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## Wing venation and Distal-less expression in *Heliconius* butterfly wing pattern development

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**Abstract** Here we show that major color pattern elements of *Heliconius* butterfly wings develop independently of wing venation. We recovered a hybrid *Heliconius* displaying a mutant phenotype with a severe vein deficiency. Although this butterfly lacked most of its wing veins, the large, melanic banding patterns typical of the genus were conserved across the entire wing. The only obvious correlation between vein reduction and pigment patterns was a loss of vein-associated melanin stripes near the distal margin of the wings. We examined the expression of the eyespot-associated transcription factor Distal-less in a banded and a spotted species of *Heliconius* and found no obvious relationship between protein expression and the band or spot patterns typical of the genus. Together, our results suggest that the melanic bands and spots in *Heliconius* are unlikely to be derived from an eyespot determination system. We propose that major elements of *Heliconius* wing pattern formation are based primarily on a complex, whole-wing proximodistal axis system.

**Keywords** Eyespot · Evolution · Nymphalid ground plan · Mimicry · Development

### Introduction

Many butterfly wing pattern elements have boundaries or symmetries related to vein positions; however, the relationship between vein development and the formation of many specific color pattern elements remains an open question. For this report, we took advantage of a wing vein mutation to assess the role of wing venation in the development of wing patterns characteristic of the nymphalid genus *Heliconius*. We also examined expression of Distal-less, a protein associated with wing pattern development, to consider the developmental and evolutionary nature of some *Heliconius* wing pattern elements.

Wing vein precursors, also known as proveins, appear to play at least four roles in color pattern determination: dislocation, induction, disruption, and intervenous organization (Fig. 1). Dislocation occurs when prepatterns that are parallel to the wing margin lose their continuous quality due to spatial fragmentation by proveins. A good example of this phenomenon comes from the *Papilio xuthus veins-reduced* mutant, where the loss of wing veins results in normally dislocated marginal band patterns merging into continuous bands running parallel to the wing margin (Koch and Nijhout 2002). Various models of the dislocation effect have been previously proposed (Koch and Nijhout 2002; Nijhout 1991; Schwanwitsch 1925). It has been hypothesized that dislocation may be due to proveins acting as barriers to a morphogen originating from the wing margin (Koch and Nijhout 2002).

Inductive signals that originate from proveins themselves are likely required for determination of venous color patterns. Venous patterns typically have lines of symmetry along wing veins, although their widths along the proximodistal axis may vary dramatically. Evidence for vein-dependent pattern induction comes again from the *P. xuthus veins-reduced* mutant, where the loss of venous stripes is correlated with the loss of wing veins (Koch and Nijhout 2002).

Another function of proveins is that of pattern disruption. With this effect, pattern elements running parallel to

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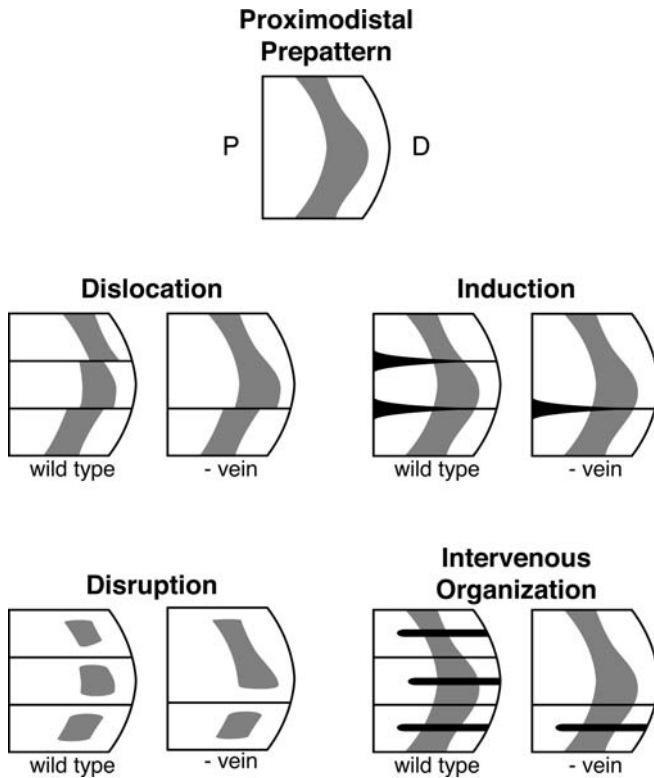
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**Fig. 1** Effects of vein development on determination of butterfly wing pigment patterns. Nascent wing veins can induce patterns or alter prepatterns through dislocation, induction, disruption, or intervenous organization effects. Each effect is summarized in the context of a hypothetical proximodistal prepattern and also in the context of a loss-of-vein mutation

