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Effects of diet and temperature on monarch butterfly wing morphology and flight ability

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Abstract

Background While global change research has greatly expanded in recent years, it remains unclear how environmental change will impact the mobility of many organisms. Flight is an important mode of transportation that affects ecological functions, including mate location, foraging, and migration. However, the effects of increasing temperature and diet quality on flight remain largely unknown. Here, we explore the effects of rearing temperature and larval diet quality on the flight ability of an iconic and ecologically threatened migratory insect, the monarch butterfly, *Danaus plexippus*.

Experimental Design Monarch larvae were reared at two temperatures (25 °C and 28 °C) and on three milkweed species with varying phytochemistry (*Asclepias incarnata*, *Asclepias syriaca*, and *Asclepias curassavica*) in a fully factorial experiment. We tested flight ability using an automated flight mill, which measured cumulative flight distance, duration, and instantaneous velocity.

Results Higher rearing temperatures reduced monarch flight ability, and larval diet quality influenced forewing morphology. Dietary milkweed with higher cardenolide concentrations (*A. curassavica*) induced shorter, wider forewings whereas milkweed with low to intermediate cardenolides (*A. incarnata* and *A. syriaca*) induced longer, narrower forewings, which are considered better for gliding flight used during migration.

Implications for Insect Conservation Our results provide evidence that projected increases in temperature and the subsequent expansion of tropical milkweed (*A. curassavica*) into the central breeding range of eastern North American migratory monarchs could reduce migration success. Further research is needed to identify mechanisms explaining the effects of diet and temperature on monarch flight ability and fitness, to ensure that appropriate conservation strategies are employed to preserve migratory populations.

Keywords *Danaus plexippus* · Environmental change · Migration · Flight ability · Plant secondary metabolites · Conservation

Introduction

Background

Insights gained from movement ecology remain critical to the practice of conservation, informing status assessments and recovery plans for mobile species. However, movement ecology studies rarely address conservation and management

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directly (Fraser et al. 2018). Given rapid environmental change, there is a pressing need to directly connect this field to conservation and better anticipate the highly variable responses of mobile species in our changing world. Already, many species are experiencing poleward range shifts as a result of increasing global temperatures (Forsman et al. 2016). Critically, the ability to find and adapt to suitable new environments depends largely on dispersal capability (Robillard et al. 2015). Climate change can also cause phenological asynchrony of migratory populations and their food sources (Mayor et al. 2017), which can be detrimental to population reproductive success (Lany et al. 2016). Therefore, the effects of climate change on movement ecology should be accounted for in conservation efforts for mobile taxa.

Flight is an essential form of movement for many organisms that affects important ecological functions, including dispersal, migration, mate location, and predator avoidance. For example, variability in individual flight endurance can impact population dispersal (Kautz et al. 2016). Flight patterns can be broadly divided into two categories: powered and soaring (gliding) flight. Powered flight is the use of flapping to generate aerodynamic force, whereas soaring involves the exploitation of air currents and wind patterns to sustain an animal aloft (Hedenström 1993). Powered flight increases migration distance, and the ability to exploit thermal drafts for gliding and soaring flight furthers these positive effects (McKay et al. 2016; Watanabe 2016). Higher levels of powered flight increase the likelihood that individuals within a species will locate a genetically distinct mate (de Block and Stoks 2007; Andersson 1994). In some species, active searching or fighting for a mate requires considerable flight endurance, and males that exhibit high flight endurance are more likely to successfully mate (Gyulavári et al. 2014; Kehl et al. 2015). This indicates that greater flight endurance can provide a reproductive advantage. Evasive flight maneuverability helps prey to avoid predators (Jantzen and Eisner 2008), and powered flight often gives winged predators an advantage when hunting aerial prey (Helms et al. 2016).

Given the fitness importance of flight ability for volant animals, anything that inhibits or decreases flight ability should subsequently decrease individual fitness and potentially threaten population survival. There are several environmental factors that can influence flight ability including food quality and climate. These are particularly influential in insects, as insect development and performance are tightly linked with nutrition (Waldbauer 1982) and temperature (Damos and Savopoulou-Soultani 2012).

Food quality, or the quantity and composition of nutrients available to feeding organisms, is especially important during consumer development. Larval diet quantity and quality affect adult flight ability (Reim et al. 2019) and morphology (Zahran et al. 2018) in several insects, though the mechanisms driving these relationships are not well understood. The availability and composition of larval diet food sources influences morphological traits that can impact flight performance such as adult body size (Portman et al. 2015; Reim et al. 2019), as well as wing size and shape (Cendra et al. 2014; Johnson et al. 2014; Stoks 2001; Reim et al. 2019; Yama et al. 2019). Diet quality can also influence the development and metabolism of flight muscles (Gunn and Gatehouse 1988; Portman et al. 2015), which may help to explain larval diet-induced changes in flight ability.

Environmental variables such as temperature (Zhang et al. 2016; Kunz et al. 1995) and atmospheric gas concentrations (Decker et al. 2019; Murray et al. 2013) can indirectly influence insect flight ability by altering the dietary nutrient content. Moreover, rising global temperatures impact the habitat ranges (Dinh et al. 2016) and developmental periods (Tan et al. 2010) of some flying insect species. Subsequently, these organisms are exposed to new environmental conditions that could negatively impact flight muscle mass and flight ability. Variations in temperature can directly affect insect behavior, physiology, and movement. Higher temperatures impact insect feeding behavior (Bhattacharyya et al. 2019), and often correspond with faster larval development (Murray et al. 2013; Lemoine et al. 2015; Moriyama and Numata 2019), which can affect adult body size and morphology (Smith et al. 2000). Insect distribution (Flinn and Hagstrum 2011) and flight frequency (Bonsignore and Bellamy 2007) have also been shown to fluctuate with changes in temperature. Additionally, increased temperatures as a result of urbanization influence adult body size (Merckx et al. 2018) and larval survival (Kaiser et al. 2016) in Lepidoptera.

Given the rapidity of global environmental change, it has become urgent to understand how the flight ability of organisms will respond to changing environmental conditions. Here, we explore the effects of increasing temperature and changes in diet quality on the flight ability of an iconic migratory insect, the monarch butterfly (*Danaus plexippus*). Over the last several decades, monarch populations have experienced a drastic decline in North America, with an ~80% decrease in the eastern migratory overwintering population since 1993 (Semmens et al. 2016), and a striking > 99% decrease in western migratory overwintering numbers since the 1980s (Pelton et al. 2019). In 2014, public concern for these population reductions resulted in a petition to the U.S. Fish and Wildlife Service to classify monarchs as a threatened species under the Endangered Species Act (Center for Biological Diversity 2014), and there remains substantial interest in monarch conservation nationwide.

