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Morpho morphometrics: Shared ancestry and selection drive the evolution of wing size and shape in *Morpho* butterflies

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Butterfly wings harbor highly diverse phenotypes and are involved in many functions. Wing size and shape result from interactions between adaptive processes, phylogenetic history, and developmental constraints, which are complex to disentangle. Here, we focus on the genus *Morpho* (Nymphalidae: Satyrinae, 30 species), which presents a high diversity of sizes, shapes, and color patterns. First, we generate a comprehensive molecular phylogeny of these 30 species. Next, using 911 collection specimens, we quantify the variation of wing size and shape across species, to assess the importance of shared ancestry, microhabitat use, and sexual selection in the evolution of the wings. While accounting for phylogenetic and allometric effects, we detect a significant difference in wing shape but not size among microhabitats. Fore and hindwings covary at the individual and species levels, and the covariation differs among microhabitats. However, the microhabitat structure in covariation disappears when phylogenetic relationships are taken into account. Our results demonstrate that microhabitat has driven wing shape evolution, although it has not strongly affected forewing and hindwing integration. We also found that sexual dimorphism of forewing shape and color pattern are coupled, suggesting a common selective force.

KEY WORDS: Microhabitat, *Morpho*, morphometrics, phylogeny, sexual dimorphism, wing morphology.

Understanding the factors that drive phenotypic diversity is central to evolutionary biology. Three such factors have been proposed, depending on the level at which phenotypic diversity is examined (e.g., Breuker et al. 2006): functional factors, either in functional morphology studies or in population biology, when selective aspects (i.e., adaptation) are considered; historical factors, in macroevolutionary comparative studies, to assess the relative contributions of shared ancestry and selection to phenotypic evolution; and developmental factors, in evo-devo studies that mostly attempt to provide a proximal (developmental) explanation of the phenotypic diversity. Integrative approaches are needed to combine these perspectives and achieve a more comprehensive explanation of phenotypic evolution.

Several traits across model systems have received attention, such as the vertebrate skulls and teeth and the arthropod appendages. Insects' wings, with their stunning morphological diversity, are particularly interesting. They have been investigated at various scales and from various perspectives, including the evolution and development of color patterns in butterflies and *Drosophila* (e.g., Beldade and Brakefield 2002; Joron et al. 2011; Arnoult et al. 2013); wing development, mostly in *Drosophila* (e.g., de Celis 2003), but also and increasingly in other groups (e.g., Loehlin and Werren 2012; Xu et al. 2015); morpho-functional perspective on wing performances in relation to their structure (Dudley 2002; Park et al. 2010; Kovac et al. 2012), and also at macroevolutionary scales (e.g., Grimaldi and Engel 2005; Bai et al. 2012; Oliver et al. 2012).

The main function of butterflies'—and insects'—wings is obviously flight. Flight is involved in a large panel of behaviors,

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including foraging, courtship, predator escape, aerial dispersal, and patrolling behavior (Dudley 2002). Size and shape are obvious determinants of wing physical properties, affecting drag or lift, for example, and thus strongly influence flight behavior (Wootton 1992; Berwaerts et al. 2002; Dudley 2002). Habitat- or predator-driven selection acting on flight behaviors may therefore lead to ecologically adapted wing shapes and sizes. The different functions of fore and hindwings during flight may have led to different pattern of size and shape diversification (Klingenberg et al. 2001). Natural selection may also drive sexual dimorphism, when flight behavior differs between males and females of a single species.

Signals carried by butterfly wing colors and sizes are used in mate choice (Naisbit et al. 2001; Merrill et al. 2011) and in the determination of the quality of potential mating partners (Breuker and Brakefield 2002; Robertson and Monteiro 2005). Hence, sexual selection is also a potential driver of sexual dimorphism. Many species exhibit male-biased sex ratio, which suggests the existence of an intense male mate competition (Allen et al. 2011). The evolution of traits such as wing size, the intensity of coloration, or UV-reflective patches may be driven at least partly by sexual selection (Kemp 2007; Morehouse and Rutowski 2010).

Butterfly fore and hindwings originate from two separate imaginal discs that mostly grow during the last larval instar and the pupal stage. Although they are jointly influenced by the circulating hormones in the hemolymph (e.g., Klingenberg and Nijhout 1998), they are developmentally autonomous. Autonomy of wings might result in a limited amount of covariation among the adult structures. In other words, one can expect the two pairs of wings to constitute two developmental modules in which groups of traits evolve relatively independently from each other and from other modules (Breuker et al. 2006). It is also known that the two pairs of wings can play different roles in flight (Grodnitsky et al. 1994) possibly adding a degree of functional independence. On the other hand, the two wing imaginal discs are serially homologous structures and their developmental genetic bases overlap to a large extent (Carroll et al. 1994; Keys et al. 1999). In addition, the joint contribution to flight is expected to generate some degree of functional integration. Hence, assessing the integration of wings and the extent to which it relates to ecology is a fundamental question to understand the evolution of butterfly wings.

The neotropical genus *Morpho* (Nymphalidae: Satyriinae) comprises 30 species that typically harbor large iridescent blue wings. This genus is a remarkable example of phenotypic diversity in terms of wing color, size, and shape (Le Moult and Réal 1962, 1963; Blandin 1988, 1993, 2007a,b). Two major flight behaviors have been reported from field observations. Some species fly very high above ground, up to the canopy whereas others fly close to the ground within the first forest strata in the understorey (DeVries et al. 2010; Michael 1911). Previous publications emphasized that *Morpho* species probably diverged early in their history into these two microhabitats (DeVries et al. 2010; Penz et al. 2012). Using a subset of species, DeVries et al. (2010) suggested that microhabitat shift might have influenced wing shape evolution through changes in flight behavior. Such behavioral and ecological divergence during the history of the clade therefore offers an excellent opportunity to investigate the effect of habitat-driven natural selection on the evolution of wings. Observations of females in the field and presence in collections are very scarce compared to those of males suggesting either a strongly biased sex ratio or a strong divergence of behavior and/or microhabitat between sexes. Penz et al. (2015) found that in *Morpho achilles* males have higher individual dispersal rates than females. If behavioral differences that involve flight such as dispersal exist, they can potentially drive wing sexual dimorphism. Moreover, sexual color dimorphism is highly heterogeneous across the clade, from species exhibiting only slightly different wing color patterns between sexes to species harboring highly contrasted color patterns (Fig. 1). This strong dimorphism, in addition to vivid coloration of males in many species, suggests the existence of strong sexual selection, which could potentially contribute to wing size and shape variation among sexes.

