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Did Adult Diurnal Activity Influence the Evolution of Wing Morphology in *Opoptera* Butterflies?

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Abstract

The butterfly genus *Opoptera* includes eight species, three of which have diurnal habits while the others are crepuscular (the usual activity period for members of the tribe Brassolini). Although never measured in the field, it is presumed that diurnal *Opoptera* species potentially spend more time flying than their crepuscular relatives. If a shift to diurnal habits potentially leads to a higher level of activity and energy expenditure during flight, then selection should operate on increased aerodynamic and energetic efficiency, leading to changes in wing shape. Accordingly, we ask whether diurnal habits have influenced the evolution of wing morphology in *Opoptera*. Using phylogenetically independent contrasts and Wilcoxon rank sum tests, we confirmed our expectation that the wings of diurnal species have higher aspect ratios (ARs) and lower wing centroids (WCs) than crepuscular congeners. These wing shape characteristics are known to promote energy efficiency during flight. Three *Opoptera* wing morphotypes established a priori significantly differed in AR and WC values. The crepuscular, cloud forest dweller *Opoptera staudingeri* (Godman & Salvin) was exceptional in having an extended forewing tip and the highest AR and lowest WC within *Opoptera*, possibly to facilitate flight in a cooler environment. Our study is the first to investigate how butterfly wing morphology might evolve as a response to a behavioral shift in adult time of activity.

Introduction

As butterflies use flight for activities that are directly linked to survival and reproduction, selection operating on flight performance for particular behaviors or activities will have an effect on wing morphology (see Dudley 2000 for a review). Butterfly flight behavior can be modified depending on their various activities, and some of these behaviors are characteristic of particular butterfly groups or sex. For example, while most butterflies land on the substrate to feed, swallowtails (Papilionidae) usually hover when feeding on flower nectar, a behavior that expedites movement between flowers (Stone *et al* 1988). Chemically protected nymphalids such as some members of the Ithomiini (Danainae) and *Heliconius* (Heliconinae) have a slow and predictable flight

that resulted from the evolution of a suite of wing and body morphology attributes (Chai & Srygley 1990, Srygley & Chai 1990, Marden & Chai 1991). Male and female reproductive activities typically require different flight behaviors like territorial displays in males (Rutowski 1991, Wickman 1992) and searching for oviposition sites in females. Such differences in flight behavior and mass allocation (females have heavier abdomens) can lead to the evolution of wing shape dimorphism (Srygley 2001, DeVries *et al* 2010, Cespedes *et al* 2015).

The interplay between microhabitat use and flight behavior has also been shown to influence the evolution of wing morphology. In some species of *Morpho* (Satyrinae, Morphini), males spend a considerable amount of time performing patrolling flight at the canopy level, and they have longer wings that are aerodynamically efficient for

gliding (DeVries *et al* 2010). Furthermore, genera in the tribe Haeterini (Satyrinae) like *Cithaerias* butterflies that glide in ground-effect have significantly longer wings than their mid-story *Dulcedo* relatives that utilize flapping flight (Cespedes *et al* 2015). Elongate wings that have high aspect ratios and low wing centroids produce the best gliding performance by maximizing lift and reducing drag (Dudley 2000). In both *Morpho* and *Cithaerias*, male wings are more elongated than those of females, reflecting differences in flight behaviors between sexes and possibly promoting energy efficiency in prolonged male territorial displays (DeVries *et al* 2010, Cespedes *et al* 2015).

The Neotropical tribe Brassolini (Satyrinae) includes ca. 100 species in 17 genera (Penz 2007) with the adults of most species being crepuscular (active at dawn, dusk, or both; Fruhstorfer 1912, DeVries 1987), a behavioral trait thus considered ancestral for the tribe. Diurnal habits have evolved in two taxa, *Opoptera* and *Dasyophthalma* (Casagrande & Mielke 2000, CMP pers. obs.), and although not quantified, field observations suggest that diurnal brassolines may spend proportionally more time flying than crepuscular species. As flight is costly, the amount of time spent on the wing clearly affects the energy budget of flying organisms (Chai & Dudley 1996). Based on flight energetics and biomechanics, we might expect that the wings of diurnal brassolines would show more energy-efficient properties (higher aspect ratio, lower wing centroid) than those of their crepuscular relatives.

