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Butterfly Wing Pattern Evolution Is Associated with Changes in a Notch/Distal-less Temporal Pattern Formation Process

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Summary

In butterflies there is a class of "intervein" wing patterns that have lines of symmetry halfway between wing veins. These patterns occur in a range of shapes, including eyespots, ellipses, and midlines, and were proposed to have evolved through developmental shifts along a midline-to-eyespot continuum. Here we show that Notch (N) upregulation, followed by activation of the transcription factor Distal-less (Dll), is an early event in the development of eyespot and intervein midline patterns across multiple species of butterflies. A relationship between eyespot phenotype and N and Dll expression is demonstrated in a loss-of-eyespot mutant in which N and Dll expression is reduced at missing eyespot sites. A phylogenetic comparison of expression time series from eight moth and butterfly species suggests that intervein N and Dll patterns are a derived characteristic of the butterfly lineage. Furthermore, prior to eyespot determination in eyespot-bearing butterflies, N and Dll are transiently expressed in a pattern that resembles ancestral intervein midline patterns. In this study we establish N upregulation as the earliest known event in eyespot determination, demonstrate gene expression associated with intervein midline color patterns, and provide molecular evidence that wing patterns evolved through addition to and truncation of a conserved midline-to-eyespot pattern formation sequence.

Results and Discussion

Butterfly eyespots provide a prime example of how a novel character system may arise through the evolutionary recruitment of developmental genes and then diversify under the influence of natural selection [1–3]. During development, eyespot pigment patterns are induced by a long-range signal that originates from a group of focal cells at the center of the eyespot [1]. In late last-instar wing discs, several molecules normally associated with axis specification are expressed in focal cells, and it is proposed that these molecules are involved with activating the focal signal [2]. Of these focal molecules, the transcription factor Dll is of particular interest because the gene encoding it is genetically linked to eyespot size [4]. While gene expression studies have provided insight into eyespot development, little is known about how gene expression may be associated with the evolution and development of noneyespot patterns.

Intervein pattern elements, those with centers of symmetry halfway between wing veins, occur in a range of shapes, including eyespots, tapered ellipses, and midlines, with gradients of intermediate shapes occurring both within and between species [1]. Based on these adult phenotypes, Nijhout proposed that midline patterns are developmental precursors of circular eyespot patterns [5] and that the observed gradient of intervein pattern morphologies could be explained by evolutionary changes in the timing of a common underlying developmental process [1]. The observation that Dll expression passes through an intervein midline stage before forming an eyespot focus [6] increases interest in this idea; however, there has been little comparative work done to test the molecular basis of this model across species. In this study we compared the expression of Dll and the receptor molecule N in a variety of butterflies and moths in order to explore the relationship between prepattern regulation and the evolution of wing patterns.

Eyespot Phenotype Is Associated with N and Dll Expression

We hypothesized that the N signaling pathway may be an upstream component of the focal determination process because ectopic expression of activated N in *Drosophila melanogaster* imaginal discs is sufficient to cause expression of Dll [7]. N is a membrane bound receptor that plays several roles during *D. melanogaster* wing development. Its functions that are best understood in this context include defining the dorsoventral boundary [8, 9] and defining intervein tissue via a lateral inhibition interaction with its ligand Delta [10]. In pupal butterfly wings, there is evidence that N-mediated lateral inhibition may be involved with organizing wing scales [11]. During a lateral inhibition process, N expression tends to increase over time as a result of a local positive-feedback mechanism [8, 10, 12].

To test for an association between N and Dll expression and eyespots, we examined the localization of N and Dll in late last-instar wing imaginal discs of three species of eyespot-bearing nymphalid butterflies: *Vanessa cardui* (Figure 1E), *Junonia (Precis) coenia* (Figure 1K), and *Bicyclus anynana* (Figure 1Q). In all three of these species there is a perfect correlation between presence of forewing and hindwing eyespots and late last-instar N and Dll focal expression. To further characterize the

