The Evolution of Flight and Wing Shape in *Heliconius* Butterflies

Lucie Mariecke Queste

Doctor of Philosophy

University of York

Biology

March 2020

Abstract

Understanding how ecological traits are driven by divergent selection and identifying the factors that shape their evolution can provide important insight on species evolution. Wing shape is an important component of flight behaviour, which is thought to have been one of the main drivers of insect diversification. However, wing shape has also evolved in response to a number of other selective pressures. The *Heliconius* genus provides an excellent system to study different evolutionary processes owing to our extensive knowledge of their ecology and well-developed genomic resources. They are famous for their aposematic colour patterns, a classic example of Müllerian mimicry, but evidence suggests that flight and wing shape are also involved in the mimetic signal. In this thesis, I present an integrative approach to understanding the evolution of wing shape in *Heliconius*. Using phylogenetically corrected ecomorphological analyses I have demonstrated genus wide convergence of wing shape between mimics and habitat types, with different selective regimes acting on the two wings. These patterns are strongly driven by the silvaniform, which mimic Ithomiine species. Secondly, I used experimental manipulations of wing shape to determine whether differences between two sister species, *H. elevatus* and *H. pardalinus*, with divergent colour patterns, can explain differences in flight behaviour. I found that wing shape is correlated with flight measurements but does not appear to drive differences in flight. Finally, I carried out Quantitative Trait (QTL) Loci analyses to identify the underlying genomic structure of wing shape in F2 crosses of *H. elevatus* and *H. pardalinus* and identified two QTLs associated with wing shape. Overall, I demonstrate the importance of identifying the interactions between different ecological factors and underline the need to understand how wing shape and flight are connected, as well as provide some of the first results on the genetic basis of wing shape.

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Acknowledgements

This thesis could not have been completed without the involvement, support and advice from many different people.

First of all, I would like to thank my supervisor, Kanchon Dasmahapatra, who first gave me the opportunity to do a summer placement and MSc project in his lab back in 2014, which marked the beginning of my work on *Heliconius* and for which I am incredibly grateful. He invested his time to teach me lab procedures and bioinformatics, gave valuable input on experimental designs and analyses throughout the PhD as well as comments on manuscripts and drafts; and towards the end advised me on job applications and interviews. His input and contributions through the good times (and the bad) have helped me drive this project forward. Neil Rosser, who became my unofficial supervisor out in the field, also ended up being an invaluable and constant source of advice and support during both the MSc and the Phd. I have learnt a lot about *Heliconius* and *Passiflora* from him; without his input the entire experience would not have been the same.

I would then like to thank Peter Mayhew, my co-supervisor, who's contribution not only improved the contents of this thesis, but also improved the process of getting it done. My PhD advisors, Daniel Jeffares and Peter Young also ensured that the project was carried out in the best conditions. Finally, Owen McMillan and Robert Srygley, provided invaluable advice on field experiments while out in Panama.

My project has always been carried out with the support and friendships of the various lab members that have come and gone, these are Jake Morris, Bruna Cama, Lewis White, Josie Monaghan (who proofread my introduction), Melissa Minter and Michael Lawson. The J1 corridor in general has always been a friendly and welcoming environment to work in. I have spent my PhD surrounded by brilliant people always willing to share stories, opinions and advice on matters relating to my project as well as other aspects of academia, over a cup of coffee. Monica Bandeira and the rest of the Graduate Office have also played an important role in ensuring the smooth running of my, and other students' projects.

The *Heliconius* community is a valuable source of information and collaboration, I have many people to thank in this community for support in the field, contributions of specimens or just general advice and input on my project. In no particular order, I

would like to thank Nicola Nadeau, Nate Edelman, Mathieu Chouteau, Melanie McClure, Monica Arias, Stephen Montgomery, Ronald Mori, Oscar Paneso, Diana Almeida, Chi-Yun Kuo, Monica Monllor, Laura Hebberecht López, Fletcher Young, Jamie Musgrove, Lina Gabriela, Edgardo Santiago, Sebastian Mena, Kelsey Byers, Luca Livraghi, Leila Shirai, Gabriela Montejo-Kovacevich, Andre Freitas, Gilson Moreira, Mathieu Joron and Chris Jiggins.

Various students have contributed to this project during undergraduate or MSc projects, including Christian Alessandro Perez, Jarreth Caldwell, Marina Perbone Leon, Lauren Fryer, Mizwar Mukhlis and Edd Page. I am grateful for their work and the experience I had in overseeing it.

Finally, I would like to thank my examiners, Violaine Llaurens and Calvin Dytham for taking the time to read through my thesis. They provided important insight to all aspects of the work, from experimental design and analysis to the writing and ideas included in these pages. Discussing my project with them during my viva was very enjoyable (despite being done remotely!) and I would have happily kept going for longer!

Finally, on a more personal note, I could not have done this PhD without my parents' endless support and unwavering faith in my abilities, they have always encouraged me to pursue my dreams and how far I've come is a testament to their parenting. My brother and sister, who are constant sources of inspiration, have been cheering me on since the beginning, helping me with both the PhD and life alongside it. Finally, friends in York and from further afar have accompanied me throughout this PhD, and words cannot express how grateful I am to them for their support and patience.

Declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as references. Where the work that contributed to this thesis was undertaken by someone else this has been indicated below:

Landmark placement and wing measurements on scanned wing images for the phylogeny chapter were carried out by myself and two undergraduate students from the University of York, Marina Perbone Leon and Lauren Fryer. Landmark placement for the QTL chapter was carried out by Mizwar Mukhlis during an undergraduate project at the University of York.

RAD library preparations were carried out by myself, Bruna Cama, Michaela Nelson and Kanchon Dasmahapatra at the University of York.

Linkage map generation was carried out by Neil Rosser (Harvard) and Kanchon Dasmahapatra (University of York). Neil Rosser advised on the QTL analysis and contributed a custom script to calculate and combine LOD scores from families separately.

Chapter 1

Introduction

1.1. The Ecological Context of Speciation and Adaptation

A major focus of the study of evolutionary biology is understanding how diversity and species arise through the processes of adaptation and speciation. In the past 50 years, evolutionary biologists have moved away from geographical based modes of evolution in light of the abundant evidence for divergence with gene flow (Smadja & Butlin, 2011). Today, it is accepted that populations and species lie on a continuum of divergence and that natural selection plays an important role in driving speciation (Schluter, 2009). In this section, I will briefly review the alternative modes of speciation, and discuss the role of ecological adaptation in driving species diversity.

1.1.1. Mechanisms of Speciation

Speciation can broadly be understood as arising from two processes, selection and neutral, stochastic evolution (Coyne & Orr, 2004; Nosil, 2012). Stochastic processes involve mechanisms such as the build-up of Dobzhansky-Müller incompatibilities (DMI), as well as models of speciation by drifts. Dobzhansky-Müller incompatibilities arise from natural accumulation of mutations through time in different populations (Dobzhansky, 1936; Müller, 1942; Orr & Turelli, 2001). Theoretical models of the evolution of DMIs have been met with a large amount of empirical evidence, especially in the *Drosophila* system (Coyne & Orr, 2004) but also in a number of other plant and animal systems (Coyne & Orr, 2004; Welch, 2004). Models of speciation by drift, however, have received less support, in part due to the theoretical difficulty of drift overcoming selection across adaptive troughs to reach new adaptive peaks (Coyne & Orr, 2004; Turelli *et al.*, 2001).

Recently, evolutionary biologists have moved away from stochastic modes of speciation with a renewed interest in the role of natural selection, as first described by Darwin (1859). Speciation from selection can be categorised by two processes,

ecological speciation and mutation order speciation. Mutation-order speciation occurs when two isolated populations in similar environments accumulate different adaptive alleles which lead to genetic incompatibilities, essentially leading to DMIs; divergence from new adaptive mutations is also less sensitive to population size, unlike drift which weakens with growing population (Mani & Clarke, 1990). Laboratory experiments (Cohan & Hoffmann, 1989; Lenski *et al.*, 1991) have demonstrated the potential of mutation order speciation. However, it is difficult to conclusively attribute examples in nature to mutation order speciation as species could be responding to undetected divergent aspects of habitats (Nosil, 2012; Schluter, 2009).

In contrast, under a process of ecological speciation, divergence occurs in response to selection in different habitats, known as adaptive divergence; divergent selection then leads to reproductive isolation when mating is closely linked to habitat, differences in mating preferences, or intrinsic and/or extrinsic selection against hybrids (Nosil, 2012). These isolating mechanisms act both at the pre-zygotic and post-zygotic stage and complete isolation occurs as a result of the accumulation of these barriers (Sobel & Chen, 2014). For example, evidence for ecologically driven divergence was found between two sympatric *Heliconius* butterfly species, *H. elevatus* and *H. pardalinus*, displaying strong pre-zygotic isolation; divergence was identified across a suite of important ecological traits including host preference, colour pattern preference during mate choice, and pheromones, all considered to be involved in overall isolation (Rosser *et al.*, 2019).

Different mechanisms of speciation are not necessarily mutually exclusive, and more than one process can be involved at the same time or at different stages of divergence. For example, in a comparative analysis across 17 species of *Anolis* lizards, authors determined the relative contributions of isolation-by-distance (increased divergence with increasing geographical distance or presence of geographical barriers) and isolation-by-environment (increased divergence with increasing ecological dissimilarity) to neutral genetic divergence. The results found that both mechanisms contributed to divergence, however, geographical distance and physical barriers contributed more to genetic divergence (Wang *et al.*, 2013). Sexual selection is also a type of selection that acts to increase reproductive fitness and is not necessarily considered a stand-alone process of speciation but can act both as part of mutation order and ecological speciation (Schluter, 2009). Sexual selection falls within ecological speciation if the traits under sexual selection also

confer a selective advantage in their respective environments. For example, the cichlid fish of Lake Victoria have evolved in different light environments from varying levels of turbidity in the water, and species from the different environments were found to have different sensitivities to coloured light which coincide with female driven assortative mating in response to different male colourations (Maan *et al.*, 2006). However, despite the potential involvement of different mechanisms, natural divergent selection and ecological adaptation are widely agreed to play a major role in shaping species differences.

1.1.2. Ecological Adaptation

Adaptive divergence is an important driver of phenotypic disparity and speciation and is driven by many factors. Populations and species are known to adapt to abiotic factors which can lead to populations adapting to their local environment such as the rock pocket mouse, where strong selection for camouflage maintains the polymorphism across different substrate colours (Hoekstra *et al.*, 2005). Abiotic factors vary across climates and geography whereas biotic factors include all interactions with other organisms, such as competition, predation, and host use. Insects provide many examples of divergent adaptation to biotic factors due to their strong associations with host plants, with textbook examples in aphids (Via, 1999), stick insects (Nosil, 2007), and races of the apple fly (Feder *et al.*, 1998). Different factors may also interact in shaping adaptive divergence and studying adaptation requires untangling these effects.

Understanding adaptation also requires identifying the factors that limit the effect of selection. Adaptation occurs as a result of selection acting on phenotypes underlined by standing genetic variation or new mutations; however, different genetic factors may limit adaptation (Futuyma, 2010). For example, populations may lack suitable genetic variation and mutations may be rare; furthermore, complex phenotypes may be underlined by many genes and new mutations may not have large enough effects or only have epistatic effects (Futuyma, 2010). Other sources of constraint are phenotypic and genetic correlations or covariations of traits. Developmental constraints can also limit adaptation when traits are determined by common ontogenetic processes (Arnold, 1992). In these cases, selection for one trait may be too weak to overcome selection for another. Conflicting selective pressures can even

act on single traits, the *Eurosta* fly gall size represents a trade-off between chances of parasitism and bird predation, larger galls are less parasitized but more easily detected by birds (Weis & Gorman, 1990).

Adaptive radiations provide an excellent system to study adaptation. They are characterised by the rapid evolution of phenotypic disparity within a single lineage ultimately leading to an array of species or ecologically distinct forms, adapted to a variety of environments (Schluter, 2000). Classic examples include the radiation of Darwin's finches in the Galapagos, where different beak shapes have evolved in response to different food sources (Grant & Grant, 1989), or the radiation of cichlids in the East African Great Lakes, where habitat divergence led to natural selection driving divergence in mouth morphology and sexual selection driving colouration (Kocher, 2004).

Identifying potential covariation between traits and understanding how they respond to their environment is very important to understand adaptation. For example, in the stickleback radiation, evidence of adaptation has been reported across different environments in populations inhabiting lakes or streams, or exploiting different resources such as benthic and limnetic populations (McKinnon & Rundle, 2002). Recent genomic analyses have even demonstrated the importance of standing genetic variation and chromosomal inversions in marine and freshwater environments, enhancing our understanding of adaptive evolution (Jones *et al.*, 2012). However, in Norwegian populations of stickleback, convergent evolution of phenotypes in similar environments, suggesting adaptation, was explained by allometric relationships between traits (Voje *et al.*, 2013). In another study of sticklebacks occupying varying lake environments from different watersheds in Canada, convergence between morphological traits varied a lot, being absent in certain populations and occurring at relatively high levels in others (Kaeuffer *et al.*, 2012). The authors suggested this could represent a lack of divergent selection acting on traits or could be a result of a failure to measure the appropriate ecological variable driving trait adaptation. Therefore, a thorough understanding of the ecology and habitat of organisms, as well as correlations between traits, is necessary to understand patterns of evolution and adaptation.

1.1.3. Methods in studying role of ecology during divergence

There are broadly two approaches to understand evolution and particularly the role of ecology during divergence. The first, is a “real time” approach, where evolution can be observed “in action”. This can occur through experimental set ups in the laboratory where selection to different conditions are measured, which forms the basis of experimental microbial evolution. This allows the study of evolution in controlled condition, and clear conclusions can be drawn on the cause and consequence of observed processes. However, extrapolating findings to natural populations can be tricky as many more factors are involved in the wild, and such experiments are generally restricted to organisms with very short generation times.

Real time evolution can also be observed in the wild, where adaptation to natural changing conditions are measured over long periods of time. A classic example is that of the peppered moth, *Biston betularia*, in which the melanic form gained fitness advantages over the pale morph during the industrial revolution (Kettlewell, 1955, 1956), resulting in an increase in frequency which reversed after the Clean Air Act in 1956 (Clarke *et al.*, 1985). Another example from a study that spanned over only 30 years, is that of a population of Edith's checkerspot butterfly, *Euphydryas editha*, which adapted to a new, human introduced host ribwort plantain, *Plantago lanceolata*, and eventually went extinct as a result of land use change (Singer & Parmesan, 2018).

Such systems are ideal for understanding evolution in the context of ecology but in general such studies are difficult as examples of such rapid evolution are rare and require long periods of monitoring. The second approach to understanding the role of ecology in evolution is to decipher past processes through the interpretation of present patterns in nature; however, it can be difficult to disentangle the different mechanisms involved, and the timing and relative role of each. In this thesis, I will focus on this second approach, which can be applied at every level of organisation, from the molecular to the macro-scale.

At the macroscale, ecomorphological studies have made huge strides with the development of methods that incorporate phylogenies to understand the relationship between phenotypes and their environment (Losos & Miles, 1994). These types of studies have investigated ancestral states of phenotypes, the number of times traits have evolved, patterns of evolution and adaptation, the role of common ancestry and

selection, as well as correlated evolution of traits (Losos & Miles, 1994). One assumption often made by these analyses is that convergence of phenotypes between species or populations subject to similar pressures is evidence of adaptation (Losos, 2011; Losos & Miles, 1994), such as the striking convergence of Arctic animals in their white pelage.

However, there are conditions under which phenotypes can repeatedly evolve in different population without a role of adaptation (Losos, 2011), either by chance or shared constraints on the production of variation, sometimes referred to as phylogenetic niche conservatism (Losos, 2008). Repeated evolution of webbing across *Bolitoglossa* salamander species was investigated to assess the role of adaptation in driving this trait; morphometric analyses of foot shape revealed conserved allometric relationships between webbing and body weight across all but one species (Jaekel & Wake, 2007). Therefore, functional adaptation of webbing was evident in only one species but was found to be a result of developmental pathways held in common with other traits in the other species. Non-adaptive convergence of traits can arise if correlations exist between the studied trait and a trait under selection. Adaptation is still involved, but the trait under investigation is not directly under selection. The incorporation of phylogenetic information to statistical methods can help identify whether associations between phenotypes and habitat arose by chance or as a result of other processes (Losos, 2011). Studies using a phylogenetic framework can, therefore, provide important information on patterns of evolution of traits across species in different habitats. However, it is important to inform such analyses with tests for selection at the species or population levels.

To understand ecological divergence, it is important to confirm that phenotypes have indeed evolved in response to divergent selection. Several types of experiments can be used to answer such questions. For example, reciprocal transplant experiments or common garden experiments have been used for decades as tests for adaption to environments (Nosil, 2012). In guppies, crypsis is more strongly selected for in individuals from habitats with predators, but increased conspicuousness is selected for mating in habitats with no predation. Using common garden experiments, fish in ponds with predators were found to display fewer spots than fish in predator free environments after several generations (Endler, 1980). Experiments identifying the effects of phenotypic variation on fitness can help determine a role of selection, such as the importance of hind leg length on sprint speed in Phrynosomatid lizards (Bonine

& Garland, 1999) or varying crushing abilities of labrid fish jaws (Wainwright, 1991). Evolution requires that phenotypes under selection be underlined by heritable genetic variation, therefore, understanding the genomic and genetic basis of phenotypic variation can further inform us on the role of selection.

We can understand the role of ecology in evolution and past processes by investigating the underlying genomic architecture of traits. For adaptive divergence to lead to reproductive isolation, associations need to be made between traits under divergent selection and traits involved in reproductive isolation (Nosil, 2012; Smadja & Butlin, 2011). Associations can be a result of particular characteristics of the gene causing isolation, such as pleiotropy (when one gene affects two traits), physical linkage between genes, or even as a result of particular features of the chromosomes such as chromosomal inversions, translocations, centromeres or sex chromosomes (Feder *et al.*, 2013). Traits under divergent selection involved in assortative mating, known as "magic traits", where both natural and sexual selection are acting (Servedio *et al.*, 2011) overcome the need for strong associations between traits. These associations between traits can lead to areas of divergence, sometimes referred to "islands of divergence" which build up and eventually lead to divergence across the genome (Via, 2012).

Incomplete lineage sorting in recently diverged species pairs can also results in variation in levels of divergence across the genome; and selective sweeps of new mutations can rapidly reduce genetic variation at specific loci which therefore appear strongly diverged (Cruickshank & Hahn, 2014). Formal measurements of divergence and levels of gene flow are necessary to discriminate between these different processes. Evidence of selective sweeps, or high ratios of non-synonymous to synonymous mutations are also genomic patterns used to identify areas under selection (Vitti *et al.*, 2013), however, these patterns are not specific to ecological adaptive divergence. Overall, the array of methods used to study evolution at different levels of organisation are complimentary and using multiple approaches are needed to confidently answer questions on the role of ecology during divergence.

1.1.4. Mimicry

Mimicry is a well-known example of convergence. Bates first identified the existence of mimicry, whereby individuals benefit from protection when displaying patterns or

colours similar to that of a co-occuring, defended species, known as Batesian mimicry (Bates, 1862). This was further studied by Müller, who differentiated the case where mimicry occurs between two defended species and therefore individuals from both can benefit from sharing the cost of predation, thereafter, known as Müllerian mimicry (Wallace, 1882). Mimicry is found across a range of taxa in many different forms; in many harmless snake species, the colours are similar to those observed in the coral (from the genera *Micrurus* and *Leptomicrurus*), and avoidance of these colours has even been shown to be innate in certain bird species (Smith, 1975) clearly demonstrating a benefit of Batesian mimicry. The evolution of cryptic mimetic patterns is also frequently observed; many insects can be mistaken for twigs or leaves to the untrained eye, with fossil evidence of a katydid species showing that this type of mimicry dates back to the Permian (Garrouste *et al.*, 2016). Furthermore, mimicry is not limited to the animal kingdom, mimicry is also observed in many plants, whereby shapes and colour attracts pollinators or deter herbivors (Barrett, 1987)

Mimicry is also not limited to visual signals and can also be found in auditory and olfactory cues. Furthermore, the mimetic signal can extend to multiple traits as a more complex signal will not only be easier for predator to learn but also be harder for Batesian mimics to copy (Srygley, 1999). For example, in tests carried out on flower preference in bumble bees, generalisation of the model to the mimetic, non-rewarding flower, was greater when visual cues were accompanied by scent cues (Kunze & Gumbert, 2001). In the wild, there are varying levels of complexity of mimetic signals, ranging from simple colour pattern mimicry such as in the coral snakes (Greene & McDiarmid, 1981) to examples such as in the parasitoid wasp, *Gelis agilis*, in which morphology, behaviour and scent are under selection for mimicry of the black garden ant, *Lasius niger* (Malcicka *et al.*, 2015).

Both Batesian and Müllerian mimicry depend on encounter rates and learning ability of predators but involve very different evolutionary processes. In Batesian mimics, mimicry is under negative frequency dependent selection; an increasing number of harmless individuals mimicking the signal will decrease the protection provided by the warning signal.. This leads to an evolutionary “arms race” between mimics and models, the latter which does not benefit from this parasitic mimicry (Joron & Mallet, 1998). Müllerian mimicry, on the other hand, is driven by positive frequency dependent selection; both model and mimic benefit from sharing the costs of predation. Frequency dependent selection was empirically shown in an experiment

using models of Müllerian mimetic *Heliconius* butterflies to measure attack rates on different morphs (Chouteau *et al.*, 2016). The results showed a clear effect of abundance on attack rate, rarer individuals suffering more from predation. This effect was not due to inherent signal quality as the same patterns suffered different levels of predation across localities, depending on their local abundances.

Differences between Müllerian and Batesian mimicry also lead to different expectations on the evolution of diversity of colour pattern between and within mimetic species. For Batesian mimicry, diversity in mimetic patterns is expected to occur as a result of the negative dependent selection; high frequency of mimics of one pattern will suffer from a diluted signal and strong selection will favour new mimetic patterns appearing in undefended populations (Joron & Mallet, 1998; Mallet & Joron, 1999). However, in Müllerian mimicry, new mimetic patterns are expected to suffer from higher predation. New patterns arising within Müllerian will be unknown to predators and strongly selected against. However, in the *Heliconius* system, a genus butterflies with bright aposematic colour patterns, species of Müllerian mimics display a variety of different patterns, both within and between species, with co-occurring patterns within localities (Joron & Mallet, 1998; Mallet & Joron, 1999). Variation in population density and geographic and temporal variation in pressure from predation could allow novel phenotypes to arise and be maintained enough to then undergo positive frequency section (Mallet, 2010; Mallet & Joron, 1999) and strong frequency dependent selection may ease shifts across geographical locations (Chouteau *et al.*, 2016). Finally, the experiment using models of different morphs found that over a certain threshold of abundance, predator learning was “saturated”, therefore weakening selection for convergence of the more abundant patterns; this can help explain the co-occurrence of several mimetic patterns in a given location (Chouteau *et al.*, 2016).

An interesting case of intra-specific variation in mimicry is sex-limited mimicry (where only one sex is mimetic) driven by sexual selection or differential natural selection between the sexes (Joron & Mallet, 1998). Sex-limited mimicry, specifically female-limited mimicry, is mostly found in Batesian mimics in butterflies (Mallet & Joron, 1999), although examples exist such as in *Chrysobothris humilis*, a species of beetle (Hespenheide, 1975), which is an unusual case of male limited mimicry. Sexual selection is usually considered as an important driver of female limited mimicry, either through female choice or male-male competition (Ohsaki, 2005). However, spatial

segregation, different behaviours due to life history or physiological trade-offs (for example, aerodynamic constraint from egg load) may also lead to sex limited mimicry if males and females are subject to different selective pressures (Kunte, 2009). For example, evidence of higher predation rates was found in the non-mimetic female morph of *Papilio polytes*, compared to the mimetic females, non-mimetic males and the model, *P. aristolochiae* (Ohsaki, 1995), suggesting unequal predation pressures between males and females.

Sexual dimorphism is not expected to occur in Müllerian mimicry as selection should favour mimicry between the sexes as well as between co-mimics. However, from the observation that males and females of *Heliconius numata* fly at different heights in the canopy, Joron (2005) presents an extension of Müller's model of mimicry which includes partial behavioural or spatial mimicry between males and females. Results from the model show that habitat segregation between the sexes can promote mimicry by reducing the proportion of (female) mimics within the model's habitat, thereby increasing the benefits of mimicry in the mimetic sex, outweighing the costs of weaker or non-existent mimicry in the other sex (in this case, the males). Therefore, the same factors driving sexual dimorphism in Batesian mimics, could also be causing sexual dimorphism, albeit more subtly, in Müllerian mimics. Although sexual dimorphism in colour pattern is generally rare in *Heliconius* (Brown, 1981), differences in habitat and behaviour may translate to differences in flight behaviour (which will be discussed later), which may in turn be involved in the mimetic signal. Interestingly, H. numata females appear to have slightly larger black patches (*personal communications*, Llaurens) and the species *Heliconius demeter* and *Heliconius nattereri* do display sexually dimorphic colour patterns, coupled with sexually dimorphic wing shapes in *H. demeter* (no data is available on wing shape in *H. nattereri*) (Rosser *et al.*, 2019). Furthermore, males and females of *H. nattereri* has been reported to have different flight habits (Brown, 1981). Different foraging behaviours and energy expenditure related to flight were found in *Heliconius charithonia* between males with different mating strategies, and females (Mendoza-Cuenca & Macías-Ordóñez, 2005). Little is still known about the intraspecific dynamics that shape mimicry, and traits involved in mimicry may be found to be constrained by other factors, even between males and females of Müllerian mimics.

1.2. Flight and wing shape

Flight is believed to have been one of the main drivers of insect diversification, enabling the colonisation of new ecological niches (Dudley, 2002). Flight is energetically costly trait but can confer many fitness benefits such as further dispersal, access to a wider range of food sources, and increased escape ability from predators among other things. Flight is also a complex trait, driven by anatomy, physiology and wing shape (Dudley, 1990), and many studies have focused on the role of wing shape for flight performance (for example, Betts and Wootton, 1988; Srygley, 1994, 1999; Berwaerts *et al.*, 2002). Understanding the evolution of wing shape means understanding how wing morphology correlates with anatomy and physiology to drive flight, but also requires understanding the suite of ecological factors that have driven wing shape morphology. Indeed, wing shape has been shown to be under selection from a number of ecological drivers, usually resulting in trade-offs between different functions.

1.2.1. Wing aerodynamics during flight

Flapping wings are an excellent example of convergence for driving flight across insects and vertebrates, such as birds and bats. One common mechanism used by insects and vertebrates during flapping flight is leading edge vortices, which are vortices that form on the leading edge of wings at high angles of attack (Chin & Lentink, 2016). This unsteady mechanism was thought to be restricted to insects, however, airflow measures in wing tunnel studies have identified leading edge vortices during flight in bats (F. Muijres *et al.*, 2008) and birds (Muijres *et al.*, 2012); leading edge vortices were also identified using swift wing models in water tunnels (Videler *et al.*, 2004).

However, there are considerable differences between insect flight and vertebrate flight. Insects bodies are, on average, substantially smaller than those of vertebrates, and insects fly at slower speeds with higher wing beat frequencies (Dudley, 2002). These differences mean that insect bodies are subject to very different forces of drag and lift than vertebrates (Chin & Lentink, 2016). Another important difference is that bird and bat wings include bones and muscles that can be actively deformed, whereas insect control wing deformation solely from muscles at the wing base

(Dudley, 2002). Apart from hummingbirds, whose flight resembles that of insects (Warrick *et al.*, 2005), vertebrate wings generally flex during the up-stroke meaning that most of the lift generated occurs during the down-stroke (Chin & Lentink, 2016). In contrast, insects use complex wing motion, involving rotation (Dickinson *et al.*, 1999) to generate lift during both the up and down-stroke using horizontal stroke-plane.

Measures of wing beat frequency (using high speed cameras) and other flight related morphologies in species across different insect orders have shown strong phylogenetic clustering of these traits, suggesting that variation in flight (and associated traits) is strongly linked to evolutionary history (Tercel *et al.*, 2018). This study also confirmed associations between wing loading and wing beat frequency in insects. However, despite common trends, insect wing morphology and flight can vary in many ways.

Insect flight can be bimotoric, such as in Odonata (dragonflies and damselflies) where both the forewing and hindwing are involved in driving flight (Dudley, 2002). Flight function can be lost to different extents in one of the wings resulting in anteromotorism (forewing driven flight) or posteromotorism (hindwing driven flight) (Dudley, 2002). This is reflected in the morphology of the wings; with insects displaying homonomous wings where both forewing and hindwing retain similar morphologies and function, or heteronomous wings, which are more differentiated. Examples of these different types of flight and associated wing morphologies span across a continuum. At one extreme, the Diptera display anteromotorism with hindwings reduced to halteres, whereas Coleoptera use posteromotorism with forewings having evolved into protective elytra. In Lepidoptera, flight is anteromotoric, however, the wings still form a continuous plane and act mostly in synchrony (Le Roy *et al.*, 2019), and the hindwing retains some aerodynamic function. Manipulations in moth and a butterfly species have demonstrated that flight could not be sustained without the forewing and when the hindwing was removed flight ability was maintained but manoeuvrability was affected (Jantzen & Eisner, 2008). The diversity of insect morphology, size and behaviour has resulted in different flight behaviours such as gliding, flapping or hovering, all of which are subject to different aerodynamic expectations (Dudley, 2002).

Flight in Lepidoptera is characterised by flapping and gliding, used for different types of behaviour and to different extents across taxa (Le Roy *et al.*, 2019). Gliding flight

is common and is usually associated with more sustained, long distance flying as it is energetically less costly than flapping flight. Wings with higher aspect ratios (more elongated wings) are generally thought as being better for gliding flight, whereas lower aspect ratios (rounder wings) are usually associated with increased manoeuvrability during flapping flight (Betts & Wootton, 1988; DeVries *et al.*, 2010). Clear evidence for this exists for example in migratory and non-migratory populations of Monarch butterflies; non-migratory populations had smaller and rounder forewings and these differences were maintained in common garden experiments, which suggests this is genetically determined (Altizer & Davis, 2010).

Flapping flight is important for other behaviours such as take-off, hovering and climbing (Le Roy *et al.*, 2019), and is more difficult to explain using steady state aerodynamic mechanisms (Dudley, 2002). The use of "quasi-steady" models found an important role for moments of area during flapping flight, where larger proportion of area away from the base of wing produced stronger lift forces (Ellington, 1984; Le Roy *et al.*, 2019). However, all the properties of wing shape and size act in synchrony with other anatomical and physiological aspects of insects and cannot be considered independent of each other. In an experiment measuring the flight performance of *Drosophila* flies with genetically modified wings, flight agility improved in several mutants, albeit at the cost of flight efficiency likely due to a trade-off between the aerodynamic and mechanical aspects of flight (Ray *et al.*, 2016). In another study across species in the Heliconiini tribe, a group of neotropical butterflies, strong correlations were found between the 2nd moment of area and the position of centre of body mass; in turn, the position of the centre of body mass in relation to the wing base was proposed to be important for flight manoeuvrability (Srygley, 1994; Srygley & Dudley, 1993). Therefore, wing shape alone does not explain differences in flight.

1.2.2. Non-aerodynamic drivers of flight and wing shape

A wide variety of wing shape morphologies can be observed in insects today, often reflecting trade-offs between different pressures from habitat, behaviour and energetic costs (Le Roy *et al.*, 2019). Ecomorphological comparative analyses have played an important role in identifying patterns of divergence in wing shape and flight metrics; a first step towards understanding the selective drivers at play. Large scale changes across latitudes and elevations have been shown to be an important driver

of wing shape divergence (Klepsatel *et al.*, 2014; Montejo-Kovacevich *et al.*, 2019; David Outomuro & Johansson, 2011). Micro scale habitat segregation can also lead to differences in wing shape; higher aspect ratios were found in *Morpho* species associated with more sustained flight in the canopy compared to understory species, and wing morphologies associated with gliding flight in Haeterini butterflies were found for species using "ground effect" gliding flight (Cespedes *et al.*, 2015). However, differences between habitats can also reflect differences in other aspects of insect ecology. For example, changes in host or food density may affect flight requirements for foraging throughout the day, and equally, food type affects nutrient intake, which may in turn limit the energy spent during flight (Le Roy *et al.*, 2019).