the wing margin are subdivided and locally altered by provein-dependent processes. One particularly illustrative example of the disruption effect may be seen in the *Bicyclus anynana Cyclops* mutant, where the loss of a wing vein causes two adjacent eyespots to merge into a single continuous pattern that is anteroposteriorly elongated (Brakefield et al. 1996). In the case of *Cyclops*, larval expression of the eyespot-associated transcription factor Distal-less was elongated across the position of the missing wing vein in a manner resembling the final eyespot color pattern. This implies that the disruption effect occurs during prepattern formation, prior to diffusion of the eyespot morphogen. Other examples of disruption have been described from *Speyeria aphrodite*, *Battus philenor*, and *Papilio polymnestor* (Nijhout 1991). It has been proposed that disruption may be due to developing veins acting as morphogen sinks (Nijhout 1991).

Intervenous organization is a less well-understood function of proveins in color pattern determination. The key examples of this effect are the *intervenous* pattern elements, which are proximodistally elongate patterns that occur halfway between wing veins (Schwanwitsch 1924). The *B. anynana Cyclops* mutant, which lacks a longitudinal wing vein, shows a corresponding loss of Distal-less intervenous midline expression in the affected area (Brakefield et al. 1996). This result, while not entirely

conclusive, may be interpreted as demonstrating the dependence of intervenous prepatterns on the presence of appropriately spaced longitudinal veins. A mutant *Papilio (Druryeia) antimachus*, with an extra cross-vein, shows a disruption of an intervenous midline pigment pattern at the site of the cross-vein (Nijhout 1991), providing further evidence that proveins provide positional information for intervenous patterning.

Superficially, disruption and intervenous organization effects can be difficult to distinguish without the benefit of vein mutants or molecular pre patterning markers. Developmentally, however, these two processes are quite different. Disruption alters the expression of proximodistal patterns, while intervenous organization can apparently establish pattern elements independently of the proximodistal axis system.

Butterfly wing vein mutants are exceedingly rare, yet can be highly informative about the processes underlying color pattern determination. We were fortunate to recover a *Heliconius* mutant lacking most of its wing venation. This mutant allowed us the opportunity to assess the role of vein development in the formation of specific *Heliconius* color pattern elements. Comparative analysis of Distal-less expression patterns further allowed us to address previous models of color pattern formation and homology in the genus. The data we present here provide evidence for a whole-wing proximodistal pattern-formation system of a complexity unprecedented in insects. This discovery is of general significance for understanding the development and evolution of lepidopteron wing patterns.

## Materials and methods

### Mutant isolation

The wing vein deficient butterfly that we recovered was a spontaneous mutant isolated from a *Heliconius cydno* × *Heliconius pacheus* backcross to *H. pacheus* in a greenhouse colony at the University of Texas, Austin. The individual was spread, mounted, and digitally photographed and is currently in the collection of L.E. Gilbert at the University of Texas, Austin. The “wild-type” hybrid shown for comparison was female number 36 from F<sub>2</sub> brood J61.

### Immunohistochemistry

Detection of Distal-less protein in fifth-instar *H. cydno* and *H. hecale* wing discs was accomplished using a previously described protocol (Brunetti et al. 2001). We utilized a rabbit polyclonal primary antibody to *Junonia (Precis) coenia* (Lepidoptera: Nymphalidae) Distal-less (Panganiban et al. 1995), and a goat anti-rabbit Cy2 secondary antibody (Jackson Laboratories). We visualized whole-mount wing discs with a confocal microscope. The staining patterns presented here were replicated in each species at least three times, and minus-primary controls

were run for the time stages presented here. Staging of imaginal discs followed Reed and Serfas (2004).

