during September-November 2021 using a tethered flight mill in a windowless room with a mean temperature of 25 ºC between 0900 and 1800 h. Following at least 1 day after eclosion, we attached lightweight steel wires to the dorsal thorax of each monarch under study (see *Wire attachment* above). Given the logistical difficulty of flying all monarchs at the same age, we ensured there was no bias in the selection process for each flight trial. Each monarch flown on the mill was randomized, so the age of the monarch was also randomly selected. We assembled flight mills for flight trials following (Bradley & Altizer 2005; Fritzsche McKay *et al.* 2016). The flight mills consist of a 12-inch (30.48 cm) base, a 21.8-inch (55.4 cm) support rod, a frictionless pivot, a 3 ft (91.4 cm) carbon rod (3 mm in diameter), three small counterbalance weights, and a photogate "flag" consisting of a 0.02 cm wide piece of lab tape fastened to the end flight arm. We used two flight mills (one 75 cm and another 79 cm from the pivot to the end of the arm; total arm length 91.44 cm). Monarch selection for a flight trial was randomized regardless of age, diet, population, or other characteristics. The photogate flag passed through a photogate sensor (PASCO® wireless photogate PS-3225) as the monarch flew, which uses the interruption of an infrared beam as the flag passes through to transmit motion-related information to a computer connected via Bluetooth and PASCO® Capstone software. Prior to the flight trial, we weighed the monarch with the wire attachment using a digital scale. Next, we measured resting oxygen consumption rate (see *Oxygen consumption rate* below). To initiate a flight trial, we attached the monarch to the carbon rod of the flight mill. Prior to releasing the monarch for flight, we checked the positioning of the monarch's body to assure it was horizontal.

Once the monarch flew a full rotation, we initiated data collection via the PASCO® Capstone software. We allowed all monarchs to fly to exhaustion but no more than 60 min due to logistical constraints. Throughout the trial, if the monarch ceased flight for 10 consecutive seconds, we used a small portable fan to lightly blow air on the monarch from behind (in the direction of flight) to stimulate flight. This encouraged the monarch to resume flying. We considered a trial completed when the monarch failed to resume flapping after three consecutive "blows" separated by 10 seconds of gliding. If a monarch refused to fly, we placed the monarch on sponges with 20% honey water to encourage feeding and we attempted to fly that individual the next day. Immediately after the flight concluded, we measured oxygen consumption to attain post-flight metabolism measurements. Following post-flight metabolism measurements, we weighed monarchs with the wire attachment, we removed the steel wire attachment from the thorax of the monarch and weighed the wire itself.

Flight metrics

For each monarch we used data captured by Capstone software to calculate the average velocity through the photogate (meters per second), duration (minutes), distance traveled, and power. Total distance (in meters) traveled was quantified as the number of revolutions of the circle a monarch flew, with radius equal to the length of the carbon rod. We calculated power (units were watts or joules per second) by dividing the estimated kinetic energy of the monarch's flight (kinetic energy = ½ x mass x velocity^2) and then dividing the kinetic energy by the time the monarch flew. A higher-powered flight may signify a higher mass butterfly with a slower velocity or a lower mass butterfly with a larger velocity (although velocity was the higher contributing variable). We considered a successful flight to be at least 15 minutes.

Oxygen consumption rate

To estimate flight-associated metabolism, we measured the pre- and post-flight oxygen consumption of a monarch using an O_2 Sensor (The Vernier O_2 Gas Sensor O2-BTAm Beaverton, Oregon) (Schroeder *et al.* 2020). We obtained the data via Logger Lite 1.9.4 software (Vernier Software & Technology). We calibrated the oxygen sensor prior to data collection and normalized it by ensuring it was always kept upright. For pre-flight metabolism (before any flight mill activities were initiated), we placed a monarch inside of a plastic 50 mL vial, placed the long cylindrical portion of the O_2 sensor into the vial to establish a tight seal and covered the setup with a dark cloth to reduce stress on the animal. We then recorded the pre-flight oxygen consumption for each animal for 5 minutes. Following data collection, we removed the monarch using fine-tip forceps and attached it to the flight mill. For post-flight oxygen consumption, we flew monarchs on the flight mill (see above) and immediately following the completion of a flight trial, we placed the monarch in the plastic vial, sealing the vial with O_2 sensor, and measuring the change in oxygen for 5 minutes. To estimate oxygen consumption rate, we calculated the slope of the relationship between time and oxygen level recorded. We removed the first 30 seconds of the data given the disturbance of the sensor at the start of data collection and calculated the slope for the following 2-minute interval.

Wing morphometrics

To assess the wing area and aspect ratio of the forewing, we scanned the dorsal and ventral sides of both the right and left wings with a Canon® CanoScan LiDE 210 flatbed scanner and processed the images with ImageJ 1.52k (https://imagej.nih.gov/ij/) (Alaidrous *et al.* 2022). Forewing area, length and width were calculated by using the “measure” tool on ImageJ. Forewing aspect ratio was quantified as wing length divided by the width. Larger wing aspect ratio indicates a more elongated wing and is thought to be more adaptive for long-distance soaring flight, while small aspect ratio is more characteristic in non-migratory, resident populations (Flockhart *et al.* 2017).

Results

The proportion of monarchs that emerged higher among Puerto Rican monarchs (t=3.88, df = 111, p <0.001), however it did not differ between plant species diets (t =1.57, df = 111, p = 0.12). Puerto Rican monarchs weighed less (t = -3.76, df = 6.19, p < 0.01), had smaller wing area (t = -4.92, df = 8.32, p = 0.001), and had lower wing aspect ratio (t=-4.21, df = 8.60, p = 0.002).

Table S1. Number of monarchs used in each experiment, the percent of individuals emerging, and number of individuals successfully flown.

		Eastern North American	**Puerto Rican**	**Total**
A. curassavica	**Initial number of caterpillars**	30	32	62
	% emerged	63.3%	84.4%	
	Successfully flown	12	23	35
A. incarnata	**Initial number of caterpillars**	20	22	42
	% emerged	80.0%	95.5%	
	Successfully flown	9	16	25

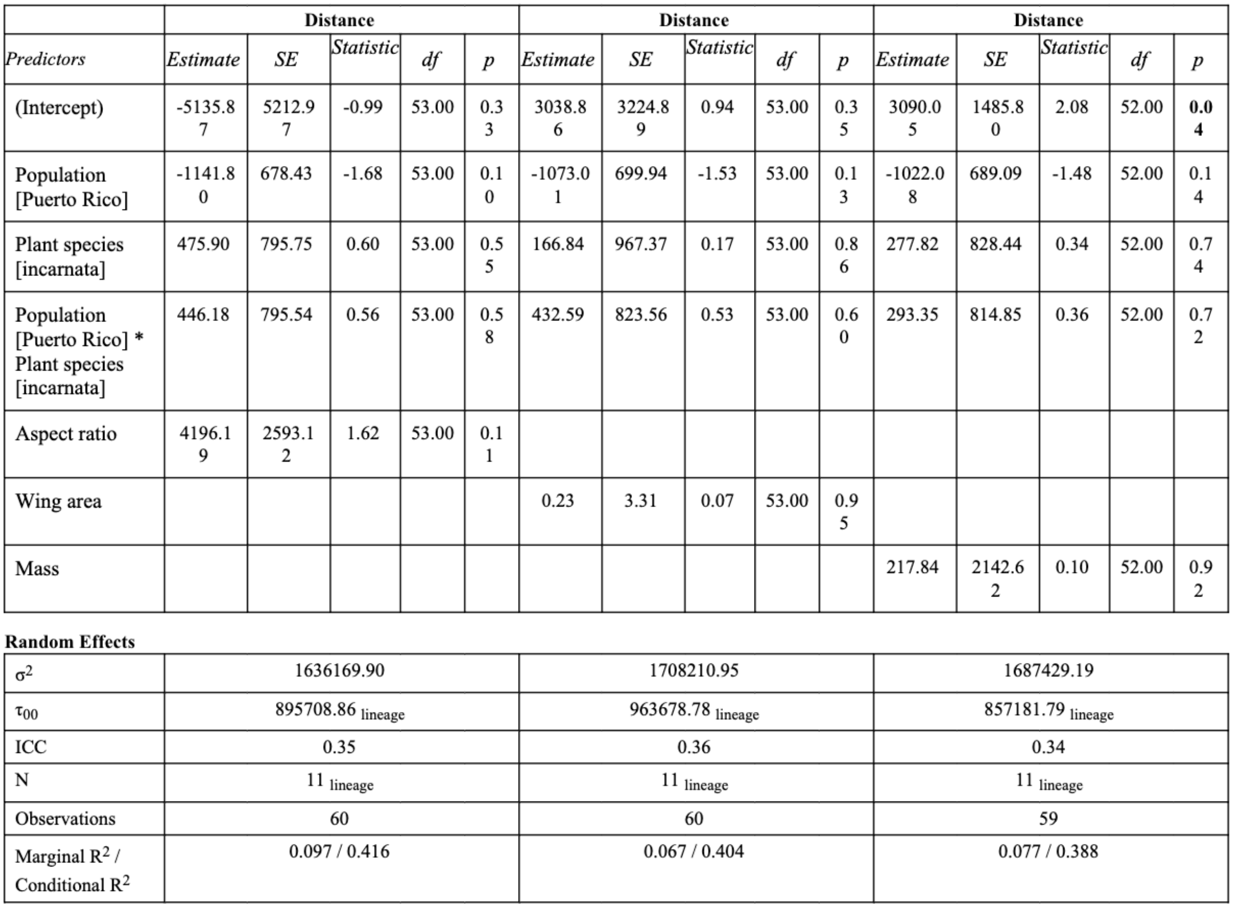
Table S2. Full model results examining the impact of population, plant species, interaction between population and plant species, and the wing aspect ratio, wing area, or mass on flight speed. As shown, none of the models resulted in statistical significance.

