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Source: Florida Entomologist, 101(2) : 195-202

Published By: Florida Entomological Society

URL: https://doi.org/10.1653/024.101.0207

Geomorphic morphometric differences between populations of *Speyeria diana* (Lepidoptera: Nymphalidae)

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Abstract

The wing shape of butterflies can be used as a character to measure individual butterfly and population-level differences in adult forms. We conducted a geometric morphometric analysis of wing shape for *Speyeria diana* (Cramer & Stoll 1777) (Lepidoptera: Nymphalidae), a species currently threatened by extirpation across portions of its range. We photographed and digitized 243 pinned *S. diana* specimens from natural history museums in North America and Europe to compare wing shape and size across the historical and current distribution. We used principal components analysis (PCA) to compare variation in landmarks on forewings and hind wings. We used the principal component scores in a multiple analysis of variance (MANOVA) to test for differences, and interactions, between male and female specimens, eastern and western specimens, low and high elevation specimens, and specimens collected before and after 1950. We found significant differences between wing shape in male and female specimens, with male forewings being narrower and more elongated, possibly making them well suited for patrolling behavior. Female forewings were more rounded and wider, possibly reflecting their life history, because much of their life is spent on the forest floor in search of oviposition sites. We found significant regional differences in male forewing shape, reflecting the long-term geographic and genetic separation between these groups. We also found significant differences in female forewing shape between low and high elevation sites. *Speyeria diana* female forewings from high elevation populations were narrower than low elevation populations, indicating that these females may be more mobile than those from low elevations with wider forewings. There were no effects of yr since capture of specimens. The wing shape of western populations of *S. diana* appears to be adapted for low dispersal, supporting other recent findings that also indicate western populations of *S. diana* are both spatially and genetically isolated. We conclude that the future of *S. diana* will require the thoughtful preservation of well-connected high elevation habitats, especially in the western distribution where dispersal is more limited than in the east.

Key Words: butterfly; wing; conservation; Diana fritillary

Resumen

Se puede usar la forma del ala de las mariposas como una caracterista para medir las diferencias individuales de las mariposas y las diferencias al nivel del población en las formas adultas. Se realizó un análisis morfométrico geométrico de la forma del ala para *Speyeria diana* (Lepidoptera: Nymphalidae) (Cramer y Stoll 1777), una especie actualmente amenazada por la extirpación en partes de su rango geográfico. Tomamos fotografias y digitalizamos 243 especímenes de *S. diana* depositadas en museos de historia natural de América del Norte y de Europa para comparar la forma y el tamaño de las alas a través de su en la distribución histórica y actual. Utilizamos el análisis de componentes principales (ACP) para comparar la variación en sitios específicos de las alas anteriores y posteriores. Utilizamos los puntajes de los componentes principales en un análisis múltiple de varianza (ANOVA) para evaluar las diferencias e interacciones entre especímenes machos y hembras, especímenes orientales y occidentales, especímenes de elevación baja y alta y especímenes recolectados antes y después de 1950. Encontramos diferencias significativas entre la forma del ala en especímenes masculinos y femeninos, con las alas anteriores masculinas siendo más estrechas y más alargadas, posiblemente haciéndolos muy adecuados para el comportamiento de patrullaje. Las alas anteriores femeninas fueron más redondeadas y más anchas, posiblemente reflejando su historia de vida, ya que gran parte de su vida transcurre en el suelo del bosque en busca de sitios de oviposición. Encontramos diferencias regionales significativas en la forma masculina de las alas anteriores, lo que refleja la separación geográfica y genética a largo plazo entre estos grupos. También encontramos diferencias significativas en la forma de las alas delanteras femeninas entre sitios de baja y alta elevación. Las alas anteriores de poblaciones de *Speyeria diana* encontradas en sitios de alta elevación fueron más estrechas que las poblaciones de baja elevación, lo que indica que estas hembras pueden ser más móviles que las de elevaciones bajas con alas anteriores más anchas. No hubo efectos del año desde la captura de especímenes. La forma del ala de las poblaciones occidentales de *S. diana* parece estar adaptada para una baja dispersión, lo que respalda otros hallazgos recientes que también indican que las poblaciones occidentales de *S. diana* están espacialmente y genéticamente aisladas. Concluimos que el futuro de *S. diana* requerirá la preservación cuidadosa de hábitats de alta elevación bien conectados, especialmente en la distribución occidental donde la dispersión es más limitada que en el este.

