



WING SHAPE VARIATION ASSOCIATED WITH MIMICRY IN BUTTERFLIES

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Mimetic resemblance in unpalatable butterflies has been studied by evolutionary biologists for over a century, but has largely focused on the convergence in wing color patterns. In *Heliconius numata*, discrete color-pattern morphs closely resemble comimics in the distantly related genus *Melinaea*. We examine the possibility that the shape of the butterfly wing also shows adaptive convergence. First, simple measures of forewing dimensions were taken of individuals in a cross between *H. numata* morphs, and showed quantitative differences between two of the segregating morphs, f. *elegans* and f. *silvana*. Second, landmark-based geometric morphometric and elliptical Fourier outline analyses were used to more fully characterize these shape differences. Extension of these techniques to specimens from natural populations suggested that, although many of the coexisting morphs could not be discriminated by shape, the differences we identified between f. *elegans* and f. *silvana* hold in the wild. Interestingly, despite extensive overlap, the shape variation between these two morphs is paralleled in their respective *Melinaea* comimics. Our study therefore suggests that wing-shape variation is associated with mimetic resemblance, and raises the intriguing possibility that the supergene responsible for controlling the major switch in color pattern between morphs also contributes to wing shape differences in *H. numata*.

KEY WORDS: *Heliconius*, mimicry, morphological evolution, polymorphism, wing shape.

Many prey species that are unpalatable to predators have evolved bright warning colors and patterns that advertise their unprofitability (e.g., Summers and Clough 2001; Cortesi and Cheney 2010). Studying tropical butterflies, Bates (1862) recognized that these warning signals may be mimicked by other species that are otherwise defenseless; by doing so they benefit from predator avoidance. Natural selection favors those individuals that most closely resemble the unpalatable species, but protective resemblance is not limited to the convergence of a palatable “mimic” to a distasteful “model.” Following Bates, Müller (1879) described relationships in which two unpalatable species converge on the same warning display; both of these “Müllerian” mimic species can contribute to educating a naïve or forgetful predator but the number of individuals attacked from either species is reduced,

leading to a per capita survival benefit. “Comimicry” therefore arises when mutual survival benefits of resemblance between multiple unpalatable species result in selection on all members toward a shared phenotype.

In some species of butterflies, as well as in other taxonomic groups, protective resemblance may not be limited to coloration. Accurate mimicry often involves a suite of morphological and behavioral traits modulated through natural selection which further help to protect individuals from predatory attack (Fisher 1930; Sherratt and Beatty 2003). For example, the convoluted, looping flight characteristics of droneflies (*Eristalis tenax*) have been interpreted as being among the behavioral adaptations that improve the resemblance of these flies to honeybees (Golding et al. 2001). Indeed, Bates (1862) suggested that mimicry in insects should

extend to their physical motions, so a consequence of such selection may be a convergence of locomotory behavior as well as color patterning. In a study of wing motion in butterflies, Srygley (2007; Srygley and Ellington 1999a) found that this appears to be true for species of the genus *Heliconius*: two comimetic species, *Heliconius cydno* and *Heliconius sapho*, beat their wings more slowly and with a more symmetrical motion than their respective sister species, the comimics *Heliconius melpomene* and *Heliconius erato*. Because these features are more strongly determined by mimicry group than the lineage from which they derived, wing motion appears to be an important component of the overall mimetic signal and may serve to identify mimics before the predator can see details of the wing pattern (Srygley 2007).

Wing shape is an important determinant of flight ability, and functional constraints exist that limit the variation seen in nature. For example, the leading edge of the aerofoil must be strongly reinforced to provide rigidity across the wingspan, and its aerodynamic shape is critical for effective flight (Maxworthy 1981; Wootton 1992). This constraint has been highlighted in studies of wing morphology among heliconine species, in which variation generally increases from the anterior to the posterior of the forewing (Strauss 1990). Despite such restrictions, considerable variation is seen in both wing size and shape across the Lepidoptera and may be related to flight height, maneuverability, migratory habits or other complex behaviors (Betts and Wootton 1988; Dudley 1990; Berwaerts et al. 2008; Altizer and Davis 2010; DeVries et al. 2010). Within *H. erato*, measurements of wing length taken by Strauss (1990) varied by close to 20% either side of the mean, which translates into a much larger divergence in area measurements and demonstrates that substantial size differences exist within a single species. *H. erato* is polymorphic across its range and exists in mimicry rings with other *Heliconius* species. Whether the differences in size found by Strauss represent common natural within-species variation or adaptive variation associated with the different races of *H. erato* was not investigated. Furthermore, despite the importance of shape to the appearance and locomotion of mimetic butterflies, few studies have directly tested for differences of wing shapes between pairs of comimics (Srygley 1999).

In this study, we examine wing shape in *Heliconius numata*. Heliconines were among the study species of both Bates and Müller, and were central to the theories they developed. In particular, *Heliconius* species are noted for their radiation of color-pattern forms across Central and South America: a single species may display multiple patterns across its distribution range and accurately mimic sympatric species in any given locality (Brown et al. 1974; Sheppard et al. 1985). *Heliconius numata* is unusual because it is a Müllerian mimic that is locally polymorphic (Brown and Benson 1974). In some localities up to seven different forms, or morphs, may be found, each being an accurate mimic of a

Table 1. *Heliconius numata* morphs and their recognized *Melinaea* comimics.

