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Evolutionary redeployment of a biosynthetic module: expression of eye pigment genes *vermilion*, *cinnabar*, and *white* in butterfly wing development

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SUMMARY Ommochromes are common among insects as visual pigments; however, in some insect lineages ommochromes have evolved novel functions such as integument coloration and tryptophan secretion. One role of ommochromes, as butterfly wing pigments, can apparently 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. To help understand how this subcellular process appeared in a novel context during evolution, we explored aspects of ommochrome pigment development in the wings of the nymphalid butterfly *Vanessa cardui*. Using chromatography and radiolabeled precursor incorporation studies we identified the ommochrome xanthommatin as a *V. cardui* wing pigment. We cloned fragments of two ommochrome enzyme genes, *vermilion* and *cinnabar*, and an ommochrome precursor transporter gene, *white*, and found that these genes were transcribed in wing tissue at relatively high levels during wing scale development. Unexpectedly, however, the spatial patterns of transcription were not associated in a simple way with adult pigment patterns. Although our results suggest that the evolution of ommochrome synthesis in butterfly wings likely arose in part through novel regulation of *vermilion*, *cinnabar*, and *white* transcription, they also point to a complex relationship between transcriptional prepatterns and pigment synthesis in *V. cardui*.

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 pattern formation in butterfly wings, especially in the context of eyespot and intervenous 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 Gilbert 2004; 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). To date, expression data have been published for only a few pigment synthesis genes in butterflies. Transcripts representing the pigment synthesis enzymes henna (pterin associated) and vermilion (ommochrome associated) were recently sequenced from a *Bicyclus anynana* wing cDNA library (Beldade et al. 2005). In the swallowtail butterfly *Papilio glaucus*, transcripts for the melanin synthesis enzyme dopa decarboxylase (DDC) were reported to be spatially localized to melanic pattern elements late in pupal development (Koch et al. 1998). Likewise, transcripts for the pterin synthesis enzyme GTP CH 1 were observed to be localized coincident with presumptive pterin pigment patterns during pupal wing development in *Junonia (Precis) coenia* (Sawada et al. 2002). The apparent spatial correlations between *DDC* and *GTP CH 1* gene expression and pigment synthesis strongly suggest that some 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 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. As a first step in exploiting this opportunity, we 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 as eye pigments (Linzen 1974). A genetic understanding of ommochrome synthesis in *Drosophila melanogaster* eyes provides candidate genes and a preliminary model to assess in butterfly wings (Fig. 1).

The first step in ommochrome synthesis is establishing a supply of the 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,

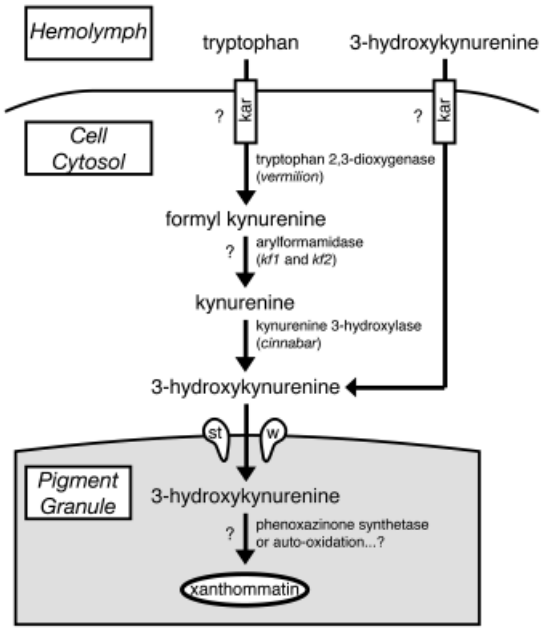

Fig. 1. A model of ommochrome synthesis in *Drosophila melanogaster* eyes. Question marks denote aspects of the model that are highly speculative in the context of butterfly wing pigment development. "w" and "st" are abbreviations for the transporter molecules white and scarlet. See text for details.

it has been proposed 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 also plausible that arylformamidase may not be required for ommochrome synthesis at all. Arylformamidase mutants have not been described from *D. melanogaster*, and sequences resembling arylformamaidase genes known from other species are not evident in the *D. melanogaster* genome database at this time.

Kynurenine 3-monooxygenase (EC 1.14.13.9), encoded by the *cinnabar* gene, catalyzes that 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, Linzen (1974) argued that the formation of xanthommatin may occur through nonspecific 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 catalyst for xanthommatin formation may be, its activity is associated with pigment granules themselves (Phillips et al. 1973).

Delivery of ommochrome precursors into pigment granules involves activity of the white/scarlet transporter. White and scarlet form a heterodimer ATP-binding cassette 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, however, exactly which precursor(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 identified in butterfly wings (Linzen 1974; Gilbert et al. 1988; Nijhout 1991, 1997), little is known about the genetic basis of ommochrome development in scale cells. In order to test the applicability of the *D. melanogaster* model to butterfly wings, we sought to determine if key elements of this model occur in butterfly wings. In this study we identified xanthommatin as an ommochrome pigment found in red wing scales of the nymphalid butterfly *Vanessa cardui* and described transcription patterns of the *vermilion*, *cinnabar*, and *white* genes in developing wings using quantitative RT-PCR and in situ RNA hybridization.

MATERIALS AND METHODS

Rearing and staging

V. cardui larvae and artificial diet were obtained from stocks maintained by Carolina Biological Supply Company. Larvae were fed on artificial diet and both larvae and pupae were maintained at 25°C. Experimental individuals were staged by the length of time after pupation. Time-based staging should only be considered approximate because of individual variation in the relationship between developmental time and developmental stage.

