

REVIEW

The diversification of *Heliconius* butterflies: what have we learned in 150 years?

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Abstract

Research into *Heliconius* butterflies has made a significant contribution to evolutionary biology. Here, we review our understanding of the diversification of these butterflies, covering recent advances and a vast foundation of earlier work. Whereas no single group of organisms can be sufficient for understanding life's diversity, after years of intensive study, research into *Heliconius* has addressed a wide variety of evolutionary questions. We first discuss evidence for widespread gene flow between *Heliconius* species and what this reveals about the nature of species. We then address the evolution and diversity of warning patterns, both as the target of selection and with respect to their underlying genetic basis. The identification of major genes involved in mimetic shifts, and homology at these loci between distantly related taxa, has revealed a surprising predictability in the genetic basis of evolution. In the final sections, we consider the evolution of warning patterns, and *Heliconius* diversity more generally, within a broader context of ecological and sexual selection. We consider how different traits and modes of selection can interact and influence the evolution of reproductive isolation.

Introduction

No single species or clade can provide a model for understanding evolutionary processes across the diversity of life. Nevertheless, some taxonomic groups have become so well studied that they have contributed to a

broad range of evolutionary questions. The Neotropical *Heliconius* butterflies are one such group (Box 1). Early evolutionists were drawn to the group's striking wing-pattern mimicry. Divergent lineages have repeatedly converged on the same bright warning patterns (Fig. 1), an observation that led Henry Walter Bates to develop mimicry theory (Bates, 1862). Bates further interpreted differences between different geographic populations as support for Darwin's hypothesis of species mutability (Darwin, 1859). Subsequent studies

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Box1: Major discoveries in *Heliconius* and challenges for the future

(a) Advances:

- Experimental evidence for positive frequency-dependent selection maintaining local warning patterns in the wild. This provides an excellent example of local adaptation maintained by strong natural selection.
- Evidence for widespread gene flow across the species barrier.
- Discovery of horizontal transfer of colour-pattern alleles permitting adaptive introgression, and evidence that this may lead to reproductive isolation and hybrid trait speciation.
- Identification of major genes involved in mimetic shifts, and the demonstration of homology between colour-pattern genes across distantly related *Heliconius* as well as other Lepidoptera.
- Demonstration that chromosomal inversions are associated with the evolution of supergenes.
- Evidence for a key role of gene regulatory evolution (as opposed to coding sequence divergence) in generating morphological diversity.
- Experimental evidence that warning patterns are 'magic traits' (that is traits under divergent ecological selection which also contribute to nonrandom mating, and which are hypothesized to facilitate speciation with gene flow).

(b) Challenges:

- A greater understanding of the ecology of mimicry: What are the predators? How do they learn? When does predation occur? How does toxicity vary between taxa and individuals?
- How do selection, introgression and recombination interact to create the genomic patterns we observe between divergent taxa?
- Understanding the sequence of evolution of adaptive traits, which together characterize the differentiated species we observe. What traits are initially important, are other traits selected through their reinforcement role?
- Understanding the origins and the maintenance of diversity in mimicry. Why are new patterns emerging? What contributes to their initial establishment? What contributes to the maintenance of polymorphisms?
- Understanding the structure and origins of supergenes, and the build-up of differentiated alleles controlling complex variation.
- Geography of speciation: Have species evolved in the face of gene flow or are the porous species boundaries we now observe recent?
- Development of new methodologies to allow functional tests of candidate loci underlying phenotypic variation.
- Understanding the role that behavioural flexibility played during diversification.
- A greater understanding of chemical signalling in reproductive isolation.
- Addressing the genetics of more complex traits, including behavioural traits that contribute to reproductive and ecological isolation.

focused on taxonomy and the evolution of mimicry, the genetic basis of divergent colour patterns, and ecology and behaviour. More recently, genomic and developmental studies have played an important role in evolutionary debates about the genetic architecture of adaptation and speciation. Here, we integrate findings from these studies to assess the contribution *Heliconius* have made to our understanding of evolutionary processes.

The Heliconiini radiation and the nature of species

Heliconius and nine smaller genera form the Neotropical tribe Heliconiini (Nymphalidae: Heliconiinae), most closely related to the Indopacific *Cethosia* and the Palaeotropical Acraeini (Wahlberg *et al.*, 2009). Analysis of both morphological (Brown, 1981; Lee *et al.*, 1992; Penz, 1999) and molecular data (Lee *et al.*, 1992;

Brower, 1994; Brower & Egan, 1997; Mallet *et al.*, 2007; Kozak *et al.*, 2015) has resulted in a highly resolved and taxonomically complete phylogeny across the tribe (Fig. 1). Dating using molecular clocks implies that the tribe first appeared 24.8–29.0 Ma, and *Heliconius*, the most speciose genus, arose 10.5–13.4 Ma (Kozak *et al.*, 2015). In the past, *Heliconius* biogeography was frequently discussed with reference to the Pleistocene refugium theory (Turner, 1965; Brown, 1979), which proposes high rates of allopatric speciation resulting from fragmentation of tropical forests. Since then, refugium theory has been widely criticized (Nelson *et al.*, 1990; Whinnett *et al.*, 2005; Dasmahapatra *et al.*, 2010), and the time-calibrated phylogeny indicates that much of the Heliconiini species diversity significantly predates the Pleistocene (Kozak *et al.*, 2015).

A simple diversification-by-allopatry model seems insufficient to explain the radiation of Heliconiini, espe-

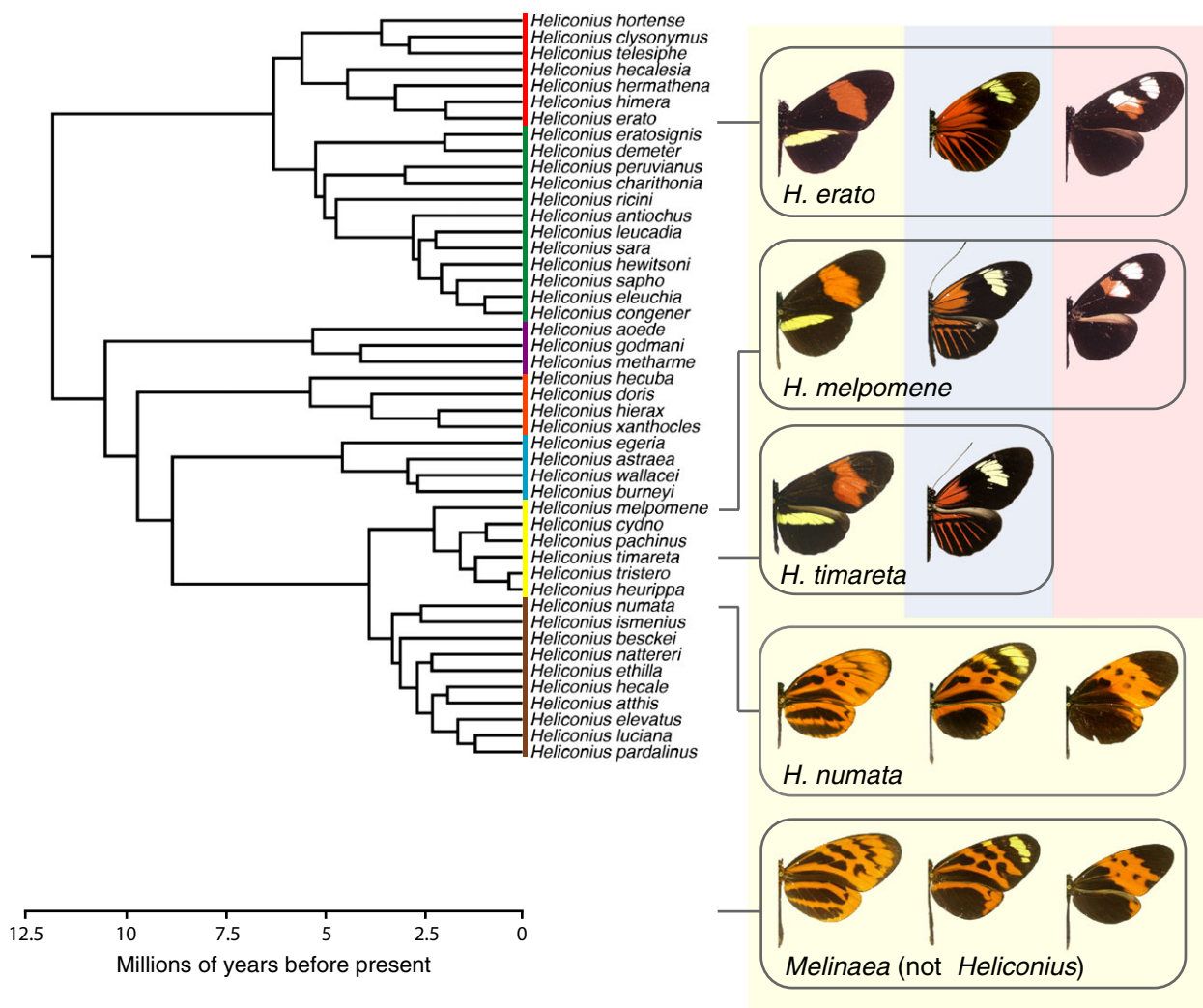


Fig. 1 *Heliconius* mimicry in its phylogenetic context. Mimicry is observed between closely related *Heliconius* species (e.g. *H. melpomene* and *H. timareta*), between distantly related *Heliconius* species (e.g. *H. melpomene* and *H. erato*) and between *Heliconius* and heterogeneric species (e.g. *H. numata* and *Melinaea* spp.). Coloured background boxes indicate taxa that co-occur geographically. Vertical colours indicate subclades: erato = red; sara/sapho = green; aoe = purple; orange = doris; wallacei = blue; melpomene = yellow; silvaniform = brown. Phylogeny after Kozak *et al.* (2015).

