

POPULATIONS OF MONARCH BUTTERFLIES WITH DIFFERENT MIGRATORY BEHAVIORS SHOW DIVERGENCE IN WING MORPHOLOGY

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The demands of long-distance flight represent an important evolutionary force operating on the traits of migratory species. Monarchs are widespread butterflies known for their annual migrations in North America. We examined divergence in wing morphology among migratory monarchs from eastern and western N. America, and nonmigratory monarchs in S. Florida, Puerto Rico, Costa Rica, and Hawaii. For the three N. American populations, we also examined monarchs reared in four common environment experiments. We used image analysis to measure multiple traits including forewing area and aspect ratio; for laboratory-reared monarchs we also quantified body area and wing loading. Results showed wild monarchs from all nonmigratory populations were smaller than those from migratory populations. Wild and captive-reared eastern monarchs had the largest and most elongated forewings, whereas monarchs from Puerto Rico and Costa Rica had the smallest and roundest forewings. Eastern monarchs also had the largest bodies and high measures of wing loading, whereas western and S. Florida monarchs had less elongated forewings and smaller bodies. Among captive-reared butterflies, family-level effects provided evidence that genetic factors contributed to variation in wing traits. Collectively, these results support evolutionary responses to long-distance flight in monarchs, with implications for the conservation of phenotypically distinct wild populations.

KEY WORDS: *Danaus plexippus*, image analysis, long-distance migration, population divergence, wing morphology.

A number of morphological traits have been shown to affect the flight performance of migratory animals (Roff 1991; Dingle 1996; Alerstam et al. 2003). In birds and insects, greater wing size, more elongated wings (i.e., those with a higher aspect ratio), and a lower ratio between body mass and wing area (i.e., lower wing loading) are associated with a higher capacity for long-distance flight (Dingle et al. 1980; Dingle 1981; Monkkonen 1995; Calmaestra and Moreno 2001). Because the demands of long-distance flight represent an important selective force operating on the physical characteristics of species, different evolutionary outcomes are expected among species or populations with different migratory tendencies.

Most research on adaptive morphology and flight capacity has involved comparisons across multiple species that differ in

their migratory behavior (Roff 1991; Winkler and Leisler 1992; Dudley and Srygley 1994) or between distinct sedentary and dispersal phenotypes of wing-dimorphic species (Dingle 1996). In butterflies, cross-species comparisons show that flight speed is positively correlated with body size, thorax size, and wing loading (Dudley and Srygley 1994). Although fewer studies have examined variation in flight morphology within wing-monomorphic species, evolutionary changes in flight morphology have been demonstrated for a handful of butterflies (including *Hesperia comma*, *Melitaea cinxia*, and *Pararge aegeria*) in relation to range expansions and colonization success (Berwaerts et al. 1998; Hill et al. 1999). Collectively, these studies show that individuals from more fragmented landscapes or colonizing populations have larger body mass and thorax size, and suggest that

morphological changes can indeed result from selection on flight ability.

Monarch butterflies (*Danaus plexippus* L.) have a wide distribution that ranges from southern Canada to Central and South America and throughout the Caribbean Islands (Ackery and Vane-Wright 1984). More recently, monarchs have colonized Australia, Hawaii, and other Pacific islands following the introduction of their milkweed host plants (Vane-Wright 1993). Monarch populations show extreme variation in their migratory behavior, making them a suitable system for studying geographic variation in flight morphology. In eastern North America, monarchs undergo long-distance two-way migration, traveling 3500 km or longer from breeding to overwintering sites (Urquhart and Urquhart 1978; Brower and Malcolm 1991; Brower 1995; Howard and Davis 2009). In spring, the same butterflies that winter in Mexico mate and fly north to recolonize their breeding range (Van Hook 1993; Howard and Davis 2004). A second population in western North America migrates a shorter distance to overwinter along the coast of California (Tuskes and Brower 1978; Leong 1990; Frey et al. 1992; Nagano et al. 1993; Brower 1995). In tropical and subtropical locations such as South Florida and Caribbean and Pacific Islands, monarchs breed year-round and do not migrate, although some work has shown an influx of North American migrants into S. Florida and Cuba during the large eastern population's annual fall migration (Knight et al. 1999; Dockx et al. 2004).

Despite decades of study and large differences in their migratory tendencies, there is little quantitative information on morphological differences among wild monarch populations. Wing length was reported to differ among wild monarchs sampled from North and South America and Caribbean locations (Beall and Williams 1945), but this study found little variation within North America and was conducted decades before the migratory ecology of monarchs was fully known. More recently, Dockx (2007) showed that among monarchs captured in Cuba, migrants originating from northern latitudes had larger and more angular wings than those identified as year-round residents. However, limitations of these past studies are that they focused solely on wild-captured monarchs (and did not include data on individuals reared in a common environment) and they explored a narrower range of morphological traits predicted to influence flight ability.

The goal of our study was to examine variation in flight morphology among monarch butterflies from six different populations: migratory monarchs from eastern and western North America, and nonmigratory monarchs from S. Florida, Puerto Rico, Costa Rica, and Hawaii. Like other large-bodied insects, migrating monarchs use powered flight to disperse within the boundary layer (Dudley 2000) and also rely on upward convection and prevailing winds to reduce energy expenditure during migration (i.e., gliding flight, Gibo and Pallett 1979; Gibo 1986). We therefore predicted that monarchs from long-distance migra-

tory populations would have larger and more elongated forewings to increase flight surface area and reduce wingtip-induced drag. If migrating monarchs rely heavily on gliding flight, we predict that this would select for lower wing loading (ratio of body size to wing area) in migratory populations. Alternatively, if migrants rely heavily on powered flight (and have high energetic demands), this could select for larger body size, which may or may not increase wing loading, depending on whether wing area increases as well. To provide further evidence for evolutionary change, we examined monarchs from eastern and western migratory and S. Florida resident populations reared under common environmental conditions. In this latter analysis, we investigated the contribution of genetic factors to differences in wing morphology among populations and tested for a heritable basis of within-population variation.

