THE EVOLUTION OF PATTERN FORMATION IN BUTTERFLY WINGS

by

Robert Dale Reed Jr.

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and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Lisa M. Nagy	5/21/04 date
John W. Little	5/21/04 date
Danny L. Brower	5/21/04 date
Diana E. Wheeler	5/21/04 date
David R. Maddison	21 May 2004 date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director: Lisa M. Nagy 5/21/04 date

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I dedicate this dissertation to my parents, Dale and Donna Reed, for their work on my own evolution and development.

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ABSTRACT

I employed a comparative gene expression approach to address the evolution of butterfly wing pattern formation at several levels, including early pattern determination and pigment gene regulation during late development. Expression analysis of the receptor molecule Notch suggested several previously unknown roles for Notch signaling in butterfly wing patterning. Notch upregulation was found to precede the activation of the transcription factor Distal-less during early eyespot color pattern determination. A phylogenetic comparison of expression time series from multiple moth and butterfly species suggested that changes in a Notch / Distal-less temporal pattern formation process were associated with the gain and loss of both eyespot and midline color patterns during wing pattern evolution. Notch expression was found to occur in a grid pattern in the butterfly wing epithelium shortly after pupation. This observation, together with previous expression and simulation studies, support a Notch-mediated lateral inhibition model of wing scale organization. Tryptophan-derived pigments, including the ommochromes, are a derived feature of nymphalid butterfly wings. These pigments appear in wing scales shortly before adult emergence. I found that multiple genes in the ommochrome biosynthetic pathway were expressed in the wings of some nymphalid butterflies. Additionally, transcriptional regulation of genes encoding the ommochrome synthesis enzymes vermilion and cinnabar was found to be associated with the polymorphism and development of forewing band patterns in the mimetic butterfly *Heliconius erato*.

CHAPTER 1

PATTERN FORMATION IN BUTTERFLY WINGS

INTRODUCTION

Butterflies are creatures of the sunlight that use wings adorned in color to communicate with the world around them. The wings of butterflies are genetic banners that display a species' identity and intentions, while betraying its historical relationship with the universe around it. Wing patterns can be used to hide from or confuse would-be predators, to advertise distastefulness, or to attract mates and other conspecifics. It is easy for humans to identify with these highly visual animals because of their habits and lifestyles. Butterflies can be common in many habitats, including cities, at certain times of the year. They live very visible lives associated with flowers and sunshine, and who among us is not jealous of a butterfly's ability to soar through the air? The process of butterfly metamorphosis has also attracted attention throughout the ages as a powerful symbol for growth and renewal. Given that most insects fly, exploit plants, and metamorphose, why the overwhelming popular fascination with butterflies? The colorful wings, of course!

The aesthetic interest in butterflies throughout human history is alone sufficient to justify the study of butterfly wings. For well over 150 years, however, butterfly wings

have been used as models to formulate and demonstrate a number of fundamental scientific concepts. A butterfly whose wings resemble a dead leaf is, for many, the first image that comes to mind at the mention of crypsis. A vomiting jay bird with brightly colored butterfly wings laying at its feet is the poster child for aposematism. And of course there is mimicry; what marginally curious North American child cannot relate the tale of the viceroy and monarch?

In addition to these textbook case studies, some biologists would recognize various other intellectual breakthroughs stemming from work on butterfly wings. The concept of Müllerian mimicry, where multiple distasteful models converge on a phenotype, owes much to work on various butterflies. Studies of wing scales have produced insight on how structural properties of organisms can produce color through interference and diffraction mechanisms. A great deal of work regarding mate choice and sexual selection have revealed how these phenomena can shape animal morphology and the developmental mechanisms underlying those morphologies. At the molecular level, butterfly wing patterns have produced some of the most well-known and oft-cited examples of gene circuit recruitment. More recent studies examining the effects of artificial selection on wing patterns promise to become modern classics concerning the interplay of genetic variation and constraint in phenotype evolution.

Butterfly wing patterns are beautiful, interesting, and scientifically important. Despite the interest in wing patterns, however, relatively little is known about how they form, and even less is known about how the development of these patterns has evolved. Gaining a better understanding of wing patterning, especially at the molecular level, will

make greatly facilitate more integrative research. Identifying loci and gene networks that are responsible for producing specific patterns will allow researchers to ask questions about the genetic interface between natural selection and phenotypic change, about how novel morphologies arise and how the processes underlying those morphologies originated.

In this dissertation I address the topic of pattern formation in butterfly wings at several levels, and the chapters are arranged in an order consistent with the temporal sequence of pattern formation events in butterfly wings. In Chapter 2 I investigate the early process of eyespot determination in larval wing discs, and explore how changes in this process are associated with the evolution of eyespot and eyespot-related patterns in moths and butterflies. Chapter 3 covers the mechanism by which scale cells are arranged in parallel rows in early pupal wings, while Chapters 4 and 5 focus on gene expression associated with pigment development and polymorphism, primarily in late-stage pupal wings. Each chapter covers a different aspect of pattern formation and realization in butterfly wings; together they provide a glimpse into how multiple pattern generating mechanisms may interact through time and space to produce a complex adult butterfly wing from a small cluster of imaginal disc cells. This introductory chapter briefly outlines the stages of butterfly wing development and the main elements of wing color patterns, providing a context for interpreting the data outlined in the rest of this dissertation.

STAGES OF BUTTERFLY WING DEVELOPMENT

Larval development

The majority of butterflies advance through five larval stages, a pupal stage, and an adult stage. Butterfly wings develop from small clusters of cells, known as imaginal discs, that develop during the larval stage. The wing imaginal discs are very small, yet detectable, in pre fifth-instar lepidopteran larvae. Before the fifth-instar, the wing discs appear to be small clusters of morphologically undifferentiated cells attached to tracheae (respiratory tubes) that run parallel to the large lateral plurisegmental tracheal trunks in the second and third thoracic segments (Snodgrass 1993). These basal connecting tracheae will eventually be the source of the tracheae that follow the wing veins of the adult wings. Butterfly wing discs grow as flattened epidermal "pouches", with the two epidermal layers forming the dorsal and ventral wing surfaces. This mode of wing disc development has also been noted in ants (Abouheif and Wray 2002) and beetles (Powell 1904; Powell 1905), and appears to be the ancestral mode of wing development in insects. It is important to note here that butterfly wing disc development is much different in the fly *Drosophila melanogaster*, whose wing discs undergo a complex process of eversion and folding during late development to produce the two surfaces of an adult wing from a single epithelial sheet.

Dramatic wing disc growth occurs during the last larval instar. In fifth-instar wing discs, genes known to be involved with axis specification in *D. melanogaster* wings are also expressed in butterflies in function-consistent patterns (Carroll et al. 1994). In

butterflies, *apterous* transcription was found to be associated with the dorsal epidermis, *wingless* with the wing margin, *invected* with the posterior compartment, and *scalloped* with the entire wing. It has also been found in butterflies that Ultrabithorax functions to determine the identity of the hindwing, versus the forewing, consistent with its function in haltere specification in *D. melanogaster* (Weatherbee et al. 1999). Without belaboring details, these expression patterns suggest that axis- and appendage-specification are conserved (with a few exceptions) between *D. melanogaster* and butterflies.

One notable aspect of larval butterfly wing development is the determination of wing shape through cell-death around the wing margin (Nijhout 1991). Along the dorsal-ventral boundary of the larval wing discs is a strip of peripheral tissue extending from the margin to a distinct line of cells known as the border lacuna. Lacunae are lines where the cells in the dorsal and ventral epidermal layers are basally separated, forming a sort of "tunnel" between the epidermal layers. In fifth –instar wing discs the peripheral tissue is known to express transcripts of the intercellular signaling molecule *wingless* at a later time in the fifth-instar (Carroll et al. 1994). After pupation, the peripheral tissue undergoes programmed cell death, and the adult wing shape then follows the line of the original border lacuna (Nijhout 1991; Nijhout 1994).

As with the adult wing margin, the identity of the adult wing veins is also presaged by a system of lacunae that begin developing within the first day after the fifth-instar molt. Throughout fifth-instar wing disc development, tracheae progressively grow out from the basal tracheal mass, "invading" the lacunae. The progress of tracheal growth into the lacunae provides a series of temporal landmarks by which larval wing disc

development may be staged (Miner et al. 2000). In late fifth-instar wing discs, many small, branching tracheoles grow out from the large wing vein-associated tracheae, accessing all regions of the wing disc epidermal layers in between the vein lacunae. Little is known about the molecular basis for lacuna development or tracheal growth in butterfly wings. Data reported in Chapter 2, however, suggest that the Notch receptor may be implicated in specifying non-lacuna "intervein" tissue – consistent with its role in *D. melanogastor* wing vein development (Huppert et al. 1997).

Some elements of the adult color pattern are thought to be determined in larval wings discs. The data supporting this consist primarily of gene expression patterns that correlate with adult color patterns. Almost all of the data relate to gene expression associated with eyespot patterns. Specifically, in late fifth-instar wing discs the transcription factor Distal-less, as well as several genes in the Hedgehog pathway, are expressed in cells that will become the center of the eyespot pigment patterns (Carroll et al. 1994; Brakefield et al. 1996; Keys et al. 1999). Also at this time, *wingless* transcription correlates with two red bands near the base of the wing of *Junonia coenia* (Carroll et al. 1994), although nobody has pursued this finding any further, to my knowledge.

Pre-pupa and pupation

Before pupating, butterfly larvae undergo a pre-pupal phase in which the animals sit motionless, then hang from a silk string before shedding the skin. During this ~12 hr period the wings grow at an exceptionally rapid pace, filling much of the larval thorax

and thoracic legs, causing the thorax to expand in a most uncomfortable-looking manner. The growth of the pupal epidermis during the pre-pupal phase occurs in such a way that the wing discs are placed outside the epidermis, and are attached to the pupal epidermis at their bases. Details of the tissue growth associated with how the larval imaginal discs move outside the nascent pupal epidermis then attach to that epidermis are not entirely clear. More descriptive work is required on this front. After the pre-pupa sheds its skin, the dorsal surfaces of the forewing, and some of the hindwing, produce the pupal cuticle from the peripodial membrane that surrounds the wings.

Pupal development

The wing epidermal bilayer in early pupae is very thin and delicate. In the day after pupation the polyploid scale-forming cells are determined in parallel rows, while extensive cell death occurs throughout the epidermis (Nijhout 1991; Galant et al. 1998). This time is also important for eyespot pattern development because cells from the center of the presumptive eyespot patterns are capable of inducing ectopic eyespots when transplanted to other parts of the wing (Nijhout 1991). This result strongly implies that concentric circular eyespot pigment patterns are produced by a morphogen that diffuses through the wing epidermis in early pupae (Fig. 1-1). Subsequent to the period of the inductive signal, expression of the transcription factors Distal-less, Engrailed/Invected, and Spalt occur in concentric circles that mirror the adult eyespot phenotype (Brunetti et al. 2001). Together, these data suggest that the specific adult color pattern of the eyespot is determined in the early pupa.

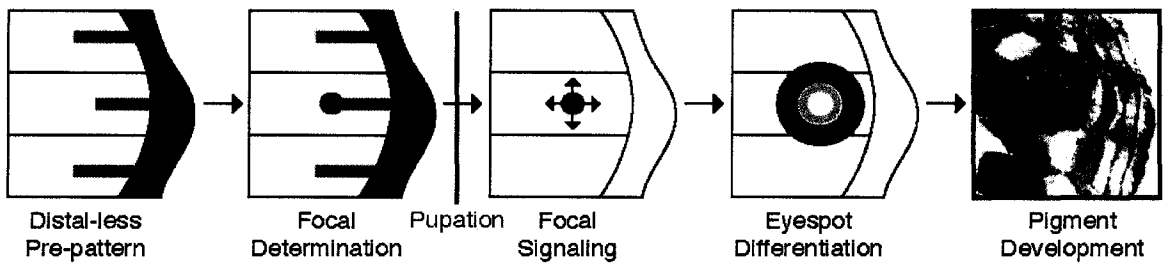
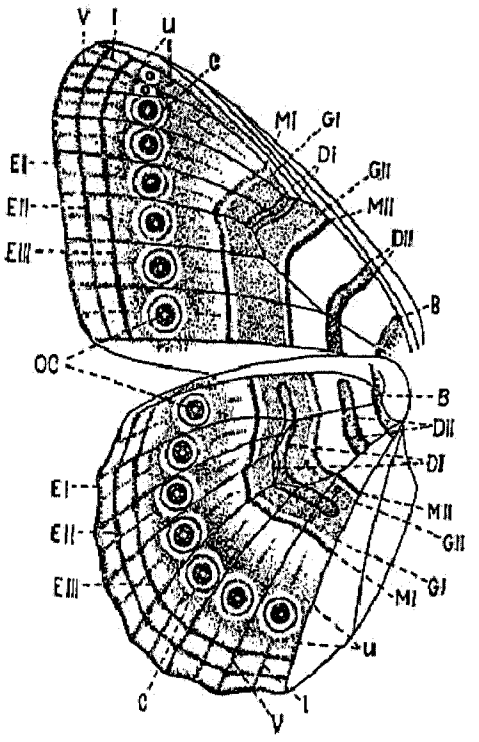


Fig. 1-1. A model of eyespot patterning in butterfly wings. The diagram represents a portion of a developing wing, with four wing veins shown as black lines, and the margin peripheral tissue at the right. In the fifth instar wing discs the developmental transcription factor Distal-less, represented in green, is expresses in the distal portion of the wing, and in an intervein midline halfway between wing veins. At the proximal terminus of this intervein midline a cluster of cells that will become the eyspot focus express Distal-less as well. After pupation, these cells produce a diffusible morphogen that induces eyespot formation. Before pigments development, the expression of transcription factors like Distal-less, Engrailed/Invected, and Spalt have been seen to express in patterns correlated with the concentric color rings in eyespots.

Several days after pupation, when the scale-forming cells have differentiated, scales begin to mature. In all species so far examined, scale maturation occurs in complex spatiotemporal patterns that correlate with adult pigment patterns. Scales that will bear the "colored" pterin, ommochrome, or papiliochrome pigments mature first, followed by scales that will bear melanin (Nijhout 1991; ffrench-Constant and Koch 2003). It is striking how the scales of different pattern elements mature at different times, and is reminiscent of a child's paint-by-number game. The differential timing of scale development across the wing epidermis has been proposed to be a mechanism of pattern determination in the case of swallowtail butterflies (Koch et al. 2000b; ffrench-Constant and Koch 2003). To date, all published examples of scale and pigment development in butterflies suggest that the pigment synthesis is coincident with scale maturation. After all the scales and pigments have fully matured, the adult butterfly emerges from the pupa and inflates and dries its wings. At this point the scale cells have died and consist only of sculpted and pigmented bits of cuticle arrayed like shingles upon the wing membrane.

THE NYMPHALID GROUND PLAN

The evolution of butterfly wing patterns has been a matter of great interest for many years, and a sizable literature has developed surrounding the topic. An entire book exists on the subject (Nijhout 1991), as well as several recent reviews (Brakefield 2001; Beldade and Brakefield 2002; McMillan et al. 2002; Brakefield and Monteiro 2003), and a comprehensive review of the topic is beyond the scope of this introduction. In my view,



Proximodistal pattern elements

EI,EII,EIII	Externae	Marginal and sub-marginal bands
U	Umbra	Parafocal elements
OC	Ocellata	Eye-spots
C	Circulus	Outer ring of eye-spot
MI,MII	Mediae	Central symmetry system
GI,GII	Granulatae	Central symmetry system
DI	Discalis I	Discal spot
DII	Discalis II	Basal symmetry system
B	Basalis	Wing root band

Anteroposterior pattern elements

V	Venosa	Venous stripe
I	Intervenosa	Intervenous stripe, intervein midline

Fig. 1-2. The nymphalid ground plan from Schwanwitsch (1924). The table gives Schwanwitsch's names for specific pattern elements, as well as terms used in the modern literature (most of which are attributable to Nijhout,1991).

the most important thread uniting all work on butterfly wing patterns is the model of pattern homologies known as the nymphalid ground plan (NGP). Accordingly, for this introduction I will briefly outline the concept of the NGP, and its significance for understanding pattern development.

There is an incredible diversity of butterfly wing patterns, and many of the constituent motifs comprising these patterns are seen in various forms across many different butterfly species. In 1924 Schwanwitsch proposed what we now call the NGP, a formal model of pattern homologies that describe the similarities between wing patterns (Schwanwitsch 1924). The NGP is a remarkable statement that goes far beyond simply proposing homologies between some morphological characters; it summarizes a discrete system of developmental potential and constraint that can be explored using comparative, evolutionary, and developmental approaches. Although other slightly different interpretations of the NGP exist (Schwanwitsch 1924; Süffert 1927; Nijhout 1990; Nijhout 1991), I generally prefer Schwanwitsch's model because of its simplicity, comprehensiveness, and consistency with gene expression patterns.

Schwanwitsch proposed two primary classes of pattern elements in butterfly wings: those that form parallel to the wing veins, and those that form perpendicular to the wing veins. Among the vein-associated patterns are the *venosa* elements that co-localize with the veins themselves, and then the *intervenosa* patterns that are located between veins (Fig. 1-2). Mutants of the swallowtail butterfly *Papilio xuthus* lacking wing veins show a loss of *venosa* color patterns, suggesting that veins are required for the induction of these patterns (Koch and Nijhout 2002). Little is known about the development of

intervenosa patterns, beyond the observation that the eyespot-associated transcription factor Distal-less expresses in intervein midline patterns prior to eyespot determination (Brakefield et al. 1996).

Patterns perpendicular to wing veins can be separated into several categories, moving from the distal margin of the wing proximally. Closest to the wing margin are a set of *externae*, the number of which may vary between species. Nijhout (1991) proposes that these patterns form through the action of diffusible signal from the wing margin, an hypothesis based on the observation that cautery of the wing margin typically results in the marginal bands "adapting" to the damage, following perfectly the healed margins of damaged discs. More proximally there are other systems of bands, including the *umbra*, which flank the chain of eyespots proximally and distally. Schwanwitsch considered *ocellata*, also known as the eyespots, to belong to the proximodistal set of pattern elements, however it is my own belied that the eyespots represent intersection points between a proximodistal band prepattern and an *intervenosa* prepattern. Proximal to the eyespot-related patterns are the *media* and *granulata* bands, forming the "central symmetry system" *sensu* Nijhout. At the base of the wing is a rare pattern element known as the *basalis* that is only occasionally seen in butterflies, and tends to be more common in moths. There are also the *discalis* bands associated with the discal wing cell, known otherwise as the "discal spot" and/or the "basal symmetry system".

Excepting the eyespot, the development of which is described above, little is know about these developmental bases of any of these proximodistal pattern elements. It is of great interest to understand how these patterns develop and evolved, for they appear

to represent an entirely novel pattern formation system whose parallel is unknown in any other insect wings.

A major theme in butterfly wing pattern evolution especially relevant to this dissertation is that of polymorphism and mimicry. Many butterfly species have wing pattern polymorphisms that allow them to mimic other distasteful species in order to gain a modicum of protection from predators. In many cases, the geographic distribution of pattern polymorphisms are tightly correlated with the boundaries of mimicry rings involving multiple species of Lepidoptera. Butterfly systems as found in *Heliconius*, where some of the genetic basis of wing pattern polymorphisms is understood, provide an enticing opportunity to study the developmental basis of the origin and diversification of morphological traits of unambiguous adaptive value. In *Heliconius*, it appears that most of the mimetic wing patterns are derived from the proximodistal band patterns of the NGP, although the wing pattern homologies between *Heliconius* and other butterflies are often not entirely clear.

RESEARCH PRIORITIES

If this brief introduction makes anything clear, it is that the knowledge of molecular basis of pattern formation in butterfly wings is quite limited at this time, despite the existence of a sizable body of related morphological, comparative, and ecological work. Even more limited is our understanding of the developmental basis of wing pattern evolution. Some priorities for future study include:

Post-axial pattern formation

While Carroll et al. (1994) have described gene expression patterns suggesting that anteroposterior and dorsoventral axis formation is generally conserved between butterflies and *Drosophila*, less is known about the processes responsible for downstream pattern elaboration. Significant patterning systems novel to lepidopteran wings include delimitation of the margin apoptosis zone, organization of scale cells, and determination of the basic elements of the NGP. Further work determining candidate gene expression patterns would likely be the most efficient way to gain more insight into this topic.

Gene networks underlying non-eyespot patterns

As described above, much more is known about the process of eyespot color pattern formation than for any other type of color pattern. It would be useful to know what gene networks or processes underlie the development of other pigment patterns. Candidate genes studies have obviously been successful for associating the activity of specific developmental genes with pattern formation, and should continue on this front. Genetic mapping studies and gene expression comparisons using microarrays with polymorphic species or mutants may prove useful as well.

Pigment development

While some biochemical studies have been done to identify butterfly wing pigments, little is known about the specific genes or molecules underlying the synthesis of these pigments. Work from *Drosophila* eyes offers a number of pigment synthesis

candidate genes, and expression studies on these genes would be an important first step in understanding how they are regulated and may function in pattern development.

Phylogenetic studies

One of the most exciting opportunities provided by a developmental understanding of the NGP is the possibility to understand the biological basis of the origin and evolution of multiple morphological characters. Newly available phylogenies now make it possible to reconstruct the evolution of specific wing pattern elements in a rigorous way, and even to test the historical associations of gene expression with color pattern elements. These kinds of exercises may allow us to focus the search for specific developmental genetic novelties underlying phenotypic change.

Functional molecular studies

Gene expression studies have identified a number of genes and pathways potentially involved in butterfly wing patterning, however it has been difficult to test any specific hypotheses of gene function due to a lack of manipulative experimental techniques in butterflies. RNAi gene knockdown techniques by myself and other labs over the years have so far been of limited success. As well, antisense morpholinos have not worked and pathway inhibitors have so far produced unclear results (S.B. Carroll, pers. com.). Attempts to use a Sindbis virus to ectopically express genes have been confounded because wing tissue appears to be resistant to infection (S.B. Carroll, pers. com.). While much effort has been given to failed attempts at functional tests of gene

function in butterflies, many more techniques remain to be tried. The recent germline transformation of a butterfly using transposable elements may be the breakthrough needed on this front (Marcus et al. 2004).

The research outlined in this dissertation attempts to address several priorities outlined above. Chapter 2 addresses gene expression associated with non-eyespot patterns in the context of the phylogenetic history of intervein midline and eyespot pattern formation in moths and butterflies. In the category of post-axial patterning, Chapter 3 provides the first evidence that Notch signaling may be implicated in the organization of scale cells into parallel rows. The last part of this dissertation deals with pigment development in butterfly wings, with Chapter 4 describing the expression of pigment synthesis genes in Painted Lady butterflies, and Chapter 5 identifying changes the regulation of pigment genes associated with wing pattern polymorphism in mimetic *Heliconius* butterflies.

The comparative data presented here provide a strong foundation for future experimental work on butterfly wing pattern development. Now that manipulative genetic techniques in butterflies are beginning to mature, the candidate genes I have identified and the data and models I have developed as a graduate student will provide key starting points for future lines of research into the evolution and development of pattern formation in butterfly wings.

CHAPTER 2

BUTTERFLY WING PATTERN EVOLUTION IS ASSOCIATED WITH CHANGES IN A NOTCH / DISTAL-LESS TEMPORAL PATTERN FORMATION PROCESS*

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 it has been proposed that they evolved through developmental shifts along a midline-to-eyespot continuum. In this chapter I show that Notch (N) upregulation, followed by activation of the transcription factor Distal-less (Dll), is an early event in the development of both eyespot and intervein midline patterns across multiple species of butterflies. A genetic association between eyespot phenotype and N/Dll expression is demonstrated by a loss-of-eyespot mutant in which N/Dll expression is repressed at missing eyespot sites. A phylogenetic comparison of expression time series from eight lepidopterans suggests that intervein N/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

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* An abbreviated version of this chapter has been accepted for publication as: Reed, R.D., and M.S. Serfas. Butterfly wing pattern evolution is associated with changes in a Notch / Distal-less temporal pattern formation process. *Current Biology*

pattern that resembles ancestral intervein midline patterns. In this chapter I establish N upregulation as the earliest known event in eyespot determination, demonstrate gene expression associated with non-eyespot color patterns, and provide evidence supporting the hypothesis that intervein patterns have evolved through addition to, and truncation of, a conserved midline-to-eyespot molecular pattern formation sequence.

INTRODUCTION

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 (Nijhout 1991; Beldade and Brakefield 2002; McMillan et al. 2002). 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 (Nijhout 1991). In late last-instar wing discs, several molecules normally associated with ateroposterior and proximodistal axis specification are expressed in focal cells, and it is thought that these molecules are involved with activating the focal signal (McMillan et al. 2002). Of these focal molecules, the transcription factor Dll is of particular interest because the gene encoding it is genetically linked to eyespot size (Beldade et al. 2002). While gene expression studies have provided insight into eyespot development, little is known about how the expression patterns of the same genes may relate to the evolution and development of non-eyespot 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 (Nijhout 1991). Based on these adult phenotypes, Nijhout proposed that midline patterns are developmental precursors of circular eyespot patterns (Nijhout 1990), and that the observed gradient of intervein pattern morphologies could be explained by evolutionary changes in the timing of a common underlying developmental process (Nijhout 1991). The observation that Dll expression passes through an intervein midline stage before forming an eyespot focus (Carroll et al. 1994) increases interest in this idea, however there have been no previous reports of gene expression associated with non-eyespot color patterns to test the model. In this chapter I compare the expression of Dll and a putative upstream activator, the receptor molecule N, in a variety of butterflies and moths in order to explore the relationship between genetic pre-pattern regulation and the evolution of wing patterns.