## Results and discussion

### Conservation of major proximodistal color pattern elements despite loss of wing veins

The mutant *Heliconius* we recovered showed a wing vein phenotype similar to that described as *veins-reduced* from the swallowtail butterfly *P. xuthus* (Koch and Nijhout 2002). We speculate that the vein reduction we observed was due to a genetic mutation, although we were unable to generate crosses to directly test this idea. As with *veins-reduced* in *P. xuthus*, the aberration manifested itself as a loss of branching in longitudinal veins, coupled with a severe distal truncation of the remaining veins (Fig. 2). In the forewing of the *Heliconius* mutant, the major subcosta, radius, cubitus, and anal veins occurred shortened and unbranched (Fig. 2b). The subcosta, cubitus, and anal veins were limited to the proximal half of the wing, with the radius vein extending further distally than the other veins. Also retained was the cross-vein joining the radius and cubitus. None of the veins reached the wing margin, and media veins appeared to be absent. The mutant hindwing had vein abnormalities similar to the forewing (Fig. 2b).

Despite radical differences in wing venation between wild-type and vein-deficient butterflies, the majority of the adult color pattern was conserved (Fig. 3). Of particular interest was the conservation of the melanic band patterns arrayed along the proximodistal axis of the forewing. Five major melanic pattern elements were visible on the ventral forewing (Fig. 3a–c). The most distal pattern was a black element that followed the distal curve of the wing margin. Proximal to this were two black band patterns, the proximal-most of which represented the “forewing distal

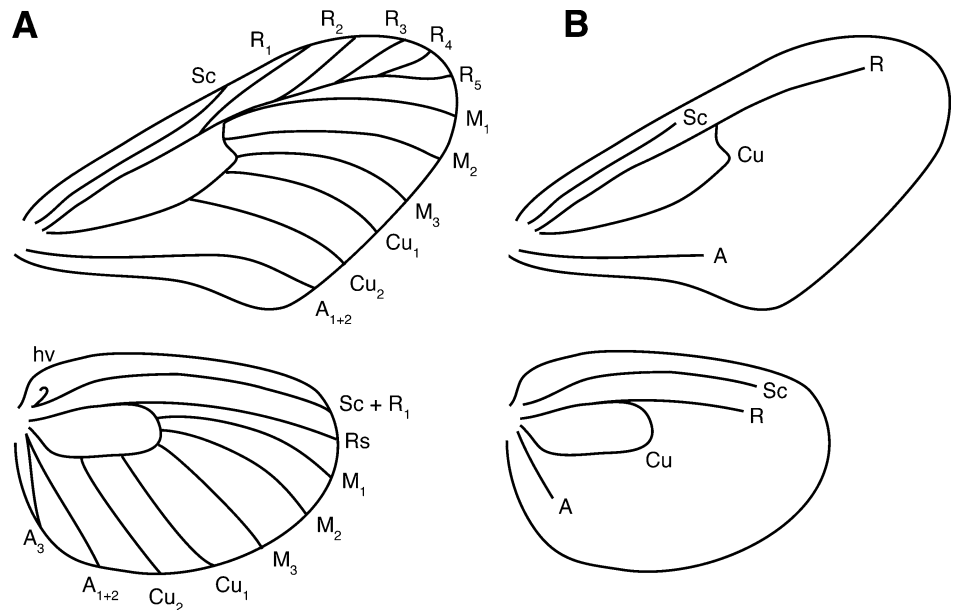
shutter” element as described by Gilbert (2003). Associated with the discal cross-vein joining the radius and cubitus was a small melanic pattern, and in the proximal third of the wing there was a large melanic region that may represent multiple overlapping semi-independent pattern elements (Gilbert 2003). The dorsal forewing pigment pattern was similar to the ventral pattern, except for that the two most distal pattern elements were not obviously distinguishable (Fig. 3e–h), possibly due to overlap of the pattern elements.