RNA extraction

Pupal wings and larval imaginal discs were dissected in cold phosphate-buffered saline (pH = 6.8). Wing tissue was either immediately transferred to cold Trizol (Invitrogen, Carlsbad, CA, USA) and homogenized for immediate RNA extraction, or stored in RNA later (Ambion Inc., Austin, TX, USA) at −20°C before RNA extraction. For the time series RT-PCRs, forewings and hindwings from a single individual were pooled before RNA extraction. This wing pooling may have masked potential wing-specific transcript abundances in subsequent analyses. For the bisection RT-PCRs, distal and proximal regions of the forewings from single individuals were pooled before RNA extraction. Total RNA was extracted using Trizol and subsequently purified using RNeasy columns (Qiagen, Valencia, CA, USA). Total RNA was run on an agarose gel to verify the absence of degradation products.

Thin layer chromatography

The method used for thin layer chromatography (Nijhout 1997) extracts and separates tryptophan-derived pigments from dried butterfly wings. 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 14 k rpm, forming a pellet of wing scales and membrane tissue. The supernatant was dried in a speed-vac for 1 h 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-OHK were spotted onto a silica gel 60 F254 thin layer chromatography plate (Merck, Whitehouse Station, NJ, USA). The references utilized were not found to be present in the butterfly wings examined by Nijhout (1997). The plate was run in 3:1 phenol:water developing solvent. R_f values were calculated as the ratio of the distance migrated by the sample to the distance traveled by the solvent front. Because R_f values from different studies may vary slightly depending on chamber conditions, we ran a set of four references (amaranth, bromphenol blue, napthol blue black, and 3-OHK) to control for consistency with respect to Nijhout (1997). Separated compounds were isolated from TLC plates, extracted with acid methanol using the above protocol, resuspended in methanol, and analyzed with a spectrophotometer.

Tryptophan incorporation assays

To assay the relative levels of tryptophan incorporation into scale cells, we followed the protocol of Nijhout and Koch (1991). Late-stage pupae were injected in the abdomens with 2 µl of 0.05 µCi/µl ^{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 6 h of emergence. Dorsal and ventral scales were peeled off of wing membranes using plastic packing tape, and the basal scale surfaces were covered with plastic wrap. The basal side of the scale mounting, still covered with plastic wrap, was exposed to Kodak X-Omat autoradiography film (Rochester, NY, USA) for 9 days at −80°C. After exposure, the mounted scales were removed and the film was developed with an automated processor.

Gene cloning and sequence analysis

cDNA was prepared using total RNA pooled from fifth (last)-instar wing discs and pupal wings. *actin* was sequenced from a randomly selected clone from this mixed-staged wing cDNA pool. *vermilion* was amplified from using nested PCR with degenerate primers. The first amplification, utilizing primers 5′-GARYTN-TGGTTYAARCARATH and 5′-NARNSWRTCDATRTCCAT, produced no products visible with an agarose mini gel. An aliquot of this reaction was subsequently used as a PCR template with the nested degenerate primers 5′-CAYGAYGARCAYYTNTTYATH and 5′-RTCNARRAANACYTTRTA, producing a product of the desired size that was then excised from an agarose gel and cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). *cinnabar* was PCR amplified using the degenerate primers 5′-AT-GATGATHGCNYTNCCNAAYCARG and 5′-RTARTTRTA-CATNGCNARRTC and then cloned. *white* was amplified using two rounds of partially nested CODEHOP PCR (Rose et al. 1998). The first round, utilizing primers 5′-GCNMGNTGYGCNTAY-GTNCARC and 5′-AGTCCAGGCCGGTGGTNGGYTCRTC, produced multiple gel-visible products, including a product of the desired size that was excised from the gel. This product was then used as a template for a CODEHOP PCR using the primers 5′-GGTTCCGGCGCCGGNAARWSNAC and 5′-AGTCCAGGC-CGGTGGTNGGYTCRTC, producing several products including one of the desired size which was excised and cloned. Automated sequencing was performed on all clones 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, we used PAUP* (Sinauer Inc., Sunderland, MA, USA) to produce maximum parsimony bootstrap consensus trees (1000 replicate) of known lepidopteran, dipteran, and coleopteran amino acid sequences sharing overlapping regions with the *V. cardui* data. Sequences from the beetle *Tribolium castaneum* were designated as outgroups. 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), *Anopheles gambiae* "ENSANGP00000019055" (XP_311177.1), *D. melanogaster* "actin 3" (A03000), *Caenorhabditis elegans* "act-4" (NP_508842.1), *Homo sapiens* "ACTB Protein" (AAH12854.1). *vermilion* sequences used for alignment were: *Plodia interpunctella* (AAR24625.1), *Aedes aegypti* (AAL37360.1), *An. gambiae* (XP_312204.1), *T. castaneum* (AAL15464.1), *D. melanogaster* (NP_511113.1). *cinnabar* sequences used for alignment were: *Bombyx mori* (BAB62418.1), *Ae.*

aegypti (AAO27576.1), *Anopheles stephensi* (AAL40890.1), *T. castanuem* (AAL15465.1), *D. melanogaster* (AAC47351.1). *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).

V. cardui sequences were deposited in GenBank with the following accession numbers: *actin* (DQ005627), *vermilion* (DQ005628), *cinnabar* (DQ005629), and *white* (DQ005630).

