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Sex-specific costs of melanization in a widespread butterfly species

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Abstract

Coloration and patterning are fundamental components of fitness and yet there exists considerable intraspecific variation in pigmentation across the animal kingdom. Much of this patterning is regulated by melanin, one of the most common classes of pigments. Understanding the causes and consequences of melanin plasticity informs how an organism's environment shapes fitness via variation in coloration. Although we have some understanding of how variables such as temperature, resource quality, and density affect melanin expression, intraspecific variation in melanization can also be caused by correlations between melanin produced at different stages of an organism's lifetime, for example between juveniles and adults. If melanin production is uncorrelated between juveniles and adults, this suggests that the genetic or molecular pathways governing juvenile and adult melanin expression are decoupled. A positive correlation between juvenile and adult melanization may suggest resources acquired by juveniles that contribute to melanin production also benefit adults, whereas a negative correlation may suggest that melanin is costly to produce and expression in juveniles trades off with expression in adults. Understanding the relationship between juvenile and adult melanization helps to uncover mechanisms underlying the production of this ubiquitous and multi-functional molecule. Here we leverage conspicuous intraspecific variation in coloration in the monarch butterfly to quantify correlations between juvenile and adult melanization. We used 11 host plant species and two temperatures to generate phenotypic variation in juvenile melanization. We tracked individual monarchs to adulthood and quantified the relationship between juvenile melanization and adult growth, size, and melanization. There were minimal effects of host plant species and large effects of temperature on juvenile melanization. Overall, darker caterpillars grew more slowly and developed into adults with rounder wings. Both sexes had similar larval coloration; however, in females but not males, caterpillars with higher proportion of black turned into adults with less black pigment in their forewings, and these wings were also larger. This

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study demonstrates trade-offs in melanization in female but not male monarchs and suggests sex-specific costs of melanin expression in this species. Lastly, the sensitivity of melanin production to temperature suggests that ongoing climate change may have important effects on female monarch fitness.

KEYWORDS
Danaus plexippus, melanin, milkweed, monarch butterfly, temperature, trade-offs

INTRODUCTION

Insect coloration and patterning are fundamental determinants of fitness because they function in mate recognition (Suárez-Tovar et al., 2022), predator avoidance (Svádová et al., 2009), and thermoregulation (Laakso et al., 2021). The pigmentation underlying much of the color variation is related to melanin production. Melanin is the most common class of pigments found in animals (Badejo et al., 2020; Britton & Davidowitz, 2023b; Fuzeau-Braesch, 1972) and is responsible for gray to black coloration (eumelanin) and yellow to red coloration (pheomelanin) (Britton & Davidowitz, 2023b). Melanin also functions in insect immune responses via wound healing and encapsulation of foreign particles (Zdybicka-Barabas et al., 2025). Intraspecific variation in melanin production should thus have direct implications for insect fitness. Indeed, darker yellow mealworm beetles (*Tenebrio molitor*) were more resistant to infection by the fungus *Metarhizium anisopliae* (Barnes & Siva-Jothy, 2000), darker "melanic" morphs of the white-lined sphinx moth (*Hyles lineata*) warmed up faster in colder temperatures (Britton & Davidowitz, 2023a), male *Colias* butterflies with darker wings spent more time in flight at low temperatures than males with lighter wings (Guppy, 1986), and "melanic" forms of the two-spotted ladybug (*Adalia bipunctata*) had higher mating success (Brakefield, 1984). Given the broad importance of melanin, a better understanding of the causes and consequences of melanin plasticity can help quantify the relationship between pigmentation and fitness.

Intraspecific variation in melanization can be affected by factors such as photoperiod, density, substrate color, temperature, and diet (Britton & Davidowitz, 2023a, 2025; Lorrain-Soligon et al., 2025; Phelps et al., 2024; Silva et al., 2016; Stoehr, 2010; Stoehr & Donahue, 2025), and the effects of the latter two factors have been particularly well characterized. Studies examining the effect of temperature often test the thermal melanism hypothesis, which states that at colder temperatures, darker individuals have an advantage over lighter individuals because the former can heat up faster (Clusella Trullas et al., 2007). The Asian lady beetle (*Harmonia axyridis*), cabbage moth (*Mamestra brassicae*), moths in the family Geometridae, the Green-veined white butterfly (*Pieris napi*), black swallowtail caterpillars (*Papilio polyxenes*), and monarch caterpillars (*Danaus plexippus*) all exhibited higher melanization at colder temperatures (Batool et al., 2024; Davis et al., 2005, 2022; Goulson, 1994; Günter et al., 2019; Hazel, 2002; Heidrich et al., 2018; Kutama et al., 2024; Tseng et al., 2023). Exceptions to this pattern also exist; wing melanism was positively correlated with temperature across 23 Californian populations of Ranchman's tiger moth (*Arctia virginalis*; Pepi et al., 2023).

The effect of diet on melanization has also been explored, although its effects are not as generalizable as those of temperature. Melanin is a large nitrogen-rich molecule and it is directly (via tyrosine) or indirectly (phenylalanine) synthesized from amino acids obtained from the organism's diet (Ethier et al., 2015; Talloen et al., 2004). Consequently, melanin is thought to be costly to produce and its expression is predicted to be reduced under poor diets (Britton & Davidowitz, 2023b; Ethier et al., 2015; Ottocento et al., 2024). Despite this expectation, the effect of diet on melanin production appears to be highly variable with some studies reporting no or minimal effects on wing coloration (Atterholt & Solensky, 2010; Blount et al., 2023; Johnson et al., 2014; Ottocento et al., 2024; Stoehr et al., 2024) and others demonstrating reduced pigmentation when organisms are fed poorer diets (Ethier et al., 2015; Lee et al., 2008; Lindstedt et al., 2020; Talloen et al., 2004). A review paper on the topic suggested the inconsistent effect of diet on melanization may be due to among-study variation in how diet is manipulated (e.g., changes in protein, fatty acids, quantity) in combination with variation in how melanin is measured (e.g., size of spots, darkness, intensity), as well as which life stages are examined (Britton & Davidowitz, 2023b).