MATERIALS AND METHODS

Wing disc staging

Butterfly wing discs were staged according to the progress of tracheal development as previously outlined (Miner et al. 2000). Specifically, Stage 0 is defined as the time point immediately following the fourth- to fifth-instar molt. Stage 1 is characterized by the initial extension of the radius trachea. Stage 1.5 is characterized by

the initial extension of the media and cubitus tracheae. Stage 2 is defined as when the distal tips of the growing tracheae have reached the border lacuna. At Stage 2.5 the costal and subcostal tracheae have grown dramatically, and the remainder of the tracheae have begun branching along the border lacuna. At Stage 3 growth of tracheoles from the primary wing vein tracheae takes place across the wing disc. I here define Stage 3.5 as being characterized by extensive tracheole growth in the wing epidermis, and the formation of a continuous line of tracheae along the border lacuna. Stage 4 represents the early pre-pupal stage wing disc, which is characterized by its very large size and slightly melanized peripodial membrane.

Immunohistochemistry

Antibody double-stains were produced using a previous protocol (Brunetti et al. 2001) with monoclonal mouse antibody C17.9C6 to the *D. melanogaster* N intracellular domain (Fehon et al. 1990), and a polyclonal rabbit antibody to the *J. coenia* Dll protein (Panganiban et al. 1995). The C17.9C6 antibody was evidently specific to the N receptor in butterflies as it has produced staining patterns consistent with N's known roles in *D. melanogaster*, including sensory cell organization (Reed 2004), wing vein patterning (unpublished), and wing margin specification (unpublished). High magnification confocal images of C17.9C6 staining in butterfly wing epithelia demonstrate signal strictly localized to apical cell membranes (see below), 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, pers. comm.), suggesting that this antibody may be effective across multiple insect orders. Anti-mouse Cy3 and anti-rabbit Cy2 secondary antibodies (Jackson Laboratories Inc.) were used for staining and samples were imaged on a confocal microscope.

Lepidoptera phylogeny

To provide an evolutionary framework for interpreting the collected gene expression patterns, I generated a supertree of lepidopteran relationships using the MinCut algorithm (Semple and Steel 2000) as modified by R.D.M. Page (Page 2002) in the software package "supertree v. 0.2.0". The topologies sampled for supertree construction were previously generated from both morphological (Minet 1991; Penz and Djunijanti 2003) and molecular data (Regier et al. 1998; Wahlberg et al. 2003). In order to process family- and superfamily-level information in our analysis, I nested genera of interest into the appropriate taxonomic categories as polytomies. For the analysis I included only taxa that were relevant to this study, with the exception of the heliconiine genus *Vindula* which I included to help resolve intra-nymphaline relationships.

The input trees were as follows: (((Papilionoidea), Bombycoidea), Gelichioidea) (Minet 1991), with insertion of nested genera as (((*Bicyclus*, *Vindula*, *Agraulis*, *Heliconius*, *Vanessa*, *Junonia*, *Pieris*), *Manduca*), *Pectinophora*); (((Nymphalidae), Pieridae), Sphingidae) (Regier et al. 1998), with insertion of nested genera as (((*Bicyclus*, *Vindula*, *Agraulis*, *Heliconius*, *Vanessa*, *Junonia*), *Pieris*), *Manduca*); (*Bicyclus*, ((*Vindula*, *Heliconius*), (*Vanessa*, *Junonia*))) (Wahlberg et al. 2003); and((*Vindula*,

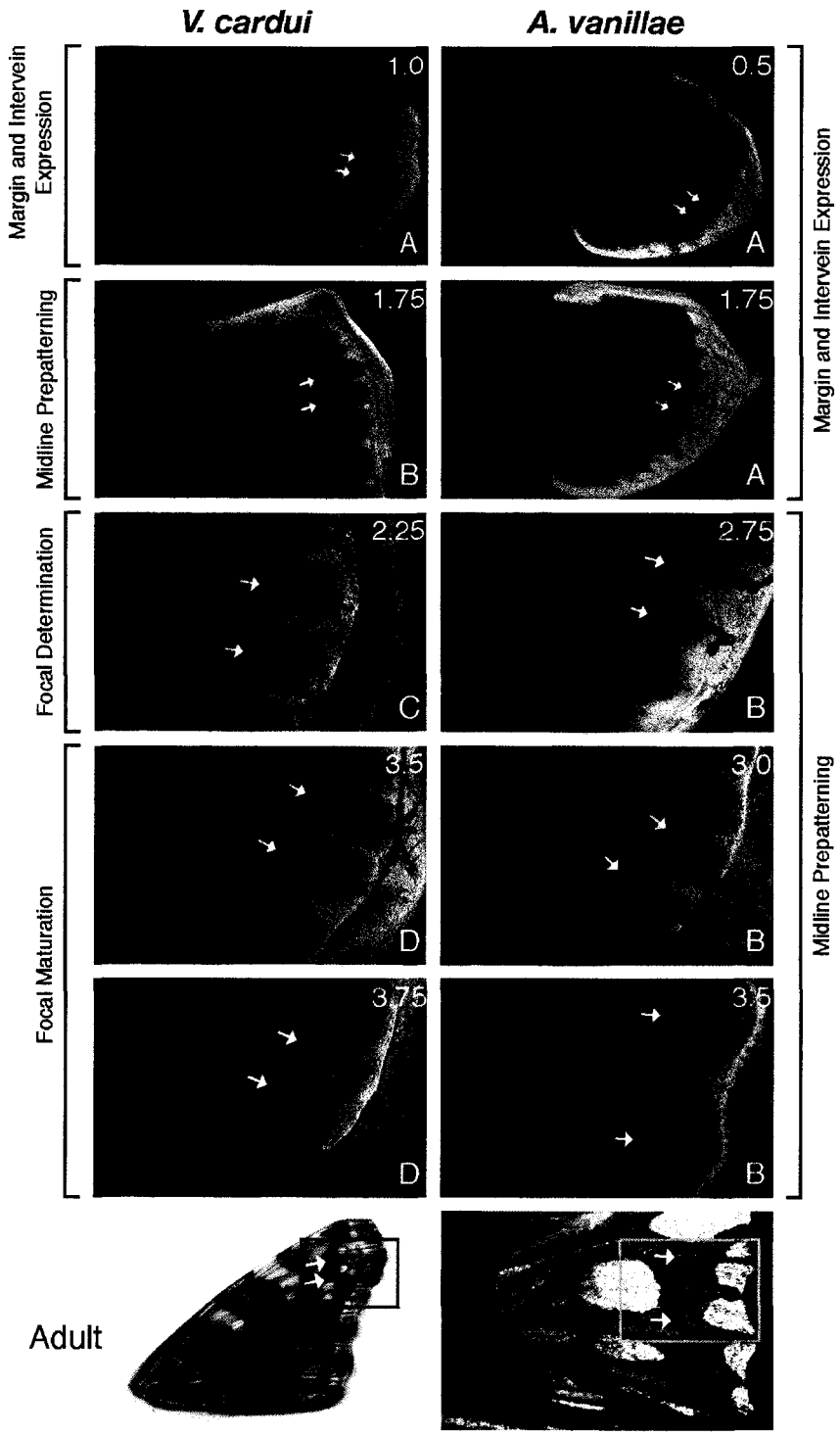
(*Agraulis*, *Heliconius*))) (Penz and Djunijanti 2003). The resulting topology was ((((*Bicyclus*, ((*Vindula*, (*Agraulis*, *Heliconius*)), (*Junonia*, *Vanessa*))), *Pieris*), *Manduca*), *Pectinophora*). The MinCut fit of our supertree was 1, reflecting the fact that there was no conflict between the sampled topologies for our taxa of interest.

RESULTS

N and Dll expression is associated with eyespot phenotype

To test for an association between N, Dll, and eyespots I examined the expression of N and Dll in late last instar wing imaginal discs of three species of eyespot-bearing nymphalid butterflies: *Vanessa cardui* (Fig. 2-1), *Junonia (Precis) coenia* (Fig. 2-2), and *Bicyclus anynana* (Fig. 2-2). In all three of these species there was a perfect correlation between presence of forewing and hindwing eyespots and late last-instar N and Dll focal expression. Confocal sections showing N expression in dorsal versus ventral eyespots on *V. cardui* show clearly that these foci were not dorsoventrally aligned (Fig. 2-3). This finding would imply that the positioning of eyespots may occur independently on the abutting dorsal and ventral wing surfaces.

Fig. 2-1 (*on following page*)Time series of N and Dll expression in the eyespot-bearing species *V. cardui*, and the intervein midline-bearing species *A. vanillae*. In the eyespot focus of *V. cardui* N upregulation occurs prior to Dll activation. In contrast to *V. cardui*, eyespot foci never form in *A. vanillae*, and the intervein midline pattern is maintained until pupation. Numbers in the upper-right panel corners indicate stages of wing disc differentiation (Miner et al. 2000). Letters in lower-right panel corners indicate stage of N pre-pattern development as coded in Fig. 2-3. White arrowheads mark homologous wing veins between panels for a particular species.


V. cardui
A. vanillae
Margin and Intervein Expression
Midline Prepatterning
Focal Determination
Focal Maturation
Margin and Intervein Expression
Midline Prepatterning
1.0
A
0.5
A
1.75
B
1.75
A
2.25
C
2.75
B
3.5
D
3.0
B
3.75
D
3.5
B
Adult

To test the genetic relationship between N and Dll expression and eyespot phenotype, I examined the effects of the *B. anynana* eyespot mutant *missing* on the expression of N and Dll. *missing* greatly reduces or eliminates eyespots in two specific hindwing-cells (Brakefield 2001). "Wing-cells" are regions of wing epithelium bounded by wing veins and the wing margin. In *missing* mutants, focal N and Dll expression was repressed in the eyespot-lacking wing-cells (Fig. 2-4). N and Dll expression is therefore genetically associated with eyespot phenotype.

N upregulation precedes Dll activation in eyespot determination

To determine the spatiotemporal relationship between N and Dll in focal determination I produced time series of N/Dll double stains from *V. cardui* (Fig. 2-1), *J. coenia* (Fig. 2-2), and *B. anynana* (Fig. 2-2). I found that focal N upregulation preceded Dll activation with a lag time of approximately 1.5 stages (equivalent to 12 to 24 hours, depending on temperature and individual variation). The spatiotemporal relationship between N and Dll may be outlined in four primary phases (Fig. 2-1).

1. *Margin and intervein expression.* N expression occurs at moderate levels across the wing disc, except for in the presumptive vein tissue (lacunae) where N is downregulated. Early during this phase Dll expression occurs only along the wing margin, but progressively moves proximally following the upregulation of N along the intervein midline.

2. *Midline pre-patterning*. In each wing-cell N is upregulated along the intervein midline, 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*. N expression is increased in foci, which is later mirrored by Dll. This stage is illustrated in better detail in Fig. 2-5, which represent time points when N has formed a focus prior to focal Dll expression.

4. *Focal maturation*. N and Dll express strongly in foci and fade 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* (Keys et al. 1999).

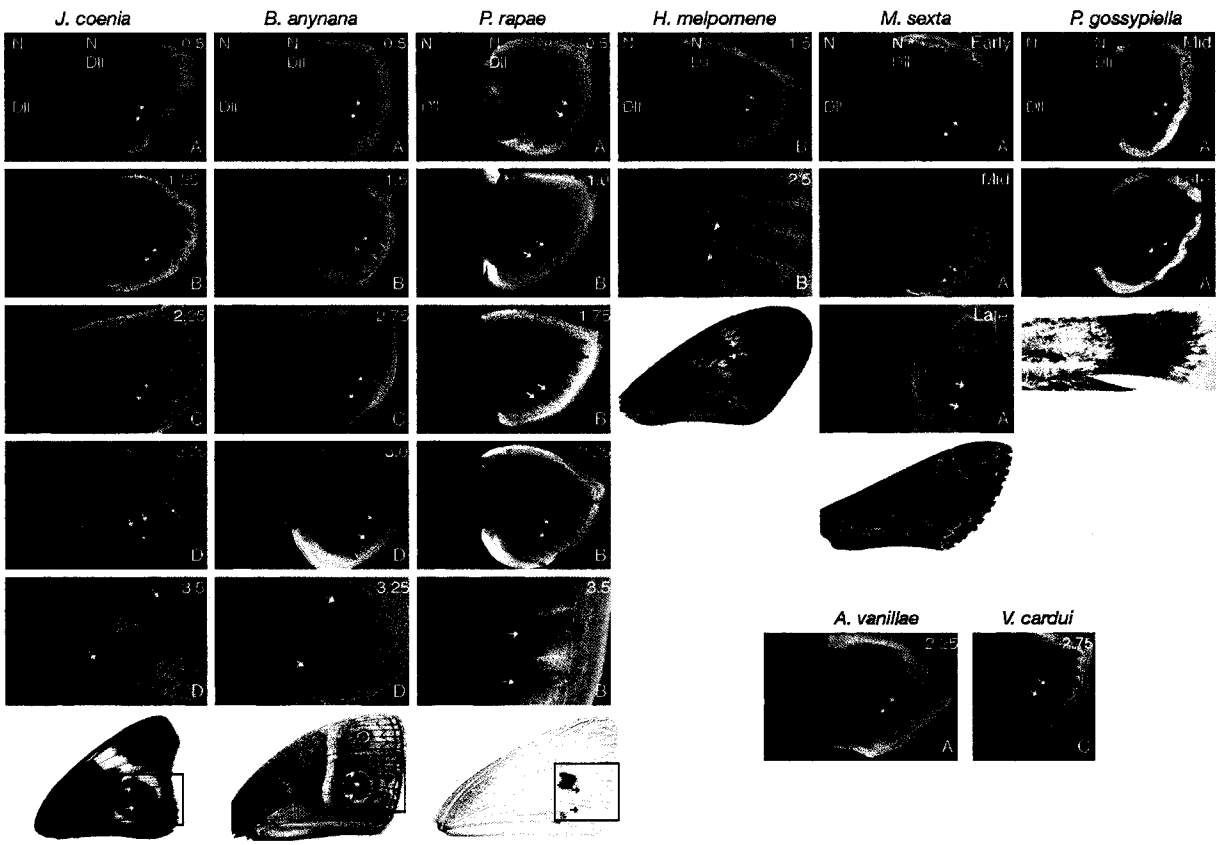


Fig. 2-2 Staged time series of N and Dll expression in *J. coenia, B. anynana, P. rapae, H. melpomene, M. sexta* and *P. gossypiella* and supplemental time points for *V. cardui* and *A. vanillae*. White arrowheads mark homologous wing veins between panels for a particular species.

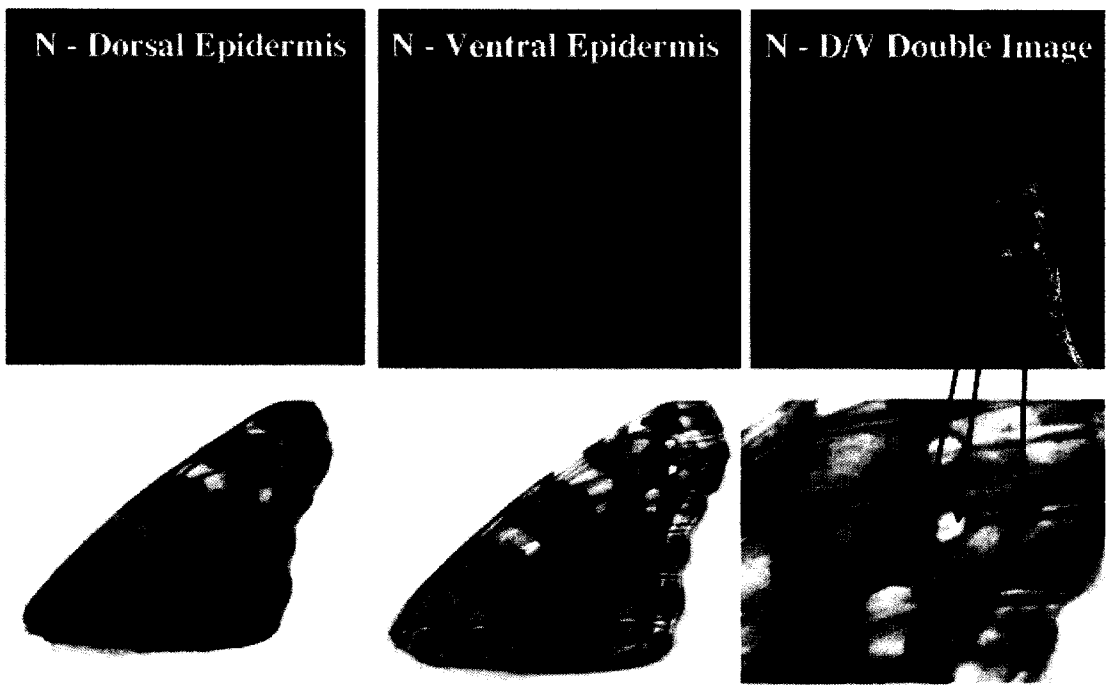


Fig. 2-3 In *V. cardui*, focal N expression is not aligned between the dorsal and ventral epithelial layers. Furthermore, N expression in the dorsal layer may possibly correspond to non-eyespot pattern elements.

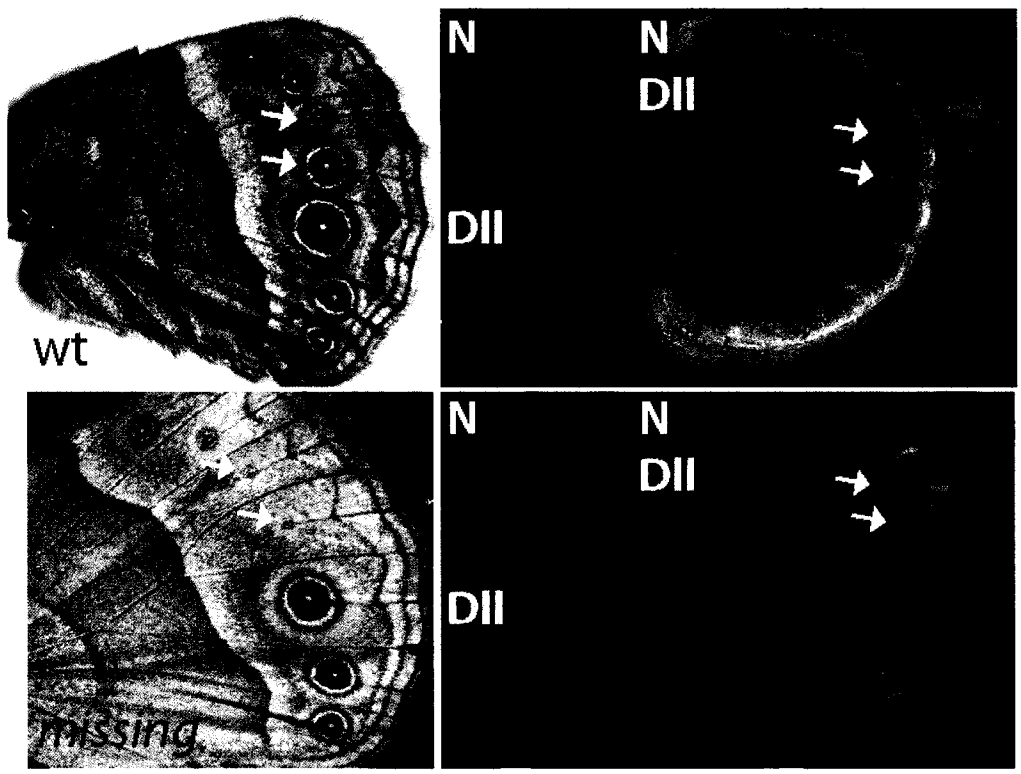


Fig. 2-4 Eyespot phenotype is associated with N and Dll expression. The *B. anynana missing* mutant has greatly reduced or missing eyespots in two adjacent hindwing cells (Brakefield 2001), along with an associated repression of N and Dll expression. Arrows mark foci with repressed gene expression in *missing* mutants.

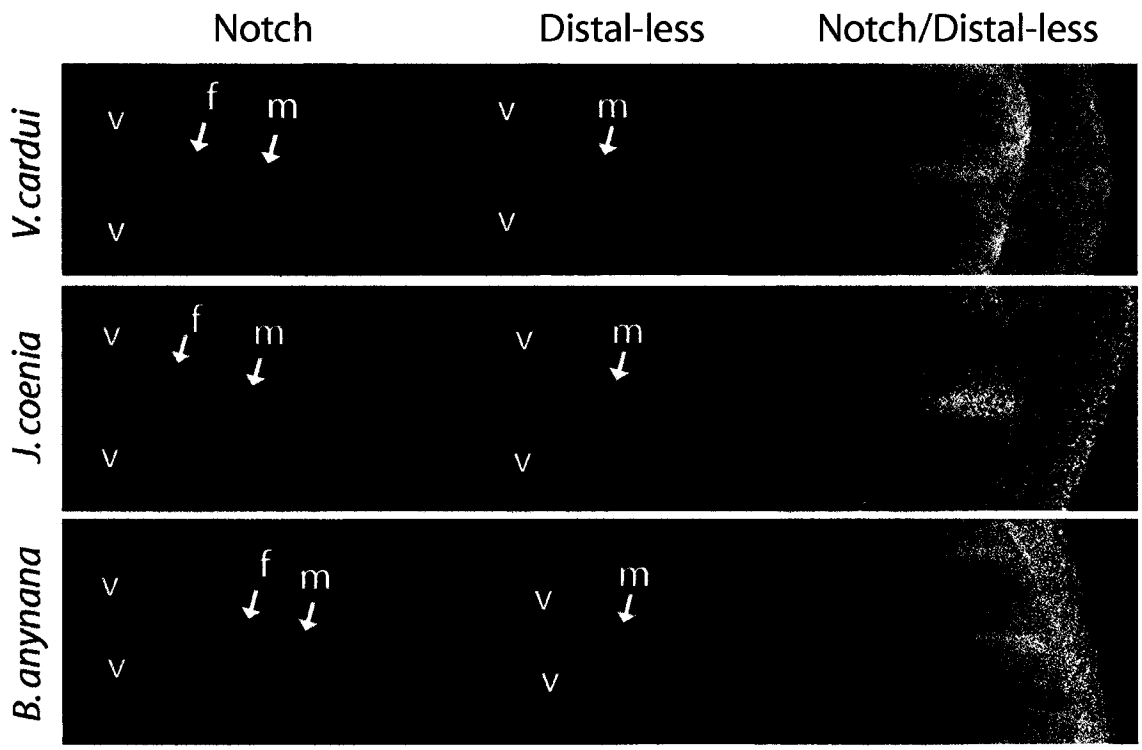


Fig. 2-5 N precedes Dll in nascent eyespot foci. These N/Dll double stains are from the focal determination stages of the three eyespot bearing butterflies also represented in Figs. 1 and 2. Darker areas labeled "v" represent presumptive wing veins, "f" marks location of foci, and "m" labels the intervein midline of gene expression.

Intracellular localization of N is associated with Dll activation

In wing discs of late fifth-instar *J. coenia* (Fig. 2-6) and *V. cardui* (not shown) discrete bodies of N localize adjacent to the nuclei of Dll expressing cells. This phenomenon is most easily observed during the focal maturation phase when N and Dll are co-localized into a discrete focus, and the intervein midline of expression has faded. At this stage, N is expressed at a relatively low level over most of the wing disc and intracellular N localization can be seen throughout regions of the wing disc where Dll expresses near the margin. In focal cells where Dll expression is especially high, however, N bodies appear to stain more intensely and are positioned more basally in epithelial cells (Fig. 2-6).

N and Delta co-localize in the eyespot focus

To test for a spatial relationship between the expression of N and its ligand Delta in the context of eyespot determination, I assayed Delta expression during focal maturation in *V. cardui* (Fig. 2-7). In Stage 2.5 wing discs (a time point when N forms a discrete focus without an intervein midline), Delta expressed in a midline with a proximal widening that appears to spatially coincide with the N focal expression. This inference of N and Delta co-expression is based on measurements of stain images from right- and left-side wing discs dissected out of the same individual. A Notch / Delta double stain would be the best way to test for co-localization, but lacking such data I must provide the caveat that the observed N and Delta domains may actually be offset slightly.