	Speed					**Speed**					**Speed**				
Predictors	*Estimate*	*SE*	*t value*	*df*	*p*	*Estimate*	*SE*	*Statistic*	*df*	*p*	*Estimate*	*SE*	*Statistic*	*df*	*P*
(Intercept)	0.63	0.22	2.89	53	**0.01**	0.37	0.13	2.73	53	**0.01**	0.20	0.06	3.37	52	**<0.001**
Population [Puerto Rico]	0.01	0.03	0.43	53	0.67	0.02	0.04	0.45	53	0.66	0.05	0.03	1.52	52	0.13
Plant species [incarnata]	**-0.07**	**0.03**	**-2.31**	**53**	**0.02**	**-0.06**	**0.03**	**-2.11**	**53**	**0.04**	**-0.06**	**0.03**	**-2.20**	**52**	**0.03**
Population [Puerto Rico] * Plant species [incarnata]	0.01	0.03	0.36	53	0.72	0.01	0.03	0.16	53	0.87	-0.00	0.03	-0.06	52	0.95
Aspect ratio	-0.19	0.11	-1.73	53	0.09										
Wing area						-0.00	0.00	-0.89	53	0.38					
Mass											0.08	0.09	0.90	52	0.37

Random Effects

σ^2	0.00	0.00	0.00
τ_{00}	0.00 $_{\text{lineage}}$	0.00 $_{\text{lineage}}$	0.00 $_{\text{lineage}}$
ICC	0.38	0.25	0.32
N	11 $_{\text{lineage}}$	11 $_{\text{lineage}}$	11 $_{\text{lineage}}$
Observations	60	60	59
Marginal R^2 / Conditional R^2	0.233 / 0.522	0.221 / 0.415	0.257 / 0.498

Table S3. Full model results examining the impact of population, plant species, interaction between population and plant species, and the wing aspect ratio, wing area, or mass on distance traveled.



	Distance					**Distance**					**Distance**				
Predictors	*Estimate*	*SE*	*Statistic*	*df*	*p*	*Estimate*	*SE*	*Statistic*	*df*	*p*	*Estimate*	*SE*	*Statistic*	*df*	*p*
(Intercept)	-5135.87	5212.97	-0.99	53.00	0.33	3038.86	3224.89	0.94	53.00	0.35	3090.05	1485.80	2.08	52.00	**0.04**
Population [Puerto Rico]	-1141.80	678.43	-1.68	53.00	0.10	-1073.01	699.94	-1.53	53.00	0.13	-1022.08	689.09	-1.48	52.00	0.14
Plant species [incarnata]	475.90	795.75	0.60	53.00	0.55	166.84	967.37	0.17	53.00	0.86	277.82	828.44	0.34	52.00	0.74
Population [Puerto Rico] * Plant species [incarnata]	446.18	795.54	0.56	53.00	0.58	432.59	823.56	0.53	53.00	0.60	293.35	814.85	0.36	52.00	0.72
Aspect ratio	4196.19	2593.12	1.62	53.00	0.11										
Wing area						0.23	3.31	0.07	53.00	0.95					
Mass											217.84	2142.62	0.10	52.00	0.92

Random Effects

σ^2	1636169.90	1708210.95	1687429.19
τ_{00}	895708.86 $_{\text{lineage}}$	963678.78 $_{\text{lineage}}$	857181.79 $_{\text{lineage}}$
ICC	0.35	0.36	0.34
N	11 $_{\text{lineage}}$	11 $_{\text{lineage}}$	11 $_{\text{lineage}}$
Observations	60	60	59
Marginal R^2 / Conditional R^2	0.097 / 0.416	0.067 / 0.404	0.077 / 0.388

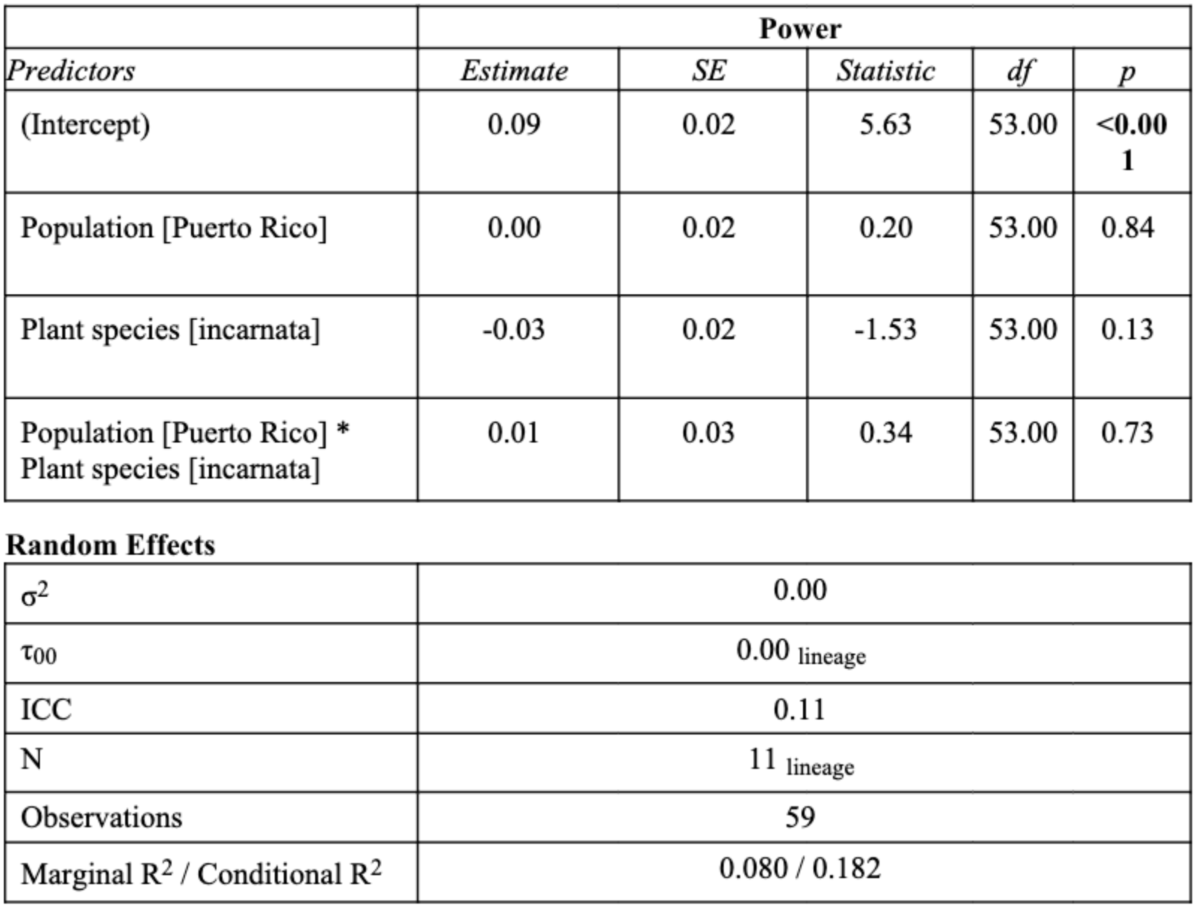
Table S4. Full model results examining the impact of population, plant species, interaction between population and plant species, and the wing aspect ratio, wing area, or mass on flight duration.

	Duration					**Duration**					**Duration**				
Predictors	*Estimate*	*SE*	*Statistic*	*df*	*p*	*Estimate*	*SE*	*Statistic*	*df*	*p*	*Estimate*	*SE*	*Statistic*	*df*	*p*
(Intercept)	-62.70	61.00	-1.03	53.00	0.31	57.86	36.93	1.57	53.00	0.12	60.40	17.15	3.52	52.00	**<0.001**
Population [Puerto Rico]	-11.15	7.65	-1.46	53.00	0.15	-10.33	7.97	-1.30	53.00	0.20	-10.06	7.82	-1.29	52.00	0.20
Plant species [incarnata]	-0.84	8.07	-0.10	53.00	0.92	-6.45	10.25	-0.63	53.00	0.53	-6.05	8.55	-0.71	52.00	0.48
Population [Puerto Rico] * Plant species [incarnata]	6.67	9.10	0.73	53.00	0.47	6.66	9.51	0.70	53.00	0.49	5.53	9.40	0.59	52.00	0.56
Aspect ratio	57.52	30.41	1.89	53.00	0.06										
Wing area						-0.01	0.04	-0.16	53.00	0.88					
Mass											-13.45	25.15	-0.54	52.00	0.59

Random Effects

σ^2	229.95	244.30	242.45
τ_{00}	67.50 $_{\text{lineage}}$	73.35 $_{\text{lineage}}$	61.70 $_{\text{lineage}}$
ICC	0.23	0.23	0.20
N	11 $_{\text{lineage}}$	11 $_{\text{lineage}}$	11 $_{\text{lineage}}$
Observations	60	60	59
Marginal R^2 / Conditional R^2	0.083 / 0.291	0.039 / 0.261	0.043 / 0.237

Table S5. Full model results examining the impact of population, plant species, interaction between population and plant species on flight power. Because mass is included in the power calculation, no morphometric is included in the model.

