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Complex interspecific and intersexual patterns of wing design in *Heliconius* butterflies

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Abstract. In butterflies, interspecific and intersexual variation in wing design morphology modifies aerodynamic efficiency and individuals' flight costs. Wing design involves traits like wing shape and size, which determine flight performance and behavioral ability, which determine individuals' flight performance and behavioural capacity under their ecological and reproductive requirements. In this context, evolutionary adjustments in wing shape are expected to be adaptive responses to specific flight requirements that allow acrobatic maneuvers to evade predators, for females reduce the costs of takeoff and flight maintenance linked to the additional weight of eggs and ovarioles, or for males improve flight efficiency during costly and long courtship displays. Here, the intra- and interspecific variation in wing shape of butterflies in the genus *Heliconius* Kluk, 1780. was analyzed using geometric morphometrics techniques, as well as its association with i) intersexual differences in flight requirements, ii) mating system, and iii) the presence of co-mimic pairs. The results highlight the relevance of sexual selection associated with mating systems and life history traits as the predominant force that promotes the patterns of sexual dimorphism in wing shape observed in *Heliconius*. In this context, both sexes exhibit contrasting wing shapes according to the mating strategies (i.e. pupal mating vs courtship species). However, there are also remarkable intersexual wing shape differences, potentially associated with the pressures that impose egg production on females. Additionally, *Heliconius* species that form co-mimic pairs also show a strong wing shape convergence despite belonging to different subclades. The results highlight the complexity of traits involved in the evolutionary designs of wings in Lepidoptera.

Introduction

Selective pressures are expected to result in adaptive responses in individual morphological and/or physiological traits that contribute to behavioral performance and fitness within particular ecological or reproductive contexts (Arnold 1983; Altshuler et al. 2004; Huyghe et al. 2005; Bishop 2007; Mendoza-Cuenca and Macías-Ordóñez 2010). Flight is recognized as the most costly locomotion behavior (e.g. costs of sustaining flight and developing flight apparatus) and represents a crucial evolutionary innovation in insects that provides substantial advantages while imposing metabolic and biomechanical pressures (Norberg 1994; Kohane and Watt 1999; Dudley 2002). These are reflected in the increase of metabolic rates with the increased flight activity and the higher basal metabolic rates observed in flying insects compared to species with other means of locomotion (Reinhold 1999).

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In that context, it has been suggested that natural selection has shaped the evolution of wing morphology to reduce flight costs in response to behavioral innovations and types of habitats, suggesting adaptive morphological divergences associated with microhabitat conditions (DeVries et al. 2010; Chazot et al. 2016). Natural or sexual selection may also drive wing phenotypic variation when critical flight behaviors (e.g., foraging, courtship, and predator evasion) are associated with interspecific and intersexual differences (Melville and Swain 2000; Irschick and Garland 2001; Lailvaux et al. 2004; Van Dyck 2004; Berwaerts et al. 2006; DeVries et al. 2010). Then, it is relevant to evaluate how trade-offs between selection pressures and biomechanical limitations may impact the morphological evolution of wings.

Wing design defines many butterflies' aerodynamic responses, such as flight performance, maneuverability, flight behavior, and, therefore, individual fitness (Ellington 1991; Srygley 1999; Srygley and Kingsolver 2000; Berwaerts and Van Dyck 2004; Berwaerts et al. 2006). Since flight is used to carry out a large diversity of behaviours, wing shape evolution in butterflies could be adaptive responses to the aerodynamic challenges imposed by different types of flight (Chai and Srygley 1990). Consequently, evolutionary forces, such as natural and sexual selection, acting independently, synergistically, or antagonistically are expected to optimize butterfly flight design, depending on the behavioral repertoires used to fulfill their survival and reproductive requirements (Chai and Srygley 1990; Marden and Chai 1991; Hall and Willmott 2000; Berwaerts and Van Dyck 2004; Mendoza-Cuenca and Macías-Ordóñez 2005, 2010).