The genus *Opoptera* contains eight species (Fig 1; Penz 2009) and includes crepuscular and diurnal species. Within *Opoptera*, the *syme*-clade includes the diurnal *Opoptera syme* (Hübner), *Opoptera sulcius* (Staudinger), and *Opoptera fruhstorferi* (Röber), which occur exclusively in the Brazilian Atlantic Forest. Their conspicuous dorsal colored markings and paler wing background are consistent with diurnal activity. The *aorsa*-clade includes the crepuscular *Opoptera aorsa* (Godart), *Opoptera hilaris* Stichel, *Opoptera arsippe* (Hoppfer), *Opoptera bracteolata* Stichel, and *Opoptera staudingeri* (Godman & Salvin), and although members of this clade are mostly Amazonian, *O. aorsa* occurs in the Atlantic Forest and *O. staudingeri* occurs in Central America and Mexico. Thus, *Opoptera* represents an appropriate group to ask if a behavioral shift to diurnal habits has influenced the evolution of wing morphology. To address this question, wing aspect ratio and centroid were calculated for the overlapped forewing and hind wing pair, which simulates wing position in flight (DeVries *et al* 2010). We examined shape divergence of the wing pair using independent contrasts to account for phylogenetic non-independence, and these analyses were complemented with non-parametric statistics aimed at comparing *Opoptera* clades and wing morphotypes. We found that diurnal and crepuscular species differed in wing aspect ratio and centroid and

discuss our results within the context of other studies on butterfly flight behavior and morphology.

Material and Methods

Species and samples

In addition to forming two sister clades (Penz 2009), the species of *Opoptera* can be divided into three morphotypes (Fig 1). The three diurnal species in the *syme-clade* have noticeably rounded forewing apices and lack hind wing tails (morphotype-1). Four of five crepuscular species in the *aorsa-clade* have truncated forewing apices (although variable between species) and prominent hind wing tails (morphotype-2). *Opoptera staudingeri* possesses different characteristics from the other four members of its assemblage by having a narrow forewing apex and no hind wing tail (morphotype-3). We examined 33 male specimens of all eight *Opoptera* species (sample sizes varied from one to 11; Online Supplementary Material S1) that permitted analytical comparisons of clades and morphotypes.

Data acquisition

Wing measurements were analyzed from photographs taken of the dorsal and ventral surfaces of each specimen with a Cannon G9 digital camera mounted on a tripod. A grid with 5-mm divisions was placed in frame for each photograph.