Study system

Monarch butterflies are Lepidopteran insects belonging to the tropical and subtropical subfamily Danainae (Family Nymphalidae) (Brower and Malcolm 1991), found across North and Central America and Indonesia, as well as in pockets of South America, Europe, and Australia (Freedman et al. 2020; Zalucki and Clarke 2004). As larvae, monarchs are specialist herbivores that feed on milkweed plants, mostly in the genus *Asclepias*, which contain varying concentrations of toxic steroids known as cardenolides (Malcolm and Brower 1989). Monarchs sequester cardenolides as larvae and store them as chemical defenses against vertebrate predators (Brower and Malcolm 1991). Moreover, cardenolides act as a natural antibiotic against infection by a neogregarine parasite *Ophryocystis elektroscirrha* (Phylum Apicomplexa); higher dietary cardenolide concentrations

correspond with lower spore load and longer lifespan in individuals infected by *O. elektroscirrha* (de Roode et al. 2008; Gowler et al. 2015). However, high cardenolide concentrations in larval diet can also decrease both uninfected larval survival rate and adult lifespan (Tao et al. 2015). Further, larval diets with high concentrations of cardenolides and foliar nitrogen decrease larval growth rate (Tao et al. 2014), though high leaf nitrogen on its own increases larval growth rate (Couture et al. 2015). The cardenolide concentration in larval diet can also affect the shape and symmetry of adult monarch forewings (Berns 2012; Decker et al. 2019). Generally, high dietary concentrations of cardenolides are beneficial for monarch fitness when large populations of natural enemies are present, but in cases where populations of natural enemies are small, the negative effects of consuming cardenolides may outweigh the benefits.

Most North American monarch populations undergo an extraordinary migration, with the eastern migratory population traveling over 3000 km to overwinter in central Mexico (Urquhart and Urquhart 1978). Overwintering populations begin mating in mid-January, matings become more frequent through mid-February followed by colony dispersal and northward spring migration (Brower et al. 1977). Monarchs then recolonize North America in successive migrant generations, with overwintered individuals laying eggs in the southern United States, and their offspring moving farther north (Cockrell et al. 1993). It should be noted that some populations in warmer areas like Arizona, southern California, and Florida breed throughout the year and either do not migrate or migrate moderate distances to overwinter (Altizer et al. 2014; Urquhart et al. 1968).

Like many other lepidopterans, monarch larval development is temperature-dependent, and recent studies have shown how rising global temperatures affect larval development and performance. For example, time to pupation decreases with increasing temperatures in situ (Lemoine et al. 2015; Zalucki 1982). Moreover, elevated temperatures alter the chemistry of dietary milkweed; foliar nitrogen levels increase, causing larvae to grow larger (Couture et al. 2015). Overall, monarch fitness increases with larval temperature up to about 28 °C, after which fitness starts to decline (Nifosi 2016; Faldyn et al. 2018).

Here, we investigate the effects of larval diet quality and rearing temperature on the fitness of monarch butterflies. We performed a factorial experiment where larvae were reared in controlled chambers at either ambient (25 °C) or elevated (28 °C) temperatures, and fed foliage from one of three milkweed species (*A. incarnata, A. syriaca,* and *A. curassavica*). We then assessed the effects of our temperature and diet treatments on monarch flight ability, wing shape, body mass, wing loading, wing aspect ratio, and larval energy reserves (Berns 2012; Altizer and Oberhauser 1999; Altizer and Davis 2010; Davis et al. 2012; Clements 1955; Hanley et al.

2013; Davis 2014). We expected the temperature treatments to influence larval growth rate, energy reserves, and body mass (Lemoine et al. 2015; Couture et al. 2015; Davis et al. 2005; Davis 2014), and the chemical composition of larval diet to impact monarch wing shape, larval energy reserves, and body mass (Berns 2012; Johnson et al. 2014; Tompkins and Kotiaho 2001; Couture et al. 2015). Specifically, we expected larval growth rate to increase with temperature (Lemoine et al. 2015), and for monarchs reared at higher temperatures to exhibit lighter integument wing pigmentation (Davis et al. 2005), which indicates larval energy reserves (Davis 2014). Furthermore, we expected monarchs reared on the same host plant to have wing shapes more similar to one another than those reared on a different milkweed species (Berns 2012); specifically, monarchs reared on milkweed with lower cardenolide concentrations were expected to have longer, thinner wings than those fed higher cardenolide milkweed (Decker et al. 2019).

In summary, we address the following questions:

1. How do temperature and diet quality influence the flight ability of monarchs?
2. What are the relative contributions of wing morphology, larval energy reserves, and body mass to variation in flight ability?

Materials and methods

Experimental design

We performed a fully factorial experiment to measure the separate and interactive effects of larval diet quality and larval temperature on flight ability. Monarchs were reared on three species of milkweed and at two temperatures, resulting in a total of six treatment groups. The two temperatures chosen for this experiment were 25 °C and 28 °C because an increase from 25 to 28 °C is expected to occur in the central range of migratory monarchs in eastern North America within the next 30 years (Nifosi 2016; Faldyn et al. 2018). The three milkweed species we chose were *A. incarnata, A. syriaca,* and *A. curassavica* (swamp, common, and tropical milkweed, respectively). We chose the three milkweed species for their differences in foliar nitrogen content and cardenolide concentration. Of the three species, *A. incarnata* has the lowest cardenolide concentration, *A. syriaca* is intermediate, and *A. curassavica* is the highest; *A. incarnata* and *A. curassavica* exhibit high foliar nitrogen levels (*A. incarnata*: $3.68 \pm 0.26\%$, *A. curassavica*: $3.32 \pm 0.26\%$) and *A. syriaca* exhibits low levels ($1.62 \pm 0.28\%$) (Tao et al. 2014). For all treatment groups, larvae were reared under 16:8 h light:dark, and dietary milkweed was replaced with fresh leaves ad libitum during larval development.

Milkweed propagation

The milkweed plants used in this experiment were purchased as seed from Victory Seeds in Molalla, OR and American Meadows in Willison, VT. Seeds were surface-sterilized and then cold-stratified for six weeks before they were germinated on wet filter paper; seeds of the tropical milkweed, *A. curassavica*, do not require cold stratification. Germinated seeds were planted in individual 20 cm pots with SunGro Metro-Mix 380 and grown for four months under 16:8 h light:dark in a greenhouse at the University of Michigan Matthaei Botanical Gardens in Ann Arbor, MI. Temperatures in the greenhouse during this period ranged from 21 to 37 °C. Milkweed plants were watered twice daily and fertilized weekly using SunGro Technigro 20–9-20 at 100 ppm until transportation to the monarch rearing facility for their use as larval host plants.