Quantitative real-time PCR

Total RNA was extracted from wing tissue as described above and treated with the DNase *DNA-free* kit (Ambion Inc.), after which cDNA was synthesized using a TaqMan reverse transcription kit (Applied Biosystems Inc., Foster City, CA, USA) with polyT primers. Locus-specific primers (Table 1) were used for SYBR Green (Applied Biosystems Inc.) real-time PCR reactions carried out in an ABI Prism 7000 Sequence Detection System machine (Applied Biosystems Inc.). Relative transcription levels were calculated using a standard curve from a control dilution series. *vermilion*, *cinnabar*, and *white* data were normalized using *actin* transcription levels. Three (whole-wing series) or four (bisected forewings) replicates were averaged for each time point. RNA-only negative controls were run for each experiment to rule out genomic DNA contamination.

In situ hybridization

Sense and anti-sense 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 $\sim$ 300 bp fragments by treatment with HCO_2 at 60°C for 48 min. 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 prehybridization buffer (50% formamide, 0.1% Tween 20, and 5 × 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 30 min. Discs were washed five times with cold PBT (PBS with 0.1% Tween 20), incubated 2 min in 25 µg/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 postfix (5% formaldehyde in PBT) for 20 min. Discs were then washed five times in PBT, twice in 1:1 PBT/prehybridization buffer, once more in prehybridization buffer, and then incubated in 55°C prehybridization buffer for several hours. RNA riboprobes were heat denatured for 5 min at 80°C, and diluted to $\sim$ 0.4 ng/µl in 100 µl of hybridization buffer (prehybridization buffer with 1 mg/ml glycogen). 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 prehybridization buffer with the final wash going overnight. Discs were washed once in 1:1 PBT:prehybridization buffer, then four times in PBT, and incubated in a 1:2000 dilution of mouse anti-digoxigenin antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) at room temperature for 2 h. This was followed by 10 washes in PBT, and two washes in alkaline phosphatase developer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris, pH 9.5, in 0.1% Tween 20). Discs were developed in the dark using BCIP/NBT (Roche, Indianapolis, IN, USA) 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 opposite-wing sense probe negative controls.

Table 1. Primers used for quantitative PCR

Gene	Primer sequences
actin	
Forward	5′-CGATCGCTTGCAGAAGGAAATCAC
Reverse	5′-AGATCCACATCTGTTGGAAGGTCG
vermilion	
Forward	5′-TATTTACGACCGGCGTCTGGTTTC
Reverse	5′-TGATTCTATGGCTTCCGGGTCATC
cinnabar	
Forward	5′-TCCACTTGTAGCTGTTAAGTGCCG
Reverse	5′-GTCTTCAAAGCCAGCGTTCATTCC
white	
Forward	5′-TGGATCGGCATATACCATACGCAC
Reverse	5′-TTCACCACCGGATATACCCTTCAG

RESULTS

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

To help determine which ommochromes were present in *V. cardui* wings, we performed TLC analysis on extracts of dissected red color pattern tissue from dried forewings. We identified three compounds using Nijhout's (1997) ommochrome extraction and separation protocol: (1) $R_f = 0.16$, 450 nm peak; (2) $R_f = 0.37$, 450 nm peak; and (3) $R_f = 0.57$, 420 nm peak. The migration and absorbance spectrum of the band at $R_f = 0.37$ was consistent with xanthommatin (Nijhout 1997). The absorbance of the band at $R_f = 0.16$ suggests it might represent dihydro-xanthommatin, although our R_f was slightly higher than that observed by Nijhout ($R_f = 0.13$). The identity of the band at $R_f = 0.57$ remains unknown and no similar compound was observed in Nijhout's (1997) work with another nymphalid. This compound was a light red color and may represent a previously undescribed wing pigment. None of the observed bands had R_f values similar to those of the ommochromes ommatin-D ($R_f = 0.29$) or rhodommatin ($R_f = 0.23$), ruling out the presence of detectable quantities of these compounds in *V. cardui* wings.

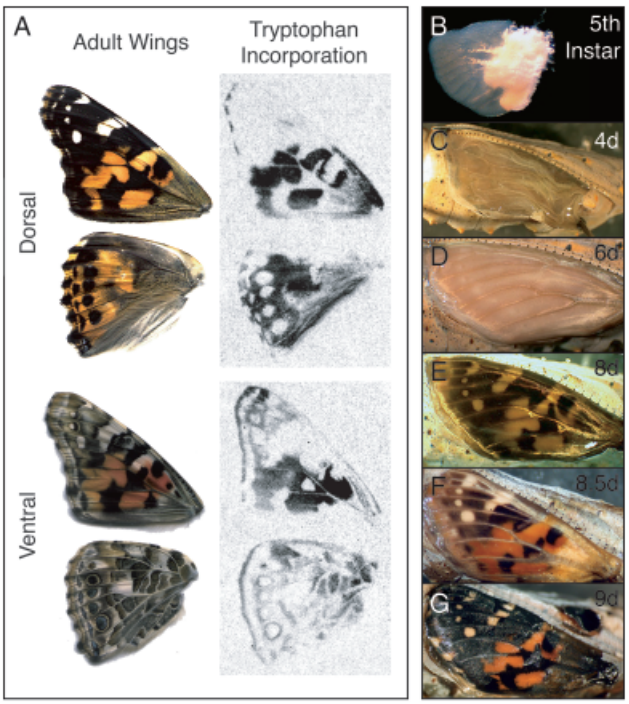

Fig. 2. Tryptophan incorporation and pigment development in *Vanessa cardui* pupal wings. (A) Radiolabeled tryptophan injected into the hemolymph of late-stage pupae is incorporated into red and beige wing scales, as illustrated by the dark regions in the autoradiographs. (B–G) A time series of wing development with approximate days after pupation given. Note that black melanin pigments appear before colored ommochrome pigments.