Intraspecific variation in melanin production can also be caused by trade-offs in melanization during different developmental stages of the organism. If producing melanin during larval stages reduces the availability of melanin precursors in the adult stage, it is possible that elevated melanization during juvenile stages may result in reduced melanization in adulthood (Lindstedt et al., 2016).

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However, because larval tissue largely dies during butterfly metamorphosis (Tettamanti & Casartelli, 2019) and adult tissues form from imaginal disks (clusters of cells that develop into adult organs) or other undifferentiated cells (Aldaz & Escudero, 2010; Whitman & Agrawal, 2009), melanin production in larvae may be decoupled from adult melanization. Given the many functions of melanin, in particular for insects, a better understanding of whether juvenile melanization affects the expression of this molecule in adults can help illuminate how environmental conditions experienced by juveniles affect adult fitness via changes in pigmentation and patterning. Despite the potential for covariation in melanization between different developmental stages, few studies have examined the relationship between juvenile and adult melanization: Talloen et al. (2004) found no relationship between pupal color and adult wing melanization in the speckled wood butterfly and Davis et al. (2005) reported a negative relationship between larval melanization and female wing melanization when data were grouped by family, but the opposite pattern for male monarchs. Whether these trait correlations are also found at the individual level in monarchs is unknown.

Monarch butterflies are an ideal study organism in which to examine the effects of juvenile melanization on adult pigmentation and patterning because both caterpillars and adults exhibit clear variation in melanization. Caterpillars have characteristic white, yellow, and black stripes and the thickness of the black stripes increases at higher latitudes and lower temperatures (Batool et al., 2024; Davis et al., 2005, 2022; Tseng et al., 2023). Monarch wing melanization can be measured as the proportion of black coloration (Atterholt & Solensky, 2010; Davis et al., 2012) or the darkness of the black sections of the wing (Davis et al., 2012; Johnson et al., 2014). This study provides some insight into this topic by examining how variation in the monarch's biotic and abiotic environment is associated with caterpillar melanization, and how this trait in turn correlates with adult melanization, size, growth rate, and wing morphology. Associations between melanization, size, and wing morphology may also be relevant as predictors of migration ability in monarchs: for example, Davis et al. (2012) showed that red pigmentation is correlated with migration timing, Davis et al. (2023) found that white wing spots were conducive to long-distance migration, and numerous studies have demonstrated connections between wing size, wing shape, and migratory status (e.g., Altizer & Davis, 2010; Freedman & Dingle, 2018). Although melanin contributes to several pigments, here we examine the amount of black pigment in caterpillars or adult wings. For simplicity we refer to this black pigment as "melanin" or "melanization" throughout the paper.

Given the known effects of temperature on monarch melanization and known effects of diet on melanization in several insect species, here we generate variation in monarch caterpillar melanization by rearing caterpillars: (1) in the greenhouse on one of 11 species of milkweed, or (2) in the laboratory at 22 or 32°C. We first tested how food type and temperature individually affected caterpillar and adult melanization as well as wing area, wing shape, and growth rate. We then combined the data from both components of the study to examine covariation between caterpillar melanization and adult traits. The thermal melanism hypothesis states that under cooler conditions, increased melanism enables more effective thermoregulation and vice versa. This hypothesis as applied to monarch larvae predicts that larvae reared under lower temperatures will be darker than those reared under higher temperatures. Based on known interspecific differences in nutritional quality (e.g., C:N ratios and cardenolide content) among milkweed species (Ladner & Altizer, 2005; Zalucki et al., 2012), we also expected caterpillars to show milkweed-specific growth rates. Furthermore, if melanin production is affected by food quality, we predicted positive correlations between growth rate and melanization. With respect to correlations between juvenile and adult melanization, if melanin production in juveniles is costly, as has been reported in several other insect species (Lee & Wilson, 2006; Talloen et al., 2004; Windig, 1999), we expected to see negative correlations between juvenile and adult melanization, as well as between juvenile melanization and traits such as wing size and growth rate. Finally, negative correlations between juvenile and adult melanization could also be driven by stage-specific fitness effects of melanin, for example, if pigmentation functions primarily in thermoregulation in juveniles but in anti-predator signaling in adults.

METHODS

Monarch source

Migratory monarchs in reproductive diapause were collected by the de Roode lab from St. Marks, FL, in November 2022, and kept at Emory University. In April 2023, surviving butterflies were mated, and their offspring were reared on either *Asclepias incarnata* or *Asclepias curassavica* before being sent to Bellingham, WA, as third instars (USDA Permit no. 526-23-125-89818). They were then brought to Vancouver, BC (USFWS Export Declaration 2023339512), where they completed their development in the University of British Columbia (UBC) greenhouse on *Asclepias fascicularis*. Butterflies from four unique maternal families eclosed. These butterflies were checked and confirmed

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to be free of the parasite *Ophryocystis elektroscirrha* (OE) and were mated to generate eggs for use in subsequent experiments. Resulting larvae were a mix of full siblings (i.e., same maternal family and same sire), half siblings (i.e., different maternal family but same sire), and unrelated individuals.

Part A: Effect of milkweed species on monarch melanization and growth

To test the effect of plant species on monarch growth and melanization, we first generated eggs by letting $n = 5$ mated adult females oviposit on potted *A. fascicularis* in a large cage. Eggs were placed in 500 mL plastic containers, stored in growth chambers (~25°C), and monitored. Once hatched, neonate caterpillars were placed on live milkweed plants within 12 h. Our initial goal was to rear each monarch on a different plant. However, at the start of the experiment, some plants were not big enough to sustain a monarch through its entire development. In these cases, two individuals were placed on a single large plant in separate mesh bags. The numbers of individual plants used per milkweed species and the number of monarchs per plant are summarized in Appendix S1: Table S1. Mesh bags were placed around the section of the plant, or around the entire plant, that the caterpillar was assigned to grow on (Appendix S1: Figure S1a). Each caterpillar was placed on a large branch, or on an entire plant, with enough leaf tissue to support their growth through all five instars. Greenhouse temperatures were set to 26:24°C and featured natural photoperiod conditions (~15:9 L:D during the period of the experiment).