<i>Heliconius numata</i> morph	<i>Melinaea</i> comimic
<i>H. n. f. arcuella</i>	<i>M. marsaeus phasiana</i>
<i>H. n. f. aurora</i>	<i>M. marsaeus rileyi</i>
<i>H. n. f. bicoloratus</i>	<i>M. marsaeus mothone</i>
<i>H. n. f. elegans</i>	<i>M. satevis cydon</i>
<i>H. n. f. silvana</i>	<i>M. ludovica ludovica</i>
<i>H. n. f. tarapotensis</i>	<i>M. menophilus ssp. nov.</i> and <i>M. satevis tarapotis</i>

different ithomiine butterfly (Table 1, and see Joron et al. 2011 Fig. 1; Joron et al. 1999). The color patterning on the wings of these different *H. numata* morphs varies considerably, but the switch between them is controlled by a single genetic locus, a supergene known as *P* (Brown 1976; Joron et al. 2006, 2011). Reduced recombination at this locus, maintained by chromosomal inversions, prevents the segregation of different color-pattern alleles (Joron et al. 2011); together with a hierarchy of dominance relationships, this ensures that intermediate and nonmimetic forms rarely occur in the wild (Joron et al. 2006). Here, we examine the possibility that the morphs of *H. numata* differ not just in their wing pattern but also in their wing shape.

First, we analyzed the forewing shape of individuals from an experimental brood of *H. numata*. The brood segregated for three alleles at *P*, producing color-pattern morphs *H. numata f. elegans*, *f. aurora*, and *f. silvana*, allowing between-morph comparisons to be made. Wing shape was first characterized using simple wing metrics including area, axis length, circularity (*C*), aspect ratio (*AR*), and wing moments. Geometric morphometric approaches (landmark analysis and elliptical Fourier analysis of outline) were then applied to investigate explicit shape variation more comprehensively. Finally, we extended our wing measurements to a large sample of wild-caught specimens from natural populations of *H. numata* and ithomiine butterflies, with the aim of identifying correlations between the wing shape of different *H. numata* morphs and their comimetic species.

Methods

HELICONIUS NUMATA AND MODEL SPECIMENS

Analyses of wing shape were performed on a single *H. numata* brood, which has been described elsewhere (Joron et al. 2006). Offspring of the brood had four different genotypes: $P^{aur}P^{sil}$, $P^{aur}P^{ele}$, $P^{ele}P^{sil}$, and $P^{sil}P^{sil}$. Because of the $P^{ele} > P^{aur} > P^{sil}$ dominance hierarchy that exists between alleles of *P*, these genotypes correspond to three phenotypic morphs: *elegans*, *aurora*, and *silvana*, with subtle wing pattern differences seen between $P^{aur}P^{ele}$ and $P^{ele}P^{sil}$ individuals (Jones et al. 2012). For the

extended analysis of wing measures we used specimens of *H. numata* and their *Melinaea* species comimics caught around the towns of Yurimaguas and Tarapoto in Peru between 1999 and 2007. Supplementary *Melinaea ludovica ludovica* were collected in French Guiana in 2009.

MEASUREMENT OF WING SHAPE

The detached wings of specimens were photographed with a Nikon D90 digital camera with 105 mm vibration reduction macro lens in a laboratory-made photo studio designed to eliminate shadows. Damaged wings were excluded from our analyses. To obtain measurements of wing parameters in the brood and wild-caught individuals, the BoneJ plug-in (Doube et al. 2010) for ImageJ (Abramoff et al. 2004) and custom scripts in Matlab (Mathworks) were used. First, we fitted an ellipse to forewing images to obtain a measure of the length (R_I) in pixels of the longest axis (axis I) and the length (R_{II}) of the axis at 90° to this (axis II). We also recorded outside perimeter length (P , in pixels) and total area (S , pixels²). From these parameters, we calculated AR and C :

Aspect ratio:

$$AR = \frac{4R_I^2}{S}.$$

Circularity:

$$C = 4\pi \frac{S}{P^2}.$$

To make a further comparison of wing shape, we measured the third moment of wing area. Moments of area have been used previously in the analysis of wing shape (Weis-Fogh 1973; Ellington 1984a; Betts and Wootton 1988), and may be calculated manually by dividing the length of the axis into regular rectangular strips each of equal diameter (dr) and of distance r from the wing base. Measuring chord length (c) at the center of each strip, the k th moment of wing area (S_k) can be calculated:

$$S_k = 2 \int_0^R cr^k dr.$$

Here, we used measures of area, axis length, and the third moment of area to calculate the nondimensional radii (\hat{r}_3) corresponding to this moment (Usherwood 2001):

$$\hat{r}_k(s) = \sqrt[k]{\frac{S_k}{SR^k}}.$$

Moments of area depend on the distribution of the chord along the wing and therefore represent the wing shape. Because the first, second, and third moments are highly correlated (Ellington 1984a), we have used just the third moment, which is the most sensitive to small variations in wing length and gives a measure of the lopsidedness of the distribution. For measurements from axis I, larger radii are found for wings in which more

of the area is positioned away from the wing base and closer to the wing tip (Ribak et al. 2009). For axis II, these radii relate to the distribution of area between the anterior and posterior of the wing. Moments were calculated with r increments of 1 pixel.

For geometric morphometric analysis of wing shape, the tpsDig2 software (Rohlf 2010) was used to capture 20 2D-landmark coordinates. On each wing, 20 landmarks were placed at locally defined distinct points around the wing such as the convergence points of veins or the intersect of a vein and the edge of the wing (Fig. 1D). Procrustes superimposition was used to normalize the coordinates. Finally, elliptical Fourier analysis was used to quantify the complete outline of each wing (Lestrel 1989), using custom scripts developed in Matlab following the directions of Kuhl and Giardina (1982). In the analysis of the experimental brood, the wings were registered for orientation using selected landmark points (Fig. 1D) and normalized for size using the landmark coordinates. In the extended study of wing shapes of both *H. numata* and *Melinaea* species, coordinates were not used because of differences in venation between the two divergent groups. Instead, a custom masked-alignment procedure in Matlab was used for registration. This procedure was based on maximizing the area of wing overlap and minimizing the area of nonoverlap when wing images were superimposed on one another. Recursive alignment of wings continued until alignments were stabilized and a mean wing mask was produced. Similar procedures have been applied elsewhere (e.g., Bergmeir and Subramanian 2009).