We injected radiolabeled tryptophan into the hemolymph of pupae and assayed spatial patterns of tryptophan incorporation into adult wing scales (Fig. 2A). On the dorsal surface of both the forewing and hindwing, the spatial patterns of tryptophan incorporation appeared to be 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 of undescribed pigments in the tryptophan–ommochrome pathway (Nijhout and Koch 1991). In any case, all red scales from *V. cardui* wings displayed high relative levels of tryptophan incorporation, consistent with the hypothesis that the red color is attributable to tryptophan-derived ommochromes.

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

Pigment development in *V. cardui* scales begins a few days before adult emergence (Fig. 2, B–G). At approximately 6 days after pupation (AP) the first color patterns are visible on the dorsal forewing as opaque white spots running in a line parallel to the distal wing margin (Fig. 2D). The five anterior spots are retained in the adult wing pattern, whereas the posterior spots eventually disappear into a field of melanin. The major melanin patterns typically appear by approximately 8 days AP (Fig. 2E). Within the last 24 h before adult emergence, the ommochrome pigments appear (Fig. 2F), with melanin around the edge of the wing and along wing veins maturing last (Fig. 2G).

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

We sequenced cloned fragments of the *V. cardui* orthologs of *actin*, *vermilion*, *cinnabar*, and *white*. A 267 bp sequence of an *actin* gene corresponding to the 65 C-terminal amino acids, along with a portion of the 3′ untranslated region of the mRNA, was identified; however, the amino acid similarity with other animals was too high to allow for informative phylogenetic analysis. A 675 bp *V. cardui vermilion* clone coding for 224 amino acids (Fig. 3), and a 429 bp clone encoding 142 amino acids of a *V. cardui cinnabar* gene were sequenced (Fig. 4). Alignment and phylogenetic relationships suggest that these sequences both represent orthologs of their *D. melanogaster* namesakes. 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, because of its phylogenetic relationship to the other *white* sequences known from insects (Fig. 5). The *white* clone contains a 301 bp fragment of the gene, encoding 100 amino acids.

High levels of *vermilion*, *cinnabar*, and *white* transcription during scale development

Quantitative RT-PCR analyses using staged all-wing RNA pools were done to determine the relative levels of *vermilion*, *cinnabar*, and *white* transcription during late-larval and pupal wing development (Fig. 6). The 2–8 days AP forewings shown in Fig. 6 are the actual tissue samples used for the RT-PCR experiments. The transcription of all of these genes was observed to occur, albeit at relatively low levels, during fifth-instar larval wing development—long before the differentiation of scale cells or the synthesis of pigments. Gene expression was observed to decrease in prepupal wings but increase again 2 days AP during early scale differentiation (Nijhout 1991; Galant et al. 1998; Reed 2004). *white* showed a significant increase in transcription between 6 h AP and 2 days AP, whereas *vermilion* and *cinnabar* showed similar significant increases in expression 4 days AP.

Spatially complex patterns of pigment gene transcription

We performed in situ RNA hybridizations to determine the spatial distribution of *vermilion* and *cinnabar* mRNAs in fifth-