Monarch rearing and handling

The monarchs used in this study were the outcrossed grand-progeny of butterflies purchased from Monarch Watch in Lawrence, KS and collected wild from St. Marks, FL and Cheboygan and Washtenaw Counties, MI. A total of 234 larvae were placed in individual 118 mL plastic containers containing cuttings from one of the three milkweed species (*A. curassavica, A. incarnata,* and *A. syriaca*) and then assigned a location within an incubator at either 25 or 28 °C. Upon reaching the third instar of larval development, individual larvae were transferred to larger (473 mL) plastic containers and reared to pupation. Two days after pupation, pupae were weighed to determine any preliminary effects of larval diet on fitness; fecundity in Lepidoptera increases with body mass (Klemola et al. 2008; Calvo and Molina 2005; Oberhauser 1997). Pupae were then transferred to taller (946 mL) plastic containers to minimize wing damage or deformation upon eclosion.

Post-eclosion, monarchs were placed into a chamber at 25 °C to ensure identical environmental conditions for wing drying. After 24 h, monarchs were sexed and weighed using pre-weighed 5.75×9.5 cm glassine envelopes labeled with ID, sex, dietary milkweed species, and eclosion date. Mass 24 h post-eclosion was used as a measurement of larval energy reserves (Oberhauser 1997; Davis 2014).

Prior to flight trials, adult monarchs were kept in labeled glassine envelopes in an incubator at 14 °C to ensure wing preservation. Monarchs were fed a solution of 10% honey water until satiation at 2, 6, and 8 days post-eclosion and were flown on day eight, immediately following their third feeding. Of the original 234 larvae placed in cups, 138 survived to flight (Table 1), likely due to consistent contamination and inadvertent infection with *O. elektroscirrha* (OE) across all treatments.

Flight trials

Following the methods of Bradley and Altizer (2005), an automated flight mill was constructed using a lightweight carbon rod 122 cm in length and 3.175 mm in diameter threaded through a steel pivot to minimize the effects of friction, and with movable counterbalances to account for variations in individual monarch weight. A flag was attached to one end of the carbon rod to interrupt an infrared beam emitted by a photogate. Using Logger *Pro* software (version 3.14), the photogate recorded the elapsed time between and instantaneous velocity of each rotation, as well as the cumulative flight distance and duration. A live monarch butterfly was attached to the carbon rod using lightweight steel fishing wire, restricting its flight to uninterrupted circles 4.08 m in circumference, and flown to exhaustion (Bradley and Altizer 2005). Here, exhaustion is defined as the point at which a monarch's continuous flight was interrupted for longer than 10 s.

Flight trials were performed in an environmental chamber maintained at 25 °C and controlled for air movement, humidity, and light. Four days post-eclosion, monarchs were removed from their glassine envelopes and an ultra-light steel wire attachment (AFW Stainless Steel Trolling Wire, Test: 7 kg, Length: 92 m) approximately 9 cm in length was glued to the dorsal side of their thorax using rubber cement. Wires were weighed immediately before attachment, and the average wire mass was 0.0628 g (range = 0.0568–0.0829 g).

Table 1 Mortality and survival of monarchs by diet, temperature, and sex

		Dietary milkweed species											
		A. curassavica				*A. incarnata*				*A. syriaca*			
		Original # Larvae	Survived to flight			Original # Larvae	Survived to flight			Original # Larvae	Survived to flight		
			M	F	Total		M	F	Total		M	F	Total
Larval rearing temperature	25 °C	45	11	17	28	44	9	13	22	40	13	10	23
	28 °C	34	9	15	24	35	8	11	19	36	11	11	22

Monarchs were then returned to their glassine envelopes and the 14 °C incubator for another 2 days. Six days post-eclosion, following their second feeding, monarchs were placed in a 0.6 m³ flight cage at 25 °C to allow for acclimation to environmental flight conditions for 48 h before flight. A minimum of 8 butterflies of each sex were flown in each of the six treatment groups (Table 1), and the total flight distance and duration, as well as the average and maximum velocity, were measured for each butterfly. Individual velocity was calculated by taking the mean of the instantaneous velocities recorded by the photogate at 30-s intervals. Average velocity for each individual butterfly was then calculated as the overall mean of the 30-s averages, and maximum velocity as the maximum value of the 30-s averages, following the model of Bradley and Altizer (2005).

Monarchs were weighed immediately before and after flight trials and the recorded change in mass during flight was then divided by the individual distance flown to estimate the amount of energy used during flight. Following flight trials, individuals were returned to their glassine envelopes and placed in an incubator at 14 °C until death to measure longevity.

Wing analysis

Wings were excised from the body *post mortem* and kept in glassine envelopes in a freezer at − 23 °C until each pair was scanned at a resolution of 600 dpi using an Epson Perfection V33 scanner. Scanned wing images were saved as JPEG files and uploaded into Adobe Photoshop CS6. Images were then scaled in Adobe Photoshop by tracing 1 cm (10 mm) on a ruler in the image and calibrating a pixel-to-millimeter ratio for distance measurements. After image scaling, the total combined surface area of forewings and hindwings (mm²), as well as forewing length from wing apex to thorax insertion (mm) and width at the longest axis perpendicular to the length measurement (mm) were measured (Fig. 1). The length and width measurements were then used to calculate the aspect ratio (length / width) of both the left and right forewings for each individual monarch. Total wing surface area and body mass measured 24 h post-eclosion were then used to calculate wing loading, a common aeronautical measure indicative of flight performance and maneuverability, defined as the ratio of total wet body mass to wing surface area (Altizer and Davis 2010).

In addition to basic metrics of forewing morphology, landmark-based geometric morphometric analyses were utilized to discern more subtle differences in shape between treatments (Zelditch et al. 2012; Adams et al. 2004). Scanned wing images (JPEG) were uploaded into the landmark digitization program, tpsDig2 (SUNY, Stonybrook) for geometric morphometric analysis. Wing images were scaled using a digitizing tool in the tpsDig2 program by tracing one centimeter on a ruler in the image (Sherratt 2016). Each forewing was assigned 19 landmarks (Fig. 2). Semi-landmarks were then added to each wing specimen using a curve-tracing tool to trace the outside of each wing. Curves traced the top of the wing from landmark 18 to 17 and the bottom of the wing from landmark 19 to 6 (Fig. 2). The "resample by length" function was then used so that each curve contained the same number of semi-landmarks—25 each for the top (C1) and bottom (C2) of every forewing (Berns 2012).