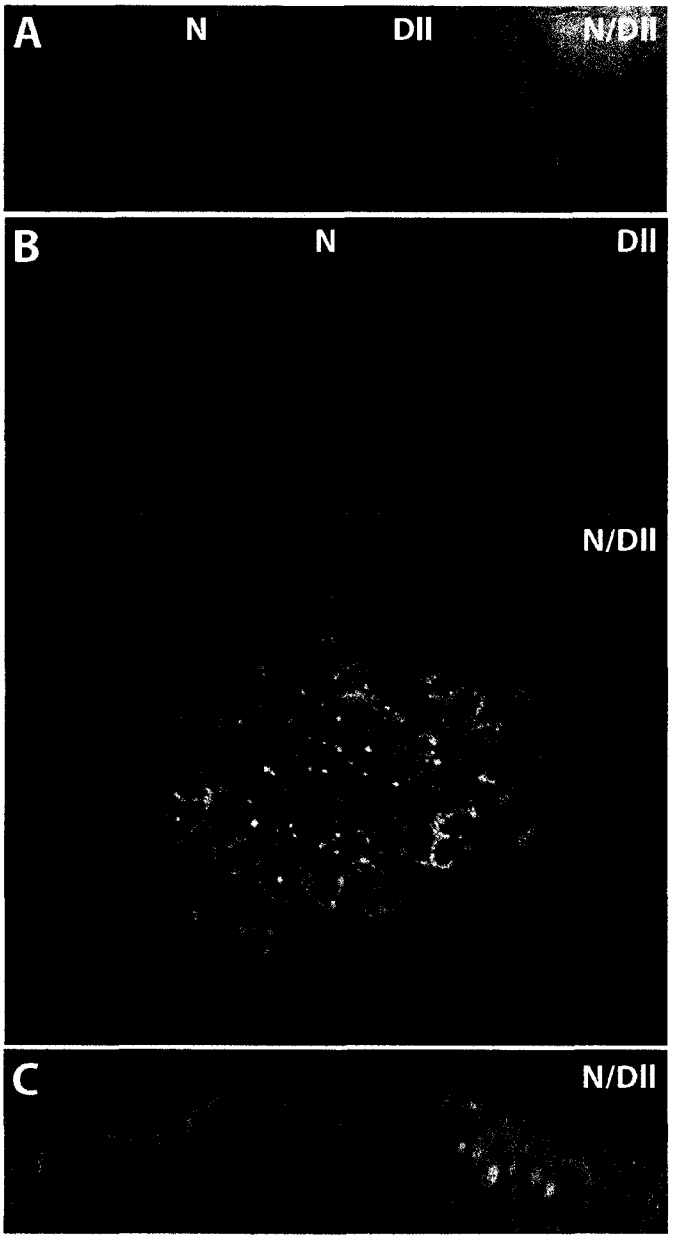


Fig. 2-6 Intracellular localization of N in Dll-expressing cells. **A.** N and Dll are co-locaized in the mature *J. coenia* focus. **B.** N localizes in discrete bodies in focal cells. This image is a confocal section of a slightly convex epithelial layer, so the center of the circle represents more basally located features such as the green Dll stained nuclei, while staining patterns towards the outside edge of the circle are located more apically in the epithelium. **C.** A three-dimensional reconstruction of a section through the eyespot focus made using a series of z-sections. Note that the N-bodies are located immediately adjacent to nuclei, and cells with brighter Dll staining have more basally located N-bodies.

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, one may nevertheless answer some questions about eyespot evolution using selected exemplar taxa. Specifically, I was interested in determining if a secondary loss of eyespots in a lineage is associated with a change in the N/Dll pre-patterning process. To address this I examined two species from the nymphalid subfamily Heliconiinae: *Agraulis vanillae* and *Heliconius melpomene* These species belong to a monophyletic subtribe called the Heliconiiti (Penz 1999; Penz and Djunijanti 2003), 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 pre-pattern phases appear similar to those in other butterflies (Fig. 2-1). In this species, however, midline definition occurs relatively slowly and development only reaches the midline pre-pattern phase by pupation. Furthermore, intervein midline gene expression does not fade as during focal maturation in eyespot-bearing species. The midline expression patterns correspond with orange midline pigment patterns on both the hindwing (Fig. 2-1) and forewing (not shown).

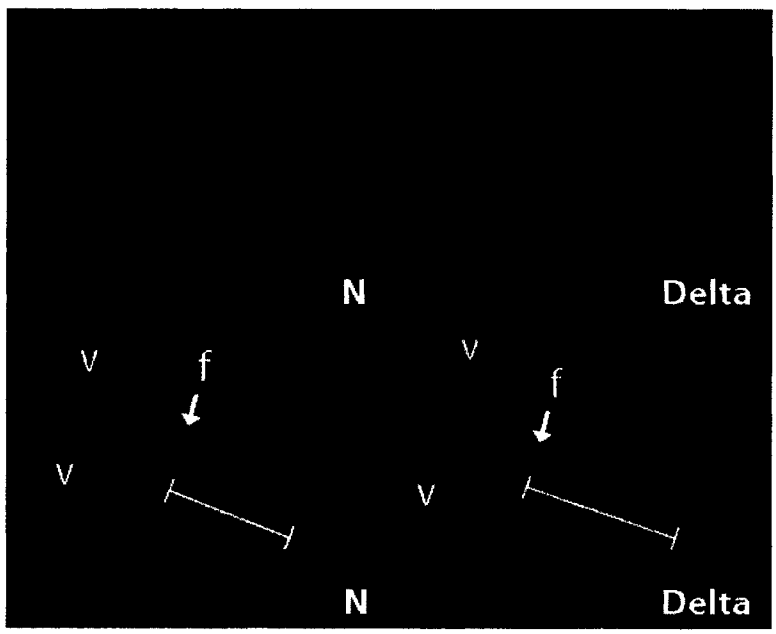


Fig. 2-7 N and its ligand Delta are co-expressed in the eyespots focus. These expression patterns are from left and right wing discs removed from the same Stage 2.5 *V. cardui.*

In last-instar *H. melpomene,* N and Dll express in intervein midline patterns (Fig. 2-2). 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 a forewing wing-cell that matches N and Dll expression (Fig. 2-2, between white arrows in *H. melpomene* panel). 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 identical between species both bearing and lacking these patterns (including *H. cydno*, *H. erato*, and *H. hecale*, unpublished data). These observations suggest that expression of the midline pigment pattern is regulated downstream of, or in parallel with, N/Dll pattern formation.

Moths lack N/Dll intervein midline expression

In order to better estimate the phylogenetic point-of-origin of N/Dll pre-patterns, I 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.* The adult color patterns of these three species appear to be relatively simple and lack any obvious eyespot or midline patterns (Fig. 2-2)

A time series of N/Dll stains in *P. rapae* (Fig. 2-2) resembles the time series from *A. vanillae,* in that N and Dll form persisting intervein midline patterns and eyespot foci never develop. Interestingly, however, *P. rapae* does not display a midline pigment pattern in the adult. Midline pigment patterns are found in many pierid species, suggesting that, as with *Heliconius*, the mechanism governing gain or loss of midline

pigment patterns may be downstream of, or in parallel with, the N/Dll prepattern system. It is arguable whether true eyespots occur among the pierids; in the family there are no patterns that consist of vein-crossing concentric circles or ovals. The black chevron on the forewing of *P. rapae* is unlikely to be a true eyespot because in some individuals the spot expands to form a dark band parallel to the proximal-distal axis of the wing. Such bands can be affected by wing veins to form patterns bearing a superficial similarity to small eyespots (Koch and Nijhout 2002). I observed no focal expression of N or Dll in the wing-cell bearing the black spot.

In the moths *M. sexta* and *P. gossypiella,* early N and Dll expression resembles initial margin and intervein expression in butterflies. In late stage *M. sexta* wing discs, Dll forms vaguely defined proximal extensions along the wing veins themselves. In *P. gossypiella* I have not detected expression of Dll in intervein tissue (Fig. 2-2). 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. As discussed below, further sampling of basal butterfly families and moth outgroups would help clarify the point of origin of the midline pre-pattern. It should be noted that published expression patterns for the monarch *Danaus plexippus* and the *B. anynana cyclops* mutant (Brakefield et al. 1996) do not show Dll midline expression, however no time series data have been published for either of these examples so it remains unknown if a midline was simply expressed earlier or later than the published time points.

DISCUSSION

Developmental implications of N and Dll expression

The gene expression patterns reported here provide useful markers for the wing pattern formation process and hint at mechanisms that may underlie these processes; however, the developmental significance of the pre-patterning sequence remains unknown. 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 non-coincidental relationship between formation of midlines and foci, however with the current data I cannot determine if the midline/focus relationship is causal or if these pre-patterns are both simply downstream of an as-of-yet unknown coordinate system. It is possible that the transient midline expression of N and Dll in some species does not have a function, but is simply an evolutionary "left-over" that appears briefly during a sort of ontogenetic recapitulation of wing pattern formation.

On a cellular level, it would be of great interest to decipher the functional regulatory relationships between N, Dll, and the other molecules implicated in eyespot development. I initially 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 (Kurata et al. 2000). N is a membrane-bound receptor that plays several roles during *D. melanogaster* wing development. Its functions best understood in this context include

defining the dorsoventral boundary (de Celis and Bray 1997; Micchelli et al. 1997), and defining intervein tissue via a lateral inhibition interaction with its ligand Delta (Huppert et al. 1997). In pupal butterfly wing discs, N-mediated lateral inhibition is thought to be involved with organizing wing scales (Reed 2004). During the lateral inhibition process N is self-activating, resulting in a feedback-assisted local increase in expression (de Celis and Bray 1997; Huppert et al. 1997; Artavanis-Tsakonas et al. 1999).

If lessons learned from *D. melanogaster* also apply to butterflies, there are several precedents for N signaling being upstream of Dll activation. Preliminary evidence for this include (1) expression of activated N is sufficient to cause expression of Dll in *D. melanogaster* imaginal discs (Kurata et al. 2000), (2) N expression precedes Dll expression in eyespot foci, and (3) N localizes intracellularly in Dll-expressing foci.

Expression of the N ligand Delta supports a reaction-diffusion model of N pattern formation

Eyespot development computer simulations based on reaction-diffusion models have supported the notion that a lateral inhibition-like process could underlie focal determination (Nijhout 1990; Nijhout 1991). Indeed, the time series of N expression from eyespot-bearing butterflies bear a superficial resemblance to Nijhout's reaction-diffusion model simulations, with a midline preceding the formation of a spot. It is enticing then to consider the possibility that N may be directly involved in a pattern formation process similar to that in the simulation studies. The Nijhout simulations consist of an activator that diffuses from wing veins and interacts with an inhibitor that begins in an evenly

distributed state. For a certain set of simulation parameters, the time course of activator distribution is very similar to the observed expression patterns of N and Dll. This similarity was previously noted for Dll (Nijhout 1994), but because N is a molecule involved with intercellular signaling it is plausible it may be functioning in a capacity similar to the simulated molecules. I attempted to assess this idea by examining the expression of the N ligand Delta during focal determmination.

There are two primary N ligands described from *D. melanogaster*: Delta and Serrate (Artavanis-Tsakonas et al. 1999). While I am unaware of any existing antibodies that recognize Serrate in Lepidoptera, a monoclonal antibody to the *D. melanogaster* Delta extracellular domain (Qi et al. 1999) produced consistent and repeatable staining patterns in butterfly wing discs. If N and Delta were interacting in a strict lateral inhibition relationship, it would be expected that their respective domains of expression would be relatively exclusive of one another. Alternatively, if the two molecules were associated with activator- and inhibitor-like qualities as in the Nijhout model, their two expression patterns would be spatially similar. In fact, the observed N and Delta expression patterns are consistent with the latter of these models (Fig. 2-7). Finding that the N and Delta domains both show a superficial resemblance to the Nijhout model raises questions about how the N / Delta system may work to produce reaction-diffusion like patterns. Recent evidence suggesting that Delta may participate in long-distance signaling through filopodial extensions (Joussineau et al. 2003) provides a possible lead for explaining the significance of N and Delta expression in butterfly wings.

Intracellular localization of N

The observation of intracellular N localization (Fig. 2-6) was an unexpected result of this work. To my knowledge, there have been no previous reports of N localizing in a discrete, nucleus-associated body in any organism. There are several possible explanations for this observation. The canonical model of N function requires that the N intracellular domain be cleaved upon activation and translocated to the nucleus to activate transcription of target genes (Artavanis-Tsakonas et al. 1999). The antibody used in this study recognizes the N intracellular domain, and it is plausible the localization of signal near the nucleus is associated with translocation of cleaved N. This scenario would be even more plausible if it was shown that Dll transcription is a directly activated by N signaling. Other possible explanations might be that the staining represents N being synthesized in the endoplasmic reticulum, or that N is being sequestered intracellularly for an unknown purpose. Further work is required to distinguish between these possibilities. When manipulative genetic techniques in butterflies such as transformation, ectopic gene expression, and gene knockouts are developed, there are a host of molecular developmental hypotheses to test.

Evolution of a character system

In Fig. 2-8 I present the pattern development data in the context of lepidopteran phylogeny and draw several conclusions regarding the evolution of intervein pre-patterning in butterfly wings. First, the temporal order of N/Dll expression states is conserved in all the taxa examined. Also, as outlined above, the formation of a discrete

intervein midline appears to be a synapomorphy of the sampled butterflies. What, then, may one conclude about the origin of focal gene expression? Given the 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 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 I would favor the latter model because of the high frequency of eyespots throughout nymphalid lineages unsampled by our study, especially basal heliconiines like *Vindula.*

In a phylogenetic context the gene expression data fit into a body of evolutionary literature concerning "heterochrony": the study of how changes in developmental timing may underlie morphological evolution. In particular, the data suggest three heterochronic events that have occurred in the evolution of the wing pre-patterning sequence: (1) the terminal addition of the intervein midline to the sequence, (2) the terminal addition of the focal determination and focal maturation stages to the sequence, (3) the loss in the Heliconiiti of the focal determination and maturation phases. The first two of these events would be considered "acceleration", where the earlier states of development have been shortened to compensate for the terminal addition of a new ontogenetic state. The secondary loss of focal expression states in the heliconiiti would be an example of "retardation", or neoteny, where the timing of ontogenetic states has been delayed relative to pupation, and the terminal gene expression states are no longer expressed.

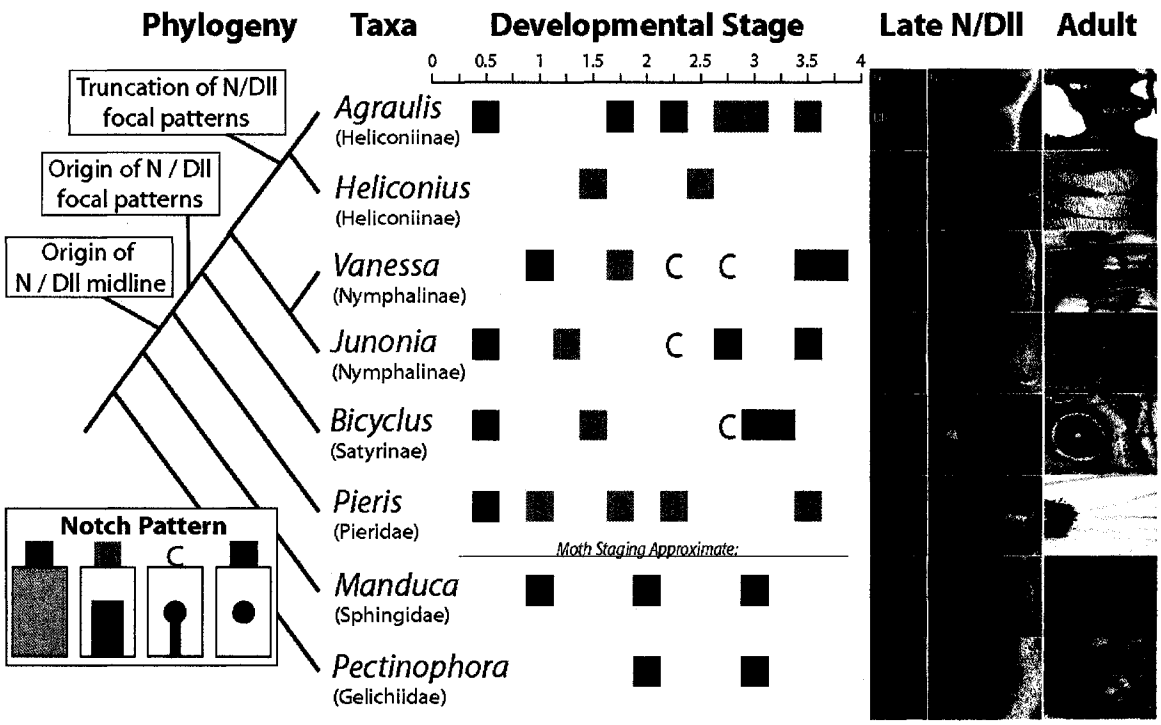


Fig. 2-8 Changes in the N and Dll pattern formation process are associated with the evolution of wing pattern elements. Colored boxes marked A through D represent the state of N expression at the sampled time points. Intervein midline gene expression is a derived characteristic of the lineage leading to the butterflies, and focal gene expression appears as a terminal addition to pre-patterning 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. Our tree is a strict consensus of previous higher-level moth and butterfly phylogenies (Nielsen 1989; Regier et al. 1998; Kristensen and Skalski 1999; Wahlberg et al. 2003). All nodes in our phylogeny have been well supported by morphological and/or molecular data and are not considered contentious

I would speculate on two possible mechanisms for the heterochronic shift of N/Dll prepatterning in the heliconiiti. One possibility is that a decrease in the strength or diffusion rate of self-inductive morphogenic signals has resulted in a retardation of pattern formation. In this "local neoteny" scenario (McKinney and McNamara 1991) predicted by the Nijhout model, the pre-patterning sequence is composed of dependent ordered states. Alternatively, there may have been regulatory changes in the heliconiiti that specifically repress the focal determination and maturation stages and lengthen the intervein midline stage. This model would not necessitate that stages in the sequence be functionally dependent on each other. Most heterochronies are speculated to be of this latter regulatory type (West-Eberhard 2003).

The theory of heterochrony, which traces back to Haeckel (Haeckel 1866) and was expanded formalized by de Beer in 1930 (de Beer 1930), is fundamental to the study of morphological evolution (de Beer 1930; Gould 1977; McKinney and McNamara 1991; McNamara 1995; West-Eberhard 2003). Despite the description of many morphological examples of evolutionary heterochrony there are few data on the molecular basis of this phenomenon, particularly in a phylogenetic context (Wray and McClay 1989; Kim et al. 2000; Skaer et al. 2002). The data presented in this chapter offer a potential case study in how heterochronic changes in a conserved molecular pattern formation system may be associated with evolutionary changes in a discrete morphological character.

CONCLUSION

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 pre-patterning process supports earlier models that these intervein pattern elements are produced by a common developmental circuit. 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 evolutionary heterochrony at the level of molecular pattern formation. In sum, these data illustrate how the evolution of a discrete character may occur through terminal addition to, and truncation of, a conserved molecular developmental sequence.

ACKNOWLEDGMENTS

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mutant. This work was supported by the U.S. National Science Foundation grant DEB 0209441.

CHAPTER 3

EVIDENCE FOR NOTCH-MEDIATED LATERAL INHIBITION IN ORGANIZING BUTTERFLY WING SCALES*

SUMMARY

In this chapter I present gene expression data that implicate a Notch mediated lateral inhibition process in the spatial organization of butterfly wing scales. During early pupal development the receptor molecule Notch is expressed in a grid-like pattern in the wing epithelium, resulting in parallel rows of uniformly spaced cells with low Notch expression. Previous work has shown that these low-Notch cells express a homolog of the proneural transcription factor *achaete-scute* and develop into scales. All of these observations are consistent with the *Drosophila* model of Notch mediated bristle determination and support the hypothesis that bristles and scales share an underlying patterning mechanism.

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INTRODUCTION

The majority of moth and butterfly wings are covered with thousands of flat, overlapping scale cells (Fig. 3-1A). These scales may serve multiple functions including thermoregulation, pheromone dispersal, and color pattern formation (Scoble 1992). Mayer (Mayer 1896) noted the developmental similarity between lepidopteran scales and insect sensory bristles and concluded that the two structures are homologous, an hypothesis supported by subsequent observations (Overton 1966; Wigglesworth 1972; Galant et al. 1998). There is molecular evidence for scales being evolutionarily derived from bristles in that a homolog of the *achaete-scute* (*ac-sc*) transcription factors is expressed in scale forming cells (SFCs) of the nymphalid butterfly *Junonia (Precis) coenia* (Galant et al. 1998). *ac-sc* expression promotes the determination of neural precursor cells, including bristle precursors, in *Drosophila melanogaster* (Calleja et al. 2002), and the expression of an *ac-sc* homolog in SFCs suggests that bristles and scales share an underlying specification mechanism (Galant et al. 1998). In this report I explore the possibility that scales and bristles may be spatially organized through a shared mechanism.

In most butterfly species, wing scales occur in parallel rows (Nijhout 1991). Perhaps not coincidentally, notal bristles of *D. melanogaster* and many other muscomorph flies are also spaced in parallel rows (Simpson et al. 1999). Bristle spacing in *D. melanogaster* is organized through lateral inhibition activity of the Notch signaling

pathway (Artavanis-Tsakonas et al. 1999; Simpson et al. 1999). In this process, cells expressing Delta, the membrane-bound Notch ligand, activate the Notch receptor in neighboring cells thereby triggering a positive feedback increase of Notch expression in those neighboring cells. The resulting pattern consists of spaced, isolated Delta-expressing cells surrounded by Notch-expressing cells. Because Notch activation results in repression of *ac-sc*, only the central Delta-expressing cells express *ac-sc* and are specified as neural precursors. On a larger scale, this process contributes to generating a pattern of bristles spaced in rows on the fly's notum. Significantly, detailed mathematical models have demonstrated that a Notch-like lateral inhibition process is sufficient to explain the morphological differentiation of butterfly SFCs during early pupal development (Honda et al. 2000).

Because of (1) the common ancestry of wing scales and sensory bristles, (2) the similar requirements of scales and bristles to be patterned on an epidermal monolayer, (3) the expression of the Notch target *ac-sc* in SFCs, and (4) the applicability of lateral inhibition models to patterns of SFC differentiation, it was apparent that Notch signaling was a strong candidate for playing a role in scale organization. To assess this I examined the expression of Notch in butterfly wings during early SFC determination.

MATERIALS AND METHODS

Immunohistochemistry

Antibody stains were performed on forewing tissue of 16h pupal *Heliconius erato petiverana* (Nymphalidae) using a previous protocol (Brunetti et al. 2001). Antibodies for the double stain were a mouse monoclonal antibody C17.9C6 that recognizes the Notch intracellular domain (Fehon et al. 1990) and a rabbit polyclonal antibody that recognizes the transcription factor Distal-less (Panganiban et al. 1995). Secondary staining was done using Cy3 conjugated goat anti-mouse and Cy2 conjugated goat anti-rabbit antibodies (Jackson Laboratories). Slide mounted samples were visualized on a confocal microscope.

In larval moth and butterfly wing discs, the C17.9C6 Notch antibody localizes in patterns consistent with Notch's known role in vein and margin patterning (unpublished observation). Furthermore, high magnification images from larval wing disc stains show C17.9C6 localization on the apical cell membranes, consistent with Notch's identity as a membrane-bound receptor (unpublished observation). Other preliminary data show that the C17.9C6 Notch antibody stains developing grasshopper legs in patterns similar to those described from *Drosophila* (B. Blachuta, pers. com.). Together, these observations suggest that the C17.9C6 Notch antibody effectively recognizes the Notch molecule across multiple insect orders.

The use of a Distal-less antibody as a nuclear marker in this study was possible because the Distal-less protein is expressed over a portion of the *H. erato* pupal wing

epidermis. Preliminary data suggest that this expression may be associated with the adult color pattern, but further work is required to establish this with certainty.

Scanning electron microscopy

For the scanning electron micrograph a melanic portion of an adult *H. erato* forewing was dissected from a deceased butterfly. Scales were carefully brushed off some of the wing membrane to reveal the underlying socket arrangement. The sample was mounted dorsal side-up on an aluminum stub with adhesive tape and then sputter coated with 30nm of gold. The sample was visualized on a scanning electron microscope at 170x magnification and the image was captured on Polaroid film.