In this study, we embrace a macroevolutionary perspective to identify the processes that have driven the diversification of wing size and shape in the genus *Morpho*, by assessing the relative role of multiple factors. Although the genus has received recent attention to resolve phylogenetic relationships (Penz and DeVries 2002; Cassildé et al. 2010, 2012; Penz et al. 2012; Blandin and Purser 2013), a completely resolved phylogenetic tree is still lacking. Here, we generate a comprehensive species-level molecular phylogeny. Then, we assess phenotypic diversity by measuring size and shape variations of both fore and hindwings of a large sample of individuals, including males and females from all species of the group. After checking whether wing size and shape exhibit phylogenetic signal, we address the following questions. (1) To which extent have the evolution of wing size and shape been driven by different microhabitat use? If different flight behaviors have been selected in each microhabitat, we expect size and shape to diverge between microhabitats and to match the morpho-functional requirements associated with different behaviors. Typically, we expect species flying in the canopy to be associated with elongated wings allowing sustained gliding flight, whereas species flying in the understorey should exhibit more rounded wings allowing more maneuverability (Dudley 2002). We further investigated the strength of microhabitat selection by testing whether fore and hindwings are integrated as the result of functional constraints driven by microhabitat use. If microhabitat use has selected particular combinations of fore and hindwings, we expect the covariation to be structured by microhabitat. (2) Are there sexual size and shape dimorphisms, and if so do they display

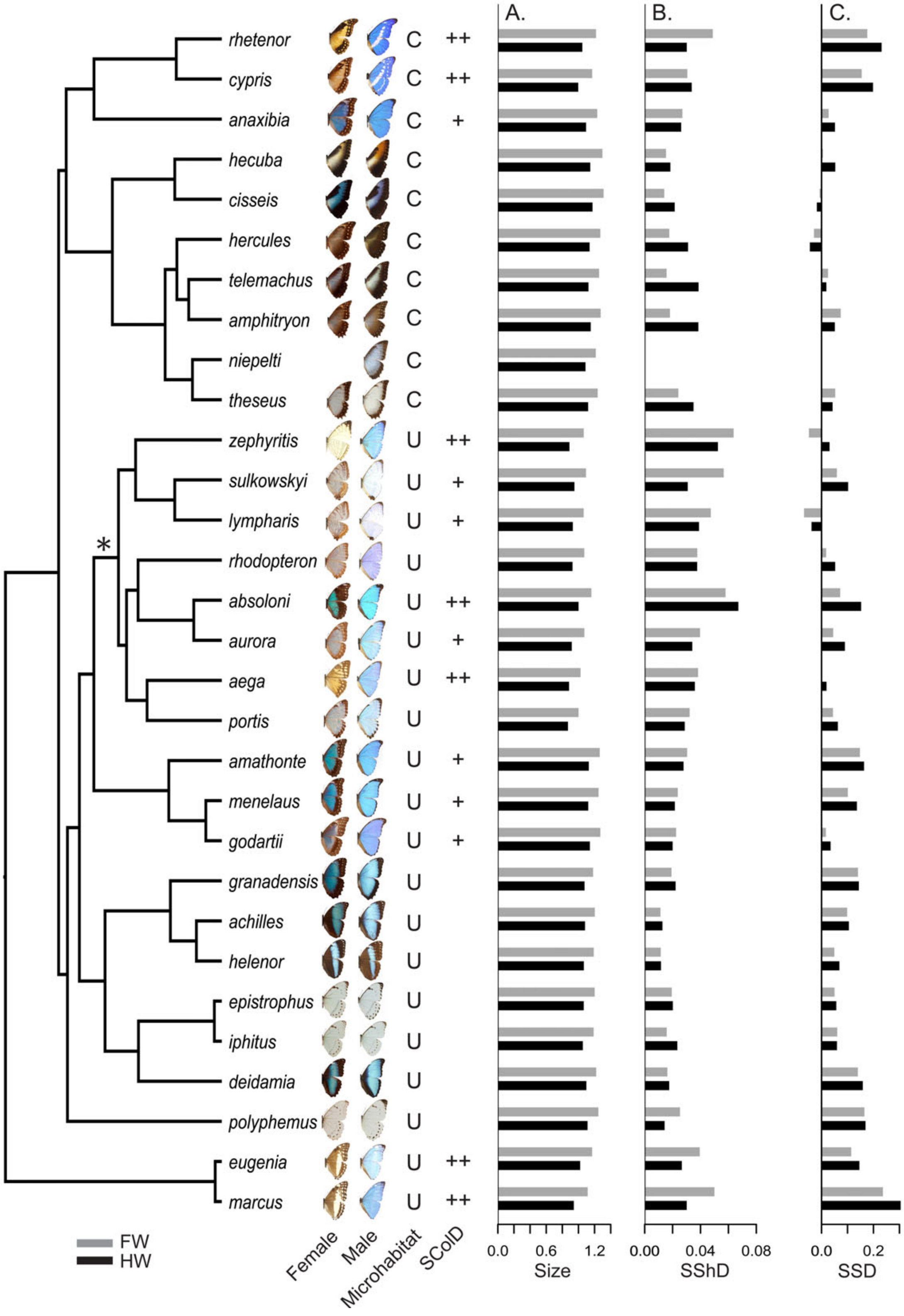

Figure 1. Molecular phylogeny of the genus *Morpho* showing wing size, sexual size dimorphism, and sexual shape dimorphism for each species. Species were classified into microhabitats (C, canopy; U, understorey) and sexual color dimorphism classes (SCoID strong = ++, intermediate = +). Histograms represent the logarithm of centroid size (A: *Size*), the intensity of sexual shape dimorphism (B: *SShD*), and the intensity of sexual size dimorphism (C: *SSD*). Forewing data are colored in gray, hindwing data in black. The star indicates the root of the *sulkowskyi* clade.

phylogenetic signal? Are size and shape dimorphisms associated with microhabitat, suggesting a difference in flight behavior between sexes? Finally, are size and shape dimorphisms correlated with color dimorphism? Color dimorphism may result from either natural or sexual selection, which may also act on wing size and shape, and we expect the different sexually dimorphic traits to be correlated.

