

## Original Article

## Forewing pigmentation predicts migration distance in wild-caught migratory monarch butterflies

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Surprisingly, little is known about how the environment influences the production of the iconic orange coloration of the monarch butterfly (*Danaus plexippus*). Previous research under controlled laboratory conditions has shown that the temperature during larval development influences the color of monarch wings, where females raised in warm conditions had a greater proportion of melanization, whereas males raised in warm conditions had a lower proportion of melanization. These melanin-based colors have been found to increase flying ability in Lepidoptera, and recent experiments have found that monarchs with redder forewings flew greater distances than monarchs with less intense coloration. We examined whether wild-caught monarchs captured in the Great Lakes region exhibited geographic polyphenism by using stable isotopes to estimate natal origin, and hence rearing temperature, spectrophotometry to measure forewing coloration, and image analysis to estimate shape. We found that monarchs from the Gulf Coast were more melanized than monarchs from the Great Lakes, and southern male monarchs were more saturated than northern male monarchs. This supports previous research suggesting that colors that absorb more solar energy allow for greater flying ability but contradicts the patterns we expected based on natal temperature. Interestingly, this effect of color on migration distance was independent of wing shape. We provide the first evidence that the coloration of wild monarchs influences their migration ability over a continental scale, and we suggest that these differences in color may benefit the cohort of monarchs destined for long-distance migration to their wintering ground.

**Key words:** flying ability, monarch butterfly, natal origin, thermoregulation, wing color.

## INTRODUCTION

Coloration in animal tissue can serve a number of important functions related to fitness, such as signaling individual quality or health, or avoiding predation (Milinski and Bakker 1990; von Schantz et al. 1999; Stevens and Merilaita 2009). In many species, there is a remarkably wide range of individual variation in color (Baker and Parker 1979; Brakefield and French 1999; Jablonski and Chaplin 2000; Hofreiter and Schoneberg 2010). Some of this variation can be attributed to individual quality (Amundsen and Pärn 2006; Cotton et al. 2006), whereas some may result from broadscale environmental factors that influence color expression independent of individual quality (Marchetti 1993; Horak et al. 2000; Gomez and Thery 2004; Gosler et al. 2005). Migratory animals present a

unique opportunity for understanding coloration because colorful tissues can be formed during one period of the annual cycle but then influence factors related to fitness during subsequent periods (Norris et al. 2004). For example, many birds molt colorful feathers used in mate choice several months prior to breeding, thousands of kilometers away on the nonbreeding grounds (Norris et al. 2007; Reudink et al. 2009). Within a migratory population, individuals may have originated from a variety of locations, and variation in coloration may be attributed to their natal environment or individual quality. However, identifying factors that influence coloration has been challenging because of the difficulty of directly following individuals over multiple periods of the year (Norris et al. 2007).

The unique migratory behavior of Monarch butterflies (*Danaus plexippus*) makes them one of the most recognizable butterflies in the world. Each year, eastern North American monarchs recolonize their breeding range from high-altitude overwintering sites in central Mexico over a series of successive generations.

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Interestingly, there is evidence that monarchs originating from Mexico adopt different recolonization strategies (Malcolm et al. 1993). Many overwintering adults only migrate as far as southern Texas or the Gulf Coast before they reproduce and die, leaving the subsequent generation to migrate north and breed. However, some overwintering monarchs may continue migrating substantial distances north before laying their last eggs of the season, with an estimated 10% reaching the extreme northern portion of their breeding range (Miller et al. 2012). Because of this variability in migration distances throughout the breeding period, a northern breeding population may be comprised not only individuals from Mexico born the previous year but also first- or second-generation individuals that were born in a variety of areas further south on the breeding grounds (Miller et al. 2012).

Monarch butterflies also possess a recognizable series of bright orange patches on their wings, and previous work has suggested that these orange patches are most likely colored by an orange ommochrome (Kayser 1985; Nijhout 1991; Davis et al. 2012). Recent experiments under captive conditions have shown that males with more saturated wing color are more successful at mating than less saturated males (Davis et al. 2007). In addition, this research found an interaction between temperature and sex such that females reared in warm conditions had a greater proportion of dark pigmentation on their forewings, whereas males had a lower proportion of dark pigmentation on their forewings (Davis et al. 2004, 2005). We will refer to this as the “temperature hypothesis.”