RESULTS AND DISCUSSION

Notch expression is consistent with a lateral inhibition model

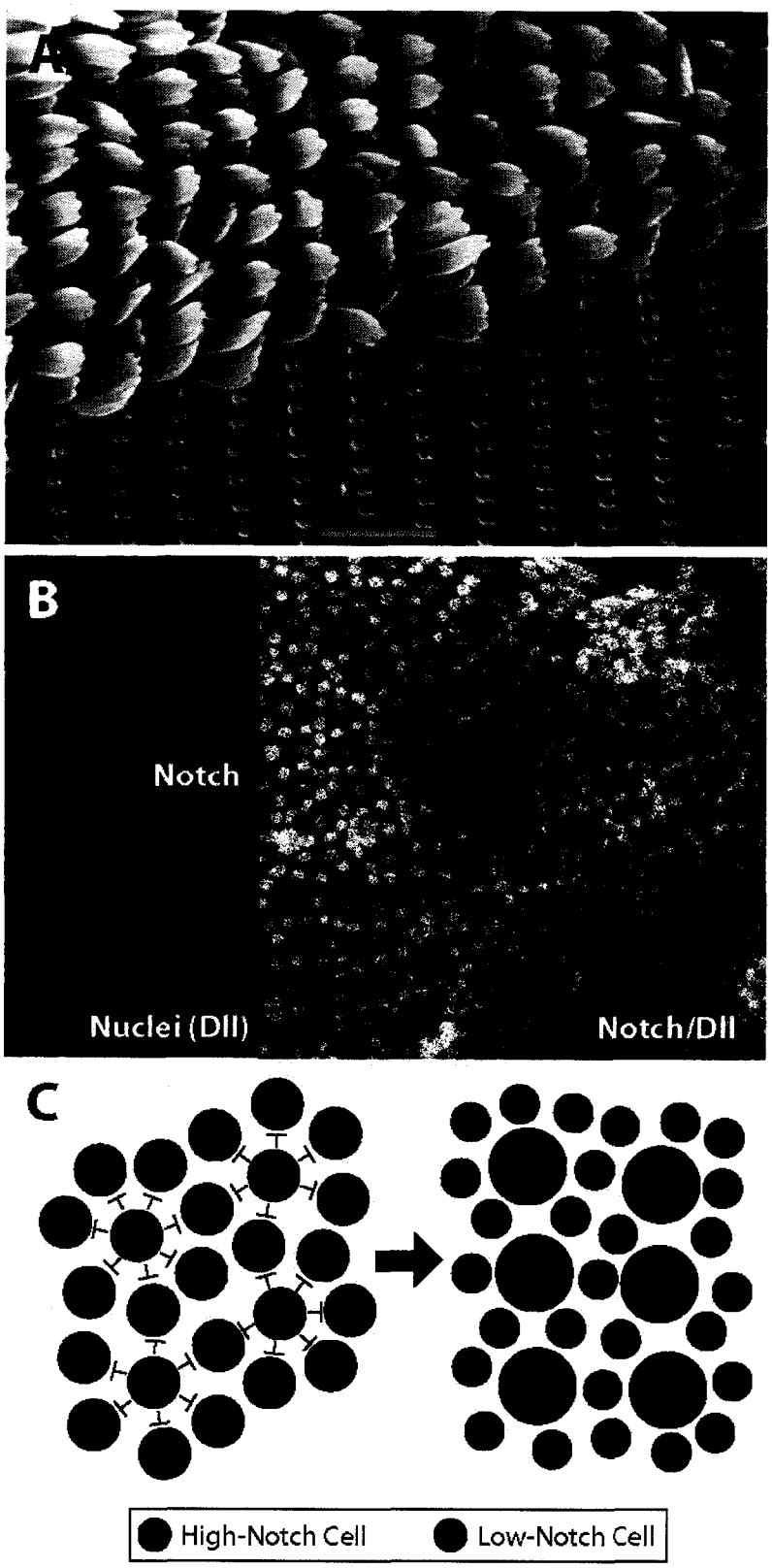
16h after pupation, when SFCs are known to be determined in nymphalid butterflies (Galant et al. 1998), Notch is upregulated in a grid-like pattern in the butterfly wing epidermis (Fig. 3.1B). This results in parallel rows of uniformly spaced cells with downregulated Notch expression. Consistent with molecular and theoretical models of lateral inhibition (Simpson et al. 1999; Honda et al. 2000), low-Notch cells never neighbor each other and are always encircled by 7-10 high-Notch cells. The low-Notch cells also have larger nuclei and diameters than the high-Notch cells (inferred from the nuclear stain, Fig. 3-1B), unambiguously identifying these cells as the nascent polyploid

SFCs (Nijhout 1991; Galant et al. 1998). While the grid-like pattern is consistent across the wing epidermis, there is variation in the specific number of cells surrounding each SFC, indicating a certain level of stochasticity in the SFC patterning process. It would be predicted that a membrane bound Notch ligand such as Delta is expressed in the low-Notch cells, however this could not be determined because a Delta marker has not yet been developed for butterflies.

The observed Notch expression pattern is suggestive of a process that may produce a repetitive and highly ordered arrangement of specified cells from a morphologically undifferentiated epithelium. In a pure lateral inhibition model the degree to which SFCs are organized in parallel rows appears to be primarily dependent upon two variables: (1) the size of the nascent SFCs during the lateral inhibition process, and (2) the ratio between the number of SFCs and their surrounding undifferentiated cells (Honda et al. 2000). Although Honda et al. (2000) did not thoroughly explore the relationship between parameter space and the emergent parallel organization of SFCs, their simulation with the lowest values for the two above parameters produced a SFC pattern similar to that observed in Fig. 3-1B. It is worth noting that differentiation of SFCs appears to occur simultaneously across the wing, ruling out the possibility that developmental asymmetry between wing regions is a mechanism for row formation.

In sum, the association between Notch expression and previous theoretical, developmental, and gene expression studies strongly implicates a Notch mediated lateral inhibition process in the spatial organization of SFCs in butterfly wings (Fig. 3-1C).

Fig. 3-1 (*on following page*) Notch expression supports a lateral inhibition model of butterfly wing scale determination. **A** Scales in most butterflies are arranged in parallel rows, as seen in this electron micrograph of a *Heliconius erato* forewing. Scales towards the bottom of the micrograph have been brushed off to reveal the parallel arrangement of sockets. Both scales and sockets are derived from a mother scale forming cell (SFC) during pupal development. **B** 16h after pupation, during early SFC differentiation, Notch is upregulated in a grid across the wing epithelium of *H. erato.* The cells with low Notch expression are nascent SFCs. Nuclei are stained by an antibody to the transcription factor Distal-less. **C** The grid-like Notch expression pattern is consistent with the lateral inhibition model of neural precursor determination from *D. melanogaster,* and with the previously described expression of *ac-sc* in SFCs 24h after pupation in the butterfly *J. coenia.* In this hypothetical model, low-Notch cells inhibit expression of *ac-sc* genes in neighboring cells while uninhibited *ac-sc* expression in low-Notch cells results in an SFC fate.


A
B
Notch
Nuclei (Dll)
Notch/Dll
C
High-Notch Cell
Low-Notch Cell

Scale and bristle organization: a developmental basis for parallel evolution?

In most moths, wing scales are not organized in any apparent pattern beyond being evenly spaced (Nijhout 1991). Most parameter combinations applied to the Honda et al. (2000) lateral inhibition model produce a semi-random arrangement of SFCs reminiscent of ancestral moth scale patterns. Pending Notch expression data from moths, it is reasonable to speculate that in moths an ancestral Notch pattern generator produces an evenly spaced, but otherwise unorganized, arrangement of SFCs. Subsequently, selection may have acted on "parameters" of SFC development to tune the lateral inhibition process to produce parallel rows as in butterflies.

There are several unrelated butterfly lineages in which scales do not form parallel rows (Nijhout 1991), suggesting that the scale patterning system may be evolutionarily labile. Furthermore, there is cryptic variation between lepidopteran families in early SFC development. In the nymphalids *J. coenia* and *H. erato,* SFCs occur in distinct parallel rows when they are first differentiating (Fig. 3-1B; (Nijhout 1991; Galant et al. 1998). In contrast, initial SFC differentiation in the pierid *Pieris rapae* appears to occur in a spatially random pattern, with SFCs only later becoming arranged into rows through an as-of-yet unknown process (Yoshida and Aoki 1989; Honda et al. 2000). In the sphingid moth *Manduca sexta,* one of the minority of moths with parallel scales, SFCs become arranged into rows through cell migration (Nardi and Magee-Adams 1986). Inferring the evolutionary history of lepidopteran scale patterning awaits a more thorough phylogenetic treatment, but ultimately may provide an excellent context for studying the evolution of a patterning system.

On a broader scale, the evolutionary transition(s) from random to parallel scales on the lepidopteran wing is remarkably similar to the transition from random to parallel bristles on the dipteran notum (Simpson et al. 1999). Another similarity between bristle and scale evolution is the derivation of row spacing. Aligned SFCs in *P. rapae* and *H. erato* are spaced by only one or two cells, which is likely the ancestral condition. In contrast, aligned SFCs in *J, coenia* are spaced by two to four cells (Nijhout 1991; Galant et al. 1998; Koch et al. 2003). This presumably derived state in *J. coenia* is similar to the spacing of fly bristle rows by four to five cells. How rows may become spaced in the context of Notch mediated lateral inhibition is unknown.

The similar sequence of innovations in the evolution of bristle and scale pattern development is striking, and is suggestive of parallel evolution. This begs the question: can the mechanistic qualities of a conserved signaling pathway promote the evolutionary re-emergence of stereotyped, but non-homologous, patterns?

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CHAPTER 4

EVOLUTIONARY REDPLOYMENT OF A BIOSYNTHETIC MODULE: EXPRESSION OF EYE PIGMENT GENES *CINNABAR*, *VERMILION*, AND *WHITE* DURING BUTTERFLY WING DEVELOPMENT

SUMMARY

Ommochromes occur ubiquitously among insects as visual pigments; however, in some insect lineages ommochromes have evolved novel functions such as integument coloration and tryptophan secretion. Of interest here is the role of ommochromes in butterfly wing pigmentation, a role that can be traced to a single origin in the family Nymphalidae. The synthesis and storage of ommochrome pigments is a complex process that requires the concerted activity of multiple enzyme and transporter molecules, and it is worthwhile to understand how such a subcellular process may, through evolution, occur in a novel context. To this end, I have undertaken work to understand the regulation of ommochrome genes in developing butterfly wings. In this study I verify the presence of the ommochrome xanthommatin in the wings of the nymphalid *Vanessa cardui*. From this species I then cloned fragments of two ommochrome enzyme genes, *vermilion* and *cinnabar*, and an ommochrome precursor transporter gene, *white*. I found that these genes were transcribed in pupal wings at high levels during early scale cell development. I propose that the evolution of ommochrome synthesis in butterfly wings arose in part through novel regulatory elements that lead to different patterns of

activation of *vermilion*, *cinnabar*, and *white* in wing tissue. I conclude this chapter with a model of ommochrome development in butterfly wing scales.

INTRODUCTION

Butterfly wing patterns have long served as a model for studying the ecology, evolution, and development of morphological traits (Nijhout 1991; Beldade and Brakefield 2002; McMillan et al. 2002). Over the last decade, work on a number of genes involved in developmental regulation and cell signaling have shed light on the early stages of molecular pattern formation in butterfly wings, especially in the context of eyespot color patterns (Carroll et al. 1994; Brakefield et al. 1996; Keys et al. 1999; Brunetti et al. 2001; Beldade et al. 2002; Koch et al. 2003; Reed and Serfas 2004). In contrast, however, comparatively little is known about the regulation of the pigment synthesis genes presumed to be downstream of patterning genes.

Four major classes of butterfly wing pigments have been identified: melanins, flavonoids, pterins, and ommochromes (Nijhout 1991). Each of these classes contains pigments of multiple different colors. To date there are butterfly gene expression data for only two pigment synthesis enzymes related to the melanin and pterin classes, respectively. In the swallowtail butterfly *Papilio glaucus,* transcripts for the melanin enzyme dopa decarboxylase (DDC) are spatially localized to melanic pattern elements late in pupal development (Koch et al. 1998). Likewise, transcripts for the pterin synthesis enzyme GTP CH 1 are localized coincident with presumptive pterin pigment patterns during pupal wing development in *Junonia (Precis) coenia* (Sawada et al. 2002).

The finding that the transcription of enzyme-coding genes is spatially correlated with enzyme activity and pigment synthesis strongly suggests that pigmentation patterns are determined in part by local transcriptional regulation of pigment synthesis genes. Furthermore, the observation that during early pupal development, spatial expression of the transcription factors Distal-less, Engrailed/Invected, and Spalt correlates with adult pigment patterns (Brunetti et al. 2001) makes it reasonable to speculate that these transcription factors may be intimately involved with the activation of pigment synthesis genes.

Pigment synthesis is a highly orchestrated process requiring the activity of multiple transporter and enzyme molecules, and it is of interest to know how such a complex biochemical process might fall under the control of a set of developmental patterning genes during evolution. Butterfly wing patterns provide an opportunity to survey, at multiple levels, how patterning and effector genes may develop new interactions to produce novel morphologies. To exploit this opportunity, I here initiate work examining the expression of genes implicated in ommochrome pigment synthesis in butterfly wings.

Ommochrome synthesis in a fly's eye

Ommochromes are widespread among insects, and they are utilized primarily as eye pigments (Linzen 1974). Fortunately, a genetic understanding of ommochrome synthesis in *Drosophila* eyes provides candidate genes and a preliminary model to explore in butterfly wings (Fig. 4-1).

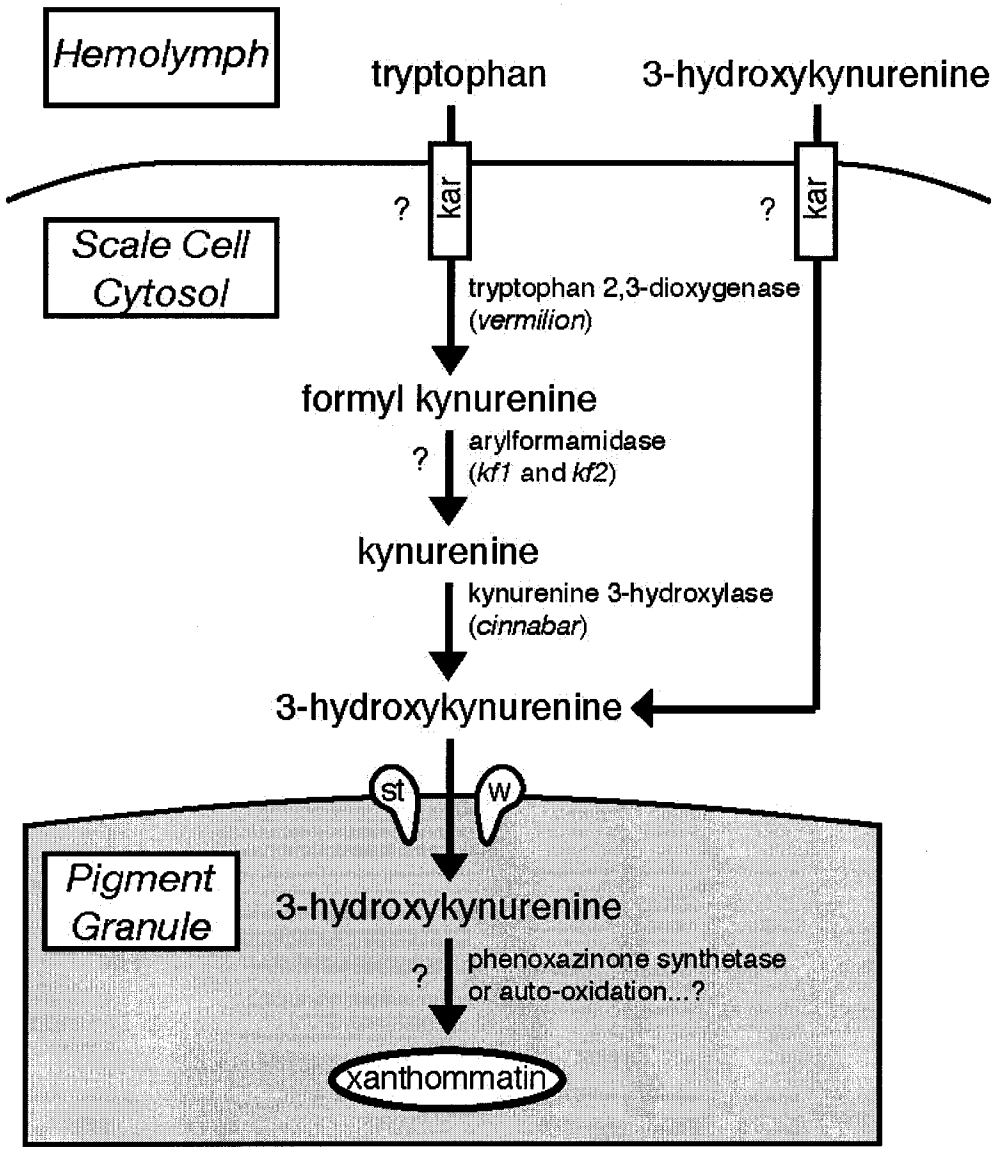


Fig. 4-1. A model of ommochrome synthesis in butterfly wing scales. This model is based primarily on work from ommochrome synthesis in *D. melanogaster* eyes. A more detailed justification of each element in the model is provided in the discussion. Question marks denote highly speculative aspects of the model. "w" and "st" are abbreviations for the transporter molecules white and scarlet.

The first step in ommochrome synthesis is establishing a supply of the amino acid precursor tryptophan. Work done on ommochrome development in *D. melanogaster* eyes implicates the aromatic amino acid transporter karmoisin in this process (Sullivan et al. 1974; Tearle 1991; Dow 2001). Functional studies on TAT1, the putative mammalian ortholog of karmoisin, suggest that this transporter is located on cell membranes and efficiently transports aromatic amino acids, including tryptophan and similar molecules (Kim et al. 2001).

The processing of tryptophan into an ommochrome pigment occurs in several steps. First, the *vermilion* gene product, tryptophan 2,3-dioxygenase (EC1.13.11.11), catalyzes the initial conversion of tryptophan to formylkynurenine. Next, it is believed that in *D. melanogaster* the hydrolysis of formylkynurenine into kynurenine is catalyzed by arylformamidase (also known as kynurenine formamidase, EC 3.5.1.9) (Moore and Sullivan 1978). Unfortunately, the sequences for the putative *D. melanogaster* arylformamidase genes *kf1* and *kf2* have not yet been characterized. The hydrolysis of formylkynurenine into kynurenine is known to occur spontaneously (Linzen 1974), so it is plausible that arylformamidase may not actually be required for ommochrome synthesis. Interestingly, arylformamidase mutants have never been described from *D. melanogaster*, and sequences resembling arylformamidase genes known from other species are not evident in BLAST searches of the *D. melanogaster* genome database.

Kynurenine 3-monooxygenase (EC 1.14.13.9), encoded by the *cinnabar* gene, catalyzes the transformation of kynurenine into 3-hydroxykynurenine (3-OHK). The *in vivo* mechanism of the transformation of 3-OHK into xanthommatin is poorly

understood. Isolated fractions from *D. melanogaster* eyes (Phillips et al. 1973) and butterfly wings (Nijhout 1997) that catalyze this transformation have suggested the possible existence of a specific phenoxazinone synthetase enzyme. In contrast to this view, however Linzen (1974) argues that the formation of xanthommatin may occur through non-specific activity in systems of high oxidation potential. A gene encoding phenoxazinone synthetase has not yet been characterized from an insect, and the *D. melanogaster* genome shows no obvious signatures of such a gene. Whatever the identity of the catalyst for xanthommatin formation may be, its activity is associated with the pigment granules themselves (Phillips et al. 1973).

Delivery of ommochromes and/or their precursors into pigment granules involves activity of the white/scarlet transporter. white and scarlet form a heterodimer ATP-binding cassette ("ABC") transporter that localizes to the surface of pigment granules in *D. melanogaster* ommatidia (Mackenzie et al. 2000). This transporter has been implicated in tryptophan and kynurenine uptake (Sullivan and Sullivan 1975; Sullivan et al. 1980), and may also be involved in 3-OHK uptake (Howels et al. 1977; Mackenzie et al. 2000). It remains unknown exactly which precursor molecule(s) white/scarlet transports into *D. melanogaster* pigment granules, or which biosynthetic steps take place in the granules versus the cell cytoplasm.

Although ommochrome pigments have been chemically identified in some butterfly wings (Linzen 1974; Gilbert et al. 1988; Nijhout 1991; Nijhout 1997), little is known about the genetic basis of ommochrome synthesis in scale cells. In order to test the applicability of the *D. melanogaster* model to butterfly wings, I set out to determine if

key elements of this model may be found in butterfly wings. In this study I identified xanthommatin as a primary ommochrome pigment found in red wing scales of the nymphalid butterfly *Vanessa cardui* and verified expression of the *vermilion*, *cinnabar*, and *white* genes in developing wings using quantitative PCR and *in situ* hybridization. With these data in mind, I extended the *D. melanogaster* model of eye ommochrome synthesis into the context of butterfly wing development.

MATERIALS AND METHODS

RNA extraction

V. cardui larvae and artificial diet were obtained from Carolina Biological Supply Company. Pupal wings and larval imaginal discs were dissected in phosphate buffered saline (pH = 6.8). Wing tissue was either immediately transferred to cold Trizol (Gibco) and homogenized for immediate RNA extraction, or briefly stored in the transcript preservativr RNA*later* (Ambion Inc.) at –20°C prior to RNA extraction. Total RNA was extracted using Trizol and subsequently purified using RNeasy columns (Qiagen). Total RNA was run on a gel to verify the absence of degradation products.

Thin layer chromatography

The method used for thin layer chromatography was based on Nijhout (1997). Red portions of dried adult *V. cardui* wings were dissected and pooled. This tissue was homogenized and sonicated in acidified methanol (0.5% HCl). Tissue slurries were

centrifuged for 5 min at 14k rpm, forming a pellet of wing scales and membrane tissue. The supernatant was dried in a speed-vac for 1 hr at 45°C, and the pellet was resuspended in methanol. The resuspended sample, as well as the references amaranth, bromphenol blue, napthol blue black, and 3-hydroxykynurenine were spotted onto a silica gel 60 F254 thin layer chromatography plate (Merck). The plate was run in 3:1 phenol:water developing solvent. I utilized a ninhydrin stain to assay for the presence of pigment binding proteins. One day after developing, the TLC plate was dipped in a 95% ethanol solution containing 1% acetic anhydride and 0.3% ninhydrin.

Tryptophan incorporation assay

To assay the relative levels on of tryptophan incorporation into scale cells, I followed the protocol of Nijhout and Koch (1991). Abdomens of late-stage pupae were injected with 2ul of 0.05 uCi/ul ^{14}C-labeled tryptophan diluted in insect saline (pH=6.9). Injections were performed using pulled glass needles fitted to a Hamilton micro-syringe. Adults were frozen within six hours of eclosion. Dorsal and ventral scales were peeled off of wing membranes using plastic packing tape, and the basal scale surfaces were covered with food plastic-wrap. The basal side of the scale mounting, still covered with plastic-wrap, was exposed to Kodak X-Omat autoradiography film for nine days at –80°C. After exposure, the mounted scales were removed and the film was developed in an automated processor.

Gene cloning and sequence analysis

cDNA was prepared using total RNA pooled from fifth (last) instar and pupal *V. cardui* wing discs. Degenerate PCR primers were designed from available insect sequences and a nested PCR strategy was used to amplify and clone cDNA fragments into a TOPO TA vector (Invitrogen). Positive clones were PCR screened for proper insert size by using M13 vector primers. Automated sequencing was performed using vector primers and sequence identities were screened using nucleotide-protein translated Blastx (NCBI). Clone sequences showing a high degree of similarity to *D. melanogaster vermilion*, *cinnabar*, *white*, and *actin* were translated and aligned to similar sequences from other taxa using ClustalW. To verify the phylogenetic positions of the putative *vermilion*, *cinnabar*, and *white* sequences, I used PAUP* 4.0b10 (Swofford 2003) to produce maximum parsimony bootstrap consensus trees (1000-replicates each, full heuristic searches with 10-replicate random branch addition) of the known insect amino acid sequences, designating as an outgroup sequences from the beetle *Tribolium castanuem*. Full-length amino acid sequences, where available, were used for phylogenetic analyses, with incomplete regions of the *V. cardui* data being coded as missing data. *actin* sequences used for alignment were: *Heliothis virescens* "actin" (AAK52066.1), *A. gambiae* "ENSANGP00000019055" (XP_311177.1), *D. melanogaster* "actin 3" (A03000), *Caenorhabditis elegans* "act-4" (NP_508842.1), *Homo sapiens* "ACTB Protein" (AAH12854.1). *cinnabar* sequences used for alignment were: *Bombyx mori* (BAB62418.1), *Aedes aegypti* (AAO27576.1), *Anopheles stephensi* (AAL40890.1), *T. castanuem* (AAL15465.1), *D. melanogaster* (AAC47351.1). *vermilion* sequences used

for alignment were: *Plodia interpunctella* (AAR24625.1), *Ae. aegypti* (AAL37360.1), *Anopheles gambiae* (XP_312204.1), *T. castaneum* (AAL15464.1), *D. melanogaster* (NP_511113.1). *white* sequences used for alignment were: *B. mori* (AAF61569.1), *Ae. aegypti* (AAC04894.1), *An. gambiae* (XP_310530.1), *D. melanogaster* (CAA26716.1), *T. castaneum* (AAL40947.1).

Quantitative real time PCR

Total RNA extracted from the four wings of a single individual was treated with the DNA-free kit (Ambion, Inc.), after which cDNA was synthesized using a TaqMan reverse transcription kit (Applied Biosystems, Inc.) with polyT primers. Locus-specific primers (Table 4-1) were used for SYBR Green (Applied Biosystems, Inc.) real-time PCR reactions carried out in an ABI Prism 7000 Sequence Detection System. Relative transcription levels were calculated using a standard curve from a cDNA standard dilution series. *cinnabar*, *vermilion*, and *white* data were normalized using expression levels of an *actin* control. Three technical replicates were averaged for each time point. RNA-only negative controls were run for each experiment.

