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Transcriptome-Wide Differential Gene Expression in *Bicyclus anynana* Butterflies: Female Vision-Related Genes Are More Plastic

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Abstract

Vision is energetically costly to maintain. Consequently, over time many cave-adapted species downregulate the expression of vision genes or even lose their eyes and associated eye genes entirely. Alternatively, organisms that live in fluctuating environments, with different requirements for vision at different times, may evolve phenotypic plasticity for expression of vision genes. Here, we use a global transcriptomic and candidate gene approach to compare gene expression in the heads of a polyphenic butterfly. *Bicyclus anynana* have two seasonal forms that display sexual dimorphism and plasticity in eye morphology, and female-specific plasticity in opsin gene expression. Nonchoosy dry season females downregulate opsin expression, consistent with the high physiological cost of vision. To identify other genes associated with sexually dimorphic and seasonally plastic differences in vision, we analyzed RNA-sequencing data from whole head tissues. We identified two eye development genes (*klarsicht* and *warts* homologs) and an eye pigment biosynthesis gene (*henna*) differentially expressed between seasonal forms. By comparing sex-specific expression across seasonal forms, we found that *klarsicht*, *warts*, *henna*, and another eye development gene (*domeless*) were plastic in a female-specific manner. In a male-only analysis, *white* (*w*) was differentially expressed between seasonal forms. Reverse transcription polymerase chain reaction confirmed that *warts* and *white* are expressed in eyes only, whereas *klarsicht*, *henna* and *domeless* are expressed in both eyes and brain. We find that differential expression of eye development and eye pigment genes is associated with divergent eye phenotypes in *B. anynana* seasonal forms, and that there is a larger effect of season on female vision-related genes.

Key words: phenotypic plasticity, photoreceptor, pigmentation, opsin, CRAL-TRIO domain protein, Sex combs reduced.

Introduction

Eyes are metabolically expensive tissues. A rare study of the energetic requirements of photoreceptor cells found that up to 8% of a fly's resting metabolic rate was consumed by their eyes (Niven 2014). Given the high cost of vision, it is unsurprising that when organisms colonize new environments with low to no light their eyes often degenerate, presumably to free up energetic resources for reallocation to other tissues (Fong et al. 1995). As an example, flies kept in captivity have smaller eyes due to a reduction in facet number, which reduces photoreceptor energy consumption (Tan et al. 2005). Another more extreme example is the colonization of caves and underground habitats that generally leads to eye reduction or loss in a broad range of species through parallel evolutionary changes in key genes contributing to visual atrophy (Culver and Pipan 2009). In many of these cave-adapted animals, changes in the expression of eye development, phototransduction, and eye pigment genes have been associated with eye size reduction or loss. A transcriptomic analysis of a cave-adapted beetle, *Ptomaphagus hirtus*, showed the absence of some eye pigment and photoreceptor genes in head

transcriptomes to be correlated with reduced compound eyes (Friedrich et al. 2011; Friedrich 2013). Similarly, in the fish genus *Sinocyclocheilus*, photoreceptor genes were downregulated in a cavefish species relative to a surface species (Meng et al. 2013). The expression of developmental genes, which can either repress or promote gene expression depending on developmental context, can be more complex. For instance, the expression of *hedgehog* was significantly lower in the eyes of the cave amphipod *Gammarus minus*, relative to surface species (Aspiras et al. 2012), whereas in the Mexican blind cavefish, *Astyanax mexicanus*, higher expression of a *hedgehog* ortholog was found to drive eye degeneration (Yamamoto et al. 2004). Although extreme examples of eye loss suggest fixed and potentially irreversible genetic changes (pseudogenizations or even gene deletions) that enhance fitness by reducing investment in vision, phenotypic plasticity in visual systems is a relatively unexplored form of adaptation (likely involving more subtle changes in the regulation of gene expression levels) that may also be an evolutionarily important mechanism for coping with the high cost of vision.