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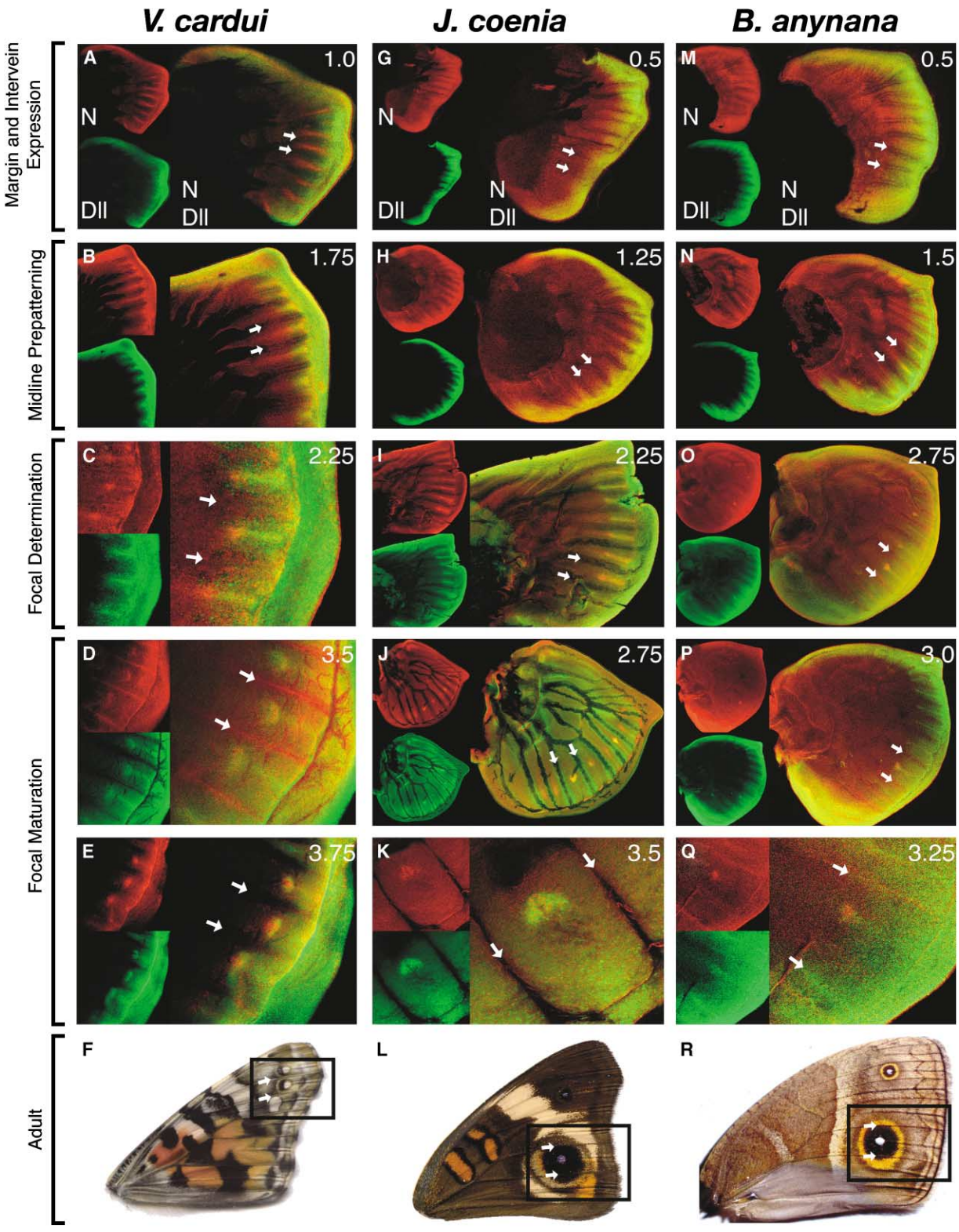

Figure 1. Spatial and Temporal Patterns of N and Dll Expression during Eyespot Development

White arrows provide spatial reference between panels for a specific species by marking homologous wing vein positions. Forewings from three eyespot-bearing species are represented: *Vanessa cardui* (A–F), *Junonia coenia* (G–L), and *Bicyclus anynana* (M–R). We divide these expression patterns into four stages of prepattern formation: margin and intervein expression (A, G, and M), midline prepatterning (B, H, and N), focal determination (C, I, and O), and focal maturation (D, E, J, K, P, and Q). Numbers denote stages of wing disc development.