Other behaviours such as migration have also been shown to affect wing shape, as long-distance flight will necessarily have a high energetic cost. As mentioned earlier, migratory populations of Monarch butterflies have more elongated wings (Altizer & Davis, 2010) as expected for more efficient flying in Lepidoptera (Le Roy *et al.*, 2019). However, in ecomorphological analyses of dragonfly wings, migratory species did not fit this prediction, instead, the greatest difference for these was the presence of a lobe in the hindwing (Johansson *et al.*, 2009). Dragonflies have very different flight mechanics, using both forewing and hindwing that can beat out of phase, and these differences may explain different adaptation to similar pressures. Therefore, understanding taxonomy specific anatomy and aerodynamics is essential when studying the drivers of wing morphology.

In some species of insect, the wings may also play an important role in signalling, particularly to predators. Bright colour patterns can be used to signal unpalatability or conversely, be used for crypsis. These different behavioural strategies for predator avoidance have important repercussions for flight and wing shape. Studies across a range of palatable and unpalatable butterflies measured body and wing morphologies consistent with slower, less erratic flight in the unpalatable species; potentially allowing easier capture that minimises damage, giving a chance for predators to "taste" the toxins and release the prey (Srygley, 2004). These results were consistent with other tests showing that palatable butterflies were better at evading predators (Chai & Srygley, 1990). These different adaptations to predators also had important consequences for relative mass distribution in the abdomen and thorax. Controlling for phylogeny, measures across neotropical butterflies identified correlations with palatability that were positive for thoracic mass and negative for

abdominal mass (Srygley & Chai, 1990). Therefore, it seemed that there was a trade off in palatable butterflies, investing more in thoracic mass to increase evasiveness at the expense of abdominal mass, associated with food storage and reproduction. Differences in mass allocation were also found between sexes, reflecting different constraints from reproduction. Therefore, complex interactions of multiple ecological factors shape wing morphology.

1.2.3. Measuring flight and wing shape

Measuring flight and wing shape presents different sets of difficulties. Many studies of flight in insects have used tethered individuals or wind tunnels, which are questionable in terms of how well they represent natural behaviour (Le Roy *et al.*, 2019). Studies in large insectaries are supposedly better to measure natural flight behaviours; still, these may not be perfectly representative of natural conditions as suggested by a study comparing flight speeds of butterflies in large insectaries compared to butterflies released over a lake and followed by a boat (Dudley & Srygley, 1994). Furthermore, flight can be measured using different variables, such as wing beat frequency, flight speed and wing amplitude, flight trajectories, and flight can vary depending on different behaviours (Srygley, 2007). These different aspects of flight may be correlated, for example we know that flight speed is a product of wing beat frequency and wing loading (Tercel *et al.*, 2018). However, flight parameters may have evolved independently in response to different selective pressures. Therefore, the choice of flight measurement used in a study may have big impacts on the patterns observed. Nevertheless, the development of high-speed cameras has allowed a better quantification of flight parameters both in laboratory condition and in the wild, and new multi-camera videography methods are also being used to better understand flight trajectories of complex behaviours (Le Roy *et al.*, 2019).

While wing shape is easier to quantify, such quantification has also benefitted from the development of new methods. Often, wing shape is reduced to univariate traits using Fourier analyses, area, length or aspect ratio. However, these have been criticised as inadequate for understanding complex traits such as wing shape (Betts & Wootton, 1988). Geometric-morphometrics use landmark-based methods to quantify complex morphological traits, and although results may depend on landmark choice and placement, they allow thorough quantifications of subtle shape changes

(Klingenberg, 2010). Furthermore, the development of multivariate alternatives to ecomorphological comparative analyses has meant that we can better understand the evolution of complex morphological traits, such as wing shape (Klingenberg, 2010).

Measuring the effects of wing shape on flight includes all the difficulties of quantifying the traits separately with the added challenge of identifying the causal relationship between the two. Correlations have been identified between flight and wing shape using broad measures such as the effect of wing area on flight (Tercel *et al.*, 2018); however, few studies have investigated the role of flight on wing shape using multivariate methods. An association between wing shape measured using geometric morphometrics and escape ability was attributed to differences in flight in damselflies (Outomuro & Johansson, 2015), but without a clear causation. Other studies looking at subtle changes in wing shape have generally looked for associations between different ecological factors (Le Roy *et al.*, 2019). More studies are required to identify causal links between wing morphology and flight. Several studies have investigated the direct effect of wing damage on flight in butterflies (Fernández *et al.*, 2014), and although informative, these studies do not investigate the selective advantage of subtle changes in wing shape between species.

1.3. The *Heliconius* System

Part of Heliconiini tribe, the *Heliconius* genus represents a rapid and recent adaptive radiation of 46 species that split from the *Eueides* genus about 10.5-13.4 Mya (Kozak *et al.*, 2015). The publication of two reference genomes and the amenability of *Heliconius* for large scale genomic analyses has made possible the use of advanced genetic methods, developed in model systems, to answer questions in wild populations. This, coupled with abundant information on their ecology gathered for over 150 years, has allowed evolutionary biologists to investigate the role of ecology during species divergence, the mechanisms at play during the early stages of species divergence, and the role of hybridisation and introgression in speciation. In these next sections, I will review our knowledge of the ecology and genetics of *Heliconius* and highlight some of the areas that need further investigation.

1.3.1. The ecology of *Heliconius*

The *Heliconius* genus has been studied for over 150 years, having first been noticed for their striking wing pattern mimicry. Toxicity has evolved in *Heliconius* species, crucial in Müllerian mimicry, though natural production and sequestration from their host plants the *Passiflora* (Merrill *et al.*, 2015). The *Heliconius* species display a variety of colour patterns, which are somewhat partitioned geographically (Mallet, 1993). The diversity of colour patterns can also be seen within species, for example *Heliconius melpomene* and *H. erato* include subspecies with patterns belonging to several different mimicry rings and the mimics from the two species co-occur across their distribution (Hines *et al.*, 2011; Rosser *et al.*, 2012). Spectacular polymorphism is also observed within *H. numata*, with patterns mimicking those of different Ithomiine species, although in this case the patterns are generally referred to collectively as the silvaniform mimicry ring (Brown, 1981).

Colour pattern has also been shown to be involved in mate choice with a multitude of studies now showing assortative mating in response to paper models or using wings with pheromones washed off. Conspecific preference was observed in experiments between *H. melpomene* and *H. cydno* (Jiggins et al,, 2001), between *H. cydno* and *H. pachinus* (Kronforst *et al.*, 2006), in *H. heurippa* (Mavárez *et al.*, 2006), between *H. elevatus* and *H. pardalinus* (Rosser *et al.*, 2019) and even among races of *H. melpomene* (Jiggins *et al.*, 2004) and *H. erato* (Muñoz *et al.*, 2010). The dual role of wing patterns in survival and mate choice has granted it the term of “magic traits” (Merrill *et al.*, 2015), however, other traits are involved in mate choice, as evidenced by the fact that sympatric mimetic species do not interbreed (Mérot *et al.*, 2017). Pheromones likely play an important role in *Heliconius* mate choice, and mate preference experiments between mimics of *H. melpomene* and *H. erato* showed that *H. erato* was able to discriminate between the two, probably using scent as a cue (Estrada & Jiggins, 2008). Furthermore, experiments blocking the pheromone release from androconial regions in males showed that these were discriminated against by females compared to controls (Darragh *et al.*, 2017).

Although famous for their colour pattern, *Heliconius* display a fascinating array of ecological traits. For example, the *Heliconius* are unique among butterflies in their ability to assimilate amino acids from pollen feeding (Gilbert, 1972). This likely explains their surprising longevity and ability to sustain a stable rate of egg production

throughout their lifetime. *Heliconius* butterflies also exhibit “trap-lining” behaviour, where males have been observed visiting the same host plants sequentially in search of mates and some species also perform communal roosting (Finkbeiner *et al.*, 2012; Gilbert, 1975). Evidence suggests that plasticity in *Heliconius* brain development plays a role in these complex behaviours (Merrill *et al.*, 2015). Indeed, comparisons between reared and wild caught butterflies as well as between young and old individuals showed significant age and experience dependent expansion of the mushroom bodies, a region of the brain involved in learning and memory (Montgomery *et al.*, 2016).

1.3.2. Genomic resources in *Heliconius*

Recent advances in molecular genetic techniques have allowed huge advances in our understanding of the genetics of wild populations as genetic information can now be extracted from smaller amounts of genetic material and fewer individuals. With these advances, information about the ecology has been integrated into studies of *Heliconius* genetics to understand these interact and drive evolution, however, most of this work has focused on the colour patterns. The publication of two reference genomes in *H. melpomene* (Dasmahapatra *et al.*, 2012; and the updated version, Davey *et al.*, 2016) and *H. erato* (Van Belleghem *et al.*, 2017), as well as the recent publication of 20 de novo genome assemblies (Edelman *et al.*, 2019) has allowed genome wide studies across the phylogeny. Studies in *Heliconius* have been able to show the role of introgression between pairs of species (Dasmahapatra *et al.*, 2012; Martin *et al.*, 2013) and more recently across the phylogeny (Edelman *et al.*, 2019; Kozak *et al.*, 2018), with evidence of adaptive introgression of important colour pattern genes (Wallbank *et al.*, 2016; Zhang *et al.*, 2016), underlying the importance of gene flow during speciation.

Research in *Heliconius* has also shown ways in which species can resist the homogenising effects of gene flow with the example of the *P* supergene in *H. numata* (Mathieu Joron *et al.*, 2011). The *P* supergene is a chromosomal inversion which includes allelic combinations of colour pattern genes that maintain the numerous polymorphisms of the species. These types of chromosomal inversions are not necessarily ubiquitous in *Heliconius* (Davey *et al.*, 2017) and other factors may be involved. Other research in *Heliconius* has focused more on the role of islands of

divergence, with a study in different pairs of *H. melpomene* and *H. timareta* finding increasing numbers of peaks of divergence with increasing levels of divergence (Nadeau *et al.*, 2012). Some of these peaks were also found to coincide with known colour pattern genes, suggesting these regions are under strong selection.

Major colour pattern loci have been identified in *Heliconius*, most famously the *WntA*, *cortex* and *optix* loci on chromosomes 10, 15 and 18 respectively (Martin *et al.*, 2012; Nadeau, 2016; Reed *et al.*, 2011). These loci control important aspects of colour pattern, such as red forewing band and dennis presence (*optix*) or yellow band presence (*cortex*) in *H. melpomene* and *H. erato,* as well as yellow and red band shapes in *H. melpomene*, *H. erato* and *H. cydno* (see Merrill *et al.*, 2015; Morris *et al.*, 2019 for further detail). These colour pattern genes have major effects on phenotype variation and represent genomic hotspots of adaptation in both the *H. melpomene* (Baxter *et al.*, 2010) and the *H. erato* (Counterman *et al.*, 2010) clades, displaying signatures of selection acting over long periods of time with ongoing gene flow. Furthermore, these loci are characterised by narrow peaks of reduced recombination. Male colour preference for red in females has been found to be associated with the *optix* loci (controlling red pattern elements) showing how important these regions are for speciation (Merrill *et al.*, 2019; Merrill *et al.*, 2011). Mate preference for yellow pattern elements has also been found in the region containing the *wingless* gene in *H. cydno* and *H. pachinus* (Kronforst *et al.*, 2006). Therefore, many of the factors favouring divergence with gene flow can be found within the *Heliconius* radiation, and research continues to identify the genetic basis of important reproductive barriers such as pheromones (Byers *et al.*, 2020). However, little is known still of the genetic basis of traits such as flight and wing shape and whether these traits are also found within these genomic regions under strong selection.

1.3.3. Wing shape and flight in *Heliconius*

Colour pattern mimicry is under strong selection in *Heliconius*, and intermediate and foreign patterns have been shown to be more strongly predated in controlled experiments (Arias *et al.*, 2016; Chouteau *et al.*, 2016). The study of mimicry in *Heliconius* has mainly been limited to colour pattern, however, due to its central role

in fitness, selection may be expected to drive mimicry in other traits, such as flight or wing shape (Srygley, 1999).

Flight and wing shape mimicry have been shown in a number of *Heliconius* species. The first evidence of mimicry in flight related traits was found in convergence of centre of body and wing mass (Srygley, 1994). A similar study was then carried out on a subset of the *Heliconius* species measured in 1994 to account for phylogenetic relatedness and interspecific variation (Srygley, 1999). Measures were taken in two pairs of sister species, *H. cydno*, *H. melpomene* and *H. sapho*, *H. erato*, each pair containing one species from two separate mimicry groups; convergence in the position of the centre of wing and body and wing centroid was stronger between the mimics than between the sister species. Direct measures of flight such as wing beat frequency and asymmetry (difference in time spent during the up and downstrokes) were then made in these four species, again showing stronger convergence between mimics (Srygley & Ellington, 1999; Srygley, 1999). Finally, this convergence was found to be maintained across different behaviours (Srygley, 2007).

Other studies have focused on wing shape; Jones *et al.* (2013) found that differences between the morphs of *H. numata* correlated with shape differences in the corresponding models which they mimic (species of the *Melinaea* genus of the Ithomiini tribe) using wing aspect ratio as a measure of forewing, though hindwing shape was not analysed. Other studies using morphometrics have reported convergence in wing shape between mimics in the forewing of *H. erato phyllis*, *H. besckei*, *H. melpomene burchelli*, and *H. melpomene nanna* (Rossato *et al.*, 2018a) and in the forewing and hindwing of *H. melpomene* and *H. timareta sub-species* (Mérot *et al.*, 2016). Sexual dimorphism was also found in between some species (Rossato *et al.*, 2018a; Rosser *et al.*, 2019).

These studies have shown evidence for a role of mimicry in flight and wing shape evolution. However, these have not always included a robust control for common ancestry and are limited in terms of numbers of species studied. Furthermore, most of these did not address the relationship between wing shape variation identified and flight. Whether mimicry is driving wing shape convergence independently to flight (because of selection favouring visual mimicry) or whether flight convergence is explained by wing shape convergence (because of selection for behavioural mimicry) between mimics is still unknown. Flight and wing shape differences are also expected to be driven by other factors such as habitat and possibly energetic costs of different

types of behaviours, which may differ between sexes (Rossato *et al.*, 2018b). Furthermore, these drivers may differ between forewing and hindwing. A recent study also showed the effect of adaptation to elevation in forewing shape between pairs of *Heliconius* species (Montejo-Kovacevich *et al.*, 2019), though, again, hindwing shape was not analysed. Ecological factors may drive selection in different directions, constraining the evolution of a traits along specific axes (Rossato *et al.*, 2018a; Rossato *et al.*, 2018b). Further investigations are needed to identify the different factors, and their interactions, involved in driving wing shape and flight evolution in a mimetic butterfly.

1.4. Thesis Aims

Understanding adaptation in an ecological context is central to understanding the process of speciation. Studying traits such as flight or wing shape, which are integral parts of species fitness in their habitat, can help us understand the evolutionary drivers shaping species diversity. Furthermore, identifying the underlying genomic architecture of such traits can allow us to understand how species evolve, and the *Heliconius* genus offers an ideal system for studying such processes. In this thesis, I take an integrated approach to studying the evolution of wing shape in *Heliconius* butterflies. The second chapter investigates the dynamics of wing shape evolution across the Heliconiini tribe in order to identify the ecological drivers and selective pressures acting on this trait. Specifically, I investigate the relative roles of mimicry and habitat, as well as other factors, on wing shape and try to determine whether these factors are different for forewing and hindwing shape. Using experimental manipulations in Chapter 3, I aim to understand the relationship between flight and wing shape in *H. elevatus* and *H. pardalinus* which are closely related, sympatric species with divergent colour patterns, in order to understand whether wing shape differences can explain observed differences in flight. Finally, in the fourth chapter I use Quantitative Trait Loci (QTL) analyses to understand the underlying genomic architecture of wing shape variation between *H. elevatus* and *H. pardalinus*. With these results I aim to support evidence of a role of divergence with gene flow and islands of divergence between the two by determining whether QTL for wing shape can be found within areas of high divergence, such as the regions containing important colour pattern genes.

Chapter 2

Aerodynamic constraints limit wing shape mimicry in Heliconiini butterflies.

Abstract

Identifying the ecological factors driving selection is fundamental to understanding how species diversify. Wing shape is an important trait in insects as it is involved in flight aerodynamics; however, wing shape has also evolved in response to a number of other ecological factors, sometimes leading to antagonistic selective pressures. In this chapter, I investigate the roles of mimicry and habitat type on the evolution of wing shape in *Heliconius* butterflies and species of the wider Heliconiini tribe. Over 600 individuals from 54 species were sampled across the Heliconiini tribe for geometric-morphometric analysis of wing shape. Controlling for phylogenetic relatedness I found that butterflies species displaying the same mimetic colour patterns also converge in wing shape, and that this mimicry of wing shape is stronger in the hindwing than the forewing. These results are consistent with evidence that flight is mainly driven by the forewing in Lepidoptera, and therefore may be subject to stronger aerodynamic constraints. These patterns are mainly driven by the silvaniform mimicry group which mimics butterflies of the Ithomiini tribe, which diverged from the Heliconiini ~90 Mya. I present evidence that the silvaniform wing shape divergence is explained by a convergence to the ithomiine wing shape and that females converge more strongly than males, suggesting stronger aerodynamic constraints on male wing shape. Together, these results reveal the complexity of interactions between different ecological drivers and the importance of ecomorphological studies across species.

2.1. Introduction

Species evolve and adapt in response to selective pressures in their environment. Understanding the evolutionary history of phenotypic traits means understanding which factors have driven their evolution, and how these have interacted. Ecomorphological studies use analyses to correlate patterns of morphological variation with patterns of ecological differentiation (Losos & Miles, 1994; Schluter, 2000). Strong phenotypic convergence between environments can occur as a result of adaptation to common pressures, even leading to incorrect assignment of species when no other data is available; for example, phylogenetic and morphological analyses in the lacertid lizard radiation found that species considered sister taxa were part of distantly related lineages; the error occurred due to strong morphological convergence between species inhabiting cluttered versus open habitats (Edwards *et al.*, 2012). Incorporating phylogenetic data in ecomorphological studies can help distinguish between convergence in phenotypes due to common ancestry and selection (Losos & Miles, 1994).

Phenotypic convergence between similar environments or ecological variables, controlling for common ancestry, has therefore been used as a strong indicator of adaptation (Schluter, 2000); however constraints can limit the role of selection. Natural selection acts on genetic variation, and lack of suitable genetic variation or low mutation rates can limit a population's ability to adapt (Futuyma, 2010). Constraints on the evolution of one trait can also occur if selection is strong on another trait controlled by common genetic or developmental pathways (Arnold, 1992); this can result in trade-offs between the traits. Traits may be under complementary selection from different drivers, whereby a trait value is beneficial for different aspects of fitness; but traits can also be under conflicting selection, in which case two evolutionary drivers select for opposing trait values (Agrawal *et al.*, 2010). Trade-offs are often considered between correlated traits, such as trade-offs between traits enhancing survival over reproduction, but conflicting evolutionary drivers can also act on single traits, for example, in the *Eurosta* fly, gall size represents a trade-off between chances of parasitism and bird predation, larger galls are less parasitized but more easily detected by birds (Weis & Gorman, 1990).

Phenotypic convergence can be limited by many factors and care should be taken when identifying such patterns. Constraints can be inherited from ancestral species, therefore biasing the evolution of traits in a certain direction in the descendent taxa (Agrawal *et al.*, 2010); similar phenotypes evolve not because they represent the optimum adaptation but because of limits on alternative phenotypes. In this case however, strong correlations with ecological factors might not be expected. Phenotypic convergence between habitats can also, in theory, arise purely by chance but the use of statistical analyses combined with ecomorphological studies can help exclude this possibility (Losos, 2008).

Furthermore, identifying the specific traits under selection and the ecological drivers involved can also be difficult, as the trait of interest might be changing as a result of correlations with other traits under selection. In a study of comparing morphological traits across 74 populations of sticklebacks, authors tried to identify evidence of convergence between similar environmental factors; however, due to strong allometric relationships between morphological traits and body, adaptation could not be ascertained as driving these traits' evolution (Voje *et al.*, 2013). Only the length of dorsal spines seemed decoupled from body size. Furthermore, evidence of adaptation could still not be found for body size across the measured habitats, suggesting that the ecological factors chosen in the study did not include drivers of this trait. In-depth knowledge of the study organism and its ecology is therefore very important when carrying out ecomorphological analyses.

Ecomorphological studies can thus be an excellent tool for identifying patterns of selection, however, as mentioned, conclusions on the specific dynamics of trait evolution need to be made carefully. Still, these can provide direction for further investigations on the phenotypes of interest. For example, common garden experiments or direct measures of the effects of variation in a trait on fitness can complement ecomorphological studies. For example, reduced colour spots in guppies evolved in the presence of a predator in just a few experimental generations (Endler, 1980). Here, a clear causal relationship was established between the environment and the phenotypic response. Finally, failing to identify convergence in a particular phenotype on a wider scale does not exclude adaptive benefits. More focused studies across pairs of species, or even within species, can provide important information about species' adaptation.

Wing shape in insects is an interesting trait in the context of ecology and adaptation. Flight is thought to have been one of the major drivers of insect diversification, allowing colonisation of new ecological niches. Flight is driven in part by anatomical and physiological characteristics of the insect body, but studies have also shown the importance of the aerodynamics of wing shape during flight (Dudley & Srygley, 1994; Dudley, 2000; Berwaerts *et al.*, 2002 among others, and reviewed in butterflies in Le Roy *et al.*, 2019). For example, wings with higher aspect ratios are usually associated with more efficient, sustained flying, whereas shorter rounder wings have been associated with increased manoeuvrability (Le Roy *et al.*, 2019). Other studies have also identified the importance of distribution of wing area along the wing (known as moments of area) in determining flapping ability in insects (Ellington, 1984). Depending on the organism, flight can also be driven more strongly by the forewing or the hindwing, known as anteromotorism or posteromotorism, respectively (Dudley, 2002). Therefore, the shape of the forewing and hindwing may be subject to different aerodynamic pressures (Breuker *et al.*, 2007; Jantzen & Eisner, 2008; Outomuro *et al.*, 2016).

Different habitats can select for different flight behaviours and wing shapes, for example, species of *Morpho* butterfly that occupy different heights in the canopy have been shown to have different wing shapes, and these were attributed to differences in flight characteristics (Chazot *et al.*, 2016). Different dispersal needs are also expected to select for different flying strategies, which can also translate into variation in wing shape, as seen in dispersing and non-dispersing populations of Monarch butterflies (Altizer & Davis, 2010). Behavioural differences may also require different flight abilities, such as in species of damselflies which have different wing morphology according to their mate guarding or dispersal behaviour (Johansson *et al.*, 2009). Therefore, selection on flight behaviour can promote the evolution of wing morphology.

Wings have also evolved in response to a number of other factors, which explains the extraordinary diversity of wing morphologies observed in extant insects. In extreme cases, wings have even lost flight function, such as the elytra in Coleoptera, used for physical protection and mostly kept stationary during flight (Dudley, 2002). These elytra often display stunning colours that might have been promoted through their signaling effect; these patterns can be involved in mate choice, such as the wing colouration in male damselflies species of the Calopterygidae family, but can

also be correlated with defenses and evolve as a response to predators, such as in *Heliconius* butterflies where colour patterns are involved in both aposematic signalling and mate choice (Merrill *et al.*, 2015). This has also led to the evolution of mimicry in wings, either in Batesian mimics which benefit from the model's defence, or in Müllerian mimics which share the cost of predation (Bates, 1862; Wallace, 1882). Crypsis has also evolve multiple times in insects and frequently involved selection on both wing shape and wing colour pattern, such as the leaf like crypsis in katydids (Mugleston, 2016). Furthermore, wing movement may also be used for thermoregulation in a number of taxa, for example, orchid bees buzz their wings when perched (Stern & Dudley, 1991). Examples of non-flight related wing adaptations are abundant and selection for these can often result in constraints or trade-offs on wing morphology.

Antagonistic selection can occur both at the levels of genes and individuals, resulting in variation in phenotypes withing and between species. Different individuals within populations can display different wing morphologies and flight characteristics associated with different behaviours. This is common in males with alternative mating strategies; for example, a study in different species of damselflies identified variation in forewing morphology between territorial and non-territorial males; however, territorial morphologies were also expected to be more energetically costly which was compatible with findings on the importance of physiological condition in territorial behaviour (Outomuro *et al.*, 2014). In a mark-recapture study in damselflies, wing shape was measured in males along with survival and reproductive success. Antagonistic natural and sexual selection was found as different forewing shapes were associated with higher survival and mating success, respectively (Outomuro *et al.*, 2016). Comparisons between palatable non-mimetic and mimetic butterfly species with the unpalatable model species found evidence of locomotor mimicry in the Batesian mimics, however, these species suffered an aerodynamic cost, suggesting a trade-off between flight efficacy and mimicry (Srygley, 2004). These examples highlight the complexity of the selective regimes acting on wing morphology.

Adaptive radiations are excellent study systems to understand the role of ecology in adaptation. Adaptive radiations refer to lineages characterised by the rapid increase of phenotypic disparity in response to adaptation to different niches (Schluter, 2000), and *Heliconius* butterflies are an example of such a radiation. The genus is found

across most of South and Central America (Figure 2.1) and is known for its numerous species which have diversified in response to local adaptation to their environment (Merrill *et al.*, 2015) and display an impressive diversity of bright of colour patterns. These colour patterns have been extensively studied due to their dual role in mimicry and mate preference and can be broadly categorised into “mimicry rings”, which are groups of co-occurring species or subspecies displaying a similar mimetic pattern (Mallet & Gilbert, 1995). Some of these mimetic wing colour patterns are found in other taxa, such as in *Chetone* moths, or ithomiine butterflies which diverged from the Heliconiini 90Mya. Species across ithomiine genera display the same brown/yellow mottled colour patterns as the silvaniform patterned *Heliconius* and are believed to be the models promoting the evolution of mimetic coloration in *Heliconius* species (Brown, 1981; Elias *et al.*, 2008).

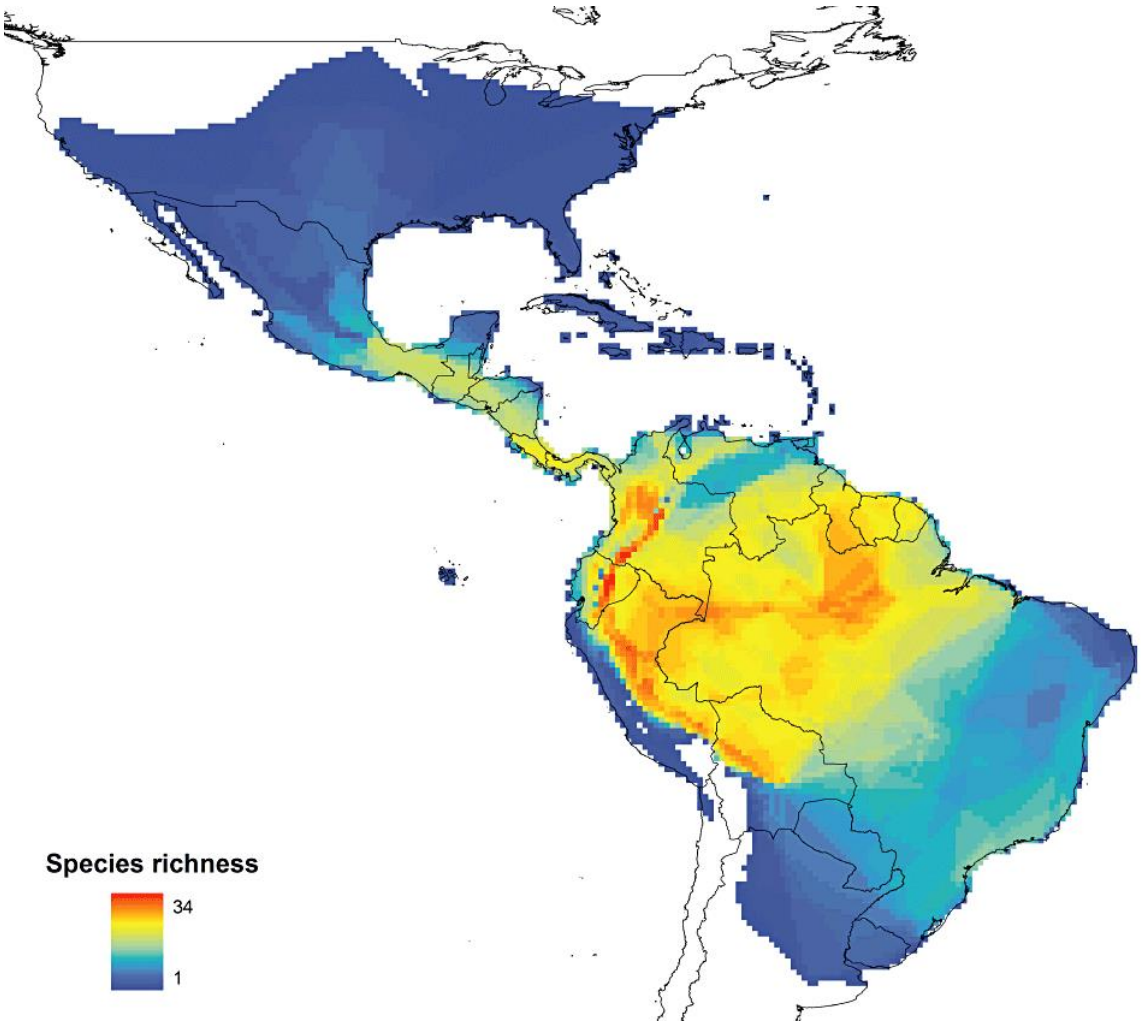


Figure 2.1. Map of *Heliconius* species richness across South and Central America taken from Rosser *et al.* (2012).

Colour pattern mimicry is central to the survival of *Heliconius* and experiments using artificial models have shown increased attack rates on non-local morphs (Arias *et al.*, 2016). Colour pattern mimicry is driven by strong positive frequency dependent selection (Chouteau *et al.*, 2016), and several studies have investigated the role of other traits in the mimetic signal. Indeed, signals involving multiple traits are expected to evolve, as increased complexity can help predator learning and also be more difficult for Batesian mimics to copy (Srygley, 1999). Comparisons between two pairs of *Heliconius* species (*H. cydno*, *H. sapho*, *H. melpomene* and *H. erato*) belonging to two distinct mimicry rings found greater convergence between co-mimics than between sister taxa for several measures of wing morphology and flight, including position of centre of wing and body mass, wing centroid position, and wing beat frequency across multiple behaviours (Srygley, 1999; Srygley, 2007). Interestingly in this case, body mass and wing length were maintained within sister species, which means wing shape and flight convergence between mimics occurred despite constrained wing loading (ratio of wing length to body mass), which has been shown to be involved in flight traits such as wing beat frequency across many taxa (Tercel *et al.*, 2018). This emphasizes the complexity of understanding the suite of traits involved in driving flight in insects. Additional evidence of wing shape mimicry has been found in other comparisons of *Heliconius* species (Jones *et al.*, 2013; Mérot *et al.*, 2016; Rossato *et al.*, 2018a), suggesting that wing shape is probably part of the mimetic signal, either because of increased visual mimicry or as part of behavioural mimicry due to its aerodynamic effects on flight (Srygley, 1999).

These studies in *Heliconius* have only focused on a few species, not always with a robust control for phylogeny and sometimes only using single measures of wing shape, such as aspect ratio, and only analysing the forewing shape. Furthermore, evidence suggests that other factors are also involved in wing shape evolution. Sex differences have been found in wing shapes of several *Heliconius* species (Rossato *et al.*, 2018a; Rosser *et al.*, 2019) which could be due to different life histories, behaviours, or small-scale habitat segregation between sexes. Another study compared forewing shape across pairs of species adapted to different elevations, and found that both within and across species, wings were rounder at higher elevations (Montejo-Kovacevich *et al.*, 2019), although this study did not control for difreences between mimicry rings. Therefore, wing shape in *Heliconius* seems to have been driven by multiple ecological factors.

In this chapter, I carry out ecomorphological analyses of wing shape across the *Heliconius* and *Eueides* genera. The *Eueides* is the sister genus of *Heliconius*, and their common wing venation patterns allows for comparisons using landmark based morphometric analyses. These analyses aim to identify the relative contributions and potential constraining effects of different ecological factors (mimicry, habitat type and elevation) on wing shape, while controlling for the effects of shared common ancestry and allometry using wing area. Due to the dominant role of the forewing compared to the hindwing in Lepidopteran flight, I will first test the hypothesis that mimicry has a relatively larger effect on hindwing shape, whereas habitat type is a stronger driver of forewing shape due to stronger constraints from flight aerodynamics. Second, I will test whether species in the silvaniform mimicry ring have the most divergent wing shape as this is expected to be converging towards the ithomiine wing shape. Finally, I will test whether there is any evidence of sexual dimorphism in wing shape mimicry.