Plasticity in eye morphology can indicate phenotypic plasticity in vision, and may be accompanied by plasticity in expression of vision-related genes. Developmental phenotypic plasticity is the ability of a single genotype to create different (fixed) phenotypes in response to environmental cues that are usually experienced early in development of the organism or even earlier in the maternal environment (Schlichting and Smith 2002). Adaptive plasticity evolves when populations live in recurrent fluctuating environments producing different phenotypes in each that have a higher fitness in their respective environments (Moran 1992). Plasticity can evolve and play a role in evolutionary processes, such as adaptation and speciation. For example, plasticity evolves through standing genetic variation or de novo mutation resulting in canalized traits or changes in plasticity to optimize fitness (Nijhout 2003; West-Eberhard 2003; Pigliucci et al. 2006; Crispo 2007). Phenotypic plasticity can thus affect the probability and direction of genetic evolution and may drive diversification (Price et al. 2003; Aubin-Horth and Renn 2009; Pfennig et al. 2010). Additionally, plasticity may play a role in adaptation through a direct influence on reproductive isolation and the promotion of evolutionary responses by nonadaptive plasticity (Fitzpatrick 2012). Furthermore, although some plastic traits in an individual can be adaptive, other plastic traits accompanying them may be maladaptive (Steinger et al. 2003; Ghalambor et al. 2007). Developmental phenotypic plasticity is distinct from phenotypic flexibility, where individuals are flexible and can acclimate to changing environments. An example of visual flexibility has been found in fish, where the type of opsin expressed in an individual fish retina will shift as the fish ages (Hofmann et al. 2010). Another example of physiological changes in vision is the circadian regulation of opsin expression levels linked to diurnal light-dark cycles (Sasagawa et al. 2003; Spaethe and Briscoe 2005; Battelle et al. 2013). Here, we explore phenotypic plasticity in vision of *Bicyclus anynana* butterflies whose distinct cohorts live in recurrent fluctuating environments in Africa, consisting of a dry season (DS) followed by a wet season (WS) (Brakefield and Reitsma 1991), and whose alternative vision phenotypes have been proposed to be adaptive (Everett et al. 2012).

Bicyclus anynana is a plastic sex-role reversed species that exhibits phenotypic plasticity in wing pattern morphology and sexual behavior. The WS form has conspicuous eyespots and a pale band on its wings, whereas the DS form has cryptic coloration and reduced eyespots (Brakefield and Reitsma 1991; Brakefield et al. 2009; Monteiro et al. 2015). In the laboratory, the WS and DS season wing forms are induced by rearing butterflies at 27 and 17°C, respectively (Koch et al. 1996; Kooi and Brakefield 1999). *Bicyclus anynana*'s courtship behavior is controlled by temperature experienced during pupal development and early adulthood (Prudic et al. 2011; Bear and Monteiro 2013). In conditions resembling the WS (27°C), males court females but in the DS (17°C), females court males (Prudic et al. 2011). In addition, wet season females (WSF) and dry season males (DSM) exhibit choosy behavior. Females choose mates based on UV-reflectance of

the dorsal forewing eyespot pupils (Robertson and Monteiro 2005); choosy males (DSM) and choosy females (WSF) prefer mates with intact UV pupils (Prudic et al. 2011). UV-reflectance brightness in these ornaments is highest in wet season males (WSM), followed by DSF, WSF, and finally DSM (Everett et al. 2012). WSM also have larger eyespot centers compared with DSM, whereas there is no difference between female seasonal forms (Prudic et al. 2011).