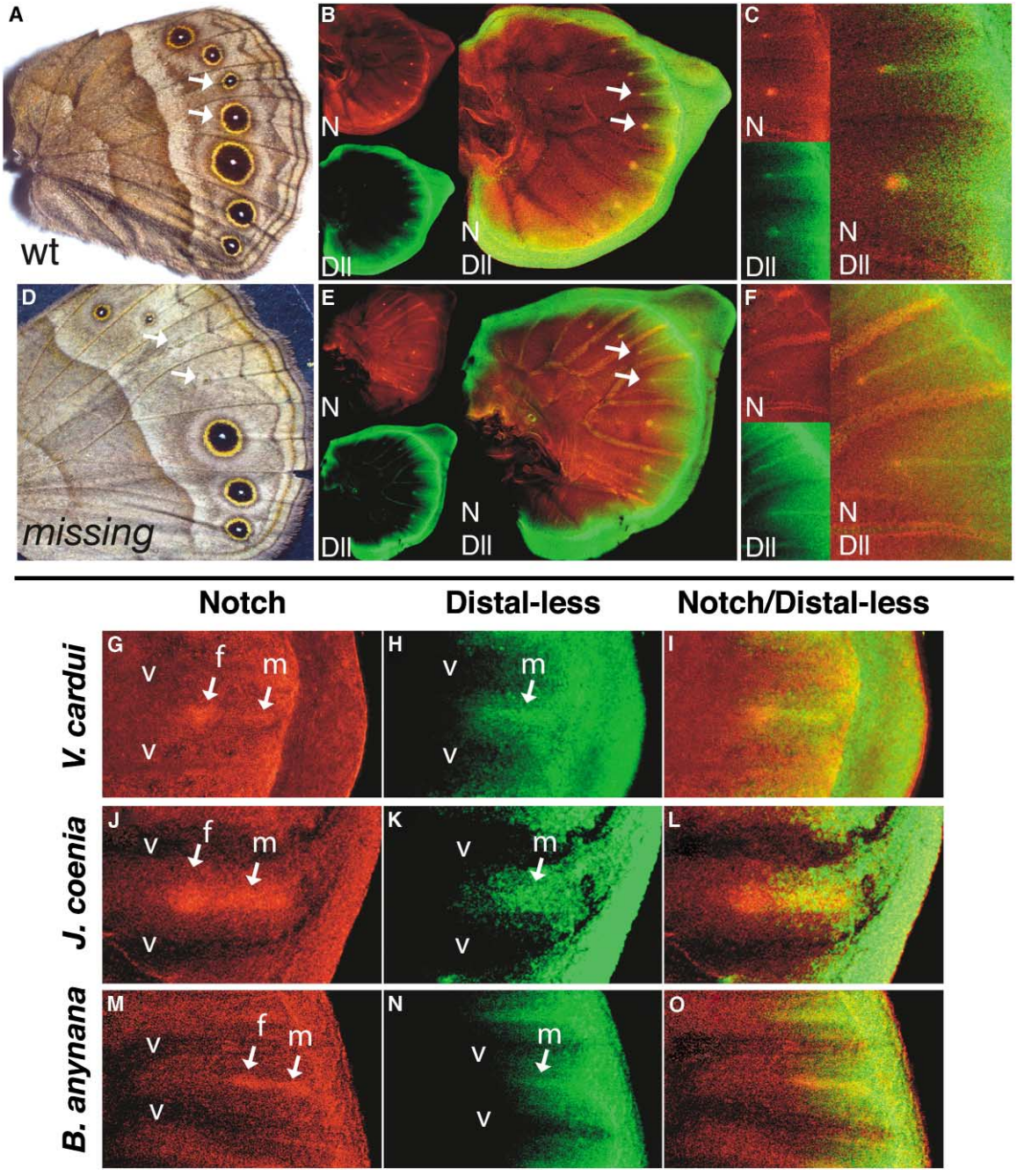

Figure 2. N and Dll Expression Is Associated with the Eyespot Phenotype, and N Is Upregulated Prior to Dll Activation in Developing Eyespot Foci

In wild-type *B. anynana* hindwings (A), N and Dll were detected in mid-fifth instar wing discs in focal patterns associated with all eyespots (B), including those affected by the *missing* mutant (C). The *missing* mutant of *B. anynana* shows a dramatic reduction in the size of two specific eyespots (arrows) on the ventral hindwing of the adult (D). Antibody stains for N and Dll in wing discs from *missing* larvae demonstrate a corresponding reduction of both N and Dll in the developing foci of these two eyespots (E and F), but not in the unaffected eyespots (E). High-magnification images N (G, J, and M) and Dll (H, K, and N) staining during early focal determination in *Vanessa cardui* (G–I), *Junonia coenia* (J–L), and *Bicyclus anynana* (M–O) reveal a stage where N is expressed in foci, while Dll is limited to the intervein midline (I, L, and O). Abbreviations: v, vein; f, focus; and m, midline.