For the purposes of this study, only forewings were digitized for wing shape and size analysis (geometric morphometrics and aspect ratio), although the surface area of both forewings and hindwings was used for the calculation of wing loading. Forewing shape and size analysis was prioritized over that of hindwings due to the anteromotoric nature of Lepidopteran flight (Jantzen and Eisner 2008), as well as the specific positioning of forewings covering hindwings that is seen during soaring flight in monarchs (Altizer and Davis 2010). Further, previous work established that milkweed chemistry only
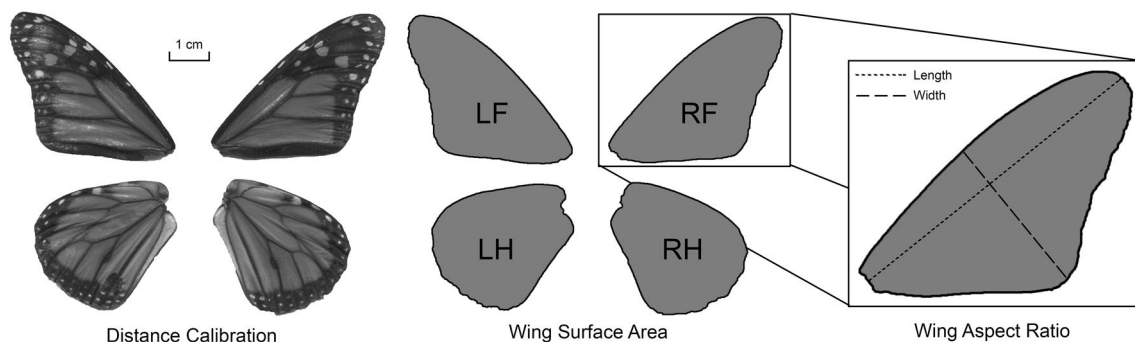

Fig. 1 Raw wing image pixel-to-distance calibration was used to measure the total combined wing surface area (mm²) as the sum of the surface area of the left forewing (LF), right forewing (RF), left hindwing (LH), and right hindwing (RH). The right forewing close-up depicts the length (mm) from wing apex to thorax insertion and width (mm) at the longest axis perpendicular to the length measurement, which were used to calculate aspect ratios (length/width)
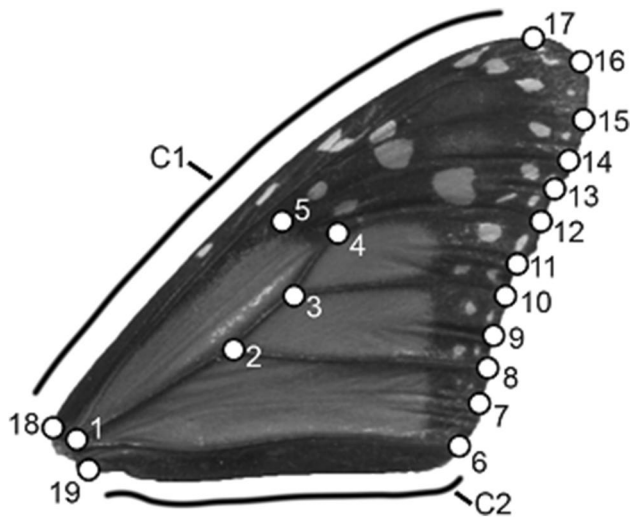
Fig. 2 Landmarks (1–19) and semilandmark curves (C1 & C2) used for geometric morphometric analysis of monarch wings

affects forewing shape (Berns 2012), and forewing morphometrics have been prioritized in prior monarch studies (Decker et al. 2019).

Cardenolide content

After wing images were scanned and uploaded, wing cardenolide concentrations were measured using the methods described by Tao and Hunter (2015). The entire left forewing of each monarch was weighed using an electronic microbalance in preparation for chemical analysis. After weighing, each wing was placed in a microcentrifuge tube, ground using a tissue lyser, and then extracted in 1 mL of methanol. Samples were then vortexed for 10 s each and centrifuged. After centrifugation, the supernatant was evaporated at 45 °C until dry. Samples were then resuspended in 150 μL of methanol and analyzed using reverse phase high-performance liquid chromatography, with 0.15 mg/mL digitoxin as an internal standard (UPLC, Waters Inc., Milford, MA, USA). Running time for each sample was 9 min. Peaks were detected using a diode array detector, which recorded absorbance spectra from 200 to 300 nm. Because cardenolides typically absorb light symmetrically between 216 and 222 nm, peak integration at 218 nm results in optimal quantitative estimation of this compound class (Wiegrebe and Witchl 1993). Total cardenolide concentrations were calculated as the sums of the individual cardenolide peak areas separated by UPLC, corrected by the concentration of an internal digitoxin standard, and normalized by the dry sample mass.

Parasitic infection

Despite sterilization of eggs and host plants, many of the monarchs in this experiment were found to be infected with *Ophryocystis elektroscirrha* (OE). Post-eclosion, the infection status of adult monarchs was assessed by rubbing a clear sticker along the abdomen and examining the sticker for dormant spores using a Leica S8AP0 magnifying scope set to $8.0 \times$ magnification (Altizer et al. 2000). A 10×10 mm grid was placed over the densest spore area and each 1 mm^2 cell in the grid was given a score of 0–4 for percentage of coverage by spores (0 = 0%; $1 \leq 25\%$; $2 = 25$–50%; $3 = 50$–75%; $4 \geq 75\%$). This score was then averaged over the entire grid for each individual butterfly as an estimate of infection.

Flight performance and life history

General linear models (SAS version 9.4) were used to test for effects of diet and temperature treatments on monarch flight performance. In all models, diet, temperature, sex, and spore load were fixed effects. The five response variables of flight performance were total flight distance (m) and duration (min), average and maximum velocity (km/h), and mass lost per unit distance flown (μg/m). Flight distance, duration, and mass loss were log-transformed for analysis to approximate normality of residuals.

Effects of diet and temperature treatments on wing characteristics (forewing aspect ratios, wing loading, and cardenolide content), and life history traits (pupal mass, butterfly mass, larval period, pupal period, and longevity) were also investigated using general linear models. Next, the influence of wing characteristics and life history on flight performance were determined using general linear models with butterfly traits as predictor variables and flight parameters as response variables. Parasite spore load was also plotted against all flight parameters, wing characteristics, and life history values to discern any unintended effects of disease on monarch flight and fitness.