2.2. Methods

2.2.1. Quantifying Wing Shape

I used specimens from collections dating back to 1989, from regions across Peru, Panama, Suriname, Ecuador and Brazil, aiming to gather information for five males and five females for each species. The ventral side of each wing was digitised using a flatbed scanner at 300 dpi; I used landmark based geometric-morphometrics analyses to measure wing shape (see Appendix 1A for landmarks based on Jones *et al.*, 2013; Mérot *et al.*, 2013) using TpsDig2 (Rohlf, 2006). Landmarks were placed on 558 individuals for the forewing and 542 individuals for the hindwing spanning across 67 taxa from 46 species (including eight *Eueides* species; see Appendix 1B for full details of specimen numbers). For analyses using semi landmarks (comparing *Heliconius* and ithomiine wing shapes), I placed 32 and 27 equidistant semi-landmarks around the forewing and hindwing outlines, respectively. The outline started and ended at the wing base. Measures of forewing and hindwing area were taken from 509 and 485 individuals (areas could not be measured from all individuals as a scale bar was not always included with the wing scan) using TpsDig2. Landmark coordinates were extracted and Procrustes analyses were used to adjust for size and orientation using the R package Geomorph (Adams *et al.*, 2019). Unless stated otherwise, all further analyses were carried out on the Procrustes adjusted coordinates on the forewing and hindwing separately. Principal component analyses (PCA) were carried out with plotTangentSpace() to visualise the mimicry rings across the morphospace.

2.2.2. Mimicry Ring and Habitat Type

To understand the effect of colour pattern mimicry on the evolution of wing shape, I assigned each taxon to one of seven mimicry rings. Most taxa fit into clearly defined groups which are commonly used in the *Heliconius* community- “Dennis Rayed”, “silvaniform”, “Blue”, “Postman”, “Orange” and two other groups I called “Red and White”, where the pattern is similar to that of the Postman but the colours on the fore and hindwing are reversed, and the “Black and White”, such as *H. charithonia* (see

Figure 2.2). These groupings broadly follow the mimicry classification in Brown (1981); assignment to these groups is presented in the appendix (Appendix 1C). For two species, *H. doris* and *H. timareta*, data were collected for morphs/sub-species that belong to different mimicry rings. I therefore used the morph for which I had most specimens but compared the effects of mimicry and habitat on a separate dataset containing the alternative morphs and found no difference in the results. *Heliconius heurippa* and certain *H. melpomene melpomene* displayed unique patterns not mimicking any other species in the dataset and were therefore excluded from analyses testing for effects of mimicry.

Habitat information reported in the literature was used to create the habitat dataset (Brown, 1981; Devries, 1987; Jiggins *et al.*, 1996; Rosser *et al.*, 2015; Rosser *et al.*, 2012) and habitat types were further classified into out into four groups, “Open Forest”, “Closed Forest”, “Forest Edges and Gaps” and “Field and Scrubs”. Habitat data were not available for *H. eratosignis*, *E. heliconioides*, or for separate sub-species of *Heliconius*. For altitude, I average the recorded altitudes of collected specimens for each species using data from (Rosser *et al.*, 2012), in the case of *H. melpomene* and *H. erato* subspecies, I used the average values of the correct subspecies. Finally, for the phylogenetic analyses, I used the time calibrated Bayesian phylogeny of the Heliconiini tribe with outgroups, estimated using 20 nuclear and 2 mitochondrial markers (Kozak *et al.*, 2015).

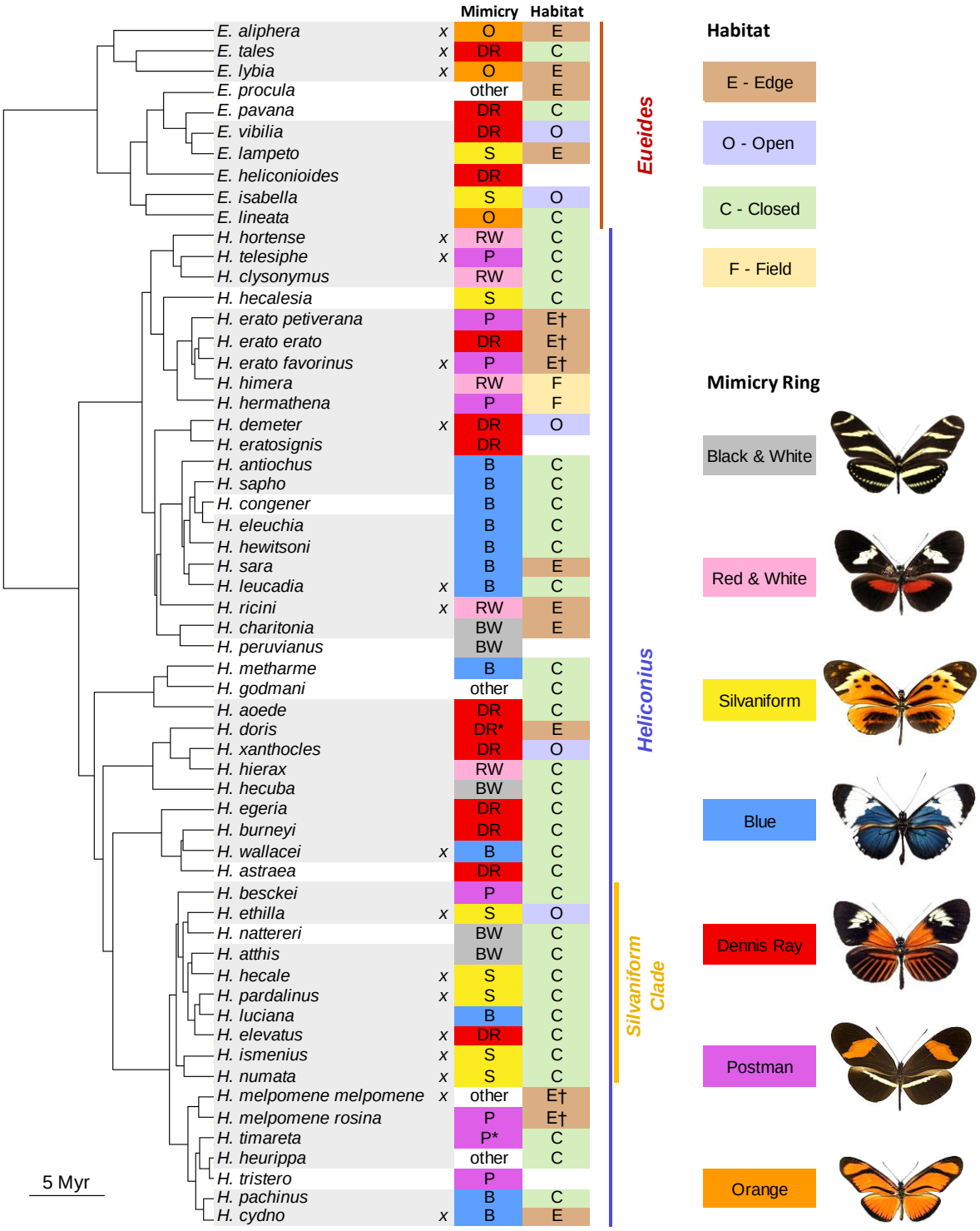


Figure 2.2. Phylogeny of the *Eueides* and *Heliconius* genera (Kozak *et al.*, 2015) with species and subspecies included in the evolutionary rate models highlighted in grey. Species used in the comparison with the outgroups are marked with an "x". The first column represents the mimicry ring each taxon is assigned to; "other" represents a pattern that does not fit in one of the seven mimicry rings used. Patterns marked with * represent taxa which include more than one pattern for which I chose the one with more individuals. The second column represents one of the four habitat types; † represents habitat allocation in sub-species based on species wide data.

2.2.3. Testing the effect of Sex, Mimicry and Habitat type

To identify sexual dimorphism in wing shape I removed all specimens with undetermined sex as some did not include a record of sex and were too faded to confidently assign (see Appendix 1B for sample sizes for males and females). I carried out linear models with residual randomization in a permutation procedure (RRPP) using 1000 permutations with the RRPP R package (Collyer & Adams, 2018) with a mixed effect model design to identify differences between sex (fixed effect) while accounting for variation among species (random effect) (Collyer & Adams, 2018).

I carried out a Canonical Variate Analysis (CVA) on individuals from *Heliconius* and *Eueides* species, assigning these to groups based on mimicry and habitat information separately, with the R package Morpho (Schlager, 2017) to determine how well wing shape could discriminate between different mimicry groups or habitat types. In CVAs, species are given prior groupings which are used to determine how well individuals fit within that classification (posterior probabilities) after maximizing the difference between groups.

Finally, analyses using phylogenetic correction are carried out on the average value of species across measured individuals. Therefore, I verified that the intraspecific variation was less than interspecific variation using a mixed effect model to ensure that the average value was representative of groups. The linear model with RRPP (1000 permutations) used individuals as a fixed effect and species as a random effect.

2.2.4. Phylogenetic signal, Models and Rates of Evolution

I first wanted to determine the level of constraints on wing shape evolution, either from common ancestry or from common genetic or developmental pathways between the two wings. I used the average wing shape across individuals per species to determine the level of phylogenetic constraint on wing shape. Using the R package geomorph (Adams *et al.*, 2019), I calculated the phylogenetic signal, Kmult, a variant of the K-statistic appropriate for multivariate data (Adams & Collyer, 2018), of forewing and hindwing shapes. The phylogenetic integration of the two wings

(degree of phylogenetic morphological covariation using Phylogenetic Least Squares) was also calculated (Adams & Collyer, 2018).

I investigated different models of wing shape evolution to understand the how wing shape has evolved across the phylogeny and whether different events caused shifts during its evolution. I compared the fit of seven models of evolution to wing shape using the R package mvMorph (v. 1.1.0) (Clavel *et al.*, 2015) on the PCs that explained over 95 % of variation for forewing and hindwing (16 and 13 PCs, respectively) to reduce the dimensionality of the data. I compared models of Brownian Motion (BM) and Early Burst (EB) across the phylogeny as well as shift models between two BM rates and from a BM to an EB model. I mapped the shift at the split between the *Heliconius* and *Eueides* clades, and due to the striking divergence of the silvaniform individuals (visible from the PCA analysis, Figure 2.3), I also included a shift before the split of the silvaniform clade , which includes all of the silvaniform patterned *Heliconius* species (referred throughout as the silvaniform, different to the silvaniform clade), along with others (see Figure 2.2). Models were compared using the sample size-corrected Akaike information criterion (AICc) from the mvMorph output.

The size of the dataset was a limiting factor when using the MvMorph models, therefore I also used the compare.evol.rates() function in geomorph (Adams *et al.*, 2019) to identify differences in rates of evolution between groups. This was used to compare the rates of evolution between *Heliconius* and *Eueides* species and between the silvaniform clade and the rest. I also compared *Heliconius* and *Eueides* to the basal taxa of the Heliconiini tribe to determine whether the rate of wing shape evolution increased in *Heliconius*, matching the diversification of colour patterns. To achieve this, I used semi-landmarks on one individual of 6 basal species (*Agraulis vanilla*, *Dryas iulia*, *Dryadula phaetusa*, *Dione juno*, *Dione moneta*, *Philaethria dido*, *Podotricha telesiphe*) and in 24 individuals from 18 species (see species marked with an “x” in Figure 2.2) of the *Heliconius* and *Eueides* (6 randomly sampled individuals across the four quadrants of the PCA, Figure 2.3, to sample the entire morphospace).

2.2.5. Phylogenetic Correction

I measured the relative effect of mimicry, habitat and elevation on wing shape differences while controlling for common ancestry to identify the main drivers of wing shape evolution. Analyses with phylogenetic correction were carried out in the R package RRPP using the linear model with generalized least-squares (GLS) estimation with a marginal sum of squares computation (Collyer & Adams, 2018); Procrustes coordinates were used as the dependent variable and mimicry, habitat type and altitude were included as dependent variables as well as centroid size to control for allometric effects, without interactions. From these analyses, I compared the R^2 and *p* values of the dependent variables to understand their roles in wing shape. Due to differences in sex in the hindwing, I ran separate models on female and male subsets of the dataset; however, differences in R^2 values were comparable between sexes and analyses were carried out on the whole dataset, with males and females combined.

To characterise differences between each mimicry and habitat group, I carried out pairwise *post hoc* tests on the linear model fits with the pairwise() function in the RRPP package which gives pairwise distances (of wing shape similarity) between least squares means of mimicry and habitat groups, separately.

2.2.6. Testing for Convergence with the Ithomiini

The strongest differences in wing shape were found to be between the species displaying the silvaniform pattern and the rest of the mimicry rings. Unlike the other mimicry groups, the members of the silvaniform mimicry ring mimic species of the Ithomiini. Therefore, a strong divergence in wing shape away from the other *Heliconius* species could be driven by convergence towards an ithomiine wing shape. To test this, I sampled single individuals from 29 species of Ithomiini across seven genera. These were *Melinaea*, *Mechanitis*, *Forbestra*, *Tithorea* and *Hypothyris*, which only include silvaniform patterned species, and *Hyposcada* and *Napeogenes* which also included non-silvaniform species. I chose to favour number of species over replicates per species to sample the morphospace across the entire ithomiine sub-family. I used a sub-sample of the *Heliconius* and *Eueides* specimens to compare against the ithomiine specimens. To ensure that the entire morphospace

was represented, I used a standard random number generator to sample individuals from each quadrant of the PCA (Figure 2.3) and then carried out further sampling to ensure that at least one male and one female of each species was included.

Since the Heliconiini with Ithomiini have different wing venations, to compare wing shapes I used a semi-landmark approach. Landmark coordinates were adjusted using Procrustes analyses. I then used a PCA using prcomp() to visualise the differences between silvaniform and non-silvaniform individuals of the *Heliconius* and *Eueides* species. The coordinates of the ithomiine individuals were then centred and rotated to the *Heliconius*/*Eueides* morphospace using the rotation matrix from the *Heliconius*/*Eueides* PCA.

To formally test for the effects of mimicry groups (silvaniform and non-silvaniform), family (Heliconiini and Ithomiini) and their interaction, I carried out linear models with RRPP on the Procrustes coordinates with mimicry and family as independent variables. The pairwise() function was used on the model output to test if the silvaniform Heliconiini were more similar to the ithomiines than the non-silvaniform individuals. Centroid size was included in the analysis to control for allometry.

Based on the PCA of Heliconiini and Ithomiini (see Figure 2.4), female silvaniform heliconiine individuals appeared to cluster more closely with the ithomiines than male silvaniform heliconiine. Therefore, I also tested for effects of sex in the *Heliconius* and *Eueides* silvaniform specimens, using linear models with sex as a fixed factor and species as a random factor to account for the variation from this. To carry out pairwise analyses between males and females from silvaniform and non-silvaniform mimicry rings with Ithomiini, I assigned each of these to a separate group and used this as a factor in a linear model which was then used for the pairwise analysis. Again, centroid size was included in the model to account for allometric variation.

2.2.7. Sexual dimorphism in silvaniform

I found sexual dimorphism in the wing shape of the silvaniform individuals, with females mimicking the ithomiines more strongly. To test whether this pattern is explained by female driven mimicry or results from a trade-off in shape for mimicry in males, I tested whether or not *Heliconius* silvaniform species also exhibit sexual dimorphism in their colour patterns. I expect that under a scenario of female driven

mimicry, differences in shape between sexes would also be matched by differences in colour pattern. However, if sexual dimorphism is limited to wing shape, then this could suggest that wing shape mimicry is constrained in males.

To examine colour pattern variation, I scanned the dorsal side of the wings of at least four males and four females of *H. pardalinus butleri*, *H. pardalinus sergestus*, *H. hecale* and *H. ethilla*. *Heliconius ismenius* was excluded as there were not enough female specimens and *H. numata* was also removed due to a difficulty in choice of morph type. The wings were landmarked as previously described, landmarks were aligned and the dark brown colours quantified (defined as R55, G35, B30, with a cut off of 0.1) with the patLanRGB() function from the R package patternize (Van Belleghem *et al.*, 2018). Dark brown was used as this was the most consistent colour across species and was the best colour to discriminate patterns. Male hindwings have androconia (wing region where pheromones are released), however, these are not visible due to overlap with the forewing, apart from during courtship. Therefore, the androconial regions were removed from the analysis on both forewing and hindwing using setMask(). The colour pattern was run through patPCA() and the PCs that explained >95 % of variation were then analysed using a linear model with RRPP, with a mixed effect design. Sex and species were included as fixed effects, species was included as a random effect to account for variation among species on sex differences and centroid size was included to control for allometry. I carried out the same analysis on wing shape using the PCs that explained >95 % of variation for direct comparison of the effect of sex on shape and colour.

2.3. Results

2.3.1. Quantifying Wing Shape

The first principal components of the PCA of forewing (PC1 = 22.7%) and hindwing (PC1 = 33.2) largely explain the curvature of the outer margin of the wings (see changes along PC1 and PC2 in Figure 2.3). There appears to be clustering of species within mimicry groups, however, the silvaniform group is by far the most divergent, and this is especially visible in the hindwing, with both *Heliconius* and *Eueides* silvaniform species diverging away from the other mimicry groups.

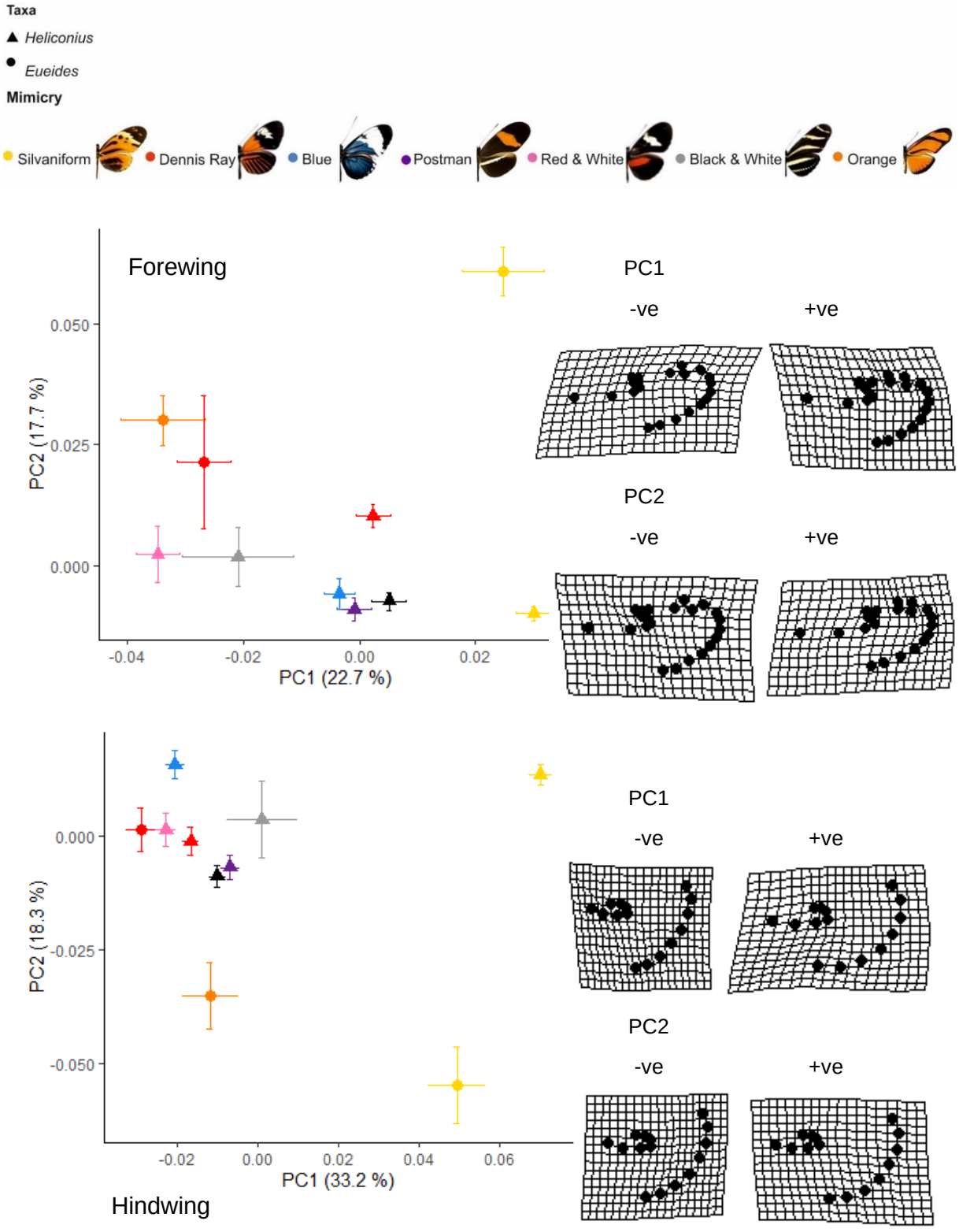


Figure 2.3. Mean and standard errors of the first two principal components of the different mimicry groups of *Heliconius* (as triangles) and *Eueides* (as circles) species for forewing (top) and hindwing (bottom). The black symbols represent specimens that do not fit into one of the seven mimicry rings. On the right are shown the deformation plots along PC1 and PC2.

2.3.2. Testing the effect of Sex, Mimicry and Habitat

I initially tested for sex differences using linear models with a mixed effect model design on the forewing and hindwing and found a significant effect of sex when accounting for variation among species in hindwing shape ($R^2 = 0.04$, $p = 0.001$) but not in forewing shape ($R^2 = 0.01$, $p = 0.052$). I also wanted to determine how reliably specimens fit into different mimicry rings and habitat types based solely on wing shape, therefore, I carried out a CVA and compared the posterior probabilities of specimens (Table 2.1). In the forewing, the overall classification accuracy was relatively high, with 84 % and 74 % accuracy for mimicry groups and habitat types respectively. In the hindwing, the values were a bit lower with an overall classification accuracy of 73 % and 71 % for mimicry groups and habitat types respectively.

Phylogenetic analyses use single measures per branch tip, therefore, I wanted to determine whether a species' average wing shape was an accurate representation of each species. I carried out linear models with a mixed effect model design to determine whether differences within species (or between individuals of each species) explained a significant amount of variation after accounting for variation explained by species differences. There was no significant effect of individual variation within species in both forewing ($R^2 = 0.01$, $p = 0.493$) and hindwing ($R^2 = 0.01$, $p = 0.440$) shape suggesting the amount of variation within species is less important than variation between, and therefore using species average was appropriate for further phylogenetic analysis.

Table 2.1. Percentage of individuals correctly assigned to mimicry group or habitat type (posterior) based on prior classification. Reading from left to right, the values represent the percentage of individuals in a mimicry group (in the prior classification column) that were classified as being most similar to individuals of the different mimicry ring (in the posterior classification). The first value corresponds to the classification based on forewing shape and the second value is the classification based on the hindwing shape. For example, 82.4 % of individuals in Blue mimicry ring were correctly identified as Blue (for forewing shape) and 1.2 % were identified as Black and White. Note, both *Heliconius* and *Eueides* species are included in the Dennis Rayed and silvaniform groups, and the Orange group only includes *Eueides* species.

		Posterior classification							
		Blue	**Black & White**	**Dennis Rayed**	**Orange**	**Postman**	**Red &White**	**Silvaniform**	**none**
Prior classification	**Blue**	82.4 - 67.1	1.2 - 1.3	7.1 - 7.6	0 - 0	8.2 - 15.2	1.2 - 8.9	0 - 0	0 - 0
	Black & White	5.3 - 11.8	89.5 - 64.7	0 - 0	0 - 0	0 - 0	5.3 - 5.9	0 - 5.9	0 - 11.8
	Dennis Rayed	10.1 - 4.2	0 - 1.4	73.9 - 74.6	8.7 - 4.2	4.3 - 7	0 - 7	0 - 0	2.9 - 1.4
	Orange	0 - 0	0 - 0	0 - 0	93.8 - 100	0 - 0	0 - 0	0 - 0	6.3 - 0
	Postman	9.5 - 14.3	3.2 - 7.9	0 - 7.9	0 - 0	84.1 - 60.3	1.6 - 6.3	1.6 - 3.2	0 - 0
	Red & White	2 - 11.4	0 - 2.3	0 - 15.9	0 - 0	16.3 - 4.5	75.5 - 65.9	2 - 0	4.1 - 0
	Silvaniform	0 - 1.1	0 - 1.1	1.1 - 0	0 - 2.1	2.1 - 8.4	0 - 0	95.8 - 86.3	1.1 - 1.1

		Closed	**Edge**	**Field**	**Open**	**none**
	Closed	73.4 - 68.1	8.3 - 9.9	9.9 - 9.9	7.3 - 12.1	1 - 0
	Edge	8.5 - 13.6	71.3 - 70.5	6.4 - 12.5	10.6 - 2.3	3.2 - 1.1
	Field	8.3 - 25	0 - 0	91.7 - 75	0 - 0	0 - 0
	Open	13.3 - 18	9.2 - 1	1 - 5	75.5 - 76	1 - 0

2.3.3. Phylogenetic signal, Models and Rates of Evolution

I measured the phylogenetic signal of wing shape to determine whether the trait was phylogenetically constrained. Kmult is a measure of phylogenetic signal where a value of 0 represents no signal, <1 indicates less similarity then expected under a model of Brownian Motion, and >1 indicates more similarity than expected under Brownian Motion (Adams, 2014). I found a significant phylogenetic signal for both forewing (Kmult = 0.44, $p = 0.001$) and hindwing (Kmult = 0.37, $p = 0.001$), suggesting that closely related species have more similar wing shapes than expected at random. I measured the level of phylogenetic integration between the fore and hindwing to investigate the hypothesis that the wings are under different selective pressures. Forewing and hindwing shapes were significantly correlated (r-PLS = 0.604, $p = 0.019$). Although significant, the phylogenetic signal remains low which suggests that factors other than common ancestry may be driving wing morphology. This is the same for the correlation between forewing and hindwing shape, despite probable common genetic or developmental pathways controlling the two wing shapes, the r-PLS value is still relatively low, therefore selective pressures may differ between the forewing and hindwing or differ in strength between the two.

I initially tested the fit of different models of evolution to identify patterns and shifts in rates of wing shape evolution in our dataset. Models including a rate shift did not reach a reliable estimate (hessian value = 1), probably due to the relatively low number of species included in the dataset as these models usually require very large data sets (Cooper *et al.*, 2016). The BM and EB models reached reliable estimates (hessian value = 0), with the BM model having the lowest AICc value for both forewing (ΔAICc = 80.75) and hindwing (ΔAICc = 37.31) which meets the assumptions of Browninan motion of the phylogenetic analyses used in this chapter.

Using the function compare.evol.rates() I was able to compare evolutionary rates between groups despite a relatively small sample size. Comparing evolutionary rates on the Procrustes output showed no significant difference between *Heliconius* and *Eueides* in the forewing (Rate Ratio = 1.06, $p = 0.83$) or the hindwing (Rate Ratio = 1.24, $p = 0.54$). There was also no difference in evolutionary rate between the silvaniform clade and the rest in the forewing (Rate Ratio = 1.03, $p = 0.91$). However, the silvaniform clade did show a greater than two-fold increase in the rate of hindwing shape evolution relative to other taxa (Rate Ratio = 2.21, $p = 0.01$) which

is consistent with the strong divergence observed in Figure 2.3. The rate of wing shape evolution was not significantly different between the advanced Heliconiine (*Heliconius* and *Eueides*) and the basal Heliconiine for the forewing (Rate Ratio = 1.53, $p = 0.26$) or the hindwing (Rate Ratio = 2.8, $p = 0.24$).

2.3.4. Phylogenetic Correction

To compare the effect of mimicry ring, habitat type and altitude on forewing and hindwing shape with phylogenetic correction, I carried out a linear model with GLS estimation and marginal sum of square computation. Due to the anteromotorism of Lepidoptera, I expected forewing shape to be more strongly affected by habitat type, whereas hindwing would be more strongly driven by mimicry due to reduced aerodynamic constraint. Overall, the results meet these expectations, and the R^2 and significance values from the tests are presented in Table 2.2. Mimicry explained a larger percentage of shape variation in the hindwing compared to the forewing, but habitat type explained a similar amount of shape variation. Altitude only had a significant effect in the hindwing (with a marginally non-significant effect in the forewing), but the amount of variation explained was very small. I also find that butterflies with similar wing shapes also have similar wing areas, but once again the amount of variation explained by wing area was very small.

Table 2.2. Proportion of wing shape variation explained (R^2) by mimicry, habitat, altitude and wing area from the linear model with GLS correction for forewing and hindwing shape. The variation of each factor is calculated accounting for variation from other factors and after controlling for common ancestry.

Forewing				**Hindwing**			
	Df	**R^2**	**Pr(>F)**		**Df**	**R^2**	**Pr(>F)**
mimicry	6	0.19	0.008	mimicry	6	0.35	0.001
habitat	3	0.11	0.010	habitat	3	0.09	0.020
altitude	1	0.03	0.093	altitude	1	0.03	0.052
centroid size	1	0.04	0.056	wing area	1	0.03	0.091

Pairwise analyses from phylogenetic models were then carried out for mimicry ring and habitat type separately (Table 2.3). In the comparison of mimicry rings, the strongest differences were found between the hindwing shapes of the silvaniform and the other mimicry groups. In the habitat comparisons, differences were found in forewing shapes between species found in different habitat types, while no such differences were found in the hindwing shape.

Table 2.3. Pairwise distances between least square means of wing shape for A. mimicry rings and B. habitat types for forewing (lower, left hand corner) and hindwing (upper, right hand corner). Colours from green to red represent a scale from more similar to more differentiated wing shapes.

A.

	blue	black & white	dennis rayed	orange	postman	red & white	silvani-form
blue		0.05	0.03	0.05	0.03	0.02	0.08
black & white	0.03		0.05	0.05	0.05	0.04	0.06
dennis rayed	0.05	0.05		0.04	0.04	0.03	0.07
orange	0.05	0.05	0.05		0.04	0.04	0.08
postman	0.03	0.03	0.05	0.05		0.02	0.07
red & white	0.05	0.05	0.06	0.04	0.03		0.08
silvaniform	0.05	0.05	0.03	0.06	0.05	0.06	

Hindwing

Forewing

B.

	Closed	Edge	Field	Open
Closed		0.02	0.06	0.03
Edge	0.03		0.05	0.05
Field	0.06	0.06		0.07
Open	0.04	0.04	0.09	

Hindwing

Forewing

2.3.5. Testing for Convergence with the Ithomiini

I used semi-landmarks to compare specimens of the Heliconiini and Ithomiini to determine whether the divergence in wing shape in the silvaniform ring could be explained by a convergence towards the ithomiine species that they mimic. I sampled 33 individuals from 29 species of ithomiines (one of each, except for two species for which I had two individuals and one with three individuals). Of these, 27 specimens belonged to the silvaniform mimicry ring, while 6 specimens belonged to non-silvaniform rings. I sampled 139 Heliconiini specimens with at least one male and one female for each species; 57 specimens (*Heliconius*, n = 51; *Eueides*, n = 6) belonging to the silvaniform mimicry ring, and 82 specimens (*Heliconius*, n = 68; *Eueides*, n = 14) to non-silvaniform mimicry. Figure 2.4 shows the first two PCs from the PCAs of forewing and hindwing of Heliconiini with the ithomiine wing shapes plotted onto heliconiine morphospace.

For the analyses, one silvaniform Ithomiine, and three non-silvaniform Heliconiine were removed for lack of scales to measure centroid size. For both forewing and hindwing, the natural log of centroid size has a significant effect on shape explaining 0.12 and 0.03 of the proportion of shape variation of the two wings, respectively, which is expected as some genera within the Ithomiini are much smaller than Heliconiini. In both forewing and hindwing there is a clear separation between the silvaniform and non-silvaniform individuals within the Heliconiini, with the silvaniform Heliconiini clustering with the Ithomiini individuals. From the results of the linear models, I found significant differences between the forewing shape of the silvaniform and non-silvaniform mimicry rings ($R^2 = 0.1$; $F = 22.3$, $p = 0.001$), as well as differences between family (Heliconiini vs Ithomiini) ($R^2 = 0.04$; $F = 9$, $p = 0.001$), and the interaction between the two is also significant ($R^2 = 0.05$; $F = 12.7$, $p = 0.001$). In the hindwing, mimicry ring explains a much larger percentage of shape variation than in forewing ($R^2 = 0.4$; $F = 141.2$, $p = 0.001$), with family ($R^2 = 0.1$; $F = 33.6$ $p = 0.001$) and the interaction ($R^2 = 0.03$; $F = 11$, $p = 0.001$) also having significant effects. The results from the pairwise analyses support these findings, with smaller distances between the mimicry groups means than between family means (Table 2.4).