STATISTICAL ANALYSIS

Differences in wing length, AR, and other measures between morphs and between sexes were analyzed with analysis of variance (ANOVA). Two-tailed t -tests with a Bonferroni correction were used post hoc to determine which groups showed significant differences (a conservative correction method included three morphs, both sexes, and a total of six measurement parameters being tested; $n = 36$). For geometric morphometric data, following Procrustes superimposition in tpsDig2, we used MorphoJ (Klingenberg 2011) to perform principal component analyses (PCA) to find the axes of variation in shape. Multivariate analyses of variance (MANOVA) were performed to test for significant effects of sex and morph, and canonical variate analyses (CVA) were used to find the shape features that best distinguish these groups. Elliptical Fourier functions were also analyzed with PCA, followed by MANOVA: all principal components (PC) accounting for 1% or more of captured variation were included in the analysis of variance. In the analysis of wild-caught specimens, pairwise MANOVA were used to determine which species and morphs significantly differed from one another. A linear discriminant analysis (LDA), which determines the axes maximizing the differentiation between predefined groups, was then performed to analyze the mimicry association. Specifically, PC values of *H. numata*

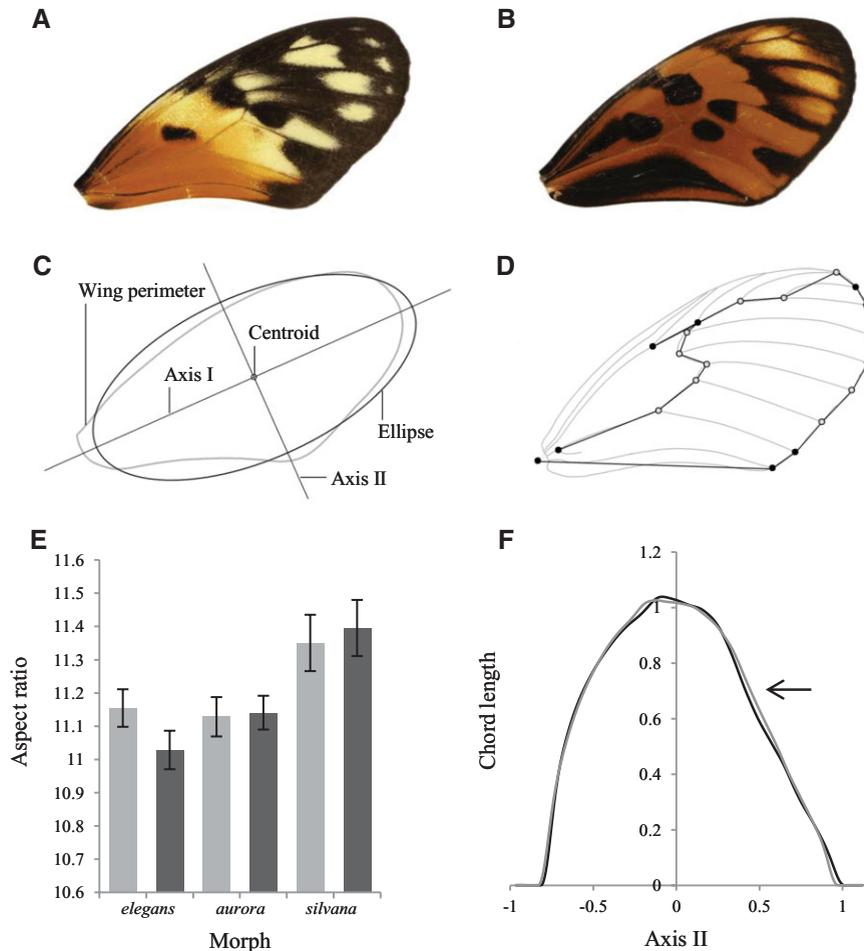


Figure 1. Forewing measurements. Two individuals from the experimental brood representing extremes of high (elongated) aspect ratio (A, female *Heliconius numata* f. *silvana*) and low (rounded) aspect ratio (B, male *H. numata* f. *elegans*). (C) Measurements taken from each wing after fitting ellipse. (D) Landmark positions used in geometric morphometric analysis of wing shape. Landmark points shaded black were used for orientation in elliptical Fourier outline analysis. (E) Plot of aspect ratio. Females shown in light gray; males in dark gray. Standard error bars are shown. (F) Curve of area distribution across the wing, using chord length taken along axis II. *H. numata* f. *silvana* wing from (A) is shown (dark gray) against that of the *elegans* wing (light gray). Arrow indicates region of the *elegans* androconia at the overlap between forewing and hindwing.

wings were used as a training set in the LDA, and PC scores of *M. ludovica ludovica* individuals were introduced to see which *H. numata* morph these wings were most closely associated with.

Results

ANALYSIS OF WING DIMENSIONS

To explore the possibility that the wing shapes of *H. numata* vary with different color-pattern morphs, we took simple measures of forewing dimensions from individuals in our experimental brood. Interestingly our results first provided evidence of sexual dimorphism in wing shape, with male forewings being significantly larger than female forewings. This was determined through measurements of both wing length and wing area ($P < 0.001$ for

both parameters). The forewings of males were also found to be marginally but significantly more circular than those of the females in the brood ($P < 0.001$; Fig. S1). Although AR was not significantly different between the two sexes, we also found evidence of sexual dimorphism when measuring the third moment of area. The moments of area incorporate shape and size components of the wing and can be presented as nondimensional radii (Ellington 1984a). The third moment of area relates to the aerodynamic moment generated by the wing (Weis-Fogh 1973; Ellington 1984b).