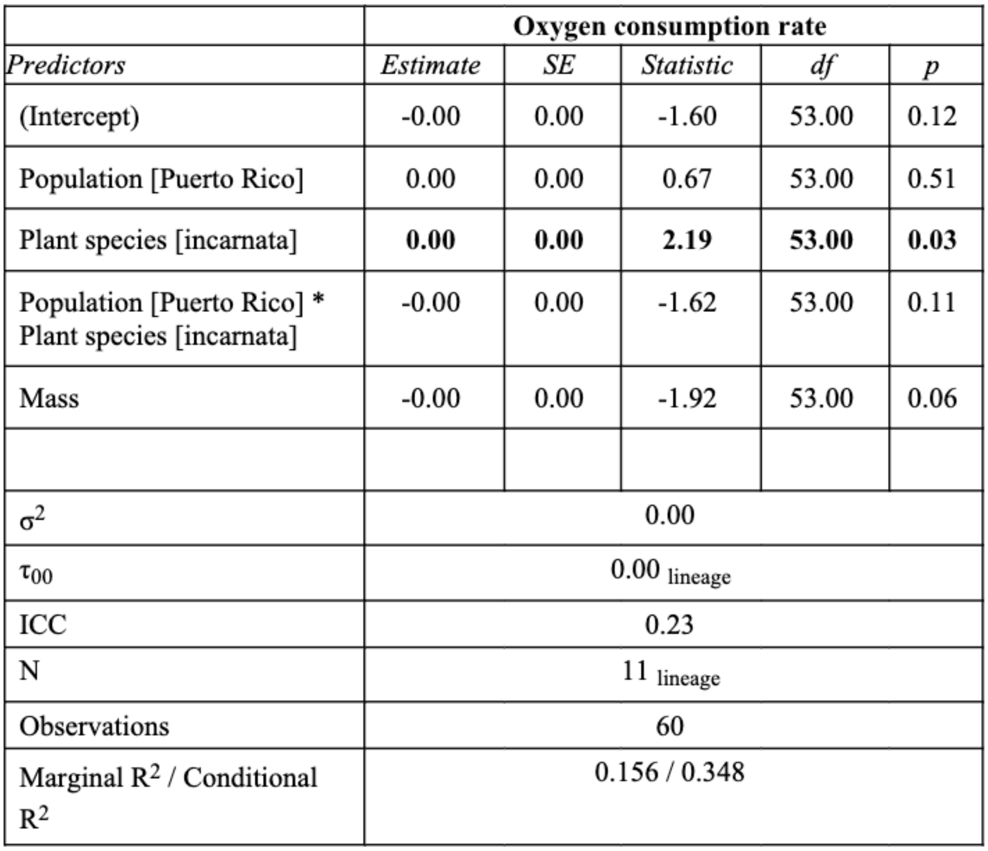


	Power				
Predictors	*Estimate*	*SE*	*Statistic*	*df*	*p*
(Intercept)	0.09	0.02	5.63	53.00	**<0.001**
Population [Puerto Rico]	0.00	0.02	0.20	53.00	0.84
Plant species [incarnata]	-0.03	0.02	-1.53	53.00	0.13
Population [Puerto Rico] * Plant species [incarnata]	0.01	0.03	0.34	53.00	0.73

Random Effects

σ^2	0.00
τ_{00}	0.00_{lineage}
ICC	0.11
N	11_{lineage}
Observations	59
Marginal R^2 / Conditional R^2	0.080 / 0.182

Table S6. Full model results examining the impact of population, plant species, interaction between population and plant species, and mass on oxygen consumption rate.



	Oxygen consumption rate				
Predictors	*Estimate*	*SE*	*Statistic*	*df*	*p*
(Intercept)	-0.00	0.00	-1.60	53.00	0.12
Population [Puerto Rico]	0.00	0.00	0.67	53.00	0.51
Plant species [incarnata]	**0.00**	**0.00**	**2.19**	**53.00**	**0.03**
Population [Puerto Rico] * Plant species [incarnata]	-0.00	0.00	-1.62	53.00	0.11
Mass	-0.00	0.00	-1.92	53.00	0.06
σ^2	0.00				
τ_{00}	0.00 $_{\text{lineage}}$				
ICC	0.23				
N	11 $_{\text{lineage}}$				
Observations	60				
Marginal R^2 / Conditional R^2	0.156 / 0.348				

Chapter 2: Monarch butterflies from two populations display similar flight performance.

Ali Ebada

Department of Biology, Emory University, Atlanta, GA 30322, USA

Introduction

Animal migration has been found to face a decline throughout the animal kingdom. Population numbers of migratory songbirds, for example, have been found to be on a steadfast decrease in recent decades and species of migratory antelope have been found to sustain population losses as high as 95% over the past two decades (Horns & Şekercioğlu, 2018). Anthropogenic stressors such as habitat loss, overexploitation, and climate change have all been suggested to affect the success of migratory species, and oftentimes these pressures significantly impact the performance and effectiveness of migratory animals to successfully complete their journeys as their fitness is disproportionately challenged.

Like many species, the size of Mexico's overwintering migratory monarch butterfly (*Danaus plexippus*) colonies has steadily declined amidst an increase in non-migratory populations, suggesting that monarch migration is an endangered phenomenon (Brower et al., 2012). As the decline unfolds, there continues to be a lack of understanding of how sedentary populations drive migration loss in terms of flight behavior and efficiency.

Previous work has demonstrated a decline in the number of overwintering monarchs in central Mexico; more specifically, studies have observed a significantly decreased, albeit steady, monarch density in Mexico that follows a 15-year downward trend (Brower et al., 2012). At the same time, the incidence of non-migratory, sedentary monarch populations that breed throughout the year has increased, especially along the Gulf of Mexico and the Atlantic Coast (Satterfield et al., 2015). These growing sedentary populations may have potentially negative consequences for monarch migration since migratory and sedentary monarchs are known to interact and could potentially exchange genes related to lower flight efficiency.

The decrease in migrating monarchs and increase in sedentary populations has been attributed to a variety of causes. For example, it has been hypothesized that there has been significant decrease in migratory eastern North American monarch populations due to increasingly extreme weather events (Flockhart et al., 2015b). Other studies suggest that there has been a decrease in the availability of milkweed and habitat loss in the monarchs' central Mexican overwintering sites (Brower et al., 2012; Flockhart et al., 2015a; Hartzler, 2010). As the migratory population has declined, the number of non-migratory, sedentary monarch populations has increased over time due to increasing average temperatures from climate change and the increased prevalence of non-native milkweed being planted by humans in residential areas (Satterfield et al., 2015). Previous studies have found that consumption of non-native milkweed influences year-round reproductive behavior, for example, and thus the planting of such milkweed could explain the establishment of sedentary, non-migratory populations (Majewska & Altizer, 2019). One such population has been established in Savannah, Georgia (Majewska et al., 2019). Beyond many aforementioned stressors that can affect successful monarch migration, Majewska et al., 2019 found that the sedentary population of monarchs in Savannah presents a significantly higher infection rate of the protozoan parasite *Ophryocystis elektroscirrha*, a highly detrimental organism that has been found to significantly impact the survival, reproductive success, and flight performance of monarchs (Altizer & Oberhauser, 1999; Satterfield et al., 2015).

By studying the sedentary population in Savannah and comparing it to nearby migratory monarchs in St. Marks, the project will investigate the effects of losing migratory behavior on the species by determining the extent to which non-migratory populations have experienced a reduced flight ability, which may be expected given that they no longer are selected to complete a long-distance migratory journey. Here, we perform a flight metric comparative analysis to compare the flight performance between monarchs from the migratory eastern North America population and monarchs from the non-migratory population found in Savannah, Georgia. The factor of a higher infection rate in sedentary monarchs in Savannah in comparison to non-migratory monarchs was also put into account in this study, where we compared monarchs not only on the basis of population origin but also on infection status.

We hypothesized in this study that monarchs from the sedentary population in Savannah would display a significantly less efficient flight performance when compared to their migratory counterparts. Additionally, we expected that monarchs that displayed significant infection by *Ophryocystis elektroscirrha* would display decreased flight performance. Ultimately, revealing flight-related shortcomings of non-migratory populations is an essential step in formulating an action plan on how to challenge the decrease in the migratory monarch population; it allows the scientific community to gain an increased understanding of how detrimental the loss of migratory behavior may be on monarchs.

Materials and Methods

Monarch Sourcing

We used monarch butterfly adults captured in St. Marks, Florida (migratory eastern North American population) in October 2022 and in Savannah, Georgia (non-migratory Savannah population) in November 2022. The eastern North American monarchs were collected during peak of Fall migration in St. Marks, Florida, a major flyway and stopover site for monarchs originating from across eastern North America. Thus, the monarchs collected in St. Marks amid migration are representative of the entire eastern North American population and were not part of the breeding population in Florida. All monarchs were screened for the parasite *Ophryocystis elektroscirrha* following Altizer et al. 2000. We considered only monarchs with spore scores of a 4 or 5 as "infected." All monarchs used in the study were adult and wild-caught; thus, there was no caterpillar rearing process comparable to the one discussed in *Chapter 1*.

Flight Trials and Metrics

Flight trials and metabolic rate acquisition were performed following Schroeder et al., 2020, identical to the protocol conducted in *Chapter 1*. A detailed description of wire attachment, flight mill, and equipment can be found under *Chapter 1 Supporting Information*. We attached a steel wire to the dorsal thorax and acclimated all monarchs to the wire for 48-72 hours in a mesh cage with 20% honey water ad libitum. Trials were performed indoors during October-November 2022 using two flight mills in a windowless room between 0900 and 1800 hrs.

We used two flight mills with identical assemblies to maximize the number of monarchs that could be flown per day. The flight mill selection for each monarch was randomized. Prior to a trial, we weighed the monarch with the wire using a digital scale and measured resting oxygen consumption rate for 5 min with an O_2 Sensor (The Vernier O_2Gas Sensor). To initiate a flight trial, we attached a monarch to the carbon rod of the flight mill. Data was collected via the PASCO® Capstone software connected to a wireless photogate which detected passage of a flag on the rod, opposite to the monarch. Monarchs flew to exhaustion but no more than 60 minutes due to logistical constraints. The 60-minute time cut-off did not limit the sample size and permitted us to fly all monarchs within one week of emergence. We considered a successful flight to be at least 5 minutes. Immediately after flight, we measured oxygen consumption for 5 minutes to attain post-flight rate and weighed monarchs with and without the wire.

For each monarch, we calculated the average velocity (m per sec), duration (min), distance traveled (m), and power (Watts). For velocity we disregarded the first five minutes of flight as monarchs appeared to fly quickly to escape the handler. Distance traveled was quantified as the number of revolutions times the circumference of the circle flown. We calculated power of each revolution by dividing the estimated kinetic energy (½ x mass x velocity^2) by time and calculated an average for the flight.

Statistical Analyses

Statistical analyses were performed using R computing language (R Core Team, 2022). We examined whether site of collection and infection status impacted speed, duration of flight, distance of flight, and power of flight. Thus, a general linear model with no random effects was run to compare the flight metrics and oxygen consumption rates between monarchs based on region of collection (eastern North American in St. Marks vs. sedentary Savannah monarchs) and infection status (infected vs. non-infected).