Mating strategies have been particularly well studied in butterflies and classified into patrolling (i.e., active searching mating behavior) and perching (i.e., sit-and-wait mate locating behavior) (Scott 1972; Rutowski 1984, 1991; Van Dyck 2004; Wiklund 2004). These behaviors have evolved by the combined effect of ecological restrictions (e.g., predation, habitat type, and foraging requirements) and the spatial and temporal distribution of receptive females in the population (Emlen and Oring 1977; Shuster and Wade 2003; Mendoza-Cuenca and Macías-Ordoñez 2010). In that sense, sexual selection using female choice or male-male competition results in behavioral and morphological traits that increase male mating success (Andersson 1994; Birkhead and Moller 1998). For example, perching males exhibit a wing design (i.e. higher wing loading and aspect ratio) that increases acceleration and flight speed at the expense of flight efficiency (i.e. power requirements of flight), while in patrolling males that engage in continuous flight activity expend more energy, wing evolution is towards a design that increases flight resistance (i.e. endurance) and flight efficiency (Wickman 1992). In addition to these morphological wing changes that reduce the cost of this mating behavior, it is also associated with performing types of flight (i.e., gliding) that are less energetically demanding (DeVries et al. 2010). Conversely, due to higher reproductive investment (i.e. gamete production) and egg loads, females have heavier abdomens and less thoracic mass. Consequently, female butterflies may have reduced maneuverability and less efficient flight (Srygley and Chai 1990; Mendoza-Cuenca and Macías-Ordóñez 2005).

Butterflies of the genus *Heliconius* are an ideal group to evaluate the overall effect of natural and sexual selection on wing shape evolution. Is the group of flying insects better studied in terms of the repercussions that selective pressures such as mimicry, mating systems, and reproductive investment in morphology, behavior, and aerodynamic performance (Chai and Srygley 1990, 2007; Marden and Chai 1991; Hall and Willmott 2000; Mendoza-Cuenca and Macías-Ordóñez 2005). *Heliconius* are sexually monomorphic species that can be grouped unambiguously based on their mating behavior into: (1) monandrous pupal mating species, in which a male locates a female pupa,

follows pupal development, and initiates mating before the receptive female begins to emerge from the pupal case; (2) polyandrous species with typical mating behavior among flying adults (Brown 1981; Gilbert 1991; Deinert et al. 1994; Hernandez and Benson 1998; Mendoza-Cuenca and Macías-Ordóñez 2010). Pupal mating is an apomorphic character relative to adult mating in *Heliconius* and the tribe Heliconiini. Each mating system is restricted to one of the two clades of *Heliconius*: the pupal mating clade PM and the non-pupal mating clade NPM (Brown 1981; Brower and Eagan 1997; Penz 1999; Kozak et al. 2015). Recent evidence supports the monophyly of the pupal mating clade and suggests that pupal mating behavior has evolved only once in the Heliconiini (Beltrán et al. 2007; Kozak et al. 2015).

Mating systems in *Heliconius* are not correlated with differences in other features, such as habitat type, foraging pattern, or oviposition and host preferences (Brown 1981; Gilbert 1991; Mendoza-Cuenca 2004). Species of both mating clades share habitats and geographic distribution, which means that they share similar predation intensities and selection on morphology and behavior. Moreover, It could be associated with the common presence of Müllerian co-mimetic pairs observed within the genus *Heliconius* Kluk., in which a PM species and an NPM species converge in colour signaling and wing size, as well as in locomotor performance (Gilbert 1991; Srygley 1994, 1999). Commonly, some species of the PM *erato-sara-sapho* sub-clade form mimetic pairs with members of the NPM *melpomene-cydno* sub-clade (Srygley 1994, 1999; Concha et al. 2019).

In this study, we used the geometric morphometric technique to disentangle the combined or conflicting action of the selective forces involved in *Heliconius*'s evolution of wing shape. This work states two specific hypotheses regarding *Heliconius*'s evolutionary forces driving wing design.

(1) If wings have been modeled by natural selection in *Heliconius*, species and sexes that share relevant ecological contexts like habitat, feeding/oviposition preferences, or anti-predator behaviour (e.g. mimetic pairs) that are associated with common selective pressures on wing design. Then, patterns of convergent wing shape are expected.
(2) If factors related to sexual selection (e.g. mating system, gamete production) have been the main evolutionary pressure on morphological design in *Heliconius* butterflies, the diversity of flight designs and intra- and interspecific morphological disparity would be limited. Then, convergence in wing shape between males of the same mating system (e.g. pupal mating) or between females would be expected since they must carry the additional load of eggs.