Materials and Methods

PHYLOGENETIC TREE

To generate the species-level tree of the genus *Morpho*, we used one representative specimen for each of the 30 recognized species (according to Blandin and Purser 2013), and 5 outgroups (*Antirrhea philoctetes*, *Caerois chorinaeus*, *Caligo telamonius*, *Opsiphanes quiteria*, *Cithaerias pireta*). We selected 119 sequences from two previously published datasets (Cassildé et al. 2012; Penz et al. 2012) and complemented this dataset with 24 additional nuclear sequences (see Supporting Information S1). The final molecular matrix contained a concatenation of one mitochondrial fragment of cytochrome c oxidase subunit 1 (COI) and four nuclear gene fragments: carbamoylphosphate synthase domain protein (CAD), elongation factor 1 α (EFIα), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and malate dehydrogenase (MDH) for a total length of 5001 bp (Wahlberg and Wheat 2008).

To find the best partition and the models associated, we ran Partition Finder (Lanfear et al. 2012) allowing all possible partitions and models implemented in Beauti version 1.7.1 (Drummond et al. 2012). The best partition contained three subsets: the first included position 1 and 2 of all genes and followed a GTR+I+Γ model, the second included position 3 of all nuclear fragments and followed a GTR+Γ model, and the third included the position 3 of the mitochondrial fragment and followed a TrN+Γ model. We ran an MCMC chain of 30,000,000 generations using Beast version 1.7.1 (Drummond et al. 2012) under a Yule process and applying an uncorrelated lognormal relaxed clock. Finally, we used TreeAnnotator (Drummond et al. 2012) to select the Maximum Clade Credibility tree with median value from the posterior distribution of branch lengths, applying a 20% burnin (see Supporting Information S2). Outgroups were pruned and the resulting tree was used in all subsequent analyses. We also cross-validated the tree topology and branch support using RAxML (Stamatakis 2014) (tree not shown).

MORPHOMETRIC DATA

Six hundred forty-two set males and 269 set females (a total of 911 specimens) from the collection of the National Museum of Natural History of Paris (MNHN, France) were used in this study. This dataset includes the 30 recognized species of *Morpho,* with males and females for all species but *M. niepelti* for which no female was available. *Morpho niepelti* was therefore removed from sexual dimorphism analyses. Pictures of both ventral and dorsal views were taken using a NikonD90 camera. Because the fore and hindwings partly overlap in set specimens, we used the dorsal view of the right forewing and the ventral view of the left hindwing. When necessary we photographed the opposite wing and took the mirrored image before digitizing it. Landmarks and semi-landmarks were used to quantify wing size and shape (see Supporting Information S3). Landmarks were placed at vein intersections and vein termini, and semi-landmarks were used to describe margin shapes and lobes of the external margins. We did not place semi-landmarks on the rear margin of the hindwing because it is generally folded on collection specimens. All landmarks were digitized using TpsDig2 (Rohlf and Slice 2010). Before starting the analyses, we checked that the measurement error was negligible (see Supporting Information S3).

For both wings, landmarks and semi-landmarks configurations were superimposed using a generalized Procrustes superimposition (Rohlf and Slice 1990) with TPSrelw (Rohlf 2014). The sliding of the semi-landmarks was done by minimizing the Procrustes distances (e.g., Andresen et al. 2000; Sheets et al. 2004). The resulting coordinates in the tangent space were used as shape data, and the log of centroid size was used as a size estimate.

All morphometric and statistical analyses were conducted using MorphoJ (Klingenberg 2011) and R (R Development Core Team, 2014). In both cases we imported the Procrustes coordinates after TPSrelw alignment, thereafter treating all positions as regular landmarks.

Note that all dimensions were used in most analyses, as statistical tests were based on permutations. In the few cases where parametric tests were performed (i.e., MANOVA for sexual dimorphism), only the first 15 axes of a PCA run on the tangent coordinates were used as shape variables, after checking that at least 95% of the shape variance was accounted for. The two wings were analyzed separately in subsequent analyses.

PHYLOGENETIC SIGNAL IN WING SHAPE AND WING SIZE

We first performed a PCA on the Procrustes coordinates for both wings. The mean position of each species within the morphospace was calculated, and the phylogenetic tree projected into it using MorphoJ (Klingenberg 2011). We also visualized the shape transformation associated with the first PCs using multivariate regression of Procrustes coordinates on the eigen-vectors (e.g., Monteiro 1999). Phylogenetic signal in wing shape was measured and tested using the multivariate permutation test implemented in MorphoJ (Tree length, Klingenberg and Gidaszewski 2010).

We calculated and tested Blomberg's K statistic (Blomberg et al. 2003) to assess phylogenetic signal in wing size using the package *picante*.

EFFECT OF MICROHABITAT ON WING EVOLUTION

Each species was assigned to a microhabitat—canopy or understorey—based on the literature (DeVries et al. 2010; Michael 1911) and field observations. We investigated how microhabitat affects the distribution of individuals and species across the morphospace, that is, whether wing shape differs according to ecological conditions. We performed a Procrustes ANOVA implemented in the R package *geomorph* (Adams and Otarola-Castillo 2013; Anderson 2001; Anderson and Braak 2003), on all individuals and including sex and microhabitat as factors. Because the interaction between sex and microhabitat was significant, we ran the subsequent analyses separately on the two sexes. For wing size, we used the phylogenetic ANOVA as implemented in the package *phytools* (Garland et al. 1993; Revell 2012) to investigate the effect of microhabitat independently of the phylogeny. For shape, we first computed the species means for each sex and then re-aligned them using the package *geomorph*. We then performed a phylogenetic Procrustes ANOVA for each sex (Adams 2014), considering microhabitat as a main factor and mean centroid size as a covariate, to account for an interspecific allometric effect.