cially considering that communities are at their most species rich within the contiguous forests of Amazonia. A general alternative to refugia is speciation driven by adaptation to local conditions. Diversification rates in *Heliconius* have been steadily increasing since the emergence of the genus in the mid-Miocene (Kozak *et al.*, 2015). This is coincident with the rise of the Andes, where today a high proportion of distinct colour-pattern races are found and the average age of species is younger than elsewhere (Rosser *et al.*, 2012). The uplift of the Andes was associated with significant environmental change perhaps fuelling increased rates of speciation, although a strong correlation cannot be demonstrated (Kozak *et al.*, 2015). Whether speciation

ever occurs in complete sympatry is unclear, although in *Heliconius* the existence of stable polymorphisms coupled with specific genetic architectures makes it plausible. A more likely scenario is that speciation is initiated in parapatry, and once populations have diverged sufficiently for coexistence, the process is completed in sympatry (Mallet *et al.*, 1998a).

What is a (*Heliconius*) species?

Although arguments over the 'correct' species concept may be sterile, examining the nature of species can help us more carefully consider the evolution of diversity. Species are often considered the fundamental unit

of biodiversity, but distinguishing between species and lower taxonomic levels has proved problematic. The widely adopted *Biological Species Concept* (BSC) (Mayr, 1942) is useful because it explicitly addresses the mechanisms (i.e. reproductive isolation) that result in the discontinuities we observe in nature. However, the strength of reproductive isolation observed between taxa, including among species and subspecies of *Heliconius*, is often broadly continuous (Mallet *et al.*, 2007; Merrill *et al.*, 2011a; Nosil, 2012). Even if we disregard a strict BSC, where genetic barriers must be absolute, the degree of reproductive isolation required for species status is arbitrary. In addition, it has been noted that if ‘gene exchange were widespread and substantial between sympatric taxa’, it would present a serious problem for the BSC (Coyne & Orr, 2004; p. 41). Recent genomic analysis of *Heliconius* species exposes just such a challenge (Martin *et al.*, 2013) (Fig. 2).

Studies of hybridization in *Heliconius* twenty years ago led to an alternative to the BSC – termed the *Genotypic Cluster Definition* (Mallet, 1995). In contrast to the BSC, Mallet’s (1995) Genotypic Cluster Definition takes a descriptive approach to species delimitation using sympatric coexistence of distinct multilocus genotypes as the defining character of a species, without prejudice as to the processes that maintain species. This genotypic cluster approach is now broadly applied in *Heliconius*, with a conservative approach to the elevation of taxa to species status. In other words, many populations with different wing-pattern phenotypes are considered subspecies, or geographic races, because they form transition zones with abundant intermediate forms where they meet. The ~30 named races of *Heliconius erato*, for example, have distinctive colour-pattern phenotypes, but belong to the same species. In contrast, the geographic replacement of *H. erato* found in south eastern Ecuador, *H. himera*, is considered a distinct species because, where the two species co-occur, hybrids are rare (but still account for ~10% of individuals) (Jiggins *et al.*, 1996). In other words, the Genotypic Cluster Definition implies that where they co-occur, ‘species’ are characterized by a bimodal distribution of traits, even if gene flow persists. By emphasizing the importance of multilocus genotypes, the Genotypic Cluster Definition is a useful tool for investigating gene flow and the maintenance of distinct species, and the genomic architecture of gene flow and divergence.

A genomewide view of porous species

The sequencing of a *Heliconius* reference genome (The Heliconius Genome Consortium, 2012), and resequencing of additional taxa (Kronforst *et al.*, 2013; Martin *et al.*, 2013), makes it possible to infer genomewide patterns of divergence. The patterns observed broadly support the adoption of a genotypic clustering approach

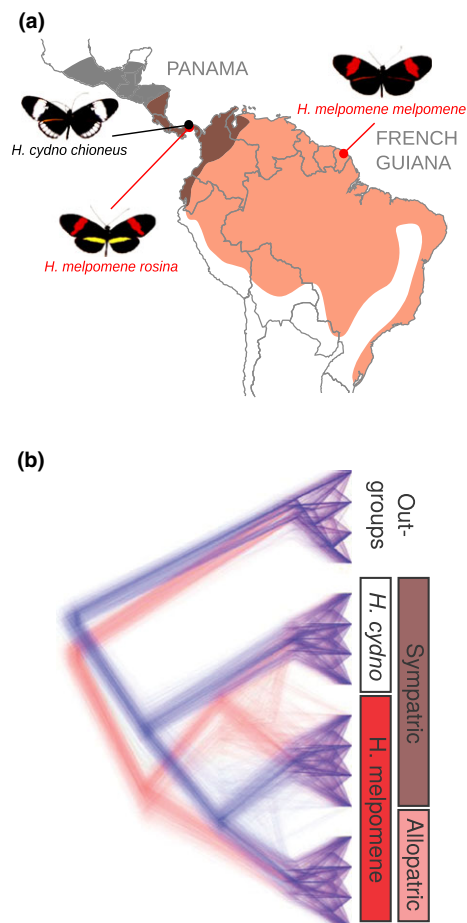


Fig. 2 Phylogenetic discordance across the ‘species’ barrier. The phylogenetic history of a species depends on which population you consider, and where in the genome you look. (a) The ranges of *Heliconius cydno* (grey shading) and *H. melpomene* (pink shading) overlap in Central America and northern South America (dark pink shading). In Panama, *H. cydno chioneus* and *H. melpomene rosina* are sympatric, whereas in French Guiana, *H. melpomene melpomene* is allopatric with respect to *H. cydno*. (b) Maximum-likelihood phylogenies for nonoverlapping 100-kb windows from throughout the genome are superimposed with branch lengths normalized. Each of the three races in A is represented by four samples. Four species from the related silvaniform clade (see Fig. 1) were used as out-groups. Trees that support the grouping of the two *H. melpomene* races as monophyletic (53%) are coloured blue. Trees that support the monophyly of the Panamanian *H. cydno chioneus* and the sympatric *H. melpomene rosina* (42%) are coloured red. Trees that support the monophyly of *H. cydno chioneus* and the allopatric *H. melpomene melpomene* (1%) are coloured green. The remaining trees in which the three races did not form distinct clades (4%) are coloured black. Reproduced from Martin *et al.* (2013).

towards species delimitation. Below the species level, low levels of divergence are observed between colour-pattern races across most of the genome, but strong differentiation is seen at a handful of loci known to be

under divergent selection (The *Heliconius* Genome Consortium, 2012; Kronforst *et al.*, 2013; Martin *et al.*, 2013). Between-species comparisons, on the other hand, reveal much higher levels of genomewide divergence despite convincing evidence that large-scale gene flow persists due to occasional hybridization (Kronforst *et al.*, 2013; Martin *et al.*, 2013). Significant phylogenetic discordance (i.e. where different parts of the genome imply different evolutionary histories) has been frequently observed in *Heliconius* (Beltran *et al.*, 2002; Bull *et al.*, 2006; Kronforst *et al.*, 2006a; Martin *et al.*, 2013). Although some level of discordance is expected due to the stochastic nature of gene coalescence, there is now strong evidence for porous species boundaries that permit gene flow for millions of years after initial divergence. For example, across 40% of its genome, *H. melpomene* from Panama is more closely related to the population of *H. cydno* with which it co-occurs than to an allopatric population of *H. melpomene* in French Guiana (Martin *et al.*, 2013) (Fig. 2).

Genomic divergence may begin at a few narrow regions containing key loci under selection, and these islands of divergence may grow as speciation proceeds, eventually expanding to encompass the whole genome (Wu, 2001; Feder *et al.*, 2012a). The narrow peaks of differentiation observed between recently diverged *Heliconius* races, and the widespread genomic divergence between species, are consistent with the early- and late-stage expectations of this islands-of-divergence model. Whether this is pertinent to our understanding of speciation depends on whether populations divergent at just a handful of narrow genomic regions do in fact represent incipient species. It has been argued that *Heliconius* wing-pattern races, which are structured at just a few loci of large phenotypic effect in otherwise genetically mixed populations, do not (Cruickshank & Hahn, 2014). However, population divergence at a few loci under strong selection is a form of reproductive isolation, albeit localized within the genome. More significant is the mass of evidence that divergence in colour pattern plays a key role – alongside the evolution of additional traits – during *Heliconius* speciation. Regardless, studies in *Heliconius* have demonstrated that selection may initially limit gene flow at discrete regions of the genome involved in adaptive divergence.