Materials and methods

This study used 203 specimens, nine individuals of each sex of the 11 *Heliconius* species (except 12 males of *H. charithonia* Linnaeus, 1767) (Fig. 1). Digital images of wings were taken from individuals in collections at the Museo de Zoología and Instituto de Biología at Universidad Nacional Autónoma de México. Additional specimens were collected around Xalapa, Mexico. We captured images of each specimen, together with a size reference (Fig. 2a), using a digital camera (Olympus 12 MP), mounted on a fixed stand (Bencher Copymate) 20 cm from the specimen with two symmetrical 300-watt quartz halogen lights to avoid shadows. We only used digital images to test our predictions since information other than the collector and collecting data were rare for the individuals in these collections.

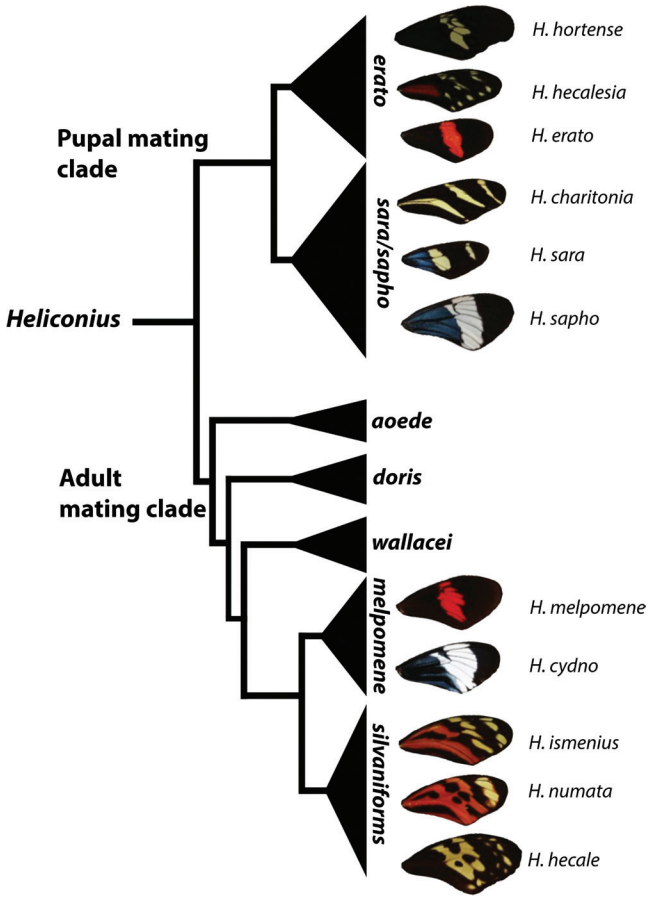

Figure 1. A simplified phylogeny of the *Heliconius* genera (Kozak et al. 2015), illustrating the wings of the studied species according to their mating systems.

We used the program TpsDig (Rohlf 1998) to register "x", "y" coordinates on 14 anatomical marks on the right forewing of each specimen. Two additional marks correspond to a "ruler" placed as a size reference (Fig. 2a). Among these anatomical marks, five correspond to landmarks (sensu Bookstein 1990), which are unambiguous and repeatable marks in all the species, and nine correspond to semi-landmarks (sensu Zelditch et al. 2012) used to describe wing contour, which are also repeatable across all species but do not correspond to any anatomical mark. Semi-landmark locations were determined by a fan-generated with the MakeFan software from the Integrated Morphometrics Package (IMP) (http://www.canisius.edu/~sheets/morphsoft.html) and were chosen because they represented the morphological wing contour adequately in those areas with a lack of homologous landmarks for all species (Zelditch et al. 2012).

We performed a Procrustes superimposition analysis (Fig. 2b) for the configuration of coordinates using the CoordGen6 program of the IMP series. We used the average configuration of all specimens as a reference (Fig. 2c). Superimposition coordinates allowed us to calculate shape variables (i.e. Procrustes distances) without size effect. We used centroid size as a size variable

