Extensive transcriptional response associated with seasonal plasticity of butterfly wing patterns

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Abstract

In the eastern United States, the buckeye butterfly, Junonia coenia, shows seasonal wing colour plasticity where adults emerging in the spring are tan, while those emerging in the autumn are dark red. This variation can be artificially induced in laboratory colonies, thus making J. coenia a useful model system to examine the mechanistic basis of plasticity. To better understand the developmental basis of seasonal plasticity, we used RNA-seq to quantify transcription profiles associated with development of alternative seasonal wing morphs. Depending on the developmental stage, between 547 and 1420 transfrags were significantly differentially expressed between morphs. These extensive differences in gene expression stand in contrast to the much smaller numbers of differentially expressed transcripts identified in previous studies of genetic wing pattern variation in other species and suggest that environmentally induced phenotypic shifts arise from very broad systemic processes. Analyses of candidate endocrine and pigmentation transcripts revealed notable genes upregulated in the red morph, including several ecdysone-associated genes, and cinnabar, an ommochrome pigmentation gene implicated in colour pattern variation in other butterflies. We also found multiple melanin-related transcripts strongly upregulated in the red morph, including tan and yellow-family genes, leading us to speculate that dark red pigmentation in autumn J. coenia may involve nonommochrome pigments. While we identified several endocrine and pigmentation genes as obvious candidates for seasonal colour morph differentiation, we speculate that the majority of observed expression differences were due

to thermal stress response. The buckeye transcriptome provides a basis for further developmental studies of phenotypic plasticity.

Introduction

Understanding the role of environmental conditions in developmental decision-making processes represents an ongoing challenge in biology. In many species, individuals that are genetically similar, or even identical, can develop into one of several phenotypically distinct morphs in response to specific environmental cues. This phylogenetically widespread mechanism, known as phenotypic plasticity, can allow rapid adaptation to the environment without relying on genetic changes between generations (Nijhout 2003; Moczek 2010; Snell-Rood *et al.* 2010; Moczek *et al.* 2011). Plasticity is particularly useful to organisms with short generation times that live in continually changing environments because it allows them to quickly react to changes by adaptively altering gene regulation during development. While much work has been carried out on the biological mechanisms and fitness advantages of plasticity in various study systems, less work has been carried out addressing what manner or magnitude of gene regulatory changes facilitate response to environmental conditions via phenotypic plasticity (Beldade *et al.* 2011).

The common buckeye butterfly, *Junonia coenia*, has been extensively studied in terms of how environmental conditions trigger morph-switching across different climatic regions (Smith 1991; Daniels *et al.* 2012). Buckeye populations in the eastern United States display two primary seasonal wing colour morphs that are determined by the photoperiod and temperature they experience during late larval and early pupal development (Smith 1991). At cool temperatures and short day lengths, such as those experienced in autumn and winter, the majority of individuals will develop a dark red wing colour, whereas at warm temperatures and long day lengths, such as those experienced in spring and summer, the majority of individuals will develop wings that are primarily of a tan colour (Fig. 1a). This seasonal plasticity probably facilitates crypsis and/or thermoregulation (e.g. Brakefield & Larsen 1984), where the red autumn form may more effectively blend in with the senescing foliage of deciduous hardwood forests of the eastern United States (Daniels *et al.* 2012) or more efficiently absorb heat. Colour morph variation in buckeyes is physiologically controlled by differences in ecdysone levels during a critical period in early pupal development (Rountree & Nijhout 1995) when high levels of ecdysone induce the tan colour morph, while low levels of ecdysone result in the red morph. Differential deposition of ommochrome pigments in wings scales during late pupal development is proposed to be the ultimate cause of colour differences, although the actual identities of all of the key pigments are still not completely certain (Nijhout & Koch 1991; Koch 1993; Nijhout 1997; Daniels & Reed 2012).

Nothing is currently known about the developmental genetic mechanisms that underlie developmental switching between the seasonal buckeye morphs. To better understand the gene regulatory basis of this phenomenon, we undertook a comparative transcriptomic approach, using RNA-seq to allow characterization of transcripts while simultaneously determining their expression levels. Because of the known involvement of ecdysone and ommochromes in wing pattern plasticity, the immediate aim of this study was to assess differential expression of genes related to endocrine and pigment pathways. We specifically sought to assess three hypotheses: (i) morph-specific gene expression differences should be highest later in pupal development, after batteries of downstream response genes (e.g. pigment genes) are activated, (ii) genes implicated in ecdysone response should show differential expression between morphs, and (iii) genes associated with ommochrome pigmentation should be upregulated in the red morph. In addition to testing these predictions, we also sought to more generally assess the number and type of transcripts differentially regulated during seasonal polyphenism to develop an initial working profile of *J. coenia*'s gene regulatory response to environmental variation.

Materials and methods

Animal rearing

Populations of *Junonia coenia* from Durham, North Carolina (35°59′19″N, 78°54′26″W), were reared in incubators and fed a standard artificial diet (Yamamoto 1969; Nijhout 1980). Two sets of conditions known to induce alternative colour morphs in North Carolina populations (Smith 1991) were applied: 21 °C with 8 h light per day for short/cool day conditions (red morph) and 27 °C with 16 h light for long/warm day conditions (tan morph).