Elsewhere, divergence between forms of threespine sticklebacks and European crows resembles the proposed initial stages of speciation (Hohenlohe *et al.*, 2010; Jones *et al.*, 2012; Poelstra *et al.*, 2014), whereas it could be argued that divergence observed between host races of *Rhagoletis* and sister species of *Anopheles gambiae* and *Ficedula* flycatchers looks more like the later stages (Lawniczak *et al.*, 2010; Michel *et al.*, 2010; Ellegren *et al.*, 2012; Hahn *et al.*, 2012). Thus, the ability to sample *Heliconius* genomes across a continuum of divergence greatly informs our understanding of speciation genomics. Nevertheless, there are arguably

no empirical examples of the transition between these two states either from *Heliconius* or from other taxa. The lack of intermediate stages may be expected if a rapid and unstable transition occurs at the species boundary (Feder *et al.*, 2012a). This is perhaps the critical transition in the evolution of species. Selection maintaining initial localized divergence may later provide opportunities for further differentiation in physically linked genomic regions ('divergence hitchhiking'; Via & West, 2008; Via, 2012; Feder *et al.*, 2012a) or across the genome more generally ('genome hitchhiking'; Feder & Nosil, 2012). Examples of the transition between the two states would therefore be important for understanding the role of these two forms of hitchhiking (Via & West, 2008; Feder & Nosil, 2012; Feder *et al.*, 2012b; Via, 2012). As more genomes are sequenced, this apparent gap in our sampling may well be overcome.

Mimicry: can selection lead to a predictable genome?

The spectacular mimicry in *Heliconius* has become a textbook example of natural selection (e.g. Barton *et al.*, 2007). Cyanogenic glycosides, either synthesized *de novo* or sequestered from host plants as larvae, render *Heliconius* butterflies unpalatable to vertebrate predators (Engler *et al.*, 2000; Cardoso & Gilbert, 2007; Engler-Chaouat & Gilbert, 2007; Hay-Roe & Nation, 2007; Chauhan *et al.*, 2013). Predators include insectivorous birds that learn the wing patterns of distasteful prey and subsequently avoid them (Brower *et al.*, 1963; Chai, 1986; Chai, 1988; Pinheiro, 1996; Langham, 2004). Unpalatable prey therefore benefit from protection against predators by displaying colour patterns similar to other unpalatable species, a widespread phenomenon known as Müllerian mimicry (Müller, 1879). Despite the difficulty of observing predation in the wild, three lines of evidence confirm the importance of mimicry as an adaptation in *Heliconius*: (i) evidence for learning and sight rejection based on previous experience from focal predators studied in cages (Chai & Srygley, 1990; Merrill *et al.*, 2012) or in the wild (Langham, 2004), (ii) higher recapture rates of released butterflies that match the local mimicry community (Benson, 1972; Mallet & Barton, 1989; Kapan, 2001) and (iii) lower attack rates on artificial butterflies matching local comimics (Merrill *et al.*, 2012; Finkbeiner *et al.*, 2014).

Estimates of selection coefficients favouring mimicry are high, whether calculated directly from the recapture rates of released butterflies [overall $s = 0.52$ in *H. erato* (Mallet & Barton, 1989); $s = 0.64$ in *H. cydno* (Kapan, 2001)] or indirectly from cline width and linkage disequilibrium measurements across hybrid zones (per locus $s = 0.13$ – 0.40 in *H. erato* and *H. melpomene*) (Mallet *et al.*, 1990; Rosser *et al.*, 2014). Each mimetic wing pattern therefore represents a towering fitness peak in

the adaptive landscape, driving convergence across a wide diversity of prey species sharing a habitat. However, despite strong evidence for intense selection for mimicry in *Heliconius*, we still have limited knowledge about the actual communities of predators, and there are difficulties in obtaining direct estimates of predation rates in the wild. This has hindered our understanding of how variation at individual loci controlling wing patterns directly affects fitness in the wild, a problem common to many systems (Barrett & Hoekstra, 2011).

What factors influence the fitness landscape?

Fitness landscapes are often simplified to a single isolated trait, but in reality they must include multiple interacting traits. *Heliconius* warning signals provide a tractable example of a phenotype comprising a composite of multiple characters, some of which may have additional functions (Sherratt & Beatty, 2003). In *Heliconius*, warning signals can involve the spatial arrangement of wing-pattern elements (see Brown, 1981; for a review), colour hue (Crane, 1954; Sweeney *et al.*, 2002, 2003; Bybee *et al.*, 2012; Llaurens *et al.*, 2014), wing shape (Jones *et al.*, 2013) and behaviour (Srygley, 1999, 2004, 2007; Finkbeiner *et al.*, 2012). The addition of new characters to warning signals can enhance their efficiency (Sherratt & Beatty, 2003; Rowe & Halpin, 2013). However, we also expect trade-offs between ecological functions; for example, mimetic flight behaviours and wing shape carry aerodynamic costs (Srygley, 1999, 2004). Wing patterns are also involved in intraspecific communication and mate recognition, resulting in conflict between mimicry and intraspecific communication (Estrada & Jiggins, 2008). The relative fitness associated with butterfly warning patterns therefore results from the resolution of synergies and trade-offs between functions (Mallet & Gilbert, 1995; Salcedo, 2010), although little is known about how these interactions affect the diversity of patterns or the coexistence of species.

Despite this, local abundance is clearly central to the fitness of specific warning patterns. Positive *number dependence* (Müller, 1879; Turner, 1984; Mallet & Joron, 1999) underpins mimicry and leads to convergence among coexisting taxa. An individual's risk of predation decreases as the local density of its warning pattern increases: as numbers increase, local predators will have had greater opportunity to associate particular patterns with unpleasant experiences and so avoid them. *Heliconius* butterflies participate in diverse but often coexisting mimicry rings (groups of species that converge on the same warning phenotype). Hence, there may be several, distinct mimetic phenotypes in any given area, all of which impose strong stabilizing selection on participating taxa. This complexity, alongside variation in local predator and prey communities, shapes a rugged adaptive landscape with distinct fitness peaks (Turner,

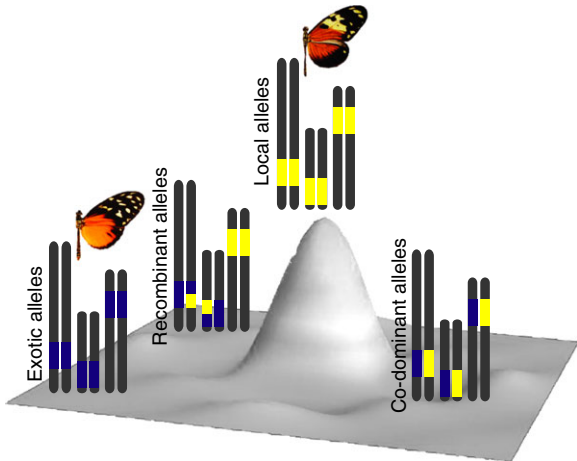
1984). A given *Heliconius* species may join many distinct mimicry rings according to local context (Brown, 1979), leading to the well-documented spatial mosaic of mimetic races observed across the Neotropics in many widespread species, the most prominent being the parallel geographic radiations of *H. erato* and *H. melpomene* (Brown, 1976; Linares, 1997) (Fig. 1). A few species, such as *H. doris*, *H. numata*, and some populations of *H. hecale*, *H. cydno* and *H. timareta*, maintain local polymorphisms (Brown & Benson, 1974; Brown, 1976; Kapan, 2001; Chamberlain *et al.*, 2009). In *H. numata*, for example, multiple forms participate in distinct mimicry rings dominated by butterflies in the tribe Ithomiini (Nymphalidae) (Figs 1 and 3), and the persistence of polymorphism appears to be maintained by a balance between migration and selection across mimicry communities (Joron *et al.*, 2001; Joron & Iwasa, 2005). Nonetheless, there are also many widespread *Heliconius* species that are monomorphic across their range, and the reasons for extreme variability in some but not all species remain unclear.

Spatial variation in prey communities is therefore crucial to the diversification of warning signals. Nevertheless, the establishment of entirely new phenotypes is paradoxical under strict number-dependent selection. One further possibility is that during periods of relaxed selection, drift may allow new variants to rise above a threshold density until mimicry selection takes over (Mallet, 1993, 2010; Mallet & Joron, 1999; Sherratt, 2006; Chouteau & Angers, 2012). Stochastic changes in the abundance of butterflies carrying warning signals, leading to turnover in the composition of prey communities, may also cause fluctuations in the direction and intensity of selection (Turner & Mallet, 1996). *Heliconius* warning patterns provide one of the most convincing examples of Wright's shifting balance (Mallet, 2010).