Materials and Methods

SAMPLE COLLECTION AND MONARCH REARING

Wing parameters were measured for 822 wild adult monarchs collected from six different populations between 1996 and 2009 (Table 1) for the purposes of assessing the prevalence of a protozoan parasite (as described in Altizer et al. 2000) or as parents to initiate laboratory experiments (e.g., Altizer 2001; Davis et al. 2005). Butterflies were stored in glassine envelopes at -20°C prior to wing measurement. We excluded data for wild monarchs that were heavily infected with the protozoan *Ophryocystis elektroscirrha*, as this parasite has been shown to reduce body mass and wing area in previous studies (Altizer and Oberhauser 1999; de Roode et al. 2007). Specifically, we excluded data for monarchs assigned to the highest spore load category as defined by Altizer et al. (2000). We also examined a total of 1019 captive-reared monarchs from four common environment experiments conducted in 1996 and 1997 at the University of Minnesota (St. Paul, MN) and in 2003 at Emory University (Atlanta, GA). In each of these experiments, full-sibling progeny from multiple mated adult females collected from two or more populations were reared simultaneously under laboratory conditions (Table 2). Rearing protocols and containers remained similar among experiments: larvae were reared at low densities (6–12 larvae) in 4.7 L plastic containers with metal screened lids at temperatures between 22 and 26°C, and containers were cleaned and fresh cuttings of milkweed (*Asclepias* spp.) were added daily until all monarchs in each container pupated. Several hours following eclosion, adult butterflies were placed in glassine envelopes and were examined for the presence of *O. elektroscirrha* using methods described in Altizer et al. (2000). For the purposes of this study, we labeled each monarch according to the experiment number (1–4; Table 2), full-sibling family (using mother ID number), source population, and sex. At the end of each experiment, adult

Table 1. Origins of wild-collected monarchs used to measure wing traits as described in Methods (abbreviations are shown for U.S. states, with county names in parentheses). Eastern and western migratory populations are from N. America.

Population	Month	Year	Locations	<i>N</i>
Eastern migratory	Jul–Aug	1996	MN (Hennepin), WI (St. Croix)	34
	Jul–Aug	1997	MN (Hennepin), WI (St. Croix)	62
	Oct	2005	GA (Clarke, Oconee)	44
	Feb	2008	Mexico wintering sites (Sierra Chincua and Cerro Pelon)	132
	Oct	2008	Georgia (Clarke, Oconee)	30
Western migratory	Feb	1997	California wintering sites (Monterey, San Luis Obispo and Santa Barbara)	97
	Jul–Sep	1997	CA, UT, NV, OR, WA, CO	162
	May	2003	CA (Santa Barbara)	14
S. Florida	Jul	1996	FL (Dade)	29
	Apr–May	2003	FL (Collier and Dade)	14
	Jul	2009	FL (Collier and Dade)	15
Hawaii	Dec	2007	HI (Hawaii, Honolulu)	31
	Jan–Feb	2009	HI (Hawaii, Honolulu, Kauai, Maui)	94
Costa Rica	Dec	2008	San Luis (Puntarenas province)	20
Puerto Rico	Oct	2008	PR (Arecibo, Cabo Rojo)	58

butterflies were frozen at -20°C . Among laboratory-reared monarchs, only adults in control treatments (e.g., not exposed to parasites and reared under conditions described above) were used for comparisons in this study. Prior to analysis, data were excluded from butterflies with damaged or deformed wings.

MEASURING WING CHARACTERISTICS

Butterflies have fore- and hindwings that together make up the entire wing surface area during flight. During soaring flight, monarchs generally position their wings so that the anterior edge of the forewing is perpendicular to the main body axis, thus covering a large portion of the hindwing (D. Gibo, pers. comm., Fig. 1). For this reason, the exposed wing surface area and shape should be

most strongly influenced by forewing rather than hindwing characteristics. We therefore quantified forewing characteristics (size and shape) for all monarchs and for a subset of captive-reared monarchs, we examined both fore- and hindwing characteristics as described below.

For monarch specimens collected or reared in 1996, 1997, and 2005–2009, we removed the entire right forewing and digitally scanned the dorsal side at 300 dpi on a Hewlett Packard Scanjet flatbed scanner (Hewlett Packard, Palo Alto, CA). For 2003 captive samples, live monarchs were individually scanned at the same resolution after chilling them on ice for 10 min; wings were then configured in a standard pinning position using weights to immobilize each monarch with the dorsal side against

Table 2. Dates and sample sizes for captive-reared monarchs from four experiments used to measure wing and body traits as described in Methods. For experiment 1, fresh cuttings of *Asclepias curassavica* and *A. syriaca* were used to feed larvae, for experiments 2 and 3, *A. syriaca* cuttings were used, and for experiment 4, cuttings from potted *A. incarnata* were fed to larvae. *N* refers to the total number of adults from which data were obtained and number of families refers to the number of mated females from which progeny were obtained. Other details of experiments 1–3 are provided in Altizer (2001), and experiment 4 is described in Davis et al. (2005). Only control treatment monarchs (those not infected with parasites and exposed to ambient laboratory temperatures) were used in this current study.