Tissue sampling

Hindwings from four developmental stages were sampled for both seasonal colour morphs. Hindwings were used for this work because they show much more extensive colour plasticity compared to forewings. The four stages sampled include imaginal discs from late stage fifth instar larvae, prepigment pupae, early ommochrome development and late ommochrome development (Fig. 1b). Two biological replicates were prepared for each developmental stage and colour morph combination, for a total of 16 samples. Each sample represented a pool of hindwings taken from multiple male and female individuals. To preserve mRNA quality, wings were rapidly dissected into RNA-later (Life Technologies, Grand Island, NY, USA), incubated for *c.* 24 h at 4 °C to allow the solution to permeate tissues and then stored at −80 °C until mRNA extraction.

RNA-seq

Total RNA was extracted from wing tissues using the RNeasy Mini Kit spin column protocol (QIAGEN, Valencia, CA, USA). TURBO™ DNase treatment (Life Technologies) was then used to degrade contaminating DNA, and mRNA was purified from the total RNA using the Oligotex mRNA Mini Kit (QIAGEN). The Ovation® RNA-seq system (NuGEN, San Carlos, CA, USA) was used to prepare double-stranded cDNA from mRNA, and this cDNA product was then purified with QIAquick PCR purification columns (QIAGEN). The purified double-stranded cDNA was sheared by a Covaris Ultrasonicator to 300 bp. The

EncoreTM NGS Multiplex System I (NuGEN) was used for final library preparation. Multiplexed libraries were sequenced as 75-bp paired-end Illumina reads or 100-bp paired-end Illumina reads. Sequencing lanes were randomized between samples.

Data analysis

Each RNA-seq data set was processed with Khmer (Brown *et al.* 2012) to even out the coverage, to remove redundant reads, and to remove errors in reads, thus reducing the data sets to 30–54% of their read counts. Reads from replicates of each developmental stage in each colour morph were pooled into eight individual sets (Table S1, Supporting information). The filtered reads were assembled into developmental stage transcriptomes with OASES v.1.2.03 (Schulz *et al.* 2012) using a range of hash lengths from 29 to 61 bp. For transcripts with more than 50 transcription fragments (transfrags) per assembly, the longest 50 per transcript were kept. The eight assemblies were consolidated with CD-HIT-EST of CD-HIT package v.4.5.7 (Li & Godzik 2006) into a reference assembly. Only transfrags longer than 1 kb were used in subsequent analyses. This semi-arbitrary length cut-off was selected to improve annotation efficiency while minimizing potential problems associated with isoform variation.

Reads were mapped to the reference transcriptome using BOWTIE v.0.12.7 (Langmead *et al.* 2009), and transcript expression levels were obtained using EXPRESS v.1.0.0 (Roberts & Pachter 2013). Using Bioconductor package EDGER v.2.4.6 (Robinson *et al.* 2010) and biological replicates for each developmental stage, differentially expressed transcripts were identified. Functional annotation and gene ontology assignment of transfrags were carried out using Blast2GO (Conesa *et al.* 2005). The transcriptome was translated to find the longest open reading frame for each transcript. ORTHOMCL 1.4 (Li *et al.* 2003) was used to identify orthologous protein clusters with *Heliconius melpomene* (The Heliconius Genome Consortium 2012) and *Danaus plexippus* (Zhan *et al.* 2011) transcriptomes.

We tested the sets of upregulated and downregulated genes of each stage-specific comparison among the morphs for gene ontology enrichment using the Fisher's Exact Test function of Blast2GO. Each of the eight sets of upregulated or downregulated genes was tested for GO enrichment with the annotations of all 16 251 transfrags in the transcriptome as the reference. A *P*-value of 0.00625 (i.e. multiple testing corrected value for *P* = 0.05) was used as the threshold to call significant expression differences between morphs. The same analysis was repeated for the upregulated and downregulated gene sets of comparisons between the developmental time points of each the two morphs. A *P*-value of 0.00417 (i.e. multiple testing corrected value for *P* = 0.05) was used as the threshold for these GO enrichment analyses.

Results

Transcriptome assembly of the wing development time-course

Because the genome of *Junonia coenia* is not currently sequenced, we performed a *de novo* transcriptome assembly using our Illumina sequence data. Multiplexed libraries yielded 41.5 million 75-bp paired-end Illumina reads and 246 million 100-bp paired-end Illumina reads.

We assembled a reference transcriptome of 77.55 Mb with 121 076 transfrags and an N50 of 687 bp. Remaining analyses were focused on the 16 251 assembled transcripts >1 kb (26.65 Mb of the transcriptome), as the longer sequences were more likely to be annotatable. The longest transfrag was 22 289 bp (Table 1). DNA sequences and expression metadata are available at NCBI GEO with Accession no. GSE54819. Assembled transcript sequences are available for download at Dryad (doi:10.5061/dryad.5n5h6), and download and BLAST searches at *butterflygenome.org*.

About two-thirds (10 787 of 16 251) of the assembled *J. coenia* transfrags clustered with at least one other butterfly transcript from *H. melpomene* or *D. plexippus* (Fig. 1c). 3517 one-to-one orthologue clusters were obtained between all three species. Forty-nine per cent of the ORTHOMCL clusters contained transcripts from all three species (5490 of 11 267). Thirty-seven per cent of the total clusters contained both *H. melpomene* and *D. plexippus*, but not *J. coenia*, transfrags (4208 of 11 267), probably representing genes not expressed during wing development. Approximately 81% of clusters with *J. coenia* transfrags also included both sequenced butterfly species (5490 of 6782), 10% of the total clusters which contain *J. coenia* transfrags clustered with one of the sequenced butterfly species but not the other (660 of 6782), while about nine per cent only clustered with other *J. coenia* transfrags (632 of 6782) (Fig. 1c).