***In situ* hybridization**

Sense and antisense digoxigenin-labeled riboprobes were synthesized using SP6 and T7 RNA polymerases with dual-promoter TOPO vectors bearing *vermilion* and *cinnabar* fragment inserts. Riboprobes were hydrolyzed into ~300bp fragments by treating with 0.1 M $NaHCO^2$ at 60°C for 48min. Hydrolyzed probes were precipitated

with sodium acetate and ethanol overnight at –20°C, pelleted, washed with 75% ethanol, pelleted again, and then air-dried. Probes were resuspended in pre-hybridization buffer (50% formamide, 0.1% Tween 20, and 5x SSC in DEPC water with 0.1 mg/ml RNAse-free salmon sperm DNA) overnight at –20°C. Dot-blot dilution series were used to determine the concentrations of the resuspended probes, and sense and anti-sense riboprobe concentrations for each gene were then equalized.

The *in situ* hybridization protocol was based on a method developed by J. Selegue and S.B. Carroll (pers. comm.). Larvae were anesthetized in ice water for 15 min, and wing discs were dissected out and immediately placed into fix (9% formaldehyde in PBS with 50 mM EGTA) for 30min. Discs were washed five times with cold PBT (PBS with 0.1% Tween 20), incubated 2min in 25 ug/ml Proteinase K in PBT, then washed twice with PBT with 2 mg/ml glycine. After washing discs twice more in PBT, the peripodial membranes were removed and the discs were then placed into post-fix (5% formaldehyde in PBT) for 20 min. Discs were then washed five time in PBT, twice in 1:1 PBT/pre-hybridization buffer, once more in prehybridization buffer, and then incubated in 55 °C pre-hybridization buffer for several hours. RNA riboprobes were heat denatured for 5 min at 80°C, and diluted to ~.4 ng/ul in 100ul of hybridization buffer (pre-hybridization buffer with 1 mg/ml gylcogen). Discs were placed in the diluted probe and incubated for 24 h at 55°C, after which they were washed five times in 55°C pre-hybridization buffer with the final wash going overnight. Discs were washed once in 1:1 PBT:pre-hybridization buffer, then four times in PBT, and incubated in a 1:2000 dilution of mouse anti-digoxigenin antibody (Jackson ImmunoResearch Laboratories, Inc.) for at room

Table 4-1. Primers used for quantitative PCR

Gene	Primer sequences	
actin	Fwd	5'-CGATCGCTTGCAGAAGGAAATCAC
	Rev	5'-AGATCCACATCTGTTGGAAGGTCG
vermilion	Fwd	5'-TATTTACGACCGGCGTCTGGTTTC
	Rev	5'-TGATTCTATGGCTTCCGGGTCATC
cinnabar	Fwd	5'-TCCACTTGTAGCTGTTAAGTGCCG
	Rev	5'-GTCTTCAAAGCCAGCGTTCATTCC
white	Fwd	5'-TGGATCGGCATATACCATACGCAC
	Rev	5'-TTCACCACCGGATATACCCTTCAG

temperature for two hours. This was followed by 10 washes in PBT, and two washes in alkaline phosphatase developer (100 mM Na Cl, 50mM MgCl2, 100 mM Tris pH 9.5, in 0.1% Tween 20). Discs were developed in the dark using BCIP/NBT (Roche) in alkaline phosphatase developer. Development was monitored until appropriate levels of colored precipitate formed, at which time the reaction was stopped with five washes of PBT with 50 mM EDTA. Discs were then mounted in 10% glycerol with 50 mM EDTA and photographed. All *in situ* hybridization experiments were conducted using a left wing / right wing sense / antisense control strategy.

RESULTS

Identification of xanthommatin in red *V. cardui* wing scales.

In order to determine which ommochromes were present in *V. cardui* wings, I performed TLC analysis on extracts of dissected red color pattern tissue. I observed three reddish-brown bands with R_f values of 0.37, 0.52, and 0.57 respectively (Table 4-2). The migration and color of the band at $R_f = 0.37$ was consistent with xanthommatin (Nijhout 1997), however the identity of the bands at $R_f = 0.52$ and $R_f = 0.57$ was unknown and such bands were not observed in Nijhout's work with another nymphalid (Nijhout 1997). None of the observed bands had R_f values similar to those of the other ommochromes described from nymphalid wings: ommatin-D ($R_f = 0.29$), rhodommatin ($R_f = 0.23$), or dihydroxanthommatin ($R_f = 0.13$). While these results suggest that ommatin-D and rhodommatin were not present in *V. cardui* wings in detectable quantities, one cannot

Table 4-2. Thin layer chromatography reveales xanthommatin signature in *V. cardui* wings

	Sample	R_f (this study)	R_f (Nijhout 1991)
References	amaranth	0.14	0.13
	bromphenol blue	0.43	0.43
	napthol blue black	0.35	0.32
	3-hydroxykynurenine	0.57	0.56
Pigments	xanthommatin		0.36
	dihydroxanthommatin		0.13
	ommatin-D		0.29
	rhodommatin		0.23
	V. cardui extract	0.37	
		0.52	
		0.57	

completely rule out the possibility that, if dihydroxanthommatin was present in the wing extracts, it was not oxidized into xanthommatin during the extraction or TLC run. To test for the possible presence of a xanthommatin binding protein, I treated the TLC plate with ninhydrin, which stains amino acid residues. Although the ninhydrin appropriately stained the 3-OHK control, there was no detectable staining associated with the xanthommatin band, suggesting that the extracted xanthommatin was not protein-bound.

To help determine the spatial distribution of ommochrome pigments in *V. cardui* wings, I injected the radiolabeled tryptophan into the hemolymph of pupae and assayed tryptophan incorporation into adult wing scales (Fig 4-2A). On the dorsal surface of both the forewing and hindwing, the spatial patterns of tryptophan incorporation appear to be perfectly correlated with only red pattern elements. In contrast, on the ventral wing surfaces, tryptophan incorporation was also seen in some brown- and tan-colored pattern elements as well as in some small patches of black pattern. Tryptophan incorporation into brown and tan butterfly wing scales has been previously noted, leading to the speculation that undescribed pigments exist in the tryptophan-ommochrome pathway (Nijhout and Koch 1991). In any case, all red scales from *V. cardui* wings displayed high levels of tryptophan incorporation, supporting the TLC results in identifying xanthommatin as a spatially specific component of red pattern elements.

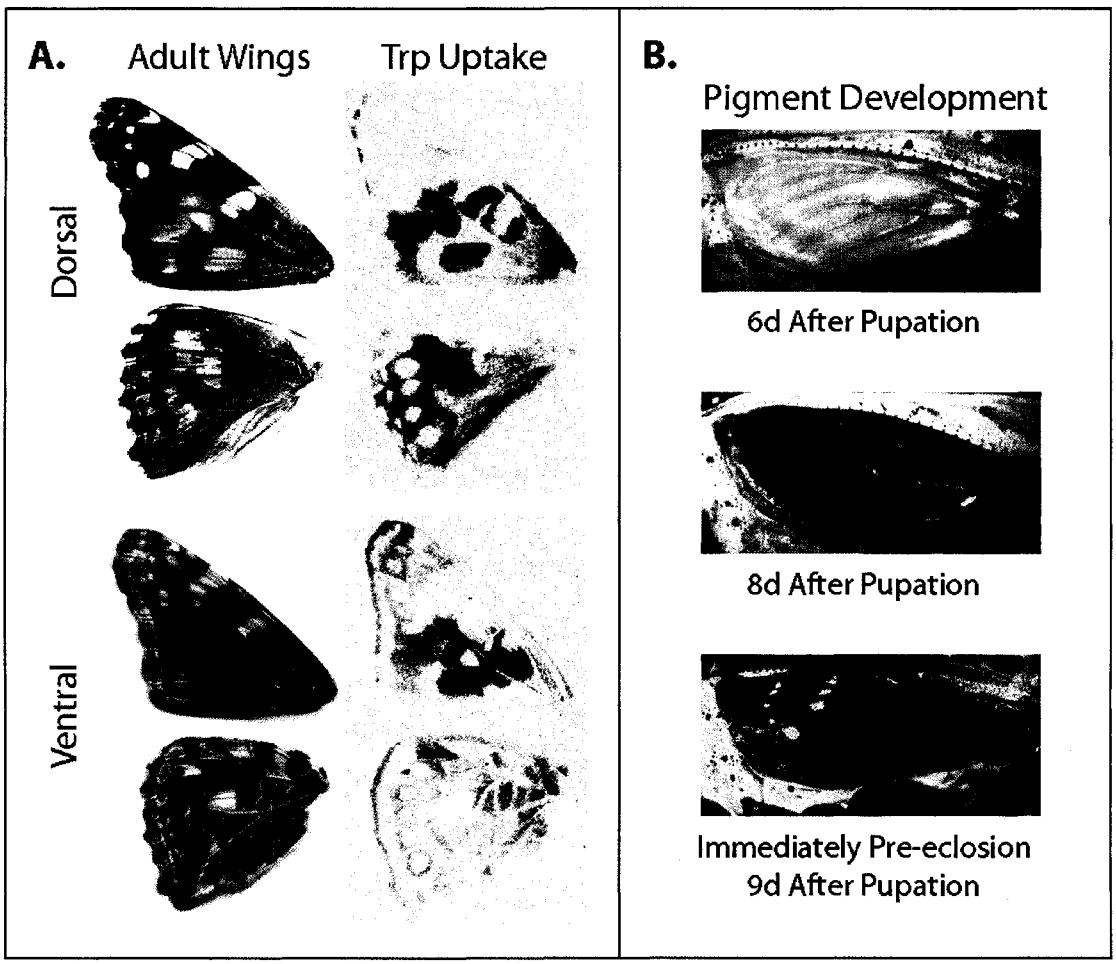


Fig. 4-2. Pigment development in *V. cardui* wings. A. Uptake of tryptophan, the ommochrome precursor, corresponds with the distribution of red scales in adult *V. cardui* wings. B. The sequence of pigment development in *V. cardui* pupal wings. Note that melanin development precedes ommochrome development.

Melanin development precedes xanthommatin development in *V. cardui* wings

Pigment development in *V. cardui* scales began a few days before eclosion (Fig. 4-2B). At 6d after pupation (AP) the first color patterns were visible on the dorsal forewing as opaque white spots running in a line parallel to the distal wing margin. The five anterior spots are retained in the adult wing pattern, while the posterior spots eventually developed as melanin pattern. By 8d AP the melanin pattern developed, and the rest of the developing scales took on a beige hue. Within the last 24hr before eclosion, the ommochrome pigments developed fully.

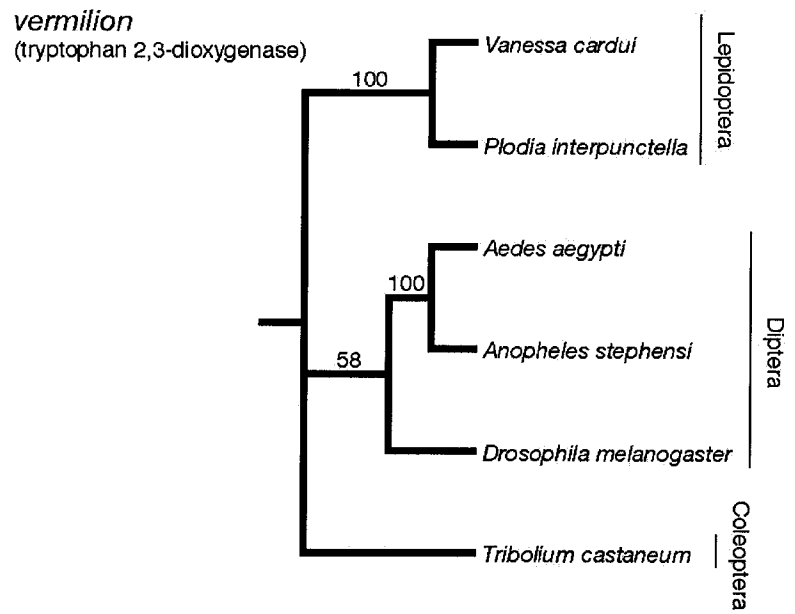
Sequencing of *V. cardui* orthologs of *actin*, *vermilion*, *cinnabar*, and *white*

I sequenced individual cloned fragments of the *V. cardui* orthologs of *actin* (used as a positive control for RT-PCRs, see below), *vermilion*, *cinnabar*, and *white*. I sequenced a 280bp fragment of an *actin* gene corresponding to the 65 C-terminal amino acids, along with a portion of the 3' untranslated region of the mRNA. Two amino acids were not predicted because of ambiguity of two nucleotides in the coding sequence. The predicted *V. cardui* amino acid sequence is more than 99 percent identical to actin sequences from other animals (Fig. 4-3). 429bp encoding 142 amino acids of a *V. cardui cinnabar* gene were sequenced. Alignment and phylogenetic relationships suggest that this sequence represents an ortholog (Fig. 4-4), and despite sequencing multiple *V. cardui* clones, no other divergent copies of this gene have been found. A 673bp *V. cardui vermilion* clone coding for 224 amino acids was sequenced, which, like the *cinnabar* sequence, appears to represent an ortholog of the *D. melanogaster* gene (Fig. 4-5).

Multiple clones representing various ATP-binding cassette transporters were sequenced from *V. cardui*. The clone analyzed in this study appears to be an ortholog of the *D. melanogaster white* gene, due to its phylogenetic relationship to the other *white* sequences known from insects (Fig. 4-6). The *white* clone contains a 301bp fragment of the gene, encoding 100 amino acids.

```
V.cardui          DRLQKEITA?APSTMKIKI?APPERKYSVWIGGSILASLSTFQQMWISKQEYDESGPSIVHRKCF
H.virescens       DRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISKQEYDESGPSIVHRKCF
D.melanogaster    DRMQKEITALAPSTMKIKIVAPPERKYSVWIGGSILASLSTFQQMWISKQEYDESGPSIVHRKCF
An.gambiae        DRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISKQEYDESGPSIVHRKCF
C.elegans         DRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISKQEYDESGPSIVHRKCF
H.triserialis     DRMQKEITAMAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISKQEYDESGPSIVHRKCF
H.sapiens         DRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISKQEYDESGPSIVHRKCF
                  **:****** ********* ******************************************
```

Fig. 4-3. Alignment of *actin* gene sequences. The fragment sequenced from *V. cardui* corresponds to *D. melanogaster* amino acid positions 312-376. 3' untranslated sequence was also recovered from our clone.



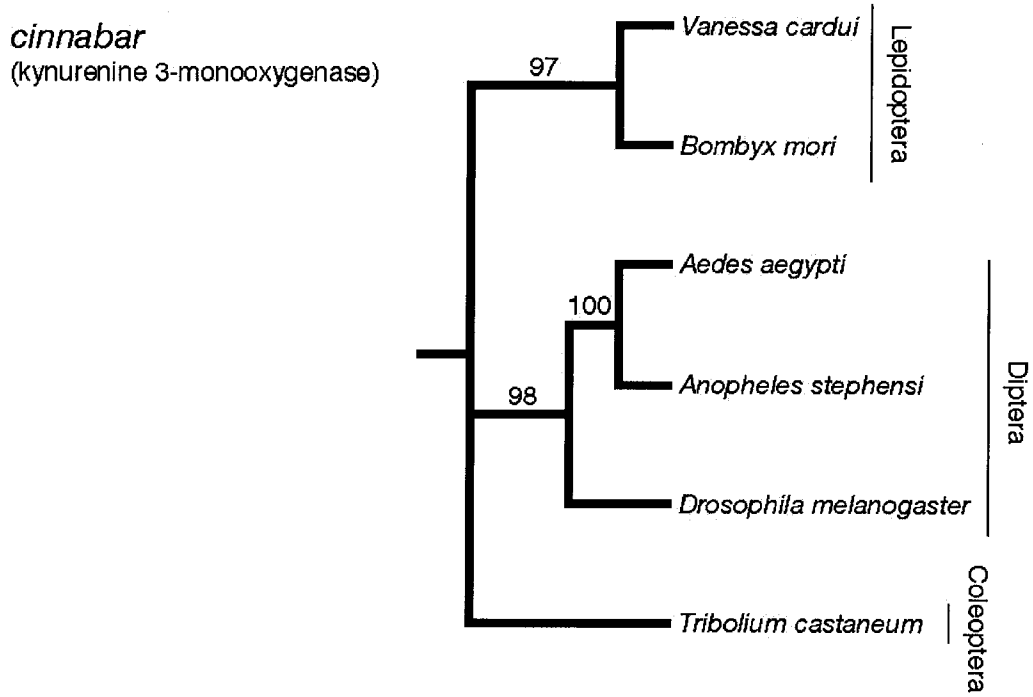

```
V.cardui            ----------------------ELWFKQIIFEVDSVRSLLDVEGLDES
P.interpunctella    RMLSAESSRPAHDEHLFIVTHQAYELWFKQIIYEVDSVRSLLDVEGLDES
D.melanogaster      CMLSEEDKRPVHDEHLFIITHQAYELWFKQIIFEFDSIRDMLDAEVIDET
Ae.aegypti          RMLSVESNKPVHDEHLFIVTHQAYELWFKQIIFELDSIRSLFSTEHMEES
An.gambiae          RMLSVEGKKPVHDEHLFIVTHQAYELWFKQIIFELDSIRDLFSTEHIEES
T.castaneuam        RLLSEQNNQPVHDEHLFIVTHQAYELWFKQIIYELDSIRNIFS-DVLEES
                                         ********:*.**:*.::. : ::*:

V.cardui            HTMEILKRLNRIVLILKLLVDQVMILETMTPLDFMDFRNYLRPASGFQSL
P.interpunctella    HTMEILKRLNRIVLILKLLVDQVMILETMTPLDFMDFRNYLRPASGFQSL
D.melanogaster      KTLEIVKRLNRVVLILKLLVDQVPILETMTPLDFMDFRKYLAPASGFQSL
Ae.aegypti          RTLEILKRLNRIVMILKLLVDQVPILETMTPLDFMDFRDFLSPASGFQSL
An.gambiae          RTLEILKRLNRIVMILKLLVDQVPILETMTPLDFMDFRDYLSPASGFQSL
T.castaneuam        QTLEILKRLNRVVLILKVLVDQVMILETMTPLDFMDFRCYLRPASGFQSL
                    :*:**:*****:*:***:***** ************** :* ********

V.cardui            QFRLLENKLGLKQALRVKYNQNYQTVFGDDPEAIESLHKSEEEPALLALI
P.interpunctella    QFRLLENKLGLKQALRVKYNQNYQTVFGDDPAAMDALHKSEEEPALLALI
D.melanogaster      QFRLIENKLGVLTEQRVRYNQKYSDVFS-DEEARNSIRNSEKDPSLLELV
Ae.aegypti          QFRLLENKLGVKTEHRVKYNQKYSEVFASDPCAIERLSITESEPSLADLV
An.gambiae          QFRLLENKLGVKSEHRVKYNQKYTEVFASDPGAIERIGTTETEPSLADLV
T.castaneuam        QFRLLENKLGVRQENRVKYNQNYSKVFGNDEKALEQIAKSEKEPSLTDLV
                    ****:*****:    **:***:*  **. *  * : :  :* :*:*  *:

V.cardui            ERWLERTPGLNTYGFNFWGKFQAAVDKLVKGDIEEAMREPNEVVRRHRLQ
P.interpunctella    ERWLERTPGLQTHGFNFWGKFQAAVNKTITDDIEAAMTEPNETVRRHRLQ
D.melanogaster      QRWLERTPGLEESGFNFWAKFQESVDRFLEAQVQSAMEEPVEKAKNYRLM
Ae.aegypti          QKWLERTPGLETNGFNFWGKFEESVEQLLADQEASAMEEEHENVKNYRLM
An.gambiae          QKWLERTPGLEQDGFNFWGKFQESVEQLLAEQEASAMSEEHENVREYRLM
T.castaneuam        QRWLERTPGLELEGFNFWGKYQKAVEKLLTEQKELAEKEEAETLKRYKLN
                    ::********:  *****.*:: :*:: :  :   *  *  *  :.::*
```

Fig. 4-4. Alignments and phylogenetic relationships of *vermilion* gene sequences from representative insects. The fragment sequenced from *V. cardui* corresponds to *D. melanogaster* amino acid positions 65-288. The tree is a 1000-replicate maximum parsimony bootstrap consensus, with the numbers over branches representing bootstrap support > 50%.

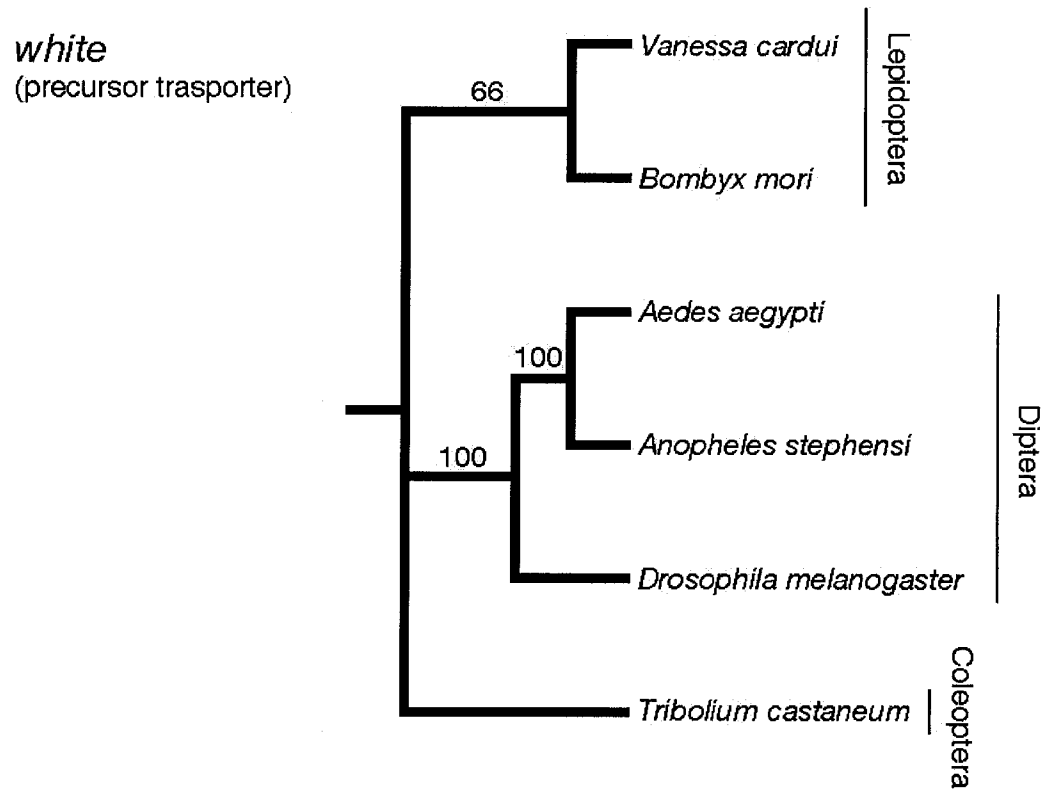



```
V.cardui          ----MMIALPNQDCSWTVTLFMPFKNFKEIDTDEKLMIFFQKYFPDSIPLIGEKKLIEDF
B.mori            RGEFMMIALPNQDCSWTVTLFMPFTHFKSLDNEDKLLKFFEKYFPDSIPLIGKQKLIADY
Ae.aegypti        RGKFMMIALPNQDRTWTVTLFMPFTNFNSIKCDGDLLKFFRTYFPDAIDLIGRERLVKDF
An.stephensi      RGQFMMIALPNQDRTWTVTLFMPFTQFHSITDPGRLIDFFRQYFPDAIELIGRERLIKDF
D.melanogaster    RNTFMMIALPNQDKSFTVTLSMPFEIFAGIQNQNDLLEFFKLNFRDALPLIGEQQLIKDF
T.castaneum       RGQFMMIALPNKDNSWTVTLFMPFGKFESLRNAAELKDFYYKTFPDAVPLIGEDLLVNDF
                  *******:* ::**** *** * : * *: * *:: ***.. *: *:

V.cardui           FGGSPSPLVAVKCRPYNVVDKALIIGDAAHAVVPFYGQGMNAGFEDCYILDELFQKHHDN
B.mori             FAGSASPLIAIKCRPYNVEDKALIIGDAAHAVVPFYGQGMNAGFEDCTILNQLFQKHHDD
Ae.aegypti         FKTRPQSLVMIKCKPYNVGGKAVIIGDAAHAMVPFYGQGMNAGFEDCTVLTELFNQHGSD
An.stephensi       FKTKPQPLVMIKCRPYHVGSKALIIGDAAHAMVPFYGQGMNAGFEDCSVLTDLFNQYGTD
D.melanogaster     FKTRPQFLVSIKCRPYHYADKALILGDAAHAMVPYYGQGMNAGMEDVTLLTDILAKQLP-
T.castaneum        FKVKPSALVSVKCKPYHVGSKFLLIGDAAHAMVPFYGQGMNAGFEDCFLLDGILERRSND
                   *   .. *: :**:** :  .* :::****** :**:******* :**  :*  :: :

V.cardui           VADILQEFSDSRWKDAFAISDLAMYNY----------
B.mori             LAKILKEFSDTRWEDTFAISDLAMYNYIEE--IRTSRSGFK---------CYMNSRIVV
Ae.aegypti         VDRILAEFSDTRWEDAHSICDLAMYNYVEMRDLVTKRSYLFRKKLDELLYWMLPNTWVPL
An.stephensi       LTRILPEFSEKRWEDAHAICDLAMYNYIEMRDLVTKRSYLLRKKLDELLFWMLPNTWVPL
D.melanogaster     LDETLALFTESRWQDAFAICDLAMYNYVEMRDLTKRWTFRLRKWLDTLLFRLFPG-WIPL
T.castaneum        IAGSIEEFSRERVEDAYAICELAMYNYVEMRDLVTRPSYRLRKFFDELLFKCMKEKWIPL
                   :   :  *:  * :*:.:*.:******
```

Fig. 4-5. Alignments and phylogenetic relationships of *cinnabar* gene sequences from representative insects. The fragment sequenced from *V. cardui* corresponds to *D. melanogaster* amino acid positions 302-443. The tree is a 1000-replicate maximum parsimony bootstrap consensus, with the numbers over branches representing bootstrap support > 50%.




```
V.cardui          -----ARCAYVQQQDLFIGTLTVREHLIFQALVRMDRHIPYAQRMRRVQEVISELALTKC
B.mori            PDALTALSAYVQQQDLFIGTLTVREHLVFQAMVRMDRHIPYAQRMKRVQEVIQELALSKC
An.gambiae        AEQLRARCAYVQQDDLFIPSLTTREHLLFQAMLRMGRDVPASVKQHRVQEVLQELSLVKC
Ae.aegypti        AEQLRARCAYVQQDDLFIPALTTREHLVFHAMLRMGKDVPKSVKMNRVNEVLQELSLAKC
D.melanogaster    AKEMQARCAYVQQDDLFIGSLTAREHLIFQAMVRMPRHLTYRQRVARVDQVIQELSLSKC
T.castaneum       SKTLASQSAYVQQDDLFIGTLTVKEHLIFQALLRMDRDISYSQRMARVEEVISDLALSKC
                        : .*****:**** :**.:***:*:*::** :.:.   :  **::*:.:*:* **

V.cardui          QNTVIGIPGRLKGISGGEMKRLSFASEVLTDPPLMFCDEPTTGLD---------------
B.mori            QNTVIGIPGRLKGISGGEMKRLSFASEVLTDPPLMFCDEPTSGLDSFMAQNVIQVLKGLA
Ae.aegypti        ADTIIGAPGRMKGLSGGERKRLAFASETLTDPHLLLCDEPTSGLDSFMAHSVLQVLKGMA
An.gambiae        ADTIIGAPGRIKGLSGGERKRLAFASETLTDPHLLLCDEPTSGLDSFMAHSVLQVLKGMA
D.melanogaster    QHTIIGVPGRVKGLSGGERKRLAFASEALTDPPLLICDEPTSGLDSFTAHSVVQVLKKLS
T.castaneum       QNTPIGILGRIKGISGGEKKRLSFAAEVLTNPKLMFCDEPTSGLDSFMALTVMQVLKEMA
                    .* **  **:**:**** ***:**:*.**:* *:*****:***
```

Fig. 4-6. Alignments and phylogenetic relationships of *white* gene sequences from representative insects. The fragment sequenced from *V. cardui* corresponds to *D. melanogaster* amino acid positions 182-281. The tree is a 1000-replicate maximum parsimony bootstrap consensus, with the numbers over branches representing bootstrap support > 50%.