Fitness valleys and genetic leaps

Evolving a new mimetic resemblance implies crossing a substantial fitness valley. This presents an additional challenge to our understanding of colour-pattern diversity within *Heliconius* (Fisher, 1930; Turner, 1977, 1984), but also an excellent opportunity to test hypotheses on how fitness valleys can be bridged. The 'two-step' theory (Poulton, 1913; Nicholson, 1927) predicts that crossing a valley can be achieved with a mutation of major effect, followed by mutations of smaller effect that refine mimicry. A long tradition of laboratory rearing and crossing experiments between mimetic forms within species has revealed that (i) phenotypic plasticity is not involved in major shifts between warning pattern phenotypes and (ii) that major colour-pattern elements (Beebe, 1955; Turner & Crane, 1962; Emsley, 1964; Sheppard *et al.*, 1985; Mallet, 1989; Linares, 1996; Jiggins & McMillan, 1997; Gilbert, 2003; Joron *et al.*,

(a) Locally monomorphic species



(b) Locally polymorphic species

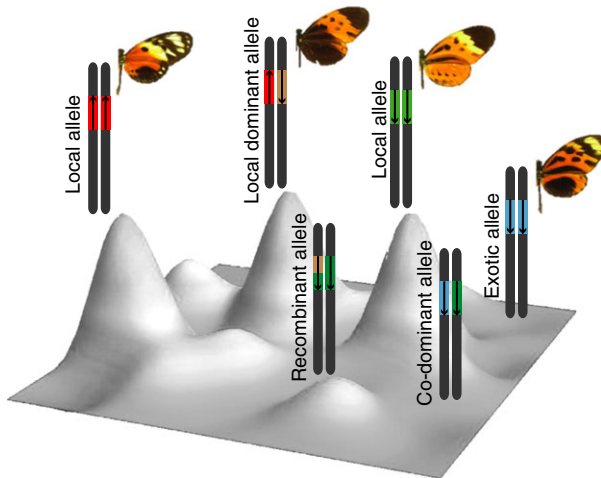


Fig. 3 Putative adaptive landscape shaping the genetic architecture of (a) monomorphic and (b) polymorphic *Heliconius* species. (a) In the locally monomorphic species (e.g. *H. hecale*), three main loci control variation in wing colour pattern. Local alleles (shown in yellow), for example from the *melicerta* subspecies, result in a locally mimetic phenotype, which resides on the local fitness peak. Exotic alleles (shown in blue), for example from the *zuleika* subspecies, as well as recombinant or codominant alleles result in locally nonmimetic phenotypes and fall into fitness valleys. (b) In the polymorphic species *H. numata*, wing colour-pattern variation is controlled by a supergene, which displays chromosomal rearrangements, represented by arrows indicating different gene orders. Several mimetic alleles coexist within a single population: local alleles shown in red, brown and green result in the locally mimetic morphs, for example *silvana*, *bicoloratus* and *tarapotensis* (left to right), which reside on local fitness peaks. Recombination is rare due to the inversions, thereby preventing genetically intermediate morphs falling in fitness valleys. Strict dominance of alleles with inverted gene order (brown) over ancestral gene order (red) allows heterozygous individuals to remain on fitness peaks. Exotic alleles, shown in blue, on the other hand result in locally nonmimetic morphs and will fall into fitness valleys. For example, the *aurora* morph shown here (far right) is allopatric with respect to *silvana*, *bicoloratus* and *tarapotensis*. Similarly, codominance between alleles sharing the inverted gene order (green and blue) displays a locally nonmimetic phenotype. Consequently, by preventing recombination, inversions with strict dominance relationships can facilitate the co-occurrence of different mimetic alleles within a single population.


2006; Kronforst *et al.*, 2006b) or even entire wing-pattern polymorphisms (Brown & Benson, 1974; Joron *et al.*, 2006) are controlled by a relatively small number of Mendelian loci (Fig. 4). The distinct phenotypes resulting from alternative alleles at these loci may represent the large-effect mutations hypothesized to bridge troughs in the fitness landscape. Modifier loci, and loci of small effect, which refine wing-pattern phenotypes, have also been documented in several *Heliconius* species (Sheppard *et al.*, 1985; Nijhout, 1991; Baxter *et al.*, 2008; Jones *et al.*, 2013; Papa *et al.*, 2013; Huber *et al.*, 2015) and perhaps represent the second step in the 'two-step' theory.

The difficulty of estimating selection coefficients for individual loci in nature, however, makes it challenging to measure the effect sizes of individual mutations in terms of fitness. In addition, we still lack information about the precise mutations at patterning loci underlying phenotypic shifts, which is required to test

whether the distribution of effect sizes of adaptive mutations reflects theoretical expectations. Advances in the functional characterization of causative loci will perhaps reveal the number, timing and effects of mutational steps involved in adaptive evolution in *Heliconius*. Differentiation of individual colour-pattern elements likely involves multiple, sequential mutations targeting the same gene(s) (McGregor *et al.*, 2007; Baxter *et al.*, 2008; Martin & Orgogozo, 2013). These may build up in separate populations and be later brought together through hybridization. As a consequence, 'ready-made' alleles of large phenotypic effect, capable of crossing deep adaptive valleys, can be made available through adaptive introgression (Gilbert, 2003; Mallet *et al.*, 2007; Pardo-Diaz *et al.*, 2012; The Heliconius Genome Consortium, 2012). Indeed, recent research has shown that mimicry in *Heliconius* can be achieved through hybridization and subsequent sharing of key loci between closely related species (Pardo-Diaz *et al.*, 2012; The Heliconius Genome Consortium, 2012). In this way, phenotypic evolution can occur through selection on extant genetic variation, rather than large-effect novel mutations. It seems likely that adaptive introgression and homoploid hybrid speciation are considerably more common than formerly believed.

(a)

Linkage group 1
K
White/yellow switch of colour pattern elements



Cosegregates with QTL for male attraction to white/yellow females (FW pattern variation)

Refs: 3–7, 32


Linkage group 2
(Red pattern variation)

Refs: 1, 8

Linkage group 7
(Red pattern variation)

Refs: 8


Linkage group 10
Ac = Sd
Melanism of FW, affecting FW band shape



Associated with female host-plant acceptance (FW & HW pattern variation)

Refs: 1, 2, 7–16, 32

Linkage group 13
Ro
Rounding of distal edge of FW band




(Red FW band shape variation)

Refs: 1, 8

Linkage group 15
Yb/Sb/N = Cr = P*
Yellow HW bar ('Yb')
Yellow/white HW margin ('Sb')
Yellow/white in FW bar ('N')

*P = *H. numata* supergene, controlling entire wing pattern (see figs. 1 & 2)



Yb Sb N 'HhN/HiN'

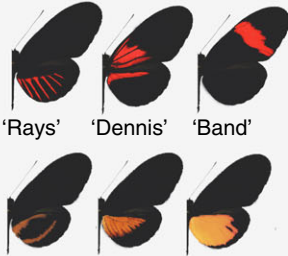
Associated with female mating probability (Yellow dots FW submarginal area)

Refs: 2, 4–8, 10–14, 17, 19–23, 32

Linkage group 17
(Yellow HW bar variation)

Refs: 14

Linkage group 18
B/D = B = Br/G
Red HW and FW colour pattern elements



B/D 'Rays' 'Dennis' 'Band'

Br

Associated with male attraction to red patterned females (white/yellow switch in FW band)

Refs: 1, 3–5, 8, 10, 12, 15, 16, 18, 21–28, 32

Linkage group 19
(FW pattern variation)

Refs: 2

Linkage group 21 (Z)
Associated with female sterility in interspecific hybrids (FW pattern variation)

Refs: 1, 2, 10, 24, 29–31

(b)