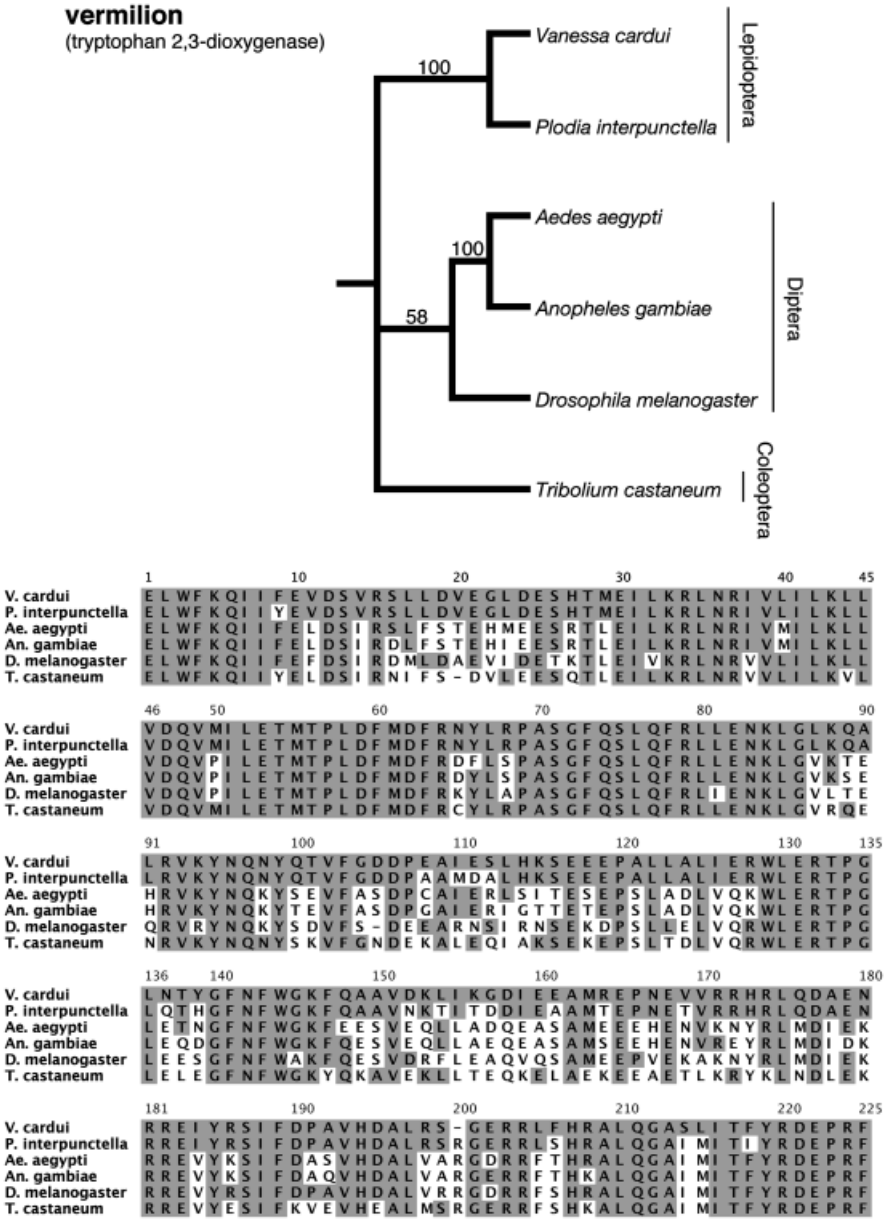

Fig. 3. Bootstrap consensus tree (1000 replicates) and alignment of vermilion amino acid sequences as inferred from nucleotide sequences from representative insects. Bootstrap support >50% shown over branches. The fragment sequenced from *Vanessa cardui* corresponds to *Drosophila melanogaster* amino acid positions 65–288.

instar wing discs (Fig. 7). We focused our in situ hybridizations only on larval wing discs because the digoxigenin antibody we used for riboprobe detection bound nonspecifically to pupal wing tissue. *vermilion* was found to be transcribed throughout the fifth-instar wing discs, with higher levels being observed in the epidermis between the wing veins (Fig. 7, A and D), whereas *cinnabar* had a somewhat more complex expression pattern (Fig. 7, B, E, G, and H). 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 (Fig. 7, B and E). At a later time point, *cinnabar* mRNA was observed to express adjacent to wing veins, with higher expression proximally (Fig. 7, G and H). We were unable to generate consistent *white* in situ expression data in larval wings, possibly because of low levels of transcription.

We sought to test for a spatial correlation between late pupal pigment gene transcription levels and adult pigment patterns. To do this we extracted RNA from tissue of bisected late-stage pupal forewings (Fig. 8). The forewings shown in Fig. 8 were the actual tissue samples used for RNA extraction. Wings were divided into proximal sections bearing large areas of red ommochrome pigment, and distal sections with limited tryptophan incorporation and little or no obvious ommochrome pigmentation. If there were a direct relationship between pigment gene transcription levels