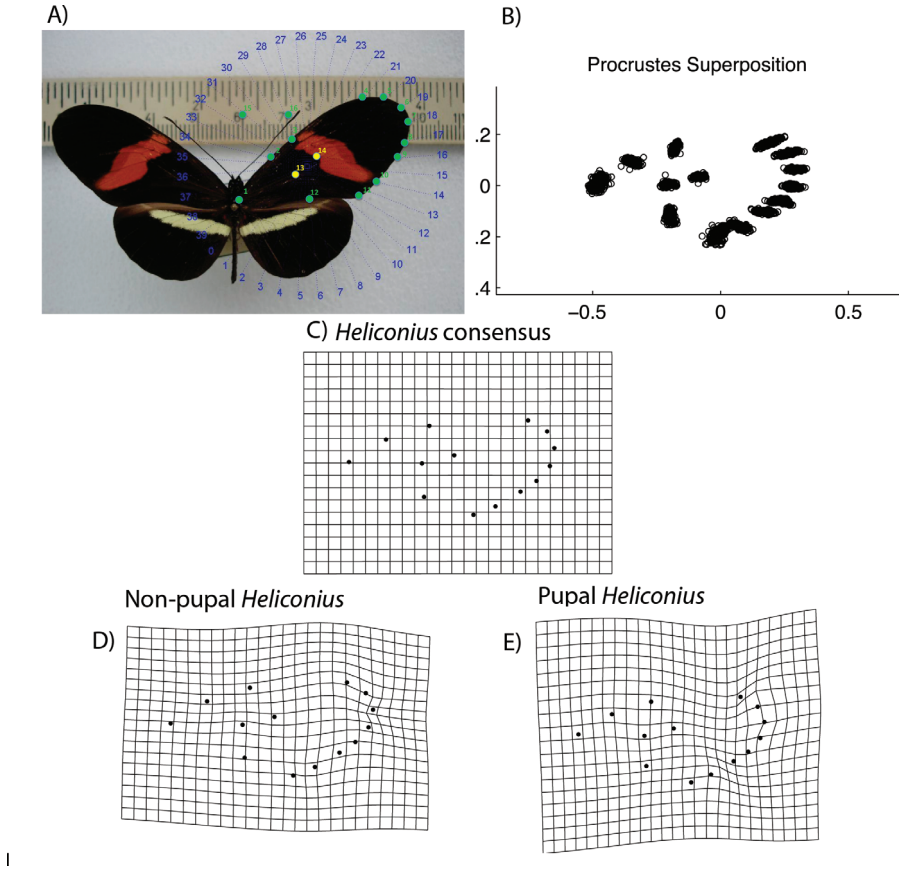

Figure 2. Geometric morphometric analysis. **A.** Configuration of landmarks using the right forewing of a *Heliconius erato* male; **B**. Shape coordinates of landmark configuration from all species (n = 203). Deformation grid for mean wing specimen for: **C**. All *Heliconius* species (i.e. *Heliconius* consensus); **D**. Non-pupal mating species; **E**. Pupal mating species.

(CS), calculated as the square root of the sum of squared distances of each landmark from the centroid of the landmark configuration. We used these shape variables to test our three predictions and evaluate the importance of different evolutionary forces in *Heliconius*'s wing shape evolution.

The relative importance of factors related to wing shape evolution, was evaluated using a full factorial Procrustes ANOVA, with the mating system, sex, and clade membership as independent variables and Procrustes variables as the dependent variable. We examined size variation using an ANOVA to compare CS differences between mating systems and sexes.

Additionally, to assess the intra- and interspecific variation in wing shape related to sexual selection traits, a canonical variate analysis was conducted using the mating system and sex as grouping factors. We used interpolation by the thin plate spline technique (Bookstein 1990; but see Zelditch et al. 2012) to visualize shape changes as landmark displacement in a deformation grid according to the mating system (Fig. 2d, e) and sex. We also conducted a morphological disparity analysis (10,000 permutations) to evaluate wing shape disparity (i.e. Procrustes variance)

among *Heliconius* species from both mating systems and sexes (sensu Foote 1990; Zelditch et al. 2012). A disparity test using the Procrustes coordinates was performed with the TwoGroup and PairDisparity software, both from the IMP series. Since clade membership may impose evolutionary constraints on wing divergence, we performed multiple regression of Procrustes coordinates against CS to examine evolutionary allometry between clades (Klingenberg 1998) and examined size variation using ANOVA to compare CS differences between *Heliconius* clades.