Caterpillars were grown on 11 different species of milkweed plants: *A. curassavica*, *Asclepias eriocarpa*, *Asclepias erosa*, *A. fascicularis*, *A. incarnata*, *Asclepias labriformis*, *Asclepias speciosa*, *Asclepias syriaca*, *Asclepias tuberosa*, *Asclepias verticillata*, and *Gomphocarpus physocarpus*. The 11 species in our study include some of the most common host plants of monarchs throughout their North American range (Woodson, 1954). Milkweed seeds were collected throughout the United States by Micah Freedman in summer 2022, germinated in the greenhouse at the UBC in early 2023, and transplanted as 6-week-old seedlings into 1-gallon (3.8-L) pots that were fertilized weekly. Previous studies on some of these species have documented among-species variation in several plant traits including cardenolide profiles and concentrations of N and P (Decker et al., 2018; Jones et al., 2019; Ladner & Altizer, 2005; Sternberg et al., 2012; Tao et al., 2014; Zalucki et al., 2012).

Once caterpillars reached fifth instars they were removed from the plant, photographed (see *Monarch imaging*), and placed back on the same plant to finish development. Pupae were removed from the plant and secured to the bottom of a 500 mL clear plastic container, which was then placed upside down on a table in a warm, sunny spot in the greenhouse (Appendix S1: Figure S1b). After butterflies emerged, they were given 4–8 h to dry and were then placed into glassine envelopes. Butterflies were then frozen at −20°C. Monarch wings were removed with forceps and prepared for scanning, measuring, and color analysis.

Part B: Effect of temperature on monarch melanization and growth

To generate monarchs for the laboratory temperature experiment, we made three crosses between unrelated families. Females oviposited on *A. fascicularis*. Eggs were collected from *A. fascicularis* and placed in groups of 10–20 in 500 mL transparent plastic containers with several *A. fascicularis* leaves. Containers were labeled with maternal family and the date eggs were laid, and placed roughly equally into each temperature treatment. Containers also had holes poked into the lid to allow for airflow. The "cold" temperature treatment was 22/16°C day/night, and the "warm" temperature was 32/26°C. Photoperiod was set at 16:8 L:D. Neonate caterpillars, or ready-to-hatch eggs (which had visible head capsules within them) were moved from the egg containers to individual 500-mL transparent plastic containers with holes poked in the lid to allow for airflow. We placed several *A. fascicularis* leaves in each container as well as a folded, damp piece of paper towel at the bottom. Milkweed leaves and the paper towel were replaced and frass was emptied ad libitum. The placement of the caterpillar containers within incubators was changed every day to minimize potential positional effects within incubators. Within the warm treatment, caterpillars experienced high mortality. Most caterpillars from the three original matings died in their first or early second instar. Subsequently, one of the original females (code EJRB1) was mated again, and her eggs placed into the warm treatment incubator to replace some of those lost. Sample sizes in the two temperature treatments were not balanced (see *Results* tables).

When caterpillars reached their fifth instar, they were removed from the incubator, photographed, and returned to their respective temperature treatments (see *Monarch imaging*). Once caterpillars pupated, the pupae were taped to the bottom of the container, which was then placed upside down in the incubator. After butterflies emerged, they were given 4–8 h to dry and transferred to individual glassine envelopes. Envelopes with butterflies

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were held in the 22°C incubator, and butterflies were fed a 5:1 ratio of water: honey water once per week. Once all butterflies from the experiment emerged, all butterflies were frozen (−20°C). After a week in the freezer, their wings were removed and stored.

Monarch imaging

For both parts of the experiment, fifth instar monarch caterpillars were photographed using a Nikon DSLR 3500 camera fitted with a Nikon 100 mm macro lens. The camera was mounted on a tripod at a fixed distance (~1 m) from the specimen. Caterpillars were photographed with a color card in frame. Caterpillars were placed on a wooden chopstick to encourage them to straighten out as much as possible. Images of caterpillars that were fully straight were used for ImageJ analysis. Butterfly wings were removed and scanned using an Epson V39 scanner. All four wings were scanned.

Quantifying caterpillar and adult melanization

We used ImageJ (Schneider et al., 2012) to quantify the proportion black on each caterpillar. Images were first converted to 8-bit (grayscale). Using the segmented line tool on ImageJ with a line width of 10, a ventral midline was drawn from anterior tentacles to posterior filaments on each caterpillar (Appendix S1: Figure S1c). At each black stripe, the segmented line was placed through the approximate midpoint of the caterpillar. From there, a plot profile of grayscale values was generated (Appendix S1: Figure S1d). A table of all points (pixels) and their corresponding gray value was generated from the plot profile. Points under a threshold of 50 were considered to be part of a black stripe. We defined caterpillar proportion black as the fraction of points under 50 out of the total.

To quantify the amount of black on adult wings, first a thresholding step was used to convert images to black and white. Brightness was adjusted to 120, the level determined to be sufficient to distinguish black from any other color. The butterflies' left forewing, or right forewing if the left was deformed or otherwise damaged, was selected. We analyzed monarch forewings because they contribute more to flight compared to hind wings (Decker et al., 2018) and because of the connection between forewing coloration and migratory ability (Davis et al., 2012, 2023). A histogram containing only the number of black pixels and number of white pixels in the wing area was generated. We define wing "percent black" as the fraction of black pixels out of the total number of pixels (Davis et al., 2005). Our threshold-based measure of percent black does not take into account the intensity of black coloration in larvae or adults; these two measures may be partly independent (e.g., Davis et al., 2005; Johnson et al., 2014).