For measurements of wing length and calculations of aspect ratio and centroid, images were processed with Adobe Photoshop CS5.1. For all dorsal images, a dot was placed at the intersection of proximal vein endings to mark both wing bases (see Fig 2a). The hind wing (ventral image, Fig 2b) was digitally cut from the original image, pasted onto the dorsal image (Fig 2a), aligned with the hind wing base dot, and rotated to overlap the forewing by placing the hind wing anterior edge immediately below forewing vein CuA2. The overlapped pair was color-changed to a uniform gray and imported into Adobe Illustrator CS5.1 where 14 concentric arches were placed onto the image to create 15 wing sectors (Fig 2c). The center of the arch configuration was placed at the forewing base, and the edge of the outermost arch was aligned with the forewing edge. We used freeware program *Image J* (http://imagej.nih.gov/ij/) to measure forewing length (from the dot at the forewing base to the edge of the outermost arch) and the area of each wing sector. The aspect ratio (AR) of a wing can be defined as the ratio of wing length to the chord (i.e., the chord adjoining two edges of a curvature; see Dudley 2000 and references therein). Measurements of wing centroid (WC) refer to the center of wing mass, which is inversely correlated to AR. These two
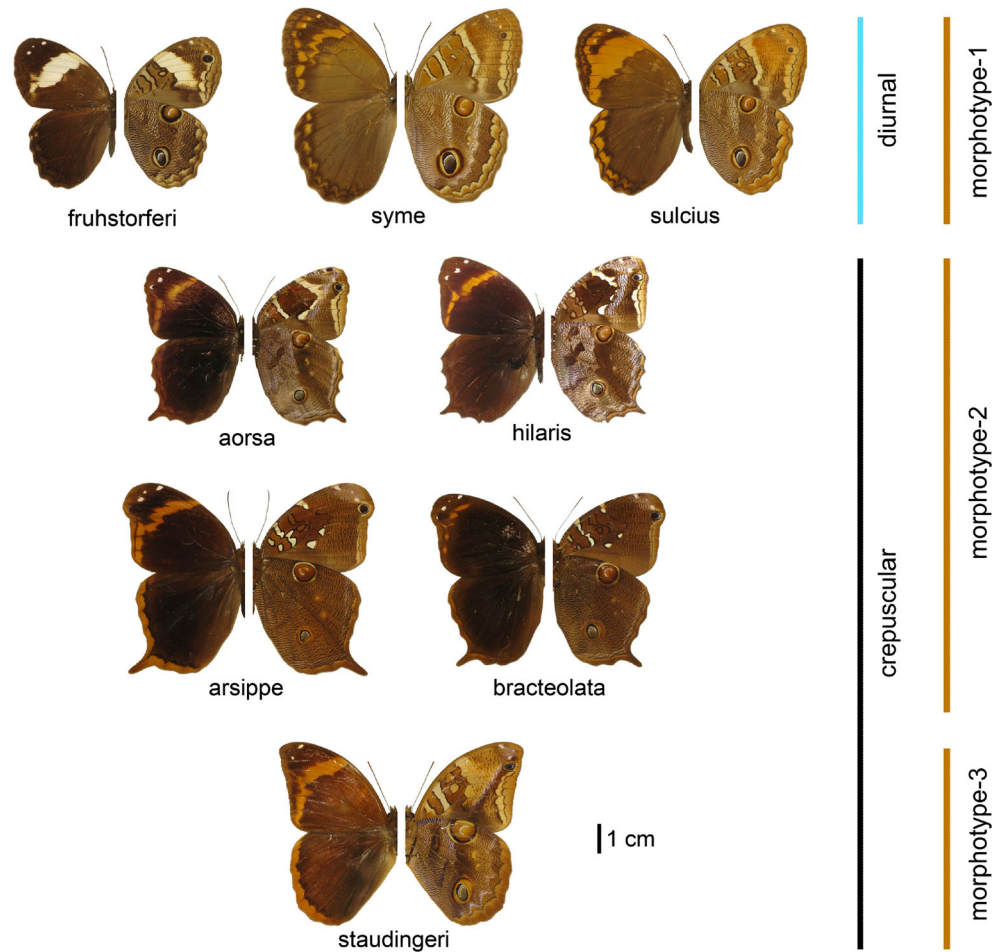
Fig 1 Species of *Opoptera* (dorsal view on the *left*, ventral on the *right*) classified by adult time of activity and three wing morphotypes.

parameters were calculated with Microsoft Excel using the equations in Ellington (1984).

Phylogeny

The morphology-based phylogeny in Penz (2009) is the only hypothesis of relationships available for *Opoptera*, and that analysis included 14 wing morphology characters. To avoid issues of non-independence of information, we re-analyzed the Penz (2009) data matrix excluding wing characters and using only characters from eye, leg, and genitalia (1, 2, and 20 characters respectively). Analysis protocol and settings followed Penz (2009). Two equally parsimonious trees were produced: one identical to those in the original analysis (Fig 3a) plus a topology in which *O. staudingeri* was sister to *O. arsippe* plus *O. bracteolata* (Fig 3b). We considered both these trees for calculations of phylogenetically independent contrasts (see below). Ancestral state reconstruction of crepuscular and diurnal flight activity was done in Mesquite (Maddison & Maddison 2009) as indicated on trees in Fig 3.