Geometric morphometrics

TPS files with digitized landmarks and semi-landmarks were loaded into R using the geomorph package (Adams and Otarola-Castillo 2013). The data was converted into a three-dimensional array and a sliders matrix was created designating how semi-landmark points would slide along the assigned curve using the "define.sliders" command. This matrix contains three columns that specify the semi-landmarks and two neighboring semi-landmarks towards which each semi-landmark will tangentially slide. Next, a Generalized Procrustes Analysis (GPA) was performed on the sliders matrix in order to remove variation in specimen position, scale, and orientation and to determine any outliers

(functions: gpagen, plotOutliers; Gower 1975; Rohlf and Slice 1990). Analysis with and without outliers provided similar results, so they were not removed from the dataset. We used a Procrustes ANOVA (function: procD.lm) to quantify the amount of variation in wing shape explained by butterfly size, sex, larval rearing temperature, larval diet quality, and the interaction between diet and temperature treatments on the Procrustes distances (Euclidean distances in tangent space) between specimens (Adams and Otarola-Castillo 2013). The Procrustes ANOVA is equivalent to a permutational MANOVA of the distance matrix (Anderson 2001). Finally, a Principal Components Analysis (PCA) plot was generated for any effect that yielded a significant result in the Procrustes ANOVA using the two axes (PC1 and PC2) which explained the majority of the variance among specimens.

Structural equation modeling

To elucidate the causal relationships between larval diet, temperature, wing morphology, health, and flight metrics, the data were fit to a structural equation model (SEM). However, due to the presence of categorical variables, a limited sample-size-to-parameter ratio (n = 138; min parameters = 9), and a poor model-data fit (RMSEA = 0.103, CFI = 0.917), it was determined that SEM was not an appropriate analysis for the data.

Results

Direct effects on flight performance

Temperature

As adults, larvae reared at 28 °C flew for shorter periods ($F_{1,132} = 5.25$, p = 0.024, Table 2), shorter distances ($F_{1,132} = 3.91$, p = 0.050, Table 2), and lost more mass per unit distance flown ($F_{1,132} = 8.73$, p = 0.004, Table 2) than did larvae reared at 25 °C (Fig. 3). In short, higher larval rearing temperatures reduce flight performance in adult monarch butterflies.

Milkweed species

Effects of diet on flight ability were much weaker than were those of temperature. There was a marginally significant interaction between diet and temperature on maximum flight velocity, where butterflies fed a larval diet of *A. curassavica* or *A. syriaca* experienced a decrease in maximum flight velocity while individuals fed *A. incarnata* experienced a slight increase in maximum flight velocity when reared at a higher temperature ($F_{2,126} = 2.77$, p = 0.066,

Table 2 The primary significant effects of (a) larval diet, (b) rearing temperature, (c) interactive effects of temperature and diet on monarch flight measures (duration, distance, and mass loss per unit distance flown), wing shape (aspect ratio), and life history (cardenolide content and pupal/adult mass)

Diet

		Aspect ratio (L/W)	
	RF	$F_{2,129} = 3.58$	p = 0.031
	LF	$F_{2,132} = 3.11$	p = 0.048
A. curassavica	RF	1.927 ± 0.010	
	LF	1.949 ± 0.012	
A. incarnata	RF	1.956 ± 0.016	
	LF	1.971 ± 0.015	
A. syriaca	RF	1.960 ± 0.012	
	LF	1.986 ± 0.017	
		Wing Cardenolides (% dw)	
		$F_{2,132} = 121.15$	p < 0.0001
A. curassavica		2.35 ± 0.17	
A. incarnata		0.40 ± 0.06	
A. syriaca		1.08 ± 0.12	
		Wing loading (mg/mm^2)	
		$F_{2,132} = 4.84$	p = 0.009
A. curassavica		0.461 ± 0.007	
A. incarnata		0.485 ± 0.009	
A. syriaca		0.475 ± 0.008	
		Pupal mass (mg)	
		$F_{2,125} = 5.96$	p = 0.042
A. curassavica		1150.4 ± 35.5	
A. incarnata		1037.4 ± 24.5	
A. syriaca		1147.7 ± 42.3	
		Adult mass (mg)	
		$F_{2,126} = 4.25$	p = 0.016
A. curassavica		447.1 ± 17.4	
A. incarnata		411.9 ± 12.8	
A. syriaca		464.2 ± 19.9	
Temperature			
		Aspect ratio (L/W)	
	RF	$F_{1,129} = 6.58$	p = 0.012
25 °C		1.958 ± 0.011	
28 °C		1.934 ± 0.014	
		Flight duration (min)	
		$F_{1,132} = 5.25$	p = 0.024
25 °C		1.87 ± 0.05	
28 °C		1.66 ± 0.05	
		Flight distance (m)	
		$F_{1,132} = 3.91$	p = 0.050
25 °C		5.48 ± 0.06	
28 °C		5.24 ± 0.06	
		Mass loss during flight (ug/m)	
		$F_{1,132} = 8.73$	p = 0.004
25 °C		1.62 ± 0.06	
28 °C		1.85 ± 0.06	

Table 2 (continued)

Diet * Temperature

		Wing cardenolides (%)	
		$F_{2,132} = 4.38$	p = 0.017
A. curassavica	25 °C	2.03 ± 0.14	
	28 °C	2.72 ± 0.19	
A. incarnata	25 °C	0.40 ± 0.06	
	28 °C	0.40 ± 0.05	
A. syriaca	25 °C	1.03 ± 0.11	
	28 °C	1.14 ± 0.13	

Mean and 95% confidence intervals shown for all metrics. Values shown for flight duration, distance, and mass loss are all logarithmically transformed. Right forewing (RF) and left forewing (LF) specified for aspect ratio measurements. Effects of sex are not reported because there was no significant impact on any of the wing shape, flight, or life history measures
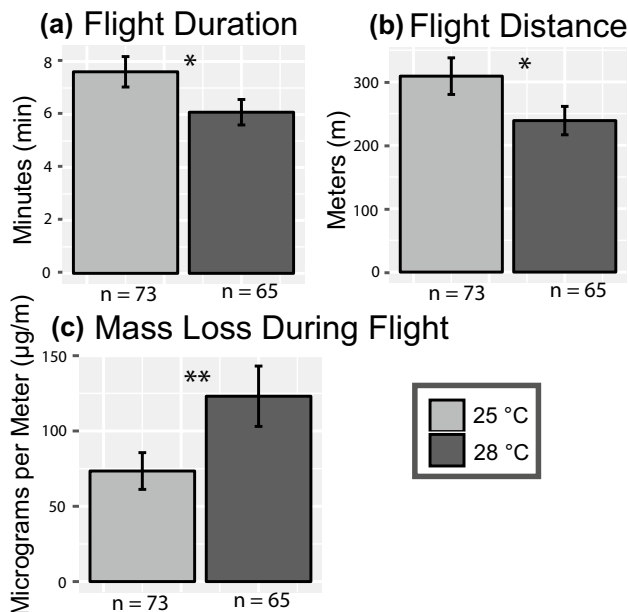
Fig. 3 Average flight duration (**a**), distance (**b**), and mass loss (**c**) for monarchs reared at 25 °C versus 28 °C. Confidence intervals represent the standard error around the mean. Asterisks above each barplot represent the significance of the temperature-induced effects on flight metrics (*p < 0.05; **p < 0.01; ***p < 0.001). Sample sizes (n) of each treatment group are provided below each barplot

Table S1). Additionally, wing cardenolide concentrations largely reflected the well-established differences in cardenolide concentrations among the milkweed species ($F_{2,132} = 121.15$, p < 0.0001, Table 2). The concentration of cardenolides sequestered from *A. curassavica* were higher when larvae were reared at higher temperatures as well ($F_{2,132} = 4.38$, p = 0.017, Table 2). Butterflies also tended to fly faster when their wing cardenolide concentrations were

high ($F_{1,136} = 4.062$, p = 0.046; Table 3), indicating that monarchs that feed on higher cardenolide milkweed may be more fit for powered flight.