Data collected

For both the greenhouse and laboratory components of the study, we collected data on caterpillar proportion black, adult percent black, forewing area, forewing aspect ratio, and growth rate. Forewing area was quantified in ImageJ by converting total pixel number to area (in square millimeters). Forewing aspect ratio was calculated as forewing length divided by width (Decker et al., 2018; Soule et al., 2020). We measured these two wing traits because larger and more elongated wings are associated with longer distance flight in monarchs (Altizer & Davis, 2010; Freedman & Dingle, 2018). Growth rate was calculated as wing size divided by development time but because of logistical constraints, development time was calculated slightly differently in the greenhouse and the laboratory components of the study. For the greenhouse study, development time was measured as the number of days from first instar to eclosion. In the laboratory temperature study, development time was measured as egg lay date to pupation. Because of these two different metrics of development time, we refrain from comparing growth rate between the two parts of the study. Additionally, often butterfly growth rate is measured as accumulated mass divided by development time (Karl et al., 2008; Kingsolver, 2000). We did not measure butterfly dry mass because it was not clear if the drying process would change wing coloration. Instead, we used wing size as a proxy of mass.

Statistical analyses

For the greenhouse and laboratory parts of the study, we use linear models to test whether host plant species or temperature explained significant variation in caterpillar proportion black, adult percent black, forewing area, forewing aspect ratio, and monarch growth rate. Sex was included in the analyses of adult traits.

Although a few monarchs shared the same plant, because sharing occurred so infrequently (Appendix S1: Table S1), we did not include individual plant id as a random effect in any of the statistical models. A quick check of the statistics revealed that including individual plants as a random effect did not change the results of any models (data not shown). We initially included a random

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effect of monarch family in the statistical models investigating the effects of plant species and temperature. We removed the random effect of family because "family" explained a negligible amount of variation in all models. The results for all models did not change with the removal of the "family" term. Summary statistics for models without the "family" term are presented in the main text (Tables 1 and 2). Summary statistics for all models with the "family" term included are available in the supplementary materials (Appendix S1: Tables S2 and S3). We used type I SS to generate ANOVA tables for all linear models and type III SS for the lmer results reported in the supplementary materials. We also re-ran all linear models (lm) with type III SS and none of the results changed (data not shown).

To quantify possible associations between caterpillar melanization and adult traits, we combined data from both studies and created bivariate plots between caterpillar proportion black and (1) adult percent black, (2) forewing area, (3) forewing aspect ratio, and (4) growth rate. Because growth rate was measured differently in the greenhouse versus the laboratory components of the study, and because growth rate of monarchs in the two temperature treatments formed two distinct non-overlapping distributions (see *Results*), we used data from only the greenhouse experiment to examine (4) the relationship between caterpillar melanization and monarch growth rate. We conducted four linear models (type III SS) with caterpillar proportion black as the predictor and each of the four traits as the dependent variable. Each model also included "sex" and "experiment type" (greenhouse vs. laboratory) as explanatory variables. Non-statistically significant interactions between main effects were removed. All statistics were conducted in R (R Core Team, 2023). ANOVA and linear models were run in base R. Data wrangling and graphics were conducted in base R and the tidyverse package (Wickham et al., 2019). Linear mixed-effects models were conducted using the lme4 and lmerTest packages (Bates et al., 2015; Kuznetsova et al., 2017).

RESULTS

Effect of host plant species

Overall, we assessed melanization of 136 monarch caterpillars grown on 11 milkweed species. There was no effect of host plant species on caterpillar proportion black ($F_{10,125} = 1.35$, $p = 0.21$) or on adult wing percent black (Figure 1, Table 1). Variation in wing size and growth rate were both explained by host plant species, but plant species did not affect wing aspect ratio. Post

TABLE 1 Summary statistics for the analyses of the effect of host plant species and sex on monarch traits.

	Adult percent black ($n = 102$)			Forewing area ($n = 102$)			Forewing aspect ratio ($n = 102$)			Growth rate ($n = 92$)		
Effect	***F***	**df**	***p***	***F***	**df**	***p***	***F***	**df**	***p***	***F***	**df**	***p***
Plant species	0.68	10, 80	0.74	2.32	10, 80	**0.02**	0.59	10, 80	0.82	4.7	10, 70	**<0.0001**
Sex	358	1, 80	**<0.0001**	1.82	1, 80	0.18	0.58	1, 80	0.45	0.22	1, 70	0.67
Sex × plant species	1.10	10, 80	0.06	1.16	10, 80	0.33	0.98	10, 80	0.46	0.75	10, 70	0.68

Note: Post hoc tests showed no significant pairwise comparisons for forewing area. Post hoc tests for growth rate are available in Appendix S1: Table S4. Sample sizes (n) are included in parentheses for each trait. Significant p-values are denoted in bold font.

TABLE 2 Summary of statistics from the lab experiment investigating the effect of temperature on monarch traits ($n = 66$ for all analyses, 49 "cold," 17 "warm").

	Caterpillar proportion black			Adult percent black			Forewing area			Forewing aspect ratio			Growth rate		
Effect	***F***	**df**	***p***	***F***	**df**	***p***	***F***	**df**	***p***	***F***	**df**	***p***	***F***	**df**	***p***
Temperature	160	1, 62	**<0.0001**	0.16	1, 62	0.69	14.1	1, 62	**0.0004**	9.2	1, 62	**0.004**	690	1, 62	**<0.0001**
Sex	1.18	1, 62	0.28	558	1, 62	**<0.0001**	0.19	1, 62	0.67	0.38	1, 62	0.54	0.15	1, 62	0.70
Temperature × sex	0.09	1, 62	0.76	57.6	1, 62	**<0.0001**	0.19	1, 62	0.66	0.45	1, 62	0.50	1.0	1, 62	0.32

Note: Significant p-values are denoted in bold font.