Measuring the third moment of area from axis I, we found a significant difference between the sexes ($P < 0.001$; Fig. S1), suggesting that there are differences in the proximo-distal distribution of wing area between males and females, with males having more of the wing area distributed closer to the wing base. Similarly,

calculating the nondimensional radii of the third moment of area measured from the perpendicular axis (axis II), we again found differences associated with sex: males had a larger radius of moment than females ($P < 0.001$), signifying that more of the wing area is positioned away from the leading edge at the wing anterior. These differences between males and females likely reflect the position of the androconial area found on the basal part of the male forewing, where it overlaps with the hindwing (Emsley 1963). The area of overlap appears to be larger in males than in females.

These wing metrics were also tested to investigate morph-associated differences in wing shape. Wing length and area were not found to significantly differ between any of the morphs in our brood, and the size trends between males and females were the same in each morph, with no significant sex-by-morph interaction. Conversely, ANOVA identified significant differences between morphs in terms of AR (Table S1 and Fig. S1). We found that the *silvana* wings had the highest ARs, indicating that they had the most elongated wings, and *elegans* individuals had the lowest average ARs (Fig. 1). However, pairwise *t*-tests did not reach significance at $\alpha = 0.05$ after applying a conservative Bonferroni correction for multiple testing. By contrast, we found statistical support for morph-related shape variation measured through the nondimensional radii.

Male and female *silvana* individuals had lower average nondimensional radii (axis II) than *elegans* individuals of the same sex, suggesting that there are indeed differences in wing shape between these morphs of *H. numata* ($P = 0.002$, significant at $\alpha = 0.05$ for males after Bonferroni correction in pairwise two-tailed *t*-tests between *elegans* and *silvana*. Not significant in females, however there were no significant sex-by-morph interactions, indicating a probable lack of power in the female analysis). Figure 1F represents these differences graphically; starting at the leading edge and moving toward the trailing edge along axis II, measurements of the wing chord are displayed in parallel for an *elegans* and a *silvana* individual. The chord measurements diverge most at the androconia.

ANALYSIS OF WING SHAPE

To further analyze the variation in wing shape that our initial measurements identified, we used a landmark-based morphometric approach, placing landmarks around and within the wings of all individuals in the brood. A PCA of the residuals from the Procrustes analysis of landmark positions found that 90% of the variance of wing shape could be captured by the first 12 PC. The first component (accounting for 21.5% of measurable variation) captured a shape change in the posterior portion of the wing subapex, whereas PC2 captured a narrowing of the discal part of the wing and extension at the apical margin. The axes of variation captured in these first two components were not strongly

associated with the variation between the sexes or the different morphs, and a plot of PC1 and PC2 did not fully separate the wing shapes by these groups (Fig. S2). However, Procrustes ANOVA found a significant difference in shape both between the sexes and between morphs ($P < 0.0001$ for each). The sex-by-morph interaction did not have a significant effect on wing shape ($P = 0.118$). Furthermore, a post hoc CVA resulted in just two CV, a plot of which shows discrete clusters with minor overlap between morphs. This analysis specifically allowed visualization of those differences that exist between them (Fig. 2), which are most clearly seen along the wing termen.

ANALYSIS OF WING OUTLINE

Finally, analysis with elliptical Fourier functions allowed measurement of the complete outline of the wing. This method was therefore sensitive to differences in wing shape that were not captured by the landmark analysis because of a lack of suitable positions for landmarks to be placed. We found that the major difference in shape was associated with sex: a principal component analysis separated in the first axis male wings from female wings, and captured the posterior extension of the basal part of the wing where males present androconial scales (Fig. 2). Plotting PC1 and PC2 revealed limited clustering of individuals by morph (Fig. 2). However, MANOVA showed that significant differences do exist between morphs, for both males ($P < 0.001$) and females ($P = 0.001$).

In summary, from measurements on an experimental brood we have found evidence of sexual dimorphism in each of the components we measured, with the exception of AR. Intriguingly, differences relating to shape and the distribution of area across the wing were not limited to the sexes but were also found between morphs. These differences are most evident between the dominant pattern morph *elegans* and the recessive morph *silvana*. Together the three approaches we have taken have allowed us to determine that the wings of the *silvana* morph have a distinct shape that is more narrow and elongated than those of the other morphs in our brood and shows less convex curvature along the termen.

ANALYSIS OF WING SHAPE IN NATURAL POPULATIONS OF COMIMICS

Wing shape parameters were measured in a larger sample of *H. numata* and their *Melinaea* comimics from a set of wild-caught specimens, to examine the possibility that the differences we have observed are associated with mimicry in natural populations. Because comimics belong to distantly related clades, the structure of wing veins is not necessarily preserved across clades, and the variation in wing landmark position is expected to reflect primarily phylogenetic divergence. This precludes using landmark-based geometric morphometrics to investigate mimetic resemblance, so we focused our extended study on the simple shape parameters