Results

Eastern North American monarchs captured from St. Marks and sedentary monarchs captured from Savannah, irrespective of infection status, displayed similar flight performance in velocity ($p = 0.2399$, Figure 1A), distance traveled ($p = 0.4467$, Figure 1B), flight duration ($p = 0.5022$, Figure 1C), flight power ($p = 0.5841$, Figure 1D), and oxygen consumption rate ($p = 0.4612$, Figure 1E). When comparing only infected monarchs based on capture location, there was a marginal difference in velocity ($p = 0.09895$, Figure 2A) and flight power ($p = 0.1194$, Figure 2D). However, all other metrics displayed similar flight performance between the two locations for infected monarchs in distance traveled ($p = 0.6321$, Figure 2B), flight duration ($p = 0.7914$, Figure 2C), and oxygen consumption rate ($p = 0.277$, Figure 2E)

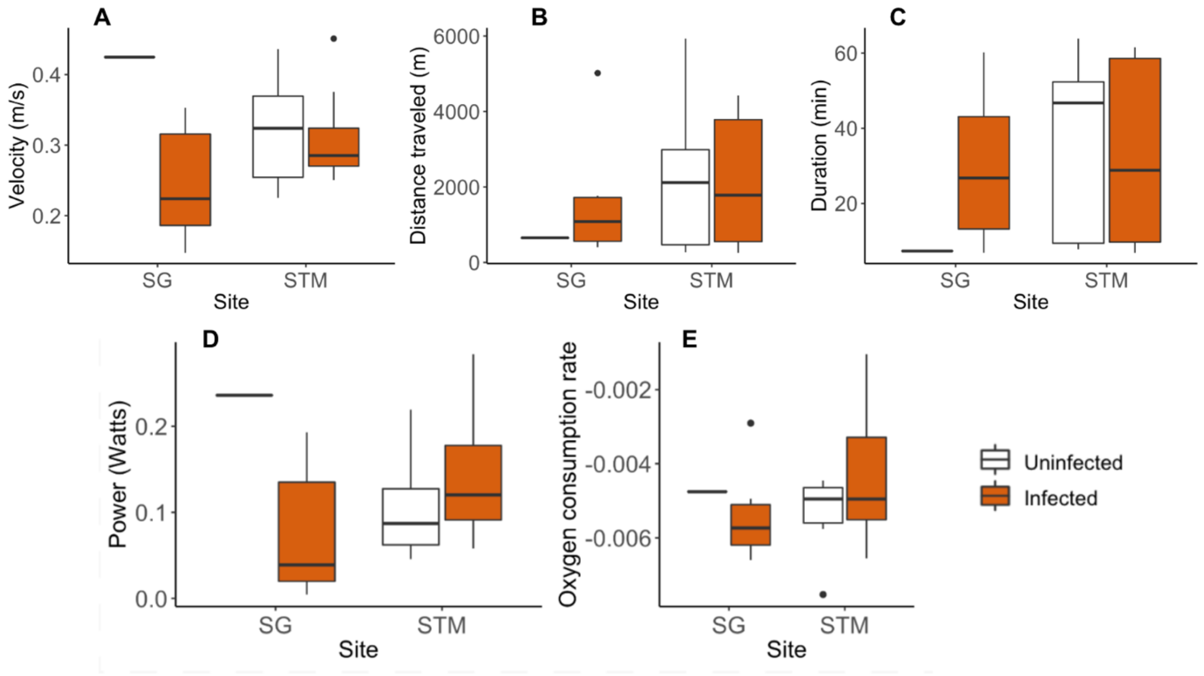


Figure 1. Flight metrics of monarchs from eastern North America (STM) and Savannah, GA (SG)*:* (A) velocity, (B) total distance traveled, (C) total flight duration, (D) flight power, and (E) post-flight oxygen consumption rate. Orange fill indicates that the monarchs were infected with OE and white fill indicates the lack of infection. The top horizontal bar represents the third quartile, followed by the median and the first quartile. Each whisker indicates the full data range.

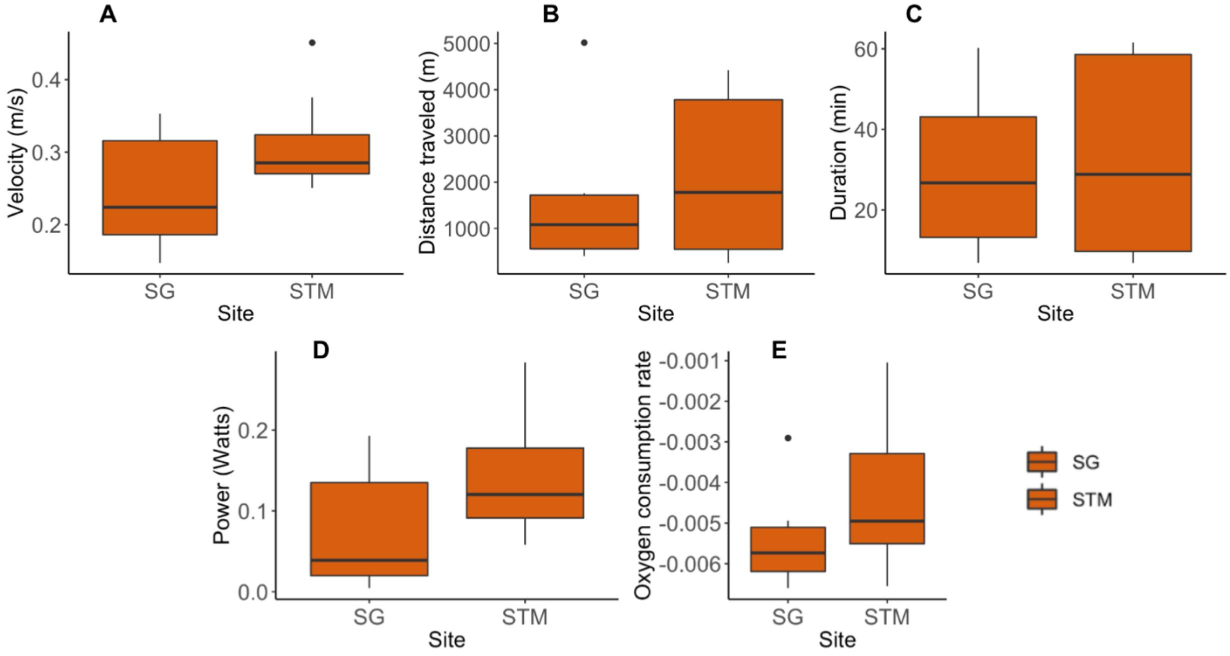


Figure 2. Flight metrics of infected monarchs from eastern North America (STM) and Savannah, GA (SG)*:* (A) velocity, (B) total distance traveled, (C) total flight duration, (D) flight power, and (E) post-flight oxygen consumption rate. The top horizontal bar represents the third quartile, followed by the median and the first quartile. Each whisker indicates the full data range.

Discussion

Our results indicate that there is generally no difference in the flight performance between migratory eastern North American monarchs and the sedentary population present in Savannah, Georgia, as measured on the tethered flight mill that we used. Many studies have found that monarchs with a particular set of morphometric characteristics, such as larger, more elongated wings with higher aspect ratios, have more successful flight performances (Satterfield & Davis, 2015). Given the results of this study, it can be inferred that monarchs originating from the Savannah population may not possess large enough physical differences to confer differential flight performance from the migratory monarchs derived from the eastern North American population. For example, there may have not been enough time for the sedentary population in Savannah and the migratory population in St. Marks to diverge and develop different traits given the recent forming of the non-migratory population. Further, there may be the existence of mixing of migratory monarchs and resident monarchs in Savannah, thus diluting any differences that may exist.

Previous studies have found, when considering the same population of monarchs, that monarchs display more successful migration when presenting lower infection scores from *Ophryocystis elektroscirrha* (Altizer et al., 2015). However, Altizer et., 2015 was an experimental study that involved the inoculation of monarchs. In contrast, our study involved wild-caught monarchs that, despite having sustained a natural *Ophryocystis elektroscirrha* infection, had made significant progress during their migratory journey. In our study, while an analytical comparison in flight performance between infected versus uninfected monarchs could have been informative, the sample size of uninfected monarchs was lacking to allow for statistical significance.

Overall, the findings of this study may suggest that the existence of non-migratory populations of monarchs may not arise due to intrinsic difference. A variety of differential phenotypes exist between the sedentary Savannah population and the migratory eastern North American population. For example, monarchs in the Savannah population exhibit year-round reproductive activity and a lack of hormonally induced reproductive diapause, a metabolic phenomenon that confers the proper allocation of metabolic resources to allow for migratory flight (Majewska & Altizer, 2019). Savannah monarchs do not migrate. This is in contrast with monarchs from the eastern North American population, which do exhibit reproductive diapause throughout migratory-inducing months (fall and winter) and do migrate. Because these phenotypic differences, we hypothesized that flight performance difference would also exist, which was not the case. Thus, rather than intrinsic differences, the increasing prevalence of non-migratory populations may be explained by extrinsic factors, including climate change, non-native milkweed planting, and habitat loss, that cause monarchs to exhibit behavioral changes that encourage a tendency to remain sedentary amid a migration season. While this study may not have found obvious, significant differences in flight performances between the populations under study, it still provides an insight into the physiological differences that may or may not exist between behaviorally distinct populations. In the future, a wing morphometric analysis will be conducted to allow for an analysis of possible anatomical differences present between the populations under study.

Chapter 3: Gone with the Wind: Testing the efficacy of two experimental orientation procedures in the eastern North American monarch butterfly.

Ali Ebada

Department of Biology, Emory University, Atlanta, GA 30322, USA

Introduction

Migration is a behavior that numerous organisms rely on to escape the selective pressures of a seasonally changing environment; the phenomenon grants access to food, resources, and sustainable climate conditions (Dingle & Drake, 2007; Merlin et al., 2012). Generally, all animals, even non-migratory ones, must possess some degree of navigational skill to function normally. For example, through a combination of a neuronal compass and landmark cues, the fruit fly (*Drosophila melanogaster*) can draw a cognitive map of its environment and precisely navigate through it (Clandinin & Giocomo, 2015). However, migratory behavior confers a selective pressure that particularly impacts the necessity of navigational ability. These migration-related orientation abilities are found across the animal kingdom, including in fish, birds, amphibians, and mammals (Milner-Gulland et al., 2011; Takahashi et al., 2022). Long-distance migration requires accurate navigational ability for an organism to successfully reach what is often a precise destination and thus many organisms display the ability to align their bodies along magnetic field lines; migratory streaked shearwater birds (*Calonectris leucomelas*), for example, have been found to possess head direction cells that function to confer an internal magnetic compass (Takahashi et al., 2022). While the physiological basis of navigation varies highly between taxa, migratory organisms have been consistently found to display these highly specialized capabilities.