relationship between N and Dll expression and eyespot phenotype, we ascertained the effects of the *B. anynana* eyespot mutant *missing* [13] on levels of N and Dll (Figures 2A–2F). *missing* greatly reduces or eliminates two specific eyespots from the hindwing (Figure 2D). We found that in *missing* mutants, focal N and Dll expression was observed at lower levels (Figures 2E and 2F) than at corresponding positions in wild-type wings (Figures

2B and 2C). N and Dll accumulation must therefore be regulated directly or indirectly by the *missing* locus, and localized inductions of N and Dll may serve as markers for the process of eyespot focus determination at a point downstream of *missing* activity.

N Upregulation Precedes Dll Activation in Eyespot Determination

To determine the spatiotemporal relationship between N and Dll in focal determination, we produced time series of N/Dll double stains from *V. cardui* (Figures 1A–1E), *J. coenia* (Figures 1G–1K), and *B. anynana* (Figures 1M–1Q). We found that focal N upregulation preceded Dll activation with a lag time of approximately 1.5 stages (equivalent to 12 to 24 hr, depending on species, temperature, and individual variation). In all three eyespot-bearing species, we observed stages of gene expression where N was upregulated in discrete focal patterns, while Dll was upregulated only in intervein midlines (Figures 2G–2O). The spatiotemporal relationship between N and Dll may be outlined in the four following primary phases. (1) Margin and intervein expression (Figures 1A, 1G, and 1M). N expression occurs at moderate levels across the wing disc, except for in the presumptive veins where N expression is relatively low. Early during this phase, Dll expression occurs only along the wing margin but progressively moves proximally after the upregulation of N along the intervein midline. (2) Midline prepatterning (Figures 1B, 1H, and 1N). Between the developing wing veins, N is upregulated in an intervein midline pattern along with an accompanying midline of Dll expression. In most species Dll expression in the midline tends to be more discretely focused than N. (3) Focal determination (Figures 1C, 1I, and 1O). N expression is increased in foci, which is subsequently mirrored by Dll. (4) Focal maturation (Figures 1D, 1E, 1J, 1K, 1P, and 1Q). N and Dll express strongly in foci while fading sequentially from the intervein midline. During the focal maturation phase, expression of genes in the hedgehog pathway have been observed in foci of *J. coenia* and *B. anynana* [14].

N and Dll Expression Is Associated with Intervein Midline Pigment Patterns in a Clade of Butterflies Lacking Eyespots

Eyespot patterns have been gained and/or lost multiple times throughout butterfly evolution, and eyespots are even seen in some moths. While a rigorous phylogenetic treatment is required to infer the specific pattern of eyespot evolution throughout the Lepidoptera, we may nevertheless answer some questions about eyespot evolution by using selected exemplar taxa. Specifically, we were interested in determining if a secondary loss of eyespots in a lineage is associated with a change in the N/Dll prepatterning process. To address this we examined two species from the nymphalid subfamily Heliconiinae: *Agraulis vanillae* and *Heliconius melpomene*. These species belong to a monophyletic subtribe called the Heliconiiti [15, 16], in which there are no obvious eyespot-bearing species, although intervein midline patterns are common throughout the group. Eyespots are found in non-Heliconiiti heliconiines, as well as throughout the rest of the Nymphalidae, suggesting that the Heliconiiti represent a secondary loss of eyespots.

In *A. vanillae* the margin and intervein expression and midline prepattern phases appear similar to those in other butterflies (Figures 3A–3F). In this species, however, midline definition occurs relatively slowly, and development only reaches the midline prepattern phase by pupation. Furthermore, intervein midline gene expression was not observed to fade as during focal maturation in eyespot-bearing species. The midline expression patterns correspond with orange midline pigment patterns on both the hindwing (Figures 3L and 3M) and forewing (data not shown).