COVARIATION BETWEEN FORE AND HINDWINGS

The covariation between fore and hindwings was estimated by the RV coefficient (a multivariate analog of the squared Pearson correlation coefficient) (Escoufier 1973; Klingenberg 2009) followed by a two-block partial-least square (PLS) regression when the RV was significantly different from zero. We followed a three-step analysis. First, we tested the covariation between forewing and hindwing shapes across all individuals. To account for a potential allometric effect, this test was performed on the residuals of a multivariate pooled within-species regression of shape on size, including all individuals. This represents the general covariation between the two wings controlling for allometric effects, regardless of species identity. Second, we tested the covariation across species controlling for allometric effects, using the residuals of a multivariate regression of species mean shapes on species mean size. Finally, we performed the covariation analysis on phylogenetic independent contrasts computed from the residuals of a pooled within-species regression, to investigate the residual covariation after correcting for phylogenetic correlations and allometry (Klingenberg and Marugan-Lobon 2013). In each case, we further investigated how covariation was structured by microhabitat.

We estimated the covariation between forewing and hindwing size using a linear regression between the log of centroid size of fore and hindwings instead of PLS, for all individuals, for species means, and for independent contrasts.

SEXUAL DIMORPHISM

We tested whether sexual dimorphism varied across species as follows. For sexual size dimorphism (SSD), we performed a two-factor ANOVA (species and sex). For sexual shape dimorphism (SShD), we ran a MANOVA including both factors (species and sex), and wing size as a covariate to test for a global effect of allometry on shape dimorphism. We used the 15 first PCA axes in the MANOVA, which accounted for 95% of the variation. Because the interaction species × sex × size was not significant, we further investigated SShD without taking into account wing size.

We then assessed sexual dimorphism for each species using three descriptors, two for shape and one for size. The vector connecting the average male and female configurations in the shape space was used as a measure of SShD. The length of this vector, measured in units of Procrustes distance, was used as an estimate of the strength of dimorphism. The orientation of the vector was used as a description of the corresponding shape change between sexes, and the angle among vectors of pairs of species was used to quantify the variation of shape dimorphism across species. The intensity of wing SSD was measured as the difference between mean female and male centroid sizes. We tested for a phylogenetic signal in the intensity of SShD and SSD using Blomberg's K statistic (Blomberg et al. 2003). We tested for a phylogenetic signal in the direction of shape dimorphism by computing and testing the RV coefficient between phylogenetic distances and pairwise angles between dimorphism vectors of species.

To test whether sexual dimorphism differed among microhabitats independently from the phylogeny, we performed a phylogenetic ANOVA (Harmon et al. 2008) on SShD or SSD and the microhabitat use.

Finally, species were sorted into categories of intensity of sexual color dimorphism (weak, intermediate, and strong, see Supporting Information S5). Phylogenetic ANOVA was used to test whether SShD or SSD was different depending on the intensity of color dimorphism.

Results

The phylogeny of *Morpho* was comprehensive and almost fully resolved (Supporting Information S2). Only the position of *M. rhodopteron* remained uncertain.

PHYLOGENETIC SIGNAL AND EFFECT OF MICROHABITAT

We detected a significant phylogenetic signal for both fore and hindwings, in both size (forewing: $K = 1.20$, $P < 0.001$; hindwing: $K = 1.08$, $P < 0.001$) and shape (forewing: Tree length $= 0.0135$, $P < 0.001$; hindwing: Tree length $= 0.0156$, $P < 0.001$).