Palabras Clave: mariposa; ala; conservación; fritilaria de Diana

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The North American Genus, *Speyeria* (Lepidoptera: Nymphalidae) (Cramer & Stoll 1777) is comprised of at least 16 species and over 100 named subspecies (Dunford 2009; De Moya 2017). All species are univoltine and exclusively use violets (*Viola* spp. [Violaceae]) as their larval host plants (Scott 1986). *Speyeria diana* is a large, sexually dimorphic member of this genus found in areas of the southeastern United States. This species is threatened by a number of factors across its range, such as habitat loss and fragmentation (Hammond & McCorkle 1983), pesticide use (Peacock et al. 1998), fire suppression (Rudolph et al. 2006), and climate change (Wells & Tonkyn 2014), and is therefore of conservation interest (Sims 2017). *Speyeria diana* has disappeared entirely from the Atlantic coastal plain, where it was first described by Cramer & Stoll (1777), and from interior lowland sites, including Indiana, Ohio, and Illinois. The species now persists in 2 disjunct parts of its former range, the Southern Appalachian Mountains in the east, and the Interior Highlands of Oklahoma and Arkansas in the west, and is shifting to higher elevations at about 18 m per decade (Wells & Tonkyn 2014). Using mitochondrial cytochrome oxidase II DNA sequences from museum and field sampled specimens, Wells et al. (2015) recently documented greater mtDNA diversity and more widespread genetic differentiation among eastern populations than western ones. In addition, using coalescent-based population divergence models, Wells et al. (2015) dated the earliest splitting of eastern and western populations at least 20,000 yr ago, during the Last Glacial Maximum. For this study, we photographed pinned *S. diana* specimens held by North American and European natural history museums in order to measure geomorphic morphometric variation in wings across the historical and current distribution of the species.

Because butterfly wings largely reflect the behavior and ecological roles of the species, variation in their shape and size can provide insight about important population-level differences. For example, wing morphology specifically provides information about the flight patterns and flight behaviors of butterflies, which can provide insight into populations' habitat suitability and dispersal rates. A major aim of evolutionary biology is addressing how and why morphology and behavior of different organisms adapt to different environments (Cespedes et al. 2015). Flight is an important character in the evolutionary success of insects, because it is essential for dispersal, courtship, and oviposition behaviors in many species. Wing shape affects flight performance in insects, and selection is expected to maximize performance of behaviors that result in the evolution of wing shape. Because wing shape contributes to the fitness of insects, optimal wing shapes for particular flight behaviors can be used to make predictions in an ecological context (Cespedes et al. 2015).

Wing shape is directly related to the ecological roles and physiological constraints of insect flight that differ between sexes (Willmott & Ellington 1997). As a result, geometric morphometric differences often are found between male and female butterflies due to their different behaviors and life history strategies. For example, *Speyeria diana* males spend a significant portion of their time searching for mates by patrolling at high speeds up and down open roadways. Alternatively, females stay relatively well hidden, spending a majority of their time searching for suitable oviposition sites near violets on the forest floor. Behavioral sexual dimorphism, such as that displayed by *S. diana*, often is associated with differences in flight morphology, which is reflected in the shape of wings (Breuker et al. 2007). *Speyeria diana* females are slightly larger on average than males, with the average male forewing of *S. diana* measuring 4.3 cm, and the average female forewing measuring 5.0 cm (Opler & Krizek 1984). Female wings are iridescent blue and white around the edges and black toward the center, while the smaller males have a bright orange and black coloration instead (Fig. 1).

Wing morphology is widely used across a variety of scientific disciplines to study insects, including taxonomic, ecological, behavioral, and evolutionary studies (De Moraes & Mescher 2004; Carreira et al. 2006; Soto et al. 2008). Wings are an especially useful trait due to their significance in so many aspects of insect life (Betts & Wootton 1988; Wootton 1992; Berwaerts et al. 2002, 2006). Wing morphology, shape in particular, can be very informative as an indicator of changing, and often stressful, environmental conditions (Hoffmann et al. 2005). Geometric morphometric methods in particular offer a comprehensive approach to the study of shape through the multivariate statistical analysis of anatomical landmarks of biological homology (Bookstein 1991; Rohlf & Marcus 1993; Adams et al. 2004). These methods preserve the information about the relative spatial arrangement of the data throughout the analysis (Zelditch et al. 2004), making it possible to

Fig. 1. Female (left) and male (right) *Speyeria diana* specimens were photographed for this study. All specimens were photographed with a cm ruler for proper scaling.

find and analyze shape variations in the organisms within and between populations (Walker & Bell 2000). Moreover, geometric morphometric analyses have high statistical power and have easily visualized results, which help with their interpretation and communication across scientific disciplines (Rohlf & Marcus 1993; Adams et al. 2004; Zelditch et al. 2004). The aim of our study was to use geometric morphometric methods to examine wing shape variation in male and female *S. diana* museum specimens collected from 1777 to 2007 across its historic and current distribution in order to better understand differential selection pressures across the distribution of this threatened species. Our results will advance the knowledge of adaptive morphology in fritillary butterflies and will help inform appropriate management of this species based on its dispersal ability and flight behavior across its range.