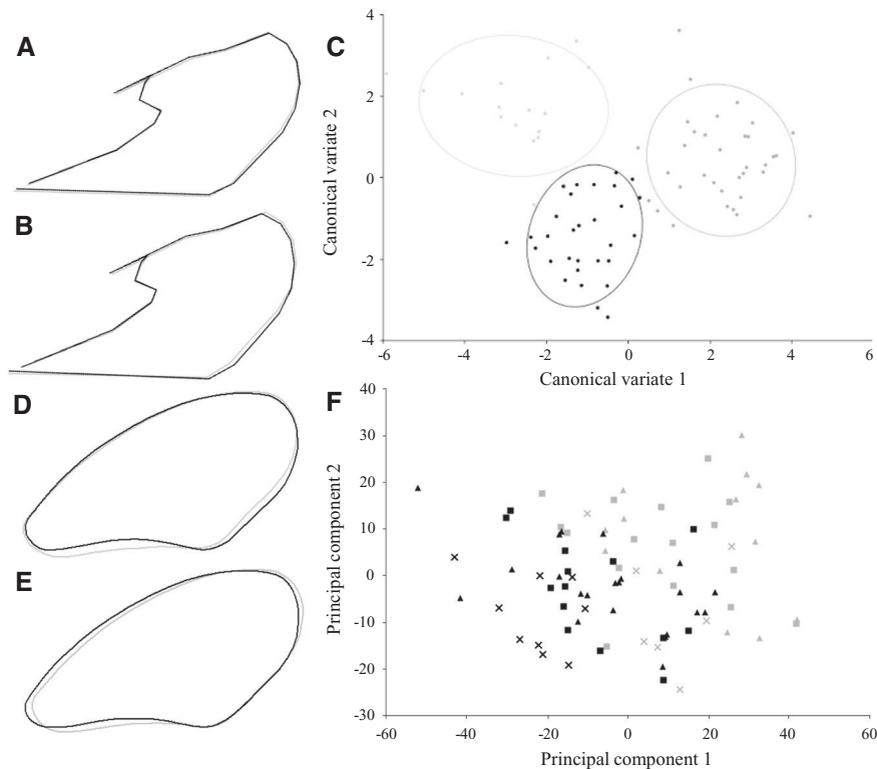


Figure 2. Variation in wing shape determined by geometric morphometric approaches. The variation captured in canonical variate 1 (A) and 2 (B) is shown (CVA using phenotype as the classifier variable for grouping), alongside a plot of the CVA output (C), with confidence ellipses fitted in MorphoJ: *Heliconius numata* f. *elegans* shown in black, *aurora* in medium gray, *silvana* in light gray. (D,E) Variation captured by elliptical Fourier outline analysis, principal components 1 (D) and 2 (E). A plot of all individuals in the brood (F) shows that PC1 is associated with the difference between male (black) and female (gray) wing shapes. *H. numata* f. *aurora* is shown with squares; *elegans* with triangles; *silvana* with crosses. In images of the wings, lowest CV/PC values are shown in gray and highest are in black.

described above and on elliptical Fourier analysis. Measuring these parameters in wild caught specimens of *H. numata*, we found consistency with the variation identified in our experimental brood; male *silvana* individuals had higher average ARs than male *elegans* individuals (Fig. 3A, $P = 0.019$, not significant after conservative Bonferroni correction), and the nondimensional radius of the third moment of area was significantly lower in both male and female *silvana* individuals than in their *elegans* counterparts ($P < 0.001$). These measurements also suggest that the *silvana* and *elegans* morphs have the most extreme wing shapes of the morphs we examined; both the mean AR and standard moment of area scores of male *H. numata* f. *arcuella*, *bicoloratus*, and *tarapotensis* were within the mean values of *silvana* and *elegans*.

Extending our analyses to the *Melinaea* comimics of *H. numata*, we found that the shape differences seen between the *elegans* and *silvana* morphs are apparently shared with the respective comimics: the forewings of *M. ludovica ludovica*, comimic of the recessive *silvana* morph, are the most elongated of all of the species we examined and have a considerably higher AR than *Melinaea satevis cydon*, the comimic of *H. numata* f. *elegans* ($P < 0.0001$ males; $P = 0.05$ females; Table S2). Unfortunately

only one female *M. ludovica ludovica* individual was available from Peru for wing measurement.

Despite considerable overlap in measures between the *Heliconius* wings and those of *Melinaea*, the former have significantly lower ARs than the sampled ithomiines ($P < 0.0001$); the wings of *H. numata* are generally less elongated in form, with wing tips that are less pointed than those of their comimic species. To more accurately capture these differences in shape, the forewing outline of each of our wild-caught specimens was captured by elliptical Fourier analysis and PCA was used to determine the major axes of variation. PC1 captured variation in the discal part of the wing, the termen and the wing apex (Fig. 3B, Fig. S3), and was associated with differences related to both sex and morph, with females having generally higher values than males and *H. numata* f. *silvana* wings having lower average values than any other conspecific morph: both female and male *silvana* significantly separated from all other morphs ($P = 0.002$ and $P < 0.001$, respectively; Fig. S4). Pairwise MANOVA also found that this morph was the only one to significantly separate from any other morph (Table S3). Similarly, the wings of the comimic *M. ludovica ludovica* had the lowest values in this PC, with male *ludovica* wings being

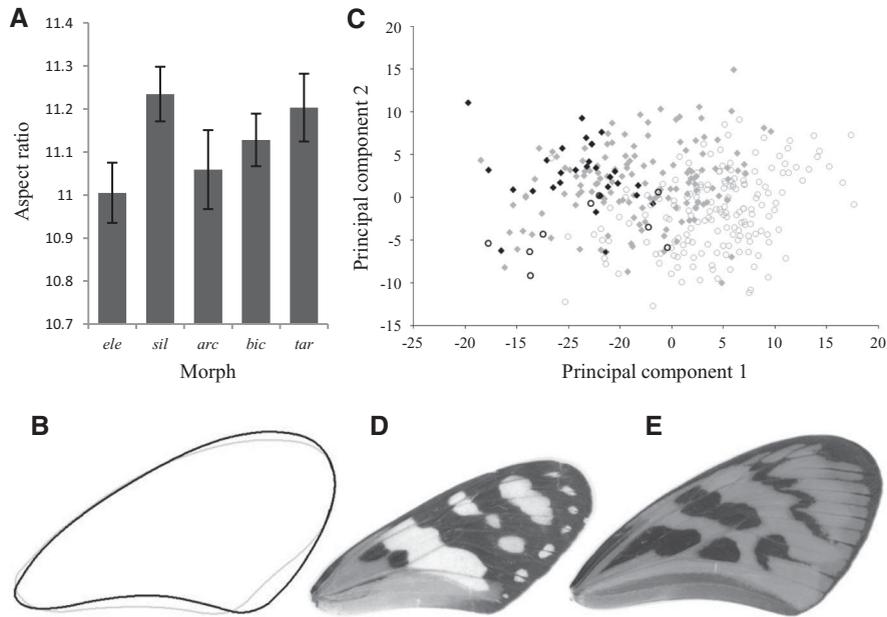


Figure 3. Bar chart of aspect ratio, measured from the forewing of wild-caught male *Heliconius numata* (A). Standard error bars are shown. (B) Reconstructed outline of wings showing extremes of PC1 of wild-individual outline analysis, with lowest value shown in gray and highest in black. (C) Plot of PC1 and PC2, displaying all *H. numata* (diamonds) and *Melinaea* (circles) wild individuals. *H. numata* f. *silvana* and their ithomiine comimics *Melinaea ludovica ludovica* are shown in black. (D) Representative low-scoring wing (male *M. ludovica ludovica*) and (E) high-scoring wing (female *M. marsaeus phasiana*).