In the hindwing, female silvaniform specimens cluster closer towards the ithomiine specimens than do the male silvaniform specimens. I carried out linear models within the Heliconiini species to test for effects of sex in the silvaniform while accounting for

species variation. In the forewing, sex had a significant effect on forewing shape (R^2 0.08, F = 10, p = 0.001) and hindwing shape (R^2 = 0.11, F = 12.4, p = 0.0017). These analyses included centroid size to control for allometry, which was significant for both forewing and hindwing, explaining 0.09 of the proportion of variation in wing shape in both wings. The results from the pairwise analysis on distance of female and male means of silvaniform and non-silvaniform compared to the Ithomiini showed that females were more similar within mimicry type, and the difference with males was stronger in the hindwing.

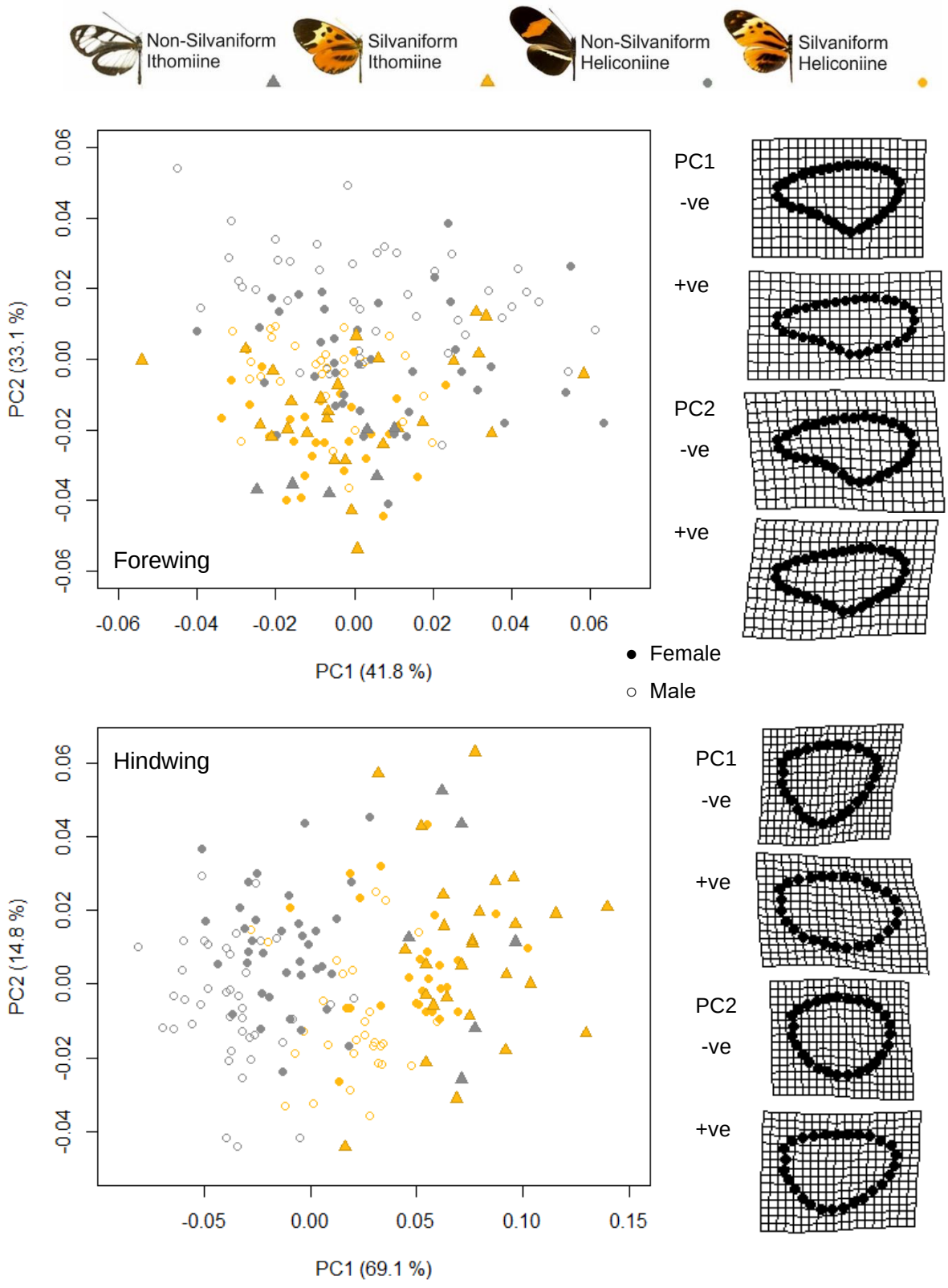
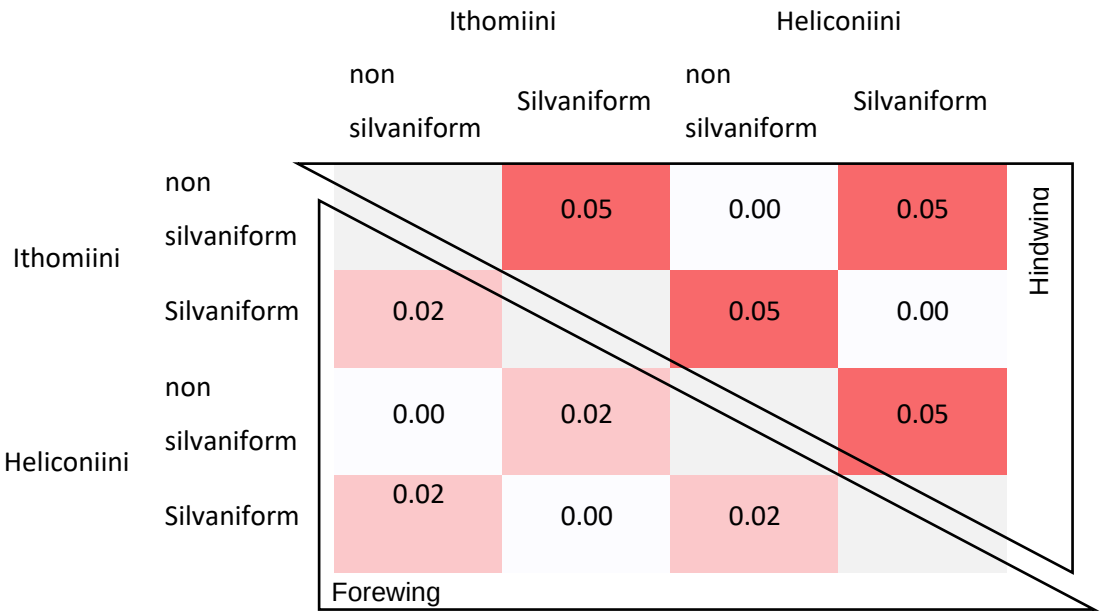


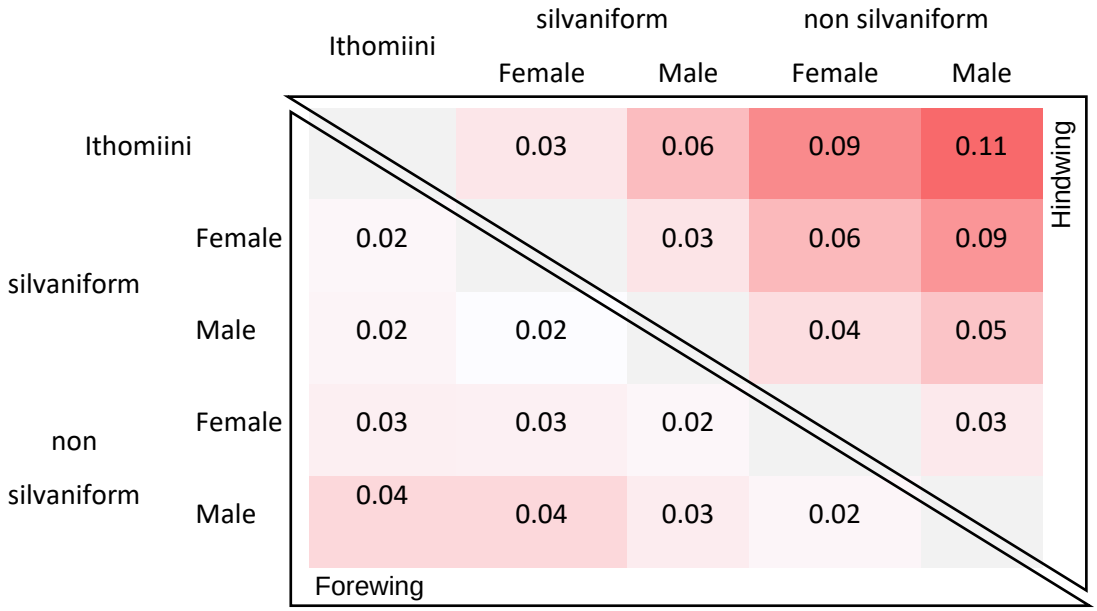
Figure 2.4. First and second principal components of the PCA on *Heliconius* (○) wing shape for forewing (top) and hindwing (bottom). Ithomiine specimens (Δ) are plotted onto the existing variation. Specimens displaying the silvaniform colour pattern are shown in yellow and specimens with different colour patterns in grey. The closed circles represent female individuals of the *Heliconius* and *Eueides* specimens, open circles represent males. On the right are shown the deformation plots along PC1 and PC2.

Table 2.4. A. Pairwise distances between least square means of wing shape of the silvaniform and non-silvaniform of Ithomiini and Heliconiini for forewing (lower, left hand corner) and hindwing (upper, right hand corner). B. Pairwise distance of group means of males and females of Heliconinni compared to the ithomiines for forewing (lower, left hand corner) and hindwing (upper, right hand corner). Colours from green to red represent a scale from more similar to more differentiated wing shapes.

A.



B.



2.3.6. Sexual dimorphism in silvaniform colour and wingshape

I analysed the colour pattern of 45 forewings and 46 hindwings of four silvaniform species and analysed the PCs explaining over 95% of variation to compare any sex differences in colour pattern and wing shape. There were no significant sex differences in colour pattern or shape of forewing, controlling for centroid size. Sex differences were only found in the hindwing shape (Table 2.5). Species explained more variation in forewing colour pattern than forewing wing shape. However, in the hindwing, species explained a similar amount of variation in both colour pattern and wing shape.

Table 2.5. Results from linear models with a mixed effect design testing for differences in colour pattern and wing shape between males and females of four silvaniform species, accounting for species variation.

		Colour Pattern			**Wing Shape**		
		df	R^2	p value	df	R^2	p value
Forewing	log(centroid size)	1	0.12	0.001	1	0.09	0.001
	sex	1	0.02	0.751	1	0.04	0.253
	species	3	0.25	0.001	3	0.13	0.001
	sex : species	3	0.05	0.495	3	0.07	0.096
		df	R^2	p value	df	R^2	p value
Hindwing	log(centroid size)	1	0.06	0.001	1	0.03	0.058
	sex	1	0.02	0.749	1	0.12	0.007
	species	3	0.36	0.001	3	0.38	0.001
	sex : species	3	0.06	0.007	3	0.03	0.518

2.4. Discussion

Species evolve and adapt in response to different aspects of their environment and ecomorphological studies have been used to identify these selective pressures and their interactions. In this chapter, I aimed to identify and characterise the ecological drivers acting on wing shape variation in a group of mimetic butterflies. Overall, the results are consistent with the notion that wing shape is involved in the mimetic signal of *Heliconius* butterflies. Controlling for common ancestry, both mimicry ring and habitat type have a significant effect on wing shape variation; and the effect of mimicry is stronger than habitat overall, and stronger in the hindwing than the forewing (Table 2.2). However, these patterns are mostly driven by the silvaniform mimicry ring (Table 2.3) which are strongly divergent in shape from the other mimicry rings, especially for hindwing shape.

These results exemplify the role and dynamics of ecological factors driving selection; both mimicry and habitat type drive forewing and hindwing shape, but their effects differ in strength between the two wings. However, it is important to note that habitat and mimicry are not fully independent, there is some geographical segregation of colour patterns since mimics co-occur and therefore, are exposed to similar habitats. I also found that compared to the hindwing, the forewing is less convergent between mimics, which could be due to stronger phylogenetic or aerodynamic constraints. Although results from Chapter 3 did not conclusively identify a stronger role of forewing shape in flight, evidence from manipulations in Lepidoptera have demonstrated that the two wings are very different in terms of their role during flight; forewing is necessary for flight whereas loss of hindwings only affected manoeuvrability in experiments removing the wings (Jantzen & Eisner, 2008). Therefore, these results are consistent with different selective regimes acting on the two wings. Other studies of wing shape have identified different pressures acting on the two wings; wing shape changes (attributed to flight performance) in damselflies with varying mate success and survival were stronger in the forewing than in the hindwing (Outomuro *et al.*, 2016). Females of the butterfly species *Melitaea cinxia* showed dispersal-related shape variation in the forewing only (Breuker *et al.*, 2007).

2.4.1. Strong divergence of the silvaniform wing shape

As mentioned, however, strong differences between mimics seem to be mostly driven by the silvaniform mimicry group, for which wing shape converges towards the Ithomiini shape. While there is no evidence to suggest that birds can detect subtle variation in wing shape or differences in flight patterns, experienced collectors can recognise different *Heliconius* species during flight. Wing shape mimicry could therefore be selected for increased visual mimicry or could be selected for behavioural mimicry through flight (Srygley, 1999). However, due to the non-independence of mimicry and habitat it is also possible that convergence in flight between the silvaniform and the Ithomiines happened as a result of adaptation to similar habitats in sympatry.

Divergence of the silvaniform is consistently stronger in the hindwing throughout all analyses. Stronger mimicry in the silvaniform hindwing could increase resemblance with ithomiine flight behaviour, particularly in aspects relevant to manoeuvrability (Jantzen & Eisner, 2008). From these results it seems that *Heliconius* forewing shape is likely more strongly constrained to retain a *Heliconius*-like shape compared to hindwing shape. Flight characteristics are not driven solely by wing shape and various studies have demonstrated the role of body mass, muscle contractions, physiology and many other factors (Le Roy *et al.*, 2019) and these do not act independently to wing shape. Indeed, genetic manipulations in *Drosophila* wing shape found an increase in cost associated with mutant wing shapes which may have resulted from a disjunction with muscle morphology. Ithomiine butterflies are different to *Heliconius* in several ways. Ithomiine butterflies tend to fly more slowly in the understory, and many species are much smaller than *Heliconius* (data for morphological and flight measures in a few *Heliconius* and Ithomiini species can be found in Dudley & Srygley, 1994 and Srygley, 2004) and these differences will probably also impact flight behaviour, either independently or in conjunction with wing shape.

Pairwise comparisons between the non-silvaniform mimicry groups show that they do not differ much in their forewing and hindwing shapes (Table 2.3). Weaker divergence in the hindwing shape of the non-silvaniform mimicry groups could occur for two reasons. Firstly, if hindwing shape is driven by mimicry of warning signal, then this weaker divergence could be explained by smaller variation in colour patterns

within the other mimicry groups compared to the silvaniform. This is very difficult to measure however, as colour variation is difficult to quantify in complex patterns involving multiple colours across two separate wings. Furthermore, we do not have a thorough understanding of the exact visual cues used by predators, although the importance of colour for predation has been demonstrated, with lower attack rates of colourful compared to achromatic models (Finkbeiner *et al.*, 2014), as well as the importance of UV colouration in predator vision (Arias *et al.*, 2016). Similarly, stronger similarities in overall body shape and size, as well as flight-related behaviours between *Heliconius* species compared to the Ithomiini, could also explain weaker divergence in the non-silvaniform mimicry rings.

2.4.2. Sexual dimorphism in the silvaniform wing shape

Interestingly, I found stronger convergence of silvaniform female wing shape with the Ithomiini. Two mechanisms could drive sexual dimorphism in silvaniform wing shape. Firstly, mimicry could be female driven, whereby the mimetic signal evolves in females first, common in female limited Batesian mimics where the mimetic pattern is derived (Kunte, 2009). Alternatively, wing shape mimicry could be constrained in males. In the former case, if females are driving mimicry, one might expect sexual dimorphism in other mimetic traits. However, in the analysis of the four silvaniform species, I found no evidence of sexual dimorphism in colour pattern although analyses of on the colours of *H. numata* have found that females have slightly larger black patches (Llaurens, *personal communications*); this discrepancy can be attributed to differences in methodology and trait being measured. However, without knowing which aspects of colour pattern are relevant to predator signalling, it is difficult to make any conclusion on the relevance of these differences. Hindwing shape was the only trait found to be different between males and females, which suggests different selective constraints on wing shape evolution for mimicry between the sexes.

Selective constraint against mimicry in males could be due to sexual selection. Males perform courtship hovers which could select for wing shapes that enable this complex flight (Dudley, 2002; Le Roy *et al.*, 2019), but there is no evidence to suggest that females use male wing shape itself as a mating signal. There is also evidence of small-scale habitat segregation between the sexes in *H. numata* (Joron, 2005),

which belongs to the silvaniform mimicry ring (and in a few other species reported in Bates, 1862) which could explain sexual dimorphism in wing shape. In the Joron (2005) study, male *H. numata* were caught on average 1.21 m higher than females, with female flight height being more similar to that of *Melinaea* species that they mimic. This is consistent with the finding that females are the better mimics; although it is difficult to determine whether this is due to increased visual mimicry or similar aerodynamic pressures from flying lower to the ground. Stronger mimicry in females is also consistent with patterns of female-limited Batesian mimicry in Lepidoptera (Kunte, 2009; Turner, 1978).

Small-scale habitat segregation between males and females is likely common in other *Heliconius* species as there is often a male bias during collections of wild individuals (pers. observation and communication). Male bias not only reflects potential habitat segregation but also different levels of activity as males spend more time flying looking for mates or guarding territories whereas females look for host plants for oviposition and may rest to avoid harassment from males. These differences and evidence for sexual dimorphism in wing shape are strongly suggestive of different morphological adaptations between males and females, subject to different evolutionary pressures.

When testing for effects of mimicry ring and habitat type in males and females across the whole of the Heliconiine, there was no evidence of stronger convergence between mimics in females. Sex differences occur depending on species' ecology and the presence of habitat segregation or behavioural differences between males and females, which might not occur consistently enough across the phylogeny for these analyses to identify. Furthermore, sex differences within species may be too subtle compared to interspecific variation and more focused analyses on a few species may be required to detect these, such as in the silvaniform analysis in this Chapter. Other analyses that identified sex differences in wing shape between *H. demeter* and *H. eratosignis* (Rosser *et al.*, 2019) or in *H. melpomene*, *H. erato* and *H. besckei* (Rossato *et al.*, 2018a) were carried out in only a few species.

2.4.3. Conclusions

Overall, these results reveal the complexity of patterns of wing shape evolution in *Heliconius* and reveal the drivers that have been most important across the entire

genus. However, more fine-scale studies will be required to understand these patterns and these may also find that other factors are important at the species level; as shown by sex differences which are apparent in more focused studies but not across the genus (although this may also be due to sampling). Also, forewing shape was shown to vary across pairs of species adapted to different elevations (Montejo-Kovacevich *et al.*, 2019); however, only a small effect of elevation was found for hindwing in this chapter. This discrepancy could be explained by different methods of measuring shape (Montejo-Kovacevich *et al.*, 2019 used aspect ratio) and larger within species sample sizes. Adaptation to elevation may also only occur in a handful of species (the majority of *Heliconius* are distributed at lower elevations) and may not be as relevant to wing shape evolution across the entire genus. This might reflect a limitation of large-scale ecological comparisons to detect important correlations at the species level. However, this does not invalidate larger scale analyses; in the wing shape analysis *H. numata* (a silvaniform), results revealed convergence with wing shape of *Melinaea* species (Jones *et al.*, 2013), but this analysis was limited to one species and did not identify the exceptional divergence of the silvaniform group as a whole, away from other mimicry rings.

Understanding the effects of habitat and mimicry is also difficult and further studies will be needed to characterise their independent contributions to wing shape evolution, as convergence in one will favour convergence in the other. Furthermore, understanding how wing shape and colour pattern evolve towards increased mimicry requires more investigation. Eyespots shape in *Bicyclus anynana* was shown to be correlated to shape changes in the corresponding wing cell suggesting common developmental pathways (Monteiro *et al.*, 2002). Therefore, wing shape and colour pattern in *Heliconius* could be similarly developmentally constrained and understanding which trait is directly driven by selection is still unclear. Studying the evolutionary drivers of traits is therefore dependent on the context and understanding their dynamics at different levels of organisation is necessary to fully understand the evolution of phenotypes such as wing shape.

In conclusion, this chapter is the first phylogeny wide study of wing shape across the Heliconiine and has demonstrated the importance of studying the evolution of complex traits at different scales. The results also reveal the contribution of mimicry ring and habitat type in driving wing shape evolution, and suggests stronger aerodynamic constraints in forewing shape. In this chapter, I also demonstrated the

striking divergence of the silvaniform mimicry groups away from other *Heliconius* groups and the convergence with Ithomiini. Furthermore, I presented results for sexual dimorphism in wing shape demonstrating that females are the better mimics. Studies using fine grain measures of habitat type will hopefully further our understanding of its relationship with mimicry. Indeed, there is some level of mimicry-based spatial partitioning in *Heliconius* (Mallet, 1993) and mimicry, ecology and phylogeny likely interact in forming mimetic communities (Elias *et al.*, 2008). The order in which these different factors have shaped the *Heliconius* radiation is still unknown.

Chapter 3

Understanding associations between flight and wing shape in *Heliconius* butterflies.

Abstract

The evolution of flight in winged insects is thought to have been one of the main drivers in their diversification. While the aerodynamics of flight strongly influences wing shape, other non-aerodynamic factors may also be driving its evolution. In addition to colour patterns, aspects of flight and wing shape are thought to be involved in the Müllerian mimicry of unpalatable *Heliconius* butterflies. In this chapter, I characterise the wing shape and flight (wing beat frequency and wing angle) of *Heliconius elevatus* and *Heliconius pardalinus* and use experimental manipulations to establish the relationship between these traits to understand how they may respond to different selective pressures. I find that these two closely related non-mimetic sister species show differences in wing shape as well as wing beat frequency and wing angles. Shape and flight parameter differences between species were stronger in the hindwing compared to the forewing, suggestive of different selective constraints acting on the two wings. Both forewing and hindwing shape were associated with flight parameters. However, the wing shape manipulations experiments suggest that species differences in flight parameters are not caused by the differences in wing shape between the species. Overall, results indicate that different factors may be independently driving the evolution of flight and wing shape in these butterflies.

3.1. Introduction

The evolution of flight is considered one of the main drivers of diversification in pterygote insects. Increased dispersal abilities have allowed access to a wider range of habitats and enabled adaption to new ecological niches (Dudley, 2002). The act of flying is a complex process involving different aspects of anatomy, physiology and aerodynamics, and can vary in characteristics such as speed, wing beat frequency, wing stroke asymmetry and wing angles. These characteristics vary among taxa, within taxa, and within individuals to suit different behaviours and ecological requirements. Differences in flight characteristics may be a consequence of different dispersal distances, navigational requirements resulting from habitat complexity, or specific requirements imposed by feeding ecologies for example (Dudley, 2002). In insects, in addition to its main role in locomotion, flight may also be shaped by factors such as sexual selection, intra and/or interspecific signalling or thermoregulation (Berwaerts *et al.*, 2001; Betts & Wootton, 1988).

Flight characteristics can be strongly influenced by wing shape and extant insects show a range of wing morphologies. For example, narrow wings (high aspect ratio) are generally associated with more efficient flight and long-distance dispersal, whereas more rounded wings (lower aspect ratio) are associated with slower, more agile flight (Betts & Wootton, 1988; Chazot *et al.*, 2016; DeVries *et al.*, 2010). However, the secondary selective forces acting on wings may result in the evolution of wing shapes that optimise fitness at the expense of flight performance. Insect flight can be generated by homonomous wings, with the hindwing and forewing sharing similar morphologies, or from heteronomous wings where aerodynamic function is lost to different extents in either the hindwing (anteromotorism) or in the forewing (posteromotorism) (Dudley, 2002). In some cases, there have been extreme changes in the morphology of the non-aerodynamic wing, such as the elytra in Coleoptera, or even loss of a wing pair such as in Diptera, where hindwings have reduced to halteres. The different flight characteristics and wing shapes adopted by insects can shed light on the ecological factors that have shaped their evolution.

Understanding how flight characteristics and wing shape are connected is the subject of much research. We know that insect flight is determined both by the aerodynamic properties of the wings and by morphological and physiological aspects of the body

(Dudley, 2002). However, the relationship between flight characteristics and wing shape is complex as the two may be subject to different evolutionary pressures, which may even be different between forewing and hindwing.

In some insects, particularly in the Lepidoptera, wings may also play a role in mimicry, where individuals from different species display the same defensive wing colour patterns (Bates, 1862). In Müllerian mimicry, the wing patterns serve as warnings, with colour patterns of multiple unpalatable species converging as a result of selective pressure from predators (Meldola, 1882; Wallace, 1882). By contrast, in Batesian mimicry, patterns of undefended species converge with those of defended species. Several studies in insects have found mimicry extending to wing shape and flight (Golding *et al.*, 2001; Kitamura & Imafuku, 2015; Silberglied & Eisner, 1969). Extension of mimicry to behaviour is expected (Bates, 1862) possibly as a consequence of selection for a more complex signal which not only improves predator recognition of unpalatable butterflies but also limiting the cost of mimicry by palatable mimics.

Mimicry has been particularly well studied in some butterfly genera such as *Papilio* and *Heliconius* (Merrill *et al.*, 2015; Nadeau, 2016), where much of the focus has been on understanding the genetics of colour patterning. *Heliconius* is a genus of ~50 neotropical butterfly species whose ecology and evolution have been strongly shaped by mimicry. All *Heliconius* are Müllerian mimics and members of a variety of mimicry rings together with basal heliconiine, ithomiine butterflies and even day flying *Chetone* moths. Distantly related *Heliconius* species can be near identical whereas sister species often display strikingly different patterns (Joron & Mallet, 1998; Mallet & Gilbert, 1995). This mimetic signal has been shown to extend to flight patterns in a few *Heliconius* species; Srygley (2007) found evidence of convergence in wing beat frequency and wing stroke asymmetry between the co-mimics of two pairs of sister species (*H. melpomene-H. cydno* and *H. erato-H. sapho*). Flight is particularly interesting in the context of Müllerian mimicry as species may experience relaxed pressure to evade predators ; as a result, Müllerian mimics have been shown to have a reduced flight muscle mass and display slower and less erratic flight than palatable butterflies (Marden & Chai, 1991; Srygley & Chai, 1990). We might therefore expect wing shape to also experience relaxed selection from predation, instead allowing mimicry to be an important driver. Indeed, studies have shown evidence of mimicry

driving convergence of wing shape between *Heliconius* species and their co-mimics (Jones *et al.*, 2013; Mérot *et al.*, 2016; Rossato *et al.*, 2018a).

Although there is some evidence for flight and wing shape mimicry in *Heliconius*, the effect of wing shape on flight parameters, and the extent to which one constrains the other, are poorly understood. In a study of four *Heliconius* species, the centres of wing and body mass were found to be correlated with wing beat frequency (Srygley, 1999). However, direct wing shape manipulations are needed to identify a causal relationship between wing shape and flight. This could help us understand whether flight and wing shape are independently selected for by mimicry, or if selection on one drives the evolution of the other.

In this chapter I measure flight and wing shape characteristics in two *Heliconius* butterfly species; *Heliconius elevatus* and *Heliconius pardalinus*. *Heliconius elevatus* and *H. pardalinus* are very closely related sister species with largely overlapping geographic distributions in the Amazon basin (Rosser *et al.*, 2012). The species differ in a range of traits that affect pre- and postzygotic isolation, including colour pattern (Rosser *et al.*, 2019). *Heliconius elevatus* has bright red and yellow pattern elements on a black background, mimicking other “dennis-rayed” heliconiine species, while *H. pardalinus* has a mottled brown/orange and yellow pattern mimicking genera from the Ithomiini as well as some *Heliconius* species (Figure 3.1). Divergence into these distinct mimetic rings has been driven by the introgression of colour pattern alleles between dennis-rayed *H. melpomene* and *H. elevatus* (Dasmahapatra *et al.*, 2012; Wallbank *et al.*, 2016). The species are also found in slightly different habitats; *H. elevatus* butterflies being found flying high in primary forests while *H. pardalinus* butterflies are generally found flying at a lower height in more disturbed forest (Rosser *et al.*, 2019). These habitat and flight preferences may exert different selection pressures on the aerodynamic properties of hindwings and forewings, which may in turn conflict with selection towards different mimicry rings.

I initially characterise the natural variation between the two species before examining the association between wing shape and flight parameters. I then investigate whether there is a causal relationship between the two by testing whether manipulating the wings of one species to the shape of the other species results in changes in flight to resemble that of the other species. I also test the relative contributions of changes in forewing and hindwing on flight characteristics, with the

expectation that forewing manipulations would have a stronger effect due to the anteromotorism of Lepidoptera.

3.2. Methods

3.1.1. Characterising the Natural Variation in Flight and Wing Shape

I measured wing shape and flight parameters of *H. elevatus* and *H. pardalinus* to estimate morphological and behavioural differences between the species. To characterise shape, wings were removed from wild-caught specimens (*H. elevatus* = 30, *H. pardalinus* = 24) around Tarapoto (Peru) and digitised using a flatbed scanner at 300 dpi. I increased the sample sizes using specimens reared in captivity in York, UK, (*H. elevatus* = 7, *H. pardalinus* = 10). Instead of using wing loading or centre of wing mass (Srygley, 1994), in the context of mimicry, I chose to use geometric morphometrics to fully characterise hindwing shape variation from 26 *H. pardalinus* (7 females and 19 males) and 31 *H. elevatus* (12 females and 14 males) samples, and forewing shape from 29 *H. pardalinus* (10 females and 19 males) and 26 *H. elevatus* (12 females and 19 males) samples. Owing to wing damage, fore and hindwing measurements were not possible for all individuals. Landmarks were placed at vein intersections (see Appendix 1A) based on similar studies in other species (Jones *et al.*, 2013; Mérot *et al.*, 2013). Coordinates were extracted from these landmarks using TpsDig2 (Rohlf, 2006). Forewing and hindwing landmarks were then adjusted for size and orientation using Procrustes analyses and analysed separately using principal component analyses (PCA) with the geomorph package in R (Adams *et al.*, 2019). Differences between sex and species were tested using ANOVA on the Procrustes output. I also calculated the wings' aspect ratio using the formula from Hill *et al.*(1999):

$$Aspect\ ratio = \frac{(wing\ length)^2}{wing\ area}$$

Wing area and length (see Appendix 1A) were measured in TpsDig2. Although considered to be a crude measure of wing shape (Betts & Wootton, 1988), aspect ratio is widely used in studies of wing shape as it can be used to predict aerodynamic efficiency. Differences in aspect ratios between sex and species were tested with linear models.

Two characteristics of flight have previously been shown to co-vary among two pairs of mimetic *Heliconius* species (Srygley, 2007): wing beat frequency and wing stroke

asymmetry. In these experiments, I measured wing beat frequency and wing angles (as a proxy for wing stroke asymmetry); this is the measure of the angle the wings make when at the highest and lowest points of the wing beat cycle (see schematic in Figure 3.1C and Appendix 2A). To measure these parameters, individual butterflies were filmed while flying freely in a large cage measuring 1.5 m (W) × 9 m (L) × 2.5m (H), using a GoPro HERO4 Black camera shooting at a rate of 240 frames per second at a resolution of 720p. Time of day and temperature were recorded at the beginning of each video. Videos were analysed using GoPro Studio 2.5.9.2658 and only straight flights (lasting at least four beats and with no dips, lifts or turns) were used for measuring flight parameters. Five flights were measured for all but two individuals. The total number of wing beats over the number of frames was counted across all flights to determine wing beat frequency. Wing angles at the down beat and up beat (see Appendix 2A) were measured using ImageJ from frames where the butterflies were directly in front of the camera to minimise parallax errors. Wing beat frequency was measured from 12 *H. pardalinus* (7 males and 5 females) and 12 *H. elevatus* (6 males and 6 females) individuals, and wing angles from 11 *H. pardalinus* (6 males and 5 females) and 11 *H. elevatus* (7 males and 4 females) individuals. Differences between the species were tested using linear models in R version 3.5.3. The effect of temperature on wing beat frequency and wing angle was tested using mixed models in *H. pardalinus* and *H. elevatus* separately with individuals as a random factor. As no effect of temperature was found, it was not included as a parameter in the other analyses. Residuals were tested for normality using the Shapiro-Wilk normality test.