In last-instar *H. melpomene*, N and Dll were expressed in an intervein midline pattern similar to *A. vanillae*, although extended proximally (Figure 3H–3J). There is an association between gene expression and pigment pattern in *H. melpomene*, where a recessive gene from the Ecuadorian race *plesseni* reveals a melanic midline pattern in the forewing that matches N and Dll expression (Figures 3H–3J). It is notable that even though expressivity of the intervein midline pigment pattern varies throughout the genus *Heliconius*, the N/Dll expression pattern appears to be similar between species both bearing and lacking these patterns (including *H. cydno*, *H. erato*, and *H. hecale*, R.D.R., unpublished data). These observations suggest that N and Dll expression is, in itself, not sufficient for midline pigment patterns in *Heliconius*.

Moths Lack N/Dll Intervein Midline Expression

In order to gain insight into the origin of the N/Dll prepatterning process, we determined the expression patterns of these proteins in the outgroup pierid butterfly *Pieris rapae* and two "higher" (ditrysian) moths: the sphingid *Manduca sexta*, and the gelichiid *Pectinophora gossypiella*.

A time series of N/Dll stains in *P. rapae* (Figures 3N–3R) resembled the time series from *A. vanillae* in that N and Dll formed persisting intervein midline patterns. Interestingly, however, *P. rapae* did not display a midline pigment pattern in the adult. Midline pigment patterns are found in many pierid species, suggesting that as with *Heliconius*, N/Dll midline expression may be associated with, but is not sufficient for, pigment midlines in adults. We observed no gene expression associated with the black chevrons on the *P. rapae* forewing.

In the moths *M. sexta* and *P. gossypiella*, early N and Dll expression resembled initial margin and intervein expression in butterflies (Figures 3T–3V, 3X, and 3Y). In late stage *M. sexta* wing discs, Dll formed vaguely defined proximal extensions along the wing veins themselves (Figure 3V). In *P. gossypiella* we did not detect expression of Dll in intervein tissue (Figures 3X and 3Y). Given the species sampling in this study, it is most parsimonius to infer that the N/Dll intervein midline originated sometime after the divergence of the sphingid lineage and before the divergence of the pierid lineage. Further sampling of gene expression patterns from basal butterfly families and moth outgroups would help clarify the