Statistical analysis

In both trees, the behavioral shift to diurnal activity occurred a single time (Fig 3), implying that activity time and wing characteristics are confounded by phylogeny. Following the approach developed by Garland *et al* (1993), we used phylogenetically independent contrasts (Felsenstein 1985) for wing AR and WC in relation to forewing length to test whether morphological changes were significantly larger at the node where the transition to diurnal activity potentially occurred.

Contrasts were calculated based on the average forewing length, AR and WC for each species, and two trees were considered (see Online Supplementary Material S2 for contrast values). We assumed a speciational model, where morphological changes are associated with speciation events (Rohlf *et al* 1990). To standardize the units of evolutionary change (expressed as branch lengths) across the entire topology, trees were made ultrametric (see method in Grafen 1989); that is, all terminals are at an equal distance from the basal node independent of the number of terminals between clades. Negative contrasts for wing length were positivized, and corresponding contrasts for AR and WC were sign-adjusted. Plots of wing AR and WC in relation to forewing
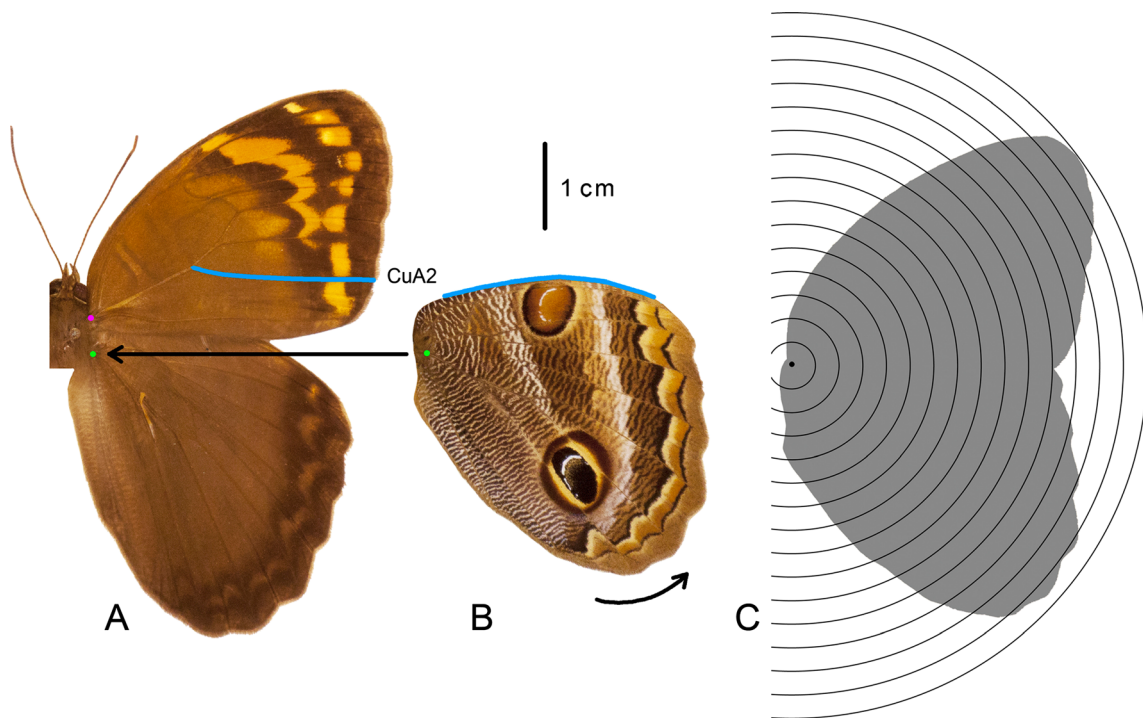
Fig 2 Image of *Opoptera syme* processed for data acquisition. **a** Pinned specimen in dorsal view, where forewing CuA2 vein is *highlighted* and both wing bases are marked with *dots*. **b** Hind wing of the same specimen photographed in ventral view, with costal margin *highlighted*. **c** Coupled wing pair, where the image of the hind wing (**b**) was overlapped with the forewing image (**a**) by matching the wing base *dots* and aligning the hind wing edge to forewing vein CuA2. *Center dot* marks the forewing base, and also the center of the concentric arches.

length were used to calculate a linear regression forced through the origin (Garland *et al* 1992), plus confidence intervals. All calculations were done in R 3.0.1 (R Core Team 2013).