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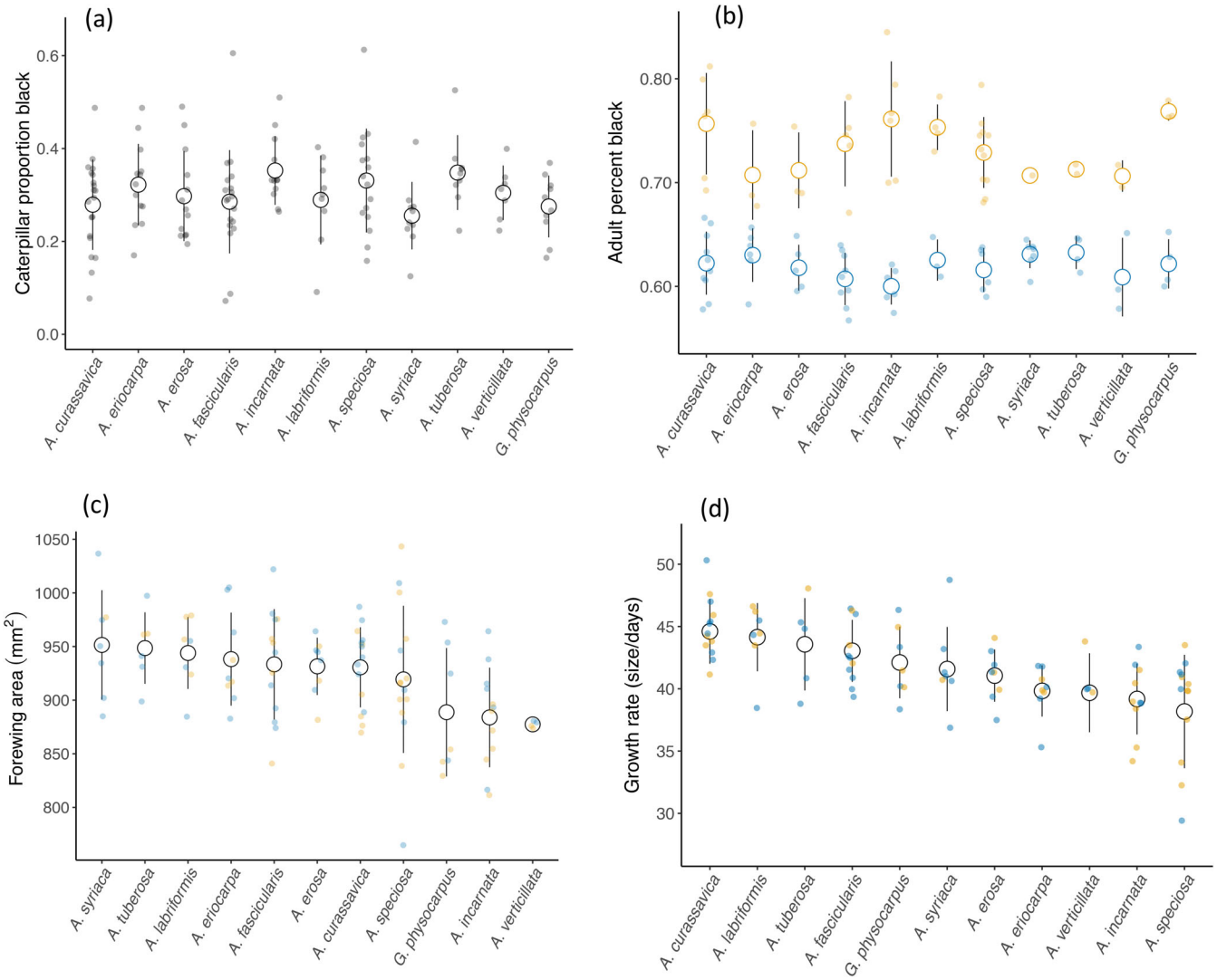


FIGURE 1 Monarch (a) caterpillar proportion black, (b) adult percent black, (c) forewing area, and (d) growth rate (forewing area in square millimeters per developmental days) when reared on different species of milkweed. Small dots represent individual monarch data points and the larger central dot is the mean value. Error bars are ±1 SD. There was no effect of milkweed species for (a) and (b) and thus the plants are sorted alphabetically. Statistics for (a) are available in the main text. Summary statistics for panels (b)–(d) are available in Table 1. The means of the sexes are plotted separately in (b) because the effect of sex is statistically significant. Blue points denote males, yellow points denote females.

hoc comparisons revealed no significant pairwise differences between plant species for wing size, but several differences were observed for growth rate. Monarchs grown on *A. curassavica* and *A. speciosa* generally grew the fastest and slowest, respectively (Appendix S1: Table S4).

Effect of temperature

Monarch caterpillars grown at warm temperatures had thinner black stripes compared to those grown at cooler temperatures (Figure 2, Table 2). The effect of temperature on adult percent black was sex-specific: warmer temperatures led to lighter wings in males (post hoc contrast: $t_{55} = 2.0$, $p = 0.048$) but females grown at warmer temperatures were darker than those grown at cooler temperatures (post hoc contrast: $t_{62} = -7.7$, $p < 0.001$). Warmer temperatures also resulted in monarchs with smaller and more elongated wings and faster growth rates.