Gene ontology groups

Gene Ontology (GO) analysis of differentially expressed transcripts indicated that there were more ontology groups significantly differentially expressed between the colour forms at the fifth instar larval stage than at any other time point, with 33 GO terms over- or under-expressed ($P < 0.00625$) compared to six GO terms at early pupal, seven GO terms at early ommochrome, and 27 GO terms at the late ommochrome and melanin pigment development stages (full list of GO terms $P < 0.00625$ in Table S2). Several GO terms with very significant enrichments of interest between the colour morphs were identified. Genes associated with structural constituents of ribosomes ($P = 1.52E-05$) and oxygen transport ($P = 1.49E-04$) were significantly upregulated in the tan morph when compared to the red morph at the fifth instar larval stage of development. Genes associated with peptidase activity ($P = 6.06E-10$) were very significantly overexpressed in the red morph at the fifth instar larval stage of development.

One hundred and sixty-three GO terms were significantly upregulated or downregulated ($P < 0.00417$), between the fifth instar larval stage and the earliest pupal development stage compared with only 26 GO terms between early pupal and early ommochrome stage, and 37 GO terms between early ommochrome and the late ommochrome and melanin pigment development stages. A full list of enriched GO terms is presented in Table S2 (Supporting information). Of particular biological interest, genes involved in the function of translation were significantly overrepresented in the downregulated genes ($P = 1.86E-17$) during early pupal development of the tan morph when compared to the larval stage of this morph. Between these stages, cellular macromolecule biosynthetic process genes were also very significantly overrepresented in the downregulated genes ($P = 1.53E-15$), as were genes associated with ribosome functions ($P = 1.48E-13$), and genes involved with cellular

macromolecule metabolic process (P = 2.09E-13). Genes associated with structural constituent of cuticle were extremely significantly overrepresented in the upregulated genes (P = 3.58E-18 and 4.41E-19, respectively), between the larval development stage and the earliest pupal stage of the tan and red morphs.

Differential expression analyses

There were 3145 significantly differentially expressed transfrags between the different stages and colour morphs (Fig. 1d and Table S3, Supporting information). 1420 transfrags were differentially expressed when comparing the tan and red colour morphs at the fifth instar stage, more than at any other time point sampled. Five hundred and forty-seven transfrags were differentially expressed between the colour morphs during early pupal development, 1231 transfrags were differentially expressed during early ommochrome development, and 854 transfrags were differentially expressed during late ommochrome and early melanin development.

Expression of ecdysone-associated genes

Of 11 ecdysone-associated transfrags identified by BLAST similarity, 10 showed significant differential expression between seasonal morphs (Fig. 2), and all but one of these showed significant upregulation specifically in the red morph. Transfrags encoding ecdysone-altering enzymes, including *ecdysone oxidase* and *3-dehydroecdy-sone 3α-reductase*, showed the most dramatic patterns of differential expression at both early and late stages of development.

Expression of pigmentation-associated genes

Based on BLAST similarity, we identified 19 transfrags potentially associated with ommochrome pigmentation, 14 of which were differentially expressed between morphs. Interestingly, 11 of these were differentially expressed in fifth instar wing discs, including significant red morph-specific upregulation of transfrags encoding ATP-binding cassette (ABC) transporters (JC_0023776, JC_0033677, JC_0036375, JC_0036375, JC_0092468, JC_0024281, JC_0028972) and monocarboxylate transporters (JC_0115080, JC_0024201, JC_0013098) (Table S2, Supporting information). Six transfrags showed differential expression during pupal development, including three transporters, *rosy*, a *sulfotransferase* (potentially involved in synthesis of sulphur-bearing ommatin-D) and *cinnabar* (kynurenine 3-monooxygenase) (Fig. 3). Interestingly, *cinnabar* was expressed at significantly higher levels in the tan morph during earlier developmental time points, but at significantly lower levels in the tan morph during late ommochrome/melanin development stage when compared to the red morph, thus suggesting a heterochronic shift in peak transcription between morphs.

Of 13 transfrags identified as melanin gene transcripts based on BLAST similarity, six showed significant expression differences between colour morphs (Fig. 4). In the red morph, there was significant upregulation of *tan* at the final stage of pupal development, and *yellow* and *yellow-e* during early ommochrome development. *yellow-h3* and *black* also showed striking patterns of specific upregulation in the red morph; however, their expression levels

were relatively low and the differential expression was not statistically significant (Table S2, Supporting information).

Discussion

Large numbers of differentially expressed transcripts

In addition to assembling the first transcriptome resource for *Junonia coenia*, we generated RNA-seq developmental time-courses for two distinct wing morphs to address the question of how developmental gene transcription can be influenced by environmental conditions. In part to make a preliminary assessment of the quality of our assembled transcripts, we used the trans-frag sequences to make comparisons of gene conservation with the postman butterfly *H. melpomene* (The Heliconius Genome Consortium 2012) and the monarch butterfly *D. plexippus* (Zhan *et al.* 2011)—two butterfly species with completely sequenced genomes. Using comparative sequence analyses, we found that about half of the gene clusters are shared by all three species. We speculate that most of the 39% of transfrags shared solely between *H. melpomene* and *D. plexippus*, and not *J. coenia*, are likely to represent genes not expressed during *J. coenia* wing development but that are present in the genome sequences. About 81% *J. coenia* transfrags were also represented in both other butterfly species, indicating a high level of conservation of the majority of genes between all three species.