Indirect effects on flight performance through wing morphology, life history, and parasitism

Wing aspect ratio

Right forewing aspect ratio was lowest for individuals fed a larval diet of *A. curassavica*, highest for those fed *A. syriaca*, and intermediate for those on *A. incarnata* ($F_{2,129} = 3.58$, p = 0.031, Table 2; Fig. 4). These trends were consistent for the left forewing ($F_{2,132} = 3.11$, p = 0.048, Table 2). In short, feeding on milkweed plants with higher cardenolide content (*A. curassavica*) resulted in shorter, wider forewings whereas feeding on milkweed with low to intermediate cardenolides (*A. incarnata* and *A. syriaca*) resulted in longer, more narrow forewings. The marginally significant temperature x diet interactions on both left and right forewing aspect ratios (left—$F_{2,132} = 2.83$, p = 0.063; right—$F_{2,129} = 2.74$, p = 0.068, Table S1) reflect that diet has a larger impact on wing aspect ratio at lower than at higher temperatures (Fig. 4).

Interestingly, temperature had a significant effect on the aspect ratio of the right forewing ($F_{1,129} = 6.58$, p = 0.012, Table 2) but not on the left ($F_{1,132} = 0.05$, p = 0.826, Table S1), indicating some asymmetry, particularly in individuals reared on *A. incarnata*. Further, individuals with longer, narrower wings lost less mass per unit distance than did those with shorter, rounder wings (left—$F_{1,136} = 4.071$, p = 0.044; right—$F_{1,136} = 8.244$, p = 0.004; Table 3), indicating a greater ability to reserve energy during flight.

Wing loading

Larval diet also influenced wing loading, with individuals reared on *A. incarnata* having the highest wing loading values, *A. curassavica* the lowest, and *A. syriaca* intermediate ($F_{2,132} = 4.84$, p = 0.009, Table 2; Fig. 5). Thus, the cardenolide content of larval host plants was negatively associated with monarch wing loading. Interestingly, flight duration increased, albeit marginally, with wing loading ($F_{1,136} = 2.832$, p = 0.067; Table 3).

Geometric morphometrics

Geometric morphometric results were qualitatively consistent with those reported above for basic measures of wing morphology. Host plant affected the shape of both the right ($F_{2,130} = 2.4672$, p = 0.012, Fig. 6a) and left ($F_{2,129} = 3.2015$, p = 0.002) forewing, with individuals reared on *A. curassavica* and *A. incarnata* being the

Table 3 F-statistics and p-values from general linear model comparisons of fitness measures and flight performance

Independent/explanatory variable	Dependent variable	F-statistic	p-value
Aspect ratio			
RF	Mass loss during flight	8.244	0.004
LF	Mass loss during Flight	4.071	0.044
Wing loading	Flight duration	2.832	0.067
Wing cardenolide content	Maximum flight velocity	4.062	0.046
Adult body mass	Mass loss during flight	8.018	0.005
	Flight duration	3.891	0.052
Parasite spore load	Wing loading	2.858	0.029

Right forewing (RF) and left forewing (LF) specified for aspect ratio measurements. Degrees of freedom = 1 and sample size = 136 for each comparison
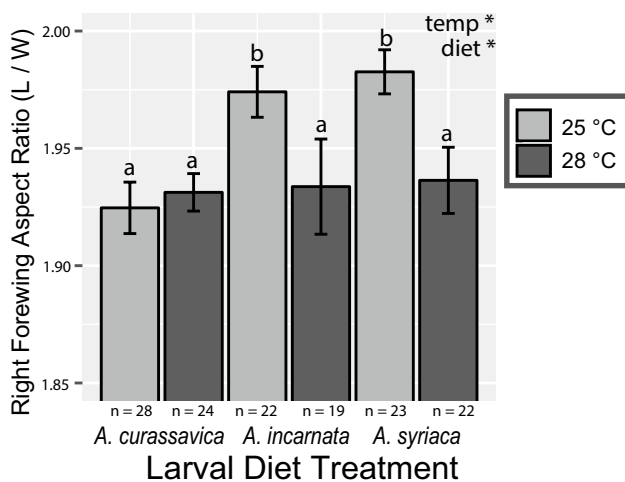
Fig. 4 Factorial comparison of the average aspect ratio (length / width) of the right forewing for monarchs fed *A. curassavica*, *A. incarnata*, or *A. syriaca* and reared at 25 °C or 28 °C. Confidence intervals represent the standard error around the mean. The significance of the temperature and diet treatment effects on aspect ratio are represented by asterisks in the upper right corner of the plot (*p < 0.05; **p < 0.01; ***p < 0.001). Statistically significant (no overlap in confidence intervals) treatment groups are represented by the letters 'a' and 'b'. Sample sizes (n) of each treatment group are provided below each barplot

Fig. 5 Comparison of the average wing loading values for monarchs reared on each of the three dietary milkweed species—*A. curassavica*, *A. incarnata*, and *A. syriaca*. Confidence intervals represent the standard error around the mean. Asterisks above the plot represent the significance of diet-induced effects on wing loading (*p < 0.05; **p < 0.01; ***p < 0.001). Statistically significant (no overlap in confidence intervals) treatment groups are represented by the letters 'a' and 'b'. Sample sizes (n) of each treatment group are provided below each barplot