Everett et al. (2012) posited that nonchoosy individuals would have relaxed selection for vision accompanied by plasticity in eye morphology or visual sensitivity due to energetic costs of maintaining enhanced vision (Niven et al. 2007; Niven and Laughlin 2008; Niven 2014). This hypothesis was partially upheld by data from female *B. anynana* but not males. Eye size measurements demonstrated that eye size is both sexually dimorphic and phenotypically plastic; males had larger eyes compared with females and WS forms had larger eyes in both sexes compared with DS forms (fig. 1A) (table 1; Everett et al. 2012). Facet number was also greater in males compared with females and in WS forms compared with DS, whereas facet size was larger in males and DS forms compared with females and WS forms, respectively (table 1; Everett et al. 2012). Most significantly for the current study, quantitative polymerase chain reaction (qPCR) showed that *B. anynana* long-wavelength (*LWRh*), blue (*BRh*), and ultraviolet (*UVRh*) opsin genes have decreased expression in nonchoosy DS female butterflies, but not in males (Everett et al. 2012). The plasticity and sexual dimorphism of *B. anynana* eye phenotypes make this a suitable system with which to 1) identify additional vision-related genes on a whole-transcriptome level, which to our knowledge has not been done in butterflies before; and 2) investigate how variation in expression levels of these genes is correlated with previously measured phenotypic differences. We hypothesized that eye developmental pathway genes would be differentially expressed (DE) between individuals with different eye sizes (i.e., males vs. females; DS vs. WS). We also hypothesized that as opsins were DE in DS and WS females, additional phototransduction genes would be downregulated in nonchoosy DSF with smaller eyes and lower opsin expression. Finally, to relate our findings in butterflies to eye development in other organisms, we examined our transcriptomes for the presence or absence of genes in several signaling pathways that may be conserved across arthropods: visual system specification, retinal determination, and photoreceptor differentiation (Rivera et al. 2010).

We used a high-throughput RNA-Sequencing (RNA-Seq) approach to examine differential expression between *B. anynana* sexes and seasonal forms to identify genes associated with divergent eye phenotypes (Wang et al. 2009; Colombo et al. 2013; Meng et al. 2013; Smith et al. 2013). We explored differences between DSF, DSM, WSF and WSM and, to validate larger effects of seasonal form on females, we compared effects of seasonal forms within sexes. A large number of genes were DE between seasonal forms and sexes, including three vision-related genes (*klarsicht* [*klar*], *warts* [*wts*], and *henna* [*Hn*]) that were DE across seasonal forms. These three