Combined data: Correlations between caterpillar melanization and adult traits

Greenhouse and lab-reared monarch data were combined to test for associations between caterpillar melanization

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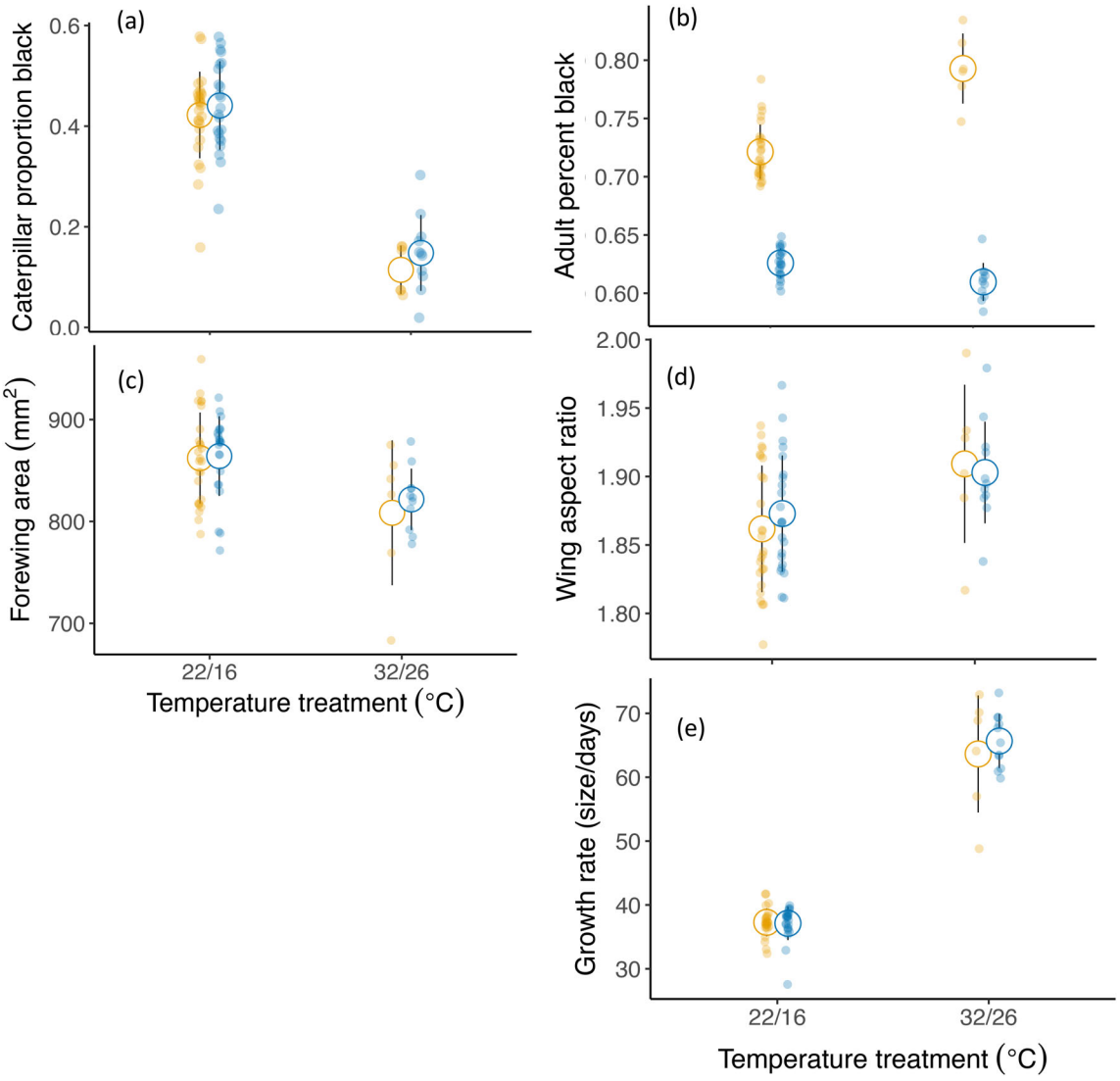


FIGURE 2 Monarch (a) caterpillar proportion black, (b) adult percent black, (c) forewing area, (d) wing aspect ratio, and (e) growth rate (forewing area in square millimeters per development time) when reared at 22/16°C day/night or 32/26°C day/night temperature. Small dots represent individual monarch data points and the larger central dot is the mean value. Error bars are ±1 SD. Blue and yellow points denote male and female monarchs respectively. Summary statistics are available in Table 2.

and monarch traits across the broadest phenotypic space. Female caterpillars with higher proportion black were less melanized as adults, but there was no relationship between caterpillar and adult darkness in males (Figure 3, Table 3). Darker caterpillars developed into adults with larger wings, but this pattern was only observed in the laboratory temperature experiment. Compared to caterpillars with less black, darker caterpillars turned into adults with rounder wings, and this effect was observed in monarchs from the greenhouse and laboratory experiments. Finally, darker caterpillars from the greenhouse component of the study grew more slowly. Because we observed a negative correlation between caterpillar and female melanization, we subsequently plotted female percent black versus forewing size and found a significant negative relationship between these two traits ($F_{1,75} = 9.3$, $p = 0.003$).

DISCUSSION

Understanding the causes and consequences of melanin plasticity provides insight into how variation in color and patterning shapes animal fitness. Here we examined the effects of diet and rearing temperature on juvenile melanization in the monarch butterfly and subsequently quantified the associations between this trait and adult melanization, wing size, wing shape, and growth rate. Overall, we documented negligible effects of milkweed species and strong effects of temperature on caterpillar