The hindwing pigment patterns of the hybrid we analyzed were simple, with the most relevant feature being a small crescent of white near the distal margin of the wing. This crescent was present on both ventral (Fig. 3a, b, d) and dorsal (Fig. 3e–g, i) wing surfaces, and likely represented a “gap” between distal and central melanin pattern elements as per the model of Gilbert (2003). Red patterns were present along the costal margin and the anal region of the proximal ventral wing surface (Fig. 3a, b). The white crescent and the red patterns were not affected in any obvious way by wing vein reduction (Fig. 3a, e, f), except for the loss of vein-associated melanin patterns transecting the crescent (see below).

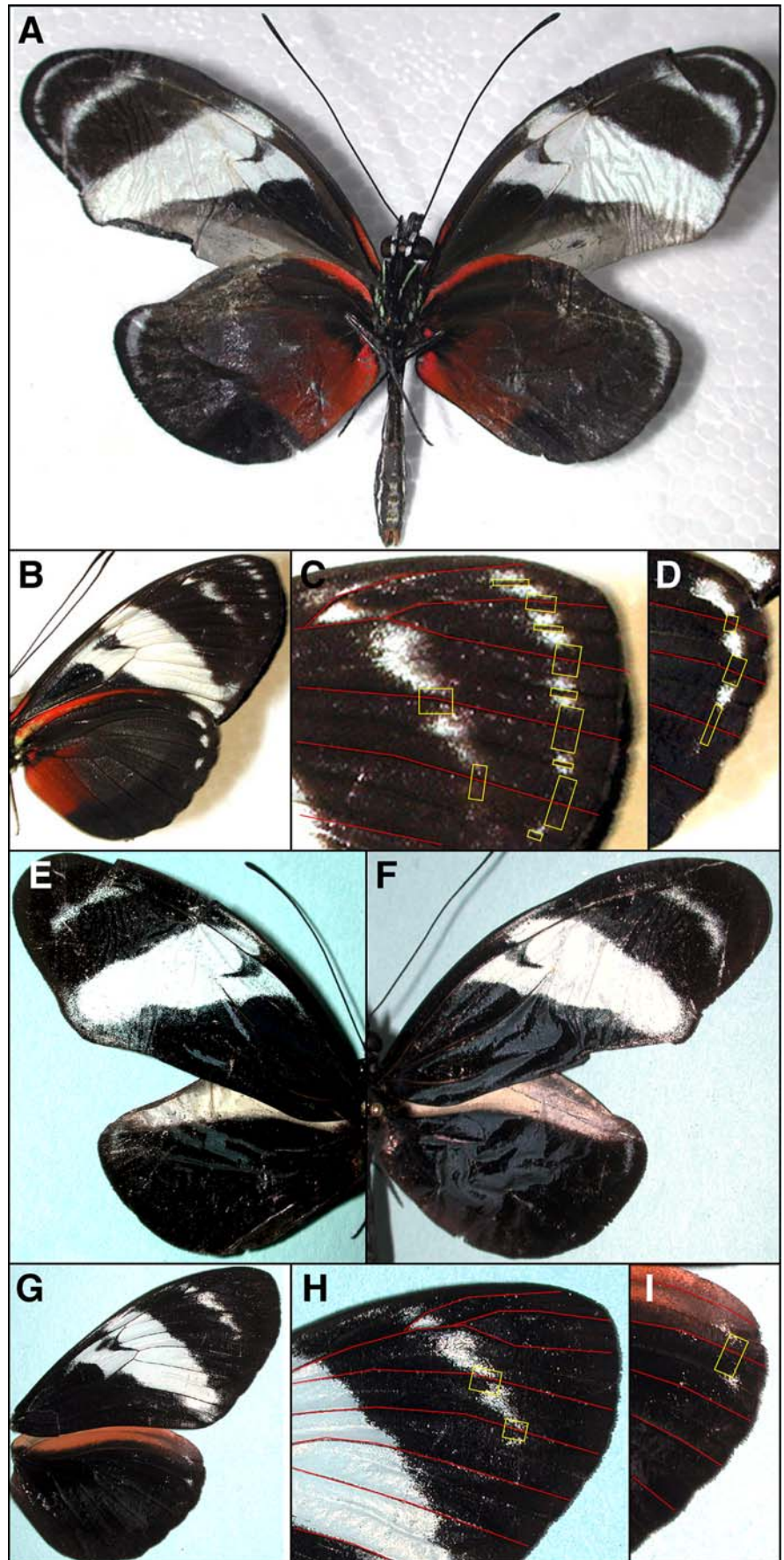
### Loss of vein-associated pigment patterns suggests a role for proveins in *Heliconius* color pattern induction and organization