In contrast to the temperature hypothesis, which suggests that forewing coloration is directly dependent on temperature, certain forewing colors may allow for greater ability to fly, which could partly explain observed color variation in migratory populations (Davis 2009), such as found in the eastern North America monarch butterfly. The “flying ability hypothesis” predicts that butterfly forewing coloration may provide an adaptive advantage for flight, because more heavily pigmented wings absorb more solar radiation and this allows ectothermic butterflies to fly at low temperatures (Watt 1969; Guppy 1986). However, this reasoning should apply to both the melanin-based and ommochrome-based pigmentation, because both black and orange butterfly wing pigments have relatively high absorption of nonreflected wavelengths (Yoshioka and Kinoshita 2006).

Interestingly, a recent experimental study provided evidence that male and female monarch butterflies exhibiting redder hues on their forewing flew farther than individuals with less red hues (Davis et al. 2012). However, Davis et al. (2012) did not find that melanization enhances flying ability, a finding that contrasts experiments conducted under natural conditions (Watt 1969; Guppy 1986), and laboratory conditions simulating natural temperature ranges (Watt 1969). The solar energy absorbed by melanin raises the body temperature of ectothermic insects and allows them to be active when ambient temperatures are otherwise too low to sustain activity (Watt 1969). In contrast to previous experiments (Watt 1969; Guppy 1986), Davis et al. (2012) used a constant room temperature and they suggested that melanin was unrelated to flying distance in their experiment because captive monarchs were not exposed to temperatures close to their lower temperature threshold (Watt 1969; Guppy 1986). Davis et al. (2012) also found that females flew farther than male monarchs. Proximately, this could be because they have a greater proportion of melanin and, ultimately, because they have been selected to

continually search for locations to lay their eggs. Interestingly, the last generation of monarchs breeding in North America, which are destined to undergo a long-distance migration to their wintering ground in Mexico, have a significantly redder hue (Davis 2009), providing some support for the hypothesis that monarch wing coloration is related to flying ability. However, no study has shown that monarch forewing pigmentation is related to migratory distance within a wild population.

In this paper, we examined variation in wing color of wild-caught migratory monarchs during the breeding season. By using stable isotopes to estimate natal origin (Miller et al. 2012) and spectrophotometry to measure color, we examined the influence of both larval environment and sex on variation in coloration of wild-caught monarchs. This technique provides a unique opportunity to examine the temperature hypothesis and the flying ability hypothesis. If temperature dictates adult coloration, we predicted an interaction between sex and natal origin such that males reared in warmer southern locations would have a lower proportion of melanin than northern males, and females reared in warmer southern locations would have a higher proportion of melanin relative to northern females (sensu Davis et al. 2005). Our method also establishes 3 distinct migratory distances. Here, monarchs that hatched in the Gulf Coast and migrated to the Great Lakes migrated farther than those that had hatched in the Central United States and migrated to the Great Lakes, which travelled farther than those monarchs that hatched and stayed within the Great Lakes region. In contrast to the predictions of the temperature hypothesis, if adult forewing color most strongly reflects flying ability, assessed here as migration distance, we expected a higher proportion of melanin and more saturated orange pigmentation in monarchs that migrated greater distances, regardless of sex. Because males have less melanin on their wings than females, we expected that the saturation of their orange pigmentation should aid migratory distance more than in females, which have predominantly melanized forewings (Davis et al. 2005; Atterholt and Solensky 2010).

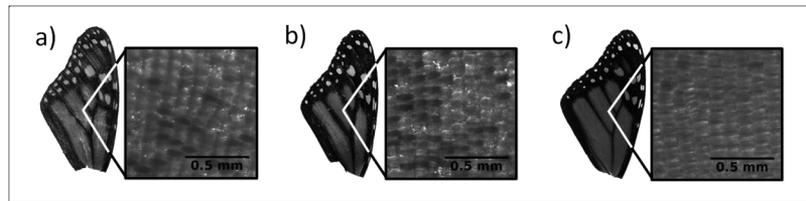
## METHODS

### Collections and natal origin

Adult monarch butterflies ( $n = 98$ ) were captured using standard sweep insect nets between May and June 2009 in Ontario, Michigan, Wisconsin, and Minnesota. In a previously published study (Miller et al. 2012), a combination of stable-hydrogen isotopes and wing wear was used to estimate the natal origin (Gulf Coast, midlatitude United States, or the Great Lakes region) and whether individuals overwintered in Mexico or were born during the sampling year. Details of the stable isotope analysis and wing wear scoring can be found in Miller et al. (2012).