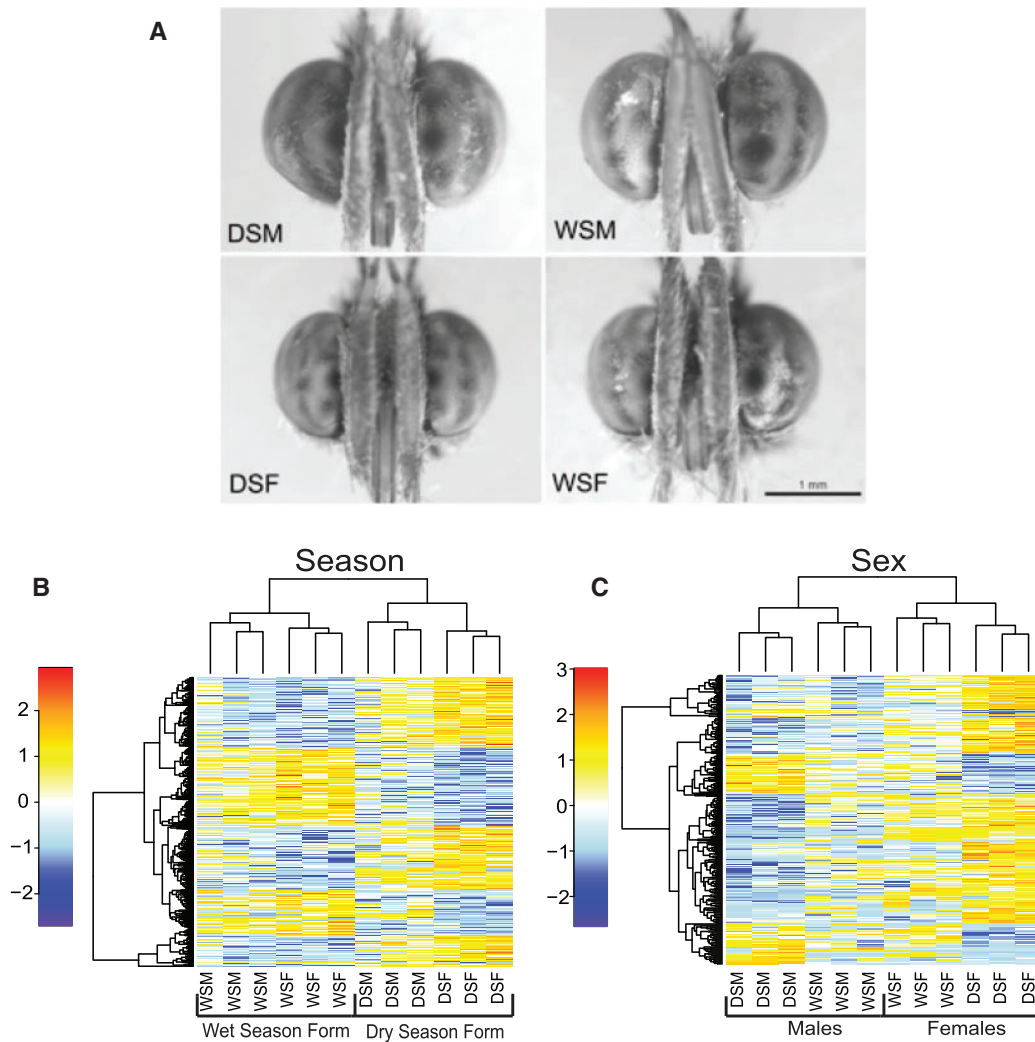


FIG. 1. Photographs of adult *Bicyclus anynana* heads and heatmaps of differentially expressed (DE) contigs by seasonal form and sex. (A) Eye size varies between wet season (WS) and dry season (DS) forms and between males (M) and females (F). Heatmaps of DE contigs by seasonal form (B) and sex (C). Color bar indicates scaled logCPM (log counts per million).

Table 1. Summary of Findings from Everett et al. (2012).

Season	Sex	Behavior	Mean Eye Size (mm ²)	Mean Facet Number	Mean Facet Size (μm ²)	Relative BRh Expression	Relative UVRh Expression	Relative LWRh Expression
Dry	F	Nonchoosy	0.87 ± 0.06	2,311 ± 326	348.8 ± 21	−0.80 ^{a,b}	0.78 ^b	0.01 ^{a,b}
Dry	M	Choosy	1.09 ± 0.065	2,727 ± 242.5	396.8 ± 13	0.61	2.01	0.76
Wet	F	Choosy	0.98 ± 0.06	2,857 ± 326	342.9 ± 21	0.83	2.39	0.38
Wet	M	Nonchoosy	1.33 ± 0.085	3,541 ± 352.5	362.8 ± 19	0.53	2.12	0.44
Global gene prediction			Eye developmental genes and eye differentiation genes will be upregulated or downregulated in males relative to females and WS forms relative to DS forms			Additional phototransduction genes will be downregulated in DSFs		

NOTE.—Opsin expression was quantified using qPCR and by normalizing to 18S rRNA then against the normalized opsin levels of a randomly picked sample using $2^{-\Delta\Delta CT}$ method (see Everett et al. 2012 for details). $N = 3$ biological and $n = 2$ technical replicates were performed.

^a $P < 0.05$ for DSF versus DSM.

^b $P < 0.05$ for DSF versus WSF.

genes in addition to *domeless* (*dome*) were DE across seasonal forms within females only, whereas a single vision-related gene (*white*) was DE across male forms. A vision-related (eye development, phototransduction, and eye pigment) candidate gene approach showed that 36 and 19 genes had

P values < 0.05 for season and sex contrasts, respectively. We find that plasticity in expression of eye development and eye pigment genes is associated with divergent eye phenotypes in *B. anynana* seasonal forms, and that season has a larger effect on female visual systems.

Table 2. Summary of the Total Number of DE Contigs and Unique GO Terms Discovered in Analyses.