The small number of *Opoptera* species is challenging for independent contrasts and regression analyses (eight species, seven contrasts). To further explore the divergence among clades and morphotypes, a non-parametric Wilcoxon rank sum test was used to compare average AR and WC of the coupled wing pair. In this case, we did not account for phylogeny, but instead presumed that the similarity between clades was due to common ancestry, and the divergence reflected evolutionary change resulting from different times of activity (diurnal vs. crepuscular). Non-parametric tests lessen (but do not remove) the analytical problems due to the phylogenetic non-independence of the data (i.e., they do not assume that the errors are drawn from the same distribution) at the expense of statistical power (see Garland & Ives 2000 for a discussion). The Wilcoxon rank sum tests should, therefore, be simply viewed as supplementary to the analyses using phylogenetically independent contrasts. Specimens and species were pooled to perform comparisons between clades and morphotypes as follows (see Figs 1 and 3): (i) between clades, *syme-clade* vs. *aorsa-clade*; (ii) within the *aorsa-clade*, morphotype-2 vs. morphotype-3; and (iii) between clades, morphotype-1

vs. morphotype-3. Wilcoxon Rank Sum tests were done in R 3.0.1 (R Core Team 2013).

Results

Members of the two *Opoptera* clades vary in activity times, and all diurnal species are descendants of a common ancestor. This precluded the estimation of ancestral time of activity for the genus based on the trees in Fig 3, so the ancestral state for the *Opoptera* root is considered ambiguous. We used independent contrasts to account for the correlation between time of activity (diurnal vs. crepuscular) and phylogeny (*syme*-clade vs. *aorsa*-clade) in our investigations of wing shape divergence.