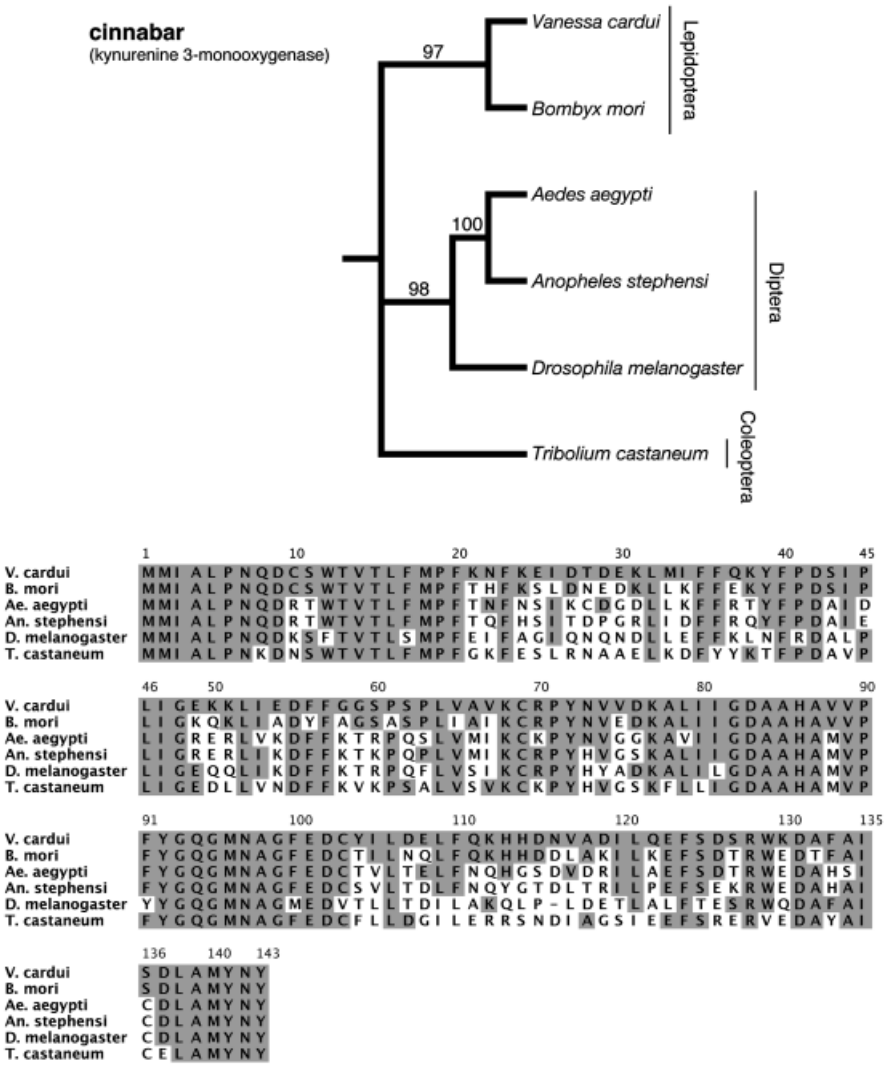

Fig. 4. Bootstrap consensus tree (1000 replicates) and alignment of cinnabar amino acid sequences as inferred from nucleotide sequences from representative insects. Bootstrap support >50% shown over branches. The fragment sequenced from *Vanessa cardui* corresponds to *Drosophila melanogaster* amino acid positions 302–443.

and ommochrome synthesis, it would be predicted that gene expression would be relatively higher in the xanthommatin-bearing proximal region of the wing. Although we observed variation in gene expression levels between stages and tissue types (Fig. 8), the data were not entirely consistent with our predictions. Transcription of all three pigment genes occurred in the proximal region of the wing during xanthommatin synthesis as expected; however, significantly high pigment gene expression also occurred in the distal region of the wing.

DISCUSSION

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, malpighian tubules, nervous systems, and waste products (Linzen 1974; Sawada et al. 1990; Sawada et al. 2000). Given the current data sampling, however, these noneye contexts appear to be derived and lineage specific. 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). We would therefore regard the synthesis of ommochromes in butterfly wing scales as an evolutionary novelty with an origin somewhere in the family Nymphalidae.

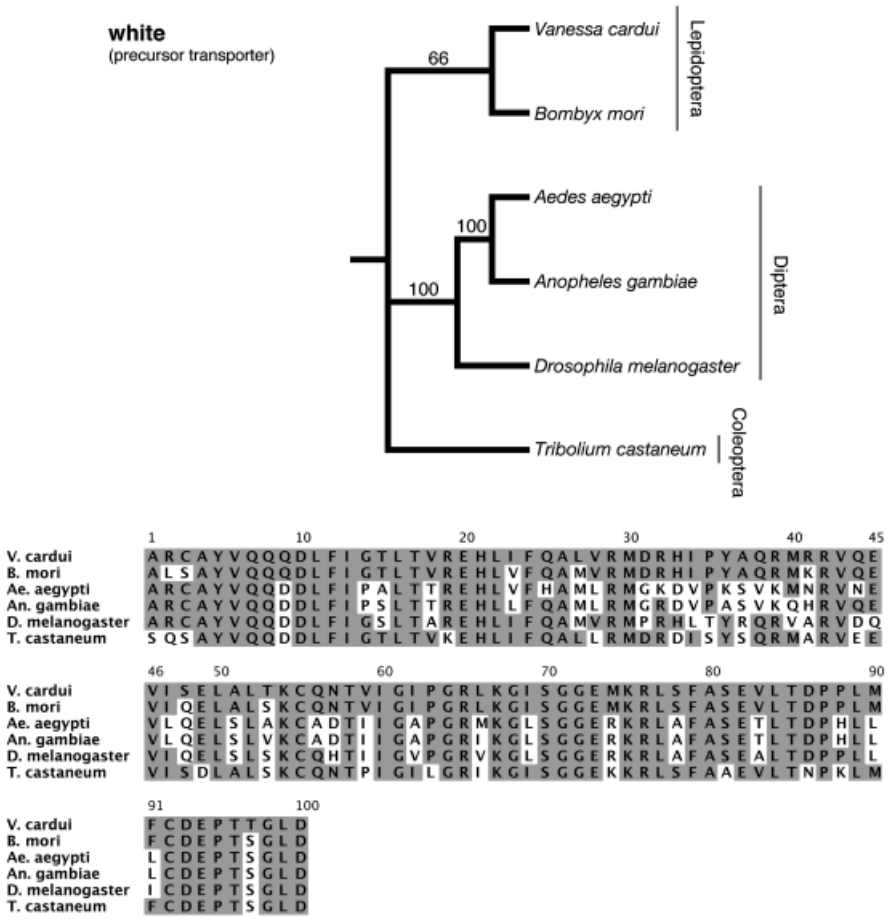

Fig. 5. Bootstrap consensus tree (1000 replicates) and alignment of white amino acid sequences as inferred from nucleotide sequences from representative insects. Bootstrap support >50% shown over branches. The fragment sequenced from *Vanessa cardui* corresponds to *Drosophila melanogaster* amino acid positions 182–281.

Future comparative work may help us to understand how novel transcriptional regulation of the eye pigment genes *vermilion*, *cinnabar*, and *white* could be associated with this innovation.

Unusual sequence of pigment synthesis in *V. cardui*

The overall sequence of pigment development in *V. cardui* is unusual compared with 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 we 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 thought. The only other wing pigment we have observed to appear after melanin is the yellow pigment 3-OHK in *Heliconius erato*, which develops its color only a few hours before adult emergence (unpublished data).

Gene expression in the context of butterfly wing ommochrome synthesis

Previous experiments with the nymphalid butterflies *Araschnia levana* (Koch 1991) and *Junonia (Precis) coenia* (Koch 1993) showed that pupal wing discs in culture could uptake tryptophan and synthesize ommochromes in vitro, suggesting that the biochemical 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.