Experiment	Population	Month	Year	No. of families	<i>N</i>
1.	Eastern migratory	May–Jul	1996	5	81
	Western migratory	May–Jul	1996	4	38
2.	Eastern migratory	Sep–Oct	1996	5	86
	S. Florida	Sep–Oct	1996	5	91
3.	Eastern migratory	Aug–Sep	1997	12	119
	Western migratory	Aug–Sep	1997	15	113
4.	Eastern migratory	Jun–Jul	2003	15	298
	Western migratory	Jun–Jul	2003	10	109
	S. Florida	Jun–Jul	2003	6	84

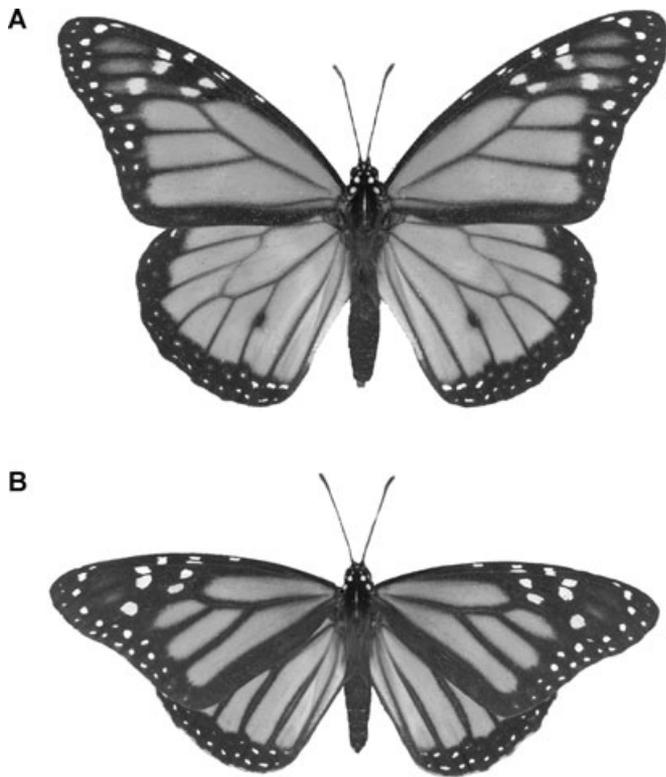


Figure 1. Scanned image of male monarch butterfly, displayed in standard pinning position (A). Female monarch shown in typical soaring flight position (B) with the anterior edge of forewing held perpendicular to the main body axis (so that fore- and hindwings are partially overlapping).

the scanner glass (e.g., Fig. 1A). Once images were captured, right forewings (or fore- and hindwings combined, for a subset of monarchs) were digitally traced, extracted, and saved separately prior to analysis.

Measurements were made using Adobe Photoshop software with Fovea Pro plugins (Reindeer Graphics, Inc., Asheville, NC) following methods described in Davis et al. (2005, 2007). We calibrated the software to compute distances (in mm) within each wing image based on the on-screen pixel-to-millimeter ratio. A total of four raw forewing traits were measured for all monarchs including (1) length in millimeter of the longest axis from the point of wing attachment at the thorax to the distal tip, (2) width in millimeter of the longest line that could be drawn perpendicular to the length axis, (3) outside perimeter length in millimeter of the entire forewing, and (4) total forewing area in millimeter². From these, we calculated two shape measures including (5) aspect ratio (length divided by width) and (6) roundness (area to perimeter ratio; $4\pi \text{ area/perimeter}^2$).

BODY SIZE AND WING LOADING

Adult wet mass, measured to the nearest milligram 24 h posteclosion using an electronic balance, was available for a total of 626

captive-reared monarchs from 1996, 1997, and 2003. Wing loading, defined as the ratio of body size to wing size, was estimated for a subset of captive-reared monarchs as wet mass (in mg)/forewing area. Also, for 491 captive-reared monarchs scanned live in 2003, we used digital image analysis to measure total body area (in mm²) including head, thorax, and abdomen combined, based on the outline of the dorsal side of the body minus the wings.

STATISTICAL ANALYSIS

Data reduction

Initial tests showed that forewing area, length, and width were all significantly positively correlated (e.g., $R_{\text{area-length}} = 0.953$; $R_{\text{area-width}} = 0.966$; $R_{\text{length-width}} = 0.876$; $N = 1841$; $P < 0.0001$ in all cases). In addition, forewing area was significantly positively correlated with hindwing area ($R = 0.919$; $N = 91$; $P < 0.001$) and the two measures of forewing shape (aspect ratio and roundness) were highly negatively correlated ($R = -0.902$; $N = 1841$; $P < 0.001$). To reduce the number of wing traits in analyses of population-level effects, measures of forewing size and shape were summarized into two variables using principal component (PC) analysis (SPSS 2007). Measurements were extracted first using data pooled across all wild-captured butterflies, and second using data from laboratory-reared monarchs from each experiment. For both groups of monarchs, we reduced forewing area, length, and width into one variable (PC-1, hereafter called PC-size) that explained 96% of the total variance (components among wild-caught monarchs: area = 0.996, length = 0.965, width = 0.975). This resulted in a number for which higher values represented butterflies with larger forewings and vice versa. For shape analyses, we entered forewing aspect ratio and roundness to generate a second composite variable (PC-2, hereafter PC-shape) that explained over 95% of the total variance (components among captive-reared monarchs: aspect ratio = 0.976; roundness = -0.976). Low values of PC-shape corresponded to blunt wing and higher values corresponded to elongated wings (Fig. 2).