To evaluate if wing shape is modeled by natural selection, avoiding any possible historical effect, we compared shape variables of mimetic pairs species versus non-mimetic species without separating by sex, assuming that predation pressures (i.e. natural selection) that could drive wing shape evolution act similarly on both sexes. Following the phylogenetic structure proposed by Brower and Eagan (1997) and by Beltrán et al. (2007), and considering the evidence of mimetic relationships within the *Heliconius* genera (Srygley and Ellington 1999a, b), we established four groups: 1) *H. erato* (L., 1758) (PM) – *H. melpomene* (L., 1758) (NP) mimetic pair (E-M), 2) *H. sapho* (Drury, 1782) (PM) – *H. cydno* Doubleday, 1847 (NP) mimetic pair (S-C) 3) Non-mimetic pupal mating species *H. hecalesia* Hewitson, 1853 *H. hortense* Guérin-Méneville, 1844, *H. sara* (Fabricius, 1793) and *H. charithonia* (PM), 4) Non-mimetic non-pupal mating species (*H. numata* (Cramer, 1780), *H. ismenius* (Latreille, 1817) and *H. hecale* (Fabricius, 1793) (NPM). We used a canonical variate analysis to compare the effect of mimicry on the intra- and inter-clade wing shape variation. A bootstrap test (10,000 permutations) was used to evaluate distances among these four *Heliconius* groups.

Results

The Procrustes ANOVA analysis revealed significant effects on wing shape in *Heliconius* from the three main factors: mating systems ($F = 38.5$; d.f. = 1; $p < 0.001$), sex ($F = 8.5$; d.f. = 1; $p < 0.001$), and clade ($F = 28.8$; d.f. = 3; $p < 0.001$). There were also significant interactions for sex-mating system ($F = 3.3$; d.f. = 1; $p = 0.021$), sex-clade ($F = 6.8$; d.f. = 1; $p < 0.003$), and mating system-clade ($F = 3.2$; d.f. = 1; $p < 0.02$), but not for the interaction mating system-sex-clade ($F = 1.71$; d.f. = 1; $p = 0.134$).

The canonical variates analysis, considering the mating system and sex showed that males and females from both mating systems significantly differed in wing shape (Axis $\lambda = 0.0730$, $\chi 2 = 191.2214$, d.f. = 72, $p < 0.01$; Fig. 3). Thin plate spline analysis indicated that the wings of pupal mating males are more elongated and narrower compared to those of non-pupal mating males (Fig. 3). Permutation analysis showed that pupal and non-pupal mating males showed a higher and significant wing shape morphological disparity (Pairwise absolute differences = 0.0022; $p = 0.003$), while pupal and non-pupal female species displayed a lower and non-significant morphological disparity (Pairwise absolute differences = 0.0017; $p = 0.06$). Multiple regression analysis showed that there was no evolutionary allometry between shape and size in these butterfly species (regression slope = 0.0001, $r = 0.03$, $p > 0.05$). that males of both mating systems were significantly isolated by wing shape.

Canonical variate analysis of our four *Heliconius* mimetic and non-mimetic groups (not considering a sex effect) revealed significant differences between groups (Wilk's Lambda = 0.0220912, d.f. = 87, $p < 0.0001$), with a low number of misclassified individuals (n = 8, percentage of misclassified = 3.47). The analysis revealed a clear mimetic effect, as the wing shape of the *erato-melpomene* group closely resembled that of the other mimetic pair, *sapho-cydno*, while both were significantly different in wing shape from the species in non-mimetic groups (PM and NPM), even considering that some of these species belong to the same subclade (Fig. 4).

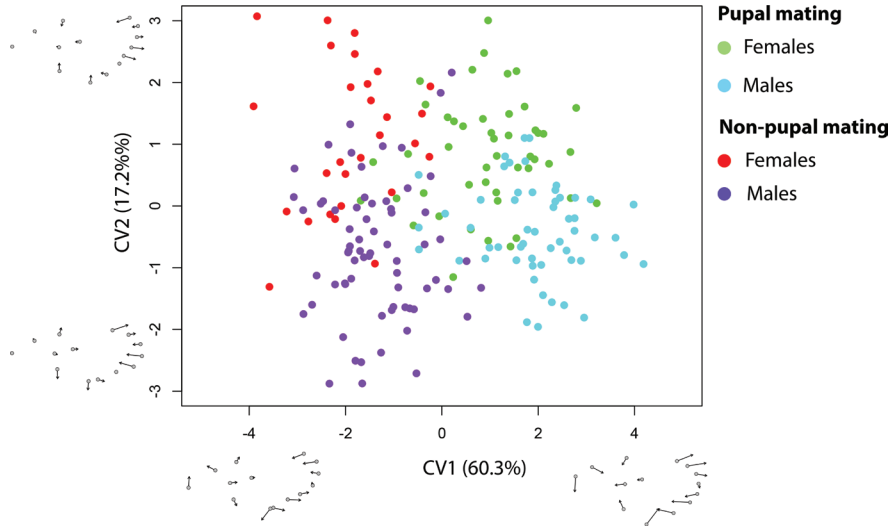

Figure 3. Canonical variate analysis of all 203 specimens, grouping by mating system and sex. The maximum and minimum deformation grid vectors are displayed along the CVA axes.