Figure 3a, b shows the linear regressions of AR and WC contrasts in relation to wing length calculated based on two equally parsimonious trees (shown below corresponding scatterplots). The trees differed only in the position of *O. staudingeri*, a member of the *aorsa*-clade that has divergent wing morphology (Fig 1). In general, contrast values were broadly dispersed, so AR regressions were not significant for either tree but the WC regressions were significant. The diurnal and crepuscular clades are joined at node 9, marking the point at which a behavioral shift possibly occurred, and selection may have led to divergence in wing shape. For the tree in Fig 3a, node 9 fell inside the confidence
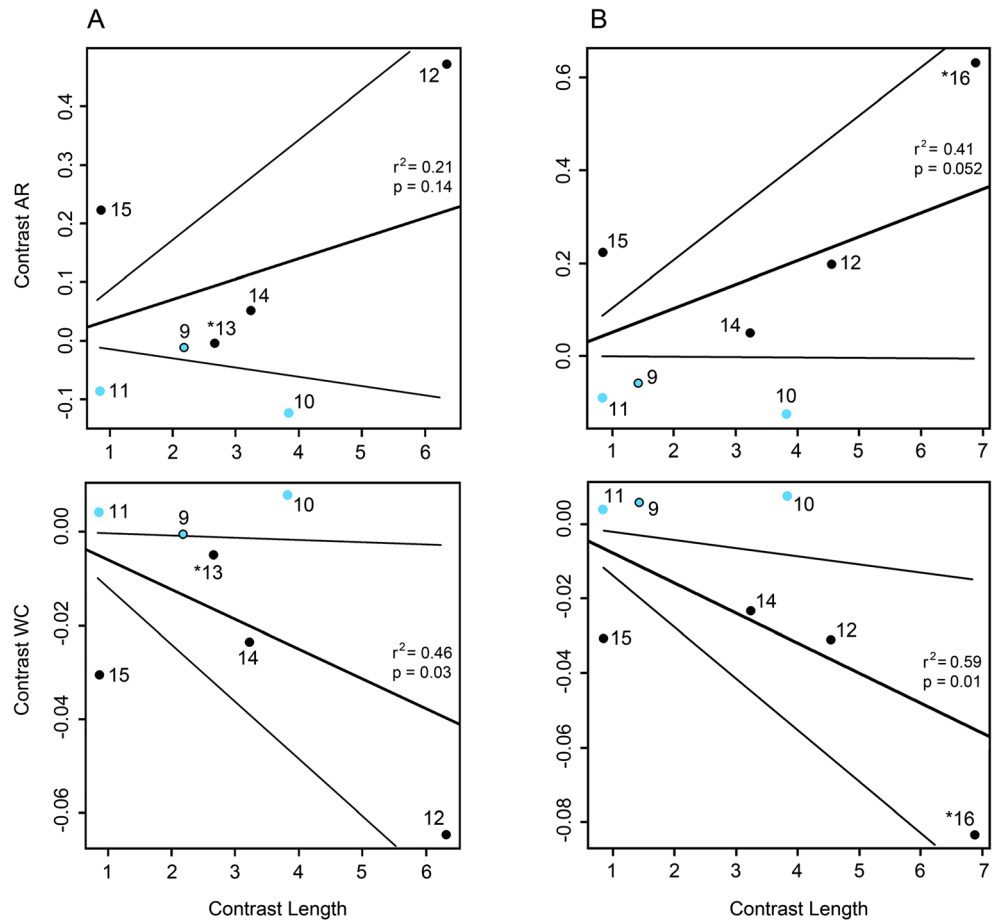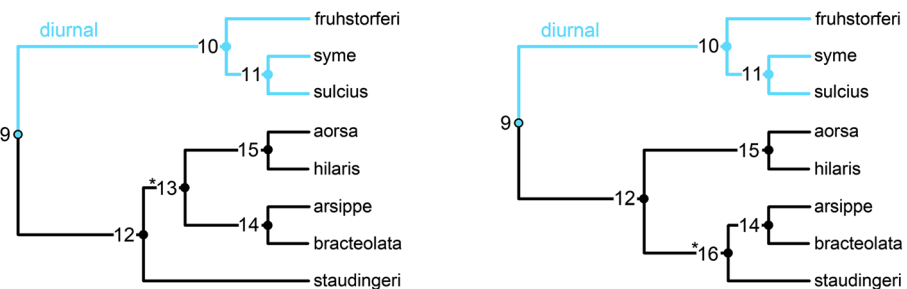
Fig 3 Scatterplots of positivized contrasts in aspect ratio (AR) and wing centroid (WC) over forewing length (FW) for their corresponding trees (*bottom* of each column). Nodes/contrasts are numbered on the ultrametric trees. Node 9 marks the behavioral shift to diurnal habits. Nodes 13 and 16, marked with asterisks, are unique to tree in **a** and **b**, respectively.

intervals for the AR contrasts but was barely significant for WC. This was not the case for Fig 3b where node 9 differs significantly from the trend set by regression lines for both AR and WC. Although agreement was not complete between analyses (Fig 3a, b), our results suggest a greater level of morphological change at node 9, and the high AR and low WC of the *syme*-clade (Table 1) might be associated with diurnal activity time. The AR and WC contrasts for *syme*-clade nodes 10 and 11 consistently fell outside the confidence intervals of the regression lines (Fig 3a, b), implying further evolution within this clade. Despite the similarity between *O. aorsa* and *O. hilaris* (Fig 1), AR and WC contrasts for node 15 unexpectedly fell outside the confidence intervals (Fig 3a, b).

Opoptera staudingeri (morphotype-3) had the highest AR and lowest WC within the genus, followed by species in the *syme*-clade (morphotype-1) and other members of the *aorsa*-clade (morphotype-2) (Table 1). The Wilcoxon rank sum tests showed that the *syme* and *aorsa*-clades differed significantly in their AR and WC, and also that the three morphotypes differ significantly from each other (Table 2).

Discussion

Most Brassolini are crepuscular (Fruhstorfer 1912, DeVries 1987), but a behavioral shift to diurnal activity has occurred in some species (Casagrande & Mielke 2000, CMP pers.