### Color analysis

To estimate color, we used a spectrometer (Jaz, Ocean Optics, Dunedin, FL) with a built-in full spectrum, pulsed xenon light source (Jaz-PX, Ocean Optics), and a diffuse reflectance standard (WS-1-SL, Ocean Optics) to measure the middle cell of the left forewing from adult monarchs (Figure 1) at a constant distance with a 90° incidence angle. We measured the middle cell 5 times, moving the spectrometer probe on each measurement. This part of the wing is generally free from tattering (Davis et al. 2007) and has been used in previous studies (Davis et al. 2007; Davis 2009). Each spectrum was visually inspected, smoothed with a



**Figure 1**

Variation in monarch forewing pigmentation. The middle cells (enlarged portion) of 3 monarch forewings illustrate the substantial variation in saturation scores, from (a) low (1.5), (b) medium (1.8), to (c) high (3.6). Each wing shows a magnification of individual scales. Worn wings (a) exhibit missing scales (omitted from our analysis and are depicted here for illustrative purposes only). Wings with relatively low saturation (a, b) display scales with both heavy and light pigmentation. On the other hand, saturated wings (c) show relatively consistent (“pure”) pigmentation across all scales.

locally weighted polynomial regression (Cleveland et al. 1992), and summarized between 300 and 700 nm with values every 5 nm. We calculated saturation as the maximum reflectance minus the minimum reflectance divided by the mean brightness (Smiseth et al. 2001; Andersson et al. 2002; Montgomerie 2006). Saturation provides an estimate of color purity (Montgomerie 2006) and has been found to correlate well with pigment concentration in some systems (Butler et al. 2011). Although we did not quantify the types or levels of pigments in the forewings used in this study, forewing saturation (i.e., color purity) is a product of both saturation of individual scales and the uniformity of pigmentation among scales, such that monarchs with lower forewing saturation generally have less saturated scales with a mixture of pigments or pigment concentrations (Figure 1). We also calculated brightness as the mean spectral reflectance from 300 to 700 nm (Montgomerie 2006).

Each monarch was also scanned on an HP PSC 750 flatbed scanner at 300 dots per inch. We used ImageJ v 1.47 (<http://rsb.info.nih.gov/ij/>) to calculate the proportion of melanin-based coloration on each forewing, as well as a metric of wing elongation. To calculate the proportion of melanin, we thresholded each size calibrated image to isolate the black from nonblack portions of the forewing, following previously published methodology (Solensky and Larkin 2003; Davis et al. 2004, 2005, 2007). In addition, previous research has found that migratory populations of monarchs have more elongated wings compared with sedentary populations that have rounder wings (Altizer and Davis 2010). It is possible that forewing coloration relates to these known differences in wing morphology. To account for this possibility, we also determined if forewing coloration was independent of forewing shape. To examine this relationship, we calculated the roundness,  $4 \times [\text{area}/(\pi \times \text{major axis})^2]$ , of each monarch forewing using the “measure” tool. Area was calculated for the entire forewing and the major axis represents the longest vector of the best fitting ellipse around each forewing. Previous research has used circularity (Altizer and Davis 2010), which is greatly impacted by even subtle wing wear (see Supplementary Data). Therefore, we report a more conservative roundness measure. For more complete information on our image analysis, see Supplementary Data.

### Statistical analysis

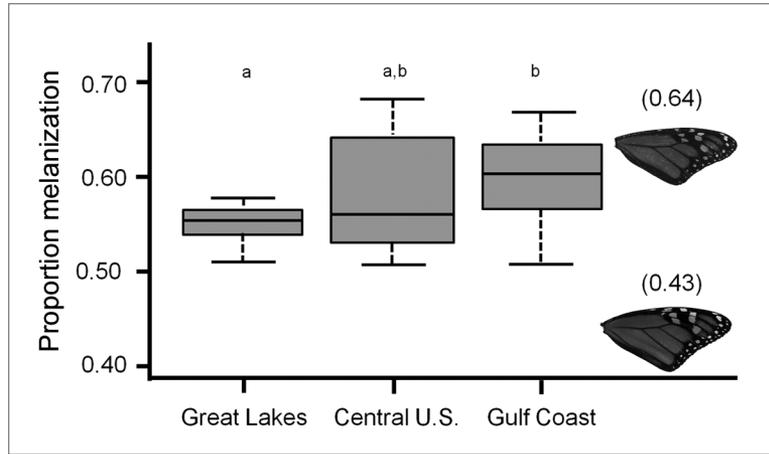
We examined how saturation was related to the proportion of black pigmentation on monarch forewings using a random intercepts mixed model, to account for male and female differences in coloration and proportion of melanin coverage. To examine if natal origin influenced monarch wing color (saturation or