significantly separated from several all other *Melinaea* species ($P < 0.001$, not shown). Among these taxa, pairwise MANOVA found that several species differed significantly from one another (Table S3).

Most interestingly, Fourier outline analysis reveals an apparent coassociation of the *silvana-ludovica* mimicry group in morphospace. The *H. numata* f. *silvana* individuals, which differed significantly from other morphs, showed moderate clustering with *M. ludovica ludovica* (Fig. 3C). To examine this clustering, LDA were performed on the principal components. Component values from the *H. numata* morphs were used in the model as a training set, and the discrimination of the Peruvian *ludovica* into the *numata* morph classes was tested. The analysis predicted that eight out of nine individuals were of the class “*silvana*” (Table S4), providing support to our interpretation of the PCA that, of all interspecies associations, the *silvana* wings most closely resemble those of *M. ludovica ludovica*. As a final examination of the resemblance between these comimics, we supplemented the *M. ludovica ludovica* specimens in our analysis with individuals collected in French Guiana where this species co-occurs with *H. numata* f. *silvana* and is relatively abundant. Outline analysis found that the wings of the Guianan *ludovica* were not significantly different in shape from those collected in Peru (MANOVA $P = 0.268$), and consistent with this the Guianan and Peruvian *ludovica* clustered together in a plot of principal components 1 and 2 (Fig. S4C). LDA performed with the expanded *ludovica* sample

size also supported the Peruvian analyses (Table S4C, S4D), indicating that there is a degree of shared wing morphology between the comimics *H. numata* f. *silvana* and *M. ludovica ludovica*, and implicating an adaptive component to the shape variation between *H. numata* f. *silvana* and other *H. numata* morphs.

Discussion

Individuals of *H. numata* do not wander far and they are much less common than their ithomiine comimics. This creates an environment that is coarse-grained through space, with different individuals living in significantly different mimetic environments (Brown and Benson 1974), and has promoted adaptive polymorphism in this species (Joron 2005). Here, we have quantified wing shape parameters in *H. numata* to investigate the possibility of adaptive variation in wing shape as well as color pattern.

VARIATION IN WING SHAPE

We have found evidence of sexual dimorphism in *H. numata* in a number of different parameters of wing shape, as well as significant differences in wing shape between mimetic morphs. As has been well characterized in studies of birds and bats (Savile 1957; Findley et al. 1972), the shape of butterfly wings varies with different flight requirements. Wings with a high AR are associated with extended flight, whereas low AR wings favor slow, agile flight and are expected of species that frequently fly in

restricted spaces or dense vegetation (Betts and Wootton 1988). For example in the monarch butterfly populations with the largest migratory distances tend to have higher AR wings than nonmigratory populations (Altizer and Davis 2010). Similarly in the speckled wood butterfly *Pararge aegeria* the higher acceleration capacities of males can in part be explained by significant differences in AR (Berwaerts et al. 2002). Minor sexual differences in wing shape may be predicted for *Heliconius*, given the differences in their life histories and the occurrence of sexual shape dimorphism in the wings of other insects (Svensson and Petersson 1987; Moore 1990; Thayer 1992; Gilchrist et al. 2000); whereas male *H. numata* patrol throughout the day at a height of 3–10 m, females tend to fly slowly, searching for oviposition sites in the understory using a weak flight similar to that of their ithomiine comimics (Brown and Benson 1974; Joron 2005). As a result, we may expect the AR of the females to be lower than in males, as with *P. aegeria* (Berwaerts et al. 2002), but we found no significant differences between the sexes in this parameter.

Sexual dimorphism in AR has been reported previously in *Heliconius*. Mendoza-Cuenca and Macias-Ordonez (2005) studied a population of *Heliconius charitonia* in which males show distinct size-related mate-locating behaviors. Pupal-mating males of this species are large and mate with females as they eclose from the pupal case, whereas smaller males fly continuously throughout the season and pursue flying adult virgin females. The mating strategy of these “patrolling” males might therefore result in selection for more efficient and controlled flight. Correspondingly, the wings of these individuals have a significantly higher AR than those of the female or the pupal-mating males, whose flight patterns more closely resemble each other. Although the use of AR avoids the potential biases of absolute measures, there are limitations to measuring wing shape with this parameter alone because differences in shape across the wing are also important (Betts and Wootton 1988): wings with extended, narrow tips would gain some of the benefits of high AR but maintain reasonably low moments of area and mass, and hence agility. Perhaps as a result, the relationship between AR and flight performance in butterflies remains unclear, with some authors reporting only weak relations between AR and flight speed (Chai and Srygley 1990; Dudley and Srygley 2004). It is also important to note that the hindwings contribute to the normal flight of a butterfly (Jantzen and Eisner 2008). The difficulty in determining precisely how the forewing and hindwing overlap in flight means that flight-surface ARs are not readily available, and may show different trends to forewing measures alone.