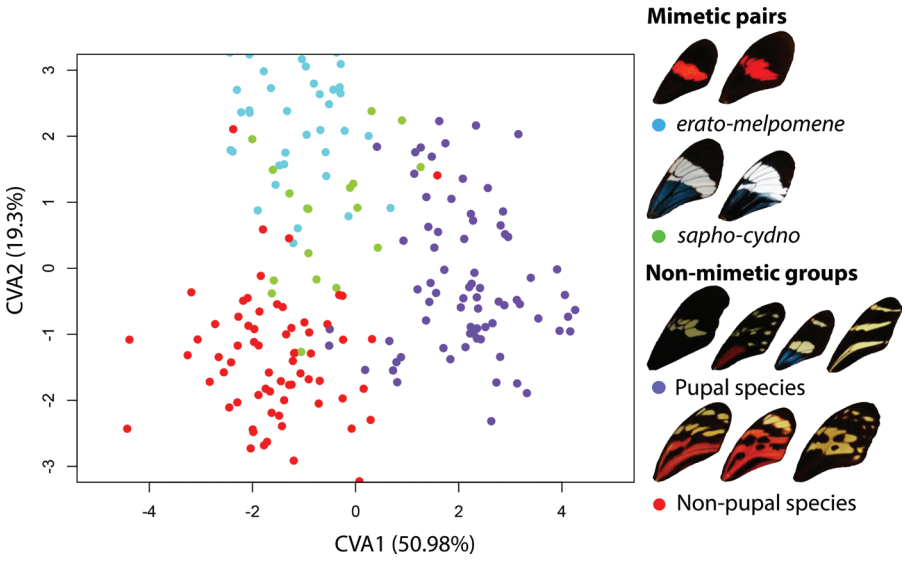

Figure 4. Canonical variate analysis of all 203 specimens, categorized into four groups. **1.** *erato-melpomene* mimetic pair (blue circles); **2**. *shapo-cydno* mimetic pair (green circles); **3**. pupal species (violet circles); **4**. Non-pupal species (red circles).

Discussion

Species and individuals within species often exhibit variation in phenotypic traits, including physiology and morphology. This variation can influence individual behaviour, performance, and fitness (Lande and Arnold 1983). Among Lepidoptera, the evolution of morphological flight design has been studied by examining how selective pressures, such as natural and sexual

selection, impact aerodynamic traits to optimize flight performance for specific behaviours (Wickman 1992; Srygley 1999; Mendoza-Cuenca and Macías-Ordóñez 2005; DeVries et al. 2010; Ramos-Pérez et al. 2020). Common examples include non-dimensionalized morphological descriptors, such as the centre of wing area and the position of the centre of wing mass (Srygley 1994, 1999), along with studies of changes in wing motion (Srygley 2007). However, to better understand the evolution of morphological complexity, it is essential to evaluate how these selective pressures affect variation and changes in wing shape design.

Flight is considered one of the costliest forms of locomotion (Dudley 2002). Butterflies depend on flight to perform most of their reproductive and non-reproductive activities; thus, it is expected that strong selective pressures have played a role in optimizing wing design based on the specific ecological context of each *Heliconius* species. If variation in forewing shape evolved under natural selection pressures, we expect: (1) multiple instances of intra- and inter-clade convergent wing shapes among sexes and species that share habitats, feeding, oviposition, and anti-predatory behaviours; or (2) since a vast majority of *Heliconius* species share a common ecological context (Brown 1981; Gilbert 1991; Estrada and Jiggins 2002; Mendoza-Cuenca, 2004), we predict very few flight designs and low morphological disparity in our sample. The results suggest that natural selection alone does not account for all the variation in wing shape observed in *Heliconius* samples. Indeed, natural selection only helps explain the greater morphological similarity between females of the two clades (see below) and the significant convergence in wing shape among species from different clades. This convergence in wing shape may be attributed to the extended Müllerian mimicry observed between non-pupal and pupal mating sub-clades, as seen between *Heliconius erato* and *Heliconius melpomene*, as well as in *Heliconius cydno* and *Heliconius sapho* (Srygley 1999, 2007; Concha et al. 2019). In addition to evolutionary convergence in colour patterns, natural selection seems to promote a remarkable convergence of wing shape between pairs of species that, based on genetic evidence, do not share a common biogeographical history (Joron and Mallet 1998). Wing shape convergence may explain the observed similarities in other parameters related to flight biomechanics, such as the centre of mass, wing mass, and wing-beat frequency (Srygley and Ellington 1999a, 1999b), while enhancing the similar performance linked to the previously described "locomotor mimicry" among co-mimic *Heliconius* species (Srygley 1994, 1999, 2007), thereby reinforcing the warning signal to avian predators.