Materials and Methods

We photographed 267 pinned *S. diana* specimens that each had accurate label information documenting the date and exact location of collection from natural history museums in North America and Europe (Table 1). Our sample included 166 males and 101 females, dating back to the *S. diana* holotype, collected from Jamestown, Virginia, USA, by Cramer & Stoll (1777), and now housed in the British Museum of Natural History (BMNH) (Table 2). Populations of *S. diana* were extirpated from this locality in coastal Virginia by the 1950s.

In butterflies, the geometric morphometric landmarks most often used to measure shape are the wing vein intersections (Breuker et al. 2007). We identified 11 vein intersections on *Speyeria* forewings, and 11 on hind wings to use as landmarks for our study (Fig. 2). Forewings and hind wings were digitized and analyzed separately for all comparisons. Landmarks were digitized on the dorsal side of pinned specimen wings using tpsDig2 software v.2.12 (Rohlf 2009). We measured variation in wing shape using geometric morphometrics based on generalized least squares Procrustes superimposition methods. Procrustes analysis examines shape by superimposing configurations of landmarks in 2 or more specimens to achieve an overall best fit (Klingenberg & McIntyre 1998). Procrustes transformation eliminates variation due to wing size and orientation of pinned specimens by scaling the specimens to a unit centroid size (Rohlf & Slice 1990). This allowed us to analyze variation solely due to differences in wing morphology (Fig. 3).

We conducted a principal components analysis using Morphologika2 v.2.5 (O'Higgins & Jones 2006) to summarize differences between the average shapes of wing landmarks. A principal components analysis works by finding the first axis that passes through the centroid, accounting for the most variation present in the data. A second axis is then formed by passing through the centroid orthogonally to the first principal components analysis, which accounts for the next greatest variation. Each rotation of the axis accounts for a principal component score, and each axis has an associated eigenvalue that describes the variation explained by the axis. The eigenvalues are then expressed as percentages of the total variation (Palmer 2000). We used the first 3 principal component scores that explained the greatest variation in forewing and hind wing shape as characters for analysis in a multiple analysis of variance (MANOVA) using the statistics program IBM/SPSS v.22.0 (IBM 2013). We used MANOVA to test for effects and interactions between sex (male or female), population location (eastern or western; low or high elevation), and population age (collected before or after 1950) on forewing and hind wing shape. We used the Wilks' Lambda test, a positive-valued statistic that ranges from 0 to 1. Decreasing values of the statistic indicate effects that contribute more to the model. We also used the Hotelling's trace statistical test, which is the sum of the eigenvalues of the test matrix. It is also a positive-valued statistic for which increasing values indicate effects that contribute more to the model. All statistical analyses were conducted with $\alpha = 0.05$.

Results

The principal components analysis performed on hind wing and forewing landmarks identified important sources of geometric morphometric variation (Fig. 4). Principal components analysis showed clear separation of male forewings ($n = 166$), which were narrower and more angular, from female forewings ($n = 101$), which were wider and more rounded (Fig. 5). Significant differences were found between male hind wings from eastern ($n = 131$) and western populations ($n = 102$) (Fig. 6), as well as female hind wings from low elevations (below 300 m) ($n = 111$) and from high elevations (above 300 m) ($n = 157$) (Fig. 7).

The first 3 principal components scores were tested with MANOVA, which showed a significant effect of sex ($F = 39.108$; $P = 0.000$), location (east or west) ($F = 41.944$; $P \leq 0.05$), and elevation (low or high) ($F = 37.443$; $P \leq 0.05$) on wing morphology (Table 3). There were no significant effects of age on male or female hind wings or forewings, and no significant effects from interactions between sex, location, elevation, or yr of collection.