Our analysis of the radii of the third moment of area found significant differences between males and females in terms of the proportion of wing area being distributed along the major axis. Males were found to have smaller radii than females, which may largely be explained by the androconial patch that broadens

the posterior of the forewing in the region that overlaps with the hindwing. This has the relative effect of placing the centroid closer to the wing base. In fact, centroid position is given by the related measure, the first moment of area, and our findings can be compared with those of *P. aegeria*, in which distance to centroid was also measured (Berwaerts et al. 2002). In this species, centroid position was also located more distally in females than in males and had a significant impact on flight performance: a centroid located more distally allows the butterfly to move air with a higher relative speed and thus generate higher aerodynamic forces per unit area (Dudley 2000). It is also expected to compromise the flight speed and maneuverability, and improve conspicuousness (Srygley 1994). We have previously noted that female *H. numata* may rely more heavily on mimetic relationships than do males; females tend to use more predictable flight patterns and less agile flight, whereas males use higher activity levels and more agile flight to escape predators (Ohsaki 1995; Joron 2005). Our findings are therefore consistent with this observation.

In addition to sex-related differences, our study has found evidence of differences in wing shape between mimetic morphs of *H. numata*. Although wings of the *aurora* and *elegans* morphs of our experimental brood did not significantly differ from one another, the *silvana* morph showed a significantly different shape when measured through a number of different indices. This discrimination of *silvana* was supported in the analysis of further individuals caught in the wild.

CORRELATION OF WING SHAPE BETWEEN COMIMICS

Detailed behavioral studies of *H. numata* are lacking, but Brown and Benson (1974) noted differences between morphs in adaptation to insectaries: their *silvana* individuals generally adapted poorly and fluttered against the roof, whereas most of the individuals of the other morph of their study, *superioris*, adapted more readily; courting, feeding, and laying eggs normally. However, those differences were not quantified and could not be replicated in insectaries with our broods. Also, no differences in flight height have been observed in the field (Joron 2005), suggesting that different mimetic morphs of *H. numata* are found in similar microhabitats. The wing shape differences reported here should therefore not relate directly to variation in microhabitat use via behavioral specialization. Instead, they appear to relate to mimetic adaptation because the quantitative differences we found between morphs are paralleled in the corresponding comimic species. Fourier-based techniques have been used elsewhere to characterize the shape of wings and features in a variety of taxa (Holwell and Herberstein 2010; Rohlf and Archie 1984), and to find evidence for morphological convergence in some groups (Ge et al. 2011). Application of Fourier outline analysis in this study suggests that the subtle variations of wing shape in *H. numata* are of adaptive

significance, and may have been selected because resemblance involves not just color patterning but also flight characteristics.

ECOLOGICAL SIGNIFICANCE

Chemically defended species will often fly slowly and in straight lines, so as to display their bright warning patterns. Batesian mimics, which are not chemically defended, are also known to fly slowly and deliberately, so accurately resemble their model species in both their display patterns and locomotory behavior, but retain the wide thoraces and powerful flight muscles from their ancestry, allowing them to adopt more rapid and erratic flight behaviors when pursued by a predator (Chai and Srygley 1990; Srygley 1994, 2004). Srygley and Ellington (1999a) described a more subtle aspect of mimetic flight behavior in Müllerian mimic species: both wing-beat frequency and wing motion symmetry could be used to discriminate the comimics *H. cydno* and *H. sapho* from a second comimicry group, *H. melpomene* and *H. erato*. Although convergence on similar wing-beat frequencies may be related to requirements of aerodynamic force, variation from nearly sinusoidal to more asymmetrical wing motions is expected to have little effect on lift production. As a result, convergence of this character is more likely to have arisen from direct selection on signaling behavior by aerial predators. Furthermore, unlike wing-beat frequency, wing motion symmetry does not shift from cruising flight to courtship or the search for a host plant: it is unconstrained by flight behavior and wing kinematics (Srygley 2007), so serves as a reliable signal that can be generalized across ecological contexts. Together with wing beat frequency it may allow predators to identify mimics before they can see details of the wing pattern. We hypothesize that the differences we describe in *H. numata* wings may be translated into the kind of subtle differences in motion that have been observed in *H. melpomene* and *H. erato*.

Studies of *Heliconius* and other butterflies have previously demonstrated that locomotor mimicry may be achieved by kinematic (Srygley 1999; Srygley and Ellington 1999a), aerodynamic (Srygley and Ellington 1999b), and morphological effects (Srygley 1994). However, Srygley (1994) found no evidence of convergence of wing shape between comimics, and suggested that this may be due to (i) conflicting selective forces operating on the wing; (ii) the evolution of dark apical margins contrasting against the bright colors of the wing, which can create false outlines to the wing that closely resemble those of the model species; (iii) a lack of sufficient selection pressure; or (iv) relatively imprecise measurements of wing shape. In this study we have used a number of precise techniques to make various measurements of wing shape. Although we have not found evidence of significant differences between most of the morphs of *H. numata*, we find clustering of these morphs among those of their respective comimics. In the case of *H. numata* f. *silvana*, wing shape is quantitatively different

and appears to be associated with parallel changes in the comimic of this form.

The correspondence in wing shape between comimics is suggestive of an adaptive significance to the variation measured, but precisely how selection acts on wing shape is not well understood. As mentioned earlier, Srygley (1999) demonstrated convergence in flight kinematics between distantly related members of two mimicry rings, presumably owing to mimicry selection. However, he could not distinguish between selection acting on flight kinematics directly and that acting on wing shape and pattern. Mimicry often involves resemblance even to minute details of wing patterns, and slight variations in a given pattern theme are usually mirrored by all comimetic species, as is seen for instance in the quantitative variations across broad geographical regions (Clarke and Sheppard 1960). The eyes of avian predators are known to have faster flicker-fusion rates than the eyes of humans, allowing them to see details of pattern in motion that would not be clearly seen by human observers (Kare 1965). It is therefore possible that shape itself could directly be under selection, either as a morphological character or through its effect on the perception of the color pattern. Alternatively, there could be selection on wing shape through its effects on flight performance, in which variations in shape parameters directly translate into distinct kinematic patterns (Betts and Wootton 1988), which may be perceived as identification cues by predators. For example, variations in centroid position can render flight more or less “fluttery” because of the constraints that wing shape impose on wing movement and air motion. We cannot at this stage distinguish between the two possibilities, but kinematic studies of the butterflies examined here would be an interesting follow-up to the shape analyses that we have presented. A study focusing on the differences between male and female flight might also be rewarding, and provide insight into any effect on flight performance caused by differences in forewing–hindwing overlap due to the presence of androconia.