Analysis of population variation

For wild-captured butterflies, we used analysis of variance (ANOVA; SPSS 2007) to examine the effects of population origin,

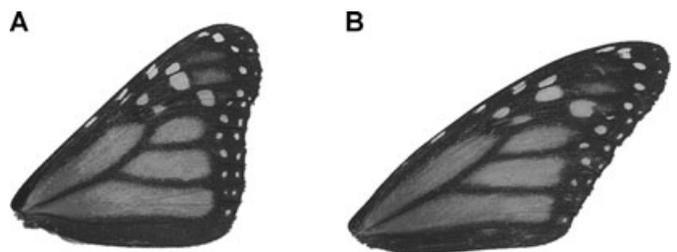


Figure 2. Forewing with a low PC-shape score, indicating a blunt-shaped or round wing (A), and a high PC-shape score indicating a narrower, more elongated wing (B).

sex, and sampling period on forewing size and shape measures (ANOVA model: morphology = population + sex + population × sex + sample). Sampling period was treated as a random effect nested within population origin. Tukey's post hoc tests were performed to test comparison of means for population origin (with significance reported at the 0.05 level).

For laboratory-reared monarchs, we used ANOVA to test the effects of population origin, sex, and family on forewing size and shape. Experiment number (1–4; as in Table 2) was treated as a fixed effect and full-sibling family was treated as a random effect nested within the population by experiment interaction (Full model: morphology = population + sex + experiment + population × sex + population × experiment + sex × experiment + family[population × experiment]). Comparison of means for population origin was again performed using Tukey's post hoc tests at the 0.05 level. For a subset of laboratory-reared monarchs, we examined population variation in body area and wing loading. As before, full-sibling family was treated as a random effect nested within the population by experiment interaction, and Tukey's tests were used for comparison of means at the population level.

Finally, to further explore genetic variation underlying forewing size and shape, we used the MIXED Procedure in SPSS to estimate the variance in PC-size, PC-shape, wing loading, and body area attributed to full-sibling family effects within each population. This procedure computes variance components (and standard errors [SEs]) using restricted maximum likelihood (REML). Because variance attributed to similarity among full siblings should reflect $\frac{1}{2}V_A + \frac{1}{4}V_D$ (i.e., additive and dominance genetic variance), this measure can indicate the relative magnitude of genetic variation underlying phenotypic variability. For analyses within each population, experiment was treated as a fixed factor, and family nested within experiment was treated as a random effect.

Results

POPULATION VARIATION AMONG WILD MONARCHS

On average, wild-caught males had larger forewings than wild females, but aspect ratio means were similar for both sexes (Table 3a). Eastern migratory males and females were slightly

Table 3. Mean forewing dimensions for (a) wild-captured and (b) laboratory-reared adult monarch butterflies from each population. Means are shown separately for males and females (\pm SE). In (a), values are pooled across butterflies caught in all years as listed in Table 2. In (b), values are pooled across experiments (1)–(4) as described in methods and in Table 2. Body area is also shown in (b) for a subset of laboratory-reared butterflies measured in 2003, and sample sizes for this measure are indicated by footnotes.

(a) Wild-captured adults						
Sex	Population	N	Length (mm)	Area (mm ²)	Aspect ratio	
Male	Eastern migratory	180	51.44 (\pm 0.19)	876.7 (\pm 6.1)	1.94 (\pm 0.00)	
	Western migratory	201	50.56 (\pm 0.17)	852.6 (\pm 5.1)	1.93 (\pm 0.00)	
	S. Florida	30	48.83 (\pm 0.46)	794.4 (\pm 12.8)	1.94 (\pm 0.01)	
	Hawaii	70	49.28 (\pm 0.21)	801.9 (\pm 6.6)	1.95 (\pm 0.01)	
	Costa Rica	13	47.92 (\pm 0.73)	762.5 (\pm 22.1)	1.94 (\pm 0.02)	
	Puerto Rico	27	45.53 (\pm 0.41)	718.2 (\pm 11.4)	1.87 (\pm 0.01)	
Female	Eastern migratory	122	51.18 (\pm 0.19)	865.7 (\pm 6.3)	1.94 (\pm 0.00)	
	Western migratory	58	49.12 (\pm 0.26)	818.3 (\pm 8.2)	1.92 (\pm 0.01)	
	S. Florida	28	48.59 (\pm 0.53)	792.9 (\pm 16.1)	1.93 (\pm 0.01)	
	Hawaii	55	48.49 (\pm 0.29)	775.9 (\pm 8.6)	1.95 (\pm 0.01)	
	Costa Rica	7	44.8 (\pm 1.56)	701.5 (\pm 41.7)	1.88 (\pm 0.01)	
	Puerto Rico	31	44.80 (\pm 0.41)	678.1 (\pm 10.2)	1.91 (\pm 0.01)	
(b) Laboratory-reared adults						
Sex	Population	N	Length (mm)	Area (mm ²)	Body area	A.R. ⁷
Male	Eastern migratory	328	51.1 (\pm 0.13)	873.3 (\pm 4.1)	167.6 (\pm 1.5) ¹	1.93 (\pm 0.00)
	Western migratory	131	50.8 (\pm 0.19)	865.6 (\pm 5.8)	154.3 (\pm 1.9) ²	1.90 (\pm 0.00)
	S. Florida	95	48.3 (\pm 0.30)	788.7 (\pm 8.7)	156.7 (\pm 1.9) ³	1.91 (\pm 0.01)
Female	Eastern migratory	256	50.7 (\pm 0.14)	864.2 (\pm 4.4)	151.0 (\pm 1.3) ⁴	1.92 (\pm 0.00)
	Western migratory	129	50.6 (\pm 0.19)	861.3 (\pm 5.9)	139.3 (\pm 1.9) ⁵	1.90 (\pm 0.00)
	S. Florida	80	47.4 (\pm 0.32)	758.9 (\pm 10.1)	141.9 (\pm 2.6) ⁶	1.92 (\pm 0.01)

¹N=152; ²N=56; ³N=49; ⁴N=145; ⁵N=53; ⁶N=35; ⁷A.R., aspect ratio.