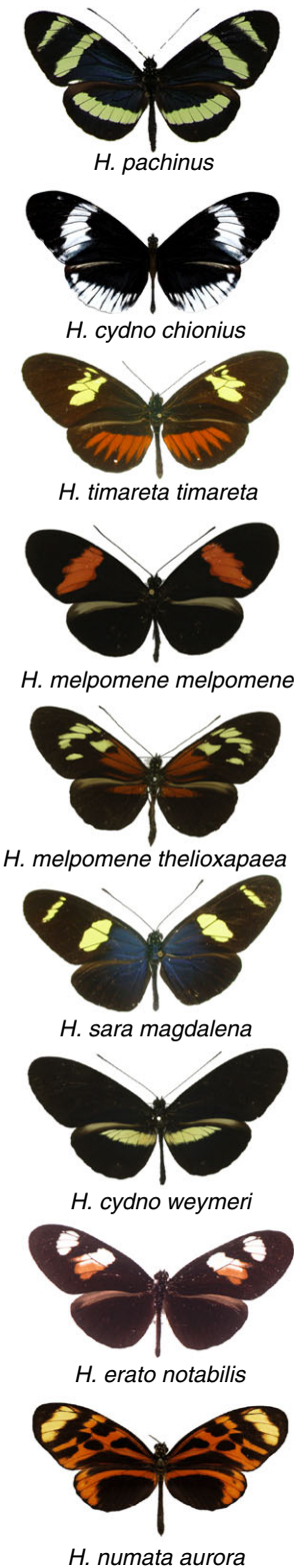


Fig. 4 Genetic architecture of phenotypic variation in *Heliconius* butterflies. (a) Distribution of loci with known phenotypic effects across *Heliconius* linkage groups. Names of loci with ‘major’ phenotypic effects are shown in bold. ‘Minor-’ effect loci associated with linkage groups are shown in parentheses. Only linkage groups with known associations with phenotypic variation are shown. Homology between loci identified in different taxa is implied by an ‘=’ symbol; in each case, the ‘*melpomene* name’ is given first. (b) Examples of pattern diversity resulting from allele substitution at these loci. References: ¹Baxter *et al.* (2008), ²Jones *et al.* (2013), ³Kapan (1998), ⁴Linares (1997), ⁵Naisbit *et al.* (2003), ⁶Joron *et al.* (2006), ⁷Kronforst *et al.* (2006a,b,c), ⁸Nadeau *et al.* (2014), ⁹Kapan *et al.* (2006), ¹⁰Mallet (1989), ¹¹Papa *et al.* (2013), ¹²Sheppard *et al.* (1985), ¹³Jiggins *et al.* (2005), ¹⁴Nijhout *et al.* (1990); ¹⁵Gilbert (2003), ¹⁶Martin *et al.* (2012), ¹⁷Merrill *et al.* (2011b), ¹⁸Merrill *et al.* (2013), ¹⁹Emsley (1964), ²⁰Ferguson *et al.* (2010), ²¹Counterman *et al.* (2010), ²²Jiggins & McMillan (1997), ²³Tobler *et al.* (2004), ²⁴Papa *et al.* (2008), ²⁵Reed *et al.* (2011), ²⁶Turner & Crane (1962), ²⁷Turner (1972), ²⁸Chamberlain *et al.* (2011), ²⁹Jiggins *et al.* (2001a,b), ³⁰Naisbit *et al.* (2002), ³¹Salazar *et al.* (2005), and ³²Huber *et al.* (2015).

The role of genetic architecture in navigating fitness landscapes

Adaptive shifts in one phenotypic axis often require correlated or compensatory change in others. Shifts between mimetic fitness peaks, for example, often require correlated changes in multiple wing-pattern elements. Where selection favours rapid change, or acts to maintain complex polymorphisms, recombination will break down the key associations between alleles underlying co-adapted traits if left unchecked. How then, are these complex, composite phenotypes maintained? In species with well-defined geographic races, the correct assortment of alleles affecting warning patterns does not require linkage between loci as this is ensured by spatial segregation [e.g. the four major wing-pattern genomic regions of *H. erato* and *H. melpomene* are fixed for different alleles in different geographic regions (Mallet, 1989; Supple *et al.*, 2013)]. In contrast, in those species where local polymorphism is maintained (such as *H. numata*), tight linkage between loci, or strong assortative mating, is required to facilitate the coexistence of multiple combinations of congruous alleles, which together produce mimetic phenotypes. ‘Supergenes’, which allow multiple functional elements to segregate as a single Mendelian locus despite recombination elsewhere, are classically associated with polymorphic mimicry (Brown & Benson, 1974; Charlesworth & Charlesworth, 1975; Turner, 1977; Joron *et al.*, 2006; Thompson & Jiggins, 2014). In *H. numata*, coexisting mimetic phenotypes are determined by the supergene *P*, at which polymorphic chromosomal inversions maintain linkage disequilibrium and protect co-adapted allele combinations (Joron *et al.*, 2011) (Fig. 3). Dominance relationships among alleles at the *P* supergene locus limit the expression of intermediate nonmimetic phenotypes when sympatric morphs interbreed (Joron *et al.*, 2006; Le Poul *et al.*, 2014). In contrast, similar supergene architectures are not observed in the sister species of *H. numata*, namely *H. ismenius* and *H. hecale*, which do not maintain local polymorphisms (Huber *et al.*, 2015). Variation in selection regime between taxa, as well as the age, tempo and genealogy of adaptation, therefore leads to different genetic architectures. For example, the contrasted phylogeographic history of

the parallel radiations in *H. erato* and *H. melpomene* suggests that the tempo of adaptation varied markedly, *H. melpomene* having recently colonized an older diversification in *H. erato* (Flanagan *et al.*, 2004; Quek *et al.*, 2010; Hines *et al.*, 2011; but see Cuthill & Charleston, 2012). However, clear predictions of genetic architectures resulting from different adaptive scenarios are lacking.

Is evolution ‘predictable’?

Mimetic warning patterns are a classic example of convergent evolution, where similar adaptive traits appear in distantly related taxa. For example, phenotypic divergence *within* both *H. erato* and *H. melpomene* has led to near-perfect convergence in warning pattern *between* geographic races of these two species, whose lineages separated ~12 mya (Fig. 1). Although very similar mimicry switches in the two lineages map to homologous genomic regions (Fig. 4), there is no evidence for sequence homology at the *cis*-regulatory regions involved in mimicry so far studied (Supple *et al.*, 2013). Thus, the evidence points towards independent evolution of the colour patterns between the lineages, albeit using similar genetic machinery. The repeated use of specific genes has been frequently observed in convergent evolution (Mundy, 2005; Coyle *et al.*, 2007), even among distantly related taxa (Arendt & Reznick, 2008). Particular features of some genes, for example their position in regulatory networks, may make them repeated targets of natural selection, and so in this sense ‘predictable’ (Stern & Orgogozo, 2009). This seems to be the case in *Heliconius*, where the recurrent evolution of mimetic phenotypes has largely been driven by evolutionary change in the same set of ‘toolkit’ genes (Fig. 4). Many of the loci that control convergent wing-pattern elements in distantly related species map to similar positions in the genome (Joron *et al.*, 2006; Baxter *et al.*, 2008; Reed *et al.*, 2011), although phylogenetic analyses imply the causative mutations are often independently derived (Hines *et al.*, 2011). Notably, the *P* locus ‘supergene’, controlling essentially all colour-pattern variation in *H. numata*, maps to the same genomic region as one of these major switch genes in both *H. erato* and *H. melpomene*, where it controls the expression of (at least superficially) very

different colour-pattern elements (Joron *et al.*, 2006). Remarkably, the homologous genomic region in *Biston betularia* (the peppered moth) controls the switch between *carbonaria* and *typica* morphs (Van't Hof *et al.*, 2013; see Gallant *et al.*, 2014, for another example). It appears that these genes are responsible for both convergent and divergent wing-pattern phenotypes not only within *Heliconius*, but also across the Lepidoptera.

Candidate genes underlying colour-pattern shifts have been identified from patterns of gene expression. Microarray, immunohistochemistry and *in situ* hybridization data have shown that expression of a homologue to the *Drosophila* homeotic gene *optix* perfectly prefigures areas of the wing fated to be red across the *Heliconius* radiation (Reed *et al.*, 2011; Martin *et al.*, 2014). A second unlinked gene, *WntA*, is associated with variation in the size and position of melanic 'shutter' elements, which often define the shape of yellow and white forewing areas (Martin *et al.*, 2012). To date, however, functional evidence is restricted to *WntA*, obtained by heparin injection, which affects the *wnt* signalling pathway (Martin *et al.*, 2012). A lack of experimental methods for full functional verification remains a major challenge for *Heliconius* research. Nonetheless, population data can assist with our inference of function. Thousands of generations of recombination in contact zones between geographic races have facilitated the identification of narrow genomic regions of high genetic divergence that control variation between distinct colour-pattern forms (Nadeau *et al.*, 2012; Nadeau *et al.*, 2013; Supple *et al.*, 2013; Nadeau *et al.*, 2014; Martin *et al.*, 2013). Strikingly, the same ~100-kb region of divergence has been found between races of both *H. melpomene* and *H. erato* within the mapped locus controlling red-colour-pattern variation (*H. melpomene* B/D and *H. erato* D). This genomic window does not contain coding sequences demonstrating that *cis*-regulatory changes (i.e. those which control the expression of adjacent genes) within this region control *optix* expression to produce convergent phenotypes in both species.