proportion melanization) or shape, we ran 3 separate linear models to predict variation in monarch wing color or shape. Because monarchs born in the Great Lakes region were caught significantly later than those born in either the Central United States or the Gulf Coast ( $F_{2,81} = 4.0$ ,  $P = 0.04$ ), we controlled for Julian capture day in each model. To exclude monarchs that overwintered in Mexico, we removed 14 monarchs with wing wear scores greater than or equal to 4 (Malcolm et al. 1993; Miller et al. 2012) and controlled for the remaining variation in wing wear in our model. Previous research has found both sex differences in adult coloration and interactions between sex and rearing conditions (Davis et al. 2005; Davis 2009), so we included an interaction between sex and natal origin in each model. To aid the interpretation of interactions, we also present Tukey’s honestly significant difference (Tukey HSD) test results from accompanying sex-specific linear models. We use a simulation-based approach to examine the significance of random effects from our mixed model and report the results of the exact restricted likelihood ratio test (Crainiceanu and Ruppert 2004). To describe the fit of the whole model, we report a goodness-of-fit estimate for the fixed effects from our mixed model (Edwards et al. 2008), as well as an approximate  $F$ -statistic from a Wald test (Draper and Smith 1998). To examine the significance of the whole model, we report the  $P$  value from a likelihood ratio test comparing the full model to a null model including only the random term.

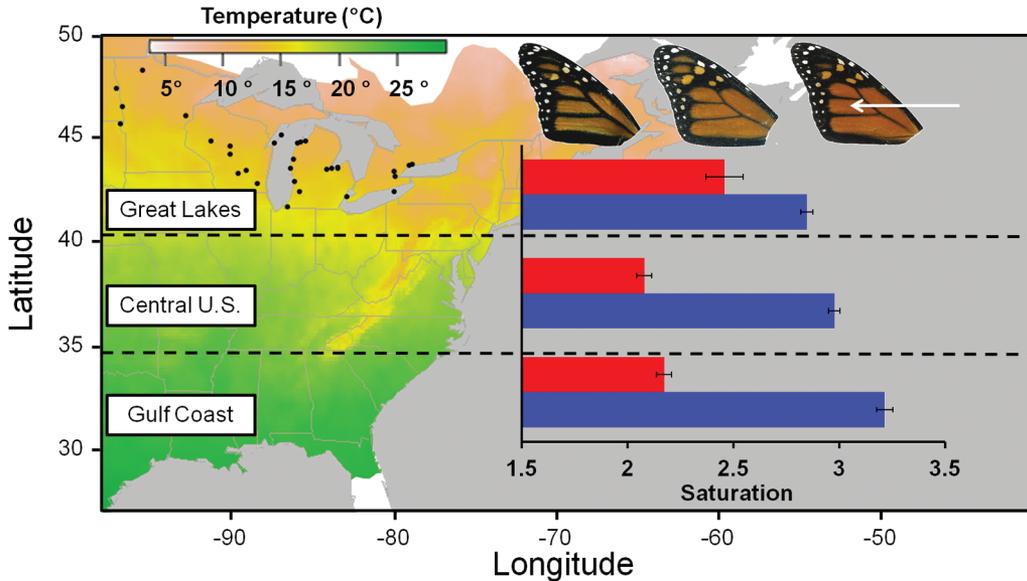
### RESULTS

Forewing saturation was positively predicted by the proportion of melanization ( $R^2 = 0.07$ ,  $F_{1,81} = 6.22$ ,  $P = 0.01$ ). Proportion of forewing melanization was effectively explained by a general linear model ( $R^2 = 0.71$ ,  $F_{11,72} = 15.6$ ,  $P < 0.0001$ ; Figure 2) with Julian day, wing wear, origin, sex, and an interaction between sex and origin. By controlling for Julian day ( $b = 0.002 \pm 0.001$ ,  $P = 0.08$ ), wing wear ( $F_{5,72} = 5.23$ ,  $P = 0.0004$ ), and sex ( $F_{1,72} = 135.29$ ,  $P < 0.0001$ ), we found that the proportion of melanization was explained by origin ( $F_{2,72} = 5.25$ ,  $P = 0.008$ ), but contrary to the prediction of the temperature hypothesis, there was no interaction between origin and sex ( $F_{2,72} = 0.49$ ,  $P = 0.61$ ).