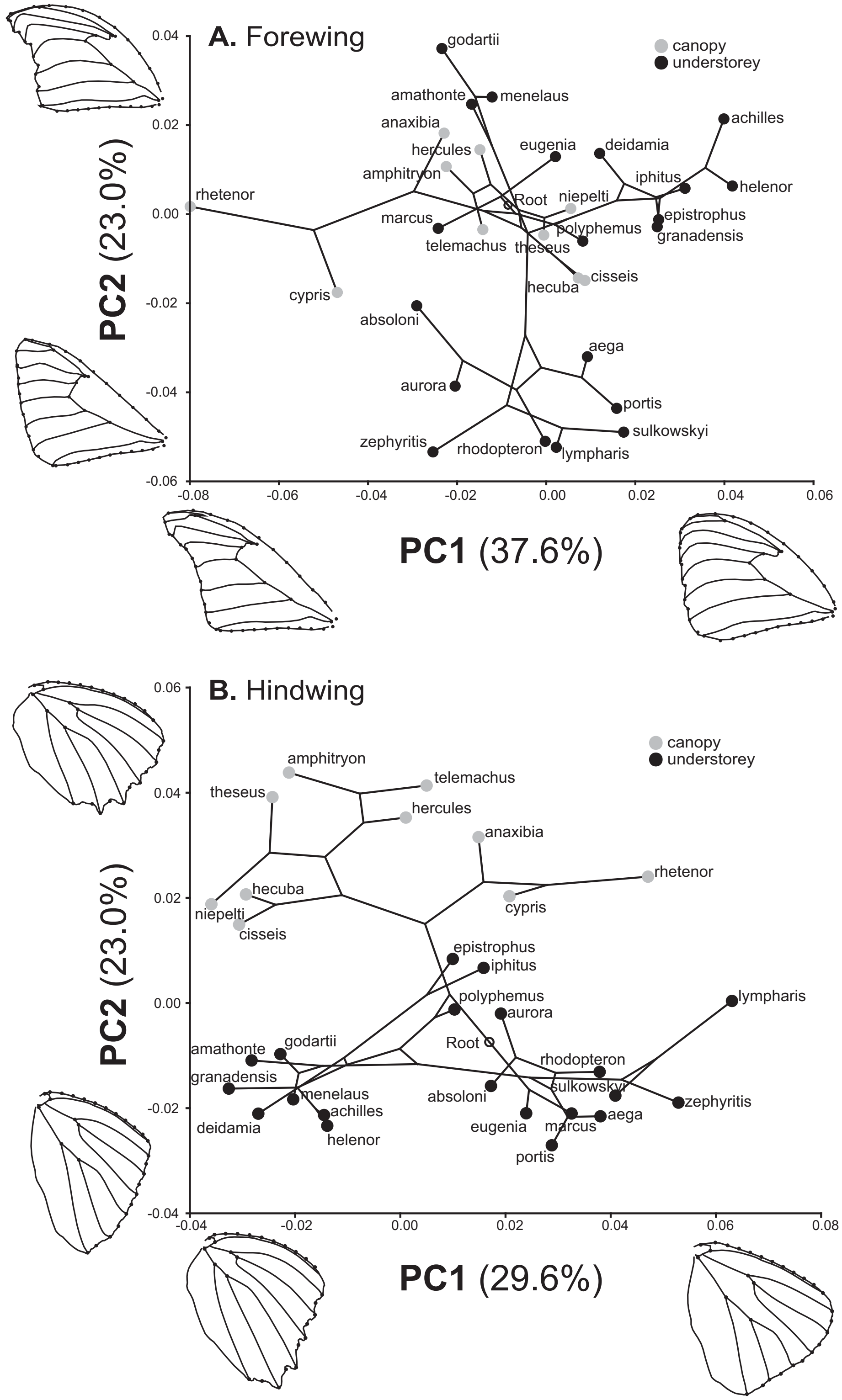

Figure 2. Principal component analysis of Procrustes coordinates (A: forewing, B: hindwing). The first two PC and the associated shape changes are shown. Only species means are displayed. The phylogenetic tree is projected into the morphospace. Gray and black points represent canopy and understorey species, respectively.

Table 1. ANOVA and Procrustes ANOVA on individual wing size and wing shape, respectively, with sex and microhabitat as factors.

Wing	Variable	Factor	F	P
FW	Shape	Sex	40.1813	0.001
		Microhabitat	136.3890	0.001
		Sex × microhabitat	7.9502	0.001
	Size	Sex	46.395	0.001
		Microhabitat	268.996	0.001
		Sex × microhabitat	5.955	0.0149
HW	Shape	Sex	18.833	0.001
		Microhabitat	181.748	0.001
		Sex × microhabitat	14.068	0.001
	Size	Sex	57.23	0.001
		Microhabitat	183.71	0.001
		Sex × microhabitat	11.27	0.0008

FW, forewing; HW, hindwing.

For forewings, the projection of the phylogenetic tree into the morphospace clearly showed that close relatives tended to cluster in the shape space (Fig. 2). The second axis appeared to be driven by a shift of the entire *sulkowskyi* clade. However, some species such as *M. rhetenor* and *M. cypris* strongly diverged from their close relatives. The first two axes did not clearly separate the species belonging to the two microhabitats. The first axis described a variation from a rounded wing to an elongated wing toward the apex. The second axis described a variation from a triangular wing to a more curved wing (Fig. 2).

For hindwings, the first PC plan appeared strongly influenced by the phylogeny, as closely related species tended to cluster (Fig. 2). Microhabitat seemed to mainly structure PC2, the entire canopy clade being clearly isolated toward positive values, the understorey clade toward negative values. *Morpho marcus* and *M. eugenia*, two understorey species that diverged from the rest of the clade before microhabitat shift, clustered with the other understorey species. The two axes described a variation from narrow elongated wings to larger but shorter wings (Fig. 2).

The ANOVA and Procrustes ANOVA testing the effects of sex and microhabitat on all individuals were significant for both size and shape, respectively, including the interaction sex × microhabitat (Table 1). Hence, we further investigated the effect of microhabitat independently on males and females.

For both wings and both sexes, the effect of microhabitat on wing size was not significant in the phylogenetic ANOVA (Table 2). For shape, habitat and size effects were significant for both wings in the Procrustes phylogenetic ANOVAs for both sexes (Table 2). However, the interaction term was not significant, meaning that the microhabitat effect on wing shape was independent from the size.

COVARIATION BETWEEN FORE AND HINDWINGS

Covariation between forewing and hindwing shape controlling for allometric effects was significant across all individuals (RV coefficient $= 0.3671$, $P < 0.0001$, Fig. 3). The first PLS axis (63.7% of covariance explained) clearly showed an effect of microhabitat. Specifically, a typical combination of forewing and hindwing shapes was associated with each microhabitat. All canopy individuals appeared in the top right corner of the graph with a very elongated triangular forewing associated with large rounded hindwing (Fig. 3). Understorey individuals were placed in the bottom left corner, with a short rounded forewing associated with narrow elongated hindwing. When measured on the species mean, the covariation remained significant (RV coefficient $= 0.4639$, $P < 0.0001$, Fig. 3) and the first PLS axis (63.4% of covariance explained) remained strongly driven by microhabitat divergence. The significant covariation measured on the independent contrasts shows that even when phylogenetic signal is taken into account, wing shapes still significantly covary (RV coefficient $= 0.4226$, $P < 0.0001$, 55.4% of covariance explained by the first axis, Fig. 3). However, none of the axes of covariation showed an association with microhabitat. This result suggests that the shape covariation associated with microhabitat detected in phylogenetically uncorrected analyses is largely due to shared ancestry.

Concerning size, a strong covariation between fore and hindwings was also significant among individuals ($R^2 = 0.95$, $P < 0.001$), species ($R^2 = 0.97$, $P < 0.001$), and independent contrasts ($R^2 = 0.95$, $P < 0.001$) without particular microhabitat structure in all three cases (see Supporting Information S4).

SEXUAL DIMORPHISM

Both species and sex effects were found significant for both wings in the ANOVAs and MANOVAs, showing that species differ in their size and shape, as do sexes (Table 3). The significant species × sex interaction for size and shape indicates that sexual dimorphism of both size and shape varies across species. However, the interaction size × sex × species was not significant. Hence, we did not further consider size as a covariate in the study of SShD.