Compared to a previous transcriptomic study that identified only 51 genes associated with colour pattern polymorphism in *Heliconius* wings (Hines *et al.* 2012), the number of transcripts differentially expressed between *J. coenia* seasonal morphs was remarkably high. This trend was strongest when comparing the red and tan morphs at the fifth instar developmental stage, which showed 1420 differentially expressed transfrags (as opposed to 547, 1231, and 854 transfrags at subsequent sampled stages). This finding that gene expression varied most strongly at the earliest sampled time point challenges our initial hypothesis that gene expression differences should be most pronounced at later stages of development when downstream effector genes are making pigments and determining scale morphology. We speculate that many of these transcriptional differences could be the result of nonpigmentation-related effects related to general thermal stress. The time for a pupa to develop under our defined cold temperature condition was about 2 weeks, as opposed to 1 week under our defined warm temperature condition. It is likely that this doubling of developmental time influenced wing cellular and metabolic processes in ways extending beyond the expected changes in wing pigment synthesis. In support of the thermal stress, hypothesis was our observation that transcripts representing genes in the oxygen transport GO group were differentially regulated between temperature conditions—a finding consistent with a transcriptional response to cold-induced hypoxia (Al-Fageeh & Smales 2006).

On another level, the unusual complexity of our data set may be due to the fact that wing pigmentation in *J. coenia* may be more complex than in *Heliconius* wings. The *Heliconius* butterfly wings for which transcriptomic data exist have simple patterns consisting of only three dominant pigments (yellow, red and black). In contrast, the wing scales of *J. coenia* form a very complex mosaic of a range of many different scale colours. Therefore, in *J.*

coenia, different individual scales in close proximity will likely have several different pigments, or relative quantities of pigments, and thus could result in a more complex gene expression profile during development.

Finally, the method we used may have contributed somewhat to the relatively high number of differentially expressed transcripts observed. RNA-seq provides increased sensitivity to detect expression level changes over microarrays, which makes it particularly useful for accurate expression analyses (Mortazavi *et al.* 2008). At the same time, RNA-seq allows detection of transcripts across the entire genome, whereas microarray studies are limited to only a subset of genes. This increased sampling in itself should lead to uncovering a potentially higher number of differentially expressed genes; however, this effect would probably not be sufficient to wholly explain the magnitude of difference between the previous *Heliconius* work and our current results.

Expression of ecdysone-associated genes: evidence for morph-specific ecdysone inactivation

Differences in ecdysone levels during early pupal development (i.e. lower in the red morph, higher in the tan morph) are directly responsible for seasonal wing pattern variation in *J. coenia* (Rountree & Nijhout 1995). Because (i) ecdysone titres themselves are determined by activity of the prothoracic gland, independent of wing development, and (ii) ecdysone levels alone are sufficient to determine wing pattern morph, we hypothesized that differential expression of genes involved with synthesis of ecdysone or reception of ecdysone signals was unlikely to be associated with plasticity, and instead we would see differential expression of genes associated with downstream ecdysone response. Indeed, to a small degree, our findings supported this idea, in that we observed differential expression of transfrags identified as genes such as *ecdysone-induced protein* and *zinc finger protein on ecdysone puffs* (Fig. 2) as opposed to synthesis genes such as Halloween-family P450s or receptors such as *ultraspiracle* or *ecdysone receptor*.

One of the more interesting findings to come from our study was a surprise, however— namely the strong morph-specific expression of ecdysone-inactivating enzymes. Specifically, multiple transfrags representing *ecdysone oxidase* and *3-dehydroecdysone 3α-reductase* were strongly upregulated during early wing development of the red morph (Fig. 2). These two enzymes catalyse an irreversible two-step inactivation of ecdysone to 3-epiec-dysteroid (Rees 1995). Therefore, high levels of these two enzymes could potentially result in reduced levels of ecdysone during the critical period in the red morph and potentially suggest a novel mechanism for localized modulation of ecdysone levels in response to environmental conditions. Future work should examine the potential roles of these genes in controlling seasonal phenotypes.

Ommochrome genes: transporters and enzymes associated with red morph

Because previous studies have identified ommochrome and ommochrome-related pigments in *J. coenia* wings (Nijhout 1997; Daniels & Reed 2012), ommochrome-associated transcripts are obvious candidates as causative agents for seasonal colour plasticity. Work in several nymphalid species has shed some light on which ommochromes genes may underlie

wing pigment development and polymorphism. Reed & Nagy (2005) showed that the ommochrome genes *vermilion*, *cinnabar* and *white* were expressed in developing wings of the nymphalid *Vanessa cardui*. Later work in *Heliconius* (Reed *et al.* 2008; Ferguson & Jiggins 2009; Hines *et al.* 2012) demonstrated a clear spatial correlation between red wing pattern elements and *cinnabar* expression, thus implicating regulation of this gene in colour pattern development. Importantly, the most recent *Heliconius* microarray work suggests that butterflies have an expanded repertoire of uncharacterized ommochrome genes compared to *Drosophila* (Hines *et al.* 2012), including several novel transporter-encoding genes. As *J. coenia* wings are known to bear ommochrome pigments, and *J. coenia* is a nymphalid such as *Vanessa* and *Heliconius*, we hypothesized that similar ommochrome pathway genes may be upregulated during development of the red seasonal morph, leading to increased quantities of red ommochrome pigments.