Discussion

We found significant geometric morphometric differences between both sexes from eastern and western populations, between male and female specimens from low (below 300 m) and high elevations (at or above 300 m), but not between male and female specimens collected before and after 1950. We detected important geometric morphometric differences in the forewings of *S. diana* male and female pinned specimens at the population-level, but not in hind wings. This makes sense, because in butterflies, flight is driven primarily by the forewings and can proceed even when the hind wings are removed (Jantzen & Eisner 2008). We predicted that differences between male and female

Table 1. Summary of *Speyeria diana* museum specimens used in this study.

Specimen source	No. of males	No. of females	No. from east	No. from west	No. from low elevation	No. from high elevation	No. pre-1950*	No. post-1950*
Carnegie Museum of Natural History	31	16	46	1	21	26	25	21
National Museum of Natural History	51	31	49	33	22	60	40	42
American Museum of Natural History	22	16	19	19	15	23	20	18
The Field Museum	29	17	46	0	22	24	26	20
British Natural History Museum	6	1	7	0	7	0	7	0
University Florida Natural History Museum	27	20	14	33	24	23	26	21

*Indicates date when specimen was captured and pinned by original collector.

Table 2. Total number of *Speyeria diana* museum specimens detailing the number of males, females, individuals from eastern populations, individuals from western populations, low elevations, high elevations, collected before 1950, and collected after 1950 used in this study.

Total specimens	Total males	Total females	Total east	Total west	Total low	Total high	Total pre-1950	Total post-1950
267	166	101	131	102	111	157	144	122

life histories would be reflected in different wing morphology between sexes, which was confirmed. Male *S. diana* specimens had narrower, more angular forewings than females, which had rounder and wider forewings. We suspect that male forewing shape in *S. diana* is related to the strong flight pattern needed for frequent patrolling, because it is known that longer, slimmer wings enhance lift in flight (Wootton 1992; Grabow & Ruppell 1995). In a study similar to ours, Cespedes et al. (2015) also used landmark-based geometric morphometrics to test whether wing shapes in the moth tribe Haeterini (Satyrinae: Nymphalidae) and their close relatives reflected observed flight patterns. They found that forewing shape differed between sexes for all taxa studied, and found male wing shapes to be more aerodynamically efficient for gliding flight than in female wings. Our results, and those of Cespedes et al. (2015), show that selection can act differently on male and female wing shapes, reinforcing the idea that sex-specific flight behaviors contribute to the evolution of sexual dimorphism (Cespedes et al. 2015).

Other studies on Lepidoptera have found variation in forewing shape based on dispersal ability (Betts & Wootton 1988; Outomoro et al. 2012). Many migratory species are known to have narrow, elongated wings for long-distance, gliding flight. Migratory populations of the monarch butterfly, *Danaus plexippus* L. (Lepidoptera: Nymphalidae), for example, have been shown to have narrower, more elongated forewings, for stronger flight, compared to the forewings of non-migratory populations, which were smaller and rounder (Altizer & Davis 2010; Li et al. 2016; Flockhart et al. 2017). We found that male forewings of *S. diana* from eastern populations were narrower and more angular than populations in the west, suggesting that males in the east demonstrate longer distance dispersal than males in the west. Dispersal ability in similar butterflies is influenced by both habitat quality (Matter & Roland 2002), and levels of habitat fragmentation (Mennechez et al. 2003). Our finding that western populations have rounder wings than those in the east suggests that western populations may have evolved less mobility due to the highly fragmented landscape located between high elevation populations, which act as refuges that inhibit dispersal.