Genes associated with wing-pattern variation in *Heliconius* are known to play important roles in other aspects of development. For example, *optix* is known to function in eye and neural development in *Drosophila* (Seimiya & Gehring, 2000) and is expressed in the optic lobe and medulla of pupal *Heliconius* (Martin *et al.*, 2014). Coding sequence evolution in such genes is likely constrained, perhaps explaining why regulatory changes are so important in colour-pattern evolution. Precise changes in tissue-specific expression avoid negative pleiotropic effects, essentially disassociating multiple developmental roles. This regulatory subfunctionalization is expected to manifest as discrete enhancer modules within *cis*-regulatory regions. We now have evidence for three regulatory modules in the B/D region of *optix* from comparative sequence analysis of multiple *Heliconius* taxa (R.W.R. Wallbank, S.W. Baxter,

C. Pardo-Diaz, J.J. Hanly, S.H. Martin, J. Mallet, K. Dasmahapatra, C. Salazar, M. Joron, N. Nadeau, W.O. McMillan & C.D. Jiggins, submitted). These appear to be specifically associated with different expression patterns of *optix* resulting in three distinct red pattern elements on the wing (rays, dennis and band – see Fig. 4). The modular nature of these enhancers means that they can be combined to produce considerable phenotypic diversity (see Fig. 4b for examples).

The position of *optix* in the wing-patterning gene network enhances its potential as a target for wing-pattern evolution in two ways. First, *optix* lies downstream of numerous genes whose spatial expression orientates wing development. These wing 'pre-patterning' factors are involved in anteroposterior, dorsoventral and mediolateral axes, vein and scale differentiation, margin determination, etc. and can theoretically be exploited in numerous combinations to drive *optix* expression in any part of the wing, providing great scope for the evolution of different patterns. Second, *optix* controls a battery of downstream genes required to produce a multicomponent structure. These include pigment enzymes such as *cinnabar*, but must also include scale structural factors such as *actins*. Scanning electron microscopy and damage-inducing experiments have illustrated that colour pattern is developmentally coordinated with scale ultrastructure (Gilbert *et al.*, 1988; Janssen *et al.*, 2001), showing that colour-pattern genes regulate downstream processes controlling both scale pigment and structure. Hence, changes in the expression of a single gene, *optix*, are sufficient to generate variation in a functioning multicomponent structure, in this case pigmented wing scales.

These findings in *Heliconius* reinforce those from other systems that particular genes tend to be repeatedly targeted by natural selection (Stern & Orgogozo, 2009; Martin & Orgogozo, 2013). This clearly demonstrates a surprising degree of predictability in genetic architecture. However, in most of these systems, including *Heliconius*, the phenotypic differences studied were known to result from changes in major-effect loci. This has had major benefits by making both the ecological and genetic foundations of adaptive traits tractable. Other traits with a more polygenic architecture may show less genetic parallelism (Hoekstra & Coyne, 2007; Stern & Orgogozo, 2009; Nadeau & Jiggins, 2010). Nevertheless, in *Heliconius* there is clearly a restricted set of loci capable of producing major phenotypic switches of ecological importance. *Heliconius* wing-pattern phenotypes evolve through multiple mutations at a small handful of loci.

Understanding adaptation within its ecological context

In addition to warning coloration, many adaptations are associated with the diversification of *Heliconius*, including behavioural and sensory changes. Microhabitat

use is related to numerous factors including dietary ecology and reproduction. Compared to most tropical taxa, these aspects of *Heliconius* have received considerable attention through ecological studies (Brown, 1981). More recently, genomic techniques have begun to provide tools that complement earlier ecological and behavioural studies. For example, recent work has characterized gene families such as the immune system genes, *Hox* genes, chemosensory proteins, or the genes involved in the detoxification of host defences (The *Heliconius* Genome Consortium, 2012; Briscoe *et al.*, 2013; Chauhan *et al.*, 2013). Variation in the evolution of gene families may indicate morphological, physiological and metabolic differences between related species that are outwardly subtle and have so far gone undetected. However, the challenge is to link genotype and phenotype for these traits and integrate genomic studies with ongoing ecological research.

What is a *Heliconius* niche?

Aside from mimicry, two relationships are central to *Heliconius* biology each of which likely involved bouts of diffuse coevolution: first, their reliance on *Passiflora* as larval host plants; and second, their reliance as adults on resources that are obtained by systematically collecting pollen (an adaptation unique to *Heliconius*) (Gilbert, 1972). In response to herbivory, *Passiflora* have evolved a high diversity of cyanogenic compounds (Spencer, 1988; Engler *et al.*, 2000; Engler-Chauat & Gilbert, 2007; Hay-Roe & Nation, 2007). *Heliconius* larvae not only detoxify these cyanogens, but can also disable the plant β -glucosidase enzymes to prevent further release of cyanide, and sequester them for their own defence as adults. Other *Passiflora* defences include trichomes to disrupt larval locomotion and feeding (Gilbert, 1971), structures that mimic butterfly eggs to deter *Heliconius* oviposition (Williams & Gilbert, 1981; Gilbert, 1982), variable leaf morphology to disrupt visual searching by gravid females (Gilbert, 1982), and extrafloral nectar production attracting larval predators, in particular ants (Smiley, 1985, 1986). In contrast, relationships with adult food plants are generally mutualistic. Preferred pollen sources, notably *Gurania* and *Psiguria*, are those that provide reliable food stations for the duration of an individual's lifespan (Gilbert, 1975). Their exploitation heavily influences individual fitness as pollen feeding supports a prolonged reproductive lifespan (Gilbert, 1972; Dunlap-Pianka *et al.*, 1977; O'Brien *et al.*, 2003), as well as the maintenance of chemical defences (Gilbert, 1972; Dunlap-Pianka *et al.*, 1977; O'Brien *et al.*, 2003; Cardoso & Gilbert, 2013). Members of the *Gurania* (~40 species) and, in particular, the *Psiguria* (~16 species) show many adaptations to attract *Heliconius* (Gilbert, 1975; Murawski & Gilbert, 1986; Condon & Gilbert, 1988). For example, the inflorescence of

P. warscewiczii produces just one flower daily over a period of several months, perfectly adapted for pollination by long-lived *Heliconius*, which learn the location and return daily.

Sensory adaptation and phenotypic diversification

The evolution of animal signals will be shaped by the environment through which the signal is transmitted and the receivers' sensory abilities. Signal receivers may include both con- and heterospecific individuals, as well as individuals at different life stages and of either sex, resulting in complex fitness trade-offs. To fully understand how signalling trade-offs are resolved in *Heliconius*, we would like to know how these butterflies perceive their environment and how their perception differs from their predators. *Heliconius* have perhaps the largest head of any Neotropical butterfly genus, predominantly due to an investment in visual neuropile (Gilbert, 1975). Visual sensitivity is shaped by an organism's ecology (Stevens, 2013), and vision in Hymenoptera, for example, is tuned to maximize perception of variation in flower colour (Chittka & Menzel, 1992). We expect *Heliconius* vision to be similarly optimized. *Heliconius* discriminate visual cues including both shape (Gilbert, 1982; Corrêa *et al.*, 2001) and colour (Swihart & Swihart, 1970; Swihart, 1972). Four opsins – light-sensitive proteins – have been identified in the compound eye of *H. erato*, with sensitivity peaks at ~355 nm (ultraviolet 1), ~398 nm (ultraviolet 2), ~470 nm (blue) and ~555 nm (longwave) (Zaccardi *et al.*, 2006; Briscoe *et al.*, 2010; Yuan *et al.*, 2010). Red filtering pigments in the ommatidia and retina further increase the range of colour discrimination by changing receptor sensitivity (Zaccardi *et al.*, 2006).

These visual adaptations may have evolved for intraspecific communication related to the evolution of mimicry (Bybee *et al.*, 2012). As *Heliconius* butterflies have strong aposematic colour signals serving both predation avoidance and mate recognition, they may be selected to use channels of communication that are not detected by predators (Bybee *et al.*, 2012; Llaurens *et al.*, 2014). In contrast to other Lepidoptera that only have one, the two *Heliconius* opsins in the UV range confer a greater sensitivity to UV reflectance (Briscoe *et al.*, 2010; Bybee *et al.*, 2012). *Heliconius* yellow wing pigments appear to have co-evolved with the additional UV opsin, as they have a much higher UV reflectance than yellow pigments in other Lepidoptera (Briscoe *et al.*, 2010; Llaurens *et al.*, 2014). Models of animal vision suggest that birds are less effective in discriminating these UV-yellows as compared to the butterflies, consistent with this being a cryptic channel of communication for *Heliconius* mate-finding that has evolved to compensate for similarities due to mimicry (Bybee *et al.*, 2012). However, the key experimental question is whether *Heliconius* can discriminate different

species-specific UV signals and therefore avoid interspecific mating and hybridization, and this remains to be demonstrated.

Does behavioural plasticity facilitate ecological adaptation and diversification?

The diversification of *Heliconius* is often associated with concordant shifts in colour pattern and habitat use (e.g. Mallet, 1993; Estrada & Jiggins, 2002; Arias *et al.*, 2008). The exploitation of novel environments may require secondary adaptations, but can be facilitated in the short term by behavioural plasticity (West-Eberhard, 2003; Pfennig *et al.*, 2010; Dukas, 2013; Lister, 2013; Snell-Rood, 2013). Behavioural flexibility may further accelerate divergence by exposing organisms to new selection regimes. In other taxa, behavioural plasticity has been linked to range and niche expansion (Sol *et al.*, 2008; Gonda *et al.*, 2009), variation in host use (Snell-Rood & Papaj, 2009; Nylin *et al.*, 2014) and mate choice (Svensson *et al.*, 2010; Westerman *et al.*, 2012).