Migratory insects have been generally found to rely on a variety of orientation mechanisms during navigation, including the observation of environmental cues and an ability to sense the Earth's magnetic field (Merlin et al., 2012; Shiozaki & Kazama, 2017). One notable example of a migratory insect, and its profound navigation ability, is the monarch butterfly (*Danaus plexippus*). These insects travel thousands of miles each year from across areas of the United States and southern Canada to escape the cooling conditions, dying host plants, and increased parasitic infection prevalence in the autumn to overwinter in central Mexico (Masters et al., 1988). Monarchs, along with other insects such as Drosophila, utilize a time-compensated sun compass; further, the butterfly species has been found to utilize magnetic cues in overcast conditions (Giraldo et al., 2018; Reppert et al., 2016). Furthermore, social interactions and geological barriers play a role in navigation in these insects (Reppert & de Roode, 2018). But while some genetic, physiological, and environmental factors that play a role in monarch migratory navigation have been thoroughly investigated in prior studies, their navigational ability remains elusive. To better understand monarch orientational capabilities, previous studies have employed a variety of experimental methods, oftentimes confined to observing monarch flight in an artificial setting. For example, past work has included the observation of monarchs in flight simulators, which indicated the existence of a time-compensated sun compass but did not suggest the presence of magnetic field sensory ability (Mouritsen & Frost, 2002). However, the observation of monarchs in an artificial setting may not necessarily give an accurate representation of monarch flight navigation, as it removes factors such as environmental obstacles and wind resistance.

In studies that employed observation of monarchs in the wild, the primary method was the use of vanishing bearings, which generally suggested that monarchs fly in a Southwesterly direction when observed in Northeastern regions of North America (Gibo, 1986; Schmidt-Koenig, 1979). However, it has been noted that the use of vanishing bearing data alone is a highly inefficient means to test navigational ability (Perez et al., 1999).

A method that has been consistently absent in the literature is the observation of confined wild monarchs in their natural setting (e.g., observing monarchs in a cage in the wild), allowing an observer to note the perching position of the monarchs to infer flight orientation.

Here we asked whether monarchs, when observed at a migratory stop over site in a cage and via vanishing bearings, display a pattern in orientation. In this study, we analyzed the eastern North American population of the monarch, which originates from areas in the United States and Canada and migrate each year to overwinter in Central Mexico. We observed a sample of this population in the migratory stopover site in St. Marks, Florida from 2019 to 2022 and placed captured adults in three distinct locations to observe if there was a significant pattern in their geographic orientation. Vanishing bearings data was only collected in 2022. In both methods, we hypothesized that monarchs would exhibit orientation that would allow navigation from St. Marks in the Florida panhandle westward along the Gulf of Mexico to successfully traverse towards central Mexico, which would present an approximate North-Northwest directionality. In this study, we aimed to present the efficacy of confined wild monarch observation and the vanishing bearing method in discerning monarch navigational orientation ability, opening the door for future research to better understand the migratory monarch phenomenon.

Materials and Methods

Experimental Location

The site of the experiment was a migratory stopover site in St. Marks, Florida amidst the peak of the eastern North American monarch migration season over the course of 4 years: October 2019, November 2020, October 2021, and October 2022. Every year (except 2020, when a subset was used), we used the same three locations coded “Coastal,” “Gazebo,” and “Lagoon” (Figure 1). The Coastal and Gazebo locations were noted for their similarity in being directly adjacent to the gulf coastline, whereas Lagoon left the monarchs moreso confined inland.

Figure 1. Satellite image of the experimental site used from 2019-2022. Three mesh cages in three distinct locations were placed at the site in St. Marks, FL. We coded the locations Coastal (1, blue), Gazebo (2, maroon), and Lagoon (3, orange). Color-coded stars indicate where the cages were approximately placed each year.

Mesh Cage Orientation Procedure

Adult eastern North American monarchs were captured and placed in each of three separate mesh cages; each mesh cage was assigned a particular experimental location. At points along the circumference of the circle, equidistant pieces of tape labeled with a number were placed, each indicating the trial number. The trial number in question was directed due North (0º). For example, in the first trial, 1 pointed due north (Figure 2). Table 1 lists the sample size and number of replicates for each mesh cage method trial. Data from the Lagoon location was not collected in 2020.

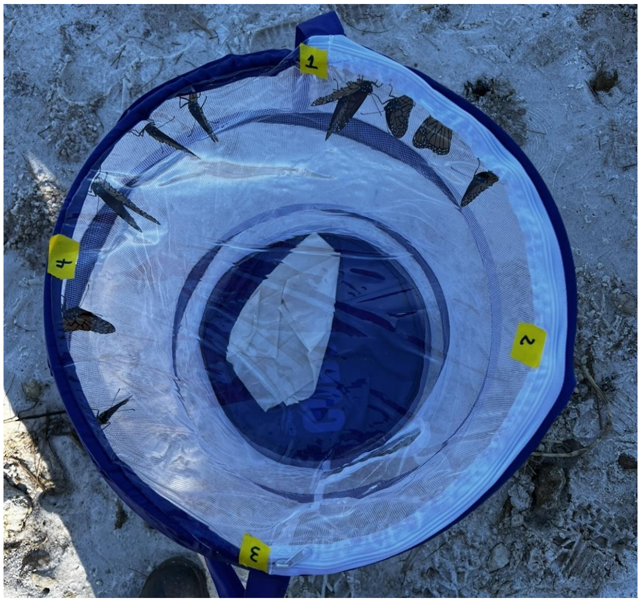


Figure 2. Mesh Cage from Gazebo, 2022. Each mesh cage contained 10 adult eastern North American monarchs. One cage per location was placed at the Coastal, Gazebo, and Lagoon locations. An observer took a picture at the conclusion of each trial to record perching position.

Table 1. Summary of the locations, years, sample size, and number of trials conducted across the four years this study was conducted. The Lagoon location was not included in 2020.

Location	Year	n	Number of Trials
Gazebo	2022	10	4
	2021	10	3
	2020	10	2
	2019	10	3
Coastal	2022	8	4
	2021	10	6
	2020	10	2
	2019	10	3
Lagoon	2022	10	4
	2021	10	3
	2020	N/A	N/A
	2019	10	3

Prior to the beginning of a trial, or between the conclusion of one and the beginning of another, the cage was lightly tapped to disturb the monarchs and dislodge them from their perching position. Following the conclusion of their disturbance, monarchs were allowed to settle for 20 minutes. At the end of the 20-minute mark, an observer took a photo and noted the position of all monarchs perched along the cage walls.

Vanishing Bearings Procedure

In 2022, following the completion of the orientation experiment using the mesh cages, 10 monarchs were released in individual order. Observers used their naked eye and a compass to note the cardinal direction of the monarch at the point when the monarch was no longer visible over the horizon, indicating its vanishing bearing. This process was repeated at all three experimental locations, with 10 monarchs being released from the mesh cage at Lagoon and Gazebo and 9 monarchs being released from the mesh cage at Coastal.

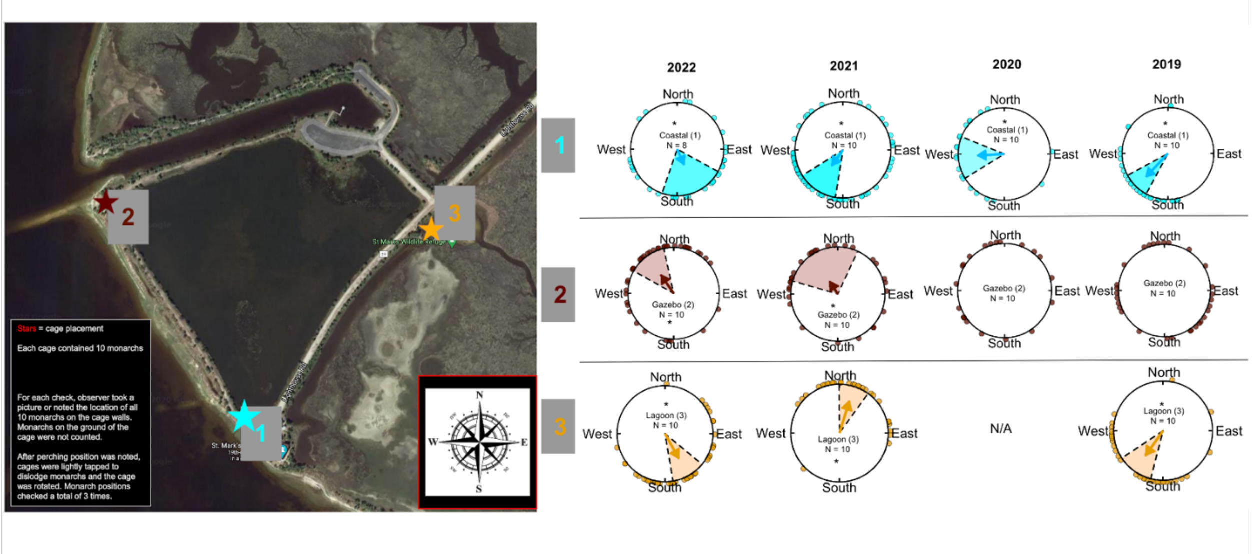
Image Compilation and Statistical Analyses

Photographs taken at the conclusion of each trial were imported and analyzed in the ImageJ software (Fiji, 2022), which was used to measure the angular position of each monarch. Each photograph was intentionally taken above the cage to display an approximate circle. The coordinates of the midpoint of each circle were measured in ImageJ, which allowed for approximation of the angle along the circle each monarch was positioned. Each position was measured in degrees, with 0° representing due North.

Statistical analyses were performed using R computing language (R core team, 2022). To analyze if monarchs displayed directionality, we asked if the group of monarchs in each mesh cage were significantly clustered, suggesting a pattern in orientation. To examine if there was significant clustering/directionality (versus a uniform distribution of the monarchs in the mesh cage), we conducted a Rayleigh's test given the circular nature of the dataset (package *circular*). The test statistic (r) for a Rayleigh's test is represented from 0 to 1 alongside a p-value. Assuming statistical significance ($\alpha < 0.05$), a Rayleigh's r-value closer to 1 suggests a stronger, more directed cluster. Furthermore, the mean orientation in degrees was recorded for each trial, alongside a 95% confidence interval. Figures were produced with arrow vectors, with the length of each vector representing the r-value from the Rayleigh's test.