3.1.2. Flight and Wing Shape Associations

After establishing that *H. pardalinus* and *H. elevatus* demonstrated significant differences in flight and wing shape, I investigated whether there were any associations between wing shape and flight parameters (wing beat frequency and wing angles) both within and between species. Flight parameters of reared individuals were measured as described above, after which the wings were collected for morphometric analysis of wing shape. Landmarks were analysed with Procrustes analyses to adjust for size and orientation in geomorph (Adams *et al.*, 2019); the output of these were analysed using ANOVA with wing beat frequency, up and down

wing angle and species as independent variables using a marginal sums of squares computation.

The association between wing aspect ratio (from forewing and hindwing separately) and flight parameters was tested using linear mixed-effects model fit by restricted maximum likelihood (REML) (Pinheiro *et al.*, 2019). Flight parameters were used as dependent variables, aspect ratio as the independent variable and species as a random effect to identify effects within species.

3.1.3. Wing Shape Manipulation

As well as testing for associations between flight and wing shape, a wing shape manipulation experiment was carried out to determine whether there is a causal relationship between the measures of flight and wing shape. In particular, I was interested in testing whether the differences in flight parameters I found between the two species were a consequence of their divergent wing shapes. Two questions were addressed in this experiment. First, what are the effects of changes in wing shape on flights? To answer this, *H. elevatus* and *H. pardalinus* individuals were manipulated either to the heterospecific shape or to the conspecific shape as a control. Second, do changes in forewing shape have a relatively larger effect on flight than changes in hindwing shape as expected for a species showing anteromotorism of flight?

An *H. elevatus* and *H. pardalinus* wing shape template, hereafter referred to as the "elevatus" and "pardalinus" templates, were made from scanned wings of an individual of each taxon. I chose to manipulate individuals based on differences explained by PC1, as this explained the most variation between species across both forewing and hindwing (Figure 3.2A). The two most extreme individuals on a plot of hindwing PC1 against forewing PC1 of the wild samples were used to generate the templates. Multiple wing templates were printed onto card, making them incrementally smaller or larger (5, 10, 15, 20, 25 and 30% of the original size) to adapt to the natural variation in butterfly size (see Appendix 2B). For any particular individual, the template used was one that minimised the reduction in wing area (the relative sizes of the forewing and hindwing were maintained). Wings shape was manipulated by cutting around the template, held over the wing, with scissors.

Manipulations were carried out in two steps. In the first manipulation, half of the individuals had the forewing manipulated, and the other half had their hindwing manipulated. In the second manipulation, the remaining wings were manipulated to produce the complete manipulation. This resulted in four treatment combinations: i) conspecific forewing first, ii) conspecific hindwing first, iii) heterospecific forewing first, iv) heteropecific hindwing first. Individuals' flights were measured three times; unmanipulated, after the first manipulation, and after the second manipulation. Two males and two females of each species were measured for each of the four treatment combinations.

To understand the effect of manipulation on flight, I analysed the change in flight parameters between the unmanipulated and fully manipulated individuals (i.e. measure of manipulated flight/measure of unmanipulated flight). I analysed the change in flight parameters between the unmanipulated and partly manipulated individuals to answer the question on the relative roles of hindwing and forewing. I used the non-parametric Wilcoxon signed-rank test on flight change to identify significant effects of "elevatus" vs. "pardalinus" template, and fore vs. hindwing manipulation, within each species. I also tested for differences in changes in flight parameters between sexes, but sex was not significant.

3.1.4. Testing the Effect of Manipulation on Other Wing Measures

Wing shape manipulation will reduce other characteristics of wing shape such as wing length and area which may in turn affect flight characteristics. I was unable to take these wing measurements before and after manipulations as measures on live butterflies are very inaccurate. Therefore, I used individuals from the flight and wing shape experiment and assigned each individual to an "elevatus" and "pardalinus" template appropriate for its wing size as was done in the shape manipulation experiment. Wing templates were scanned, and their lengths and areas measured using ImageJ. By comparing measurements of the wings and to their assigned templates I was able to investigate whether one type of manipulation had a bigger effect on one of the species compared to the other. Using GLMs I tested the effect of species and template type on the percentage difference in length and area of forewing and hindwing separately.

3.3. Results

3.3.1. Characterising the Natural Variation in Flight and Wing Shape

Heliconius elevatus had a higher wing beat frequency than *H. pardalinus* (t = -2.97, $p = 0.007$) and that females had a higher wing beat frequency than males in both species (t = -2.43, $p = 0.024$) (Figure 3.1). *Heliconius elevatus* had a larger up beat angle compared to *H. pardalinus* (t = -2.69, $p = 0.014$) but a smaller a down beat angle (t = 3.52, $p = 0.002$), and I found no sex differences in wing angle (Figure 3.1). The interaction between sex and species was not significant for any flight parameter and was therefore not included in the models.

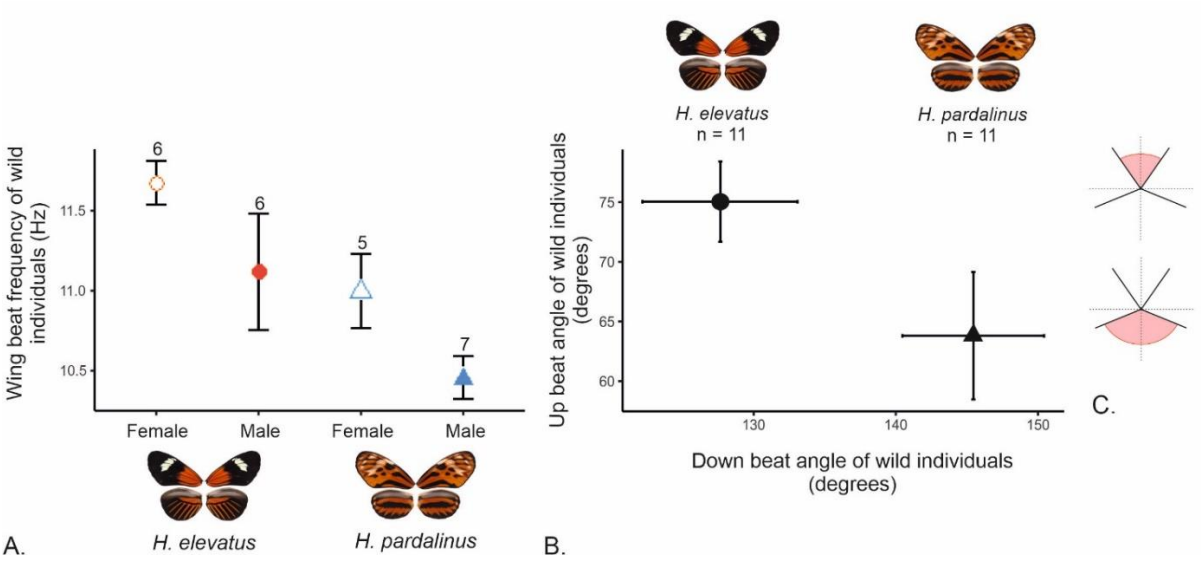


Figure 3.1. A. Wing beat frequency of *H. elevatus* and *H. pardalinus* individuals measured in Peru; sample sizes are indicated above the bars representing standard error. Wing beat frequencies were significantly different between species (t = -2.97, $p = 0.007$) and between sexes (t = -2.43, $p = 0.024$). B. Wing angles during flight of *H. elevatus* and *H. pardalinus* individuals measured in Peru, bars represent standard errors. I found significant differences between species in the down (t = 3.52, $p = 0.002$) and up (t = -2.69, $p = 0.014$) beat angles. The sexes did not show significant differences in wing angles. C. Schematic of the measured angles in red; up beat angle is shown above and down beat angle is shown below.

Wing shape was measured in 37 and 36 wild-caught individuals of *H. elevatus* and *H. pardalinus* respectively. Principal component analyses were carried out on 55 forewings and 57 hindwings; the first two principal components (PCs) of shape variation are shown in Figure 3.2. The first two PCs explained a higher proportion of

variation in hindwing shape (PC1 = 44.8 %, PC2 = 26.4 %) than in forewing shape (PC1 = 21.6 %, PC2 = 18.1 %; see also Appendix 2C for variation explained by all PCs). The warp grids from the PCAs indicate that *H. pardalinus* has rounder forewing and hindwing outer margins compared to *H. elevatus* (Figure 3.2).

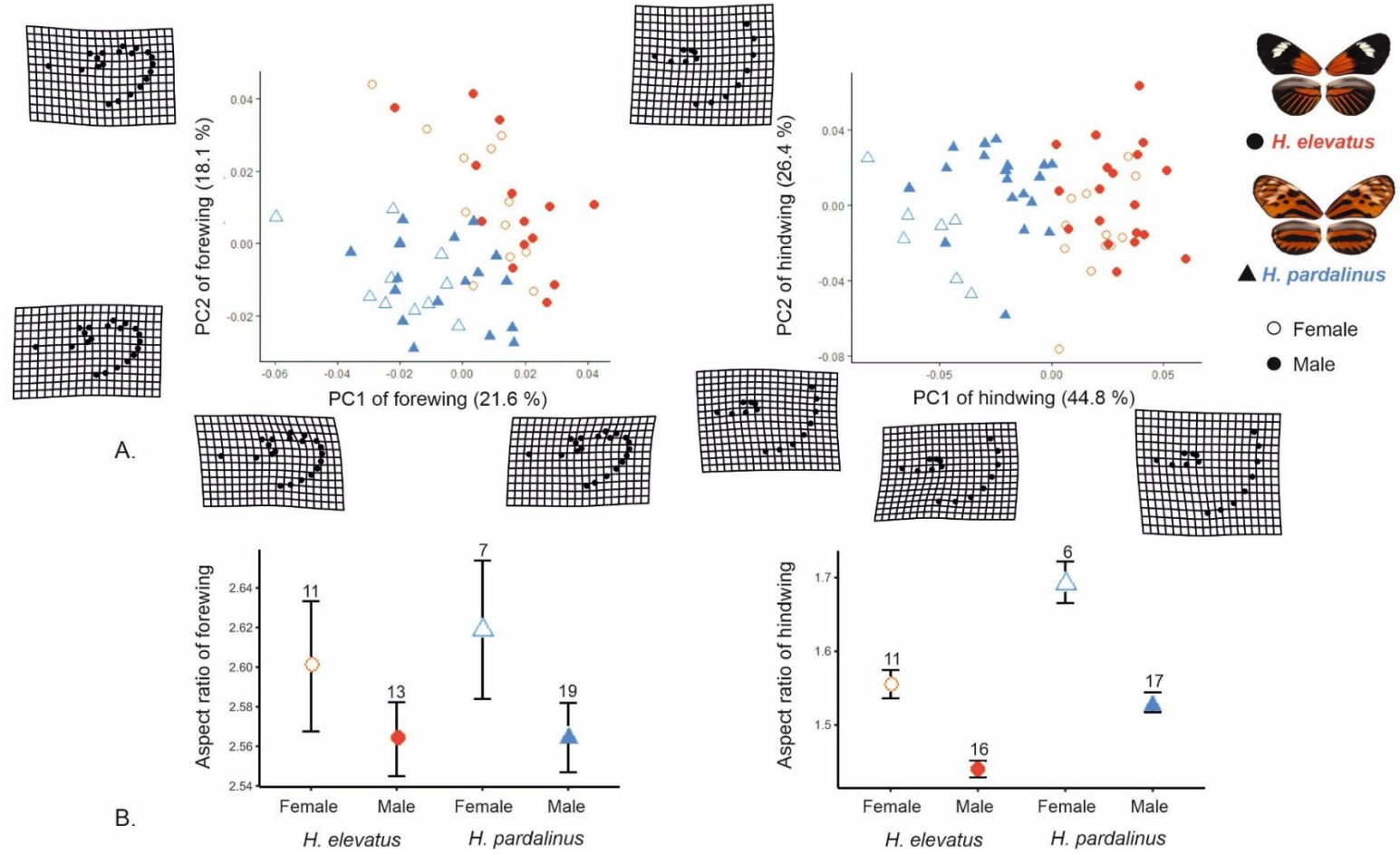


Figure 3.2. A. Principal component analysis of forewing and hindwing shape of *H. elevatus* (red) and *H. pardalinus* (blue) individuals for females (open circles) and males (closed circles). The splines show the extreme wing shape phenotypes along the first and second principal components. B. Aspect ratio of forewing and hindwing of males and females of *H. elevatus* and *H. pardalinus*. Error bars represent the standard error. The numbers denote the sample sizes of each. Aspect ratio of hindwing was significantly different between species (t = 4.86, *p* = 1.42E-05) and sexes (t = -5.24, *p* = 3.95E-06).

I carried out ANOVAs on the Procrustes output to test for differences in species and sex on the hindwing and forewing shapes and calculated the proportion of variation explained by each (R^2). There were significant effects of species ($R^2 = 0.15$; $F = 9.95$, $p = 0.001$) and sex ($R^2 = 0.04$; $F = 2.67$, $p = 0.004$) on forewing shape, as well as significant effects of species ($R^2 = 0.32$; $F = 29.64$, $p = 0.001$), sex ($R^2 = 0.086$; $F = 7.85$, $p = 0.001$) and their interaction ($R^2 = 0.014$; $F = 1.3$, $p = 0.048$) on hindwing shape.

The aspect ratios of 50 forewings and hindwings were also measured. Using linear models, I tested whether there was a significant effect of species and sex on aspect ratio (Figure 3.2). Hindwing aspect ratio was significantly larger in *H. pardalinus* compared to *H. elevatus* ($t = 4.86$, $p = 1.42E-05$) and females of both sexes had significantly larger hindwing aspect ratios than males ($t = -5.24$, $p = 3.95E-06$). No significant differences were found in any forewing measures.

3.3.2. Flight and Wing Shape Associations

To understand the relationship between flight parameters and wing shape I investigated the association between the two using ANOVA on the Procrustes output in 8 *H. elevatus* and 10 *H. pardalinus* butterflies. Species had a significant effect on wing shape, as expected from tests on wild individuals. Wing beat frequency was significantly associated with variation in wing shape of both fore and hindwings across both species. Significant associations between wing shape and the down beat angle were found in the hindwing, but not in the forewing. There were no associations between the up beat angle and wing shape. The output of the ANOVA is shown in Table 3.1, and the correlations between wing shape (PC1 and PC2 only) and flight parameters are shown in (Appendix 2D). Aspect ratio was not significantly correlated with any flight characteristic in the mixed models.

Table 3.1. Proportion of variation (R^2) in forewing and hindwing wing shape explained by the different flight parameters from the ANOVA on the Procrustes output. Significant effects are shown in bold.

		R^2	p value
Species differences			
	forewing	**0.15**	**0.001**
	hindwing	**0.30**	**0.001**
Wing beat frequency			
	forewing	**0.064**	**0.012**
	hindwing	**0.097**	**0.001**
Up beat angle			
	forewing	0.037	0.15
	hindwing	0.013	0.3
Down beat angle			
	forewing	0.043	0.08
	hindwing	**0.039**	**0.02**

3.3.3. Wing Shape Manipulation

Flight characteristics were measured before and after a succession of hindwing and forewing manipulations to either a conspecific or heterospecific shape to test for a causal relationship between species differences in wing shape and flight (Table 3.2). I measured flight parameters in 16 *H. pardalinus* individuals, one of which only has the data for the unmanipulated flight, and 19 *H. elevatus* individuals (extra individuals of *H. elevatus* were included as two were missing the final flight and there were other butterflies available).

Table 3.2. Percentage change in flight parameters after the different types of manipulation. The results from the non-parametric Wilcoxon signed rank test are given for each comparison; the significant comparisons are shown in bold.

	Average value of unmanipulated flight (st error)		**Change after single manipulation (st error)**			**Change after full manipulation (st error)**		
Wing beat frequency (Hz)	*H. elevatus* (n = 19)	11.55 (± 0.14)	forewing	4 % (± 1)	(W = 59, p = 0.28)	"elevatus" template	7 % (± 2)	(W = 42, *p* = 0.61)
			hindwing	2 % (± 2)		"pardalinus" template	1.05 (± 2)	
	H. pardalinus(n = 16)	10.81 (± 0.21)	**forewing**	**8 % (± 2)**	(W = 46, *p* = 0.040)	"elevatus" template	15% (± 3)	(W = 12, *p* = 0.072)
			hindwing	**2 % (± 1)**		"pardalinus" template	7 % (± 2)	
Up beat angle	*H. elevatus* (n = 19)	68.83 (± 2.65)	forewing	1 % (± 8)	(W = 28, *p* = 0.18)	"elevatus" template	0 % (± 4)	(W = 41, *p* = 0.97)
			hindwing	11 % (± 4)		"pardalinus" template	0 % (± 6)	
	H. pardalinus (n = 16)	54.41 (± 2.79)	forewing	-10 % (± 11)	(W = 18, *p* = 0.28)	"elevatus" template	-15 % (± 6)	(W = 123, *p* = 0.61)
			hindwing	10 % (± 8)		"pardalinus" template	-10 % (± 6)	
Down beat angle	*H. elevatus* (n = 19)	124.63 (± 1.92)	**forewing**	**-5 % (± 2)**	(W = 20, *p* = 0.043)	"elevatus" template	-9 % (± 3)	(W = 18, *p* = 0.054)
			hindwing	**3 % (± 3)**		"pardalinus" template	-4 % (± 2)	
	H. pardalinus (n = 16)	138.55 (± 3.1)	forewing	-6 % (± 4)	(W = 21, *p* = 0.46)	"elevatus" template	-8 % (± 4)	(W = 28, *p* = 1)
			hindwing	-1 % (± 0)		"pardalinus" template	-7 % (± 3)	

To understand whether species differences in wing shape could explain observed differences in flight I looked at changes in flight parameters between the first (unmanipulated) and third (fully manipulated) flights. In both species, wing beat frequency increased after manipulations, and this increase was larger for the "elevatus" template. The effect was also stronger for *H. pardalinus* individuals. But none of these changes were statistically significant. In most cases, wing angles reduced after manipulations, however, changes in up and down wing beat angles were not significantly different between manipulation types (see Table 3.2).

I compared the differences between the first (unmanipulated) and second (partly manipulated) flights to understand the relative effect of forewing and hindwing shape on flight. For *H. pardalinus*, forewing manipulation (irrespective of the type of manipulation) significantly increased wing beat frequency ($W = 46$, $p = 0.040$), while no effect was found with the hindwing manipulation. This pattern was also found in *H. elevatus* but it was not statistically significant ($W = 59$, $p = 0.277$). In *H. elevatus*, hindwing manipulation had a significantly stronger effect on down beat wing angle than forewing manipulation ($W = 20$, $p = 0.043$). A bigger effect of forewing manipulation compared to hindwing manipulation was also observed for down beat wing angles in *H. pardalinus* and up beat wing angles of both taxa, but these were not significant (Table 3.2). None of these effects remain significant after multiple testing correction.

3.3.4. Testing the Effect of Manipulation on Other Wing Measures

To identify whether wing manipulations had different effects on wing length and area of the different species, I compared the changes in these measures between the two species using the "elevatus" and "pardalinus" templates (Figure 3.3). The detailed results of the tests are presented in Appendix 2E. Overall, manipulations using the "elevatus" template had a greater effect on wing area, whereas the "pardalinus" template had a greater effect on length. The manipulations affected *H. pardalinus* wing length to a greater extent, whereas *H. elevatus* wing area was more affected. These results are consistent with expectations, changing a wing with a higher AR (like that of *H. pardalinus*) to a wing with a lower AR (like that of *H. elevatus*) reduces length and vice versa.

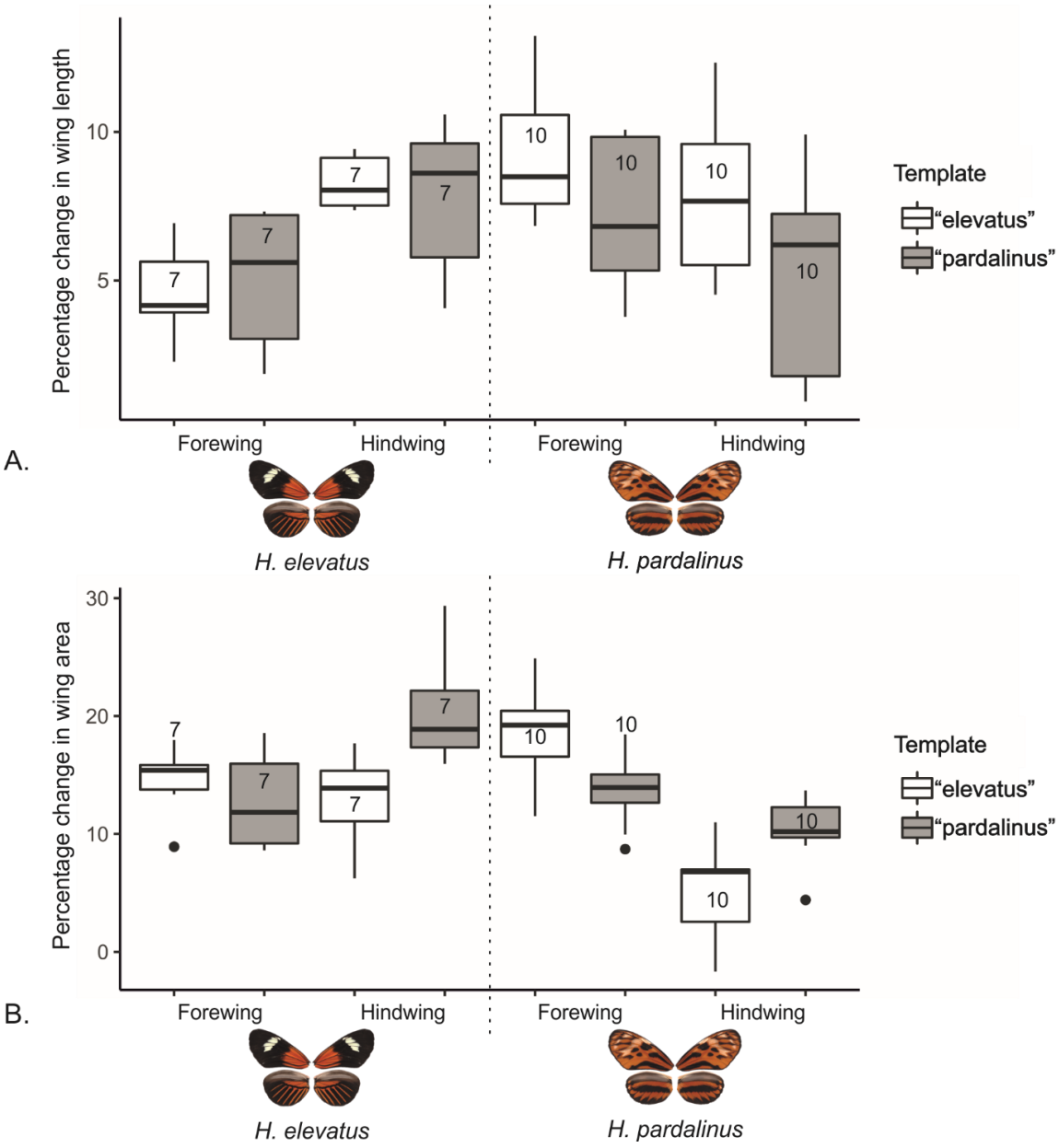


Figure 3.3. Percentage of A. wing length and B. wing area lost as a result of manipulations with either the "elevatus" or "pardalinus" template, in forewing and hindwings of *H. elevatus* and *H. pardalinus.* The numbers on the figures represent sample sizes for each treatment.

3.4. Discussion

I found significant differences in flight parameters (wing beat frequencies, up beat and down beat wing angles), and both fore and hindwing shapes between the closely related non-mimetic sister species *H. elevatus* and *H. pardalinus*. These differences could be a consequence of the two species belonging to different mimicry rings, with mimicry extending beyond wing patterning to cause closer matching between co-mimics, although this was not formally tested in the context of this study. With its dennis rayed pattern, *H. elevatus* mainly mimics several more abundant *Heliconius* species with which it co-occurs across the Amazon basin. In contrast, as the silvaniform mimicry ring that *H. pardalinus* belongs to is driven mainly by distantly-related co-mimetic ithomiine butterflies in the *Tithorea*, *Melinaea* and *Hypothyris* genera, flight and wing shape in *H. pardalinus* may be evolving in response to stronger divergent selective pressures (see Chapter 2). The differences in wing shape between the species was greater in the hindwing. This suggests that forewing shape may be subject to different aerodynamic constraints, while hindwing shape is perhaps more labile and able to respond to non-aerodynamic selection pressures such as those caused by mimicry.

In addition to being affected by mimicry, flight characteristics and wing shape may also be influenced by habitat differences between the species. *Heliconius elevatus* is generally found flying fast and high in primary forests (Brown, 1981) whereas *H. pardalinus* is usually found flying at lower levels in secondary forests. The higher tree density in secondary forest compared primary forest (Chazdon *et al.*, 2007) may select for greater flight manoeuvrability in *H. pardalinus*. Based on studies in vertebrates, more elongated wings (higher aspect ratio) are expected to be associated with more efficient, distance flying in open/canopy habitat. Whereas lower aspect ratio (more rounded) wings are more important for manoeuvrability and typical of lower flying, understory species; these predictions seem to be broadly maintained in butterflies (Betts & Wootton, 1988; Chazot *et al.*, 2016; DeVries *et al.*, 2010). Despite their higher aspect ratio, which is a less accurate measure, the warp grids from the PCA show that *H. pardalinus* wings are more rounded than those of *H. elevatus*. The latter meets the expectations of a lower flying species in secondary

forests; and less rounded wings are consistent with a faster flying species, such as *H. elevatus*.

In this chapter, both fore and hindwings were associated with flight parameters. I suppose that the forewing, being the main driver in lepidopteran flight, is used more for propulsion whereas hindwings may be more associated with smaller variations in flight patterns, as suggested by other studies where flight was maintained after the removal of hindwings but evasiveness was reduced (Jantzen & Eisner, 2008). This association between wing shape and flight could be the results of a causal relationship or independent selection on the two phenotypes from mimicry and habitat.

How mimicry and habitat affect flight and wing shape, and the order in which they act, is still unclear. Elias *et al.* (2008) found that co-mimics of ithomiine species converged in micro-habitat types and suggested that co-occurring species evolved to mimic one another, and this was followed by further convergence in other ecological traits, such as flight physiology. Flight data from a wider range of species spanning multiple mimicry rings, including mimetic and non-mimetic ithomiine species would be required to better understand the interaction of mimicry and habitat in the evolution of flight and wing shape.

In addition to species differences, I also report sex differences in flight characteristics and wing shape. While there are many reported instances of sexual dimorphism in insects (Chazot *et al.*, 2016; DeVries *et al.*, 2010; Hernández *et al.*, 2010) including *Heliconius* (Jones *et al.*, 2013; Jorge, Cordeiro-Estrela *et al.*, 2011), this study constitutes the first record of sexual dimorphism in a flight parameter of *Heliconius*, with females displaying higher wing beat frequencies than males in both the species. Sexual dimorphism can seem counter-intuitive in Müllerian mimics, but such differences might be attributed to sex differences in flight behaviour, such as oviposition in females and courting in males. In some cases, habitat segregation between the sexes (from differences in behaviour for example) could potentially increase the benefits of mimicry by reducing the proportion of mimics compared to the model in the model's habitat and alleviating pressure from predation (Joron, 2005; Turner, 1978). Flight differences can also be explained by different mass allocation due to reproductive constraints in females (Marden & Chai, 1991; Srygley & Chai, 1990). The presence of sex as well as species differences further

emphasises the importance of understanding the roles of habitat and mimicry in the evolution of wing shape and flight.

The wing manipulation experiment to test the effect of wing shape on flight yielded largely non-significant results. In all cases the shape manipulations increased wing beat frequency, likely a consequence of butterflies compensating for a reduction in aerodynamic function resulting from the loss of wing area (Kingsolver, 1999). In general, I found no effect of manipulation type ("elevatus" vs "pardalinus" shape) on the flight of the species. While the non-significant result from the manipulation experiment could be due to relatively small sample sizes leading to a lack of power in this experiment, I conclude that the species differences in wing shape, which I expect are involved in mimicry, do not in themselves explain differences found in flight characteristics between *H. elevatus* and *H. pardalinus*. Other factors, such as thorax morphology, physiology, and other aspects of wings not necessarily involved in visual mimicry may explain the observed differences in flight.

In conclusion, I find that the two closely related sister species *H. elevatus* and *H. pardalinus* show significant differences in wing shape and flight characteristics, which, as suggested by Chapter 2, could be driven by selection towards different mimicry rings and habitats. Despite complete interfertility between *H. elevatus* and *H. pardalinus*, these species rarely mate in the wild or in captivity due to the presence of multiple prezygotic barriers to reproduction (Rosser *et al.*, 2019). The species differences in flight and wing shape that may enhance pre-zygotic isolation via fine scale habitat segregation and perhaps mate choice. The differences also likely augment extrinsic postzygotic isolation with interspecific hybrids displaying intermediate flight patterns and wing shapes being selected against by predators. I also found sex differences in both wing shape and flight which could reflect differences in behaviour or sex dependent habitat segregation. Wing shape differences were stronger in the hindwing which I propose is a result of reduced constraint from flight aerodynamics. The data suggest that the differences in wing shape between the two species are not by themselves causing the observed differences in flight characteristics.

Chapter 4

QTL for wing shape identified in region of high F_{ST} in *Heliconius* butterflies.

Abstract

Understanding the genomic architecture of ecologically relevant traits can shed light on the mechanisms involved in speciation. Quantitative Trait Loci (QTL) analyses can be used to address such questions, but new developments in multivariate methods have allowed us to investigate the genomic architecture of complex traits, such as wing shape. In this chapter, I use landmark based geometric morhometrics and QTL analyses to study the genomic architecture of wing shape between two species of *Heliconius*, *H. elevatus* and *H. pardalinus*. Evidence has shown that wing shape between these species is likely under divergent selection from mimicry and habitat. The results from the univariate and multivariate QTL analyses show two different loci associated with wing shape on chromosome 20 and on chromosome 2. The QTL on chromosome 20 falls within a region of high F_{ST}, supporting a role of islands of divergence and gene flow during the divergence of these species.

4.1. Introduction

Understanding the genomic architecture of ecologically relevant traits can be a valuable tool to understand species diversification. Today, speciation is widely accepted as occurring mainly through natural selection, either through ecological speciation or mutation-order speciation (Schluter, 2009). During mutation-order speciation, species accumulate different mutations or alleles in response to similar selective pressures and reproductive isolation occurs stochastically as a result of genetic incompatibilities (Mani & Clarke, 1990). During ecological speciation, two populations diverge in response to selection from different environments or ecological niches. Reproductive isolation arises if mating probability is dependent on ecology, hybrids are maladapted, or through drift and accumulation of random mutations (Nosil, 2012). Mutation-order speciation, is unlikely to occur in the presence of strong gene flow (Nosil & Flaxman, 2011), as beneficial alleles evolving in one population will be beneficial in the other. However, in the past two decades, there has been growing evidence of gene flow during divergence (Smadja & Butlin, 2011), suggesting ecological speciation is much more common (Schluter, 2009). Despite this, understanding the mechanisms that allow populations to go from slightly divergent populations to reproductively isolated species in the face of gene flow remain poorly understood (Via, 2012).

In the genic view of speciation (Wu, 2001), the genome is not a homogenous divergent block, but instead, isolation can occur as a result of differentiation at a few key loci underlying reproductive barriers, such as mate preference traits, under divergent selection. Gene flow breaks down associations between genes in regions of the genome that are not involved in reproductive isolation, creating a landscape of heterogenous divergence across the genome (Nosil, 2012; Smadja & Butlin, 2011). Other traits can then diverge through associations with these loci involved in reproductive barriers; either through pleiotropy (when one gene influences two or more traits), tight physical linkage, or even through chromosomal structures that prevent recombination, such as inversions (Feder *et al.*, 2013; Smadja & Butlin, 2011). Certain properties of chromosomes can also reduce recombination, sex chromosomes show lower levels of recombination and recombination is also limited near the centromeres (Martin & Van Belleghem, 2017; Smadja & Butlin, 2011).

Divergent selection can then reduce gene exchange in areas around the selected loci in a process called “divergence hitchhiking” and create “islands of divergence” across the genome (Via, 2012). Ultimately, strong divergence in a few, small islands can increase and eventually lead to genomic hitchhiking where migration rate is reduced across the entire genome.