Table 1 Average forewing (FW) lengths, wing aspect ratio (AR) and centroid (WC) for *Opoptera* species, clades, and morphotypes (n = number of specimens). See Fig 1 for images of species, times of activity, and morphotype classification.

Species, clades, and morphotypes (*n*)	FW length	AR	WC
fruhstorferi (*n*=3)	37.104	2.478	0.556
syme (*n*=1)	39.586	2.413	0.561
sulcius (*n*=3)	40.037	2.366	0.563
aorsa (*n*=5)	38.243	2.312	0.575
hilaris (*n*=5)	37.783	2.212	0.591
arsippe (*n*=11)	41.122	2.261	0.572
bracteolata (*n*=2)	39.396	2.234	0.585
staudingeri (*n*=3)	45.114	2.695	0.520
syme-clade (*n*=7)	38.716	2.421	0.560
aorsa-clade (*n*=26)	40.254	2.309	0.571
morphotype-1 (*n*=7)	38.716	2.421	0.560
morphotype-2 (*n*=23)	39.620	2.259	0.578
morphotype-3 (*n*=3)	45.114	2.695	0.520

obs.). Crepuscular brassolines are mostly active at dawn and dusk but are occasionally seen feeding or puddling during the day (DeVries 1987, CMP pers. obs.). Thus, crepuscular species have a narrow window of time to perform fitness related activities such as mating and oviposition (Freitas *et al* 1997, Srygley & Penz 1999). The activity period of diurnal species, however, is less constrained and they consequently can, and likely do, spend more time flying. Given that flight is energetically costly (Chai & Dudley 1996), a greater level of activity can potentially select for more energetically efficient wings in diurnal species.

There is ample evidence that insect wing and body morphology has been refined by selection for energy efficiency

Table 2 Wilcoxon rank sum test results. Comparisons between clades and wing morphotypes were done with pooled data (*n* = number of specimens). See Fig 1 for images of species, times of activity, and morphotype classification.

Comparison	W	*p* (one tailed)
Aspect ratio (AR)		
(i) *syme*-clade vs. *aorsa*-clade (*n*=7 and 26)	150	0.008301
(ii) morphotype-2 vs. morphotype-3 (*n*=26 and 3)	72	0.0003419
(iii) morphotype-1 vs. morphotype-3 (*n*=7 and 3)	21	0.008333
Wing centroid (WC)		
(i) *syme-clade* vs. *aorsa-clade* (*n*=7 and 26)	154	0.004784
(ii) morphotype-2 vs. morphotype-3 (*n*=26 and 3)	72	0.0003419
(iii) morphotype-1 vs. morphotype-3 (*n*=7 and 3)	21	0.008333

during flight (Marden 1987, Betts & Wootton 1988, Kingsolver 1999, Kingsolver & Srygley 2000, Berwaerts *et al* 2002, 2008, Outomuro *et al* 2013a, b, 2014). In practical terms, energy efficiency resulting from a higher AR and lower WC would be beneficial to species that spend more time flying. We therefore expected that the wings of diurnal *Opoptera* species would have higher AR and lower WC than their crepuscular congeners. This expectation was generally confirmed; species of the *syme*-clade differ from those in the *aorsa*-clade in these two characteristics (Fig 3a, b; Tables 1 and 2). Nevertheless, the two equally parsimonious topologies led to slightly different results. In Fig 3b, both the AR and WC contrasts at node 9 (where the behavioral shift presumably occurred) differed from the regression line, but only the contrast for WC did so in Fig 3a. Contrasts for *syme*-clade nodes 10 and 11 consistently fell outside the confidence intervals independent of tree topology (Fig 3a, b), similarly to previous work on *Morpho* wing evolution (DeVries *et al* 2010). In that study, the node of interest marked a habitat shift to canopy flight, and while the contrast for that node represented a barely significant increase in morphological change, the node above it showed a clear change in AR and WC. In this context, further morphological evolution appears to have taken place after a behavioral shift had occurred for both *Opoptera* and *Morpho*. Finally, if diurnal *Opoptera* have a lower AR and higher WC than crepuscular ones, we might expect a similar trend in the closely related genus *Dasyophthalma*, which is indeed the case – diurnal *Dasyophthalma rusina* (Godart) male AR=2.507, WC= 0.539, *n*=2; crepuscular *Dasyophthalma creusa* (Hübner) male AR=2.379, WC=0.546, *n*=2 (CMP unpublished data). Future fieldwork should attempt to quantify duration of flight activites for diurnal versus crepuscular *Opoptera* species.