In accord with our prediction, in the red morph, we observed a significant upregulation of multiple transcripts encoding ABC and monocarboxylate transporters phylogenetically related to the ommochrome genes *white* and *karmoisin*, respectively—primarily during very early wing development (Table S2, Supporting information, Fig. 3). Upregulation of these transporters may allow butterflies to more efficiently transport ommochrome precursors into scale cells or pigment granules, thus facilitating synthesis of red pigments. Interestingly, however, we also observed that two monocarboxylate transporters were upregulated in the tan form during the ommochrome stage of development (Fig. 3), possibly suggesting a role in the synthesis of a tan morph-specific pigments.

We observed that *cinnabar* was expressed at significantly higher levels in the red morph during late pupal development when pigments are actively being synthesized. The product of *cinnabar*, kynurenine-3-monooxy-genase, catalyses the conversion of kynurenine to 3-hydroxykynurenine, which is a key intermediate in the production of red and orange ommochrome pigments including xanthommatin, ommatin-D and the recently characterized *J. coenia* wing pigment xanthurenic acid (Daniels & Reed 2012). Therefore, it is reasonable to speculate that upregulation of *cinnabar* facilitates increased ommochrome synthesis in the red morph. One unexpected finding, however, was that *cinnabar* was significantly upregulated in the tan morph early in pupal development before pigment synthesis occurs. This difference in temporal expression mirrors the temporal shift in ecdysone titres responsible for inducing the polyphenism, so it interesting to consider whether this heterochronic shift in *cinnabar* transcription may be regulated by ecdysone. More broadly, our finding that *cinnabar* is the only ommochrome enzyme transcript showing a strong association with colour variation is similar to what has been observed in *Heliconius* (Reed *et al.* 2008; Ferguson & Jiggins 2009; Hines *et al.* 2012), thus suggesting that the *cinnabar* gene may be predisposed towards a repeated role in butterfly wing pattern evolution. We also noted a complex pattern of differential expression of a *sulfotransferase* transcript (Fig. 3). This enzyme could play a role in the synthesis of sulphur-containing ommochromes such as ommatin-D—a pigment proposed to occur in nymphalids (Linzen 1974). Finally, we observed red morph-specific upregulation of the xanthine dehydrogenase gene *rosy* during ommochrome development (Fig. 3). While *rosy* is typically associated with pteridine pigmentation (Reaume *et al.* 1991), *Drosophila* mutants for this gene show significant

changes in tryptophan (including ommochrome) metabolism, although the role of *rosy* in this process is still not well understood (Kamleh *et al.* 2008).

Melanin genes: yellow-family genes and tan are associated with red morph

Work in *Drosophila* has functionally implicated a number of genes in the evolution and development of melanin pigmentation (e.g. True *et al.* 2005; Prud'homme *et al.* 2006; Jeong *et al.* 2008; Rebeiz *et al.* 2009; Wittkopp *et al.* 2009; Kronforst *et al.* 2012). Interestingly, we found that the majority of homologues to these *Drosophila* melanin genes showed elevated levels of expression during development of the red morph, and many of these trends were significant. The *yellow* family of genes in particular displayed a strong pattern of association with the red morph. Three transfrags representing *yellow* and *yellow-e* showed significant upregulation in red morphs. In addition to the *yellow* genes, the melanin enzyme gene *tan* was highly upregulated during late pigment development in red wings.

It was surprising to find that so many genes associated with black melanin pigmentation in other Lepidoptera were so strongly upregulated in red wings in our study. In *Heliconius* wing development *yellow* and *tan* are specifically associated only with melanic colour pattern elements, not red ommochrome elements (Ferguson *et al.* 2011a, b; Hines *et al.* 2012). In addition, in *Papilio xuthus* epidermal development *tan* shows strong specific spatial association with only black colour patterns (Futahashi *et al.* 2010; Futahashi *et al.* 2012). The only *yellow*-family gene previously associated with red ommochrome pigmentation is *yellow-d*, which was observed to be upregulated in red colour pattern in *H. erato* (Hines *et al.* 2012). While we did see strong upregulation of *yellow-d* during late ommochrome development in the *J. coenia* red morph, the difference did not reach statistical significance. Surprisingly, we observed that *yellow-d* was significantly upregulated in the tan morph during early ommochrome development (Fig. 3).

It was fascinating to find this association between melanin gene expression and a red phenotype, especially given that our ommochrome gene expression data are consistent with previous work that identified ommochromes as the primary red pigments in *J. coenia* (Nijhout 1997). One possible explanation is that the red coloration of the autumn morph is produced by a combination of ommochromes and red pheomelanins. Although pheomelanin pigments have not been rigorously characterized from *J. coenia* wings, there are three lines of preliminary evidence supporting their presence. First of all, labelled tyrosine—the precursor of melanin —is incorporated into scales of red regions at slightly elevated levels (Nijhout & Koch 1991). Second, acid methanol extraction of pigments from whole *J. coenia* wings does not completely remove red coloration from wings, as it does for red ommochrome wing patterns in *Heliconius* and *Vanessa* butterflies (R. D. Reed, personal observation), therefore suggesting that some of the red coloration in *J. coenia* is not due solely to ommochrome pigmentation. Third, cysteinyldopa melanin, a pheomelanin precursor, was characterized from developing *J. coenia* wings (Wakamatsu & Ito 2002). Although more work needs to be performed, our data hint at a complex relationship between the regulation of *yellow*-family genes and nonblack pigment synthesis.