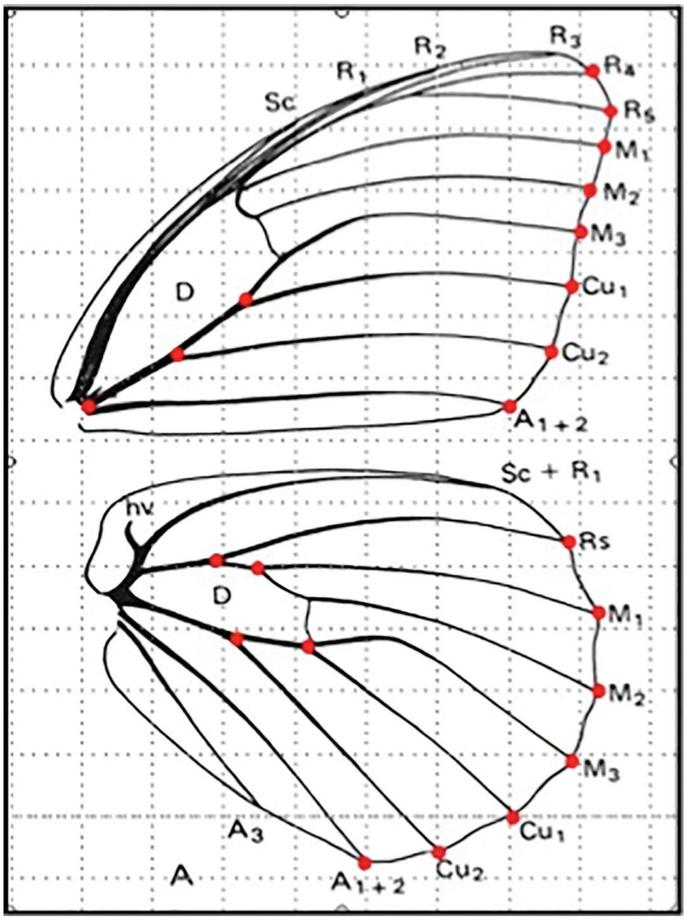

Fig. 2. Twenty-two vein intersections on the *Speyeria* wing were used as landmarks for this study based on Borror et al. 2005.

Mitochondrial DNA data support our findings of morphological differences between regions, as the eastern range of *S. diana* has been shown to harbor higher levels of genetic diversity among and between populations than those in the west (Wells et al. 2015). If narrow, angular wings are characteristic of strong flight patterns, this indicates that *S. diana* males may be more mobile in their eastern distribution, traveling more frequently between populations in search of high quality nectar plants or mates. The closely related Regal fritillary, *Speyeria idalia* Drury (Lepidoptera: Nymphalidae) has been described as a high gene flow species at a large geographical scale (in the order of hundreds of kilometers) (Williams et al. 2003), indicating that *S. idalia* demonstrates long distance dispersal across portions of its range. The distinct morphology of male *S. diana* wings, combined with genetic differentiation between males in the east and west, suggest that *S. diana* males are also a high gene flow species in the east, while those in the west are not. The combination of morphological and mitochondrial DNA differences between disjunct populations in the eastern and western distribution of *S. diana* may warrant subspecies designation, as was found with *S. idalia*, which could ultimately lead to protected status under the Endangered Species Act of 1973 (Waples 1998; Williams 2001, 2002). More data in both of these areas is necessary to pursue either of these designations for *S. diana*.

Geometric morphometric variation in wings has been attributed to environmental heterogeneity in a number of butterfly species (Vandewoestijne & Van Dyck 2011; Bai et al. 2015). We found that the forewings of *S. diana* females from high elevations were narrower than those of females from lower elevations. This is especially interesting, because *S. diana* recently has been shown to be shifting to higher elevations at a rate of 18 m per decade, while disappearing from lowland sites (Wells & Tonkyn 2014). Our results are similar to those documented in related species, such as Hernández et al. (2010), who examined variation in wing morphology between populations of the invasive potato moth, *Tecia solanivora* Polvolny (Lepidoptera: Gelechiidae) at low and high elevations in the central highlands of Ecuador. Not only did *T. solanivora* females have larger, wider wings than males, high elevation moths of both sexes had larger, narrow-shaped wings when compared to low-elevation moths. Their results suggest that variation in wing morphology is an adapted response contributing to the invasion success of the *T. solanivora* in mountainous landscapes (Hernández et al. 2010).