The crepuscular *O. staudingeri* had significantly higher AR and lower WC than members of the *syme*-clade (Tables 1 and 2). In addition to lacking hind wing tails, this species differed from other members of the *aorsa*-clade by having an extended forewing tip (Fig 1), a small change that had a large effect in wing AR and WC (Table 1). Nothing is known about the mating behavior and general biology of *O. staudingeri*, a cloud forest inhabiting species (DeVries 1987) while its congeners inhabit lowland forests (Uehara-Prado *et al* 2004). Given its occurrence at higher elevations, *O. staudingeri* is likely active at lower air temperatures than its congeners. As temperature is known to affect flight ability (Srygley 1994), we hypothesize that the high wing AR and low WC of *O. staudingeri* may facilitate flight and activity in cooler environments. However, accounting for the divergence between *O. staudingeri* and the other taxa in the *aorsa*-clade will require further empirical evidence.

Somewhat surprisingly, the contrasts for the similar species *O. aorsa* and *O. hilaris* fell outside the confidence

intervals of the regressions in Fig 3, and their average AR and WC differed more than other sister species (Table 1). To investigate this variation, we measured forewing and hind wing areas of the same specimens used for other analyses (S1) and calculated the FW:HW ratio for *O. aorsa* (0.775) and *O. hilaris* (0.763). This seemingly small difference in hind wing areas clearly had an effect on the calculations of AR and WC for these species. It has been shown experimentally in Lepidoptera that forewings play a more important role in forward flight than hind wings (Jantzen & Eisner 2008). Nonetheless, given that they may play a role in maneuverability (Jantzen & Eisner 2008) and gliding (Wootton *et al* 2000), studies focusing on butterfly hind wings are overdue.

The multiple events of evolution of hind wing tails in Lepidoptera hint that they may serve different functions in the groups where they occur. For example, in many lycaenid species, the hind wing tails are presumed to form the antennae of a "false head" that functions as a defense against predation (Robbins 1981), and Barber *et al* (2015) provide an experimental demonstration of anti-predator defense in saturniid moths with hind wing tails. Interestingly, an experimental wind-tunnel study using cut-out models of a swallowtail butterfly showed that the tails have an effect on gliding performance (Park *et al* 2010). Two groups have hind wing tails within Brassolini: *Bia* species with tails at vein CuA2 and four members of the *O. aorsa*-clade with tails located at vein M3. These have evolved independently given their structural location on the hind wing, and the fact that *Bia* and *Opoptera* are distantly related (Penz 2007, Penz *et al* 2013). Although in need of further study, the presence of an eyespot at the base of the ventral hind wing tail in *Bia* suggests an involvement in signaling (see image in http://fs.uno.edu/cpenz/bia.html, last accessed June 12, 2015). In contrast, *Opoptera* tails do not have color elements distinctive from the entire hind wing margin (see images in http://fs.uno.edu/cpenz/opoptera.html, last accessed June 12, 2015), and their function is unknown.

Butterfly wing morphology has been shaped by selection on flight performance, and studies have explored potential mechanisms driving wing shape evolution, such as migration (Dockx 2007), courtship behavior (Berwaerts *et al* 2002), gliding in the forest canopy (DeVries *et al* 2010) or in ground effect (Cespedes *et al* 2015), and also range expansion as a potential response to climate change (Hill *et al* 1999). To our knowledge, this study is the first to suggest that time of activity (diurnal vs. crepuscular) may also influence the evolution of butterfly wing morphology.

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