A general linear model with Julian day, wing wear, origin, sex, and an interaction between sex and origin explained 77% of the variation in adult monarch wing saturation ( $R^2 = 0.77$ ,  $F_{11,72} = 21.9$ ,  $P < 0.0001$ ). After controlling for Julian day ( $b = -0.01 \pm 0.006$ ,  $P = 0.06$ ), wing wear ( $F_{5,72} = 12.25$ ,  $P < 0.0001$ ), origin ( $F_{2,72} = 2.11$ ,  $P = 0.13$ ), and sex ( $F_{1,72} = 165.18$ ,  $P < 0.0001$ ), monarch wing saturation was explained by an interaction between origin and sex ( $F_{2,72} = 3.45$ ,  $P = 0.04$ ; Figure 3). We found that males ( $2.97 \pm 0.37$



**Figure 2** Saturation and proportion of melanin-based forewing coloration. Bar graphs represent the fitted proportion melanin ( $\pm$ SE) values from a linear model (see Methods) for monarchs that originated from the Gulf Coast, Central United States, or the Great Lakes. We have included 2 male monarch forewings that represent the maximum range in male proportion of melanization and display those proportions above each inset image. Tukey HSDs are indicated by letters above each bar.



**Figure 3** Monarch wing color and natal origin. Bar graphs represent the fitted saturation ( $\pm$ SE) values (saturation is unitless) from a linear model (see Methods) for males (blue bars) and females (red bars) that originated from the Gulf Coast, Central United States, or the Great Lakes. Black dots on the map represent capture locations. The colors on the map represent the mean temperature ( $^{\circ}$ C) for the months of April, May, and June based on WorldClim data (<http://www.worldclim.org>) and are depicted only within the monarch breeding range. The 3 distinct sites of natal origin assigned via stable-hydrogen isotope values (see Miller et al. 2012) are separated by dashed lines. The monarch forewings (from left to right: female, male, male) represent a range in saturation values (from left to right: 2.53, 3.22, 4.28). Each photograph was linearized with a white and black standard. The white arrow indicates the location used for measuring spectral reflectance.

were more saturated than females ( $2.15 \pm 0.34$ ). Males that originated from the Gulf Coast ( $3.21 \pm 0.31$ ) were more saturated than males originating in the Great Lakes ( $2.85 \pm 0.35$ ; Tukey HSD  $P$  value = 0.007), whereas Gulf Coast females ( $2.17 \pm 0.29$ ) and Great Lakes females ( $2.46 \pm 0.42$ ) exhibited similar saturation (Tukey HSD  $P$  value = 0.22; Figure 3).

In contrast to the models explaining forewing pigmentation, forewing shape was not predicted by a general linear model with Julian day, wing wear, origin, sex, and an interaction between sex and origin ( $R^2 = 0.07$ ,  $F_{11,72} = 0.52$ ,  $P = 0.88$ ). In addition, neither forewing saturation ( $r = 0.06$ ,  $CI_{0.95} = -0.16$  to  $0.27$ ,  $P = 0.62$ ) nor

proportion of forewing melanization ( $r = -0.10$ ,  $CI_{0.95} = -0.31$  to  $0.12$ ,  $P = 0.36$ ) was related to forewing shape.

**DISCUSSION**

In a population comprised individuals originating from different locations, many factors can impact color expression. For monarch butterflies found at the northern edge of their range, forewing color may result from variation in natal temperature, or their colors may vary by natal origin because their forewing pigments aid dispersal ability. We found that monarchs exhibit natal environment-dependent

color production such that monarchs reared in the warmer southern portion of their range had a greater proportion of melanization than monarchs reared in cooler northern temperatures (Figure 2). This finding directly contradicts previous experimental findings on rearing temperature (Davis et al. 2004, 2005); however, it provides support for the flying ability hypothesis that suggests monarch wing pigmentation enhances flying ability (Davis et al. 2012).