A large body of evidence now exists with examples of increasing size and number of islands between taxa with increasing levels of divergence (Nadeau *et al.*, 2012; Renaut *et al.*, 2012; Turner *et al.*, 2005) and with decreasing levels of geographic overlap (Kulathinal *et al.*, 2009; Martin *et al.*, 2013). However, there are also cases where evidence suggests large genomic regions, instead of “islands” are involved in early stages of divergence and where standing genetic variation is put forward as playing an important role in speciation (Michel *et al.*, 2010; Parchman *et al.*, 2013). Furthermore, caution is warranted when interpreting results from “incipient” species as we cannot be certain that complete reproductive isolation will be reached, and there are few examples of speciation at “intermediate stages” (Merrill *et al.*, 2015).

Islands of divergence can also occur without the effect of gene flow; examples show that islands can appear as a result of reduced variation within populations from selective sweeps and heterogeneous levels of recombination (Burri *et al.*, 2015; Delmore *et al.*, 2015). Recently diverged species pairs can display low levels of differentiation overall and regions of high differentiation can occur as a result of strong selection on loci within these regions (Cruickshank & Hahn, 2014). Therefore, identifying the loci involved in divergent ecological traits is central to understanding species divergence.

The *Heliconius* radiation offers an excellent opportunity to study speciation in wild populations as pairs of species or sub-species within the radiation can offer “snapshots” of different stages of divergence (Mérot *et al.*, 2017). Several studies have highlighted the importance of gene flow in the *Heliconius* radiation (Dasmahapatra *et al.*, 2012; Edelman *et al.*, 2019; Kozak *et al.*, 2018; Martin *et al.*, 2013), with examples of introgression of adaptive traits (Wallbank *et al.*, 2016; Zhang *et al.*, 2016) and even hybrid speciation (Mavárez *et al.*, 2006; Salazar *et al.*, 2005). *Heliconius* are also famous for their colour patterns, a textbook example of Müllerian mimicry (Merrill *et al.*, 2015). These colour patterns are driven by natural selection, from predator driven frequency dependent selection (Arias *et al.*, 2016; Chouteau *et al.*, 2016), but have also been shown to be used in mate choice across a range of

species (Merrill *et al.*, 2015). Additionally, studies have found linkage between colour pattern genes and genes involved in mate preference (Kronforst *et al.*, 2006; Merrill *et al.*, 2019, 2011), therefore fulfilling many theoretical predictions of speciation with gene flow (Smadja & Butlin, 2011). The genes controlling these colour patterns have also been found within regions of higher F_{ST} within species (Nadeau *et al.*, 2012), supporting the idea of genetic hitchhiking and islands of divergence (Smadja & Butlin, 2011; Via, 2012).

Compared to the genetics of wing colour patterning, the evolution of wing shape in *Heliconius* has been understudied. Wing shape is an important trait in pterygote insects as it directly influences flight aerodynamics (Dudley, 2002). Flight is thought to have been an important driver in insect evolution as it allowed the colonisation of new ecological niches. Wings have also evolved in response to a number of other selective pressures, sometimes even to the detriment of flight ability. In *Heliconius*, evidence from a few species has suggested that wing shape is also driven by Müllerian mimicry (Jones *et al.*, 2013; Mérot *et al.*, 2016; Rossato *et al.*, 2018a). My work has shown that mimicry of wing shape is likely present, to different extents, across the entire *Heliconius* phylogeny (Chapter 2). The results of Chapter 2 also indicate that variation in wing shape could be an adaptation to different habitats and to a lesser extent, altitude (Montejo-Kovacevich *et al.*, 2019b). Wing shape is therefore likely under strong divergent selection from mimicry and possibly from habitat if wing shape differences lead to habitat divergence (although the opposite may be true), suggesting that it may play an important role in ecological speciation. Furthermore, my work has shown that the shape of the forewing and hindwing appear to be driven by different selective pressures and yet nothing is known of the genetic architecture that control their variation.

Genetic mapping of phenotypic traits has been carried out in a wide variety of species for a number of traits. Until recently these analyses have focused on univariate traits, and morphological traits such as wing shape, have been summarised using single measures, such as size or length (Frary *et al.*, 2004; Manuel Pérez-Pérez *et al.*, 2002; Tanksley, 2004), or single axes from Principal Component Analyses on morphometric data (Chase *et al.*, 2002; Langlade *et al.*, 2005; Liu *et al.*, 1996). Morphological traits are complex multivariate traits, integrated within each other, meaning that variation in one aspect of shape will cause covariation in another as a result of interacting genes and developmental pathways (Klingenberg, 2010;

Klingenberg & Leamy, 2001). Reducing multivariate information to a single variable therefore leads to loss of information; hence the importance of integrating multivariate methods in genetic analyses, a method increasingly used in recent years for morphological traits (Klingenberg *et al.*, 2001; Pitchers *et al.*, 2019) and other types of multivariate data (Topp *et al.*, 2013).

Most research on the genetic basis of morphological traits have focused on model systems such as the mouse (Klingenberg *et al.*, 2001; Leamy *et al.*, 1999), as well as other systems like sticklebacks (Albert *et al.*, 2007), dogs (Chase *et al.*, 2002), and plants (Frary *et al.*, 2004; Manuel Pérez-Pérez *et al.*, 2002; Tanksley, 2004); with a majority of studies in *Drosophila* (for example, Liu *et al.*, 1996; Zimmerman *et al.*, 2000). Whether these studies have used univariate or multivariate methods, they have all consistently found several QTLs involved in shape, and sometimes more than 20. The output of this research demonstrates that shape is a polygenic trait and genetic, developmental and environmental interactions play a big role in its determination. Studies specifically on the genetic architecture of wing shape morphology, however, are primarily focused on *Drosophila* (Mezey *et al.*, 2005; Pitchers *et al.*, 2019; Zimmerman *et al.*, 2000). However, studies in other systems such as ants (Abouheif & Wray, 2002), beetles (Tomoyasu *et al.*, 2009), and butterflies (Macdonald, Martin, & Reed, 2010; Weatherbee *et al.*, 1999) have shown that genes involved in wing shape development are highly conserved among insects but variation can occur from differences in gene expression (Macdonald *et al.*, 2010).

In this chapter, I use the *Heliconius* system to study the genetic architecture of wing shape variation in the context of speciation in two *Heliconius* species, *H. elevatus* and *H. pardalinus*.. The two species belong to separate mimicry rings (Rosser *et al.*, 2019), *H. elevatus* having received its red black and white rayed pattern through introgression from *H. melpomene* (Wallbank *et al.*, 2016), and *H. pardalinus*, displaying the characteristic mottled brown, black and yellow “silvaniform” pattern (Figure 4.1). The two species show divergent wing shapes (Chapter 3), notable in the roundedness of the outer margin in *H. pardalinus*. Furthermore, the two are found in slightly different habitat, although the habitat measure used in Chapter 2 did not discriminate between their habitat types; *H. elevatus* inhabits more primary forests, whereas *H. pardalinus* is more commonly found in seasonally flooded, secondary habitat (personal observations, see Rosser *et al.*, 2019). *Heliconius elevatus* tends to fly higher up in the canopy and at a faster

speed (personal observations) and has a faster wing beat frequency (Chapter 3). The divergence in wing shape is particularly strong in the hindwing, which could be due to constraints imposed on forewing shape due to the anteromotorism of flight in Lepidoptera (Chapter 2).

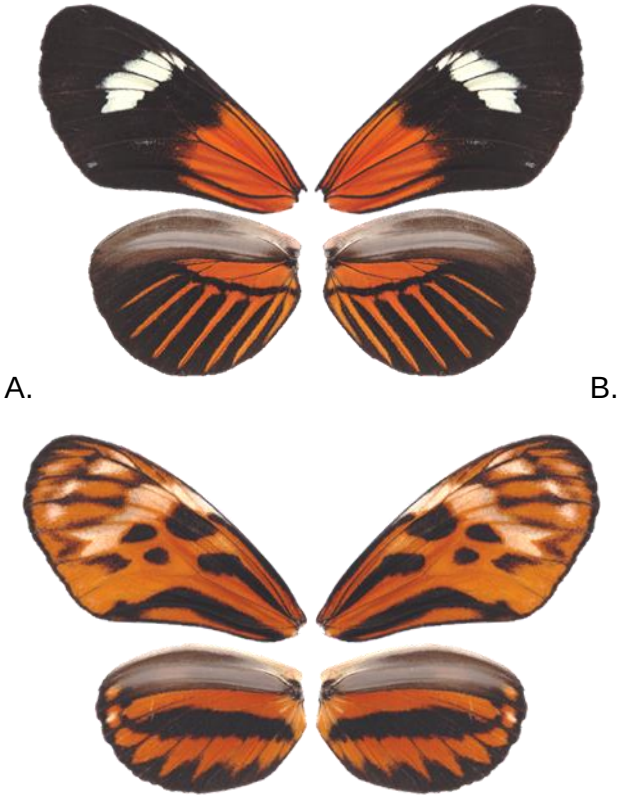


Figure 4.1. Dorsal side of *H. elevatus* (A) and *H. pardalinus* (B) wings; *H. elevatus* is characterised by the red “dennis” (forewing) and rays (hindwing) as well as the white forewing band. *H. pardalinus* is characterised by the orange/brown, yellow and black pattern of the Silvaniform.

Heliconius elevatus and *H. pardalinus* are sympatric over most of their range and show low levels of divergence in over 95% of the genome, with a few regions of high F_{ST} (Figure 4.2, Dasmahaptra, *unpublished*). Their overlapping geographical ranges combined with putative hybrids captured in the wild (Rosser *et al.*, 2019) and patterns of pre and post zygotic isolation between the two species strongly suggest divergence with gene flow (Rosser *et al.*, 2019). Furthermore, phylogenetic analyses using genome wide single nucleotide polymorphisms show that *H. elevatus* and *H. pardalinus* are paraphyletic when allopatric races of *H. elevatus* from the Guianas and of *H. pardalinus* from Peru are included (*unpublished*, Dasmahapatra). This

further supports the role of gene flow although this has not been directly tested in these species. However, certain regions of the genome are consistent with a “species” tree, where *H. elevatus* and *H. pardalinus* cluster according to the taxonomy (Figure 4.2, unpublished, Dasmahapatra). These regions coincide with areas of high F_{ST}, some of which contain genes involved in colour pattern determination (Figure 4.2, *unpublished*, Dasmahapatra). These regions therefore correspond to islands of divergence containing traits involved in reproductive isolation.

I carry out quantitative trait loci (QTL) analyses on crosses between *H. elevatus* and *H. pardalinus* to determine whether QTLs for wing shape are found within these regions of high F_{ST}, in support of the islands of divergence model of speciation. This work is part of a wider study aiming to identify the underlying genomic architecture of several important ecological traits under divergent selection between these two species (analysis ongoing). Furthermore, I expect that these QTLs may be located within regions also controlling colour patterns as these are expected to be under strong selection, and due to the role of mimicry on wing shape. I carry out these analyses on forewing and hindwing shape separately to identify potential differences due to the contrasting evolutionary pressures of the two wings.

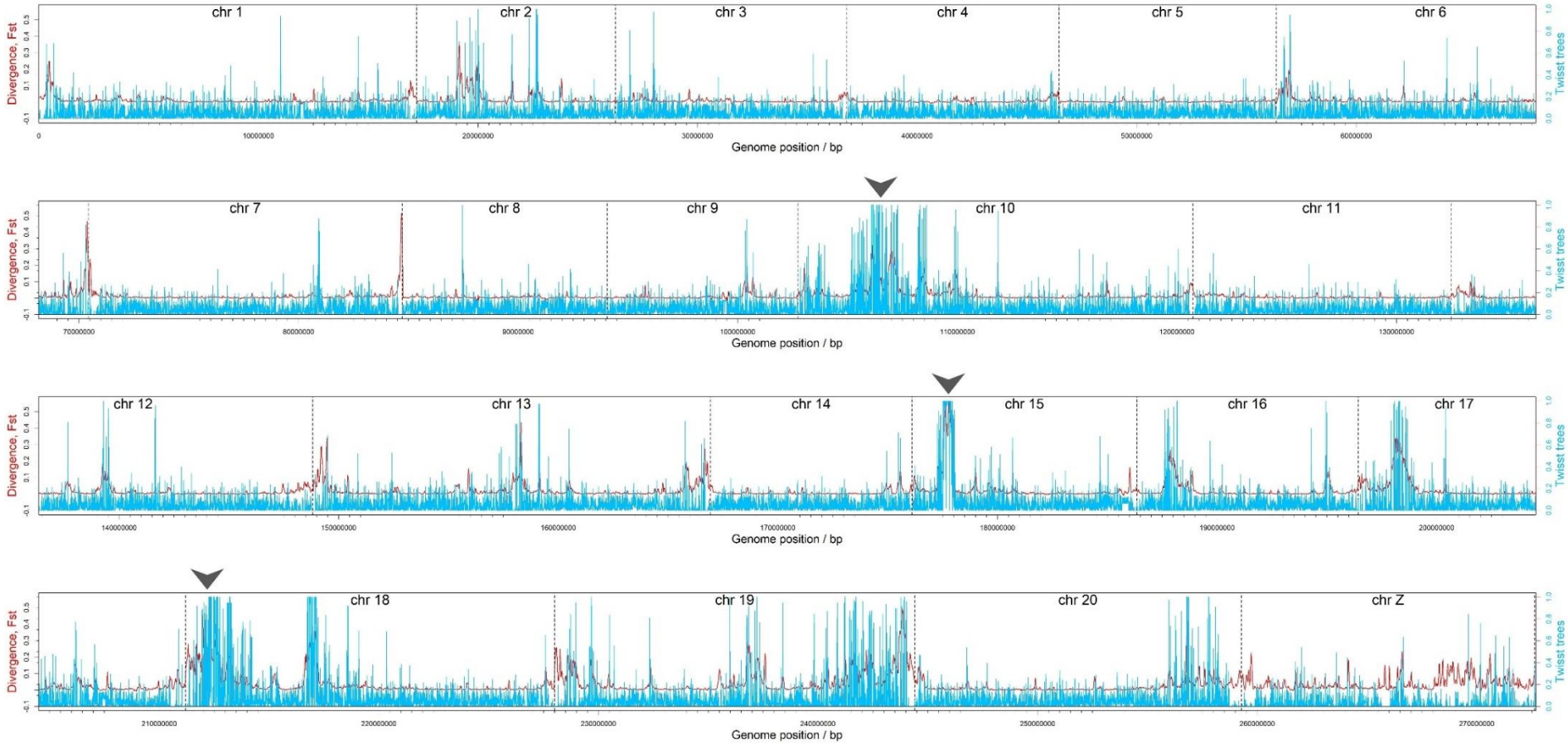


Figure 4.2. Levels of F_{ST} between *H. elevatus* and *H. pardalinus* across the genome (in red)- higher values suggest higher levels of differentiation- and tree scores from Twisst (in blue) where a value of 1 means that the location is in accordance with the taxonomic "species" tree. The regions marked with a grey arrow represent the known regions of colour pattern genes (unpublished, Dasmahapatra).

4.2. Methods

4.2.1. Collection and Rearing

Heliconius elevatus and *H. pardalinus* butterflies, hereafter referred to as *H. elevatus* and *H. pardalinus* for simplicity, collected in the region of San Martin, Peru, were used to establish stock populations in large insectaries in Tarapoto. These populations were maintained over two years with regular additions of new individuals from the wild. Populations were maintained with 10 % sugar water solutions and pollen from flowers of *Gurania*, *Lantana* and *Polianthes* species. Hybrid F1 crosses were achieved through hand-pairing, see supplementary material in Rosser *et al.* (2019). Mated females were isolated and given shoots of *Passiflora* (Passifloracea) for egg laying, typically *P. edulis*, *P. riparia* and *P. laurifolia*. Matings between F1 individuals or between an F1 and a parental species occurred through natural mating and handpairing. Parent IDs were recorded, and mated females were isolated for egg laying. Eggs were collected and larvae were reared in pots using fresh leaves of *P. edulis*, *P. riparia* and *P. serrato-digitata*.

Fathers were collected and preserved for DNA directly after mating, mothers were sampled when egg production rate reduced significantly and F2 and backcross (BC) hybrids were sampled after further phenotypic characterisation. During sampling, bodies were stored in a Dimethyl sulfoxide solution (20% DMSO, 0.25 M EDTA, saturated with NaCl) and kept in a freezer at -20°C until DNA extraction; wings were stored in glassine envelopes.

4.2.2. Quantifying Wing shape

Wing shape was quantified in the parental species and their crosses using landmark based geometric morphometrics analyses. The ventral side of the butterfly wings were scanned using a flatbed scanner at 300 dpi and landmarks were placed at specific vein intersections (Figure 4.3) using tpsDig2 (Rohlf, 2006). Landmark coordinates were adjusted for size and orientation using a Procrustes analysis from

the package geomorph (Adams *et al.*, 2019). All further analyses were carried out on the Procrustes coordinates for forewing and hindwing separately.

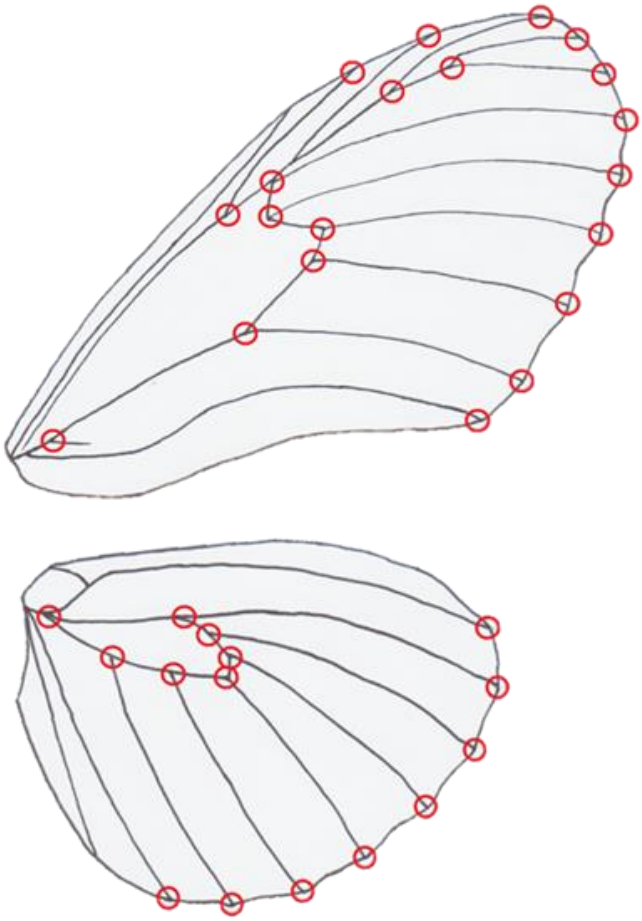


Figure 4.3. Heliconius wing venation and landmarks used to measure the forewing (20 landmarks) and the hindwing (15 landmarks) shape.

To characterise the variation in the wing shape of the crosses along the main axes of variation between the parental species, I carried out a principal component analysis (PCA) on the wild caught and reared individuals of the parental species (individuals used in Chapter 3) using prcomp() in R, centred the data from the crosses by subtracting the columns means of the parents and multiplying by the rotation matrix of the parental PCA.

I used the Procrustes coordinates to identify sex differences in the parental species and in the F2 and BC hybrids using linear models in geomorph (Adams *et al.*, 2019).

I then determined whether there was significant integration between forewing and hindwing shape (correlated variation between two morphological traits) which could suggest common developmental or genetic pathways. For this, I measured the degree of association between the forewing and hindwing Procrustes coordinates for each parental species using a two-block partial least square analysis in geomorph (Adams *et al.*, 2019). I also measured this within the crosses to determine whether the association was robust to recombination. Finally, I carried out a cluster analysis on the non-rotated PCs of the F2 crosses that explain 95 % of variation to identify potential groups, characteristic of traits underpinned by a small number of loci with major effects. The method identifies the number of clusters k where k+1 does not increase the total variance explained (V), also known as the "elbow method" (Hothorn & Everitt, 2014).

4.2.3. DNA Extraction, RAD library preparation and Linkage Map Generation

A third to a half of the thorax of each individual was used for DNA extraction using the Qiagen DNeasy "Blood and Tissue" kit. Restriction site associated DNA (RAD) libraries were prepared as described by Baird *et al.* (2008) with modifications from Hoffman et al. (2014) and sequenced using Illumina HiSeq 2500 (at FAS Center for Systems Biology, Harvard). The bioinformatics were then carried out by Neil Rosser and Kanchon Dasmahapatra; sequences were cleaned and aligned using standard bioinformatics pipelines (Dasmahapatra *et al.*, 2012) to the *H. melpomene* genome v.2.5 (Davey *et al.*, 2016) before building the linkage map in Lep-MAP3 (Rastas, 2017). The output from LepMap3 was converted to a 4-way fully informative genotype with no missing data.

4.2.4. QTL Analysis

Two methods were used to identify QTLs of wing shape in the F2 and BC crosses, using the PCs rotated to the parental PCA. The first univariate approach was carried out on only the first PC, which explains the most variation within the parents (see PC1 values in Figure 4.4), to identify QTLs that specifically explained the most difference between *H. elevatus* and *H. pardalinus* and understand the genetic

architecture of divergence between the two. I first calculated the genotype probabilities of individuals for each family at every 1cM using the hidden Markov model to deal with missing data in R\qtl (Broman *et al.*, 2003); families were then combined using the c.cross() function. I performed a single-QTL genome scans using the Haley Knott regression (Haley & Knott, 1992) which detects non-interacting (ie. epistatic interactions), dominant and additive loci. All families with at least 5 individuals phenotyped were included in the final analysis (to avoid errors from small family sizes) with family as an interactive covariate to allow for different effects of genotype in each. This was necessary as the identity of the cross grandparents were unknown therefore genotype in the Lepmap3 output could represent different parental genotypes in different families. Due to the shape differences between sexes and to control for changes due to allometry, I included sex and the natural log of Csize (measure of size from Procrustes analysis) as additive covariates.

The second approach used a multivariate alternative to the Haley-Knott regression, available from GitHub (https://github.com/nnavarro/shapeQTL), developed to map geometric morphometric data in shapeQTL (Navarro, 2015). This was analysed on combined rotated PCs of the F2 and BC crosses to understand the genetic architecture of wing shape as a whole, and not specifically the aspect of wing shape that explains most variation between the parents. This method does not support the 4-way fully informative genotypes format output; therefore, the paternal markers were extracted from the genotype information by taking the first marker (in LepMap3 output, the first marker is inherited from the father, the second from the mother) and all the maternal markers were assigned the same marker. I used the paternal cross types for analysis as maternal crosses do not recombine (typical in female lepidoptera) and the trait does not appear to be dominant (see Figure 4.12); this method is similar to using BC cross types and was necessary to carry out further analyses in shapeQTL. For hindwing shape, I used all PCs that explained over 1 % of variation which, combined, explained around 95 % of variation. The number of families included was limited by the number of individuals in the smallest family compared to the number of PCs, therefore the analysis was carried out on the 12 largest families. There were fewer forewings available for phenotyping due to natural occurring damage in butterflies, therefore I reduced the number of PCs to only include those explaining over 2 % of variation (together, explaining about 90 % of variation) in the 8 largest families.

I carried out the analyses on different variants of the multivariate dataset to ensure that results were consistent. Similar results were found when using the unrotated PCs of the crosses, however, the QTLs were clearer when the variation of the PCs was rotated to maximise variation in the parental species. To check the robustness of the results, I also ran the analyses with increasing numbers of families included, on a decreasing number of PCs, and with the other cross types (maternal and dominant) included. All analyses consistently identified a peak on chromosome 2. Finally, I carried out a univariate analysis on wing size (using log Csize) to verify that identified loci did not coincide with a QTL for size; no QTLs were identified.

I used the locations on the genome with the highest LOD scores from the univariate analysis for chromosome 20 and from the multivariate analysis for chromosome 2 to calculate the heritability of the QTLs identified based on the formula (Broman *et al.*, 2003):

$$1 - 10 - 2\,\mathrm{LOD} / n$$

Where LOD is the highest logarithm of the odds score of the significant QTL and n is the number of individuals included in the scan. The heritability is an indicator of the proportion of phenotypic variation contributed by a QTL (Tang *et al.*, 2018). In the multivariate analysis, I calculated the amount of shape variation explained by each QTL identified from the respective analyses using effectsizeShape() from shapeQTL and plotted the shape changes associated with these QTL using plot.shapeEffect().

I then tried to identify candidate genes within the 95 % CIs of the significant QTLs that could be involved in wing shape development. The genes within the corresponding QTL regions in the reference genome were characterised using protein BLASTp searches to the Swiss-Prot and nr databases. As it was difficult to extrapolate a role in wing shape from protein function, I carried out a more focused search using known genes in *Drosophila*. I searched and identified 79 genes in flyBase with phenotypes manifesting in the wing and carried out a translated BLAST (tblastx) search for the exons of these genes against the reference genome Hmel2.5 scaffolds. I then determined whether any of these genes were located within the 95 % CI of the QTLs.

4.3. Results

4.3.1. Quantifying Wing shape

Wing shape was measured in 473 individuals from F2 and back crosses, 37 *H. elevatus* individuals and 30 *H. pardalinus* individuals. Full landmarks (no missing landmark due to wing damage) were collected for 290 forewings and 406 hindwings in the crosses.

The first principal component (PC) of hindwing shape from the PCA on parental species (Figure 4.4) explained a large amount of the variation (44.8 % variation), clearly separating the two species. In forewing shape, species differences were explained by PC1 (21.7 %) and PC2 (18.1 %). The change in wing shape along PC1, using the extreme phenotypes, is shown in in Figure 4.5.

Using linear models on the Procrustes coordinates, I found significant differences between the parental species (forewing: R2 = 0.16, p = 0.001; and hindwing R2 = 0.32, p = 0.01) and between the sexes (forewing: R2 = 0.04, p = 0.003; and hindwing R2 = 0.09, p = 0.001) with a significant interaction between sex and species in the hindwing (forewing: R2 = 0.02, p = 0.658; and hindwing R2 = 0.01, p = 0.048). I found a high correlation between hindwing and forewing shape for *H. elevatus* (n = 20, r-PLS = 0.86, p = 0.003) and *H. pardalinus* (n = 21, r-PLS = 0.73, p = 0.161 although this was not significant in *H. pardalinus*. This suggests that the two wings likely have common genetic and/or developmental pathways within species.

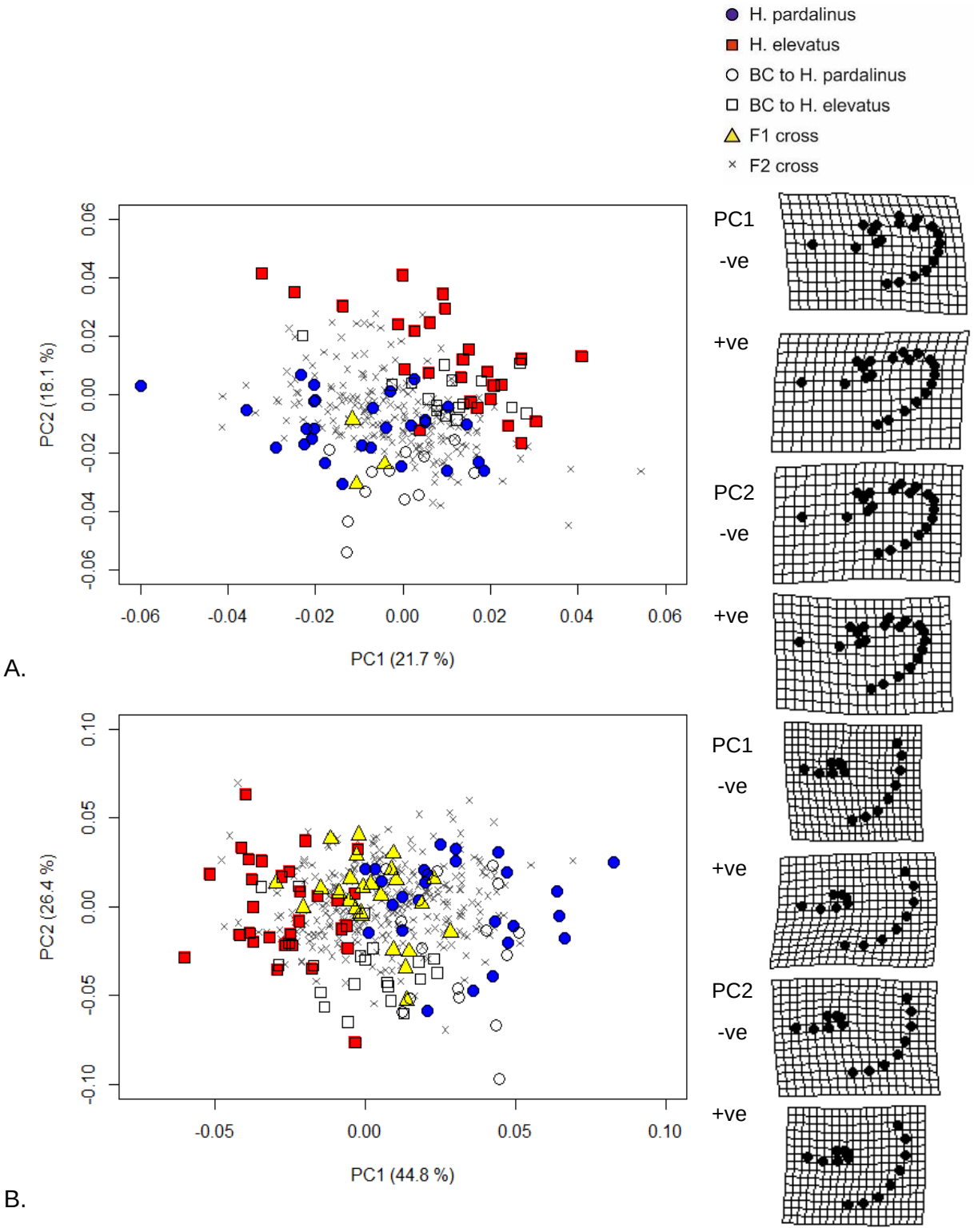
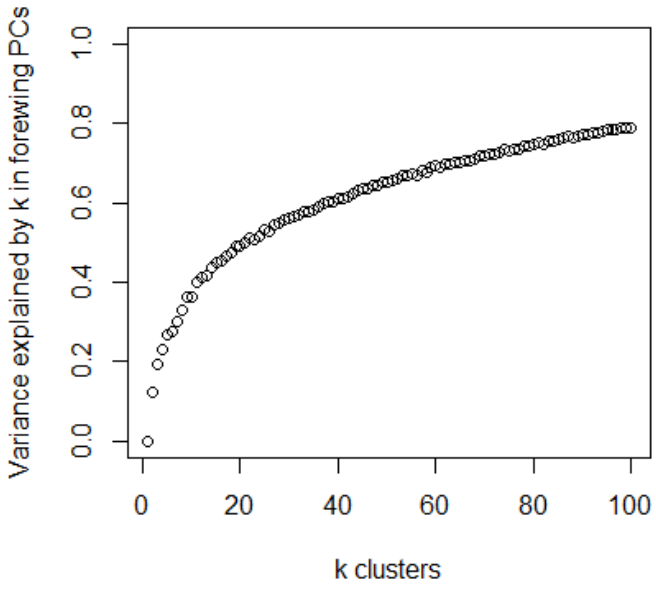
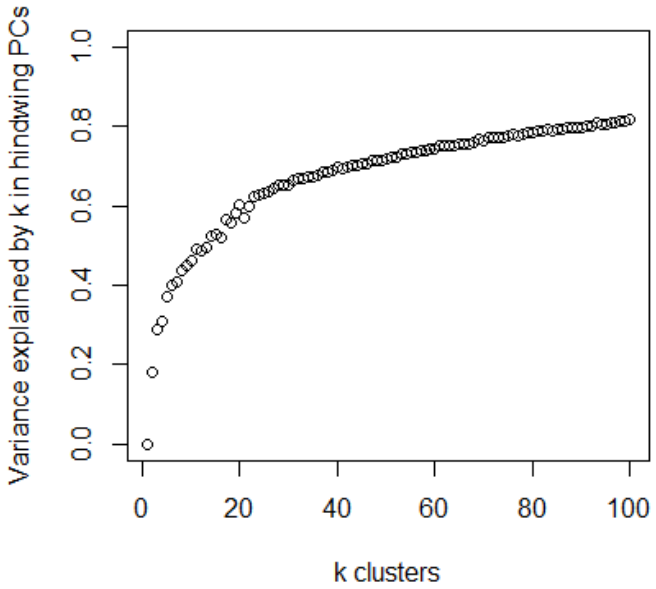


Figure 4.4. Principal component analysis of parental species, *H. elevatus* (red squares) and *H. pardalinus* (blue circles) for forewing (A) and hindwing (B). The first and second PC of the rotated PCA axes of crosses are plotted onto the parental morphospace. Different cross types are represented by different shapes and colours; backcross to *H. elevatus* (open squares), backcross to *H. pardalinus* (open circles), F1 crosses (yellow triangles) and F2 crosses (grey crosses). The proportion of variance explained by first and second PC of the parental PCA are shown in the axis labels. On the right are shown the deformation plots of the parental phenotypes along PC1 and PC2.