most different from one another in both cases. There was an effect of temperature depending on diet on both right ($F_{2,130} = 4.7818$, p = 0.001) and left ($F_{2,129} = 2.4574$, p = 0.012) forewing shape. Larval rearing temperature alone had a significant effect on the shape of the right ($F_{2,130} = 3.6650$, p = 0.006, Fig. 6b) but not the left forewing ($F_{2,129} = 1.1984$, p = 0.284), which is consistent with the forewing aspect ratio results described previously. We summarize all model results in Table 2 and Supplementary Table 1.
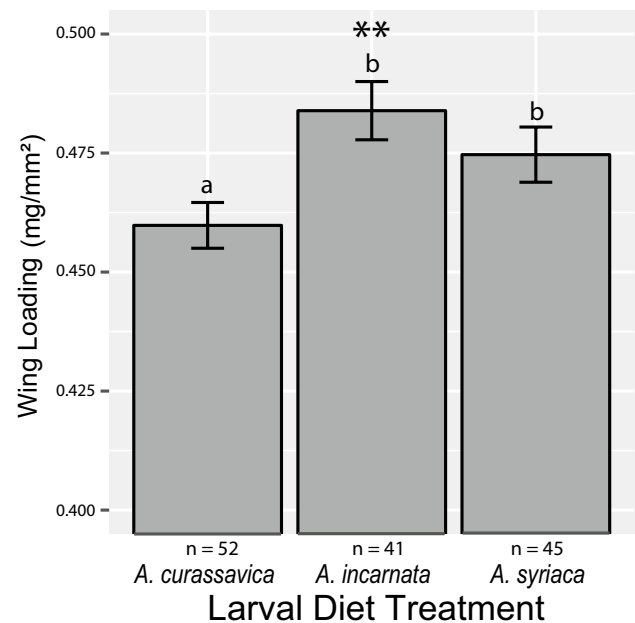
Life history

Pupal mass and adult butterfly mass were both significantly influenced by larval host plant. Monarchs reared on *A. incarnata* had the lowest pupal ($F_{2,125} = 5.96$, p = 0.042, Table 2) and adult ($F_{2,126} = 4.25$, p = 0.016, Table 2) masses, regardless of rearing temperature. Heavier butterflies also lost more mass as they flew ($F_{1,136} = 8.018$, p = 0.005, Table 3), and flew for shorter periods ($F_{1,136} = 3.891$, p = 0.052, Table 3).
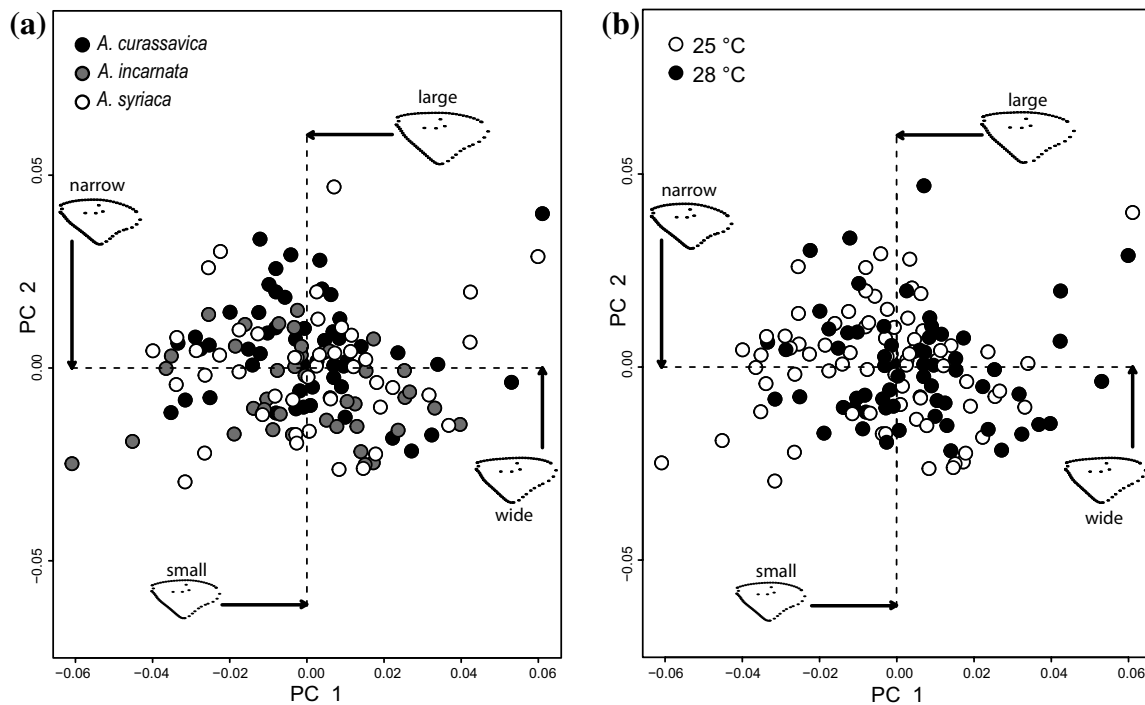
Fig. 6 Principal components plot of geometric morphometric differences in right forewing shape distinguished by **a** larval diet treatment and **b** rearing temperature. PC1 represents wing size, while PC2 represents wing shape. The arrows indicate the wing-landmark diagrams which correspond to the minimum and maximum shape differences for PC1 (min→max: left→right) and PC2 (min→max: bottom→top)

Parasitic infection

Wing loading declined significantly with increasing spore load ($F_{1,136} = 2.858$, $p = 0.029$; Table 3), reflecting a loss in butterfly mass per unit wing area.

Discussion

Our results illustrate that elevated temperatures reduce flight ability and incur greater energetic costs during flight for monarch butterflies. Specifically, butterflies reared at higher temperatures fly shorter distances and durations, while losing more energy (mass) per distance than individuals reared at lower temperatures. Therefore, rising global temperatures could be detrimental to the successful annual migration of monarch butterflies. Our results corroborate recent research showing that lower developmental temperatures improve adult insect flight ability. Fruit flies (*Drosophila suzukii*) reared at lower temperatures exhibit faster, more dynamic flight than warm-reared flies (Fraimout et al. 2018), and heat waves during larval development reduce multiple indices of flight ability in the blue-tailed damselfly, *Ischnura elegans* (Arambourou et al. 2017). Furthermore, increased temperatures during flight decrease flight duration for the true bug

Lygus lucorum (Lu et al. 2014), indicating that increased temperatures as adults may have compounding negative effects on insect flight.

Our data demonstrate a positive relationship between cardenolide sequestration and flight ability (maximum flight velocity) in monarch butterflies, indicating an indirect influence of diet quality on flight. In contrast, seasonal changes in larval host plant quality do not affect flight performance in the South American cactus moth *Cactoblastis cactorum* (Sarvaray et al. 2008). However, this study only measured the longest single flight, total distance flown, and number of flights initiated, without consideration of flight velocity, energetic costs, or the role of plant secondary metabolite sequestration in flight performance.

We predicted that larval diet quality and larval rearing temperature would affect monarch flight ability through changes in wing morphology, body mass, or energy reserves. In our study, temperature directly reduced flight ability and wing shape responded to temperature in a diet-specific manner.