Indirect evidence for the importance of learning in *Heliconius* ecology comes from the apparent expansion of the mushroom bodies in the central brain of *Heliconius* (Fig. 5; Sivinski, 1989; Montgomery *et al.*, 2015), which have repeatedly been associated with learning in other insects (Zars, 2000; Farris, 2005; Snell-Rood *et al.*, 2009). Indeed, these structures are larger (in both relative and absolute terms) in *Heliconius* than in any other Lepidoptera surveyed (Sivinski, 1989; Montgomery *et al.*, 2015). Patterns of interspecific variation and intraspecific plasticity in Hymenoptera strongly link mushroom body expansion with spatial learning in a foraging context (Withers *et al.*, 1993; Farris & Schulmeister, 2011). In *Heliconius*, the maturation of the mushroom bodies shows strong experience-dependent

effects further supporting their role in learning (Montgomery *et al.*, 2015).

Mark–release–recapture studies have demonstrated temporally and spatially faithful trap-lining behaviour in *Heliconius*, and an effect of experience on foraging efficiency and host plant visitation (Gilbert, 1975; Mallet, 1986; Murawski & Gilbert, 1986; Finkbeiner *et al.*, 2012). These data imply that *Heliconius* invest heavily in the capacity to learn distributed resources, an adaptation that facilitates efficient foraging in the complex tropical rainforest environment. *Heliconius* are also able to associate information with colour and shape (Swihart & Swihart, 1970; Gilbert, 1975), which may permit a flexible response to identifying preferred host plants or pollen resources. For the very few *Heliconius* taxa where data exist, shifts in host use appear innate (Kerpel & Moreira, 2005; Salcedo, 2011; Merrill *et al.*, 2013).

However, regions of the brain involved in primary processing of visual and olfactory information also show evidence of developmental plasticity (Montgomery *et al.*, 2015). These structures are associated with olfactory and visual learning in other taxa (Hammer & Menzel, 1998; Paulk *et al.*, 2009), and their relative size co-evolves with microhabitat and diel pattern (Montgomery & Ott, 2015). Whether behavioural plasticity facilitates diversification in *Heliconius* for now remains an open question. Nevertheless, the evidence available suggests that behavioural flexibility plays a key role in the ecology of these butterflies and may well promote shifts in habitat use.

From reproductive behaviour to reproductive isolation

Shifts in behaviour will directly influence the evolution of diversity if they result in reproductive isolation.

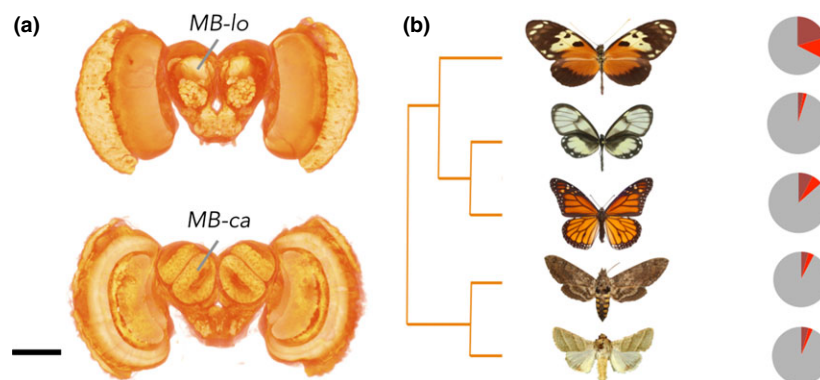


Fig. 5 Expansion of mushroom bodies in *Heliconius* butterflies. (a) 3D surface rendering of the brain of *H. hecale* (shown left, scale = 25 mm) viewed from the anterior (top) and posterior (bottom) indicating the mushroom body lobes (MB-lo) and calyx (MB-ca). Scale = 500 μ m. (b) Pie charts show the proportion of the midbrain occupied by MB-calyx (dark red) and MB-lobe+peduncles (light red) in *H. hecale*, *G. zavaleta*, *D. plexippus*, *M. sexta* and *H. virescens* (top to bottom).

Research concerning reproductive behaviours in *Heliconius* has focused on male attraction towards different female phenotypes (see Fig. 6 for the general progression of adult mating in *Heliconius*). This attention may seem misplaced as females are normally considered the more choosy sex, due to their greater investment in reproductive effort (Andersson, 1994). However, male butterflies transfer a large nutrient-rich spermatophore during mating, and in *Heliconius*, this has been demonstrated to represent a significant contribution to female reproduction (Boggs & Gilbert, 1979; Boggs, 1981, 1990). Nevertheless, despite its apparent benefits, remating appears to be a relatively uncommon event for *Heliconius* females, especially considering their extended reproductive lifespan (Boggs, 1990; Walters *et al.*, 2012). Even among polyandrous species, analyses of spermatophore counts and wing wear (as an indicator of age) suggest that the handful of mating events are temporally spaced (Walters *et al.*, 2012). Low remating rates are mediated by *anti-aphrodisiac* pheromones, which are delivered by males during mating and serve to repel subsequent suitors (Gilbert, 1976; Schulz *et al.*, 2008; Estrada *et al.*, 2011). Forced copulation in (adult) butterflies is rarely an option (Forsberg & Wiklund, 1989), and pollen feeding by *Heliconius* adults, in addition to increased vulnerability to predation during copulation, may render any benefits of multiple mating marginal. These anti-aphrodisiacs may then act as honest signals of female receptivity (Estrada *et al.*, 2011).

Whatever the underlying cause, infrequent mating by female *Heliconius* is expected to result in male–male competition for mates (Andersson, 1994). Intense competition perhaps reaches its peak in the *pupal mating* behaviours observed in species of the *erato* and *sapho* clades (Beltran *et al.*, 2007), where males mate with freshly eclosed, or even yet to eclose, females (Gilbert, 1976; Deinert *et al.*, 1994). However, the occurrence of

pupal mating seems to vary considerably between species within the *erato* and *sapho* clades (e.g. McMillan *et al.*, 1997), and its frequency in the wild remains largely unknown (but see Longino, 1984). This is an important parameter for understanding the extent to which females can exert choice in these species. More broadly, sexual selection is expected to drive the evolution of male searching behaviours that maximize the chance of securing receptive females. This, alongside male investment in the form of a nutrient-rich spermatophore, likely underlies the importance that male attraction appears to play in reproductive isolation.

A kind of magic

Speciation with gene flow is greatly facilitated if traits under divergent ecological selection also contribute to nonrandom mating (Gavrilets, 2004; Weissing *et al.*, 2011). These so-called *magic traits* (Gavrilets, 2004; Servedio *et al.*, 2011) evade the homogenizing effects of recombination, which impede the evolution of behavioural isolation when gene flow persists (Felsenstein, 1981). Although the epithet ‘magic’ was perhaps intended to suggest these types of trait were rare in nature, accumulating evidence suggests that this might not be the case (Servedio *et al.*, 2011). However, finding convincing examples has proved difficult because different traits can be strongly correlated making it difficult to distinguish their individual effects, especially with respect to ecological selection. Servedio *et al.* (2011) propose two experimental criteria: first, the putative magic trait, rather than any other correlated trait, must be subject to divergent selection; and second, the same trait, not a correlated trait, must generate nonrandom mating. The amenability of *Heliconius* colour patterns to experimental manipulation has provided an excellent opportunity to test their role in

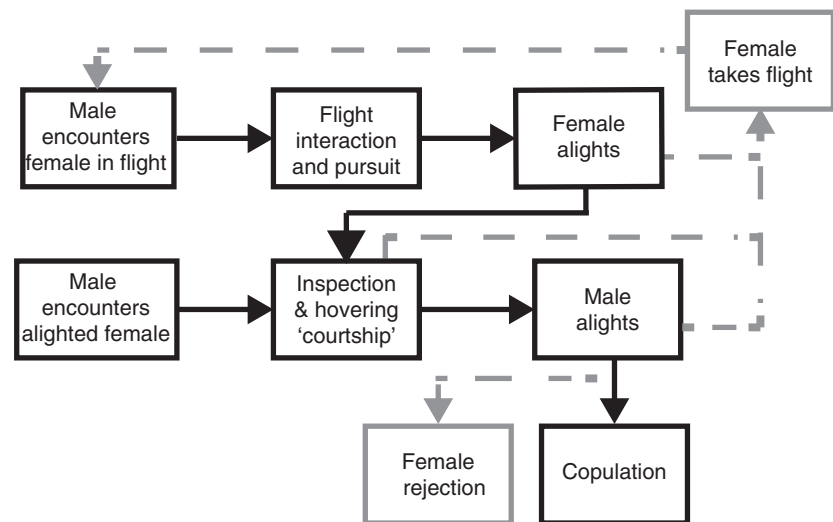


Fig. 6 Progression of adult mating in *Heliconius* butterflies (simplified from Klein & de Araújo, 2010).

reproductive isolation, and it has been argued that *Heliconius* provide the strongest empirical support for 'magic traits' (Servedio *et al.*, 2011). Specifically, experiments using paper models of *H. cydno* and *H. melpomene* and their first-generation hybrids, in addition to experiments with live butterflies and captive birds, reveal that colour pattern is under strong disruptive selection due to predation (Merrill *et al.*, 2012). In the same species, Jiggins *et al.* (2001a) have shown that individuals prefer to mate with live butterflies and court paper models of the same colour pattern. Thus, in contrast to other potential examples of magic traits, both parts of the required evidence are present.