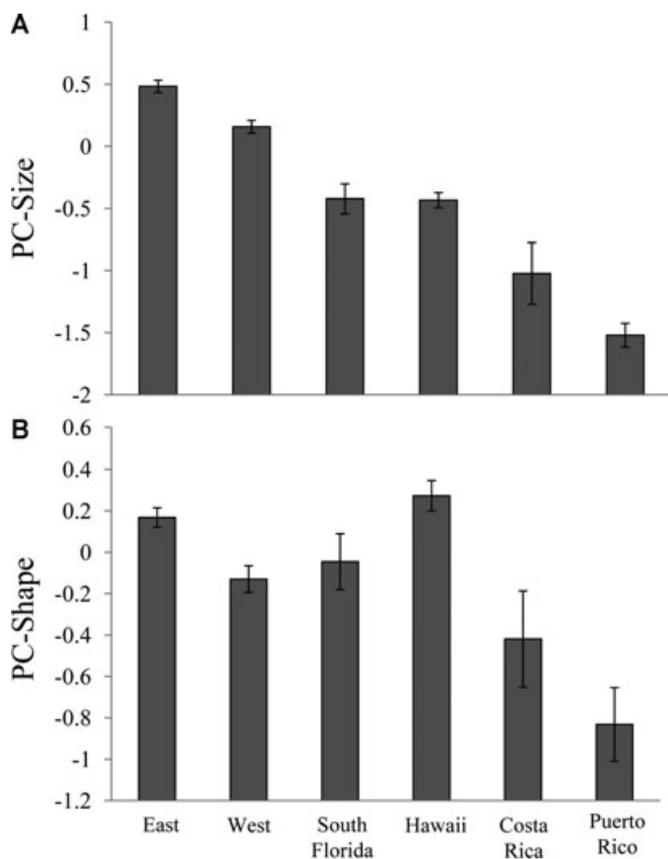


Figure 3. Differences in composite measures of forewing size (A) and shape (B) among wild monarch populations. ANOVA results are reported in the results text, and descriptions of PC-size and PC-shape are explained in the methods text. Error bars show standard errors. East and west refer to eastern and western N. American migratory populations. Note that overall means for both principal component measures were zero; bars with lower values tend to have smaller sample sizes represented as indicated in Table 1.

larger than their western counterparts, and monarchs from both migratory populations were larger than those from S. Florida, Hawaii, Costa Rica, and Puerto Rico (Table 3a). Aspect ratio means were largest among monarchs from both eastern N. America and Hawaii, indicating that butterflies from these populations had the most elongated forewings, and were smallest for monarchs from Puerto Rico and Costa Rica. Differences among wild populations in composite measures of size and shape are further illustrated in Figure 3.

Statistical comparison of wild-captured monarchs revealed significant population-level differences in forewing size and shape. ANOVA using data from wild monarchs showed significant effects of source population on both PC-size ($F_{5,9} = 27.83$, $P < 0.001$) and PC-shape ($F_{5,9} = 11.19$, $P = 0.001$). In the case of PC-size, Tukey's comparison of means showed four subsets that differed significantly (East = West > Florida = Hawaii > Costa Rica > Puerto Rico; Fig. 3A). For PC-shape, monarchs

from Puerto Rico and Costa Rica had significantly rounder wings than those from other populations (Fig. 3B). For PC-size, analysis further showed significant effects of sex ($F_{1,802} = 16.9$, $P < 0.001$), with males having larger wings than females, and sample group nested within population ($F_{8,802} = 3.04$, $P = 0.002$). For PC-shape, analysis showed a significant interaction between population origin and sex ($F_{5,796} = 4.91$, $P < 0.001$), such that males from Costa Rica had more angular wings than females, but the reverse trend was true in Puerto Rico (e.g., Table 3a).

POPULATION VARIATION AMONG CAPTIVE-REARED MONARCHS

Forewing size and shape

Among monarchs reared in common environment experiments, eastern migratory monarchs had slightly larger wings than those from the western migratory population, and S. Florida monarchs had substantially smaller forewings than those from either migratory population (Table 3b). Mean size measures for males were slightly larger than for females, but aspect ratio means were similar for both sexes. Eastern monarchs had forewings with the greatest mean aspect ratio and western monarchs showed the lowest mean aspect ratio (Table 3b). Differences among butterflies from each population in composite measures of size and shape are further illustrated in Figure 4A,B.