Temperature alone impacted the shape of the right forewing, with monarchs reared at lower temperatures having a greater aspect ratio than those reared at higher temperatures. This result corroborates recent findings that cold-reared fruit flies (*Drosophila melanogaster*) have longer wings than

warm-reared, with cold-reared also exhibiting better flight performance (Frazier et al. 2008). A similar study of another fruit fly species (*D. suzukii*) found that developmental temperature did not impact wing aspect ratio but instead wing area (Fraimout et al. 2018). However, both studies indicate that larger wings may be beneficial for insect flight.

Temperature did not directly impact body mass energy reserves in monarchs, which contrasts the minor increase in body mass observed in cold-reared fruit flies (Frazier et al. 2008). However, one could argue that higher temperatures had an indirect effect on body mass and energy reserves through the greater energetic costs (mass loss) incurred during flight. Still, the mechanism by which temperature reduces flight duration and distance in monarchs remains unclear.

Larvae fed host plants with higher foliar cardenolide concentrations (*A. curassavica*) exhibited the lowest forewing aspect ratios (rounder, wider wings), thus making them less equipped for migratory flight; organisms with more elongated (higher aspect ratio) wings have a higher capacity for energy-saving (gliding) flight required for long distance journeys (Altizer and Davis 2010). Our results support this idea, because monarchs with higher forewing aspect ratios experienced lower energetic costs (loss in mass) during flight. In fact, the empirical connection of butterfly wing morphology to energetic consumption during flight is a rare result (Le Roy et al. 2019), and one that supports widely accepted aerodynamic models (Ortega Ancel et al. 2017). With reduced energetic costs, monarchs may be capable of flying longer distances and/or duration before having to resupply energy stores.

The high-cardenolide *A. curassavica* is a tropical milkweed species that is currently undergoing a northward range expansion in response to increasing global mean surface temperature (Walther et al. 2002; Satterfield et al. 2018). Migratory monarchs that encounter *A. curassavica* may be at an increasing disadvantage as *A. curassavica* expands northward. However, the effects of larval diet on wing morphology were less pronounced when larvae were reared at 28 °C compared to 25 °C. This indicates that larval host plant quality may not influence forewing shape and potential migratory ability as strongly as global temperatures rise. Still, other environmental stressors such as lack of available milkweed can cause stunted wing growth in monarchs (Johnson et al. 2014), and lower nutritional content may result in increased levels of fluctuating asymmetry (Tompkins and Kotiaho 2001); these relationships should be investigated in the context of global change to further elucidate environmental impacts on monarch populations.

Butterflies with higher wing loading values flew for marginally longer periods, which provides evidence that individuals with larger body mass relative to wing size are better equipped for sustained flight. This supports previous research showing that high wing loading may be advantageous for migration; eastern migratory monarch populations have higher wing loading values than non-migratory or western migratory populations which do not migrate as far (Altizer and Davis 2010). Although other migratory butterfly species with high wing loading exhibit faster powered flight (Dudley and Srygley 2008), our results show no relationship between wing loading and flight speed. This contradiction may result from aerodynamic differences between tethered and untethered flight (Tanaka et al. 2005). Alternatively, the relationship between wing loading and flight duration suggests that high wing loading may be indicative of greater lipid reserves available for long-distance flight. Monarchs reared on *A. curassavica* had the lowest wing loading values, providing further evidence that a northward range expansion of this milkweed species could potentially be detrimental to the successful migration of monarch butterflies.

Pupal and adult butterfly mass were both significantly lower in individuals reared on *A. incarnata*, regardless of temperature treatment. This suggests that individuals feeding on *A. incarnata* have lower energy reserves for long distance flight. Further, this result may be indicative of lower foliar N levels in *A. incarnata*, as previous research has shown a positive relationship between foliar N levels and monarch body mass (Couture et al. 2015). Generally, monarchs reared on *A. syriaca* experience the least dramatic temperature-induced changes in flight ability, wing morphology, and life history. This may indicate that one of the most widely-distributed species of milkweed in eastern North America (Devoid 2017), could be advantageous for the resilience of wild monarch populations in the face of global environmental change.

While unintended, the prevalence of OE infection in the monarchs used in this study provides potential insights into how disease influences the fitness of surviving monarchs in a changing environment. Our study provides partial evidence that greater spore load results in lower wing loading values, and that spore load had no significant impact on flight velocity or distance. These results contradict previous research (Bradley and Altizer 2005) and are surprising given the decline in wing loading that we observed. However, it is important to note that because these monarchs were inadvertently infected, they did not receive standardized inoculation doses of the parasite, unlike previous work investigating OE infection in monarchs. Thus, a lack of controlled infection could explain the discrepancies between our results and previous studies. Another possibility is that the strain of OE infecting our monarchs may be less virulent because parasite genotypes are subject to selection favoring increased transmission (de Roode et al. 2008).

Urbanization has undeniable effects on surface temperature (Taha 1997) and host plant distribution (Miles et al. 2019), and may impact monarch flight. Some Lepidoptera

experience increased larval survival with a 1 °C increase in daytime temperature on urban heat islands (Kaiser et al. 2016). However, our data suggest that the conditions present in urban heat islands may also reduce monarch flight ability as a result of higher larval rearing temperature. English oak (*Quercus robur*) chemical defenses can also decline in urban environments, though this may be due to lack of herbivore pressure (Moreira et al. 2019). Further, urban areas have large densities of milkweed, and provide important potential habitats for monarchs (Johnston et al. 2019). Taken together, though urban heat islands may increase monarch larval survival, higher rearing temperatures could reduce the flight ability of those monarchs. However, the increased prevalence of milkweed in urban areas, combined with potential declines in defensive chemistry could counteract the negative effects of urban heat islands on migrating monarchs. Future research should investigate the impact of urbanization on milkweed chemistry and monarch mobility, as our results suggest that lower cardenolide concentrations will produce wing shapes better suited to migratory flight.

Overall, our results provide an interesting and complicated picture of the future of migratory monarch butterflies. First, projected increases of 1.5–4 °C in global mean surface temperature over the next 30 years (IPCC 2013) will likely reduce the flight ability of migratory monarchs. Second, changes in host plant availability may also reduce flight ability, however the effect of milkweed species range shifts on monarch flight will depend on the direct effects of increased temperature on host plant quality. Conservation efforts for monarchs often involve planting milkweed, and it is therefore prudent to know which milkweed host plants benefit monarchs. Our data suggest the intermediate cardenolide chemistry and nutrient quality of *A. syriaca,* the most common species of milkweed, provides a high-quality food source for migrating monarchs, and thus the preservation of this plant species should be a central focus in the conservation of monarchs.

Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interest.

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