However, if sexual selection has been the main evolutionary pressure influencing morphological design in *Heliconius* butterflies, we should observe interspecific variation in male wing shape linked to mate-locating behaviours, and in females as well, if there are strong genetic correlations between the sexes. The results indicate that pupal mating males (PM) differ in wing shape from non-pupal mating males (NPM). The thin plate spline technique shows that pupal mating males have more slender and longer forewings compared to non-pupal males. These morphological differences may directly impact a male's performance and fitness since the slender and longer wings of PMs enhance their aspect ratio, resulting in more efficient flight and improved manoeuvrability (Dudley and Srygley 1994; Berwaerts and Van Dyck 2004).

Flight design enables a pupal mating species to reduce the energetic cost of hovering, an energy-intensive form of flight (Mao and Gang 2003; Fry et al. 2005; Hedrick et al. 2015) utilized by males to assess the receptivity status of female pupae and by females while evaluating a host plant for oviposition. Wing shapes that enhance the ability to make rapid directional changes and

accelerate during flight (i.e., manoeuvrability) have been associated with potentially adaptive responses that increase the ability to escape from bird predation (Srygley and Dudley 1993) improve the capacity for responses in aerial encounters (Marden and Chai 1991), as well as performing acrobatic sexual displays (Le Roy et al. 2019) or object evasion during flight. Therefore, as described in *Heliconius* pupal mating species, for males that employ alternative mating tactics (i.e., patrolling), wing shapes that enhance manoeuvrability are an adaptive advantage when performing courtship behaviours (Mendoza-Cuenca and Macías-Ordóñez 2010). Equally important is the evidence that, across all studied pupal mating species, longer forewings offer reproductive advantages in inter-male competition, thereby influencing male mating success (Deinert et al. 1994; Mendoza-Cuenca and Macías-Ordóñez 2010).

In contrast, the wider and shorter forewings of males in non-pupal mating species would increase wing loading, airspeed (although perhaps at a greater power cost), and flight resistance. These are all essential features if a male must actively seek, locate, and court adult flying females. Additionally, it is fundamental to evaluate how these intra- and interspecific wing morphological changes could be associated with reducing the cost of different flight behaviours involved in courtship, resource acquisition, survival, and the search for oviposition plants.

The results of this study suggest that in *Heliconius*, unlike other Lepidoptera species (Wickman 1992), there does not seem to be a strong genetic correlation between the sexes. The inter-clade morphological similarity among females, in relation to males in species that lack evident sexual dimorphism, suggests an optimal female wing design driven to counterbalance their heavier abdomen due to egg load and to meet the morphological needs for oviposition host location, while male wing design is primarily limited by mate-locating behaviour.

Our findings on *Heliconius* support a broader hypothesis that mating systems may limit morphological wing design across a wide range of Lepidoptera. Specifically, male mating behaviour imposes morphological constraints on individuals, affecting their flight performance and fitness. The overall results specifically identify sexual selection as the dominant modelling force in *Heliconius*. However, the design of morphological wings is not independent of other selective pressures imposed by factors such as predation and sex-specific requirements. Divergent selection forces acting independently on female and male insect wing shapes have been described only recently (DeVries et al. 2010; Outomuro et al. 2011; Ramos-Pérez et al. 2020). However, it is crucial to conduct an aerodynamic analysis to evaluate how morphological variations impact flight performance. Additionally, the analysis should extend to include other genera within the Heliconiini to properly assess through phylogenetic comparative analysis whether wing shape is independent of evolutionary history in this group of butterflies.

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