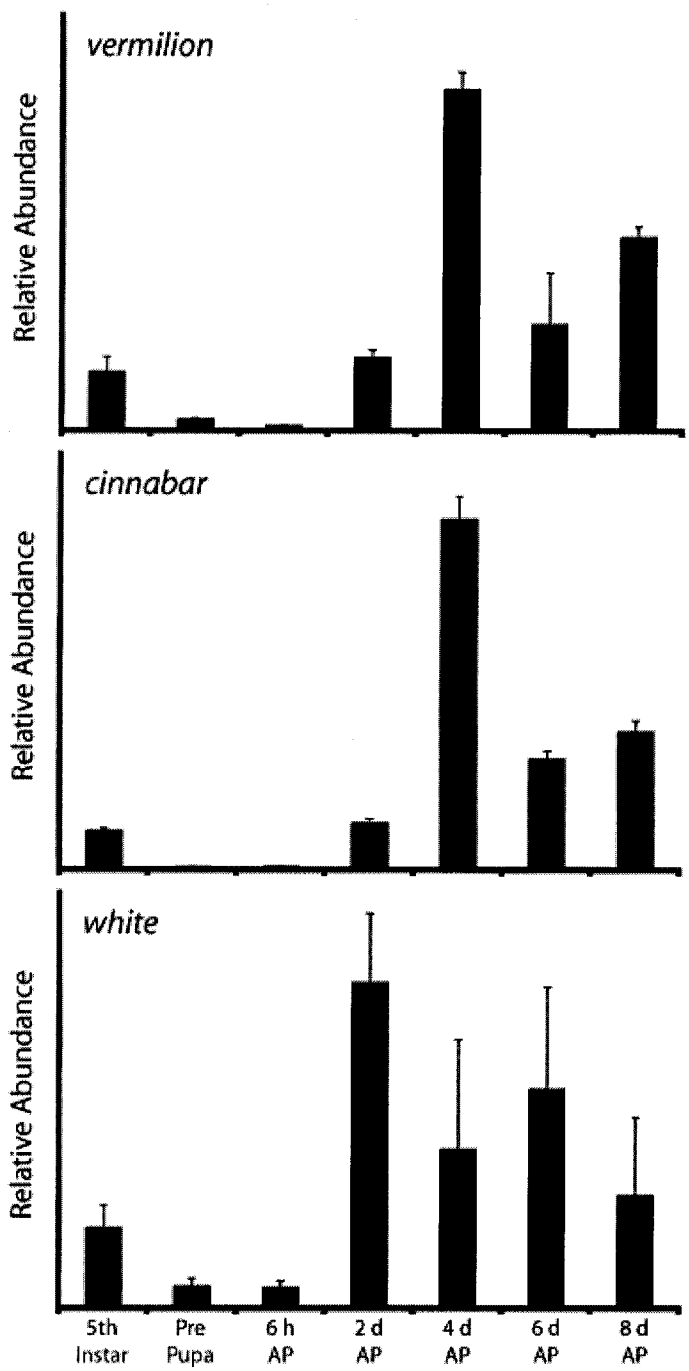


Fig. 4-7. Quantitative RT-PCR analysis of ommochrome gene transcription during *V. cardui* wing development. Total RNA was extracted from wings pooled from a single individual at a single time point. All values were calculated using a reference standard curve and normalized to levels of *actin* transcription. Each bar represents the mean of three replicates, with standard errors represented.

Quantitative PCR analysis of *vermilion*, *cinnabar*, and *white* expression

Quantiative PCR analysis using staged wing RNA pools was done to determine the relative levels of *vermilion*, *cinnabar*, and *white* expression during late-larval and pupal wing development (Fig. 4-7). Surprisingly, the transcription of all of these genes was observed to occur during fifth instar larval wing development, albeit at relatively low levels. Gene expression decreased in pre-pupal wings but increased again 2 d after pupation (AP) when scales began developing (Nijhout 1991; Galant et al. 1998; Reed 2004). *white* showed a dramatic increase in transcription between 6h AP and 2d AP, which is earlier than similar large increases in *vermilion* and *cinnabar* expression between 2 d and 4 d AP. The similar relative change in *vermilion* and *cinnabar* transcription levels over time may suggest the two genes are temporally co-regulated in some manner during pupal development.

Spatial expression patterns of *vermilion* and *cinnabar* in larval wing discs

I performed *in situ* hybridization experiments to determine the spatial distribution of *vermilion* and *cinnabar* mRNAs in fifth-instar wing discs (Fig. 4-8). I was only able to analyze expression in larval wing discs because the digoxigenin antibodies I used for riboprobe detection bound non-specifically to pupal wing scales. *vermilion* was found to be transcribed throughout the fifth instar wing discs, with especially high levels being observed in the epidermis between the wing veins, whereas *cinnabar* had a somewhat more complex expression pattern. In mid-fifth instar wing discs, *cinnabar* mRNA occurred at high levels in epidermal tissue between wing veins, with less accumulation

occurring in "spots" midway between the wing veins. As development proceeded, *cinnabar* mRNA was expressed adjacent to wing veins, with higher expression proximally.

I was unable to generate *white in situ* expression data in larval wings, possibly because *white* transcription occurs at a much lower level than *vermilion* and *cinnabar*. Although one cannot make rigorous quantitative comparisons between the RT-PCR data for the different genes, there were marked differences between the cycles-to-detection thresholds (Ct) for *white* vs. *cinnabar* and *vermilion*. For instance, the mean Cts for *vermilion* and *cinnabar* from the 4 d AP sample (i.e. the highest expression) were 29.7 and 25.8, respectively, which are both quite a bit lower than the 32.4 mean Ct for *white* 2d AP. This relatively low expression level of *white* may also help explain the larger relative standard errors in the *white* RT-PCR data (Fig. 4-7).

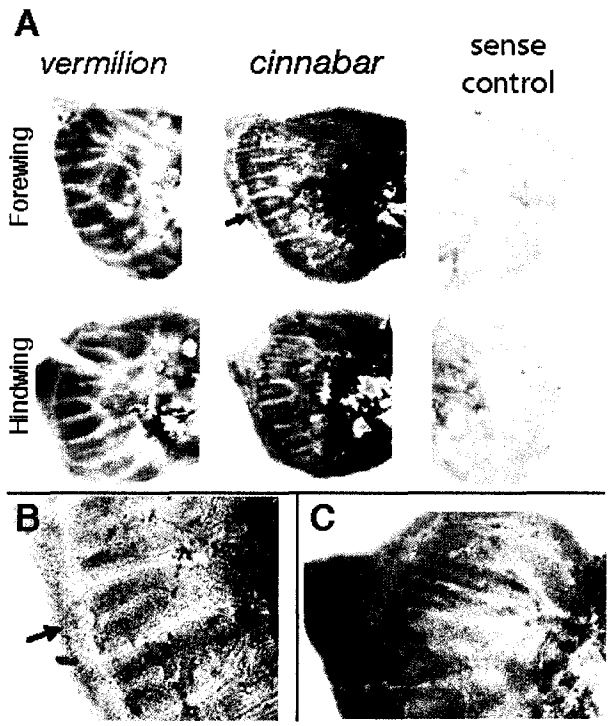


Fig. 4-8. Spatial distribution of *vermilion* and *cinnabar* mRNA in fifth instar wing discs. A. *vermilion* and *cinnabar* expression in mid-fifth instar forewing and hindwing discs. *vermilion* is expressed throughout the wing discs, while *cinnabar* has reduced accumulation of mRNA at mid-point spots in between wing veins. In late-fifth instar forewing (B) and hindwing (C) discs, the zone of *cinnabar* expression decreases proximally and in intervein midlines. The spots and intervein midlines are expression motifs also observed in the expression patterns of developmental genes Notch and Distal-less (Reed and Serfas 2004). Black arrows provide a position reference between the two forewing figures. The sense probe negative controls presented correspond to the *vermilion* stain in A, but are representative of negative control results from all larval *in situ* hybridizations.

DISCUSSION

Gene expression in the context of butterfly wing ommochrome synthesis

This study demonstrated the presence of the ommochrome pigment xanthommatin in *V. cardui* wings, and showed that genes implicated in xanthommatin synthesis are expressed in the wings before pigment development begins. Previous experiments with the nymphalid butterflies *Araschnia levana* (Koch 1991) and *Junonia (Precis) coenia* (Koch 1993) demonstrated that pupal wing discs in culture could take up tryptophan and synthesize ommochromes *in situ*, suggesting that the developmental machinery necessary for ommochrome synthesis may be found within butterfly wings themselves. Finding that the transcription of ommochrome genes occurs in xanthommatin-bearing wings provides a potential molecular genetic basis for these previous results.

A functional question arising from this study is why there is such a long lag time between ommochrome gene transcription and pigment synthesis. Expression of the ommochrome genes assayed in this study occurs in wings as early as the fifth instar, and even the major peaks in transcription occur 5-7 days before the appearance of pigments. It would be useful to know if this lag time is to due to delays in protein translation, delays in protein activity, or slow reaction kinetics of ommochrome synthesis. It is also worth mentioning that ommochrome development in *V. cardui* wings appears to be temporally coincident with ommochrome development in eyes, suggesting that an endocrine signal may be implicated in the timing of pigment synthesis.

The overall sequence of pigment development in *V. cardui* is unusual compared to other butterflies that have been studied. It has been proposed that ommochrome

development preceding melanin development is a stereotypical sequence among butterflies (Nijhout 1991; ffrench-Constant and Koch 2003). Indeed, in support of this view I have also observed ommochromes preceding melanins in *Heliconius spp.* as well as in the melitine nymphalid *Chlosyne lacinia* (unpublished data). The observation that melanin development precedes ommochrome development in *V. cardui* represents an exception to this model and argues that modes of pigment development timing in butterflies may not be as conserved as previously believed. The only other pigment pattern I have observed to develop after melanin is the yellow pigment 3-OHK in *Heliconius erato*, which develops its color only a few hours before adult emergence (Chapter 5).

A surprising finding from this study was the low level of *vermilion* and *cinnabar* transcription in fifth-instar wing discs that was not obviously associated with the adult color pattern. Although the significance of the larval-stage *in situ* expression data may be of limited usefulness for understanding the regulation of pigment development in pupal wings, they do reveal a few facts worth considering. Unlike with the temporal data, the spatial data suggest that *vermilion* and *cinnabar* are not entirely co-regulated because they both showed somewhat different distribution patterns in the epidermis. In fifth instar wing discs, *vermilion* expression appeared to be widespread across the wing discs, while *cinnabar* was expressed in a slightly more complex pattern over time.

If transcription of both *vermilion* and *cinnabar* is required for *in situ* ommochrome synthesis, at least one of their expression patterns would be predicted to change significantly by the time of scale and pigment development in order for co-

expression to occur in ommochrome-producing scale cells. Indeed, *Drosophila* wing pigment patterns are thought to be determined in part by "prepatterns" of enzyme expression (True et al. 1999). Consistent with this model, preliminary data from micro-dissected *Heliconius erato* pupal wing patterns suggest that *cinnabar* is spatially upregulated in specific ommochrome pattern elements late in pupal wing development (Chapter 5). It is also notable that the *V. cardui cinnabar* expression patterns bear some superficial resemblance to expression patterns of the developmental proteins Notch and Distal-less (Reed and Serfas 2004). In particular, the mid-fifth instar *cinnabar* expression looks much like an "inverse" of Notch and Distal-less eyespot focus expression (Chapter 2). The late-fifth instar expression also resembles an inverse pattern of the Notch and Distal-less intervein midline motif. These similarities raise the possibility that *cinnabar*, *Notch*, and *Distal-less* may all be responding to a similar wing pattern co-ordinate system.

A model of ommochrome synthesis in butterfly wings

Given the above data, along with previous work on butterflies and *D. melanogaster* eyes outlined in the introduction, I propose a hypothetical model of ommochrome synthesis in butterfly wings (Fig. 4-1). The elements of the model taken from work on butterflies include (1) the uptake of tryptophan and (possibly) 3-OHK (Koch 1993) into scale cells (2) the expression of *vermilion, white*, and *cinnabar* in wings as deduced here, and (3) the presence of ommochromes in scale cells. Additionally, there

is evidence that pigment precursor transporter *scarlet* is also expressed in the wings of the butterfly *Heliconius erato* (Chapter 5).

While several features of the model I present in Fig. 4-8 are highly speculative and have no direct support in the context of butterfly scale cells, I hope the model will serve as a useful guide for further experiments. Of particular benefit would be to identify the means of ommochrome precursor uptake into cells, determining how ommochromes are stored in developing scales, and clarifying the chemistry of the final steps of ommochrome processing. It is unknown if ommochrome-bearing pigment granules occur in developing butterfly scales; however, I have included a hypothetical pigment granule in my model because ommochromes are consistently associated with granules in the eyes, integument, and nervous systems of various other insects (Linzen 1974; Kayser 1985; Sawada et al. 1990; Sawada et al. 2000).

Evolution of ommochrome synthesis

Assays for the presence of ommochromes have been conducted in a variety of insects, many of which are summarized by Linzen (1974). The presence of ommochromes in insect eyes appears to be nearly ubiquitous, suggesting that visual filtering is the ancestral function of ommochromes in insects. There are reports from various insects of ommochromes found in other contexts besides eyes, including integument, malphigian tubules, nervous systems, and waste products (Linzen 1974; Sawada et al. 1990; Sawada et al. 2000). Given the current data sampling, however, these non-eye contexts all appear to be non-ancestral, derived characteristics of specific

lineages. To date, the only known examples of ommochromes used as wing scale pigments are from nymphalid butterflies (Nijhout 1991). Colored wing scales from several noctuid, saturniid, and sphingid moths as well as various papilionid and pierid butterflies, have all tested negative for ommochromes (Linzen 1974). I would therefore regard the synthesis of ommochromes in butterfly wing scales as an evolutionary novelty with a single origin.

I propose two non-exclusive models of evolution for scale ommochromes. In the first model, the development of ommochromes is "modular", being controlled by a conserved network of regulatory interactions. Under this model, the redeployment of the entire cadre of transporters, enzymes, and (presumed) storage granules would have occurred through one or a small number of mutations to upstream regulatory genes that would activate the entire sequence of events required to produce pigmented granules. Another "sequential" model of evolution would have the different elements of ommochrome development appearing in scales independently over time until all the elements necessary to produce stable ommochromes were co-expressed. Further comparative and functional work is needed to determine to what degree each model applies to this system, and to learn what kind of regulatory changes led to the expression of ommochrome genes in butterfly wings.

CONCLUSION

The data presented above illustrate that genes required for ommochrome synthesis in *Drosophila* eyes are transcribed in ommochrome-bearing butterfly wings. The results

of this study demonstrate that the regulation of ommochrome genes in butterfly wings is complex and defy a simple one-to-one relationship between gene expression of pigment synthesis. Several questions remain: Why are ommochrome genes transcribed so much earlier than the appearance of pigments? What is the purpose of the complex spatial gene expression patterns in larval wing discs? What are the novel pigments identified in the TLC experiment? To what extent does tryptophan processing occur in wings versus elsewhere in the insect? Our little Painted Lady may keep these secrets for a bit longer, because for the remainder of this dissertation I shall focus on pigment development in the most wondrous of butterflies: *Heliconius*.

ACKNOWLEDGMENTS

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CHAPTER 5

CHANGES IN GENE REGULATION ASSOCIATED WITH DEVELOPMENT AND POLYMORPHISM OF WING PATTERNS IN THE MIMETIC BUTTERFLY *HELICONIUS ERATO*

SUMMARY

The neotropical butterfly genus *Heliconius* has been of great interest to biologists because of the mimicry-related polymorphic wing patterns found among its constituent species. While extensive work has been done on the genetics and ecology of *Heliconius* wing patterns, little is known about how these wing patterns develop. This chapter reports work I have undertaken to begin understanding the molecular basis of wing pattern development and polymorphism in the species *Heliconius erato*. I used cDNA microarray and quantitative PCR approaches to test if changes in gene regulation are associated with the development of a colored forewing band pattern. Spatial and temporal expression patterns of selected candidate genes were then compared between two races of *H. erato* displaying a yellow / red pigment polymorphism in the forewing band. Among the candidate genes tested, transcripts encoding the ommochrome synthesis enzymes *vermilion* and *cinnabar* showed polymorphism- and color pattern-related transcription patterns, respectively. In the yellow forewing band race, *vermilion* was not expressed at detectable levels, suggesting that regulation of this gene may be involved with the yellow

/ red polymorphism. In both red and yellow forewing band races, *cinnabar* expression was spatially associated with the forewing band regardless of pigment. These findings support the hypothesis that *Heliconius* color patterns are determined in part by spatiotemporal overlap of pigment gene transcription, and suggest that evolutionary changes in *vermilion* regulation may underlie an adaptive color pattern polymorphism.

INTRODUCTION

The butterfly genus *Heliconius* has been of special interest to biologists for well over a century because of the great number of distinct mimetic wing patterns found throughout many of its species. Most *Heliconius* are unpalatable members of Müllerian mimicry rings, and the adaptive value of mimetic *Heliconius* phenotypes in nature has been demonstrated in field experiments (Benson 1972; Kapan 2001). Decades of genetic work on the species *Heliconius erato* have revealed close to a dozen wing pattern loci that behave mostly as simple two-allele Mendelian switches to define specific wing pattern elements (Sheppard et al. 1985; Mallet 1989; Nijhout 1991). Conveniently, allelic effects on wing pattern can be studied by crossing individuals from phenotypically distinct, allopatric populations. *Heliconius* wing pattern polymorphisms are of special interest to evolutionary biologists because the alleles known to determine wing patterns are not lab-generated mutants; they occur in natural populations and combinations of different alleles are maintained locally by mimicry-driven selection. Thus, the toolkit of color pattern genes in *Heliconius* is one of the richest collections of polymorphic

developmental genes of unambiguous adaptive value known to science. These polymorphism genes provide an opportunity for integrating genetics and ecology with evolution and development in a way that has been little explored. To date, however, little is known about the molecular basis of *Heliconius* wing pattern development.

The majority of *Heliconius* wing pattern elements appear to be based on a system of vein-independent proximal-distal boundaries (Gilbert 2003), and many of the color pattern genes described from *H. erato* affect the shape or color of the large "bar" and "band" color patterns. In this chapter I describe work I have done to characterize gene expression associated with the development and polymorphism of forewing band patterns in *H. erato*. I focus on forewing bands for several reasons: they are among the largest and most common pattern elements in *Heliconius*, they are important for both mimicry and mate recognition (Jiggins et al. 2001; Kapan 2001; McMillan et al. 2002), their large size makes them easy to dissect and work with, several loci are known that affect their size and color (Nijhout 1991; Jiggins and McMillan 1997), and perhaps most importantly on a larger scale, they represent a complex proximal-distal pattern formation system that appears to be unique to the Lepidoptera.

The currently popular model is that most *Heliconius* wing patterns, including the forewing band, are determined by the activity of two largely independent pattern systems: the color "background" and the black "pattern" (Nijhout and Wray 1988; Nijhout 1991). In this model, the determination of white, yellow, and orange/red pattern is controlled by one set of genes, while another set of genes controls the extent to which this background is "masked" by a melanin pattern. By shrinking or expanding the melanin pattern

elements different regions of the background are exposed, thereby creating overall color patterns that compose the band, bars, and spots seen throughout the genus. Gilbert uses the analogy of "shutters" and "windows" to describe this model, where melanin elements act as movable shutters to expose or conceal windows to the color background pattern (Gilbert 2003). To demonstrate this, Gilbert has done series of backcrosses to diminish melanin patterns, exposing extensive latent background patterns "underneath". It is significant to note that at least one forewing shutter pattern element can be red (Nijhout and Wray 1988), which demonstrates that dominant shutter pattern elements are not necessarily constrained to specifying particular pigment types.

There are three subjects covered in this chapter. The first concerns the relationship between color pattern development and the temporal sequence of scale and pigment development in *Heliconius erato* pupal wings. The second describes my use of cDNA microarrays to determine transcript levels in band- versus non-band-tissue in *H. erato petiverana*. Lastly, I report on the use of quantitative PCR (qPCR) to test for an association of specific transcripts with a red / yellow forewing band color polymorphism in the *H. erato* races *cyrbia* and *himera*. Together, I used these data to assess the hypothesis that changes in transcriptional regulation underlie color pattern development and polymorphism in *H. erato*.