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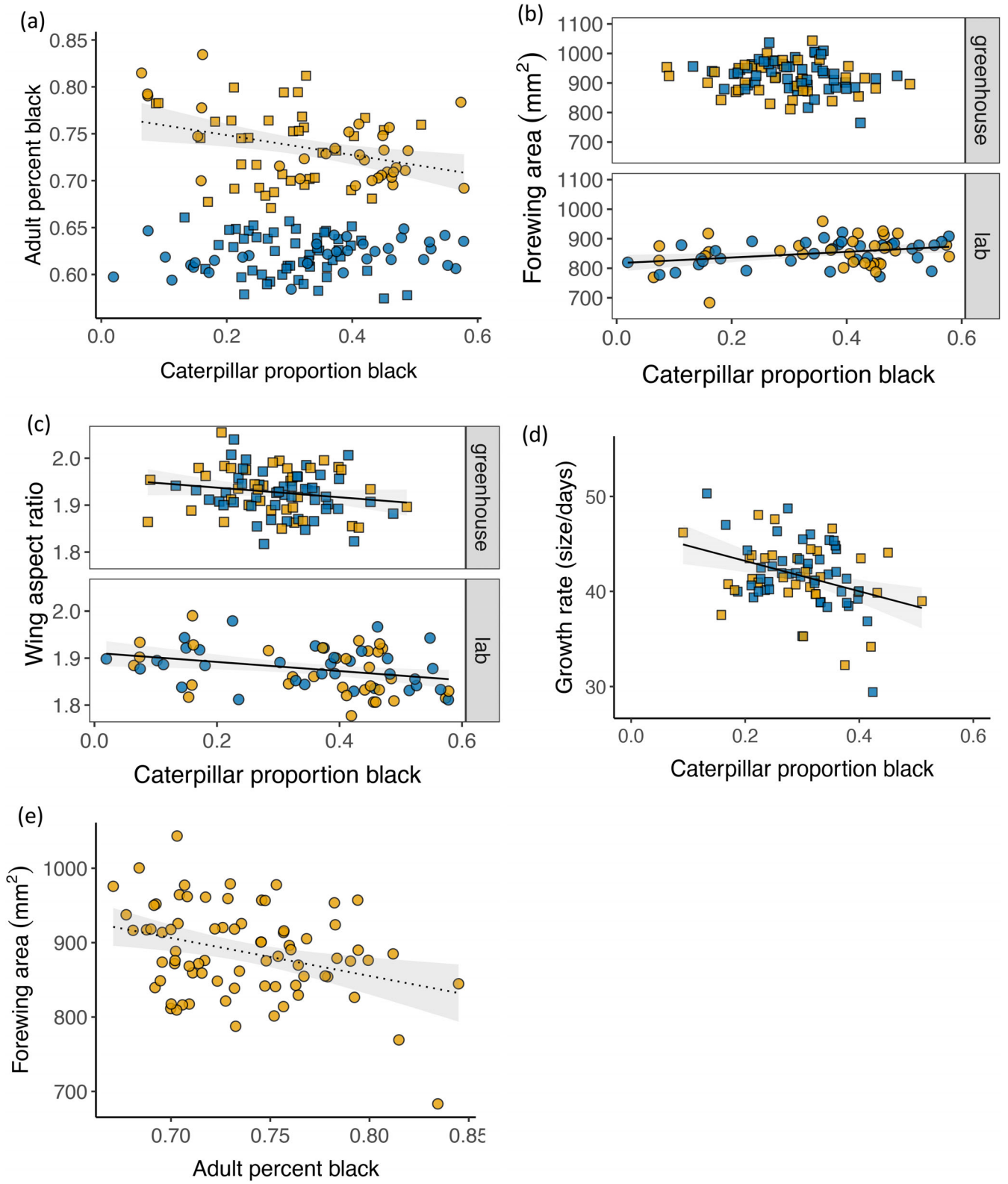


FIGURE 3 The relationship between caterpillar proportion black and (a) adult percent black, (b) forewing area, (c) wing aspect ratio, and (d) growth rate (forewing area in square millimeters per development time). Figures are faceted by "experiment type" if it or interactions with this term were statistically significant (Table 3). Yellow and blue symbols denote female and male monarchs, respectively. Squares and circles denote monarchs from the greenhouse and lab experiments, respectively. Because the caterpillar proportion black predicted adult percent black for females (a), we also examined the relationship between female percent black and wing size (e). Dotted and solid lines denote lines of best fit for females and all monarchs, respectively. Shaded areas surrounding the lines are 95% CIs. Panel (d) is plotted using squares because only the greenhouse portion of the data were used for this analysis. Summary statistics are available in Table 3.

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TABLE 3 Summary of statistics for the analyses of relationships between caterpillar proportion black and monarch traits.

	Adult percent black ($n = 153$)			Forewing area ($n = 153$)			Forewing aspect ratio ($n = 153$)			Growth rate ($n = 79$)		
Effect	***F***	**df**	***p***	***F***	**df**	***p***	***F***	**df**	***p***	***F***	**df**	***p***
Caterpillar proportion black	15.1	1, 148	**0.0002**	2.69	1, 148	0.10	9.6	1, 149	**0.002**	11.4	1, 75	**0.001**
Sex	134	1, 148	**<0.0001**	0.87	1, 149	0.35	0.002	1, 149	0.97	0.12	1, 75	0.73
Experiment type	0.46	1, 148	0.5	29.6	1, 148	**<0.0001**	32.8	1, 149	**<0.0001**	...	...	...
Caterpillar proportion black × sex	9.55	1, 148	**0.002**	...	...	...	...	...	...	1.2	1, 75	0.28
Caterpillar proportion black × experiment type	...	...	...	7.30	1, 148	**0.008**	...	...	...	...	...	...

Note: Greenhouse and lab data are analyzed together for some traits. Sample sizes (n) are included in parentheses for each trait. Significant p-values are denoted in bold font.

melanization. Furthermore, juvenile melanization was correlated with some adult traits: caterpillars with a higher proportion black developed into adults with rounder wings and had slower overall growth rates across their development. There was also a negative relationship between caterpillar and adult melanization in female monarchs but not males. Lastly, melanization was correlated with wing size in two ways: darker caterpillars developed into monarchs with larger wings (only in the lab experiment), and there was a negative relationship between wing darkness and wing size in female monarchs. Wing size and shape are tightly correlated with flight in monarchs and other butterflies (Decker et al., 2018; Soule et al., 2020). Larger wings are associated with faster and farther flight, and more elongated (less rounded) wings are better suited for sustained flight (Decker et al., 2018; Flockhart et al., 2017; Freedman & Dingle, 2018; Kerlinger, 1989; Yang et al., 2016). Our data thus suggest that melanin production in juveniles may be costly for monarch butterflies due to the negative relationships between melanization and forewing area and shape, which in turn is a strong predictor of migratory ability.