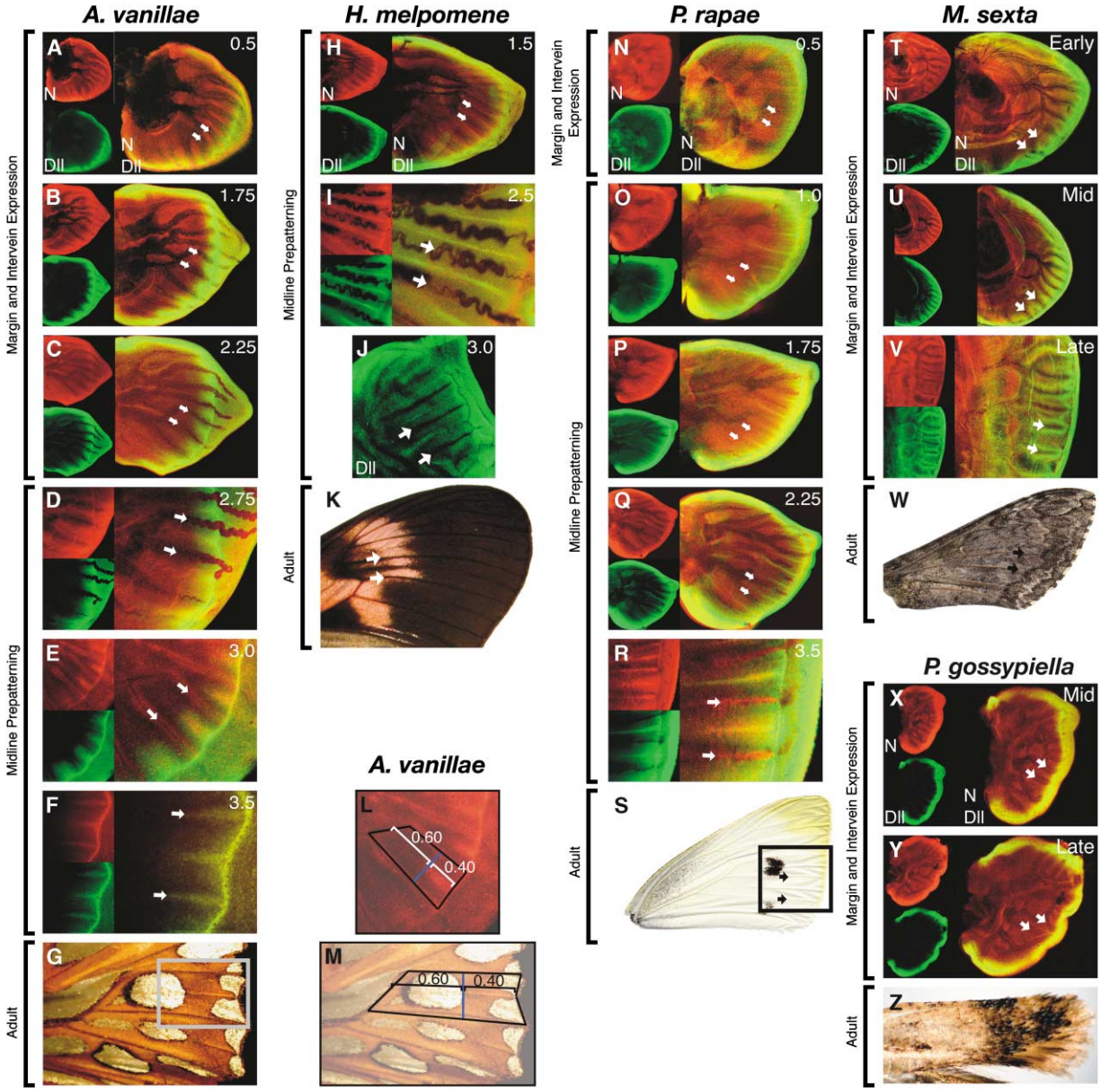

Figure 3. N and Dll Expression in Last-Instar Wing Discs of Lepidoptera Lacking Eyespots

White arrows provide spatial reference between panels for a specific species by marking homologous wing vein positions. In the last-instar hindwing of *Agraulis vanillae* (A–F), a midline of N and Dll expression (D–F) was observed that appeared to correspond with an orange intervein midline pattern in the adult wing (G). The proximal termination point of the gene expression midline was 40% of the distance from the presumptive adult wing margin to the proximal wing vein bounding the "wing-cell" compartment (L), which was identical to the proportional length of the orange midline pattern in the adult (M). In last-instar forewing discs of *Heliconius melpomene*, N and Dll are expressed in intervein midlines (H–J) that resemble a melanic intervein midline pattern in some hybrid adult butterflies (K). The pierid butterfly *Pieris rapae* (N–S) shows intervein midline expression of N and Dll in last-instar wing discs (O–R); however, no midline gene expression was detected in the moths *Manduca sexta* (T–W) or *Pectinophora gossypiella* (X–Z). Numbers denote stages of wing disc development.

point of origin of the midline prepattern. It should be noted that published expression patterns for the monarch *Danaus plexippus* and the *B. anynana Cyclops* mutant [17] do not show Dll midline expression; however, gene expression time series have not been described from either of these species, so it remains unknown if midlines may be expressed earlier or later than the sampled time points.

Wing Pattern Evolution Is Associated with the Addition and Truncation of States in a Conserved Prepatterning Sequence

We mapped our gene expression data onto a phylogeny of the Lepidoptera used in our study (Figure 4) and drew several conclusions regarding the evolution of intervein and focal prepatterning in butterfly wings. First, the temporal order of gene expression states is conserved in