Libraries Used	Contrasts	DE Contigs	GO Terms
All 12	Sex	290	77
All 12	Season	722	229
All 12	Interaction	111	23
Females only	Season	790	267

Results and Discussion

Whole-Transcriptome Expression Patterns

Assembly Statistics

RNA-Seq libraries were constructed using mRNA extracted from 0- to 3-h-old adult whole head tissue (excluding mouth parts and antennae) of 12 *B. anynana* individuals; three biological replicates of each of the four specimen types: 3 DSF, 3 WSF, 3 DSM, and 3 WSM. In total, we sequenced 12 libraries using high-throughput Illumina sequencing producing 100-bp paired-end reads. Quality trimming resulted in approximately 12 million reads per trimmed library. Several de novo assembly protocols were explored using Trinity (Grabherr et al. 2011), and the final reference assembly consisted of 43,248 contigs with an N50 of 2,299 bp. On average, approximately 85% of reads were successfully aligned to the assembly across libraries (supplementary table S1, Supplementary Material online).

To identify DE contigs between the treatment types, we fit a generalized linear model in edgeR (Robinson et al. 2010) with terms for sex, seasonal form, and a sex \times seasonal form interaction on raw count data from all 12 libraries. Using a false discovery rate (FDR) threshold of less than 0.05, we found 722 contigs that were DE across seasonal forms, 290 across sexes, and 111 showed a significant interaction between sex and seasonal form (table 2). Heatmaps for DE genes across seasonal forms (fig. 1B) and sexes (fig. 1C) showed clear groupings of gene expression for each factor and their interaction (supplementary fig. S1, Supplementary Material online). We used BLAST+ (Camacho et al. 2009) to identify homologous genes in *Drosophila* and assign gene ontology (GO) terms to our contigs (supplementary table S2, Supplementary Material online). We found 229 unique GO terms corresponding to DE contigs for seasonal form, 77 for sex, and 23 for the interaction of seasonal form and sex (table 2).

DE Genes Potentially Associated with Differences in Eye Size

Everett et al. (2012) found that WS eyes are generally larger than DS eyes and that male eyes are larger than female eyes (table 1). Functional enrichment tests were performed using Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Huang et al. 2009) for each model term (sex, seasonal form, and interaction) to group contigs with similar annotation terms into functional clusters, to identify genes associated with differences in eye size. Enrichment of DE contigs by seasonal form produced the highest number of enriched clusters (fig. 2A–F,

supplementary table S3, Supplementary Material online). Among these, we found two gene clusters, which may reflect decreased eye size in DS forms. Annotation cluster 1 included contigs annotated with insect cuticle protein structure; nine of these contigs were homologous to named cuticular protein genes. In cluster 1, 10 of 15 contigs were upregulated in DS forms (fig. 2A). In cluster 3, 13 of 21 contigs upregulated in DS forms had functions involving extracellular regions, aminoglycan and chitin metabolic process (fig. 2C). During larval development, a second stage of head tissue cell fate commitment consists of differentiation into retina or head cuticle (Friedrich 2003). These gene expression patterns may reflect a larger number of head cuticle secreting cells in DS forms. Annotation cluster 2, enriched for cellular retinaldehyde and alpha-tocopherol transfer (fig. 2B), may have a more direct effect on vision if these contigs retain a similar function to that of related gene family member *pinta*, a gene that encodes a visual chromophore binding and transport protein in *Drosophila* (see below).

Enrichment results for DE contigs across sexes resulted in four annotation clusters of genes encoding extracellular region proteins, immunoglobulin, cell adhesion, and calcium ion binding proteins (fig. 2G–J, supplementary table S3, Supplementary Material online). Fourteen of the 16 enriched DE contigs for sex were upregulated in females. Enrichment of DE contigs showing an interaction between sex and seasonal forms resulted in one significant cluster of contigs encoding endopeptidase activity proteins (supplementary table S3, Supplementary Material online). Overall, a functional enrichment of our DE contigs identified only two annotation clusters that may reflect differences observed in eye size. However, with the possible exception of annotation cluster 2 for seasonal DE contigs, enrichment tests did not detect specific vision-related clusters so we manually inspected GO terms associated with each of our DE contigs.