We found that forewing saturation was positively related to wing melanization, which supports previous findings that ommochrome synthesis was dependent on prior melanization in a Lepidopteran moth (Hiruma et al. 1984). Based on this relationship, we expect southern male monarchs reared in warmer temperatures to be less saturated (and less melanized) than their northern male counterparts, with the opposite pattern in females; however, the patterns we saw with forewing saturation (Figure 3) also contradicted the temperature hypothesis. On the other hand, if monarch wing pigmentation aids in dispersal ability, then southern monarchs, regardless of their sex, should have the greatest pigmentation. Our results support this interpretation with proportion of melanization because monarchs hatched in the south and caught in the north travelled the greatest distance, and both male and female southern monarchs had greater wing melanization.

Female monarchs move continuously in search for suitable host plants and therefore should invest more in pigmentation (i.e., melanization) that increases their flying ability, such as by increasing thermal absorption and allowing flight over a greater temperature range (Guppy 1986; Davis et al. 2012; but see Watt 1969). Recent research has shown that females fly farther than males, and redder monarchs, regardless of sex, fly farther than monarchs that are less red (Davis et al. 2012). We found that southern male monarchs had more saturated orange coloration (i.e., redder, see Supplementary Data for more details) than northern males; however, we did not find this pattern with females. This may be because females have less saturated orange on their forewings and because their wings are more melanized with a much lower proportion of orange pigmentation. Unlike captive monarchs that experienced controlled room temperature (Davis et al. 2012), the monarchs in our study experienced a natural range in temperature. Under natural conditions, melanization most likely provides a greater thermoregulatory benefit when temperatures reach a minimum threshold (Davis et al. 2012) and should allow monarchs to fly at lower temperatures. The larger, more saturated orange forewing patches of males, which do not fly as far as females (Davis et al. 2012), may provide additional thermoregulatory benefits compensating for their much lower proportion of melanin.

Although the temperature hypothesis did not explain the color variation in our data set, our findings should not suggest that monarch coloration is independent of temperature in wild populations. A large body of literature supports temperature-dependent color expression in insects (Stoehr and Goux 2008; Karl et al. 2009), and environmental factors certainly do influence coloration in monarch butterflies (Solensky and Larkin 2003; Davis et al. 2004, 2005). Instead, we suggest that most southern male monarchs could have had low melanization as previous experimentation suggests (Davis et al. 2005), but only the most melanized individuals migrated to the Great Lakes region, because this forewing pigmentation allowed for greater migratory distances. Interestingly, we did not find that monarchs travelling greater distances had more elongated wings, suggesting that thermoregulatory benefits of forewing color alone influenced these migratory distances, rather than flight mechanics.

An alternative explanation is that these color patterns are related to the species of milkweed that these monarchs were reared on. Because monarchs from all 3 regions would be subject to a variety of milkweed species, many shared between regions (*Asclepias viridis*, *Asclepias syriaca*, etc.), we find this alternative unlikely. However, it is possible that there is latitudinal variation in milkweed chemical compounds (Rasmann and Agrawal 2011). Future research should examine the influence of milkweed host on monarch coloration. In addition, parasite load is known to influence insect melanization (Wilson et al. 2001); therefore, one possible interpretation of these patterns relates to the variation of parasitism observed among monarchs during the breeding season and across their North American range (Altizer et al. 2000; Bartel et al. 2011).

These color patterns are most likely regulated by juvenile hormone, which down regulates melanin synthesis through phenoloxidase activity (Rantala et al. 2003) and steadily declines as the breeding season progresses (Lessman and Herman 1983). Therefore, the last generation of monarchs that undergoes a long-distance migration has lower levels of juvenile hormone than the breeding generation (Herman and Tatar 2001; Zhu et al. 2008). This mechanism would suggest that this migratory generation would have increased forewing melanization and saturation (due to the correlation between these 2 pigments). We suggest that this is why migratory monarchs have a redder hue than breeding monarchs (Davis 2009).

Our results support previous experimental evidence (Davis et al. 2012) and suggest that these subtle differences in coloration can manifest in large differences in migratory distances in a wild population. Such an adaptation may be vital to sustain a long-distance migration to their wintering grounds in central Mexico. Therefore, we also suggest that monarch wing pigmentation is a very important trait, particularly for the generation that undergoes a long-distance migration and postpones breeding until the following spring.

## SUPPLEMENTARY MATERIAL

Supplementary material can be found at <http://www.beheco.oxfordjournals.org/>

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