The observed negative correlations between juvenile melanization and adult traits in monarchs (especially in females) are consistent with studies reporting similar patterns between melanization and life history traits in other species (Mwando et al., 2023; Talloen et al., 2004; Windig, 1999) but inconsistent with studies documenting positive relationships between fitness and increased melanization (Mwando et al., 2023; Rajpurohit et al., 2016). Why melanin production appears to be costly in some contexts but not others is unclear but may be related to inter-experiment variation in environment quality (Stoehr et al., 2024; Talloen et al., 2004). Stoehr et al. (2024) proposed that the costs of producing resource-intensive classes of pigments such as melanin may be more prominent when resources are limited. In our study, caterpillars were given unlimited access to milkweed and yet we observed clear negative relationships between caterpillar melanization and wing size (females only) and elongation (both sexes). Johnson et al. (2014) measured the intensity of forewing darkness (potentially a better proxy for melanin investment than the measures we report) in monarchs and found that wings that were "darker" black were rounder in shape than those that were "lighter" black. This pattern was seen in both lab-reared and wild-caught monarchs (Johnson et al., 2014); we note that the percent black we observed in adult wings was lower than that reported by Johnson et al. (2014) but comparable to reported by Davis et al. (2005), highlighting that these values can vary across studies.

Together our findings suggest the presence of molecular linkages between melanin production and wing morphology, irrespective of food availability. Indeed, multiple studies have found genes involved in both melanin production and wing morphology (Martin & Reed, 2014; Matsuoka & Monteiro, 2018; Sharma et al., 2016); in particular, the compound heparin has been shown to jointly modulate wing shape and melanization in Lepidopteran through modification of WntA signaling (Martin & Reed, 2014; Sourakov, 2026). The potential of shared developmental pathways between wing morphology and melanin hints that environmentally mediated plasticity in melanization may have important consequences beyond thermoregulation and patterning.

In this study, colder temperatures produced darker caterpillars. This result supports the thermal melanism hypothesis and has been shown before in monarchs (Davis et al., 2022; Tseng et al., 2023) as well as in other insects (Goulson, 1994; Hazel, 2002). Interestingly, when data from the diet and temperature components of our study were combined, increased caterpillar melanization was negatively correlated with black pigmentation in

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female adult monarchs but not in males (despite the lack of sex-specific patterns in larval melanization). Davis et al. (2005) also examined correlations between juvenile and adult melanization. Their methods differed slightly from ours in that they reported average juvenile and adult melanization per family rather than from the same individual. Despite these methodological differences, Davis et al. (2005) also reported a negative correlation between caterpillar and adult melanization in female monarchs. However, in contrast to our study they found a positive correlation between caterpillar and adult melanization in males. Although the results differ slightly, these patterns suggest that the factors influencing melanin production are unique in male versus female monarchs. Sex-specific melanin-related gene expression was also observed in the butterfly *Papilio polytes*, suggesting that similar patterns may potentially occur in other species of Lepidoptera (Kuwalekar et al., 2020).

We initially predicted milkweed species associated with fast caterpillar growth would also produce juvenile monarchs with elevated melanin because plants able to sustain high caterpillar growth may also be able to support the synthesis of this N-rich molecule. Although growth rate and wing size varied among monarchs fed different milkweed species (Figure 1c,d), there was no effect of host plant on caterpillar or adult melanization (Figure 1a,b). Monarchs grew fastest when reared on *As. curassavica* (tropical milkweed) and slowest on *As. speciosa* (showy milkweed). We were curious whether milkweed-specific growth rates might be related to variation in plant cardenolide content. Although we did not measure plant cardenolides in this experiment, we broadly characterized the 11 species used here into one of three cardenolide levels based on previously published literature (Malcolm, 1991; Malcolm et al., 1989; Züst et al., 2019). Relative cardenolide concentration did not explain significant variation in monarch wing length or growth rate (Appendix S1: Table S5, Figure S2a,b). This result is consistent with previous findings showing that leaf cardenolides may be poorer predictors of monarch growth rate compared to other traits such as latex production or trichomes (Agrawal et al., 2015; Zalucki et al., 2012).

In summary, we have documented that environmental conditions can influence melanin production in monarch caterpillars and in turn, elevated melaninization may be costly to monarch wing morphology. Despite the robustness of these general patterns, our sample sizes are relatively limited. In the greenhouse component of the experiment, the number of caterpillars per host plant ranged from 4 to 20 (Appendix S1: Table S1). Sample sizes in the two temperature treatments were also unbalanced. We recommend future studies examine costs of melanization in multiple populations of monarchs, and with multiple types of resources in addition to host plant species, to confirm the generality of the patterns reported here. A further limitation of this study is adult melanization was only measured on the dorsal side of one forewing, possibly leaving gaps in our knowledge on total melanization. However, forewings were prioritized because flight in Lepidoptera is driven mainly by forewings (Jantzen & Eisner, 2008), and in monarchs, forewing shape and color specifically has been shown to impact flight and expected migration ability (Hanley et al., 2013; Soule et al., 2020). Despite these caveats, this study uncovers a unique finding related to negative relationships between juvenile and adult melanization in female monarchs. Our data demonstrate that female caterpillars with less melanin have darker and larger wings as adults. We also show a link between monarch rearing temperature and wing size, with smaller wings at hotter temperatures. Together these findings suggest two ways female monarch wing size is negatively affected by warming temperatures: directly through temperature and possibly indirectly through melanin-related costs. As global temperatures continue to increase, a worthwhile next step in these investigations is to more directly link decreasing overwintering monarch population sizes to temperature-mediated shifts in monarch coloration and wing morphology, especially in light of data suggesting that migration itself may be an increasingly stressful process in monarchs (Davis et al., 2024; Inamine et al., 2016).

AUTHOR CONTRIBUTIONS

All authors conceived the ideas and designed methodology; Wendy Frankel and Micah Freedman collected the data; all authors analyzed the data; Wendy Frankel and Michelle Tseng led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data (Frankel, 2026) are available from Zenodo: https://doi.org/10.5281/zenodo.22924357.

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