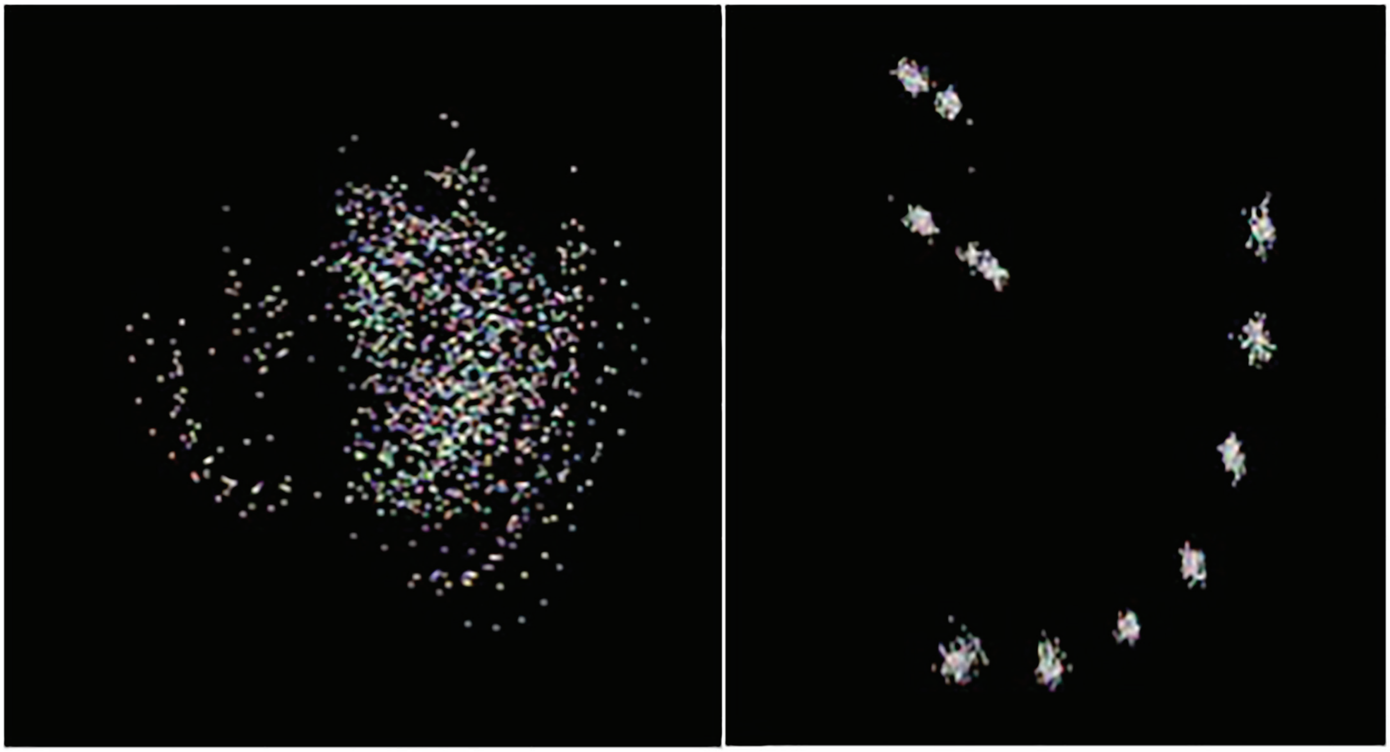

Fig. 3. Procrustes transformation on hind wing landmarks, correcting for variation due to differences in pinned specimen orientation. Before Procrustes on left, after Procrustes on right.

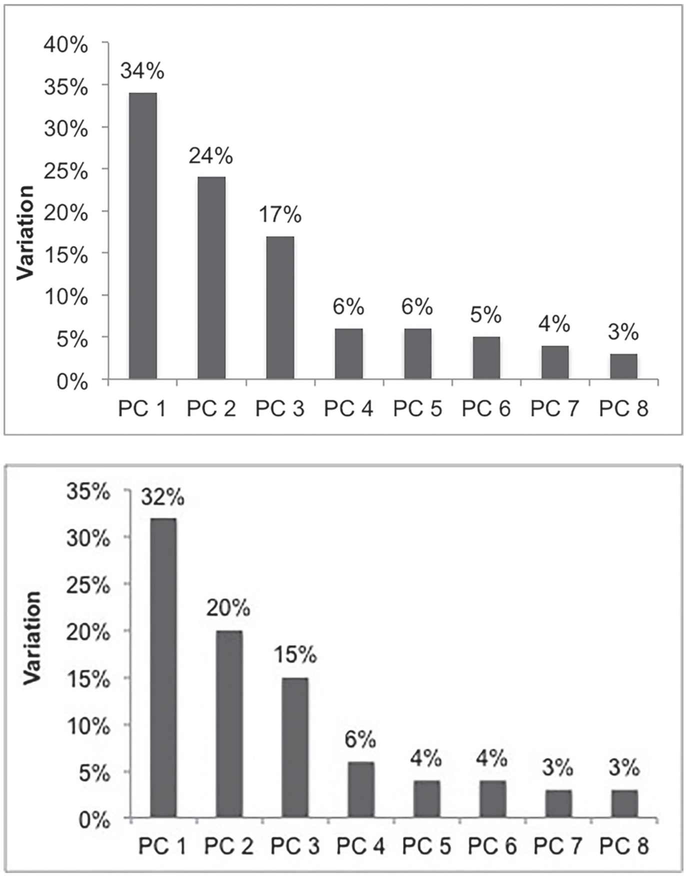

Fig. 4. Principal component scores accounting for variation in forewing (top) and hind wing (bottom) landmarks. The first three principal components scores represent the majority of variation in the data.