Results

All results derived from the mesh cage experiment are summarized in Table 2, with a model of all locations with significant clustering shown in Figure 3. All monarch groups in 2022 showed statistically significant clustering. At Gazebo, the mean orientation was 352.2º ($r = 0.4882$, $p < 0.0001$), at Coastal the mean orientation was 156.3º ($r = 0.3651$, $p = 0.013$), and at Lagoon the mean orientation was 150.8º ($r =0.5903$, $p < 0.0001$). All monarch groups also presented statistically significant clustering in 2021. At Gazebo, the mean orientation was 326.5º ($r = 0.3201$, $p = 0.0449$). At Coastal, the mean orientation was 215.8º ($r = 0.4032$, $p < 0.0001$) and at Lagoon, the mean orientation was 17.04º ($r = 0.6898$, $p < 0.0001$). In 2020, monarchs only showed statistically significant clustering at Coastal, with a mean orientation of 267.0º ($r = 0.5851$, $p < 0.0001$). In 2019, Monarchs showed significant clustering only at Coastal and Lagoon. At Coastal, the mean orientation was 223.3º ($r = 0.6998$, $p < 0.0001$) and at Lagoon, the mean orientation was 215.4º ($r = 0.6173$, $p < 0.0001$).



Stars = cage placement

Each cage contained 10 monarchs

For each check, observer took a picture or noted the location of all 10 monarchs on the cage walls. Monarchs on the ground of the cage were not counted.

After perching position was noted, cages were lightly tapped to dislodge monarchs and the cage was rotated. Monarch positions checked a total of 3 times.

Figure 3. A pictorial representation of the perching orientation of monarch groups in 2019, 2020, 2021, and 2022 for Coastal (1), Gazebo (2), and Lagoon (3) alongside a satellite map with the cardinal directions displayed on

both the satellite image and each individual cage model. Color-coded stars on the map indicate where the mesh cage was approximately placed on site by the observer, with blue representing Coastal, maroon representing Gazebo, and orange representing Lagoon. The length of the arrow vector represents Rayleigh's r-value or the strength of the clustering and orientation. Colored shading represents the 95% confidence interval. A sample size of 10 was conducted for all years and cages, except for the Coastal location in 2022. All figures except for Gazebo 2020 and Gazebo 2019 display significant clustering (significance is indicated by "*" located in each circle). Locations without significant clustering do not have a mean direction or confidence interval represented. The Lagoon location was not included in 2020, and thus is indicated as "N/A."

Data derived from the vanishing bearings trials, which were all conducted in 2022, presented that Coastal did not display significant clustering ($p = 0.5044$). Gazebo ($r = 0.7416$, $p = 0.002$, 95% CI: 208.86º - 244.38º) and Lagoon ($r = 0.7807$, $p < 0.001$, 95% CI: 221.32º - 271.27º) did display significant clustering, with a mean orientation of 230.5358º and 242.355º, respectively. Results from the vanishing bearings are summarized in a visual format in Figure 4.

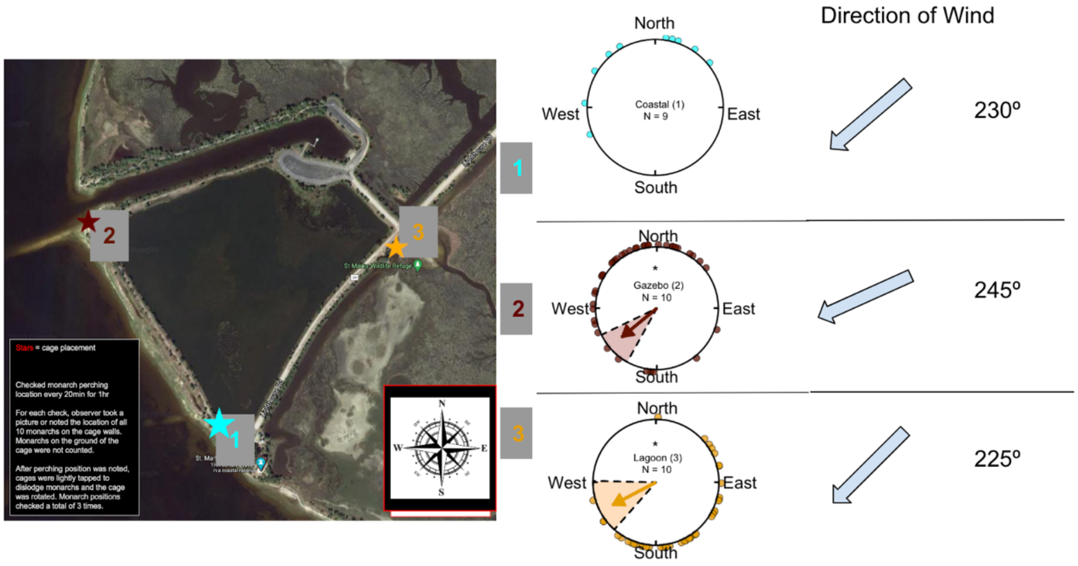


Figure 4. A pictorial representation of the vanishing bearing orientation for Coastal (1), Gazebo (2), and Lagoon (3) alongside a satellite map with the cardinal directions displayed on both the satellite image and each individual cage model in 2022. Color-coded stars on the map indicate where the mesh cage was approximately placed on site by the observer, blue representing Coastal, maroon representing Gazebo, and orange representing Lagoon. The length of the arrow vector represents Rayleigh's r-value or the strength of the clustering and orientation. Colored shading represents the 95% confidence interval. A sample size of 10 was conducted for all years and cages, except for the Coastal location. Significance is indicated by "*" located in each circle; only Gazebo and Lagoon indicated significant clustering. Locations without significant clustering do not have a mean direction or confidence interval represented. The direction of the wind at the time of the trial is represented adjacent to each figure.

Table 2. Summary of the mean orientations, Rayleigh's test statistics, and statistical significance for the three locations from 2019-2020. Rayleigh's test r-values denote the strength of directionality over 360º, with 1 being the most clustering and directionality of monarchs and 0 being a uniform distribution of monarchs. Due North was considered 0º in this model, with 90º due East, 180º due South, and 270º due West. Rayleigh's r-values and mean orientations were not considered for statistically insignificant clustering results (α = 0.05). The interval considered was a 95% confidence interval.

Location	Year	n	r	Mean Orientation (º)	Interval (º)	p-value
Gazebo	2022	10	0.4882	325.2	299.0-347.5	< 0.0001
	2021	10	0.3201	326.5	285.9-25.04	0.0449
	2020	10	0.2620	127.91	28.29-227.53	0.2563
	2019	10	0.0656	315.37	300.47-330.27	0.8807
Coastal	2022	8	0.3651	156.3	118.7-199.0	0.013
	2021	10	0.4032	215.8	189.1-237.9	< 0.0001
	2020	10	0.5851	267.0	238.3-292.0	< 0.0001
	2019	10	0.6998	223.3	208.3-241.6	< 0.0001
Lagoon	2022	10	0.5903	150.8	129.4-170.4	< 0.0001
	2021	10	0.6898	17.04	359.2-35.90	< 0.0001
	2020	N/A	N/A	N/A	N/A	N/A
	2019	10	0.6173	215.4	194.5-236.5	< 0.0001

Discussion

Our results, both in the mesh cage trials and vanishing bearings methodology, did not support our expected flight orientation of North-Northwest directionality, which would allow monarchs to traverse along the Gulf of Mexico towards their migratory destination in Central Mexico. The 95% confidence intervals for all trials, both mesh cages and vanishing bearings, did not consistently have the NNW degree direction, approximately (337.5º) within their 95% confidence interval except for only two trials: the mesh cage experiments at Gazebo in 2022 and 2021. To further investigate the breadth of data collected during the mesh cage trials, an analysis of each individual trial was observed for all three locations (Figures S1-3 in *Chapter 3*

Supporting Information). Again, the data present no clear patterns and an overall lack in consistent orientation towards the NNW direction throughout the different locations, years, and trials. Furthermore, trials conducted later in the same location did not have more significant clustering than earlier trials, indicating that the time constraint of 20 minutes placed on each trial was not insufficient to observe monarch perching orientation. Additionally, the individual trials generally follow a similar trend to the overall trend observed at each location/year. This lack of consistency throughout several years and trials of directionality observations does not provide compelling evidence that the monarchs observed in our study displayed a preference to perch or orient themselves in the expected North-Northwest direction that would allow the continuation of their migratory journey.

Previous work has consistently found that a variety of insect species display navigational ability. For example, cockroaches display antennae-based obstacle navigation and *Drosophila* has been noted to have the ability of visual landmark orientation (Fisher, 2022; Guerra, 2020; Reppert & de Roode, 2018). A variety of similar physiological attributes could explain how monarchs too navigate during migration. In addition to magnetic field awareness, sensory inputs both from the sun (e.g., a time-compensated sun compass) and the observation of environmental landmarks have been observed to affect monarch navigational ability; further, monarchs with their antennae removed lose their ability to orient themselves Southward, suggesting that the source of their orientation-related capabilities may also originate from neurological input derived from their antennae; relatedly, human-related sources of light pollution have been found to impede their migratory behavior given the interference with their sun-based environmental cue system (Guerra & Reppert, 2015; Parlin et al., 2022). The orientational capability of monarchs is not limited to long-distance migration; monarchs use cues from their environment to locate food sources. For example, monarchs have demonstrated the ability to parse out their milkweed food source amongst a diverse ecosystem based on environmental cues (Baker & Potter, 2019). In all respects, monarch spatial awareness is the consequence of a variety of traits and stimuli-response systems (Green, 2021).

However, despite the plentiful evidence of the physiological ability of monarchs to display successful navigational orientation, this study did not provide direct evidence of a consequence of the conglomeration of these traits. Rather, instead of our work providing evidence in support of monarch directionality, the data may instead suggest that the experimental means by which monarchs were observed were not effective in properly simulating monarch orientation. That is, confining monarchs to mesh cages within their migratory environment may result in a variety of confounding variables that prevent the accurate observation of monarch orientation. For example, the significant clustering that was observed in our mesh cage trials may be moreso social roosting behavior rather than the monarchs perching in their intended flying direction. Monarchs have been observed to roost in large groups in response to social cues in a variety of settings, including amidst migration (Aikins, 2022). Furthermore, the nature of leaving monarchs in a cage may subject them to avoidant-related behavior; for example, the presence or shadow of the observer themselves may influence the monarch perching position as a means of avoidance. The experimental location in St. Marks is a common tourist site, so the presence of humans in general may have influenced where the monarchs position themselves if disturbed by a person.