Within DE contigs across seasonal forms we found two contigs homologous to *Drosophila* genes that influence eye development, and an eye pigment biosynthesis gene (supplementary table S4, Supplementary Material online). *Wts* determines opsin expression in R8 photoreceptor cells in *Drosophila* that in turn differentiates pale from yellow ommatidia types, crucial to color discrimination (Mikeladze-Dvali et al. 2005). *Wts* was upregulated in DSF compared with DSM and WS forms (fig. 3A). *Klar* affects eye morphology and *klar* mutants in *Drosophila* have a rough eye phenotype driven by malformed photoreceptors (Mosley-Bishop et al. 1999); the contig homologous to this gene was downregulated in DS forms (fig. 3B). *Hn* is an eye pigment biosynthesis gene (GO:0006726) and was downregulated in DSF (fig. 3C). We did not find any vision-related genes in lists of DE contigs across sexes or displaying an interaction between seasonal forms and sex (supplementary table S4, Supplementary Material online). Although the two-factor analysis identified these three genes as being DE between seasons, close visual inspection of their plotted FPKMs (fragments per kilobase of exon per million fragments mapped) (fig. 3) revealed that it is the DS female form that is primarily responsible for the biggest magnitude change observed.

Season				Sex		
Annotation Cluster	Upregulated in DS	Upregulated in WS	Annotation Cluster	Upregulated in F	Upregulated in M	
A Annotation Cluster 1 Structural constituent of cuticle Enrichment Score: 5.400 p-value: 3.22E-08	Ccp84Ad CG1136 CG34461 CG8541 Cpr49Aa Cpr56F Cpr5C Cpr62Bb Cpr62Bc* Cpr76Bb	CG10625 Cpr49Ah Cpr50Cb Lcp65Ad Cpr66D*	G Annotation Cluster 1 Extracellular region Enrichment Score: 1.878 p-value: 2.03E-02	Ance BM-40-SPARC CG17739 cher Ch5 Impl2 vkg	Mmp2	
B Annotation Cluster 2 Cellular retinaldehyde Enrichment Score: 2.582 p-value: 9.00E-05	CG10657 CG2663 CG3823 CG5958	CG10026 CG11550	H Annotation Cluster 2 Immunoglobulin Enrichment Score: 1.678 p-value: 4.34E-03	cher Impl2 Strn-Mlck Unc-89	CG32791	
C Annotation Cluster 3 Extracellular region Enrichment Score: 2.065 p-value: 2.55E-05	Ance CG13830 CG5756 CG8483 Ch3 Ch5 Lsp1y	Mmp1 Mmp2 mfg NLaz Npc2a obst-A	I Annotation Cluster 3 Calcium ion binding Enrichment Score: 1.441 p-value: 1.50E-02	BM-40-SPARC CG33098 CG9297 Eip63F-1 Mp20 TpnC73F		
D Annotation Cluster 4 Juvenile Hormone Binding Enrichment Score: 1.984 p-value: 6.41E-03	CG10407 CG13618 CG14457	Atta PebIII CG30503 PGRP-SA Est-6 GNBP3 Hml obst-B	J Annotation Cluster 4 Cell adhesion Enrichment Score: 1.412 p-value: 5.66E-02	BM-40-SPARC CG9297 Impl2 Mp20		
E Annotation Cluster 5 Sugar transporter Enrichment Score: 1.317 p-value: 1.47E-03	CG1213 CG3168 Cyp4g15 sut4	CG10960 CG6034				
F Annotation Cluster 6 Protein dimerization activity Enrichment Score: 1.310 p-value: 4.32E-02	Gpdh	Hml Met ncd tim			U2af50	