Speyeria diana females from high elevations also may be more mobile than those from lowland populations, because females with wings built for strong flight would be most likely to make a successful shift in elevation. This adaptation may be reflective of the species shifting upward in elevation. Interestingly, we found no effect of yr of capture

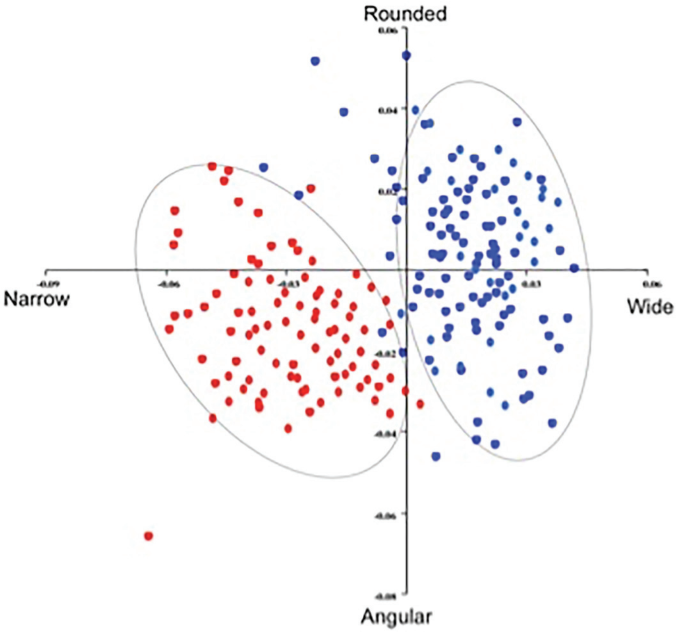

Fig. 5. Principal components analysis showing separation of male (red) and female (blue) *Speyeria diana* forewing shape. Male forewings (n = 166) were narrower and more angular than female forewings (n = 101), which were wider and more rounded.

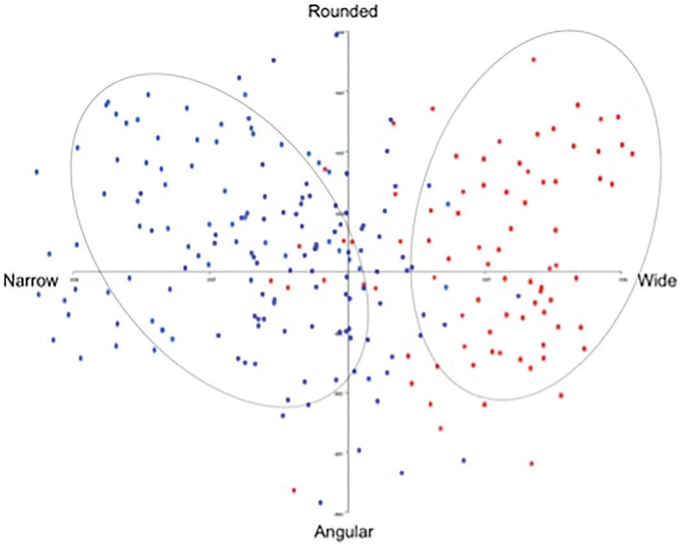

Fig. 6. Principal components analysis showing separation of male *Speyeria diana* forewings from specimens collected from eastern (blue) and western (red) populations. Male hind wings from eastern populations (n = 131) were narrower than those from western populations (n = 102).

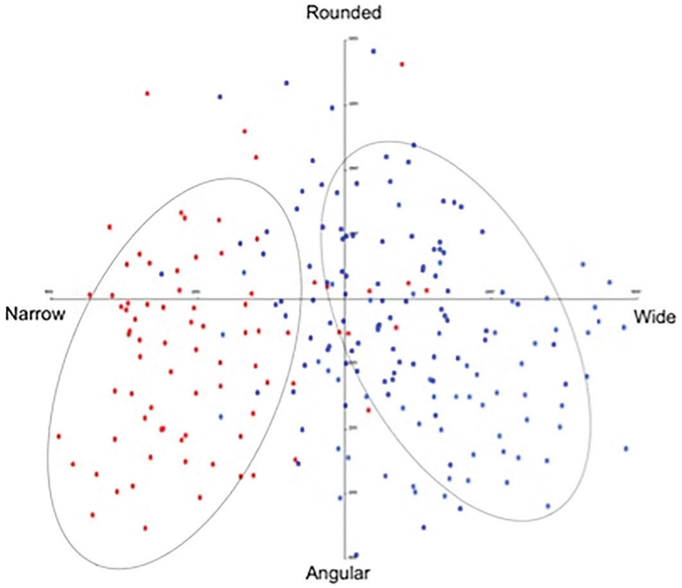

Fig. 7. Principal components analysis showing separation of female *Speyeria diana* forewings from specimens collected at low (blue) and high (red) elevations. Female forewings from low elevations (n = 111) (below 300 m) were wider than those from higher elevations (n = 157) (above 300 m).