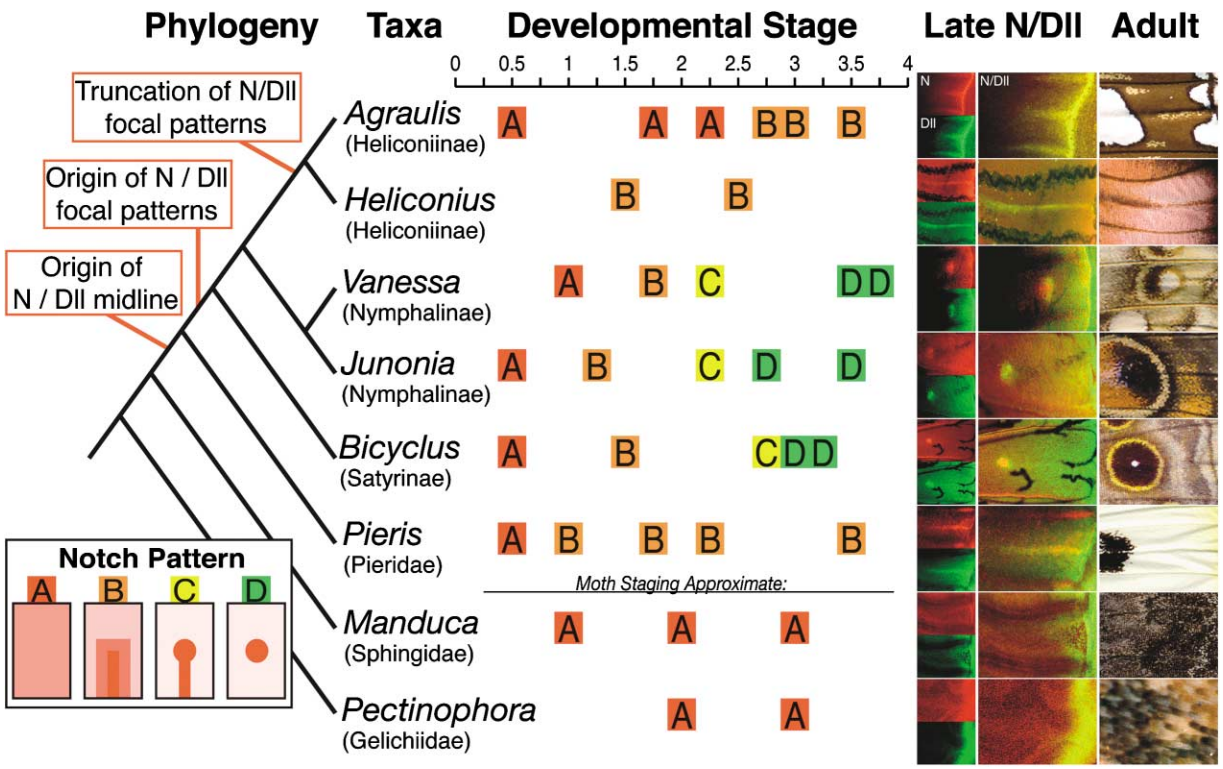

Figure 4. Changes in the N/Dll Pattern Formation Process Are Associated with the Evolution of Intervein Wing Pattern Elements

Colored boxes marked A through D encode the states of N expression at the time points presented in Figures 1 and 3, where A represents margin and intervein expression; B, midline prepatterning; C, focal determination; and D, focal maturation. Our data suggest that intervein midline gene expression is a derived characteristic of the lineage leading to the butterflies and that focal gene expression was a terminal addition to the prepatterning sequence that occurred sometime between the split of the pierids and nymphalids. In the sampled heliconiine taxa, the gene expression sequence has been truncated, with a loss of focal determination (state C) and focal maturation (state D). Both heliconiines display adult pigment patterns correlated with midline gene expression.

all the taxa examined. Second, as outlined above, the formation of a discrete intervein midline appears to be a synapomorphy of the butterfly lineage. What, then, can we conclude about the origin of focal gene expression? Given our species sampling, there are two equally parsimonious hypotheses for the evolution of focal expression patterns: (1) there were two independent origins of foci in the lineages leading to the Satyrinae and Nymphalinae, respectively, or (2) there was a gain of foci in the lineage leading to the Nymphalidae and a loss of foci in the lineage leading to the Heliconiiti. Although a greater species sampling is required to rigorously distinguish between these possibilities, at this point we would favor the latter model because of (1) the rarity or absence of eyespots (i.e., concentric circular or oval pigment patterns consistent with a focal induction model) among the basal butterfly families Pieridae, Papilionidae, and Hesperiidae, and (2) the occurrence of putative inductive eyespots in basal heliconiine genera such as *Vindula* and possibly *Cethosia*. It would be of great interest to determine the expression patterns of N and Dll in eyespot-bearing lepidopteran lineages not closely related to the Nymphalidae, such as the papilionid genus *Parnassius* or the eyespot-bearing saturniid and sphingid moths. These lineages potentially represent origins of inductive eyespot patterns evolutionarily independent from the nymphalid clade, and studying them could provide insight into the developmental basis of parallel pattern evolution.