For both fore and hindwings, phylogenetic signal in SSD was not significant (forewing: $K = 0.47$, $P = 0.130$; hindwing: $K = 0.40$, $P = 0.288$). By contrast, significant phylogenetic signal was detected for the intensity (forewing: $K = 0.99$, $P < 0.001$; hindwing: $K = 0.64$, $P = 0.003$) and the direction (forewing: RV $= 0.34$, $P = 0.002$; hindwing: RV $= 0.33$, $P = 0.002$) of SShD in both wings, suggesting that closely related species tend to present similar SShD. When accounting for this phylogenetic effect, no significant association was found between microhabitat and SShD or SSD for either wing (Table 4). By contrast, there was a significant association between SShD and sexual

Table 2. Phylogenetic ANOVAs on wing shape and on wing size, with microhabitat (and size, when applicable) as factor.

			Males		Females	
Wing	Variable	Factor	*F*	*P*	*F*	*P*
FW	Shape	Size	4.0748	**0.038**	6.1756	**0.001**
		Microhabitat	1.5092	**0.001**	1.5000	**0.001**
		Size × microhabitat	0.9464	0.339	0.8030	0.360
	Size	Microhabitat	14.8437	0.092	8.668	0.169
HW	Shape	Size	5.0963	**0.053**	6.1931	**0.001**
		Microhabitat	1.5344	**0.001**	1.1984	**0.001**
		Size × microhabitat	0.5036	0.823	1.2184	0.160
	Size	Microhabitat	10.917	0.150	5.929	0.262

The ANOVAs were performed considering sexes separately, using the species mean size and shape.
FW, forewing; HW, hindwing.

color dimorphism for the forewing but not for the hindwing (Table 4). Species with a strong sexual color dimorphism had higher forewing SShD whereas weak sexual color dimorphism was associated with weak forewing SShD. SSD was never found to be significantly correlated with color dimorphism (Table 4).

Discussion

WING MORPHOLOGY AND MICROHABITAT

Wings are involved in many functions including flight, courtship, and predator escape that impose many selective pressures on wing size and shape. Here, we focused on another ecological driver of wing evolution, microhabitat. In *Morpho* butterflies during some periods of the day, males of some species patrol high above the ground and the canopy (Michael 1911; DeVries et al. 2010). Conversely, other species mainly fly in the understorey and are usually observed patrolling in the first forest strata. Females tend to fly at similar heights as conspecific males, although they do not patrol as males do. The two microhabitats are characterized by different conditions. Canopy is more open and brighter than the understorey. Moreover, the community of predators can also vary across forest strata. Flight behavior is therefore expected to differ between canopy and understorey. For instance, the patrolling behavior of males in the canopy, even restricted in time, is often associated with straight gliding flight (Welling 1966). Consequently, wing morphology is expected to differ between microhabitats. In agreement with this expectation, we show that wing morphology is more different among microhabitats than predicted under neutral evolution. Using a comprehensive dataset, morphometric measurements on both fore and hindwings, and a well-supported phylogeny, we provide evidence that the evolution of wing shape has been driven by microhabitat use.

There is a large body of literature, either theoretical or empirical, that assesses the relationships between wing shape, flight performance, and ecology. Several studies focusing on mathematical models or living organisms inspired models have studied the shapes of wings from a functional point of view, that is, by estimating the relationship between the shape of wings and flight performances (e.g., Thomas 1996; Berwaerts et al. 2002; Park et al. 2010; Kovac et al. 2012). They notably attempted to understand the shape parameters associated with gliding flight behavior. Using butterfly models, Kovac et al. (2012) showed that shapes that maximize wingspan increase gliding performances and that the relative orientation of fore and hindwings, which changes the overall planform, also plays an important role in optimizing gliding performances (see also Wootton 1992; Lentink et al. 2007). Empirically, elongated wings have often been associated with behaviors requiring low cost flight strategies such as migration. For example, in shorebirds (Minias et al. 2015) found a negative relationship between migration distance and wing roundedness, a result also consistent with passerines (Marchetti et al. 1995; Outlaw 2011). On the opposite, shorter rounded wings generally enhance maneuverability during flight and are often associated with greater predator escape ability (Norberg and Rayner 1987; Betts and Wootton 1988; Srygley and Chai 1990; Fernández et al. 2007). For example, Srygley and Chai (1990) showed a relationship between predation escape and wing shape on 54 neotropical butterfly species. They found that butterflies with shorter and smaller wings tend to fly faster and more erratically and frequently escape from predators. By contrast, butterflies with longer wings and larger wing areas flew more slowly and were captured more easily by predators when chased (see also Svensson and Friberg 2007).

In *Morpho* butterflies, we found that species flying in the canopy exhibit fore and hindwings elongated toward the apex (increased wingspan) whereas understorey species exhibit combinations of more narrow rounded fore and hindwings. These shapes and their associations with microhabitat use are clearly congruent

Table 3. ANOVA and MANOVA on individual wing size and shape, respectively, to test for the effect of species, sex, and, when applicable, size.

Wing	Variable	Factor	*F*	*P*
FW	Shape	Size	442.64	**<0.001**
		Sex	113.11	**<0.001**
		Species	25.14	**<0.001**
		Size × sex	11.56	**<0.001**
		Size × species	2.11	**<0.001**
		Sex × species	2.19	**<0.001**
		Size × sex × species	1.04	0.303
	Size	Species	198.992	**<0.001**
		Sex	199.952	**<0.001**
		Species × sex	5.746	**<0.001**
HW	Shape	Size	964.49	**<0.001**
		Sex	181.47	**<0.001**
		Species	26.26	**<0.001**
		Size × sex	8.43	**<0.001**
		Size × species	2.13	**<0.001**
		Sex × species	2.52	**<0.001**
		Size × sex × species	1.10	0.088
	Size	Species	209.354	**<0.001**
		Sex	297.375	**<0.001**
		Species × sex	6.925	**<0.001**

FW, forewing; HW, hindwing.

with the theoretical expectations and the published observations contrasting elongated wings for gliding flight to short rounded wings for maneuverability (Dudley 2002). In the understorey, shorter rounded wings may allow an increased maneuverability and take-off speed (Lockwood et al. 1998) in a more irregular and closed environment. Canopy species, which tend to exhibit long sequences of gliding flight, are associated with wing shapes generally considered as enhancing low cost flight.