Small vein-associated melanin patterns in the distal regions of all wing surfaces were absent from the mutant (Fig. 3), suggesting that the determination of these patterns is dependent on vein development. On the ventral forewings, both venous and intervein midline (Fig. 3c) patterns present in the wild type were lost from the mutant butterfly. On the ventral and dorsal hindwings (Fig. 3a, d–f, i) and the dorsal forewings (Fig. 3e–h), venous melanin patterns were lost. Furthermore, on the dorsal forewing,

**Fig. 2** Aberrant venation in a mutant hybrid *Heliconius* butterfly. The cartoon represents an individual that was the result of backcrossing a *H. pachinus* × *H. cydno* F<sub>1</sub> to *H. pachinus*. **a** Wild-type wing venation. **b** Reduced wing venation in mutant. Vein abbreviations: *Sc* subcosta, *hv* humeral vein, *R* radius, *M* media, *Cu* cubitus, *A* anal



**Fig. 3** Effect of vein reduction on *Heliconius* wing patterns. Color pattern elements lost in the mutant are marked by *yellow boxes* and wild-type wing vein positions are marked by *red lines*. **a** Ventral wing surfaces of wing vein mutant. **b** Ventral wing surfaces of a wild-type hybrid individual with intact wing veins. **c** Ventral forewing patterns found in wild-type, but lacking in mutant. **d** Ventral hindwing patterns found in wild-type, but lacking in mutant. **e** Left dorsal wing surfaces of mutant. **f** Right dorsal wing surfaces of mutant. **g** Dorsal wing surfaces of wild-type hybrid. **h** Dorsal forewing patterns found in wild-type, but lacking in mutant. **i** Dorsal hindwing patterns found in wild-type, but lacking in mutant



black lines coincident with wing veins were lost (Fig. 3e–h).

The loss of the venous patterns (Fig. 3c, d, h, i) was consistent with the vein induction model of pattern formation, while the loss of the intervein midlines (Fig. 3c) was consistent with the intervein organization model.