The gene expression patterns we report provide useful markers for the wing pattern-formation process; however, it remains unknown what the developmental significance of the observed prepatterning sequence is. It is striking that in the eyespot-bearing butterflies examined, midline gene expression occurs prior to focal determination and that midlines always terminate proximally at the eyespot foci. These observations suggest a noncoincidental relationship between formation of midlines and foci; however, with the current data we cannot determine if the midline/focus relationship is causal or if these prepatterns are both downstream of an as-of-yet unknown coordinate system.

Conclusions

The data presented here establish N upregulation as the earliest known event in the development of butterfly eyespots. Furthermore, finding that eyespots and midlines share a similar prepatterning process supports earlier models that these intervein pattern elements are developmentally related. The observation in eyespot-bearing species that N and Dll pass through a transient, and apparently ancestral, phase of midline expression prior to focal determination raises the possibility that this developmental sequence represents a kind of evolu-

tionary heterochrony at the level of molecular pattern formation. In sum, our data illustrate how a discrete morphological character may evolve through temporal changes in a conserved molecular pattern-formation process.

Experimental Procedures

Wing Disc Staging

Butterfly wing discs were staged according to the progress a tracheal development as previously outlined [18]. Specifically, stages were as follows: 0, fifth-instar molt; 1, radius trachea extended; 1.5, media, cubitus tracheae extended; 2, tracheae reach border lacuna; 2.5, tracheal branches invade border lacuna; 3, tracheoles enter wing epidermis. Here, we define stages 3.5 and 4. In stage 3.5, extensive tracheole growth in the epidermis is visible and tracheae form a continuous line along the border lacuna. In stage 4, the wing disc has just entered the prepupal growth phase and has a slightly darkened peripodial membrane.

Immunohistochemistry

Antibody double stains were produced by using the protocol of Brunetti et al. [19] with monoclonal mouse antibody C17.9C6 to the *D. melanogaster* N intracellular domain [20] and a polyclonal rabbit antibody to the *J. coenia* Dll protein [21]. The C17.9C6 antibody is evidently specific to the N receptor in butterflies as it produces staining patterns consistent with N's known roles in *D. melanogaster*, including sensory cell organization [11], wing vein patterning (R.D.R., unpublished data), and wing margin specification (R.D.R., unpublished data). High magnification confocal images of C17.9C6 staining in butterfly wing epithelia demonstrate signal strictly localized to apical cell membranes (not shown), consistent with N's role as a membrane bound receptor. Furthermore, the C17.9C6 antibody has produced staining patterns in grasshopper legs consistent with N's role in appendage patterning in *D. melanogaster* (B. Blachuta, personal communication), suggesting that this antibody may be effective across multiple insect orders. Anti-mouse Cy3 and anti-rabbit Cy2 antibodies (Jackson Laboratories, Inc.) were used for secondary staining and whole-mount wing discs were imaged on a confocal microscope.

Lepidoptera Phylogeny

We generated a supertree of lepidopteran relationships by using the MinCut algorithm [22] as modified by R.D.M. Page in the software package "supertree v. 0.2.0" [23]. The topologies sampled for supertree construction were previously generated from both morphological [16, 24] and molecular data [25, 26]. In order to process family- and superfamily-level phylogenies in our analysis, we nested genera of interest into the appropriate taxonomic categories as polytomies. For our analysis we included only taxa that were relevant to our study, with the exception of the heliconiine genus *Vindula*, which we included to help resolve intranymphaline relationships.

The input trees were as follows: (((Papilionoidea), Bombycoidea), Gelichioidea) [24], with insertion of nested genera as (((*Bicyclus*, *Vindula*, *Agraulis*, *Heliconius*, *Vanessa*, *Junonia*, *Pieris*), *Manduca*), *Pectinophora*); (((Nymphalidae), Pieridae), Sphingidae) [25], with insertion of nested genera as (((*Bicyclus*, *Vindula*, *Agraulis*, *Heliconius*, *Vanessa*, *Junonia*), *Pieris*), *Manduca*); (*Bicyclus*, ((*Vindula*, *Heliconius*), (*Vanessa*, *Junonia*))) [26]; and ((*Vindula*, (*Agraulis*, *Heliconius*))) [16]. The resulting topology was ((((*Bicyclus*, ((*Vindula*, (*Agraulis*, *Heliconius*)), (*Junonia*, *Vanessa*))), *Pieris*), *Manduca*), *Pectinophora*). The MinCut fit of our supertree was 1, and there was no conflict between the sampled topologies for our taxa of interest.

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