Conclusion: The transcriptional profile of seasonal polyphenism

We used an unbiased whole transcriptome approach to study phenotypic plasticity in the wings of the buckeye butterfly *J. coenia*. Our work revealed a large set of transcripts that show significantly different expression levels during the development of alternative seasonal colour pattern morphs. This magnitude of transcription difference between wing morphs indicates that environmentally induced phenotypic plasticity is associated with very deep and broad shifts in developmental gene expression. This stands in marked contrast to previous work on genetic colour pattern variation, which shows an order of magnitude fewer differences in transcriptional changes. Many of the differentially expressed transcripts we identified were of unknown function, and hopefully future analyses benefitting from improved annotation and a fully sequenced genome will help determine whether they may play a functional role in phenotypic plasticity. Our focused analysis on endocrine and pigmentation genes revealed a number of candidates that may be specifically involved in differentiation of wing colour morphs. Among the interesting candidates were ecdysone-inactivating enzymes and genes related to ommochrome pigment synthesis. Surprisingly, we also found that numerous melanin genes were associated with the red colour morph, including *tan* and several members of the *yellow* gene family. This finding leads us to speculate that the red coloration in autumn *J. coenia* may be attributable to both ommochromes and red pheomelanins.

A more general question that arises from this work concerns the nature of the physiological 'switch' that underlies phenotypic plasticity. Many of the gene expression differences we observed seemed to be related to thermal response. Low temperatures are indeed stressful for buckeyes, and development time in butterflies reared at 21 °C is essentially double of those reared at 27 °C. Together, our observations raise the question to what extent seasonal morph differences are directly triggered by temperature cues, vs. to what extent they are a downstream by-product of stress and/ or delayed development that indirectly induces heterochronic shifts in hormone titres or gene expression. There is some circumstantial evidence for direct regulation of colour patterns by ecdysone in *J. coenia*, in that Koch *et al.* (2003) observed coexpression of *ecdysone receptor* and the transcription factor Distal-less in plastic eyespot patterns. This is interesting because changes in Distal-less expression are correlated with eyespot plasticity in the butterfly *Bicyclus anyana* (Brakefield *et al.* 1996). To address this question more thoroughly, we are now working to better define the relative contributions of light, temperature and critical period in buck-eye morph determination. We are also conducting comparative work between populations with different reaction norms to understand how seasonal plasticity varies between populations adapted for life at different latitudes and elevations. The insights and transcriptome resources generated through the work described here will form an important foundation for this and other future work in the buckeye system.

Supporting information

Additional supporting information may be found in the online version of this article.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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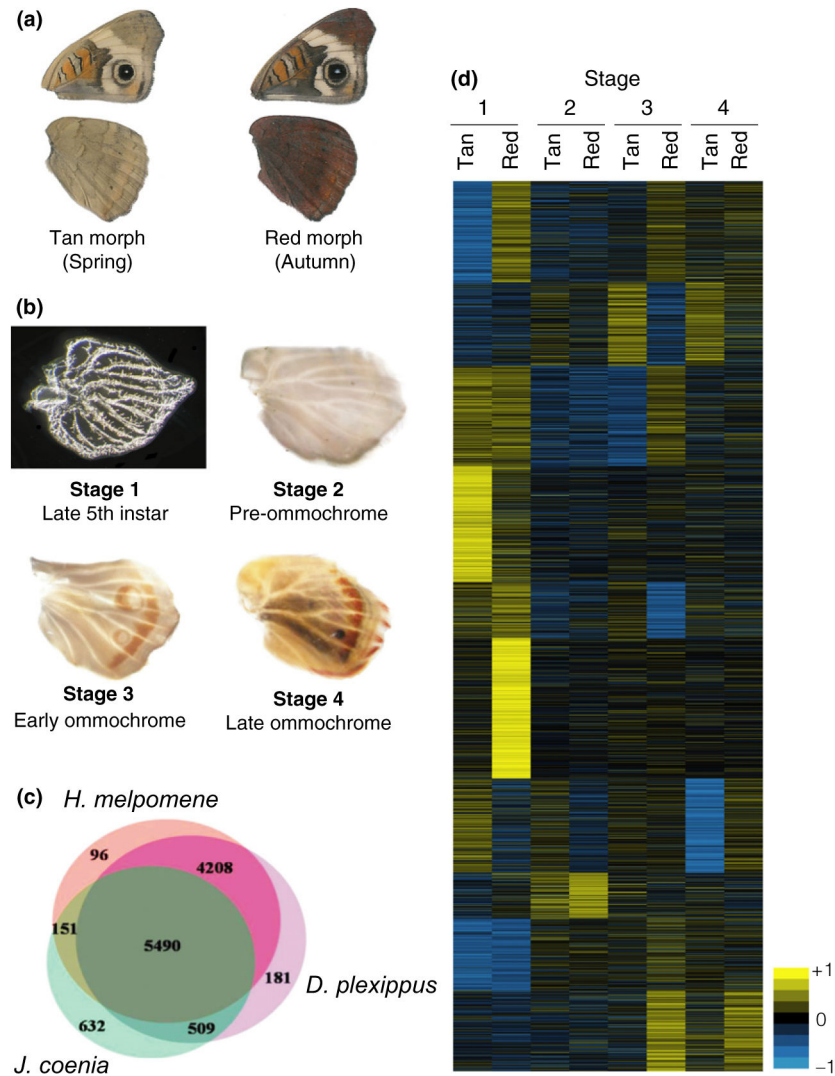
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Fig. 1.