Expression of the ommochrome genes assayed in this study occurred in wings as early as the fifth instar, and even the major peaks in transcription occurred 5–7 days before the appearance of pigments. It remains unknown why there is such a long lag time between ommochrome gene transcription and pigment synthesis. It would be useful to determine if this lag time is because of delays in protein translation or activity, slow reaction kinetics of ommochrome synthesis, or if the

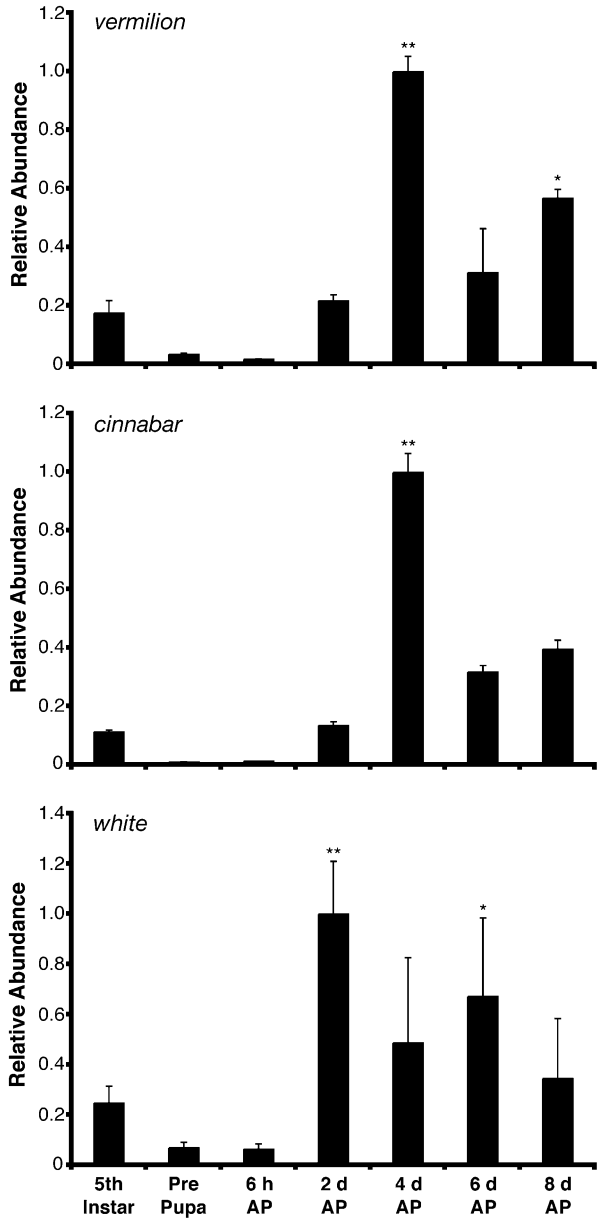

Fig. 6. Quantitative RT-PCR analysis of ommochrome gene transcription during *Vanessa cardui* wing development. Total RNA was extracted from wings pooled from an individual at the given time point after pupation (AP). All values were calculated using a reference standard curve and normalized to levels of *actin* transcription. Each bar represents the mean of three replicates, with standard errors shown. Each value is presented as the proportion of the highest relative expression. A two-tailed *z*-test was conducted to determine which expression levels were significantly higher than the mean ($^{**}P<0.001$; $^{*}P<0.05$).

ommochrome genes assayed here have additional functional roles in wing development. Alternatively, these observed expression patterns might represent "sloppy" or functionally unnecessary positive gene regulation.

Complex relationship between spatial pigment patterns and gene expression

One finding from this study was the complex association between pigment gene transcription and adult pigment patterns. Although the larval stage in situ expression data may be of limited usefulness for understanding the regulation of pigment synthesis, the patterns of gene expression in the late-pupal wings, when pigments are being synthesized, are of interest. Unlike in previous studies of nonommochrome pigment gene transcription in butterflies (Koch et al. 1998; Sawada et al. 2002), ommochrome gene expression in *V. cardui* does not show a simple one-to-one relationship with the time and place of pigment synthesis. All three genes surveyed were expressed in tissues where ommochromes are synthesized; however, the genes were also observed to express in complex patterns in larval wings long before pigment synthesis (Fig. 7), and also in late-pupal wing tissues with little or no ommochrome pigmentation