The fact that a combination of colour (hue) and movement stimulates courtship by *Heliconius* males was demonstrated as early as the 1950s (Crane, 1955). In a battery of subsequent insectary experiments involving fluttering models, made from either dissected female wings or printed paper butterflies (Jiggins *et al.*, 2001a, 2004; Kronforst *et al.*, 2006c; Estrada & Jiggins, 2008; Melo *et al.*, 2009; Muñoz *et al.*, 2010; Merrill *et al.*, 2011b, 2014), males almost invariably show a preference for their own wing pattern over that of conspecific races or other closely related species. Data from the wild are more limited (but see Chamberlain *et al.*, 2009). However, assortative mating has been shown between *Heliconius himera* and *H. erato*, which share a narrow hybrid zone (~5 km) in southern Ecuador (Mallet *et al.*, 1998b). Given male reproductive investment, selection against poorly adapted hybrids may contribute to divergent male 'preferences' through a reinforcement-like mechanism. Nonmimetic hybrids suffer increased predation (Merrill *et al.*, 2012), as well as reduced mating success (Naisbit *et al.*, 2001) and fertility (Jiggins *et al.*, 2001b; Naisbit *et al.*, 2002; Salazar *et al.*, 2005; Muñoz *et al.*, 2010), and populations not in contact with closely related taxa are less choosy (Jiggins *et al.*, 2001a; Kronforst *et al.*, 2007). However, divergent preferences are also observed between taxa where reinforcement seems unlikely (Jiggins *et al.*, 2004). The ability to visually locate potential mates at long distances may be a considerable advantage, and it seems likely that competition between males for mate location drives local preferences.

It is perhaps often overlooked that the existence of magic traits does not make speciation automatic or inevitable. Shifts in colour pattern must be accompanied by their corresponding mate preferences. In contrast to other *Heliconius* behaviours, learning appears unimportant for developing mate preferences – although these experiments are by no means exhaustive. Neither isolating males from conspecific colour patterns (either in other butterflies or on themselves) nor exposure to females of a different wing-pattern race for several days prior to testing affects courtship response (Crane, 1955; Jiggins *et al.*, 2004; Merrill *et al.*, 2011b). As offspring inherit alleles underlying both

preference and signal, a positive genetic correlation between preference and signalling loci is expected (Fisher, 1930); however, this association may be unstable if assortative mating is weak or incomplete, as might be expected during the early stages of divergence. We now know from genetic crosses that the red/white forewing switch gene distinguishing *H. melpomene* and *H. cydno* (*B* locus), as well as the white/yellow forewing switch gene distinguishing *H. cydno* and *H. pacheus* and different morphs of the polymorphic *H. cydno alithea* (*K* locus), is physically associated with major loci underlying the corresponding male preferences (Fig. 4) (Kronforst *et al.*, 2006c; Chamberlain *et al.*, 2009; Merrill *et al.*, 2011b). Whether these associations reflect pleiotropy or tight physical linkage between pattern and preference loci remains to be seen. Whatever the cause, they generate robust genetic associations that impede recombination between wing pattern and preference loci, further facilitating the evolution of reproductive isolation. These results additionally suggest a mechanism by which the introgression of colour-pattern elements, linked to the corresponding preference alleles, would directly lead to assortative mating and offer a route towards rapid hybrid speciation. Several putative examples of hybrid species in *Heliconius* are proposed to have derived from interbreeding between *H. cydno* and *H. melpomene*, and notably, the forewing of the best documented example, the Colombian species *Heliconius heurippa* (Mavárez *et al.*, 2006), involves both red and yellow pattern elements (controlled by the *B* and *N* loci, respectively). Indeed, *heurippa*-like males reconstructed by backcrossing F1 hybrids into *H. cydno* are more likely to approach and court models of their own colour pattern than either of the parental species (Melo *et al.*, 2009), showing that hybridization can very rapidly lead to premating isolation.

Are multiple components of reproductive isolation necessary to complete speciation?

The number of traits subject to divergent selection can influence speciation (Nosil *et al.*, 2009). Studies of experimental evolution in *Drosophila* suggest that in contrast to multifarious trait scenarios, selection on a single trait will typically lead only to incomplete reproductive isolation (Rice & Hostert, 1993). Similarly, in *Timema* walking sticks and *Rhagoletis* flies, the degree of reproductive isolation appears to correlate with the number of traits subject to divergent selection (Dambroski & Feder, 2007; Nosil & Sandoval, 2008). In *Heliconius*, potential isolating barriers have been considered across a range of taxon pairs and, in general support of the multifarious hypothesis, strong selection on a single trait (colour pattern) does not seem sufficient to complete speciation. Additional barriers, including additional components of sexual isolation, ecological differences (Jiggins *et al.*, 1997; Estrada & Jiggins, 2002;

Merrill *et al.*, 2013) and intrinsic post-mating barriers (Jiggins *et al.*, 2001b; Naisbit *et al.*, 2002; Salazar *et al.*, 2005; Muñoz *et al.*, 2010), clearly contribute to reproductive isolation in *Heliconius*. Indeed, the recent discovery of 'cryptic' taxa, such as *H. melpomene malleti* and *H. timareta florenci*, which show strong assortative mating despite almost indistinguishable colour patterns, necessitates this (Giraldo *et al.*, 2008; Mérot *et al.*, 2013).

An obvious, but to date largely unexplored, reproductive barrier is divergence in chemical signals. Estrada and Jiggins (Estrada & Jiggins, 2008) report that *H. erato* males can distinguish between wings dissected from conspecific and heterospecific (although comimetic *H. melpomene*) females, but that this effect disappears after wings have been washed in hexane. In addition, comimetic populations of *H. melpomene* and *H. timareta* share wing patterns but show strong premating isolation, presumably mediated by chemical cues (Giraldo *et al.*, 2008; Mérot *et al.*, 2013). Nonetheless, the chemical signals used have yet to be identified. Indeed, that female choice (in general) contributes to reproductive isolation between *Heliconius* taxa has only been shown very indirectly. In choice trials, the mating probability of female backcross hybrids between the sympatric species *H. melpomene* and *H. cydno* segregates with a major colour-pattern locus (Merrill *et al.*, 2011b). These results suggest that a locus underlying female rejection segregates with colour pattern, although the cues involved remain elusive. Overall, studies of chemical signalling in *Heliconius* are in their early stages as compared to those of visual signalling.

Conclusions

Since Bates (1862) first described mimicry, *Heliconius* butterflies have been the subject of over 700 peer-reviewed publications (<http://www.heliconius.org/publications/>). As a result, mimicry in tropical butterflies has become one of the most intriguing and powerful examples of natural selection. However, this vast body of work has yielded results beyond an ever more detailed description of a single phenomenon. Recent genomic studies, for example, challenge the conventional view of species and how they evolve. In particular, porous species boundaries may have had important evolutionary consequences by permitting the movement of adaptive alleles across the species boundary. Studies of warning patterns themselves, from analyses of differential predation to the fine-scale dissection of causative mutations, have begun to address the predictability of the genetic architecture of adaptation. Both traditional ecological and genomic studies concerning a suite of ecological adaptations, of which colour pattern is just one, allow a more holistic understanding of diversity. Finally, *Heliconius* have provided insights into how different types of selection may act, and interact,

on morphological and behavioural traits and how this can influence the evolution of reproductive isolation.

In writing this review, our aim was explicitly not to argue that *Heliconius* is a superior 'model' of evolutionary processes. Rather, we hope to present an overview of research into this genus and highlight the insights it has provided into the evolution of diversity. Research on *Heliconius* butterflies has addressed a number of evolutionary questions, but there are many to which it is not suited. Frustratingly, there remain a number of questions that we cannot currently (and may never) answer. However, research into *Heliconius* has a long and full history, the community of *Heliconius* researchers is growing, and studies of the genus are of increasing breadth. As a result, we believe research into these butterflies has made a substantial contribution to evolutionary biology. We suspect that this would be true of any genus studied in such detail, but nevertheless, and after 150 years, *Heliconius* research remains an exciting endeavour.

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Conflict of interests

The authors declare no conflict of interests.

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