Previous work has shown that melanin (black), xanthommatin and dihydroxanthommatin (orange and red, respectively), and 3-hydroxykynurenine (3-OHK, yellow), are the primary pigments in *Heliconius* (Gilbert et al. 1988). 3-OHK is a precursor of xanthommatin and dihydroxanthommatin, and both of these pigments share

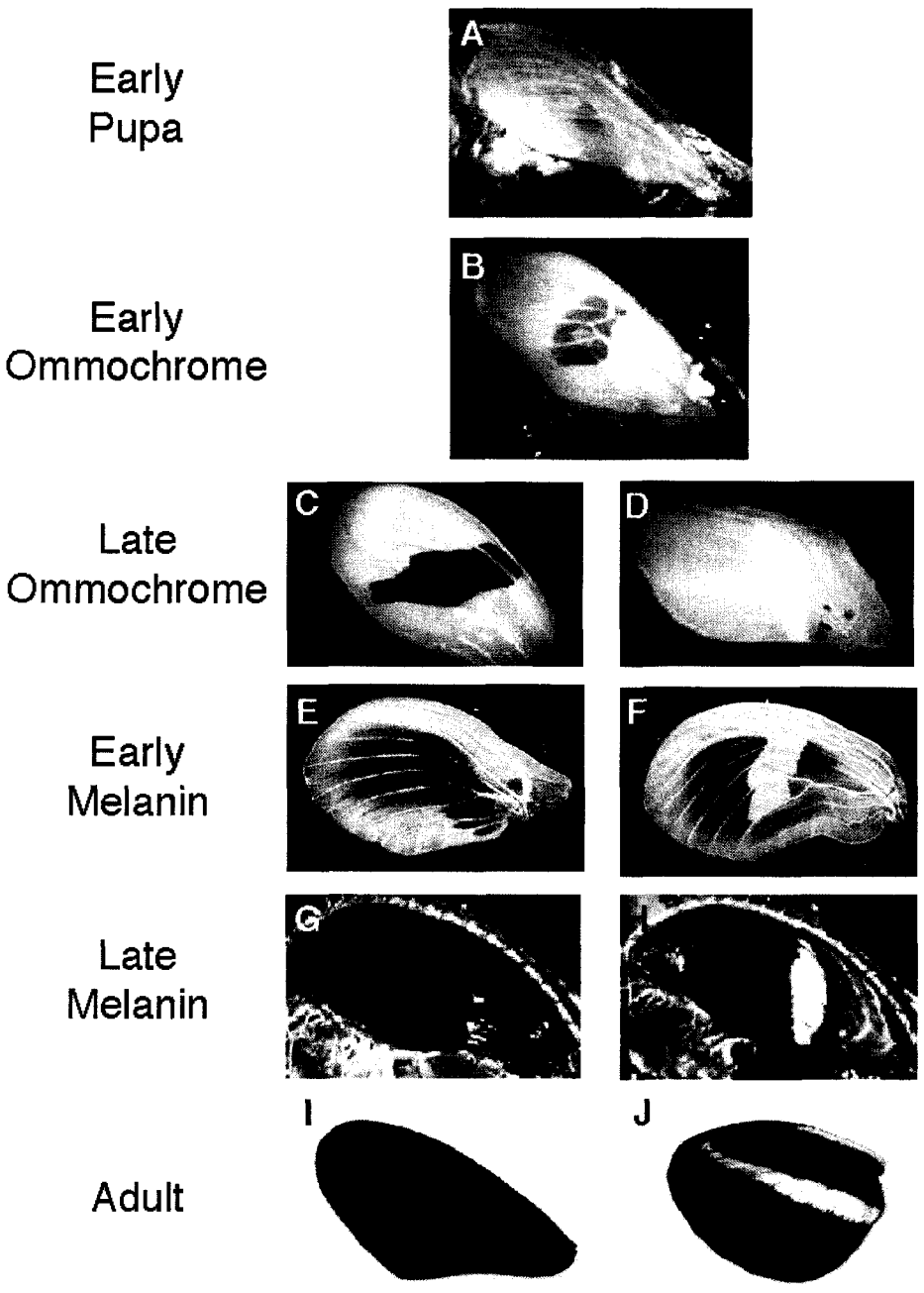
much of the same biosynthetic pathway (Linzen 1974). In most nymphalids surveyed, ommochrome development precedes melanin development in pupal wings (Nijhout 1991), with the exception of the species *Vanessa cardui* in which ommochromes appear only a matter of hours before pupation after melanin pigments have fully developed (Chapter 4). It was the first goal of the work reported here to record the temporal sequence of pigment development in the pupal wing of *H. erato*.

To screen for transcripts whose spatial expression patterns were associated with the forewing band pattern I used cDNA microarray comparisons between RNA taken from micro-dissected pattern elements. I focused these experiments on a Central American race of *H. erato* known as *petiverana* (Fig. 5-1 I, J), a race that has a large red forewing band that is especially easy to manipulate.

To test if some of the candidate genes identified from the *petiverana* microarray screen could be involved with a forewing band color polymorphism, I carried out qPCR experiments in two races of *H. erato* known as *cyrbia* and *himera* (Fig. 5-2). These races occur allopatrically in southern Ecuador and northern Peru and are separated by a narrow hybrid zone which has been studied extensively (Jiggins and McMillan 1997; McMillan et al. 1997). It is debatable whether *himera* constitutes a real species or a race of *H. erato*, but whatever the case may be, *cyrbia* and *himera* are completely interfertile, hybridize in nature, and are closely related. Two genes of major effect have been recognized to influence the forewing band phenotype in these races (Fig. 5-2). The dominant allele of the *R* locus produces a red forewing band phenotype, while the recessive phenotype is a yellow band. The dominant allele of the *Y* locus switches a yellow forewing band to

black. Some *Rr yy* backcrosses show both yellow and red scales in the forewing band divided by a boundary (Fig. 5-2). Focusing on the *cyrbia / himera* hybrid zone allowed me to test if changes in transcriptional regulation are associated with activity of the adaptive phenotypic switch gene *R*. I ultimately apply these data in formulating a general model of *Heliconius* color pattern formation.

Figure 5-1. (*on following page*) Pigment development in *H. erato* pupal wings. In early pupae the wing epithelia are thin and scales have not yet begun to develop (A). Scales fated to become yellow or red mature first (B-D), with the red pigment dihydroxanthommatin beginning to develop immediately (B), becoming progressively darker as time continues (C). After the red pigment has matured, melanic scales develop in a wave emanating from the center of the wing (E, F). At the front of this wave, intervein midline patterns of melanin expression are visible. There is a period after the ommochrome and melanin pigments have fully developed (G, H), that the yellow pigment 3-OHK is still not visually detectable in the wing (H). Sometime in the hours before adult emergence (I, J), the 3-OHK is deposited in the yellow scales (J). A: dorsal forewing of *petiverana*; B: dorsal forewing of *petiverana*; C: dorsal forewing of *petiverana*; D: ventral hindwing of *petiverana*; E: dorsal hindwing of *himera*; F: dorsal forewing of *himera*; G: dorsal forewing of *petiverana*; H: dorsal hindwing of *petiverana*; I: dorsal forewing of *petiverana*; J: ventral hindwing of *petiverana*.


Early
Pupa
A
Early
Ommochrome
B
Late
Ommochrome
C
D
Early
Melanin
E
F
Late
Melanin
G
Adult
I
J

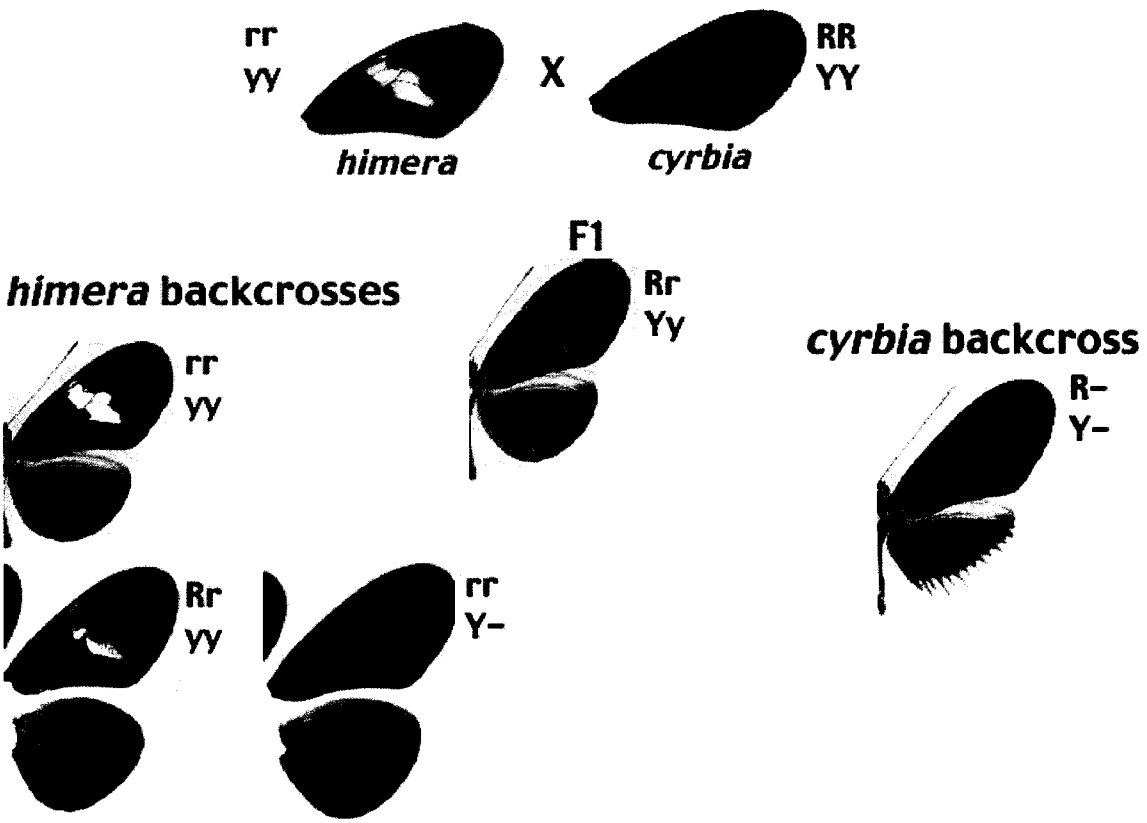


Fig. 5-2. The *R* and *Y* loci control forewing band phenotype in crosses between *cyrbia* and *himera*. The dominant allele of *R* produces a red band, while the dominant allele of *Y* causes yellow scales to become black. (*cyrbia* and *himera* hybrid images taken from Jiggins and McMillan 1997).

MATERIALS AND METHODS

Gene cloning and sequence analysis

cDNA was prepared using total RNA pooled from fifth (last) instar and pupal *H. erato petiverana* wing discs. Degenerate PCR primers were designed from available insect sequences and nested PCR was used to amplify and clone cDNA fragments into TOPO TA vectors (Invitrogren). Positive clones were PCR screened for proper insert size by using M13 vector primers. Automated sequencing was performed using vector primers and sequence identities were screened using nucleotide-protein translated Blastx (NCBI).

cDNA microarray construction

I constructed cDNA arrays representing 103 genes expressed during *H. erato* wing development (Appendix A). 81 cDNAs were ESTs randomly sequenced from a cDNA library representing multiple phenotypes and fifth instar and pupal developmental stages. 22 cDNAs on the microarray represented developmental candidate genes cloned using degenerate PCR. DNA was PCR amplified and spotted on glass Corning slides using a Virtek Chipwriter Pro. Many of the amplified ESTs, and some candidate genes, were kindly provided by W. Owen McMillan. Each array was composed of four identical subarrays, each with double spotted cDNAs. This provided a total of eight within-chip replicates of each cDNA. A list of microarray features is presented in Appendix A. All sequence data will be deposited in Genbank.

RNA extraction

H. erato were reared on *Passiflora biflora* in insectaries in Puerto Rico. Pupal wings and larval imaginal discs were dissected in phosphate buffered saline (pH = 6.8). Wing tissue was either immediately transferred to cold Trizol (Gibco) for immediate RNA extraction, or stored in RNA*later* (Ambion Inc.) at –20°C prior to RNA extraction. Tissue was homogenized and total RNA was extracted with Trizol, and purified with RNeasy columns (Qiagen). Total RNA was run on a gel to verify absence of degradation products.

cDNA preparation and hybridization for microarray experiments

For each microarray replicate, forewings were removed from three late stage *H. erato petiverana* pupae. Wings were dissected into three regions representing major color pattern elements, and the tissue samples were grouped into three respective pools from which total RNA was extracted. Reverse transcription aminoallyl labeling reactions were performed with 20ug of total RNA. The resulting cDNA was column purified and coupled to either Cy3 or Cy5 dye. Labeled cDNA was run on a gel and fluorescently visualized to verify quality of the reaction. Microarray hybridizations were performed overnight at 52°C with an Automated Hybridization Station (Genomic Solutions), and slides were visualized on an arrayWoRx Biochip Reader (Applied Precision Inc.).

I conducted two comparisons using pooled RNA extracted from pupal wings at a stage approximately one day before eclosion, when the scales have matured and red and black pigments have fully developed. The first comparison was between RNA extracted from the proximal melanic region of the wing and the red band, and the second comparison was between the red band and the distal melanic region of the wing. This sampling strategy was conducted in part because the proximal region of the wing produced almost exactly twice the amount of total RNA as the dissected band or distal tip. Each comparison was replicated twice with swapped dyes.

Microarray data analysis

Prior to data analysis, abnormal spots (*i.e.* smeared, scratched, or otherwise obscured) were visually identified and removed from the dataset. Cy3 and Cy5 spot intensities were normalized using the local background and recorded into a spreadsheet. Intensity scores from dye-flipped replicate experiments were imported into the GeneSpring program for analysis. Expression ratios for each gene were calculated using an average of the median-normalized intensities. P-values were calculated using GeneSpring, and fold changes were calculated using Excel.

Table 5-1. Primers used for quantitative PCR

Gene	Primer sequences	
annexin IX	Fwd	5'-CGGTAACTTGGAGAACGTGATCGT
	Rev	5'-TCTTCATCAGTGCCCAATCCTGAC
atet-like	Fwd	5'-TCGAACGCTGTGCAGAGATTTGTC
	Rev	5'-GGTCGTTGGCTCATCAAGGAACAA
vermilion	Fwd	5'-CCTGGAAACAATGACACCACTGGA
	Rev	5'-ATTGCAAGCTTTGGAAGCCAGACG
cinnabar	Fwd	5'-CCGGCTTCGAGGACTGTTATCTAT
	Rev	5'-CACTGATCGCAAATGCATCTCTCC

Quantitative real-time PCR

Total RNA extracted from the identical wing pattern elements from both right and left wings of a single individual was treated with the DNA-free kit (Ambion, Inc.), after which cDNA was synthesized using the TaqMan reverse transcription kit (Applied Biosystems, Inc.) with polyT primers. Locus-specific primers (Table 5-1) were used for SYBR Green (Applied Biosystems, Inc.) real-time PCR reactions carried out in an ABI Prism 7000 Sequence Detection Systems. Relative transcription levels were calculated using a standard curve from a cDNA standard dilution series. *cinnabar*, *vermilion*, and *atet-like* data were normalized using expression levels of an *annexin IX* control. Three technical replicates were executed for each time point.

RESULTS

Pigment and scale development in *H. erato*

Early pupal wing development in *H. erato* was found to be similar to that in other butterflies, with the wing tissue starting as a very thin epidermal bilayer (Fig. 5-1 A) that thickens rapidly as scale forming cells develop during the first few days after pupation (Nijhout 1991; Galant et al. 1998). Even though the parallel arrangement of scale precursor cells is determined within the first day after pupation in *H. erato* (Reed 2004), scale maturation (*i.e.* inflation and sclerotization) takes place at different times for scales associated with different color pattern elements.

There is a discrete and well-defined progression of scale maturation that occurs. The first scales to mature are those that will bear the "background" pattern. These scales appear to mature simultaneously across the dorsal and ventral surfaces of both forewings and hindwings. The scales that will bear the red ommochrome pigment dihydroxanthommatin begin developing their color early during scale maturation (Fig. 5-1 B). The color of the ommochrome-bearing scales changes during pupal development, beginning as a light orange color (Fig. 5-1 B) and progressively darkening until they are red (Fig. 5-1 C). Scales that will bear the yellow pigment 3-hydroxykynurenine mature completely without color (Fig. 5-1 D, F, H), becoming yellow only immediately before adult emergence. Scales that will remain colorless throughout the life of the butterfly develop at the same time as the presumptive red and yellow scales, yet never produce pigment (not shown). Ommochrome development in wing scales occurs at the same time as ommochrome development in the eye, and can be easily detected by shining a light through the developing pupa.

After the scales bearing the background pattern have developed and the ommochromes have darkened, the melanic scales develop in a "wave" moving outward from the center of the wing (Fig. 5-1 E, F). This wave of melanic scale development essentially fills in all regions of the wing that have not developed matured scales. At the distal front of the melanin development, there are transiently visible intervein midlines that superficially resemble Notch and Distal-less expression in larval wing discs (Reed and Serfas 2004). After all of the melanic scales have fully developed (Fig. 5-1 G, H),

and presumably only a few hours before adult emergence (Fig. 5-1 I, J), the yellow pigment 3-OHK becomes visible (Fig. 5-1 J).

In sum, the most novel finding from the above observations is that the yellow pigment, 3-OHK, appears very late in wing development after melanin development. This suggests that yellow pigments and red pigments may have different mechanisms of regulation even though they share much of the same biosynthetic pathway.

Microarray screen of forewing transcription in *petiverana*

The microarray experiments conducted on *petiverana* forewings used RNA from a relatively late time point in pupal development after scales had matured and melanin and ommochrome pigments had developed. This time point was chosen in hopes of maximizing representation of transcripts implicated in pigment development.

In the microarray comparison between proximal wing and red band RNA in *petiverana*, there were several transcripts found to have significantly different expression levels with a greater than two-fold change (FC) between tissue types (Table 5-2). A homolog of a gene encoding the putative cuticle protein TM-A1A had a significant 5.3 FC higher level of expression in the proximal region of the wing. This was the only transcript from the comparison to be found with a more than 2-fold higher abundance in the proximal region. In contrast, seven genes were expressed with > 2 FC in the red band, four of which were significant: *cinnabar*, *atet-like*, *scarlet*, and *pale*. All four of the significant genes are implicated in either the transport of synthesis of pigments and pigment precursors.

Table 5-2. Gene transcription differences in proximal versus red band wing tissue in late pupal *H. erato* .

Tissue Type	Similarity	FC	Highest	Putative function
Proximal	*TM-A1A*	5.3*	307.6	Cuticle protein
Red Band	*cinnabar*	14.5*	39.1	Ommochrome synthesis enzyme
	atet-like	9.3*	12.5	Novel ABC transporter
	vermilion	3.7	4	Ommochrome synthesis enzyme
	scarlet	2.8**	0.8	Pigment precursor transporter
	mpi	2.6	1.2	Metabolic enzyme
	pale	2.1**	104.8	Melanin synthesis enzyme
	gdph	2.0	1.1	Metabolic enzyme

*: $p < 0.05$; **: $p < 0.005$

Table 5-3. Gene transcription differences in proximal versus distal wing tissue in late pupal *H. erato* .

Tissue Type	Similarity	FC	Highest	Putative function
Proximal	*TM-A1A*	19.3**	563.2	Cuticle protein
	AF117584	6.3**	37.4	Cuticle protein
	AF117601	5.3**	47.1	Cuticle protein
	CUPP_BOMM	3.3**	531	Cuticle protein
	Frost	3.1	714	Cold response
	AF117600	3.0**	977.8	Cuticle protein
	GMPCP52	2.7**	686.6	Cuticle protein
	MAFK-like	2.7**	97.3	Transcription factor
	Trip1	2.1**	19.1	Translation initiation factor
	CG1919	2.0**	2,354	Cuticle protein
Distal	*scarlet*	6.0**	10.8	Pigment precursor transporter
	mpi	3.4	2.2	Metabolic enzyme
	atet-like	3.0	3.9	Novel ABC transporter
	ABC 2	3.0	5.6	Novel ABC transporter
	vermilion	2.9	7.3	Ommochrome synthesis enzyme
	distal-less	2.8	3.7	Transcription factor
	ABC 1	2.4*	4.9	Novel ABC transporter
	CG30042	2.3	3.9	Structural molecule
	cinnabar	2.3	6.1	Ommochrome synthesis enzyme
	white	2.2	4.1	Pigment precursor transporter
	gasp	2.1**	722.4	Chitin binding
	pale	2.0**	70.3	Melanin synthesis enzyme

*: $p < 0.05$; **: $p < 0.005$

The comparison between the proximal and distal wing RNAs also indicated a number of transcripts with > 2 FC in either tissue types (Table 5-3). In the proximal tissue there were nine transcripts with significant FCs greater than 2, all but two of which were putative cuticle binding proteins. As well, there was a transcript encoding an apparently novel transcription factor related to MAFK, and a transcript encoding a putative homolog of the *Drosophila* translation initiation factor Trip1. There were four transcripts found to be significantly upregulated in the distal tissue: *scarlet*, *gasp*, *pale*, and a novel ATP-binding cassette protein I refer to as *ABC 1*.

With both microarray comparisons taken together, *cinnabar* and *atet-like* transcripts were identified as the only genes significantly upregulated *only* in the forewing band on post-melanin *petiverana* forewings.

Pattern-specific vs. polymorphism-specific gene expression in *cyrbia* and *himera*

Given the results from the *petiverana* microarray experiments, I selected *cinnabar* and *atet-like* as candidate genes for comparison between *cyrbia* and *himera* forewings. I additionally chose to examine the expression of *vermilion* although it did not have significant upregulation in the forewing band. *cinnabar* and *vermilion* both encode enzymes implicated in ommochrome pigment synthesis, and I wanted to test the hypothesis that these genes may be co-regulated in some manner not detected by the microarray study.

Table 5-4. Candidate genes for qPCR normalization.

	Prox : Band		Prox : Dist		
Similarity	**Ratio**	**Mean Signal**	**Ratio**	**Mean Signal**	**Putative function**
AB047488	1.082	113.8	1.022	108.7	Cuticle protein
α spectrin	0.978	44.7	0.95	46.1	Actin binding
*annexin IX**	1.055	114.2	1.001	106.8	Actin binding
apoptosis inhibitor	1	39.8	0.958	35.5	Apoptosis inhibition
ciboulot	1.078	367.8	1.035	399	Actin binding
doppio fuso	1.054	509.4	0.924	487.3	poly(A) binding
polyubiquitin	0.914	89	1.022	69.3	Protein degradation tag
trans. init. factor subunit 3	1.025	487.2	1.088	725.85	Translation initiation

**annexin IX* was ultimately used to normalize qPCR data.

In order to identify suitable control genes for use the qPCR experiments, I searched the microarray data for genes that varied the *least* between tissue types across comparisons, yet were also transcribed at relatively high levels (Table 5-4). The majority of transcripts that fit these criteria encoded putative actin-binding proteins. Of these candidates, the *annexin IX* transcript turned out to produce robust and consistent signal in qPCR experiments so I chose to use this transcript for qPCR normalization.

In *cyrbia*, *atet-like* transcripts were found throughout the wing during early ommochrome development in graded distribution of transcript abundant increasing proximally to distally (Fig. 5-3 A) After melanin development, *atet-like* expression was only detected in the proximal and distal melanic regions of the wing (Fig. 5-3 C). During the early ommochrome stage in *himera*, *atet-like* was also detectable at relatively low levels in the three wing regions (Fig. 5-3 B), however, unlike in *cyrbia*, there was no detectable expression after melanin maturation (Fig. 5-3 D).

In *cyrbia*, *vermilion* transcript abundance was highest in a given tissue at the time of scale maturation. During early ommochrome development, when the red band scales were maturing, *vermilion* had a high relative abundance in the band (Fig. 5-3 E). Later, when the melanic edge scales were maturing, *vermilion* was expressed at high levels in the melanic tissue, but not the red band tissue (Fig. 5-3 G). It is this latter time point that was sampled in the *petiverana* microarray work, which may explain why the ommochrome-stage red band *vermilion* association was not previously noticed.

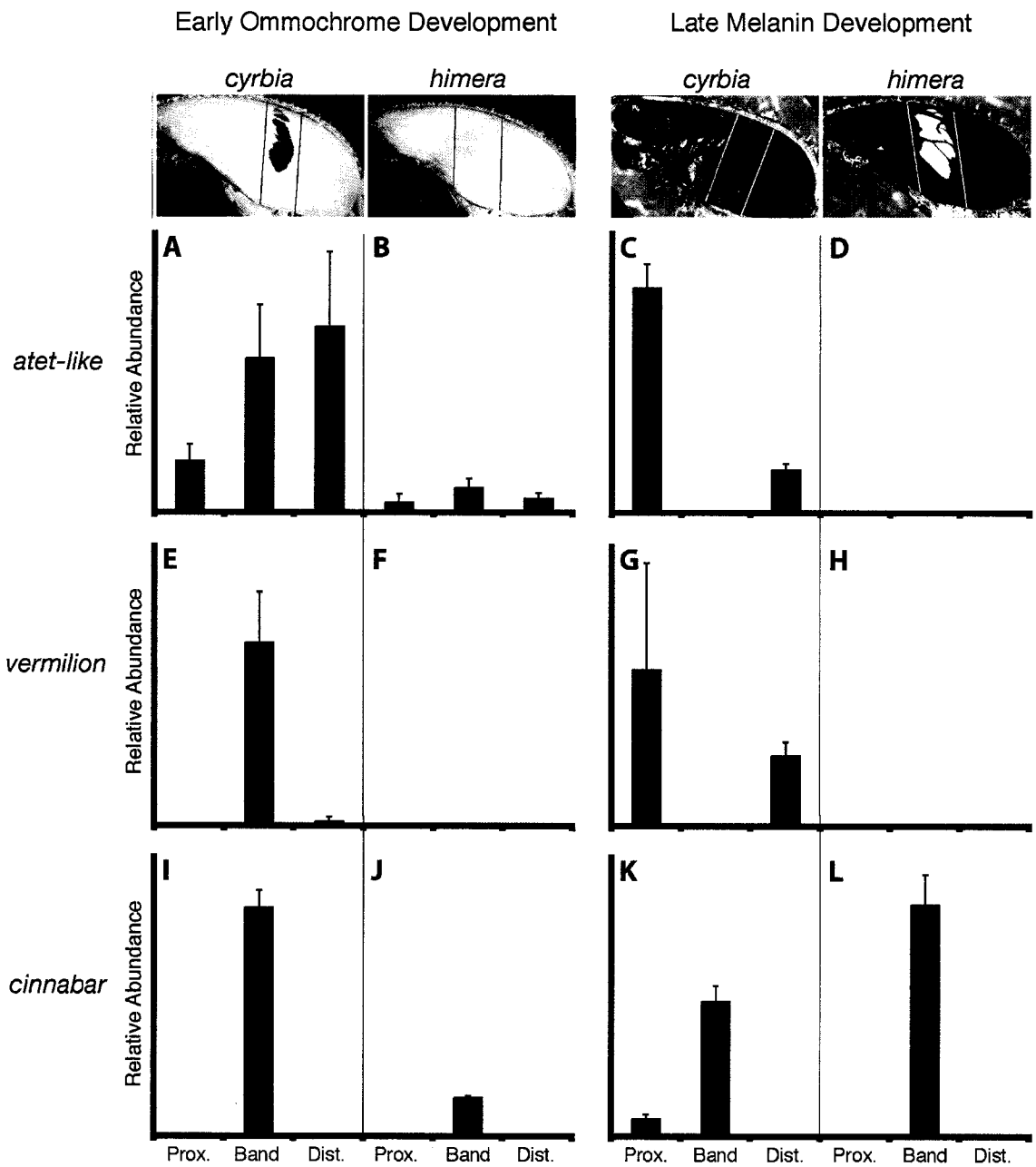


Fig. 5-3. Quantitative RT-PCR comparison of *atet-like* (A-D), *vermilion* (E-H), and *cinnabar* (I-L) transcript abundance during pigment development in *cyrbia* (A, E, I, C. G. K) and *himera* (B, F, J, D, H, L). Synthesis of the red pigment dihydroxanthommatin occurs when the transcription of all three genes spatiotemporally coincides in the forewing band (A, E, I).