Seasonal variation and wing development in *Junonia coenia*. (a) Different light and temperature conditions can induce North Carolina *J. coenia* to display extremely different seasonal morphs. (b) The four developmental stages sampled in this study: (1) late 5th instar hindwing discs, (2) preommochrome pupal hind-wings, (3) early ommochrome pupal hindwings, and (4) late ommochrome pupal hindwings. (c) Comparisons of transfrag similarity between *Heliconius melpomene, Danaus plexippus* and *J. coenia* using OrthoMCL from amino acid sequences. Note that the complete genomes have been sequenced for *H. melpomene* and *D. plexippus*, while *J. coenia* is represented only by wing tissue-specific transcripts. (d) A unified heat map illustrating the differential expression of 3145 loci between four developmental stages and two colour morphs of *J. coenia*. The diagram shows the relative expression levels from all samples compared against background levels. The relative levels of upregulation or downregulation are represented by a coloured field that ranges from yellow for highly upregulated genes to blue for highly downregulated genes.
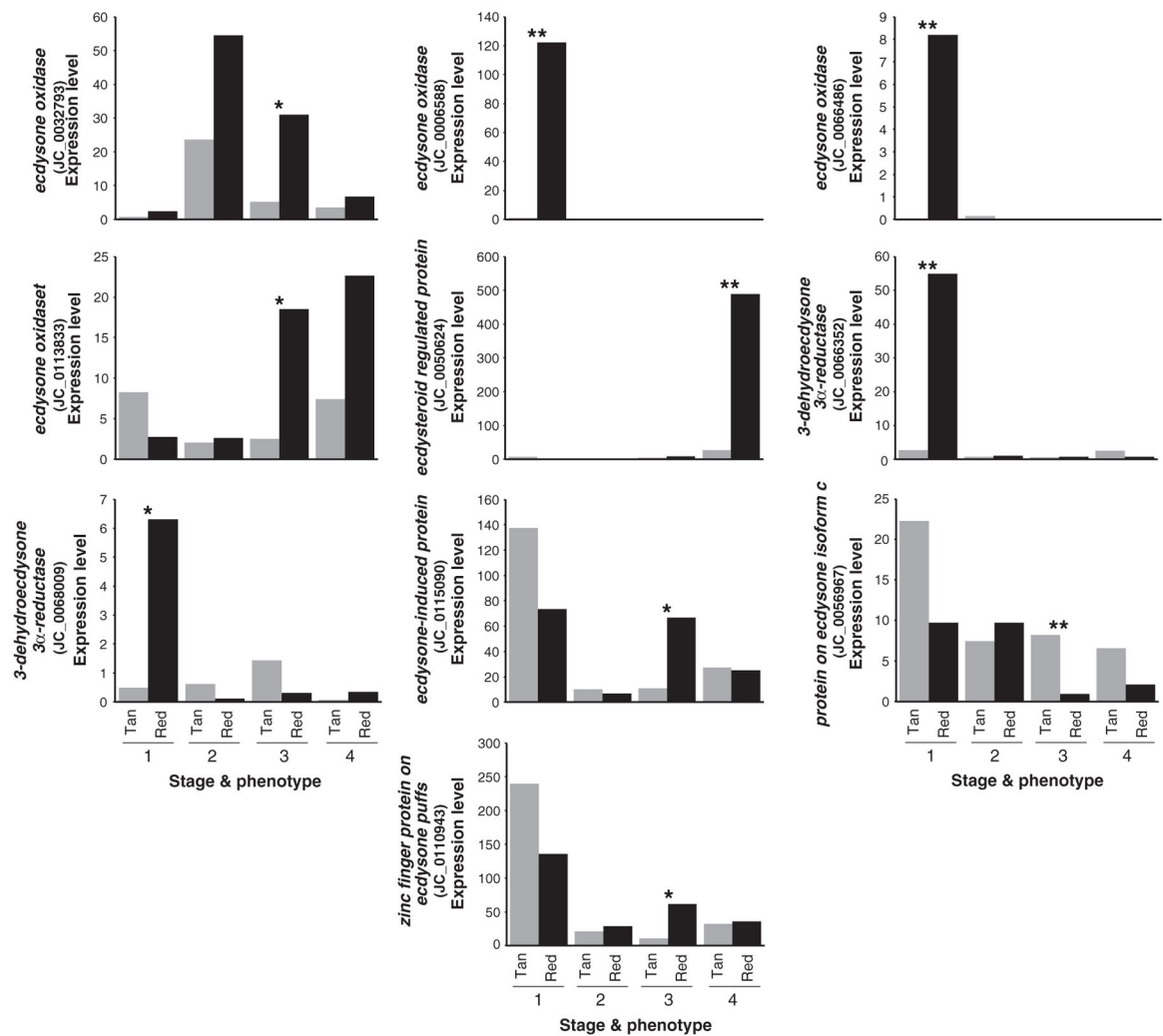
Fig. 2.