Similar to wild monarchs, analyses of captive-reared monarchs provided evidence for population-level differences in forewing size and shape. ANOVA showed significant effects of population origin on both PC-size ($F_{2,84} = 11.23$, $P < 0.001$) and PC-shape ($F_{2,84} = 3.72$, $P = 0.027$). In the case of PC-size, Tukey's comparison of means showed that S. Florida monarchs had significantly smaller forewings than western monarchs, and eastern monarchs were largest on average (East > West > Florida). Measures of PC-shape were greatest among eastern monarchs and smaller among western and S. Florida monarchs (East > Florida = West). ANOVA for PC-size also showed significant effects of experiment ($F_{3,84} = 4.31$, $P = 0.007$), and family nested within population \times experiment ($F_{84,920} = 3.15$, $P < 0.001$). For PC-shape, experiment ($F_{3,84} = 3.61$, $P = 0.016$), population \times experiment ($F_{3,84} = 5.8$, $P = 0.001$), and family nested within population \times experiment ($F_{84,918} = 2.47$, $P < 0.001$) were also significant. No significant effects of sex or population \times sex were observed for either PC-size or PC-shape.

Body area and wing loading

Males had greater body area than females, and eastern monarchs had larger bodies than western and S. Florida monarchs (Table 3b; Fig. 4C). Males also had greater measures of wing loading relative to females, and wing loading was greatest for eastern monarchs and lower for monarchs from S. Florida and western N. America (not shown). Comparison of Figure 4A,C shows that although

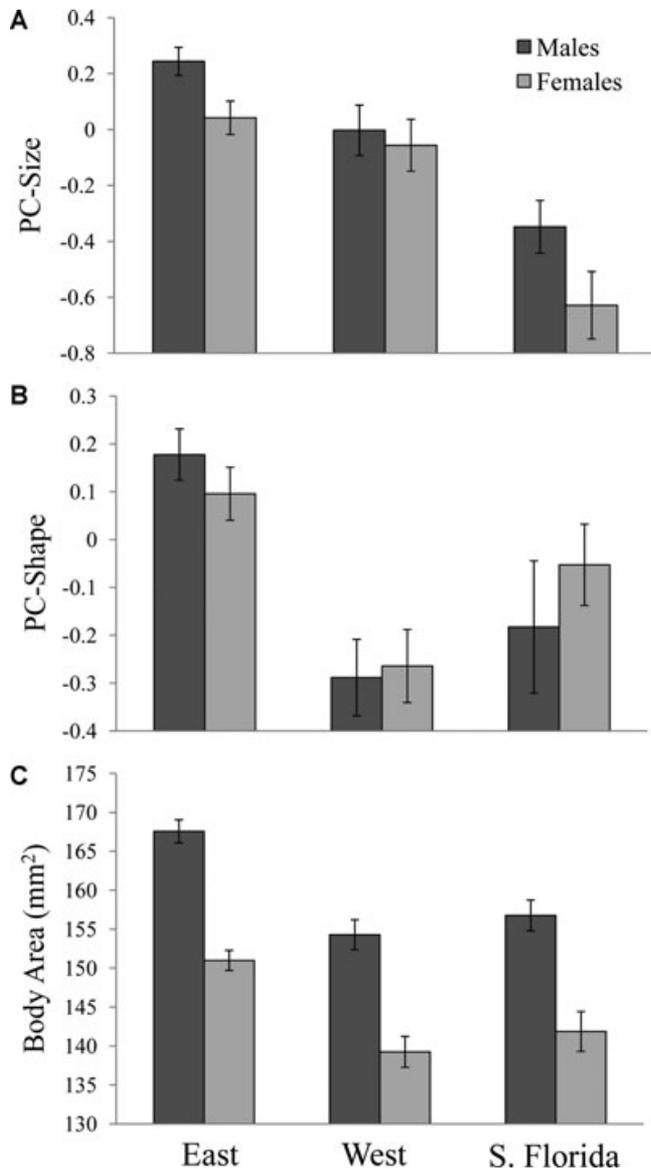


Figure 4. Differences in composite measures of forewing size (A) and shape (B) and total body area (C) for monarchs raised in common environment experiments. ANOVA results are reported in the results text, and descriptions of PC-size and PC-shape are explained in the methods text. Error bars show standard errors. East and west refer to eastern and western N. American migratory populations. Note that overall means for both principal component measures were zero; bars with lower values tend to have smaller sample sizes represented as indicated in Table 2.

S. Florida monarchs had the smallest wings, they had a similar body area to wing area ratio as eastern monarchs; by comparison, western monarchs had relatively smaller bodies for their wing surface area (Table 3b; Fig. 4).

ANOVA for body area showed significant effects of population origin ($F_{2,37} = 4.93$, $P = 0.011$), sex ($F_{1,447} = 77.7$, $P = 0.000$), and family nested within population ($F_{37,447} = 4.70$, $P < 0.001$). Tukey's comparison of means showed that measures of

body area were greatest among eastern monarchs and smaller among western and S. Florida monarchs (East > Florida = West; Fig. 4C). ANOVA for wing loading showed that sex was significant ($F_{1,545} = 67.11$, $P < 0.001$), as was experiment ($F_{3,74} = 5.37$, $P = 0.002$) and family nested within population \times experiment ($F_{74,545} = 3.13$, $P < 0.001$), but the main effect of population origin was not significant.

GENETIC BASIS FOR VARIATION IN WING MORPHOLOGY

Because family-level effects were highly significant in analyses of forewing size, forewing shape, body area, and wing loading, we used the MIXED statement in SPSS to estimate variance components attributed to effects of family nested within experiment. We note that this variance estimate does not measure heritability in the strict sense, but also includes dominance variance and both maternal and paternal effects. Each population was tested separately, and families for which fewer than three progeny had been measured were excluded from the analysis. The total number of families ranged from 11 to 37 per population for PC-size and PC-shape, from 5 to 22 for wing load_{mass}, and from 6 to 15 for body area and wing loading—with the greatest number of families represented from the eastern migratory population (e.g., Table 2).