Additionally, the lack of the expected result in the vanishing bearings data (monarchs did not fly NNW) may also be the result of confounding factors affecting monarch flight orientation. Firstly, monarchs who were released did not all horizon and vanish, thus their recorded position may not have always accurately reflected their ultimate navigational orientation. While it may appear that our data does support previous work using the vanishing bearings method, which suggested that monarchs display a Southwesterly directionality, these previous studies were done much further north, near Ontario, and thus do not accurately reflect the required direction monarchs would need to travel in the Florida panhandle to successfully reach Mexico (Schmidt-Koenig, 1979). Factors such as strong winds may have affected the positioning of the monarch within the small span of time the vanishing bearing was observed and recorded; past studies utilizing vanishing bearings have noted the issue of wind drift confounding orientational data collection (Schmidt-Koenig, 1979). Our data supports these previous findings, which show a similar directionality in the vanishing bearings as the direction of the wind at the time of the trial. Thus, in addition to mesh cage trials, our data suggests that vanishing bearings may not be an accurate means to record monarch orientation.

Chapter 3 Supporting Information

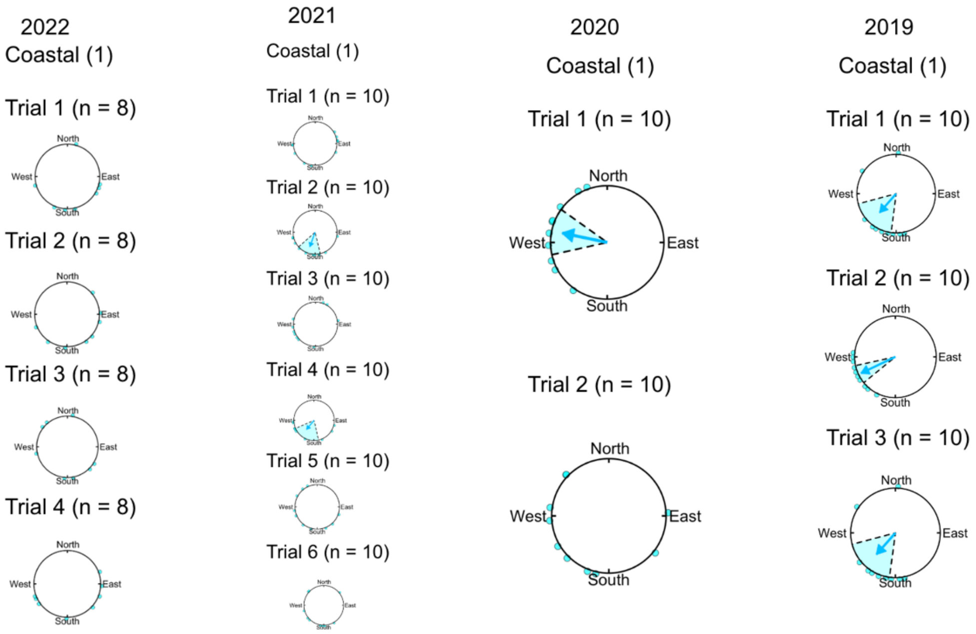


Figure S1. A pictorial representation of the perching orientation of monarch groups in 2019, 2020, 2021, and 2022 for each individual trial conducted at the Coastal location. The length of the arrow vector represents Rayleigh's r-value or the strength of the clustering and orientation. Colored shading represents the 95% confidence interval. Sample size is indicated adjacent to each trial. Only trials that presented significant clustering ($p < 0.05$) are depicted with an arrow and confidence interval shading.

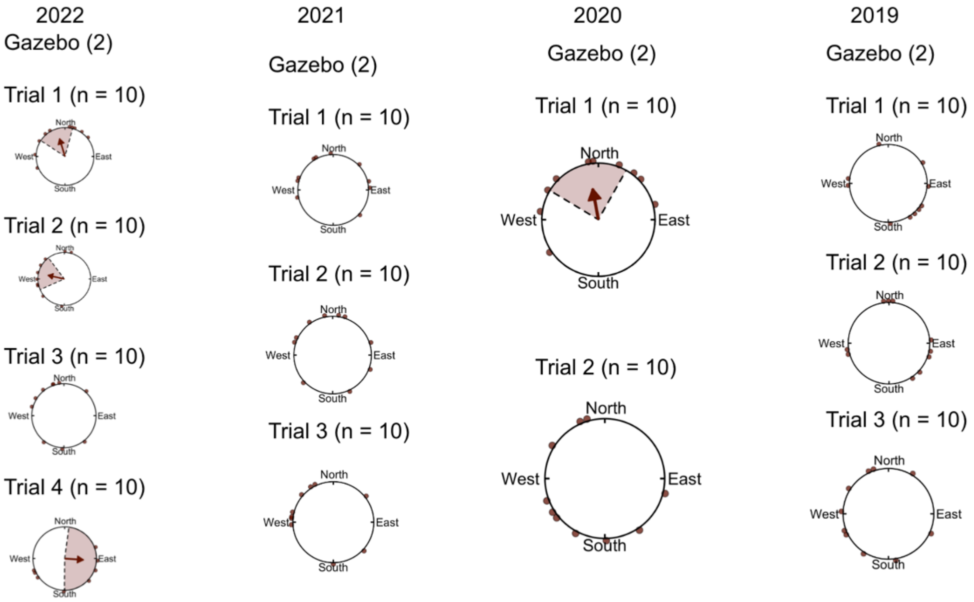


Figure S2. A pictorial representation of the perching orientation of monarch groups in 2019, 2020, 2021, and 2022 for each individual trial conducted at the Gazebo location. The length of the arrow vector represents Rayleigh's r-value or the strength of the clustering and orientation. Colored shading represents the 95% confidence interval. Sample size is indicated adjacent to each trial. Only trials that presented significant clustering ($p < 0.05$) are depicted with an arrow and confidence interval shading.

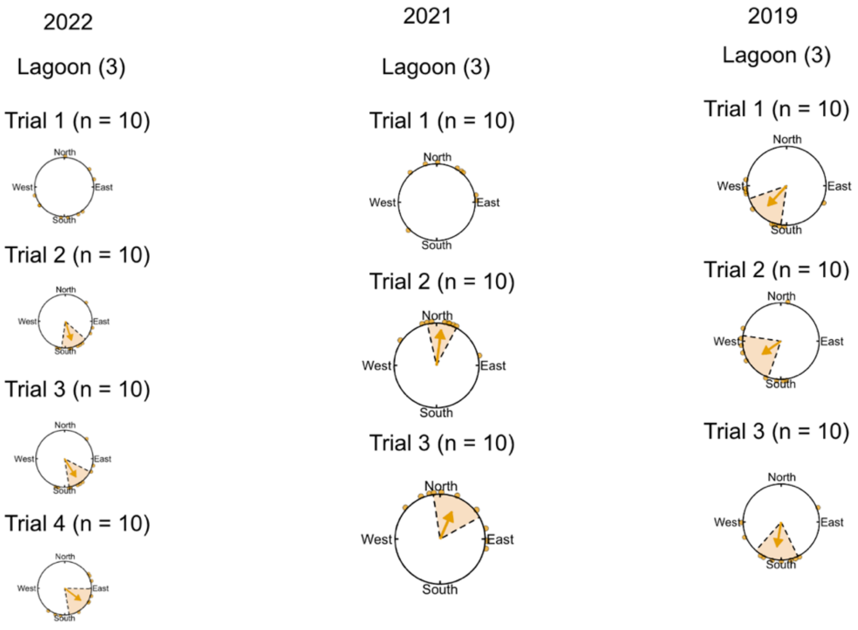


Figure S3. A pictorial representation of the perching orientation of monarch groups in 2019, 2021, and 2022 for each individual trial conducted at the Lagoon location. Lagoon was not observed in 2020. The length of the arrow vector represents Rayleigh's r-value or the strength of the clustering and orientation. Colored shading represents the 95% confidence interval. Sample size is indicated adjacent to each trial. Only trials that presented significant clustering ($p < 0.05$) are depicted with an arrow and confidence interval shading.

References

Chapter 1

Agrawal, A., Ali, J., Rasmann, S., & Fishbein, M. (2015). Macroevolutionary trends in the defense of milkweeds against monarchs: latex, cardenolides, and tolerance of herbivory. In Monarchs in a changing world: biology and conservation of an iconic butterfly. (ed. by K. Oberhauser, K. Nail & S. Altizer), pp. 47-59. Cornell University Press, Ithaca, New York.

Alaidrous, W., Villa, S.M., de Roode, J.C., & Majewska, A.A. (2022) Crowding does not affect monarch butterflies' resistance to a protozoan parasite. *Ecology and Evolution*, **12**, e8791.

Ali, J.G. & Agrawal, A.A. (2012) Specialist versus generalist insect herbivores and plant defense. *Trends in plant science*, **17**, 293-302.

Altizer, S. & Davis, A.K. (2010) Populations of monarch butterflies with different migratory behaviors show divergence in wing morphology. *Evolution*, **64**, 1018-1028.

Büyükyilmaz, E. & Tseng, M. (2022) Developmental temperature predicts body size, flight, and pollen load in a widespread butterfly. *Ecological Entomology*, **47**, 872-882.

Chen, M.S. (2008) Inducible direct plant defense against insect herbivores: a review. *Insect science*, **15**, 101-114.

Couture, J., Mason, C., Habeck, C., & Lindroth, R. (2016) Behavioral and morphological responses of an insect herbivore to low nutrient quality are inhibited by plant chemical defenses. *Arthropod-Plant Interactions*, **10**, 341-349.

Damos, P. & Savopoulou-Soultani, M. (2012) Temperature-driven models for insect development and vital thermal requirements. *Psyche*, **2012**.

Flockhart, D., Fitz-Gerald, B., Brower, L.P., Derbyshire, R., Altizer, S., Hobson, K.A., Wassenaar, L.I., & Norris, D.R. (2017) Migration distance as a selective episode for wing morphology in a migratory insect. *Movement ecology*, **5**, 1-9.