in our analysis, indicating that there is no evidence that wing morphology is changing in response to the factors driving the striking changes that have been documented in the distribution and phenology of *S. diana*. Another interesting aspect of *S. diana* populations is that females are now being collected earlier per decade by 4.3 d while males, which emerge first in spring, have not shifted their phenology (Wells and Tonkyn 2014). *Speyeria diana* females at high elevations emerging earlier in spring, before peak violet abundance, may be required to search more actively for violets, which bloom earlier below 300 m where temperatures tend to be warmer. Females with longer, more angular wings may be favored at higher elevations, because stronger flight would assist in finding more sparsely dispersed larval host plants.

The Regal fritillary, *Speyeria idalia*, the closest related species to *S. diana* (De Moya et al. 2017), is declining and at risk range-wide (Swengel and Swengel 2017). Patterns of intraspecific morphological and mtDNA differentiation have been documented between populations of *Speyeria idalia* (Williams 2001, 2002). Mitochondrial DNA sequence divergence, combined with ecological and natural-history data, suggested that a disjunct population of *S. idalia* in the east represent a remnant of a distinct evolutionary lineage of extreme conservation concern. While *S. diana* and *S. idalia* have different population structures, ecologies, and life history traits, their basic biology is similar enough to predict the 2 species should experience similar effects from genetic isolation of populations. *Speyeria diana* populations in the western distribution should be monitored especially closely to prevent further habitat loss and isolation. The distribution and interactions among *S. diana* populations is likely strongly influenced by population-specific habitat requirements.

Our results highlight the importance of examining and preserving variation, both genetic and morphometric, in natural populations before it is lost, especially in species under threat of decline. The wing shape of western populations of *S. diana* appears to be adapted for low dispersal, supporting other recent findings that also indicate western populations of *S. diana* are both spatially and genetically isolated

Table 3. MANOVA performed on first three principal components scores for 11 hind wing landmarks. Values in bold are significant ($P \leq 0.005$).

Effect	Test	Value	*F*	df	Significance
Sex	Wilks' Lambda	0.240	39.108	6	0.000
(male or female)	Hotelling's Trace	3.171			
Locality	Wilks' Lambda	0.929		6	0.048
(east or west)	Hotelling's Trace	0.077	41.944		
Elevation	Wilks' Lambda	0.877	37.443	6	0.048
(low or high)	Hotelling's Trace	0.077			
Year of Collection	Wilks' Lambda	0.872	1.118	6	0.107
(pre-1950, post-1950)	Hotelling's Trace	0.147			
Locality * Sex	Wilks' Lambda	0.97	11.326	6	0.256
	Hotelling's Trace	0.108			
Locality * Year of Collection	Wilks' Lambda	0.061	0.808	6	0.567
	Hotelling's Trace	0.065			
Sex * Year of Collection	Wilks' Lambda	0.048	0.620	6	0.714
	Hotelling's Trace	0.050			
Locality * Sex * Year of Collection	Wilks' Lambda	0.031	0.396	6	0.879
	Hotelling's Trace	0.032			

(Wells and Tonkyn 2014; Wells et al. 2015). We conclude that the future of *S. diana* will require the thoughtful preservation of well-connected high elevation habitats, especially in the western distribution where dispersal is more limited than in the east.

Acknowledgments

We would like to thank Drs. Tonkyn, Adler, and Marko for assistance designing and setting up this study. We thank Drs. Rivera for guidance and technical assistance with our methods, and Marielle Abalo and Justin Orders for help with data collection and analysis. We sincerely thank colleagues at the British Museum of Natural History, The Carnegie Museum of Natural History, the Smithsonian Natural History Museum, the American Museum, the Field Museum, and the McGuire Center at University of Florida, for allowing us access to their *S. diana* museum specimens for this and other studies. We thank Clemson University and the University of Charlotte at North Carolina for ongoing support as well.

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