Stage- and phenotype-specific expression levels (FPKM) of selected ecdysone-associated transfrags that show significant differences between induced seasonal morphs. Stages are as shown in Fig. 1b. (*$P < 0.05$; **$P < 0.005$).
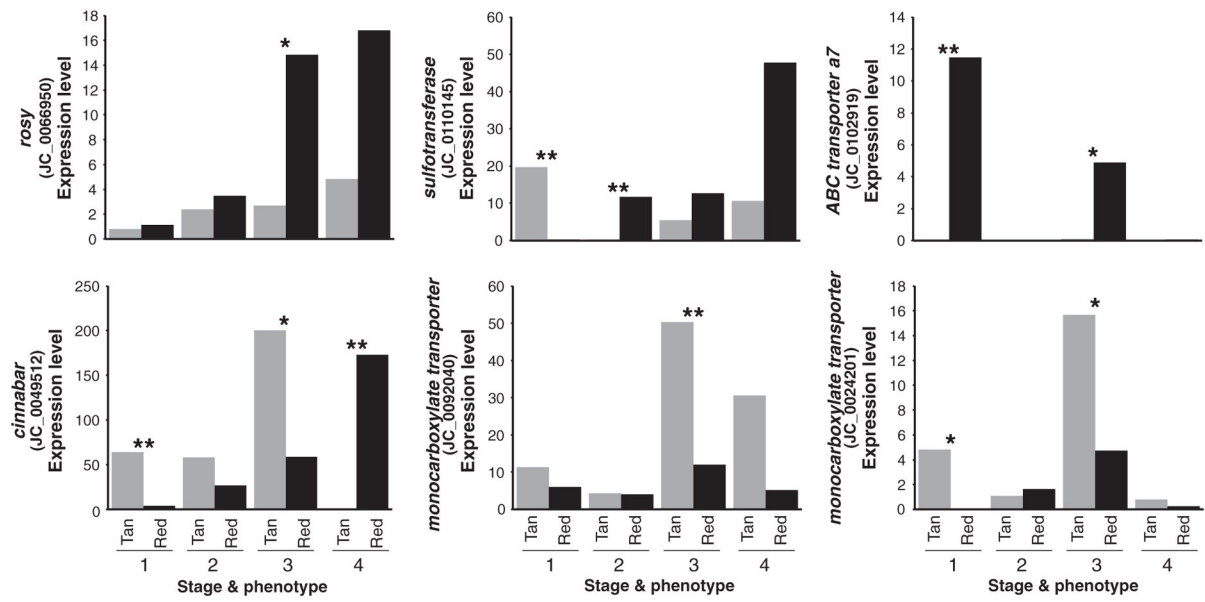
Fig. 3.
Stage- and phenotype-specific expression levels (FPKM) of selected ommochrome-associated transfrags that show significant differences between induced seasonal morphs. Stages are as shown in Fig. 1b. (*$P < 0.05$; **$P < 0.005$).
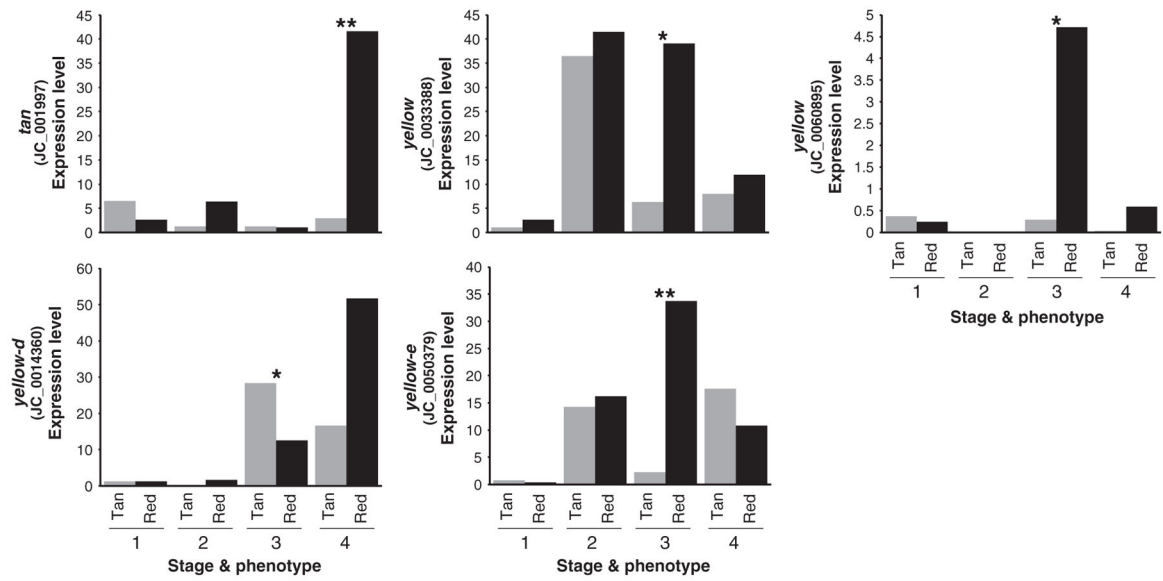
Fig. 4.
Stage- and phenotype-specific expression levels (FPKM) of melanin-associated transfrags that show significant differences between induced seasonal morphs. Only transfrags showing differences during pupal development are shown. Stages are as shown in Fig. 1b. (*$P < 0.05$; **$P < 0.005$).

Table 1

Statistics for *de novo* assembly of *Junonia coenia* wing transcriptome

Assembly size (Mb)	N50 (bp)	Contigs >N50	Longest contig (bp)	Contigs >300 bp	Contigs >500 bp	Contigs >1000 bp
77.55	687	31 057	22 289	121 076	53 707	16 251