GENETIC CONTROL OF WING SHAPE

Our study also raises the formal possibility that the *P* supergene of *H. numata* controls differences in wing shape between morphs, just as it is responsible for controlling considerable differences in wing color patterning (Joron et al. 2006). The association of genotypes at the supergene with the subtle shape variation we have captured does indeed indicate some level of involvement of *P* in the control of shape. This prospect is intriguing as it would parallel a system found in *Papilio memnon*, a Batesian mimetic species of swallowtail butterfly that offers perhaps the most striking example of within-species variation in wing shape. The hindwing “tails” of *P. memnon* contribute to the mimetic resemblance of these palatable species to their distasteful models, with tailed and tail-less forms coexisting in most populations. Early experimental crosses

between different races of *P. memnon* characterized the genetic basis of these traits and showed that a supergene is responsible for controlling both color patterning and tail presence (Clarke et al. 1968; Clarke and Sheppard 1971). In the African swallowtail butterfly *Papilio dardanus*, color patterning and tail length are controlled at unlinked loci (Clarke and Sheppard 1959, 1962), and genes with specific modifier effects have been described in *Papilio polytes* (Clarke and Sheppard 1972).

The genetic architecture controlling wing shape is complex. Studies with *Drosophila* have shown that many different genes may contribute to shape variation, and that variation in shape is relatively independent of wing size (Weber 1990; Weber et al. 2005; Carreira et al. 2011). Several of the factors that have effects on wing shape are associated with cell communication, cell-cycle control, motility, and adhesion, and include genes that have prominent developmental roles outside the wing, such as the transcription factors *invected*, *engrailed*, and *scalloped*, as well as *wingless*, *decapentaplegic*, and *vestigial* (Hidalgo 1994; Palsson and Gibson 2000; Weber et al. 2005; Baena-Lopez and Garcia-Bellido 2006; Carreira et al. 2011). The association between shape and color pattern that we have described in this study suggests that in *H. numata* the *P* genotype has a clear influence on wing morphology. Future genetic mapping with experimental broods may confirm this, and allow identification of quantitative trait loci within the supergene region. It is interesting to speculate that a developmental gene could be involved in both the control of pattern and shape in the wings of *H. numata*. For example, a recent study associated the control of black pattern elements in the wings of *H. erato* and *H. cydno* with *WntA*, a member of the *Wnt*-family of signaling ligands (Martin et al. 2012). *Wingless* has previously been identified as a morphogen of this family, and is known to contribute to wing shape in *Drosophila* and to have important effects in other aspects of development (Hidalgo 1998; Fabre et al. 2010). Neither the *WntA* or *wingless* gene is found within the *P* region, but other genes with predicted developmental effects are emerging. It should be noted here that although the *P* region has been sequenced in *H. numata* (Joron et al. 2011) and its homologous region has been described in a number of different races of *H. melpomene* (Ferguson et al. 2010; Nadeau et al. 2012; The Heliconius Genome Consortium 2012), those genes that functionally determine pattern differences in these species have not been formally identified among the multiple genes annotated in this genomic region, raising the exciting possibility that candidate genes for shape determination are yet to be discovered here. Finally, it is also worth noting that although the differences in wing shape that we have described are generally greater between sexes than between morphs, we found no sex-by-morph interaction, suggesting that *P* is not implicated in the control of wing shape determination that is relevant to sexual dimorphism.

Conclusion

The narrow wings of the heliconines and ithomines share very similar overall morphologies. However, they differ considerably from their putative sister taxa and the primitive nymphalids, which suggests that their strong similarities are derived in each lineage, in response to signaling and mimicry (Erich 1958; Strauss 1990). Through quantitative techniques we have shown that wing shapes differ between different morphs of *H. numata* and follow the trends seen in their comimetic species. Our findings therefore suggest that this convergence reflects not just the adaptation of two lineages to similar ecologies but movement toward adaptive peaks created by the selection pressures applied by avian predators. This implies that there is sufficient plasticity in wing form, within the functional constraints required for efficient flight, to overcome phylogenetic differences, just as has been suggested for variation in wing movements (Srygley 2007). Precisely how these phenotypic effects are translated into differences in flight performance and behavior, and therefore come under selective pressure by predatory species, is an interesting question for the future. Such analyses will require careful and sophisticated measurement; as noted elsewhere (Srygley and Ellington 1999a), some aspects of wing motion associated with mimetic resemblance are themselves very subtle and may not be visible to the naked human eye.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Column graphs of mean measures for experimental brood.

Figure S2. Analysis of wing shape variation captured by landmark analysis of all individuals in experimental brood.

Figure S3. Outline analysis of *Heliconius numata* morphs and *Melinaea* species collected in Peru.

Figure S4. Outline analysis of *Heliconius numata* morphs and *Melinaea* species.

Table S1. Full two-way analysis of variance results of wing parameters, including morph, sex, and sex-by-morph interaction.

Table S2. Summary *t*-test results of wing parameters of *Melinaea* species.

Table S3. Outline analysis of wild-caught *Heliconius numata* and *Melinaea* from Peru.

Table S4. Linear discriminant analysis using *Heliconius numata* wing outline PCs as a training set, to which the Peruvian *Melinaea ludovica ludovica* wings were introduced.