Goldsworthy, G.J. & Wheeler, C.H. (1989) Insect flight CRC Press, Boca Raton, Florida.

Malcolm, S.B. & Zalucki, M.P. (1996). Milkweed latex and cardenolide induction may resolve the lethal plant defence paradox. In Proceedings of the 9th International Symposium on Insect-Plant Relationships (ed. by E. Städler, M. Rowell-Rahier & R. Bauer), pp. 193-196. Springer Netherlands, Dordrecht.

Miller, N.G., Wassenaar, L.I., Hobson, K.A., & Norris, D.R. (2012) Migratory Connectivity of the Monarch Butterfly (Danaus plexippus): Patterns of Spring Re-Colonization in Eastern North America. *PLOS ONE*, **7**, e31891.

Paul, R.L., Pearse, I.S., & Ode, P.J. (2021) Fine-scale plant defence variability increases top-down control of an herbivore. *Functional Ecology*, **35**, 1437-1447.

Pocius, V.M., Cibotti, S., Ray, S., Ankoma-Darko, O., McCartney, N.B., Schilder, R.J., & Ali, J.G. (2022) Impacts of larval host plant species on dispersal traits and free-flight energetics of adult butterflies. *Communications Biology*, **5**, 1-9.

Portman, S.L., Felton, G.W., Kariyat, R.R., & Marden, J.H. (2020) Host plant defense produces species-specific alterations to flight muscle protein structure and flight-related fitness traits of two armyworms. *Journal of Experimental Biology*, **223**, jeb224907.

Portman, S.L., Kariyat, R.R., Johnston, M.A., Stephenson, A.G., & Marden, J.H. (2015) Cascading effects of host plant inbreeding on the larval growth, muscle molecular composition, and flight capacity of an adult herbivorous insect. *Functional Ecology*, **29**, 328-337.

R Core Team. (2022) *R: A language and environment for statistical computing. R foundation for statistical computing. Version 4.2.0*.

Reim, E., Eichhorn, D., Roy, J.D., Steinhoff, P.O., & Fischer, K. (2019) Nutritional stress reduces flight performance and exploratory behavior in a butterfly. *Insect science*, **26**, 897-910.

Schroeder, H., Majewska, A., & Altizer, S. (2020) Monarch butterflies reared under autumn-like conditions have more efficient flight and lower post-flight metabolism. *Ecological Entomology*, **45**, 562-572.

Scriber, J.M. (1981) Sequential diets, metabolic costs, and growth of Spodoptera eridania (Lepidoptera: Noctuidae) feeding upon dill, lima bean, and cabbage. *Oecologia*, **51**, 175-180.

Soule, A.J., Decker, L.E., & Hunter, M.D. (2020) Effects of diet and temperature on monarch butterfly wing morphology and flight ability. *Journal of Insect Conservation*, **24**, 961-975.

Tao, L., Berns, A.R., & Hunter, M.D. (2014) Why does a good thing become too much? Interactions between foliar nutrients and toxins determine performance of an insect herbivore. *Functional Ecology*, **28**, 190-196.

Wootton, R.J. (1992) Functional morphology of insect wings. *Annual review of entomology*, **37**, 113-140.

Yama, H., Soga, M., Evans, M.J., Iida, T., & Koike, S. (2019) The morphological changes of moths on Nakajima Island, Hokkaido, Japan. *Environmental entomology*, **48**, 291-298.

Zahran, N., Hamza, A., & Sayed, W. (2018) Impact of certain additives to diet on the biological and biochemical characteristics of peach fruit fly, Bactrocera zonata. *Journal of radiation research and applied sciences*, **11**, 423-428.

Zalucki, M.P., Malcolm, S.B., Paine, T.D., Hanlon, C.C., Brower, L.P., & Clarke, A.R. (2001) It's the first bites that count: Survival of first-instar monarchs on milkweeds. *Austral Ecology*, **26**, 547-555.

Zhan, S., Zhang, W., Niitepõld, K., Hsu, J., Haeger, J.F., Zalucki, M.P., Altizer, S., de Roode, J.C., Reppert, S.M., & Kronforst, M.R. (2014) The genetics of monarch butterfly migration and warning colouration. *Nature*, **514**, 317-321.

Chapter 2 and Chapter 3

Aikins, C.G. (2022) Effects of Social Cues on Monarch Butterfly Orientation and Oviposition Behavior, University of Georgia.

Altizer, S., Hobson, K.A., Davis, A.K., De Roode, J.C., & Wassenaar, L.I. (2015) Do healthy monarchs migrate farther? Tracking natal origins of parasitized vs. uninfected monarch butterflies overwintering in Mexico. *PloS one*, **10**, e0141371.

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

Baker, A.M. & Potter, D.A. (2019) Configuration and location of small urban gardens affect colonization by monarch butterflies. *Frontiers in Ecology and Evolution*, **7**, 474.

Brower, L.P., Taylor, O.R., Williams, E.H., Slayback, D.A., Zubieta, R.R., & Ramirez, M.I. (2012) Decline of monarch butterflies overwintering in Mexico: is the migratory phenomenon at risk? *Insect Conservation and Diversity*, **5**, 95-100.

Clandinin, T.R. & Giocomo, L.M. (2015) Internal compass puts flies in their place. *Nature*, **521**, 165-166.

Dingle, H. & Drake, V.A. (2007) What Is Migration? *BioScience*, **57**, 113-121.

Fisher, Y.E. (2022) Flexible navigational computations in the Drosophila central complex. *Current opinion in neurobiology*, **73**, 102514.

Flockhart, D.T., Pichancourt, J.B., Norris, D.R., & Martin, T.G. (2015a) Unravelling the annual cycle in a migratory animal: breeding-season habitat loss drives population declines of monarch butterflies. *Journal of Animal Ecology*, **84**, 155-165.

Flockhart, D.T.T., Pichancourt, J.-B., Norris, D.R., & Martin, T.G. (2015b) Unravelling the annual cycle in a migratory animal: breeding-season habitat loss drives population declines of monarch butterflies. *Journal of Animal Ecology*, **84**, 155-165.

Gibo, D. (1986) Flight strategies of migrating monarch butterflies (Danaus plexippus L.) in southern Ontario. In Insect flight: Dispersal and migration, pp. 172-184. Springer.

Giraldo, Y.M., Leitch, K.J., Ros, I.G., Warren, T.L., Weir, P.T., & Dickinson, M.H. (2018) Sun navigation requires compass neurons in Drosophila. *Current Biology*, **28**, 2845-2852. e4.

Green, D.A. (2021) Monarch butterfly migration as an integrative model of complex trait evolution. *The American Naturalist*, **198**, 142-157.

Guerra, P.A. (2020) The monarch butterfly as a model for understanding the role of environmental sensory cues in long-distance migratory phenomena. *Frontiers in Behavioral Neuroscience*, **14**, 600737.

Guerra, P.A. & Reppert, S.M. (2015) Sensory basis of lepidopteran migration: focus on the monarch butterfly. *Current Opinion in Neurobiology*, **34**, 20-28.

Hartzler, R.G. (2010) Reduction in common milkweed (Asclepias syriaca) occurrence in Iowa cropland from 1999 to 2009. *Crop Protection*, **29**, 1542-1544.

Horns, J.J. & Şekercioğlu, Ç.H. (2018) Conservation of migratory species. *Current Biology*, **28**, R980-R983.

Majewska, A.A. & Altizer, S. (2019) Exposure to non-native tropical milkweed promotes reproductive development in migratory monarch butterflies. *Insects*, **10**, 253.

Majewska, A.A., Satterfield, D.A., Harrison, R.B., Altizer, S., & Hepinstall-Cymerman, J. (2019) Urbanization predicts infection risk by a protozoan parasite in non-migratory populations of monarch butterflies from the southern coastal US and Hawaii. *Landscape Ecology*, **34**, 649-661.

Masters, A.R., Malcolm, S.B., & Brower, L.P. (1988) Monarch butterfly (Danaus plexippus) thermoregulatory behavior and adaptations for overwintering in Mexico. *Ecology*, **69**, 458-467.

Merlin, C., Heinze, S., & Reppert, S.M. (2012) Unraveling navigational strategies in migratory insects. *Current opinion in neurobiology*, **22**, 353-361.

Milner-Gulland, E.J., Fryxell, J.M., & Sinclair, A.R. (2011) Animal migration: a synthesis OUP Oxford.

Mouritsen, H. & Frost, B.J. (2002) Virtual migration in tethered flying monarch butterflies reveals their orientation mechanisms. *Proceedings of the National Academy of Sciences*, **99**, 10162-10166.

Parlin, A.F., Stratton, S.M., & Guerra, P.A. (2022) Oriented migratory flight at night: Consequences of nighttime light pollution for monarch butterflies. *Iscience*, **25**, 104310.

Perez, S.M., Taylor, O.R., & Jander, R. (1999) Testing monarch butterfly orientation in the field during the autumn migration. In 1997 North American Conference on the Monarch Butterfly, pp. 127.

Reppert, S.M. & de Roode, J.C. (2018) Demystifying monarch butterfly migration. *Current Biology*, **28**, R1009-R1022.

Reppert, S.M., Guerra, P.A., & Merlin, C. (2016) Neurobiology of monarch butterfly migration. *Annual review of entomology*, **61**.

Satterfield, D.A. & Davis, A.K. (2015) Variation in wing characteristics of monarch butterflies during migration: Earlier migrants have redder and more elongated wings. *Animal Migration*, **2**, 1-7.

Satterfield, D.A., Maerz, J.C., & Altizer, S. (2015) Loss of migratory behaviour increases infection risk for a butterfly host. *Proceedings of the Royal Society B: Biological Sciences*, **282**, 20141734.

Schmidt-Koenig, K. (1979) Directions of migrating monarch butterflies (Danaus plexippus; Danaidae; Lepidoptera) in some parts of the eastern United States. *Behavioural processes*, **4**, 73-78.

Shiozaki, H.M. & Kazama, H. (2017) Parallel encoding of recent visual experience and self-motion during navigation in Drosophila. *Nature Neuroscience*, **20**, 1395-1403.

Takahashi, S., Hombe, T., Matsumoto, S., Ide, K., & Yoda, K. (2022) Head direction cells in a migratory bird prefer north. *Science Advances*, **8**, eabl6848.