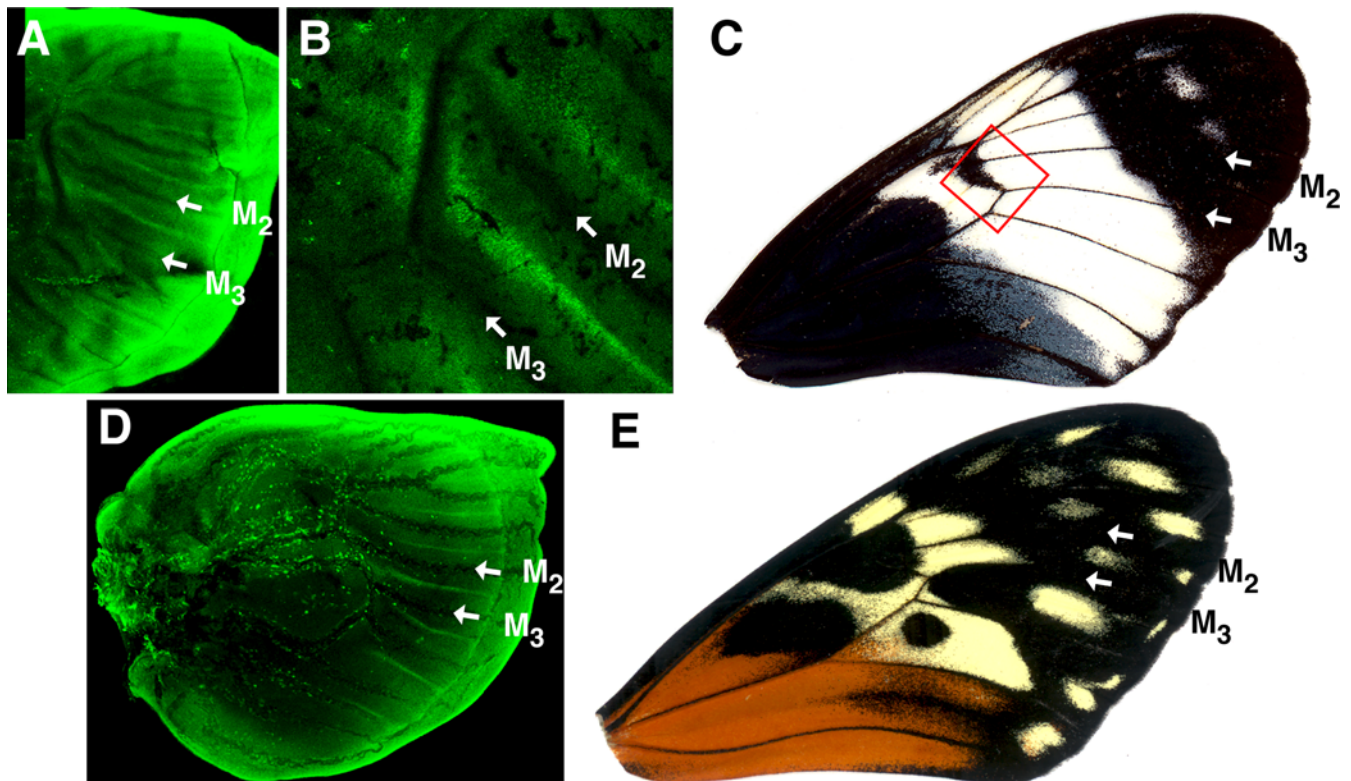
Distal-less expression in larval wing discs was not associated with melanic band or spot patterns of *Heliconius*

A major question in the study of *Heliconius* wing patterns concerns the developmental and evolutionary origins of the forewing bands. It has been proposed that forewing bands may be derived from fused “border ocelli” eyespot patterns (Nijhout and Wray 1988), or possibly from parafoveal elements (Nijhout 1991). These hypotheses were based primarily on the observation that many *Heliconius* butterflies and species from closely related genera have serially repeated black spot patterns, possibly akin to eyespots or parafoveal patterns, in place of large band patterns. To test the eyespot-homology hypothesis, we looked at the expression of Distal-less in late larval wing discs of the banded species *H. cydno* and the spot-bearing species *H. hecale*. Determination of intervein midline and

border ocelli eyespot color patterns is known to be associated with Distal-less expression across a range of butterfly species (Brakefield et al. 1996; Carroll et al. 1994; Reed and Serfas 2004), justifying our use here of Distal-less as a marker for the process underlying intervein midline and eyespot determination. However, obvious caveats regarding the use of gene expression to assess character homology may apply (Wray and Abouheif 1998). Distal-less was also a candidate for the determination of proximodistal pattern elements in general because of its evolutionarily conserved role in distal appendage specification in animals (Panganiban et al. 1997).

In late-stage *H. cydno* wing discs, at a time when eyespot foci and intervein midlines are believed to be determined in other butterfly species (Brakefield et al. 1996; Carroll et al. 1994; Reed and Serfas 2004), abundance of Distal-less protein was not obviously associated with any of the forewing band patterns (Fig. 4a), either through directly coincident expression or indirect geometrical relationships. This lack of correlation provides some evidence that band patterns may have little direct relationship with the eyespot determination network as it is currently understood.