For PC-size, family-level effects were highly significant when tested within the eastern population (variance estimate = 0.07 ± 0.02 SE; Wald's $Z = 2.85$; $P = 0.004$) and approached significance for the western population (variance estimate = 0.07 ± 0.04 SE; Wald's $Z = 1.88$; $P = 0.08$). For PC-shape, family-level effects were again highly significant among eastern monarchs (variance estimate = 0.19 ± 0.06 SE; Wald's $Z = 3.02$; $P = 0.003$) but not for western monarchs (variance estimate = 0.05 ± 0.03 SE; Wald's $Z = 1.64$; $P = 0.10$). For wing loading, variance attributed to family-level effects was highly significant for eastern monarchs (variance estimate = $6.9 \times 10^{-4} \pm 2.4 \times 10^{-4}$ SE; Wald's $Z = 2.927$; $P = 0.003$) and was also significant for western monarchs (variance estimate = $5.5 \times 10^{-4} \pm 2.7 \times 10^{-4}$ SE; Wald's $Z = 2.038$; $P = 0.042$). Full-sibling family effects for PC-size, PC-shape, and wing loading did not approach significance for analyses focused on S. Florida monarchs. Finally, measures for body area were available for only experiment 4 (conducted in 2003; Table 2). For this variable, family-level effects were significant among eastern monarchs (variance estimate = 80.23 ± 35.65 SE; Wald's $Z = 2.25$; $P = 0.024$) but not for monarchs from S. Florida or the western population.

Discussion

Consistent with past studies of migratory birds and insects (e.g., Dingle 1989; Hedenstrom and Møller 1992; Winkler and Leisler 1992; Dudley and Srygley 1994; Marchetti et al. 1995; Calmaestra

and Moreno 2001; Dockx 2007), our analyses showed that monarchs from the two long-distance migratory populations had larger forewings than their nonmigratory counterparts. In addition, nonmigratory monarchs from S. Florida, Puerto Rico, and Costa Rica had significantly rounder (less angular) wings than those from the eastern migratory population. Among monarchs reared in common environments, directional trends were similar to those based on wild-caught individuals, and were also consistent across multiple experiments. The fact that differences in mean forewing parameters persisted among progeny reared in multiple studies, and the observation of strong family-level effects with laboratory studies, strongly indicates a genetic basis for morphological differences among populations.

Ecologically significant variation in flight morphology has been demonstrated previously for a large number of insect species that differ in their dispersal tendency. For example, milkweed bugs *Oncopeltus fasciatus* can display one of three migration strategies: long-distance migration, short-distance migration, and nonmigratory status (Dingle 1981). Wings have been shown to be longest in the long-distance migratory population, and tethered flight trials showed that lineages artificially selected for longer wings had a greater propensity for flight than unselected controls (Palmer 1985; Dingle 1989). Among butterflies, species-level comparisons in British papilionids showed that wingspans of migratory species were larger than for nonmigratory species (Roff 1991). Similarly, greater flight speeds were associated with larger wingspan and thorax size in cross-species comparisons of neotropical butterflies (Dudley and Srygley 1994). Collectively, these studies suggest that the demands of long-distance flight represent an important evolutionary force operating on the physical characteristics of migratory species.

Surprisingly, the two migratory monarch populations examined here (from eastern and western N. America) appear to have adapted to the demands of migration in different ways. We found that eastern monarchs have the largest, most elongated wings (with the exception of Hawaii), and the largest bodies of all populations examined. Meanwhile western monarchs have slightly smaller and more rounded wings, and relatively small bodies given their wing area. The large and elongated forewings of the eastern monarchs are consistent with trends observed among migratory birds' wings (Winkler and Leisler 1992; Lockwood et al. 1998; Calmaestra and Moreno 2001). Specifically, past studies of bird flight indicate that the best form for long-distance gliding involves long wings with a narrower tip which enables animals to shed air vortices on the wingtips that would otherwise create drag (Kerlinger 1989).

Eastern migratory monarchs also had relatively high wing loading values. Although this could seem maladaptive for a long and energetically costly flight, research on other butterfly species suggests that high wing loading leads to faster powered flight

(Dudley and Srygley 2008), which could be advantageous during long migratory journeys. In addition, the large bodies of eastern migratory monarchs might allow them to harbor greater lipid reserves, which are known to be important for their long migration and overwintering survival (Alonso-Mejia et al. 1997; Brower et al. 2006). This is consistent with recent evidence showing how butterflies with large lipid reserves fly faster than those with smaller reserves (Dudley and Srygley 2008). In contrast, western monarchs have rounder forewings and smaller body sizes than eastern monarchs. Currently it is not known if western monarchs utilize the same flying techniques as eastern monarchs, which can alternate between powered and gliding flight (Gibo and Pallett 1979; Gibo 1986). Because the average migration distance of western monarchs is less than a third of the eastern population's journey, greater lipid stores and faster speeds may be less crucial for their migration and overwinter survival.

Monarchs from Puerto Rico and Costa Rica had the smallest and least angular forewings relative to other populations examined here, consistent with the observation that monarchs in these locations breed year-round and do not migrate. By comparison, nonmigratory monarchs from S. Florida and Hawaii had relatively small but angular forewings. Although we do not have a clear explanation for this result, it is important to note that the more recent origins of nonmigratory monarchs in S. Florida and Hawaii followed the introductions of tropical host plant species to these areas (Ackery and Vane-Wright 1984). These populations probably originated from N. American monarchs east and west of the Rocky mountains, respectively (Shephard et al. 2002). In the case of Hawaiian monarchs, it is possible that this population experienced one or more founder events in the past that influenced wing variables; in support of this idea, an otherwise rare autosomal recessive mutation that causes white wings was reported to have reached a high frequency among Hawaiian monarchs (Vane-Wright 1986).