A principal component analysis was carried out on the crosses; these values were centred and rotated to the parental morphospace (see Figure 4.4). Sex differences were found using the linear model on the F2s Procrustes coordinates (forewing: R^2 = 0.04, p = 0.001; and hindwing R^2 = 0.12, p = 0.001) and the rPLS (value for shape correlation) of forewing and hindwing was also calculated for individuals with both wings undamaged (n = 187, rPLS = 0.68, p = 0.001). The cluster analysis measured the total variance explained for k clusters (k values ranged from 1 to 100) on the unrotated PCs of the F2 crosses for forewing (PC1 explained 18.5 % of variation, 95 % explained in the first 19 PCs) and hindwing (PC1 explained 27.2 % of variation, 95 % explained in the first 15 PCs). No clear clustering was observed within the F2 individuals suggesting a polygenic architecture underlying wing shape (see Figure 4.5). Furthermore, variance of family crosses falls within parental variance (Figure 4.6) which suggest no dominance effect on wing shape.



A.



B.

Figure 4.5. Total variance explained by k clusters in the cluster analysis on PCs that explain 95 % of variation in the PCA of the F2 crosses forewing (A) and hindwing (B) shape.

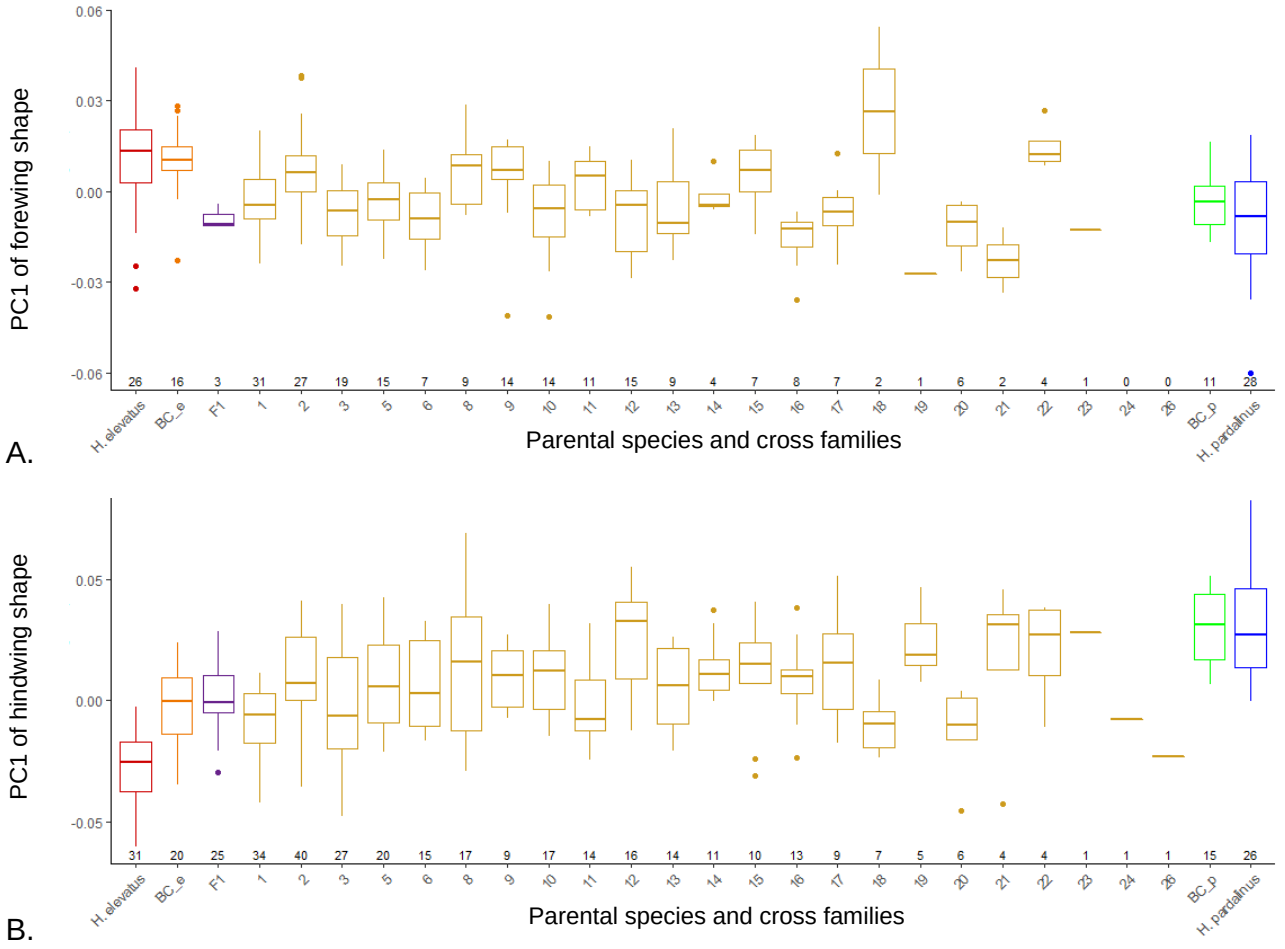


Figure 4.6. Variation in PC1 of forewing (A) and hindwing (B) shape in parental species, *H. elevatus* and *H. pardalinus*, and in each cross family. For the crosses I used the rotated PCA values. “BC_e” and “BC_p” refer to the families with individuals backcrossed to *H. elevatus* and *H. pardalinus*, respectively. The numbers along the x axis correspond the family numbers given to each F2 family in Table 4.1. The number of individuals phenotyped in each family are given above the x axis.

4.3.2. QTL Analysis

In total, 346 individuals were successfully RAD genotyped. After genetic mapping, genotype information was available from 26 F2 and two BC families (see Table 4.1 for numbers of individuals genotyped and phenotyped per family). For the hindwing, I used 20 families for the univariate analysis on the PC that explained the most variation between the parental species, giving a total of 319 individuals. The univariate analysis identified a significant QTL on chromosome 20 (see Figure 4.7 LOD = 38.29, $p < 0.001$). The 95 % confidence interval around the QTL ranged from Hmel220003_11946736 to Hmel220003_14479894 with the QTL peak found at marker Hmel220003 _12995023

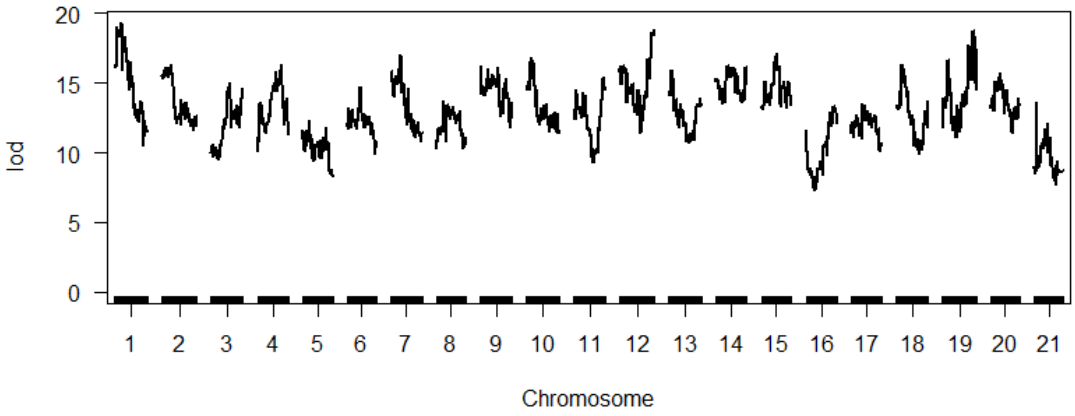
In the multivariate analysis on all PCs that explain over 1 % of variation, I included 12 families (249 individuals) and found a significant QTL on chromosome 2 (LOD = 15.08, $p < 0.05$), but did not identify a significant QTL on chromosome 20 (Figure 4.8). The 95 % CI around the chromosome 2 QTL ranged from Hmel202001_4827137 to Hmel202001_6181871 with a QTL peak at marker Hmel202001o_6165458.

In the forewing, the 8 largest families were used in the analysis including all PCs that explained over 2 % of variation, giving a total of 151 individuals for the multivariate QTL analysis. In the univariate analysis I used all families with over five individuals, giving a total of 226 individuals across 17 families. No significant QTLs were found using either method (see Figure 4.7 for the results from the univariate analysis and Figure 4.8 for the results of the multivariate analysis).

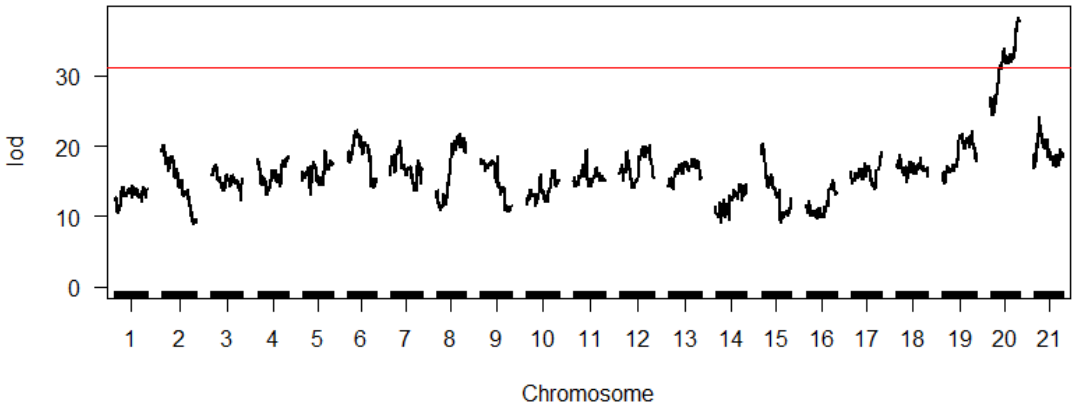
Table 4.1. Summary of numbers of individuals genotyped and phenotyped for each family. Families included in the QTL analyses, are marked with an "x" in the corresponding columns. The totals below give the numbers of individuals genotyped, phenotyped (for forewing and hindwing) and included in the different analyses. The number of PCs used in the analyses and the amount of variation explained by these is also included. Note: two families were excluded from the table as they only included one genotyped/non-phenotyped individual each. Cross type represents the species of the father-mother with the first letter corresponding to the genotype of the grand-father the second that of the grand-mother, either E for *H. elevatus* or P for *H. pardalinus.*

				Hindwing			**Forewing**		
	Cross type	**Cross type**	**Individuals genotyped**	**Individuals phenotyped**	**Univariate analysis**	**Multivariate analysis**	**Individuals phenotyped**	**Univariate analysis**	**Multivariate analysis**
family 1	F2	EP-PE	45	31	x	x	31	x	x
family 2	F2	EP-EP	44	40	x	x	27	x	x
family 3	F2	PE-PE	35	27	x	x	19	x	x
family 4	Backcross to *H. pardalinus*	PP-EP	32	15	x	x	11	x	
family 5	F2	EP-EP	29	20	x	x	15	x	x
family 7	Backcross to *H. elevatus*	EE-PE	23	20	x	x	16	x	x
family 6	F2	PE-PE	23	15	x	x	7	x	
family 8	F2	EP-EP	22	17	x	x	9	x	
family 9	F2	EP-PE	21	9	x		14	x	x
family 10	F2	EP-EP	20	17	x	x	14	x	x
family 11	F2	PE-PE	19	14	x	x	11	x	
family 12	F2	EP-EP	18	16	x	x	15	x	x
family 13	F2	EP-EP	17	14	x	x	9	x	

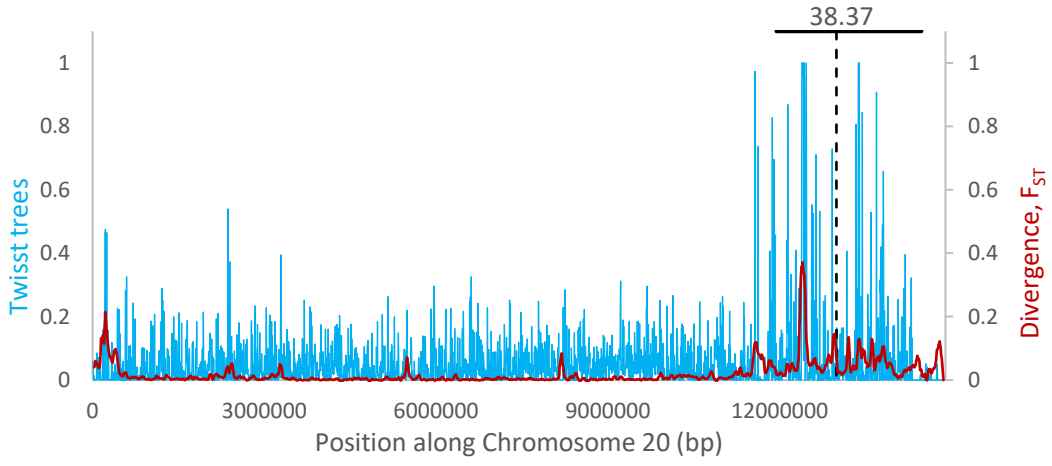
family 14	F2	PE-PE	15	11	x		4		
family 15	F2	EP-EP	14	10	x		7	x	
family 16	F2	PE?-PE	13	13	x		8	x	
family 17	F2	PE-PE	12	9	x		7	x	
family 18	F2	PE-PE	9	7	x		2		
family 19	F2	PE-PE	9	5	x		1		
family 20	F2	PE-PE	8	6	x		6	x	
family 21	F2	PE-PE	5	4			2		
family 22	F2	EP-EP	4	4			4		
family 23	F2	EP-EP	2	1			1		
family 24	F2	PE-PE	2	1			0		
family 25	F2	PE-EE	2	0			0		
family 26	F2	EP-EP	1	1			0		
Total individuals			**444**	**330**	**319**	**249**	**240**	**226**	**151**
Number of PCs used in QTL					**1**	**14**		**1**	**10**
Proportion of variation explained by PCs					**0.45**	**0.95**		**0.22**	**0.9**



A.

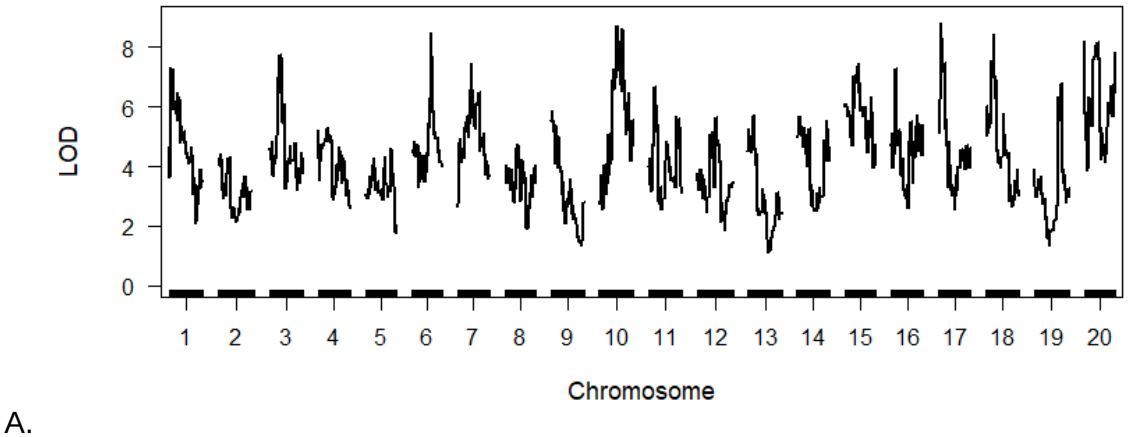


B.

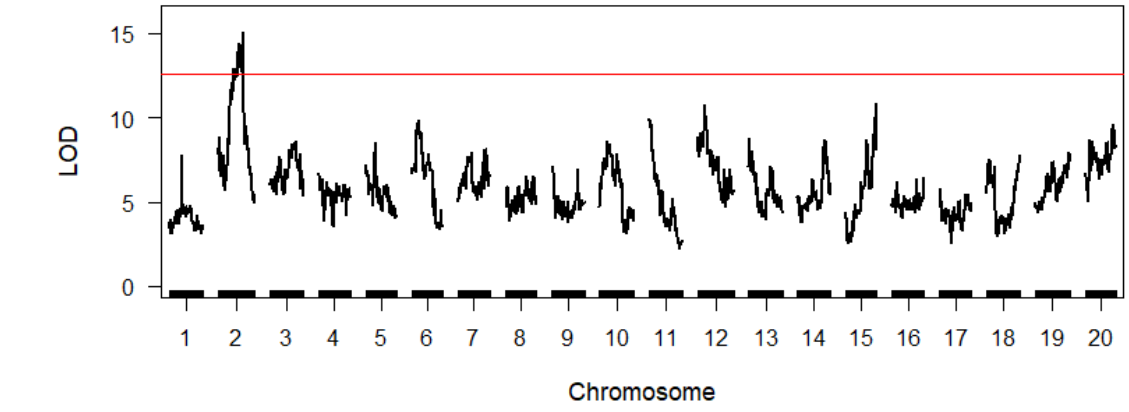


C.

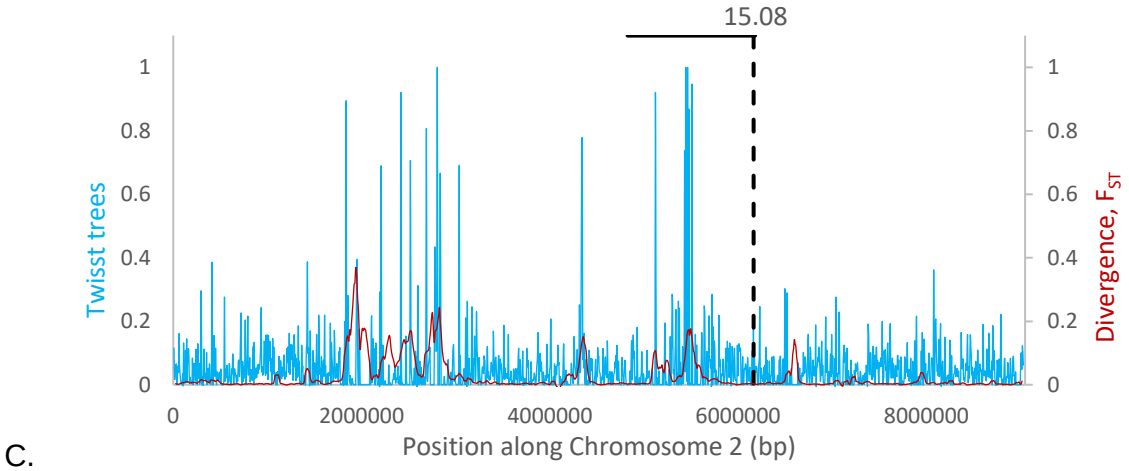
Figure 4.7. LOD scores across the 21 chromosomes from the univariate QTL analysis on the first rotated PC of F2 and backcross individuals for forewing (A) and hindwing (B). The QTL analysis was carried out on all families with over 5 individuals phenotyped (see Table 4.1 for the breakdown of families). The red line represents the 99 % confidence interval. Note that chromosome 21 corresponds to the sex chromosome, Z. C. F_{ST} values and Twisst scores for the "species" tree across chromosome 20, the dotted line shows the location of the QTL peak with the LOD score above (from B.); the 95 % CI is represented by the horizontal black bar.



A.



B.



C.

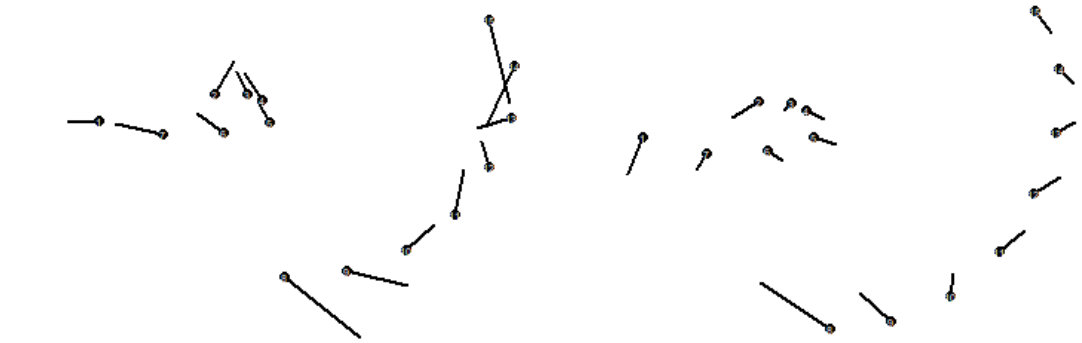
Figure 4.8. LOD scores across the 21 chromosomes from the multivariate QTL analysis using paternal marker on the rotated PCs of F2 and backcross individuals for forewing (A) and hindwing (B). The QTL analysis was carried out on 8 families in the forewing, using the first 10 PCs (about 90 % of variation explained), and on 12 families in the hindwing, using the first 14 PCs (about 95 % of variation explained), see Table 4.1 for the breakdown of families. The red line represents the 95 % confidence interval. Note that shapeQTL does not process the sex chromosome. C. F_{ST} values and Twisst scores for the "species" tree across chromosome 2, the dotted line shows the location of the QTL with the LOD score above (from B.); the 95 % CI is represented by the horizontal black bar.

The QTL on chromosome 20 was present, although not significant in the multivariate analysis (it only appears in analyses with fewer PCs and more families, although it never reaches significance) unless only the first PC and one other were included in the analysis (in this case the QTL on chromosome 2 disappeared). However, when calculating the effect size from the multivariate analysis using the percentage of total sum of squares explained by QTLs, I found that the QTL on chromosome 20 explained more of the total wing shape variance than the QTL on chromosome 2 (see Table 4.2), which I attribute to the high proportion of variation explained by PC1. The QTL effects on wing shape can be seen in Figure 4.9. I calculated the heritability of the QTLs in each analysis. In the multivariate analysis, I found a heritability value of 0.24 for the QTL on chromosome 2, and in the univariate analysis I found a heritability value of 0.42 for the chromosome 20 QTL.

Finally, I tried to identify potential candidate genes that could be involved in wing shape determination. I used the BLAST software on genes identified within the 95 % CI intervals around the QTL using the swissprot and nr databases but none of the genes had obvious functions related to wing shape in those regions. Using Flybase, one gene expressed in *Drosophila* wings was found within the QTL of chromosome 20, which is a protein coding gene called Dmel/sas, or “stranded at second” (Flybase ID: FBgn0002306). This gene is involved in axon guidance and instar larval development. The only relation to wing shape is potentially an interaction with Scer\GAL4 gene affecting muscle morphogenesis and causing a flightless phenotype (Schnorrer *et al.*, 2010).

Table 4.2. Effect size of QTLs given the log Csize and sex covariates. The percentage of the total sums of squares (SST) is the percentage of total shape variance; the percentage SS of the projected scores is the variance accounted for in the shape variable controlled by the QTL; third column the percentage of the total shape variance explained by the projected score (Navarro *et al.*, 2016).

Covariate and QTL	% SST	% SST projected scores	% explained by projected score
log Csize	0.42	13.32	3.17
Sex	0.02	22.09	0.07
Chromosome 2 QTL	0.00	20.06	0.01
Chromosome 20 QTL	0.02	16.10	0.14



A. B.

Figure 4.9. Representation of the QTL effects on wing shape at the genotype location of the QTL (identified using the multivariate analysis) (A) and at the genotype location of the chromosome 2 QTL (identified using the univariate analysis) (B). Dots represent the landmark positions used during the geometric-morphometric analysis.

4.4. Discussion

A major challenge of evolutionary biology has been to understand species divergence, especially in the presence of gene flow (Via, 2012). Gene flow breaks down associations between traits under divergent selection and/or involved in assortative mating (Felsenstein, 1981). However, different factors can reduce the homogenising effects of gene flow, such as pleiotropy, chromosomal structures or genetic hitchhiking (Feder *et al.*, 2013; Smadja & Butlin, 2011). Identifying traits under divergent selection or involved in reproductive isolation, and understanding the underlying genomic architecture, can help identify the mechanisms at play during divergence. My analysis aimed to understand the genetic architecture of wing shape between two species, *H. elevatus* and *H. pardalinus*, whose sympatry and pattern of strong pre-zygotic barriers coupled with complete interfertility is strongly suggestive of divergence with gene flow (Rosser *et al.*, 2019). The results from the QTL analysis identified two QTLs, including one in a region characterised by high levels of F_{ST} and high probabilities of fitting to a "species" tree (Figure 4.2).

Results from Chapter 2 suggest that wing shape is driven by mimicry and has evolved as an adaptation to differences in habitat. *Heliconius elevatus* and *H. pardalinus* display divergent colour patterns and inhabit slightly different habitats (Rosser *et al.*, 2019). *Heliconius elevatus* inhabits more primary forests, whereas *H. pardalinus* is more commonly found in seasonally flooded, secondary habitat; this difference is probably linked to flight differences between the two (Chapter 3). Therefore, there is strong evidence to suggest that wing shape is under divergent selection. The findings from the QTL analysis are therefore consistent with expectation of islands of divergence, where traits under divergent selection are found in regions of high divergence.

Another expectation for divergence with gene flow and islands of divergence is that traits under divergent selection also be associated with reproductive isolation and assortative mating (Merrill *et al.*, 2019; Smadja & Butlin, 2011), either through pleiotropy or physical linkage. There is no evidence to suggest that wing shape itself is used for mate choice however, if wing shape differences determine habitat type, reproductive isolation could occur through habitat segregation or reduced hybrid fitness (Nosil, 2012). This is however, unlikely to lead to sufficient levels of

reproductive isolation on its own as the two species still co-occur in some locations (Rosser *et al.*, 2019). Physical linkage to another trait involved in reproductive isolation is therefore more likely to be involved in this case.

The analysis did not identify loci in regions linked to colour pattern genes which might have been expected based on the finding that wing shape is also driven by Müllerian mimicry. Furthermore, two studies in selected lines of *Bicyclus anynana* (Monteiro *et al.*, 2002) and *veinless* mutants of *Papilio xuthus* (Nijhout, 2002) identified correlations between wing shape and colour pattern elements. The lack of wing shape QTL near colour pattern genes could therefore be a result of lack of power, but current evidence does not suggest wing shape is associated to assortative mating through linkage to colour pattern genes.

However, the results from the analysis show that the confidence interval range of the QTL on chromosome 20 overlaps over 1.5 cM with that of a QTL that explains differences in male pheromones between the two species (QTL peak found at marker Hmel220003 _12995023, ranging from Hmel220003o_10768476 to 14280705, Cama *et al.*, *unpublished* data). Just like colour pattern, pheromones have been shown to be involved in mate preference in *Heliconius* (Darragh *et al.*, 2017). Furthermore, these QTLs were identified for two families of compounds, the esters and alkenes, which are found in very low concentration in *H. elevatus* and high concentrations in *H. pardalinus* (see supplementary information of Rosser *et al.*, 2019). Therefore, the region on chromosome 20 is a possible candidate for an island of divergence, ie. an area of elevated divergence that contains traits for reproductive barriers, linking multiple traits under divergent selection, and potentially involved in reproductive isolation if linked to preference genes. The results for wing shape and pheromones will eventually be combined with QTL data on other important ecological traits, likely involved in reproductive isolation, to further investigate the possible role of gene flow and islands of divergence during the evolution of *H. elevatus* and *H. pardalinus*.

My study comprises the first analysis on the genetic basis of wing shape in *Heliconius* and Lepidoptera in general. The multivariate and univariate analyses did not identify common QTLs for wing shape, however, similar discrepancies between multivariate and univariate analyses were found in other studies owing to the fact that univariate and multivariate analyses are testing for different traits. In Pitchers *et al.* (2019), the authors compared results from a multivariate and univariate Genome Wide

Association Study and found different significant SNPs between the two, however, they also found that the univariate analysis identified SNPs that had large effects on single PCs but were “unremarkable” in the multivariate analysis. Another analysis using QTLs identified important QTLs not identified by the univariate analysis (Topp *et al.*, 2013).

From what is known about wing shape determination in other insects (Abouheif & Wray, 2002; Macdonald *et al.*, 2010; Tomoyasu *et al.*, 2009; Weatherbee *et al.*, 1999), *Drosophila* genes are a good resource on which to base further studies of the genetic basis of wing shape. However, my study did not identify any previously characterised candidate genes in the QTL regions.

The two QTLs identified were consistently found in the different analyses suggesting they are reliable loci, and both QTLs are informative about different aspects of wing shape between *H. elevatus* and *H. pardalinus*. The QTL on chromosome 20 was identified using the first axis of the rotated principal components which discriminated between the species in the hindwing shape. The variance explained by this PC was very high (44.8 %) which probably explains the higher proportion of total variation explained by the direction of this QTL (% explained by projected score in Table 4.2). Furthermore, the heritabilities of the QTLs suggest the QTL on chromosome 20 explains a larger proportion of phenotypic variation, although these are not directly comparable as the phenotypic variation in the univariate analysis is restricted to one axis. Within the direction of the respective QTLs, chromosome 20 explained a larger percentage of variation (% SST projected scores in Table 4.2). However, the QTL on chromosome 2 reflects overall changes in wing shape, not specifically involved in species differences, and so it is perhaps less relevant to understanding the genomics of divergence between *H. elevatus* and *H. pardalinus* (see the effects of the QTLs on wing shape in Figure 4.9 and difference along PC1 between the parental species Figure 4.6).

It is important to note however, that the combined effect on shape of the QTLs is less than 1 % of the total shape variation. These results are over ten times smaller than measures in the mouse skull shape (Maga *et al.*, 2015) and mandible shape (Navarro *et al.*, 2016) using the same analyses and care should be taken when making conclusions about the importance of such QTLs. Morphological traits, especially wing shape, are highly polygenic and therefore many other genes across the genome are probably involved (Klingenberg, 2010) which is consistent with the results from the

cluster analysis which failed to identify groups within the crosses. However, the small effect size could also be from a lack of power in the analysis or even strong environmental effects on wing shape.

The QTLs identified were only identified for the hindwing shape. Results from previous chapters have demonstrated the importance of studying the forewing and hindwing shapes separately, as different selective pressures act on the two. My results could reflect different genomic architectures for shape variation between the two wings, however given the strong correlation between wing and forewing shape, it is more likely that the analysis in forewing suffered from a lack of power due to reduced sample sizes. The results from the association analysis show high rPLS values between the forewing and hindwing shapes and therefore it is likely that common genes are involved in shaping their variation, especially as this association was not greatly reduced in the crosses. Furthermore, differences between the forewing and hindwing could be due to differential gene expression, like in Weatherbee *et al.* (1999) differences in wing colour pattern elements, pigmentation and scale morphology between the forewing and hindwing are due to differential regulation of the *Ubx* gene.

Another issue from the lack of statistical power due to relatively small family sizes, is the width of the confidence intervals around the QTLs. Although the confidence intervals remain within high F_{ST} regions, the large number of genes within those regions make it very difficult to identify candidate genes. This is a known pitfall of QTL analyses, which tend to identify large chromosomal regions with identification of candidate loci being difficult without prior knowledge (Zhu & Zhao, 2007). The *Dmel/sas Drosophila* gene identified within the chromosome 20 peak did not have a clear link to wing shape, although with a potential role in muscle morphogenesis it could be related to flight. This could reflect the potential link between flight and wing shape (Chapter 3). These are just speculations and further work on the genetic architecture should be carried out to identify clearer regions and candidate genes, potentially using Genome Wide Association Studies.

In conclusion, this study identifies QTLs for wing shape which, if found to be clustered with QTLs of other important ecological traits, could support a role of islands of divergence containing speciation genes and divergence with gene flow. As previously mentioned, further analyses are being carried out to identify such QTLs in other traits. Furthermore, studies of genetic patterns to confirm the presence of gene

flow across the genome would also benefit the conclusions of these analyses. My study is the first to identify regions involved in wing shape in any Lepidopteran species, albeit with low power. Analyses in other species, like other silvaniform species which display the most divergent wing shapes (Chapter 2), could provide evidence of consistency in these QTLs and help us understand the genetic architecture of this complex trait.