Distal-less was most abundant in the region around the margin of the disc, and was also seen in intervein midline patterns similar to what has previously been described from *H. melpomene* (Reed and Serfas 2004). The intervein



**Fig. 4** Distal-less expression in larval wing discs of *H. cydno* and *H. hecale* has little association with adult color patterns. Positions of media 2 and 3 are marked by  $M_2$  and  $M_3$ , respectively. **a** Distal-less distribution in dorsal epithelium of a stage 3.5 *H. cydno* forewing imaginal disc. **b** Higher magnification scan of dorsal epithelial

Distal-less staining near discal vein of a stage 3.5 *H. cydno* forewing disc. **c** Dorsal forewing of adult *H. cydno*. The red box marks the region represented in **B**. **d** Distal-less distribution in dorsal epithelium of a stage 3.25 *H. hecale* forewing imaginal disc. **e** Dorsal forewing of adult *H. hecale*

midline expression of Distal-less between media 2 and 3 extended proximally to the cross-vein of the discal cell (Fig. 4b). Just proximal of this cross-vein was a small domain of Distal-less expression, possibly associated with a melanic pattern frequently found at the discal cross-vein in *Heliconius* butterflies. This association between discal Distal-less expression and pigment pattern has been previously observed in *H. melpomene* (Reed and Serfas 2004).

In late last-instar wing discs of *H. hecale*, we did not observe Distal-less expression associated in any obvious way with the color pattern (Fig. 4c), leading us to the tentative conclusion that melanin patterns of *H. hecale* are unlikely to be derived developmentally or evolutionarily from the eyespot/intervein pattern determination network. This result is similar to that from a previous study of gene expression in the spotted heliconiine *Agraulis vanillae*, which found no association between Distal-less expression and black or silver spot patterns (Reed and Serfas 2004). Pending further data, the pattern disruption effect (Fig. 1) provides a possible alternative hypothesis for how spots on heliconiine wings may be derived from band-like prepatterns.

Implications for interpreting color pattern development and evolution in *Heliconius* and other butterflies

Previous data from functional studies on *Drosophila* (True et al. 1999) and vein aberrations in butterflies (Brakefield et al. 1996; Koch and Nijhout 2002; Nijhout 1991) suggest that proveins play an integral role in the formation of most of the known insect wing pigment patterns. The vein mutant we describe here, however, demonstrates that multiple discrete color pattern elements found in butterfly wings can be determined independently of wing venation. This finding lends support to previous theoretical models suggesting that proximodistal prepatterns can be sufficient for establishing complex whole-wing color patterns in butterflies (Sekimura et al. 2000).

The color patterns found on the wings of Lepidoptera represent a significant increase in developmental complexity compared to the wings of other insects, and the origin of the proximodistal patterning system in lepidopteron wings is a subject of great interest. Further gene expression studies should be able to begin addressing the question of whether this patterning system is a novel developmental process, or if it is derived from a conserved appendage patterning module such as that implicated in leg development.

*Heliconius* butterflies have been an important model system in ecology and evolution because of their mimicry-related wing patterns. Ongoing work on wing pattern genetics in several *Heliconius* species suggests that most wing pattern polymorphisms are determined by a few genes of major effect (Gilbert 2003; Naisbit et al. 2003; Nijhout 1991; Nijhout et al. 1990; Sheppard et al. 1985). While mimicry begs the question of wing pattern gene

homology between co-mimics, *Heliconius* also presents other puzzles in respect to lepidopteron wing pattern evolution. Namely, how are the highly derived and simplified wing patterns of *Heliconius* related to the set of butterfly wing pattern homologies known as the nymphalid ground plan?

The simplicity, large size, and polymorphic nature of *Heliconius* wing patterns could present a useful gateway into understanding the developmental basis of the nymphalid ground plan if homologies could be determined. The Distal-less expression patterns in this study provide a modicum of evidence that *Heliconius* forewing bands are not related to border ocelli eyespot elements. Hopefully, more molecular markers will be developed in the future that will allow a more comprehensive assessment of pigment pattern relationships between *Heliconius* and other butterfly taxa. In addition to developing molecular markers for other pattern elements, it would also be desirable to look at gene expression in later stages of *Heliconius* wing development. Preliminary data show that Distal-less is expressed in large areas of early pupal wings in *Heliconius erato* (Reed 2004), and it would be interesting to know if this pupal expression may be associated with specific color patterns.

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