The between-population differences in wing morphology noted here are especially interesting in light of past work indicating little or no population divergence based on selectively neutral molecular markers. Indeed, past studies using allozyme variation (Shephard et al. 2002) and mtDNA markers (Brower and Boyce 1991; Brower and Jeansonne 2004) found little evidence for population differentiation among monarchs collected from multiple sites in both N. and S. America, indicating that all New World monarch butterflies are extremely closely related. In addition, previous studies suggest a high potential for gene flow between existing populations. Specifically, migrating monarchs from southeastern Canada and the eastern United States enter S. Florida and Cuba each fall (Knight et al. 1999; Dockx et al. 2004; Dockx 2007). Because the numbers of migrants exceed the local resident populations during this time, and because migrants were reproductively active, researchers inferred a high potential

for gene flow between eastern migratory and Caribbean populations (e.g., Dockx 2002). Gene flow between eastern and western N. American monarchs has also been inferred based on anecdotal reports of monarchs in the Great Basin or the Northern Rockies (Brower and Pyle 2004), although quantitative data on mixing of populations across the continental divide is lacking (Brower 1995).

The divergent phenotypic traits reported here argue in favor of adaptive differences between monarch populations, irrespective of similarity across neutral markers. Generally speaking, evolutionary biologists have long known that spatially varying selection regimes can lead to genetic divergence and even ecological speciation, despite opportunities for substantial gene flow between subpopulations (e.g., Endler 1973; Caisse and Antonovics 1978; Mila et al. 2009; Schluter 2009; Via 2009). In the case of monarchs, it is important to note that populations inhabiting the Caribbean Islands and South America are currently recognized as a different subspecies than N. American monarchs based on their size and wing patterns (e.g., *D. p. plexippus* in temperate N. America and *D. p. megalippe* and *erippus* in Caribbean, Central, and S. America, Smith et al. 1994), even though periodic gene flow between these subspecies probably occurs (as noted above). Whether other monarch populations should be recognized as evolutionarily distinct units is a question that warrants further study, and can be informed by additional molecular work in parallel with phenotypic analyses.

Early comparisons of forewing size (indexed by wing lengths) of monarchs from multiple locations (Williams et al. 1942; Beall and Williams 1945; Urquhart 1960) found no evidence for population differences within N. America. That we discovered significant differences in wing size when others had not might relate to the use of digital image analysis to discern fine-scale differences across multiple wing traits on large numbers of butterflies. In fact, our results could be interpreted as evidence that forewing lengths alone are an incomplete measure of wing size. To illustrate this point, means presented in Table 3b indicate that eastern males have forewings that are on average 2–3 mm longer and 86 mm² greater in area than S. Florida males. If this trend were similar for both fore- and hindwings, the actual difference in wing area between individuals in these populations could be larger than the size of a standard postage stamp—illustrating that minute differences in wing length can translate into much larger area differences.

Although we focused here on predictions related to long-distance flight and wing morphology, other selective forces could influence variation in wing traits. Sex-based differences in forewing size (e.g., males were larger than females in most, but not all, captive rearing studies) indicate that sexual selection might also influence wing morphology. For example, there is growing evidence that mate selection occurs within butterflies

based on wing pattern (Knüttel and Fiedler 2001) and morphology (Wickman 1992). Within monarchs, Davis et al. (2007) found evidence that larger males (indexed by forewing area) showed greater mating success. Moreover, earlier work with monarchs showed that large males preferentially mate with large females (Frey et al. 1998). Sexual selection in favor of large wings is therefore possible in monarch butterflies, although to what degree this varies among wild populations is not known.

Family-level effects were highly significant in analyses involving captive-reared monarchs, indicating a likely genetic basis for differences in morphology within populations. When broken down by population, estimates of full-sibling family effects were significant for eastern monarchs for both size and shape, and approached significance for western monarchs (although many fewer families were available for western and S. Florida populations). Variance attributed to similarity among full siblings should indicate the relative magnitude of total genetic variation underlying phenotypic variability within populations. However, because variance among full siblings also captures dominance variance and maternal and paternal effects, data from this study cannot provide an accurate measure of heritability in the strict sense.

Much recent attention has focused on whether monarchs from different regions in N. America are biologically distinct, particularly in regards to a growing industry that involves the commercial sale and release of butterflies for special occasions. This practice has been criticized by the scientific community (Brower et al. 1995) and at present, interstate releases are regulated (and transcontinental releases prohibited) by the United States Department of Agriculture. Our study supports the current limitations on long-distance transfer monarchs, and underscores the need to further examine similarity among existing populations. Understanding genetic variability among monarch populations will also become increasingly important for future conservation efforts in light of the increasing threats that monarchs face from anthropogenic causes. In particular, the eastern migratory population is currently threatened by climate change (Oberhauser and Peterson 2003; Batalden et al. 2007) and the destruction of the oyamel fir forests that serve as the monarchs' wintering grounds (Brower et al. 2002), to the point that monarch migration is considered to be an "endangered phenomenon" (Brower and Malcolm 1991). Our study highlights the importance of long-distance migration as a selective force operating on monarch wing morphology, and suggests that the loss of overwintering habitat for eastern migratory monarchs could lead to the loss of an evolutionarily unique population.

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