In *himera* there was no detectable *vermilion* expression in the wings during ommochrome or melanin development (Fig. 5-3 F, H). This association of *vermilion* downregulation in the forewings of butterflies homozygous recessive at the *R* locus is the first evidence of the molecular activity of the *R* gene itself.

In contrast to *vermilion* expression, which appeared to be polymorphism-associated, *cinnabar* expression was strongly correlated with color pattern irrespective of developmental stage. Specifically, *cinnabar* transcript abundance was positively associated with the forewing band in both *cyrbia* and *himera* during both ommochrome and melanin development (Fig. 5-3 I-L).

DISCUSSION

Timing of scale and pigment development

My observations of *Heliconius* dihydroxanthommatin development followed the typical mode of ommochromes preceding melanins (Nijhout 1991). The change of ommochrome color from orange to red during early scale maturation (Fig. 5-1 B, C) was suggestive of xanthommatin being slowly reduced to its dark red form, dihydroxanthommatin, during scale maturation.

A novel finding from this study concerns the timing of 3-OHK appearance in yellow pattern elements. Because 3-OHK is a precursor of dihydroxanthommatin (see Chapter 4), my initial hypothesis was that 3-OHK would develop before or at the same time as xanthommatin. This hypothesis would have seemed even more plausible given

the visible progression of dihydroxanthommatin development during early scale maturation. Finding that 3-OHK develops long after dihydroxanthommatin was surprising and suggests that there is a fundamental difference in the production of 3-OHK versus ommochromes in *H. erato* scale cells. Together these observation suggest two different modes of tryptophan-derived pigment development in butterfly wings: (1) during presumptive color scale maturation, preceding melanin development, and (2) after scale maturation and melanin development, just before adult emergence.

The reason for these different modes of tryptophan-derived pigment development is unknown, but it is possible that they represent different biosynthetic strategies. In particular, the lack of *vermilion* expression in *himera* wings would lead one to speculate that tryptophan processing may not occur in the yellow scales themselves, but that kynurenine or 3-OHK are taken up into scale cells directly from the hemolymph. Indeed, there is a precedent for this hypothesis in that cultured wings of the nymphalid *Araschnia levana* were found to incorporate radio-labeled 3-OHK into ommochrome-bearing scale cells (Koch 1991). In the near future I aim to conduct further experiments to test this model in *Heliconius*, including precursor efflux/uptake assays using an *in vitro* wing culture system, and tests of pigment-associated enzymatic activity in wing extracts.

Gene expression associated with the *petiverana* forewing band

The microarray study of gene expression in the post-melanin *petiverana* forewings highlighted several color pattern-associated transcripts. Multiple mRNAs were found with significantly higher expression in the red band compared to the proximal melanic

region of the wing, all of which are potentially associated with pigment synthesis. *cinnabar* is an enzyme implicated in ommochrome synthesis and has already been reported to be expressed in xanthommatin-bearing butterfly wings (Chapter 4). Finding this gene expressed at a significantly high level in the forewing band was therefore unsurprising. *pale* encodes tyrosine 3-monooxygenase (EC:1.14.16.2), an enzyme typically associated with both melanin and cuticle development. Given this enzyme's probable function in synthesizing scale cell cuticle, one would expect *pale* to be expressed throughout all maturing scale cells during late pupal development. *pale* was also transcribed at a significantly high level in the distal region of the wing when compared to the proximal region. Activity of *pale* may be higher in the band and distal regions of the wing at the sampled time point due to the temporally progressing wave-like nature of melanic scale development.

scarlet encodes an ATP-binding cassette transporter molecule implicated in ommochrome precursor transport. It was therefore unsurprising to find it to be significantly abundant in the forewing band. It was unexpected, however, to find *scarlet* expressed in the distal melanic tissue. Although the hypothesis is speculative, the *scarlet* expression in the distal region may represent a latent color pattern "potential" obscured by the melanin pattern, an idea which is consistent with the background / "shutter" pattern model.

atet-like encodes a previously undescribed ATP-binding cassette transporter molecule that is closely related to *scarlet*, *white*, and *atet*. This transporter could potentially be involved with transporting pigment precursors, so it was of great interest to

find its transcripts associated with the red band. Several other undescribed ATP-binding cassette transporter transcripts represented on the microarray were not found to be associated with specific pattern elements.

Several genes known to be involved in ommochrome synthesis were not detected by the microarray work as being significantly associated with the red band at the time point sampled, most notably the ommochrome precursor transporter *white*, and the ommochrome enzyme *vermilion*. While both of these genes were expressed in the *H. erato* wing, neither appeared in patterns spatially associated with specific color patterns. These transcripts, however, were present in ommochrome-bearing tissue and therefore potentially available for pigment synthesis.

Pigment patterns are associated with spatiotemporal overlap of pigment gene transcription in *cyrbia* and *himera*

The enzymes encoded by *vermilion* and *cinnabar* are required for the synthesis of both 3-OHK and dihydroxanthommatin pigments (Linzen 1974). The most obvious initial prediction was that both genes would be transcribed specifically in the forewing bands of *himera* and *cyrbia*, which are pigmented by 3-OHK and dihydroxanthommatin respectively. Consistent with this hypothesis, transcription of both genes overlapped in the red forewing band of *cyrbia* during the synthesis of dihydroxanthommatin. However, an unexpected finding was that *vermilion* and *cinnabar* transcription never overlapped in the 3-OHK bearing forewing band of *himera* (Fig. 5-2 H, L)

Because *vermilion* and *cinnabar* overlap was not observed in the yellow *himera* forewing band, it is reasonable to hypothesize that 3-OHK synthesis may not take place in yellow scale cells. Indeed, *vermilion* transcripts were never detectable in *himera* wings at all, and the late stage of the last sampled time point in the qPCR experiment makes it seems unlikely that within the few hours before emergence *vermilion* would be transcribed, translated, and activated quickly enough to produce appreciable quantities of 3-OHK. As discussed above, these data would suggest then that the entire 3-OHK synthesis process does not occur in yellow scales, and that 3-OHK, or its precursor kynurenine, is taken up directly from the hemolymph. More work needs to be done to determine when , where, and how 3-OHK transport may occur.

The downregulation of *vermilion* associated with the recessive *R* phenotype raises the tantalizing possibility that *R* may be involved with activating *vermilion* in *Heliconius* wing scales, or that *vermilion* may be the *R* gene itself. To test this latter hypothesis I am now collaborating with the McMillan lab to test for genetic linkage between *R* and *vermilion.*

Another interesting observation from the *cyrbia* qPCR data was the apparent difference in *atet-like* and *vermilion* expression patterns when compared to the *petiverana* microarray results. Both *petiverana* and *cyrbia* have forewing patterns that are superficially similar, although the exact size and placement of the red band is slightly different between the two races. Patterns of *atet-like* expression in post-melanin pupal wings of *cyrbia* and *petiverana* appeared to be opposite of each other. In *petiverana*, *atet-like* was specifically upregulated in the forewing band, while in *cyrbia atet-like* was

upregulated in the distal and proximal melanic regions. This apparent contradiction between the *cyrbia* and *petiverana* results could be attributable to issues of fine-scale timing in tissue sampling. Alternatively, and possibly more interestingly, the contradiction could indicate cryptic differences in the patterning mechanisms underlying the formation of the red bands in *cyrbia* and *petiverana*. There is always the additional possibility that *atet-like* is completely irrelevant for pigment synthesis. At this point, I cannot make any firm conclusions regarding *atet-like* beyond classifying it as a candidate gene for future studies.

A model of background color pattern determination in *Heliconius*

It is not known exactly when the boundaries between the melanin pattern and the color background in *Heliconius* are established, but they must be determined prior to scale development because early scale maturation presages the melanin / color boundaries prior to pigment development. The boundaries between red, white, and yellow background pattern elements, however, could be determined during or after scale maturation. The temporal sequence of background color development mirrors the general trend in genetic dominance of the pigment types. Recent crosses between various *H. erato* races suggest that red background is dominant to white patterns, and white patterns being dominant to yellow (D.D. Kapan, pers. comm.). Given that black, and occasionally red, patterns are typically dominant to all background patterns, and that melanin boundaries are known to be established very early, the sequence of pigment development may be indicative of a progressive, temporal pattern determination sequence, where

yellow is the recessive ground state. Future experiments using timed tissue transplants between red, white, and yellow color pattern elements might help discern when background scale color is determined.

How are the boundaries between red, white, and yellow defined? I propose that these boundaries are based on the regulation of genes in the tryptophan-ommochrome biosynthetic pathway. As seen in the qPCR data, scales fated to bear dihydroxanthommatin express transcripts of all the putative ommochrome enzymes and transporters I assayed. The spatial coincidence of these transcripts occurs at the same time-point when dihydroxanthommatin is being actively synthesized. At the same time in the *himera* forewing band, the ommochrome biosynthesis pathway is essentially truncated due to a lack of *vermilion*, and xanthommatin is never produced.

vermilion transcription appeared to be correlated with scale maturation. An association between scale maturation time and pigment enzyme activity has also been noted in the context of papiliochrome development in the swallowtail butterfly *Papilio glaucus*, where temporal regulation of scale maturation has been proposed to be a mechanism for color pattern determination (Koch et al. 2000a; Koch et al. 2000b). Conversely, in *H. erato*, *cinnabar* expression is associated with the band pattern itself irrespective of the state of scale development. The interaction between the temporal pattern of *vermilion* transcription, and the spatial pattern of *cinnabar* transcription, is suggestive of a larger process of pattern formation where dynamic patterns of enzyme and transporter expression overlap like a whole-wing Venn diagram to determine the background pattern boundaries. Some backcross hybrids between *cyrbia* and *himera*

show a red / yellow boundary in the middle of the forewing band (Fig. 5-2). I would predict that this boundary represents a dividing line between red cells that express *vermilion*, and yellow cells that do not.

Because yellow pigment is unlikely to be synthesized in scales themselves, I posit that the switch between yellow and white involves the modulation of a kynurenine or 3-OHK cell-membrane transporter that is active very late in pupal development. Given that white patterns tend to be dominant over yellow, loci determining white patterns are likely to be dominant repressors of this transporter. White forewing band phenotypes were not considered in this study, but future work should be done to compare the expression of candidate transporter genes between white and yellow patterns. I have identified a number of apparently novel ATP-binding cassette transporters expressed during butterfly wing development that may potentially be involved in pigment development.

CONCLUSION

The work presented in this chapter represents an early step towards a molecular understanding of wing pattern development and polymorphism in *Heliconius* butterflies. In addition to supporting the hypothesis that changes in transcriptional regulation of pigment genes may underlie *Heliconius* wing pattern polymorphism, these data provide a solid foundation of testable molecular models of pigment pattern development. Here I have identified specific molecules whose expression patterns and functions are associated with adaptive polymorphisms that have interested biologists for over a century. There is

a great deal of further work to be done on comparative analysis of gene expression, enzyme kinetics, gene regulatory evolution, genetics, and pigment synthesis in butterflies.

Resources are now being developed for larger scale analyses of *Heliconus* wing pattern development. An EST library, large-scale microarrays, a genomic BAC library, and a genome map for *H. erato* will all aid in further characterizing genes involved in making wing patterns. The near future holds great promise for gaining an in-depth molecular perspective on how the *Heliconius* wing patterning system operates, a perspective that will be of broad significance for understanding how developmental patterning systems originate and diversify under natural selection.

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CHAPTER 6

CONCLUSION

I hope that this dissertation represents a useful contribution to the knowledge regarding the evolution of pattern development in butterfly wings. The data I have presented are primarily comparative in nature, however their interpretation is given functional significance within the larger context of experimental work done on the molecular model system *Drosophila melanogaster*. The four concepts I hope readers will take from this dissertation are as follows:

1. There are multiple roles for Notch in butterfly wing patterning.

There are a limited number of intercellular signaling pathways known to function in metazoans, and these various pathways are reused over and over again in different contexts during an animal's development. Because of this, it was not surprising to find Notch implicated in wing patterning at multiple levels *per se*, however the sheer novelty of some of the cases reported here make them interesting for several reasons.

As outlined in Chapters 2 and 3, Notch signaling appears to play a role in eyespot determination, intervein midline pre-patterning, and scale cell organization. The former two of these are of interest because they represent a function of Notch early in the development of two characters that are thought to be special innovations of the

butterflies. In the case of eyespots, Notch signaling operating upstream of a cascade of "recruited" transcription factors, an altered hedgehog circuit, the eyespot morphogen, and ultimately pigment and scale development, represents a fairly dramatic instance of evolutionary developmental recombination. Together, this cascade provides a valuable glimpse from a non-model organism into the development of a simple, discrete morphological character from its inception to its final realization,

The case of Notch's inferred role in organizing scale cells into parallel rows (Chapter 3) is evolutionarily significant because it provides a potential molecular basis for the parallel evolution of precursor cell organization in fly bristles and butterfly scales. Both of these cell types have an ancestral state of random organization, and a derived state of parallel row organization. In every aspect examined, the expression of Notch and *achaete-scute* during butterfly scale development is consistent with the known function of these genes in organizing fly bristles. It is therefore worth asking why the independent origins of row organization both appear to employ a Notch-mediated lateral inhibition process. Perhaps something inherent to the architecture of the Notch pathway increases the potential of similar patterns re-emerging throughout metazoan evolution. This concept of network structure being responsible for recurring morphological motifs deserves further investigation, and scale versus bristle organization may provide a useful model system for this purpose.

2. Discrete morphological characters may evolve through changes in the regulation of temporal pattern formation processes.

In addition to characterizing Notch's early role in butterfly eyespot determination, Chapter 2 also presented a comparative analysis of gene expression time series from multiple species of Lepidoptera. To my knowledge, this is among the first analyses of its type, and provides unusual insight into the evolution of morphology-related molecular pre-pattern formation. It was possible to trace onto an organismal phylogeny the gains and losses of specific gene expression patterns associated with the evolution of a morphological character. While the taxon sampling in this study may have been somewhat limited, as a test case it demonstrated the potential of a developmental phylogenetic approach for expanding our understanding of character evolution.

One notable observation coming from the cross-species comparison of gene expression is that, prior to eyespot focus determination, the gene expression patterns transiently pass through what appears to be an ancestral state resembling midline pigment patterns. Although it is not clear what the developmental significance of this is, if any, it is reminiscent of classic models of heterochronic character evolution. The observed sequences of gene expression may represent a kind of localized recapitulation in a molecular pattern formation process. A larger taxon sampling analyzed with more sophisticated "phylochronological" methods could provide a great deal of insight into how different modes of gene regulation may underlie evolutionary character state changes.

3. Multiple eye pigment genes are expressed in butterfly wings.

It is demonstrated in Chapter 4 that genes implicated in ommochrome pigment development are expressed during butterfly wing development. While it has been known for some time that ommochrome pigments are synthesized in butterfly wing scales, these data provide a molecular handle on the specific genes that may be involved in this process. In the larger realm of butterfly wing patterning, these genes likely represent some of the furthest downstream effector genes in the wing pattern development process. This is significant because the research community now has candidate genes associated with wing pattern development at all major points during the pattern formation process; from very early pre-pattern genes like Notch and Distal-less, to the later pattern elaboration such as Spalt and genes in the hedgehog pathway, and now the pigment synthesis genes themselves. While this collection of candidate genes probably only represents a bare skeleton of the pattern formation process, we at least now have anchor points at all the major steps of pattern formation and realization. The important processes for which we currently lack candidate genes are the long-range induction of eyespot and margin patterns, and the regulation of scale maturation.

The finding that ommochrome genes are expressed in butterfly wings also provides a new evolutionary puzzle. The ancestral function of these genes in insects is most likely for visual filtering. I know of no phylogenetic precedent for the synthesis of ommochromes in scale cells prior to the occurrence of this trait in nymphalild butterflies. Unlike the other genes associated with wing pattern development (*i.e. Notch, Distal-less*,

etc.), the ommochrome genes probably have relatively simple *cis*-regulatory logic, as they are not known to be reused in many different contexts in animal development. There is experimental evidence showing that the promoter driving eye expression of *cinnabar* in *Drosophila* is within ~1kb of the gene, and its function is conserved in distantly related mosquitoes. Because we can trace the expression of the ommochrome genes in wings to a single lineage, and because their regulation is likely to be fairly simple, ommochrome genes may provide an opportunity to study how evolutionary changes in *cis*-regulatory elements can underlie novel morphologies.

4. Gene expression associated with *Heliconius* wing pattern development and polymorphism.

Prior to collecting the data presented in Chapter 5, nothing was known about wing pattern-associated gene expression in *Heliconius*, despite the fact that these butterflies are one of the classic systems in ecology and evolution. The results of the work here provide us with several candidate genes that may play a role *Heliconius* wing patterning. Of particular interest are the pigment enzyme-encoding genes *cinnabar* and *vermilion*. *cinnabar* is the first transcript found to be associated with a specific *Heliconius* pattern element, while *vermilion* is the first transcript identified whose expression is associated with a pattern polymorphism.

These findings are significant for several reasons. From a technical perspective they illustrate that a combination of microarray and quantitative PCR techniques can be

effective in identifying pattern-associated gene expression in butterfly wings. More importantly, however, *cinnabar* and *vermilion* are the first genetic leads into the process of *Heliconius* wing pattern development. Future work on *cinnabar* has the potential to provide insight into the regulatory network underlying specification of the forewing band. With the newly available method of butterfly germline transformation, it should be possible to identify the band-specific *cinnabar* enhancer. *vermilion* is a prime candidate gene for the switch gene underlying the red/yellow wing pattern polymorphism and should be tested for linkage with the switch gene as soon as possible.

What's next?

The work in this dissertation newly implicates two molecular systems in wing pattern development: the Notch signaling pathway, and the ommochrome biosynthetic pathway. These discoveries relied heavily on the candidate gene approach, however new technologies should make it possible to identify pattern-associated genes in a more efficient and high-throughput fashion. In particular, Chapter 5 shows that microarrays can be effective for identifying pattern-specific transcripts. Fortunately, the data from Chapter 5 have made it possible to secure significant funding to develop a *Heliconius erato* EST library and high-density microarray in order to identify more candidate genes associated with color pattern development and polymorphism. A recently completed linkage map of the *H. erato* genome will make it possible to screen candidate genes for linkage with switch genes, and a new BAC library will make it possible to positionally clone wing pattern genes.

Now that we have several candidate genes in hand, and a microarray project that promises to identify more candidate genes, it will be important to develop methods to directly test hypotheses of gene function and regulation in butterflies. As mentioned in the introduction, butterflies have proven to be curiously resistant to the popular gene knock-down methods. Fortunately, germline transformation in butterflies is now possible, though the technique is labor intensive and so far has not been used to test any functional hypotheses. In theory, however, the tools are available to introduce novel elements into the butterfly genome, making it possible to test the function of specific proteins in regulatory elements in wing pattern development.

APPENDIX A – MICROARRAY FEATURES

Clone ID	Gene Name / BLAST Similarity
R121.8	Ultraspiracle
cDNA PCR	EF1a
4/27.17	pale
R57.6	white-like
R57.18	white
R57.19	scarlet
R63.22	vermilion
R24.4	cinnabar
R57.16	ATET
R57.25	ABC 2
R57.32	ABC 1
R208.5	vermilion
R215.4	cinnabar
cDNA PCR	EF1a
R166.7	pale
R121.15	cn-like
R121.14	cinnabar
R164.7	vermilion
R121.5	DDC
cDNA PCR	EF1a
WOM Clone	decapentaplegic
WOM Clone	patched
WOM Clone	scalloped
WOM Clone	distal-less
A01A02	enhancer of delta KP135
A01B03	CG8496 gene product [Drosophila melanogaster
A01E01	Cuticle protein 7 (L2-7) (L2-ACP 7),
A01F02	cuticle protein [Bombyx mori] CG15008
A01H07	dusky
A02A10	Cuticle protein 21 (LM-ACP 21).
A02D12	LD19086p [Drosophila melanogaster].
A04B08	CUP5_GALME PUPAL CUTICLE PROTEIN PCP52 PRECURSOR (GMPCP52)
A04B12	Nlaz
A04H07	yellow-c
A06E11	AF117600_1 (AF117600) putative cuticle protein [Manduca sexta]
A07D03	heparin binding glycoprotein
A07E09	CU08_LOCMI Cuticle protein 8 (LM-8) (LM-ACP 8), CG1919, CG7072
A07E12	dihydropterin reductase
A08C01	cAMP enhancer BP
A08F02	polyA BP
A08G06	Rab-RP1
A08H07	AF117584_1 (AF117584) putative cuticle protein [Manduca sexta] CG 13043
A09F06	Nlaz
A09F08	CUPP_BOMMO PUPAL CUTICLE PROTEIN PRECURSOR
A09G10	Apoptosis Inhibitor
A09G11	Rack1-P1
A10B09	bicaudal
A10B12	zinc-finger 236

Clone ID	Gene Name / BLAST Similarity
A10D10	knirps
A10E05	CU19_LOCMI Cuticle protein 19 (LM-19) (LM-ACP 19) CG1919, CG9290
A10G01	MAFK TF
A10H01	yellow
A10H02	CU1A_TENMO Larval cuticle protein A1A (TM-A1A) (TM-LCP A1A)
A11A04	Trip1
A11B06	Gasp
A11F04	EF1a
A11F09	casein kinase
A11H08	ser/threonine kinase domain
A11H12	Ras Interacting Protein
A12A12	easter
A12D03	AF117601_1 (AF117601) putative cuticle protein [Manduca sexta]
A12E02	spaghetti
A12H07	(AB047487) cuticle protein [Bombyx mori]
A12H09	cysteine string protein
A13D12	argos
A13E02	tup-like
A13F12	single-minded
A14A02	ecdysone dependent protein
A14A04	pentaxin-like
A14A05	Calnuc
A14A12	Ca Transporter
A14C08	(AB047483) cuticle protein [Bombyx mori]
A14C09	GPCR
A14D06	polyubiquitin
A14D10	integrin alpha 3A
A14E02	leonardo
A14E09	headline, laminin A
A14E10	alpha-spectrin
A14E12	astray, phosphatase
A14H12	translation initiation factor 5a
A15C03	AF117586_1 (AF117586) putative cuticle protein [Manduca sexta]
A15D07	Annexin IX
A15F01	JH esterase-like
A15G02	homothorax
A15G08	ABC subfamily E member 1
A16A08	SP1070, Notch-like
A16B01	doppio fuso
A16B08	serine/threonine kinase
A16D05	Rho1-P1
A16D08	cathepsin-B
A16E10	passover
A16F11	ciboulot
A16G05	stripe
A16H03	(AB047488) cuticle protein [Bombyx mori] CG8515
A16H09	trithorax
A17B02	DDC
A17C03	cyclin D2
A17C05	calreticulin
A17H02	Trans init factor 3 subunit
A17H10	PAST1
A18B02	Dox-A2-P1
A18C11	ubiquitin-conjugating enzyme (bendless)
A18E08	ABC Binding Protein
A18F08	Pale (TH)
A18H12	skeletor
WOM Clone	gdph
WOM Clone	mpi
WOM Clone	gpi
WOM Clone	tpi

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