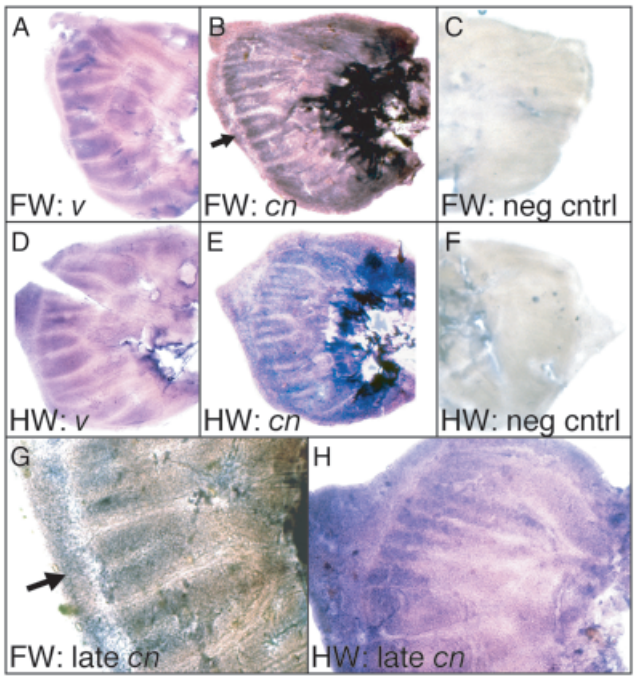

Fig. 7. Spatial distribution of *vermilion* and *cinnabar* mRNA in fifth-instar wing discs. *vermilion* (A, D) and *cinnabar* (B, E) expression in mid-fifth-instar forewing (A, B) and hindwing (D, E) discs. *vermilion* is expressed throughout the wing discs, whereas *cinnabar* has reduced accumulation of mRNA at mid-point spots in between wing veins. In late-fifth-instar forewing (G) and hindwing (H) discs, the zone of *cinnabar* expression decreases proximally and in intervein midlines. Black arrows provide a position reference between the two forewing figures. The opposite-wing sense probe negative controls (C, F) presented correspond to the *vermilion* stains in (A) and (D), but are representative of negative control results from all larval in situ hybridizations. There is accidental tissue damage to the specimen shown in (D).

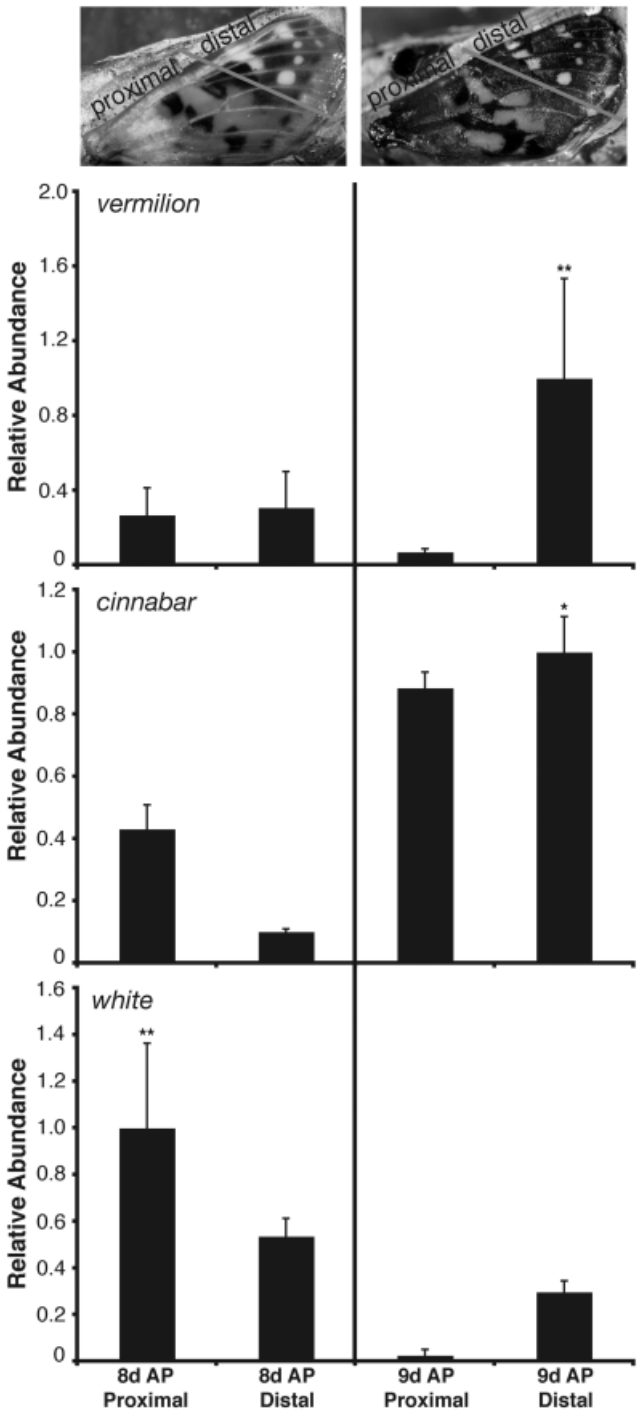

Fig. 8. Relative levels of pigment gene transcript abundance in proximal versus distal regions of late pupal forewings. RNA was extracted from the actual tissue samples shown in the figure and used for quantitative RT-PCR analysis. All values were calculated using a reference standard curve and normalized to levels of *actin* transcription. Each bar represents the mean of four replicates, with standard errors shown. Each value is presented as a proportion of the highest relative expression. A two-tailed *z*-test was conducted to determine which expression levels were significantly higher than the mean ($^{**}P<0.005$; $^{*}P<0.05$).

(Fig. 8). These results suggest that ommochrome synthesis regulation in *V. cardui* occurs at a level beyond the transcription of *vermilion*, *cinnabar*, and *white*, perhaps through posttranscriptional regulation or through regulation of other genes in the ommochrome synthesis pathway. Another possibility is that substrate availability is the limiting factor in pigment synthesis, and spatial patterns of enzyme gene transcription have little to do with determining *V. cardui* pigment patterns.

It is notable that *cinnabar* expression in larval wing discs (Fig. 7) bears 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. 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 in larval wing discs.

A hypothetical model of ommochrome synthesis in butterfly wings

The model of ommochrome synthesis in *D. melanogaster* eyes we present in Fig. 1 can be tentatively extended to butterfly wing scales. In the context of scale cells, several features of the model remain highly speculative. For instance, it is unknown if ommochrome-bearing pigment granules occur in developing butterfly scales; however, we are inclined to include granules in our working 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, 2000). The means of ommochrome precursor uptake into cells, and the chemistry of the final steps of ommochrome processing are also currently not known. This model, however, may be useful as a set of testable hypotheses for future experiments.

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