Chapter 5

General Discussion

5.1. Summary of thesis findings

Flight is an important but energetically costly phenotype in flying insects and is consequently under strong selection from different factors. While the relation between wing shape and flight has been studied extensively, there is still much to discover about the precise role of wing shape morphology on flight, the evolutionary drivers driving their evolution, and the genetic control of these complex traits. In this thesis, I used an integrative approach to study the evolution of wing shape and flight in a group of mimetic, neo-tropical butterflies, the Heliconiine. Specifically, I aimed to (1) improve our understanding of the dynamics of wing shape evolution, (2) measure the effect of wing shape differences on flight in two species with divergent colour patterns, *H. elevatus* and *H. pardalinus*, and (3) identify the underlying genomics of wing shape differences between these two species.

Chapter 2 - Aerodynamic constraints limit wing shape mimicry in Heliconiine butterflies.

Main objectives:

- Understand the role of mimicry, habitat type and elevation on wing shape.
- Determine the relative contributions of these factors in affecting forewing and hindwing shape.
- Identify the cause of the strong divergence of the silvaniform patterned heliconiine species, away from other *Heliconius* species
- Test for evidence of sexual dimorphism in wing shape mimicry.

In this chapter, I investigated the dynamics of wing shape evolution across species of the *Heliconius* and *Eueides* genera, and provided evidence of sexual dimorphism in wing shape due to stronger mimicry in females, as yet unheard of in *Heliconius*. After controlling for phylogenetic relatedness, I identified an important role for mimicry in driving wing shape evolution. Previous studies have just focussed on a few select *Heliconius* species, some only looking at forewing shape. My results emphasise the importance of separately studying both forewing and hindwings, as the two wings are clearly under different selective regimes with the forewing likely being more aerodynamically constrained. This chapter also reveals the impressive divergence of the silvaniform mimicry ring away from the other Heliconiine mimicry rings. My data indicate that this results from convergence towards the ithomiine wing shape, with evidence of stronger mimetic convergence in female Heliconiine. Overall, these results highlight the complexity of interactions between factors, including the constraining effects, driving wing shape evolution.

Chapter 3 - Understanding associations between flight and wing shape in Heliconius butterflies.

Main objectives:

- Characterise the differences in wing shape and flight behaviour between two sister species of *Heliconius*, *H. elevatus* and *H. pardalinus*, with divergent colour patterns.
- Identify correlations between variation in wing shape and flight across the two species.
- Test whether wing shape differences between the two species explain differences in flight behaviour

I identified divergent wing morphologies and flight behaviours (wing beat frequencies and wing angles) between *H. elevatus* and *H. pardalinus*, which is consistent with evidence that flight and wing shape are involved in the mimetic signal. Correlations

between wing shape and flight variables suggest either that the two phenotypes are under independent selection for mimicry (the former for visual mimicry, the latter for behavioural mimicry), or that the wing shape differences observed between the two species directly affect the measured flight parameters. The manipulations did not identify a causal relationship between the two, suggesting other wing variables probably affect flight behaviour. Overall, this chapter highlights the difficulty of understanding the relationship between flight and wing shape and evidence of stronger divergence in hindwing shape between the two species with divergence colour patterns is consistent with evidence that the two wings are under different selective regimes, perhaps in part due to their different roles during flight.

Chapter 4 - QTL for wing shape identified in region of high F_{ST} in *Heliconius* butterflies.

Main objectives:

- Identify QTLs controlling forewing and hindwing shape differences between two species of *Heliconius*, *H. elevatus* and *H. pardalinus.*
- Determine whether these QTL lie within regions of high F_{ST} to support evidence of divergence with gene flow.

Heliconius elevatus and *Heliconius pardalinus* are two sympatric species displaying patterns of pre and post-zygotic isolation consistent with divergence with gene flow (Rosser *et al.*, 2019). I have identified two QTLs controlling hindwing shape differences between these species. The QTL identified using the univariate analysis on the PC values that explained the most variation between the two species was identified in a region of high F_{ST} on chromosome 20, which coincides with a QTL for pheromones (Cama *et al.*, *unpublished*), a trait likely involved in reproductive isolation. Linkage of several important ecological traits under divergent selection could suggest a role of islands of divergence and gene flow in the divergence of *H. elevatus* and *H. pardalinus*. The QTL identified using the multivariate analysis was found on chromosome 2. The use of the two QTL methods which identified different QTL demonstrates the polygenic nature of complex morphological traits and highlights the importance of including multivariate methods to studying such traits.

5.2. Mimicry and habitat type

The results of this thesis have important implications for future investigations of the drivers of wing shape evolution. While I have identified genus wide patterns demonstrating important roles of mimicry as well as habitat type, any deviation from this pattern at smaller phylogenetic scales will therefore be informative about the pressures acting on the species studied. Further studies could also benefit from more accurate measures of habitat type, particularly looking at where butterflies fly within the forest, as has been done in other taxa (Cespedes *et al.*, 2015; Chazot *et al.*, 2016; DeVries *et al.*, 2010). Accurate characterisation of habitat type is difficult to make and would be challenging in large scale analyses such as the one carried out in Chapter 2, however, more focused analyses on a few species may help untangle the complex interactions between the different ecological factors. Such studies could also help elucidate the potential confounding effects of spatial distribution of mimicry rings to understand the extent to which these factors are linked, and possibly determine the order in which mimicry, flight behaviours, wing shape and habitat preference have evolved.

More focused studies can also help reveal other important factors not identified within this thesis, such as elevation (Montejo-Kovacevich *et al.*, 2019), which only had a negligible effect on hindwing shape. Other ecological factors may not be relevant to wing shape evolution at the scale of the genus but may be important during divergence of species over shorter evolutionary timescales. Such factors may be small scale habitat segregation, different behaviours between species or even between sexes, or even different egg laying strategies, among others.

Finally, more focused analyses in fewer species might be able to further understand how ecological factors differ between males and females and lead to sexual dimorphism. These analyses may identify how levels of sexual dimorphism vary across species and whether sexual dimorphism is limited to flight and wing shape or whether sexual dimorphism in colour pattern is more common than previously thought, even if subtle. From this, we may be able to identify the selective pressures acting on males and females as well as determine whether these are consistent across species in the genus or specific to species ecology.

5.3. Flight pattern mimicry in Heliconiini

A recurrent question throughout this thesis has been whether wing shape mimicry is selected for to increase the visual signal or selected for its aerodynamic properties for flight mimicry. An examination of four *Heliconius* species has found evidence that the mimetic signal also extends to flight characteristics such as wing beat frequency and wing angles (Srygley, 1999; Srygley & Ellington, 1999; Srygley, 2007). In Chapter 3, my experimental manipulations aimed at determining whether wing shape differences between two species in divergent mimicry rings explained differences in flight found no evidence of causation. Therefore, selection may be independently causing mimicry in flight patterns as well as wing shape. Carrying out ecomorphological analyses on flight behaviour could further our understanding of this trait (although still not conclusively identify a causative effect from wing shape).

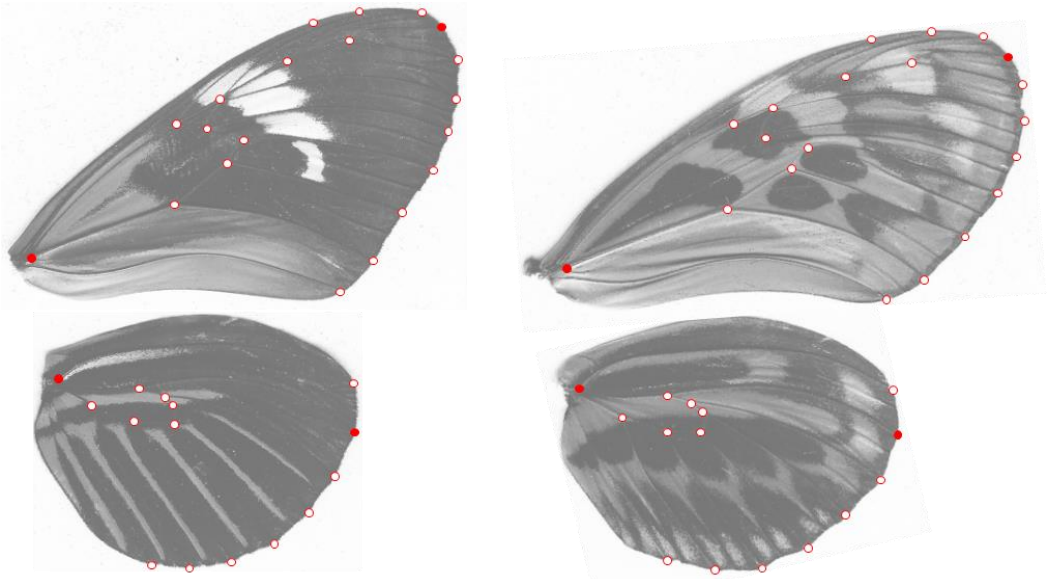
During this thesis, I also collected data for flight behaviour (wing beat frequency and wing angle measures) across the Heliconiini tribe. Data on flight were collected for 289 individuals across 36 species (including several different morphs and sub-species) from San Martin (Peru) and across Panama. Preliminary analyses carried out by myself and Edd Page (Masters student in the Dasmahapatra Lab), controlling for phylogenetic relatedness, show divergence of the taxa belonging to the silvaniform mimicry ring for both wing angles and wing beat frequency. Limited flight data in a few species of ithomiines suggest that the silvaniform patterned Heliconiini are converging towards ithomiine flight behaviour. These results are remarkably consistent with the data I present in Chapter 2 where heliconiine wing shape was also most strongly divergent in the silvaniform mimicking species, with differences driven by convergence towards the ithomiine. The flight data also show unexpectedly high variance in measured flight parameters among species of the blue mimicry ring: *H. cydno*, *H. pachinus*, *H. hewitsoni*, *H. sapho*, *H. sara* and *H. wallacei*. While these species have been categorised as members of the blue mimicry ring in this thesis due to their iridescent colour patterns, the group is in reality somewhat heterogenous; for example, *H. sara* and *H. wallacei* do not have a yellow hindwing bar. This suggests that the grouping of mimicry rings used this my thesis might not be the best way to measure colour patterns, and further work will investigate alternatives using pattern recognition with avian visual models, such as PAVO2 (Maia *et al.*, 2019) and

QPCA (van den Berg *et al.*, 2020). Also, this difference between the two types of iridescent patterns might reveal an important role of the hindwing bar in the mimetic signal and perhaps finer delimitations of colour pattern will help reveal differences between different patterns and shapes. Using the dataset from Peru and Panama will also allow us to investigate the flight of co-occuring mimics compared to distant mimics, allowing the effects of mimicry to be disentangled from possible developmental constraints between colour pattern and wing shape as well as habitat. This data set will also look for co-evolution of flight measure and wing shape across the phylogeny.

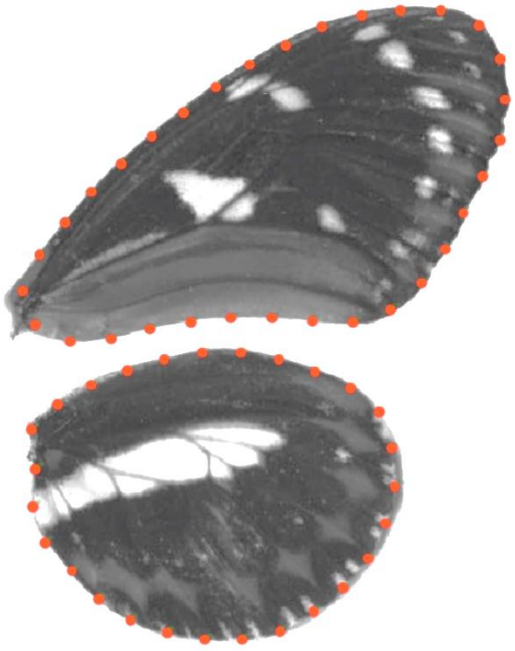
5.4. Conclusions

Together, these results have (1) identified the main drivers of wing shape evolution across the *Heliconius* and *Eueides* genera, and provided the first evidence in *Heliconius* that sexual dimorphism in wing shape is due to stronger mimicry in females, (2) improved our understanding of the relationship between flight and wing shape and (3) identified the first QTL for wing shape in Lepidoptera. These results demonstrate the importance of ecology in driving diversity, and how this can be seen using macro scale analyses, as well as through behavioural experiments and analyses of the underlying genomic architecture of ecological traits.

Appendix 1 - Supporting Information for Chapter 2



A.



B.

Appendix 1A. A. Landmarks used for geometric morphometric analysis (the forewings and hind wings of the butterflies are not scaled). The filled landmarks were used for measuring wing length. B. Semi-landmarks used to compare *Heliconius* individuals to specimens from the Heliconiini outgroups. Semi-landmarks are placed at equal distances around the outline of the wings.

Appendix 1B. Total number of specimens landmarked across all species for forewing and hindwing as well as number of males and females. Note: taxa not present in the phylogenetic tree were not included in the analyses.

			forewing			hindwing		
	Sub-species	**total**	**female**	**male**	**unsure**	**female**	**male**	**unsure**
Agraulis vanillae		7	1	5	1	0	0	0
Dione juno		6	0	5	1	0	0	0
Dione moneta		1	0	1	0	0	0	0
Dryas iulia		10	5	5	0	0	0	0
Dryadula phaetusa		10	5	5	0	0	0	0
Eueides aliphera		8	2	5	1	2	5	1
Eueides heliconioides		2	0	0	2	0	0	2
Eueides isabella		9	4	4	1	4	4	2
Eueides lampeto		3	3	0	0	1	0	0
Eueides libitina		3	2	1	0	2	1	0
Eueides lineata		1	0	1	0	0	1	0
Eueides lybia		7	2	5	0	1	3	0
Eueides tales		9	5	4	0	4	4	0
Eueides vibilia		6	2	4	0	2	5	0
Heliconius antiochus		9	4	4	1	4	4	1
Heliconius aoede		10	5	5	0	5	5	0
Heliconius atthis		7	4	3	0	3	4	0
Heliconius besckei		11	2	9	0	2	9	0
Heliconius burneyi		10	4	5	1	4	5	1
Heliconius charitonia		10	5	5	0	4	4	0
Heliconius clysonymus		9	3	6	0	1	4	0
Heliconius cydno		10	5	5	0	5	5	0
Heliconius demeter		9	4	5	0	4	5	0
Heliconius doris		4	1	3	0	1	3	0
Heliconius doris		5	3	2	0	3	2	0
Heliconius doris		1	1	0	0	0	0	0
Heliconius egeria		1	1	0	0	1	0	0
Heliconius eleuchia		11	6	5	0	6	5	0
Heliconius elevatus		8	3	5	0	5	5	0
Heliconius erato	*almafreda*	6	1	5	0	1	5	0
Heliconius erato	*amphititre*	5	1	3	1	1	3	1

Heliconius erato	*cyrbia*	9	4	5	0	5	5	0
Heliconius erato	*demophoon*	10	4	6	0	4	6	0
Heliconius erato	*emma*	10	5	5	0	5	5	0
Heliconius erato	*erato*	1	0	1	0	0	1	0
Heliconius erato	*favorinus*	10	5	5	0	5	5	0
Heliconius erato	*hydara*	10	5	5	0	5	5	0
Heliconius erato	*lativitta*	4	2	2	0	2	2	0
Heliconius erato	*luscombei*	5	1	4	0	1	3	0
Heliconius erato	*microclea*	5	4	1	0	3	1	0
Heliconius erato	*petiverana*	10	1	0	9	1	0	9
Heliconius erato	*venus*	10	5	5	0	5	5	0
Heliconius eratosignis		10	5	5	0	5	5	0
Heliconius ethilla		10	5	5	0	5	5	0
Heliconius hecale		9	5	4	0	5	5	0
Heliconius hecuba		2	0	0	2	0	0	2
Heliconius hermathena		4	3	1	0	3	1	0
Heliconius heurippa		11	4	7	0	4	7	0
Heliconius hewitsoni		7	2	5	0	0	2	0
Heliconius hierax		12	5	5	2	5	5	2
Heliconius himera		8	3	3	2	3	3	2
Heliconius hortense		9	3	1	5	3	0	5
Heliconius ismenius		10	5	5	0	5	5	0
Heliconius leucadia		2	1	0	1	1	0	1
Heliconius luciana		9	4	5	0	4	5	0
Heliconius melpomene	*aglaope*	10	5	5	0	5	5	0
Heliconius melpomene	*amarillys*	10	5	5	0	5	5	0
Heliconius melpomene	*cythera*	9	5	4	0	5	5	0
Heliconius melpomene	*melpomene*	10	5	5	0	5	5	0
Heliconius melpomene	*meriana*	1	0	1	0	0	1	0
Heliconius melpomene	*rosina*	10	5	5	0	5	5	0
Heliconius melpomene	*vulcanus*	8	4	4	0	3	4	0
Heliconius melpomene	*xenoclea*	2	1	1	0	1	1	0
Heliconius numata		44	21	22	1	21	22	1
Heliconius pachinus		12	6	6	0	6	5	0
Heliconius pardalinus	*butleri*	4	2	2	0	2	2	0
Heliconius pardalinus	*sergestus*	6	3	3	0	3	3	0
Heliconius ricini		11	5	6	0	5	6	0

Heliconius sapho		7	1	6	0	1	6	0
Heliconius sara		9	5	4	0	5	5	0
Heliconius telesiphe		8	3	5	0	3	5	0
Heliconius timareta	*DR*	7	5	2	0	5	2	0
Heliconius timareta	*thelxinoe*	10	2	8	0	2	8	0
Heliconius wallacei		9	4	5	0	4	4	0
Heliconius xanthocles		10	4	6	0	4	6	0
Philaethria dido		3	2	1	0	0	0	0
Philaethria ostara		4	0	4	0	0	0	0
Podotricha telesiphe		4	1	3	0	0	0	0
	n taxa	**603**	**259**	**313**	**31**	**235**	**277**	**30**
	78		**70**	**71**	**15**	**63**	**62**	**13**

Appendix 1C. Heliconius and Eueides taxa included in analyses of wing shape from different mimicry rings.

Pictures were taken from different web sources:

http://www.heliconius.net

https://www.aureus-butterflies.de

https://www.butterfliesofamerica.com/L/Nymphalidae.htm

https://www.heliconius.org/

http://tolweb.org/

Red and White

H. clysonymus

H. hierax

H. himera

H. hortense

H. ricini

Silvaniform

H. ethilla

H. hecale

H. ismenius

H. numata

H. pardalinus

E. lampeto

E. isabella

Dennis Ray

H. burneyi

H. aoede

*H. doris **

H. demeter

H. elevatus

H. egeria

H. eratosignis

H. xanthocles

H. erato erato

E. tales

E. vibilia

E. heliconioides

Blue

H. antiochus

H. hewitsoni

H. cydno

H. luciana

H. eleuchia

H. pachinus

H. leucadia

H. sara

H. sapho

H. wallacei

Postman

H. besckei

H. erato petiverana

H. hermathena

H. melpomene rosina

H. telesiphe

H. timareta thelxinoe*

H. erato favorinus

Black and White

H. atthis

H. charitonia

H. hecuba

Orange

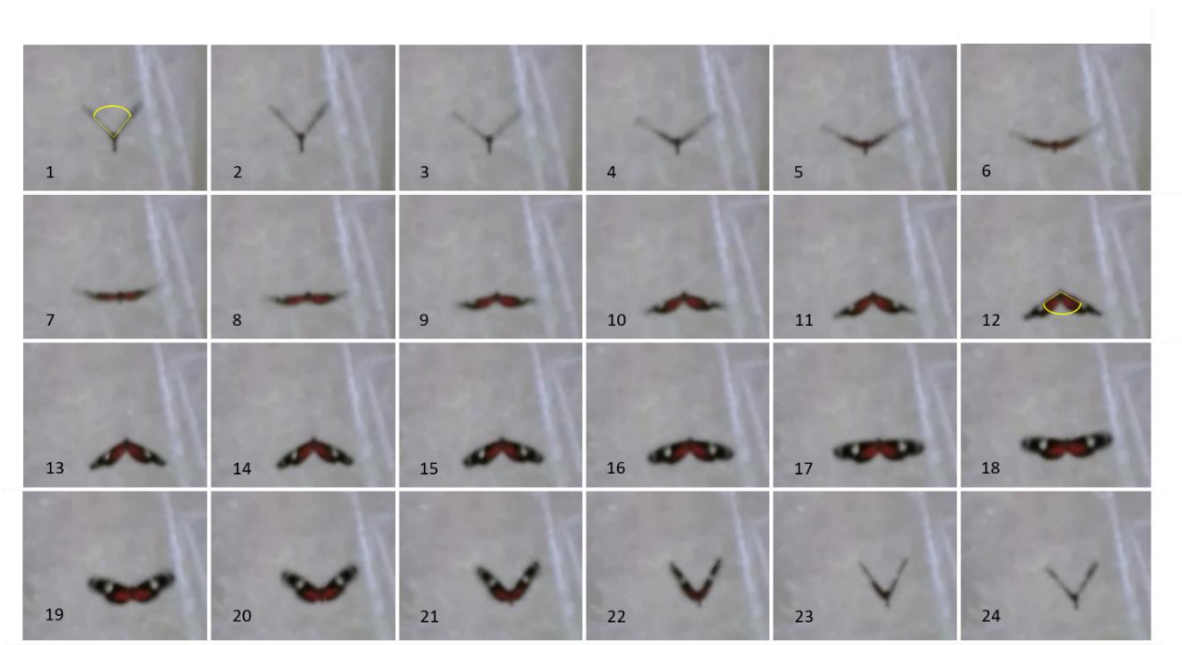
E. aliphera

E. lineata

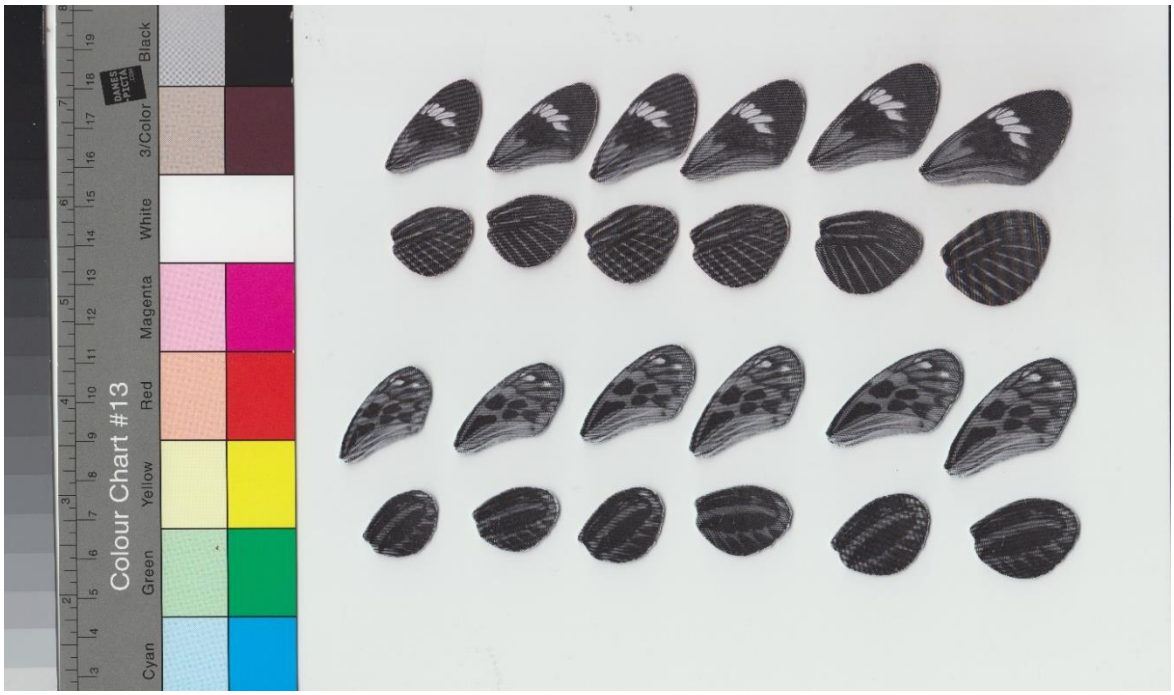
E. lybia

E. libitina

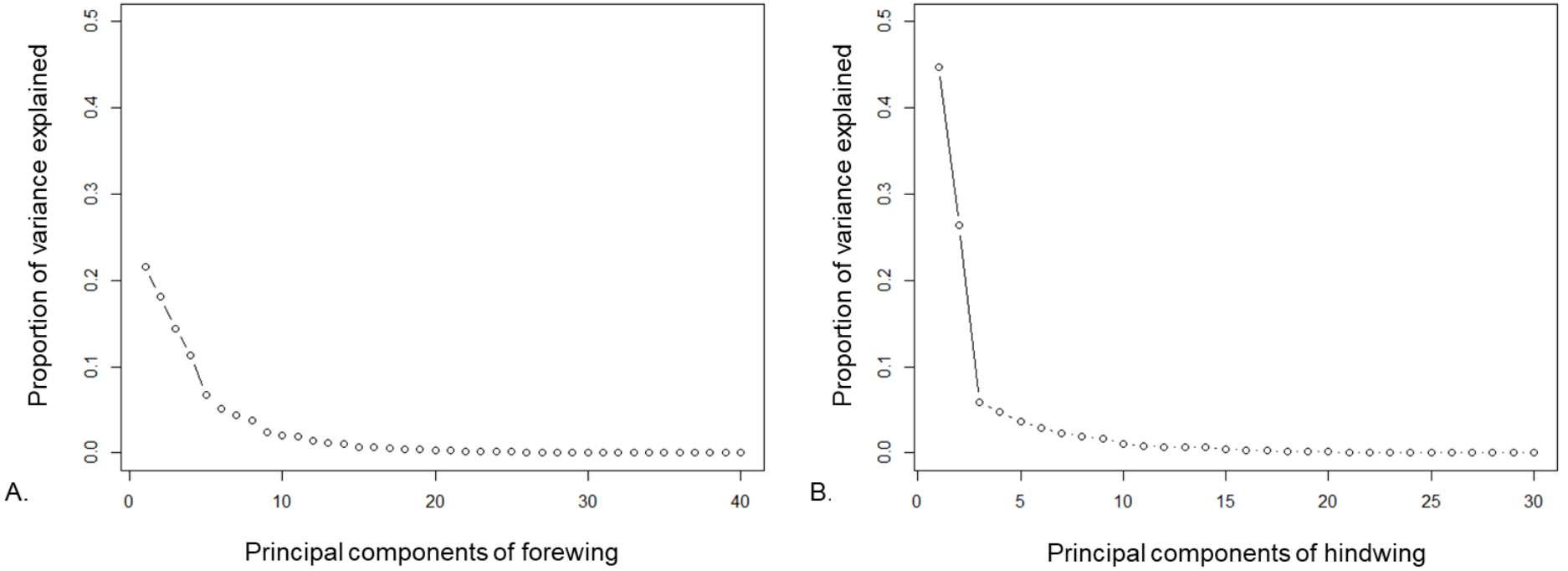
Appendix 2 - Supporting Information for Chapter 3



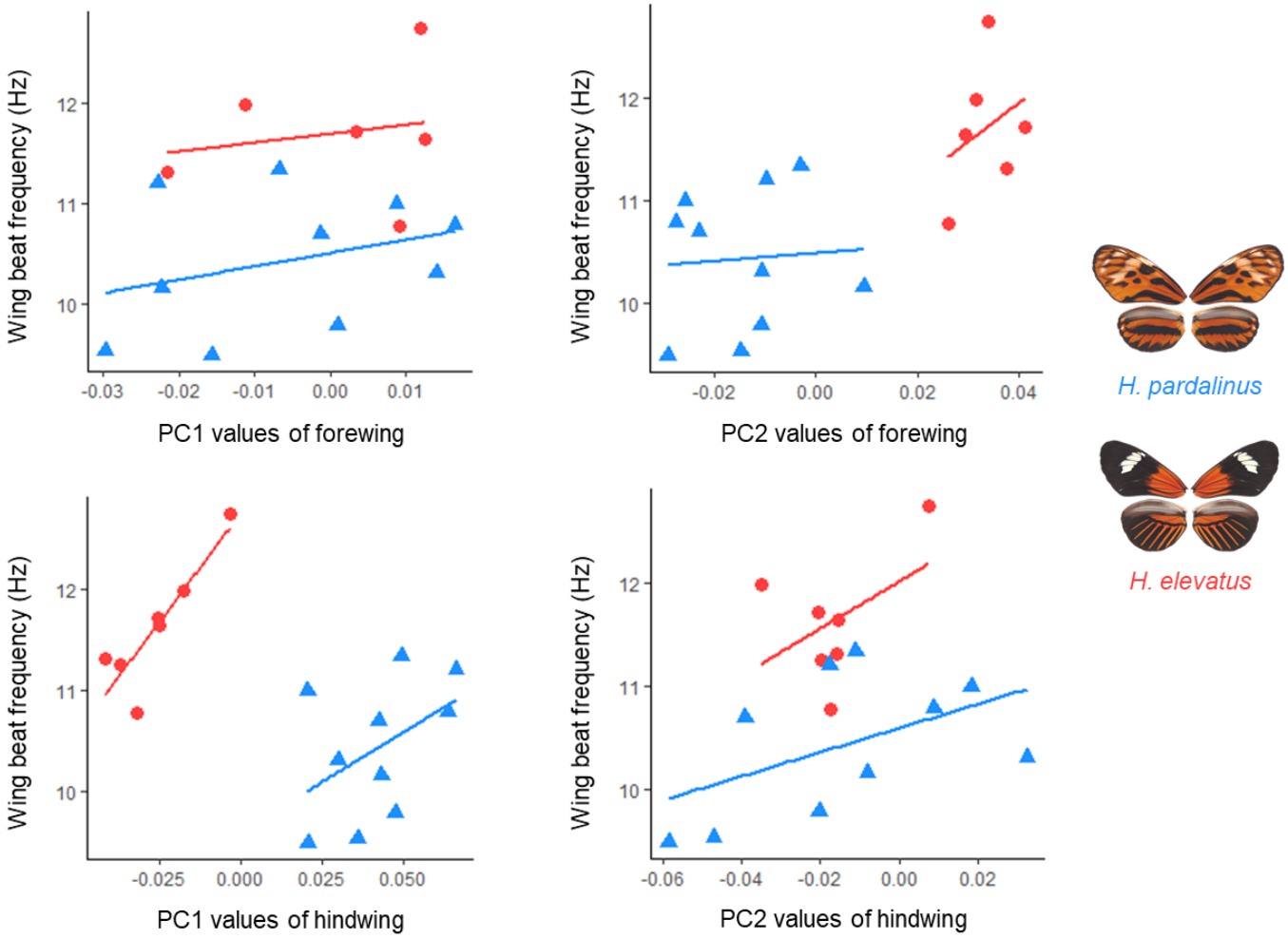
Appendix 2A. Example of footage of a butterfly flying. The 24 frames are taken in sequence for one wing beat and are equal to 1/10 of a second. In frames 1 and 12 are shown the angles that are used for the up (frame 1) and down (frame 12) beat measurements.



Appendix 2B. Templates used to manipulate butterfly wings to the “elevatus” shape (above) or the “pardalinus” shape (below).



Appendix 2C. Proportion of variance explained by the principal components of A. forewing shape and B. hindwing shape.



Appendix 2D. Proportion of variance explained by the principal components of A. forewing shape and B. hindwing shape.

Appendix 2E. Significant differences in area and wing length in the comparisons of real wings with the two types of wing templates.

	FW	HW	"elevatus" template	"pardalinus" template
Length	Manipulations in *H. pardalinus* have a larger effect ($F_{1,32}$ = 17.76, p = 0.0002)		Larger change in length in *H. pardalinus* ($F_{1,32}$ = 8.12, p = 0.008) in both wings, larger difference between FW and HW in *H. elevatus* ($F_{1,30}$ = 12.52, p = 0.001)	Interaction between species and wing is significant ($F_{1,30}$ = 5.71, p = 0.023), more length lost in *H. pardalinus* FW and *H. elevatus* HW.
Area	"elevatus" template has a larger effect on area ($F_{1,29}$ = 7.81, p = 0.009)	More area lost in *H. elevatus* ($F_{1,30}$ = 40.67, p = 5.69E10-7) and when using the "pardalinus" template ($F_{1,29}$ = 19.92, p = 0.0001)	Larger difference in area in FW ($F_{1,29}$ = 39.03, p = 9.42E-7), this difference is stronger in *H. pardalinus* ($F_{1,28}$ = 15.93, p = 0.0004)	Larger change in length in *H. elevatus* ($F_{1,30}$ = 13.32, p = 0.001), larger difference between FW and HW in *H. elevatus* ($F_{1,30}$ = 18.49, p = 0.0001)

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