We did not find any significant difference of wing size among microhabitats. Canopy species are all large and an increased total wing surface is probably involved in optimizing low cost flight behavior. By contrast, understorey species comprise a very wide range of wing size, from small *sulkowskyi*-clade species to large *amathonte*-clade species, which probably explains the absence of significant difference in wing size.

Our results are congruent with the conclusion of DeVries et al. (2010), who studied the forewings of a subset of *Morpho* species and detected a significant change of shape associated with a microhabitat shift in males, but not in females. The significant interaction between microhabitat and sex we found here implies that the effect of ecology differs between males and females. Despite this difference, we show that female wings are also affected by microhabitat use. This difference between our results and those of DeVries et al. (2010) may be due to our larger sample size: DeVries et al. (2010) only used 11 species and one female per species, whereas our dataset includes 269 females for 29 species, which have likely granted better statistical power. Whether this signal on females results from a selection by the microhabitat or a genetic correlation with male's evolution cannot be elucidated for now. The coarse microhabitat classification we used in this article, which follows that of DeVries et al. (2010), likely hides a larger diversity of flight behaviors and morphological diversity among sexes and species. For example, the understorey *M. marcus* and *M. eugenia*, which diverged early from the rest of the *Morpho*, tend to fly higher than the other understorey species. In the canopy clade, species such as *M. hecuba* use gliding interspersed with flapping flight whereas *M. rhetenor* only uses flapping flight. Further quantitative estimations of flight behavior as well as microhabitat characterization are needed to clearly refine the link between wing shape and flight performance.

FORE AND HINDWINGS: A FUNCTIONAL INTEGRATION?

In butterflies, flight is mostly driven by forewings (anteromotorism, Dudley 2002; Jantzen and Eisner 2008). Jantzen and Eisner (2008) showed that even when hindwings were removed, butterflies were still able to fly. However, their results suggested that hindwings were essential to increase flight speed and maneuverability. Functional differentiation between fore and hindwings has been reported in butterflies and damselflies (Grodnitsky et al. 1994; Outomuro et al. 2012) where different functions may have led to different evolutionary trajectories. For *Morpho* butterflies, DeVries et al. (2010) focused only on forewings and they measured the aspect ratio. Here, using morphometric tools, we measured both forewing and hindwing shapes and found that both wings were similarly affected by microhabitat, even though they are differently involved in flight. Furthermore, we also assessed the degree of integration of fore and hindwings and tested whether microhabitat affected not only wing shape and size, but also the way forewing and hindwing covary. Although a full independence of evolutionary patterns of the two wings would suggest complete modularity, a correlated evolution rather suggests that developmental and functional links are predominant and that the two wings should be considered integrated. Here, we find a strong covariation between fore and hindwings at the individual level, at the species level, and on phylogenetic independent contrasts. These results suggest that the wings are integrated rather than modular. Moreover, the covariation is structured by microhabitat meaning that individuals/species from different microhabitats exhibit different combinations of fore and hindwings. However, this pattern disappears when phylogeny is controlled for. If covariation is the result of functional constraints driven by microhabitat, it cannot be distinguished from a neutral phylogenetic signal. Other functional and developmental effects,

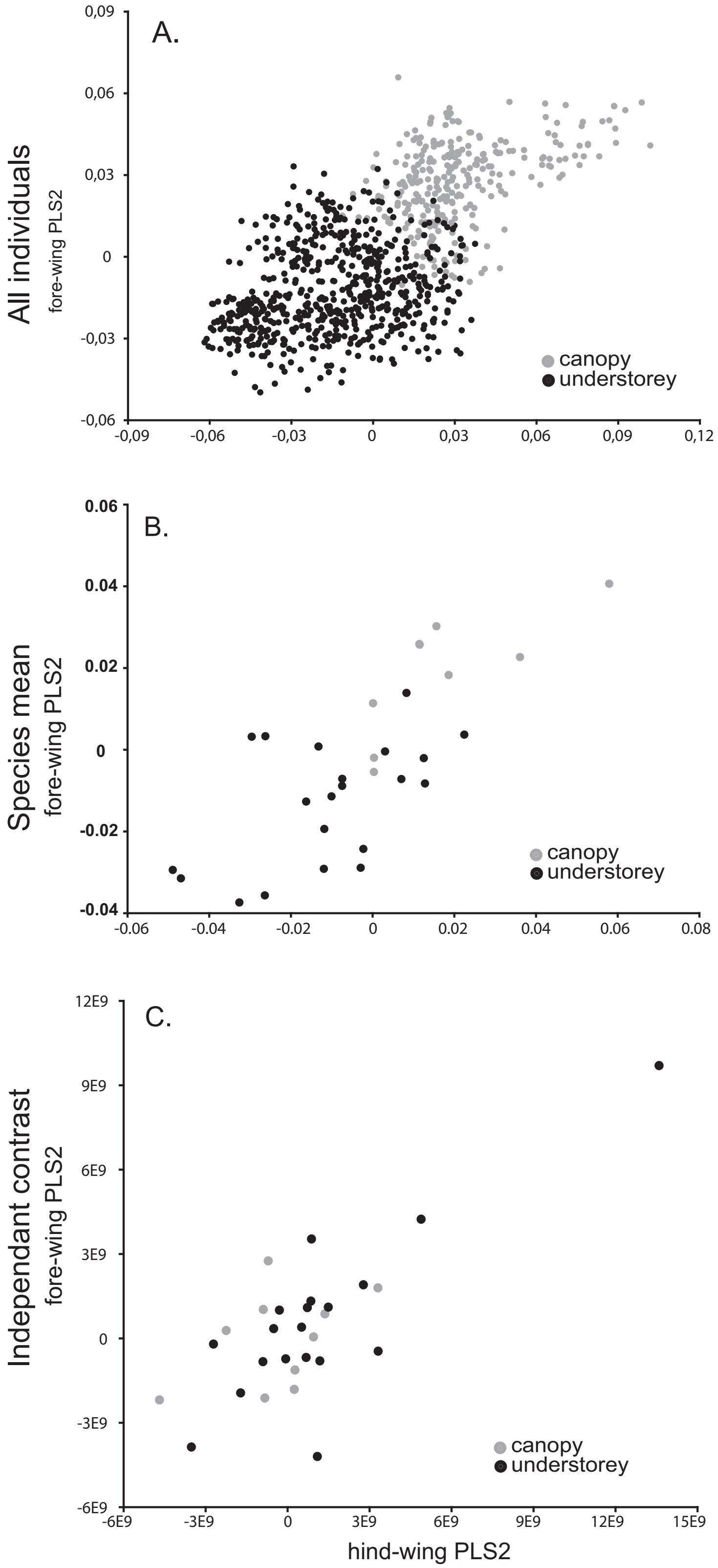

Figure 3. First PLS axis on Procrustes coordinates showing covariation between fore and hindwings after accounting for allometry. Gray and black points represent canopy and understorey species, respectively. The upper plot is based on all individuals, the middle plot on species means, and the lower plot on phylogenetic independent contrasts.

Table 4. Phylogenetic ANOVA, testing the relationship between microhabitat or sexual color dimorphism (three classes) and sexual shape or size dimorphism. *Canopy* and *understorey dimorph* are the mean SSD or SShD in each microhabitat. *Strong*, *subtle*, and *intermediate dimorph* are the mean SSD or SShD of each class of sexual color dimorphism.

	Wing	Variable	*F*	*P*	Canopy dimorph	Understorey dimorph	Strong dimorph	Subtle dimorph	Intermediate dimorph
Microhabitat	FW	Shape	2.53	0.457	0.024	0.033			
		Size	0.42	0.765	0.055	0.074			
	HW	Shape	0.11	0.88	0.031	0.029			
		Size	1.3	0.576	0.066	0.104			
Color dimorphism	FW	Shape	19.27	**0.002**			0.048	0.020	0.036
		Size	1.04	0.645			0.101	0.061	0.050
	HW	Shape	4.71	0.121			0.040	0.025	0.029
		Size	3.61	0.226			0.157	0.067	0.080

FW, forewing; HW, hindwing.

such as joint developmental pathways or wing coordination for flight, may explain the significant covariation detected between wings after controlling for the phylogeny. Additional analyses, focusing on the degree of modularity of different regions of the wings and across fore and hindwings at various levels (intra vs. interspecific; e.g., Klingenberg 2009), should provide further insight into the developmental and functional integration of butterfly wings (e.g., Frankino et al. 2007; Allen et al. 2008).

WING MORPHOLOGY AND SEXUAL DIMORPHISM

We initially hypothesized that males would be greatly affected by microhabitat given their patrolling behavior, which can involve gliding flight in canopy. By contrast, because females' behavior is less stereotyped than males', and given that DeVries et al. (2010) found that females were not affected by the shift in canopy, we expected differences in shape between canopy and understorey females to be smaller. In canopy species, gliding phases seem restricted to males and DeVries et al. (2010) did not find that canopy females were affected by microhabitat shift. Hence, we expected that canopy species would exhibit greater sexual dimorphism. Yet, we found no significant association between microhabitat and SShD or SSD. As discussed above, females generally fly in the same microhabitat as males, although they usually do not exhibit the patrolling behavior of males. Our test of the effect of microhabitat on wing size and shape shows that female wings have also been affected by the shift to canopy microhabitat, with no increase in wing size or shape dimorphism among sexes. Penz et al. (2015) found that rate of dispersal of males of *M. achilles* is higher than that of females. Different behaviors among sexes such as dispersal strategies may have affected the evolution of sexual wing shape dimorphism.

We could not identify any driver of SSD because it is not phylogenetically conserved and it does not correlate with either microhabitat or color dimorphism. Other natural selective pressures may drive SSD. In most cases, *Morpho* females are bigger than males. Selection for bigger females could be driven by increased resource allocation for reproduction (Reeve and Fairbairn 1999), whereas selection toward smaller size for males could be driven by reduced predation risk (Allen et al. 2011).

By contrast, we found that forewing shape dimorphism is higher in species that exhibit a strong color dimorphism. For example, *M. zephyritis*, whose females are white and males are blue, exhibits the strongest forewing shape dimorphism. In species with strong color dimorphism, females are often more cryptic (more brown) whereas males usually exhibit bright iridescent blue color. Specific flight behavior may be selected jointly with color crypsis, thereby influencing both wing shape and wing color evolution. Additionally, such selective pressure may jointly drive strong sexual selection by females on the expression of blue iridescence for males and forewing shape, although the link between both is unclear. A number of evidence support the role of wing coloration in mate choice in butterflies, including iridescence (e.g., Jiggins et al. 2001; Sweeney et al. 2003; Robertson and Monteiro 2005; Merrill et al. 2014). Furthermore, increased conspicuousness in males compared to conspecific females may result from female mating preferences. However, selection for iridescent males can only appear if iridescence is a good predictor of male condition, as suggested for UV iridescence in *Colias eurytheme* (Kemp and Rutowski 2007). In this context, the extreme blue iridescent patterns of the wings in *Morpho* butterflies is obviously an appealing candidate for such a scenario. However, we currently do not know whether females are sensitive to intraspecific variation in iridescence or whether iridescence in *Morpho* is condition-dependent. This prevents any further speculation on the role of sexual selection on the evolution of color patterns and how it might affect shape dimorphism.

Similarly to microhabitat, our classification of color dimorphism is not quantitative. Color dimorphism varies among species but in some cases it also varies within species. For example, *M. aega* and *M. cypris* exhibit both ochre/brown females and male-like iridescent blue females. More broadly, quantification of wing color pattern (e.g., Le Poul et al. 2014) will bring a new dimension in the understanding of the evolution of wings. Studying how color pattern covaries with both size and shape, but also among the ventral and dorsal sides, will shed light on the extent to which different dimensions of butterfly wings respond independently to different selective pressures or are instead constrained by functional or developmental components.

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DATA ARCHIVING

Genbank accession numbers are indicated in Supporting Information S1.

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Supporting Information

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

Supporting Information S1. Genbank accession numbers.
Supporting Information S2. Phylogenetic tree including posterior probabilities at the nodes.
Supporting Information S3. Morphometric data acquisition.
Supporting Information S4. Covariation between fore- and hind-wing size.
Supporting Information S5. Sexual dimorphism.