Fig. 2. Functional enrichment of DE genes. A majority of contigs were upregulated in DS forms and in females. Enrichment clusters of DE genes by season included contigs homologous to (A) insect cuticle proteins; (B) cellular retinaldehyde binding and alpha-tocopherol transport-like proteins; (C) extracellular region, aminoglycan and chitin metabolism proteins; (D) odorant and juvenile hormone binding proteins; (E) sugar transport proteins; and (F) proteins with dimerization activity and binding. Enrichment clusters of DE genes by sex included contigs homologous to (G) extracellular region, (H) immunoglobulin, (I) calcium ion binding, and (J) cell adhesion. Asterisks denote contigs that were upregulated in DSF and WSM, respectively. Gene names are based on *Drosophila* homologs.

Female-Specific Analysis

As mentioned above, both the qPCR findings of Everett et al. (2012) (table 1) and now our two-factor DE analysis suggest that female gene expression shows the largest plasticity. To further explore female-specific differential expression, we performed a single factor comparison of the two seasonal forms for females only. We found 790 DE contigs across seasonal forms (FDR < 0.05; table 2, supplementary table S4, Supplementary Material online), 555 of which overlapped with DE contigs across seasonal forms in the two-factor model (supplementary fig. S2, Supplementary Material online). A heatmap of female DE contigs across seasonal forms showed a clear grouping between seasonal forms with approximately two-thirds of contigs being upregulated in DSF (supplementary fig. S3, Supplementary Material online). A functional enrichment analysis for 267 (table 2) unique *Drosophila* homologs resulted in 13 annotation clusters (supplementary table S3, Supplementary Material online) of which there was considerable functional and gene overlap with annotations clusters 1–5 of the seasonal DE genes shown in figure 2. We visually inspected our list of female-specific DE contigs (supplementary table S4, Supplementary Material online) for vision-related GO terms and confirmed that *klar* and *Hn* were downregulated in DSF and *wts* was upregulated in DSF (fig. 3A–C). In addition to recovering these genes, we found an eye development gene DE between female seasonal forms. *Dome* is a target in the JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription) pathway that regulates compound eye size and morphogenesis (Tsai and Sun 2004) and was downregulated in DSF (fig. 3D). Taken together, *klar*, *wts*, and *dome* are candidates for investigation of their potential role in driving eye size differences. Their role

in photoreceptor differentiation and eye morphogenesis may contribute to fewer facets in DSF, and thus their DE may contribute to a smaller eye phenotype (Everett et al. 2012).

Role of Sex Combs Reduced in *B. anynana* Eye Morphology

Developmental genes, such as transcription factors, often play important roles in trait development because they directly regulate the expression of other genes. In *Drosophila*, the *hox* gene *Sex combs reduced* (*Scr*) has a sex differentiation function (GO:0007548) and has been suggested to act in sex-specific differentiation of the basitarsus tissue (Sánchez and Guerrero 2001) and to control the development of the sex-combs in male T1 legs (Tanaka et al. 2011). Here, we hypothesized that WSM display masculine expression patterns of *hox* gene expression during photoreceptor differentiation causing male-like eye morphology. We inspected our female-specific DE contigs for *Scr* (supplementary table S4, Supplementary Material online) and found it to be DE between seasonal forms. *Scr* expression was similar in WSM, DSM, and WSM, whereas it was upregulated in DSF. A one-way analysis of variance (ANOVA) comparison of FPKM between WSM and males confirmed that *Scr* expression did not significantly vary between these groups (DSM $P = 0.52$; WSM $P = 0.51$). *Scr* has been found in the maxillary and labial palps in the head segments of several insect embryos (Kokubo et al. 1997; Rogers et al. 1997; Passalacqua et al. 2010), but no detailed knowledge of its later expression domain is known for Lepidoptera. It is possible that *Scr* could be differentially regulating the expression of genes in these head segments of DSF relative to the other three forms. An eye-specific expression for *Scr* is currently not known for any insect.

To reinforce our inspection of female-specific differences between seasons, we performed differential gene expression

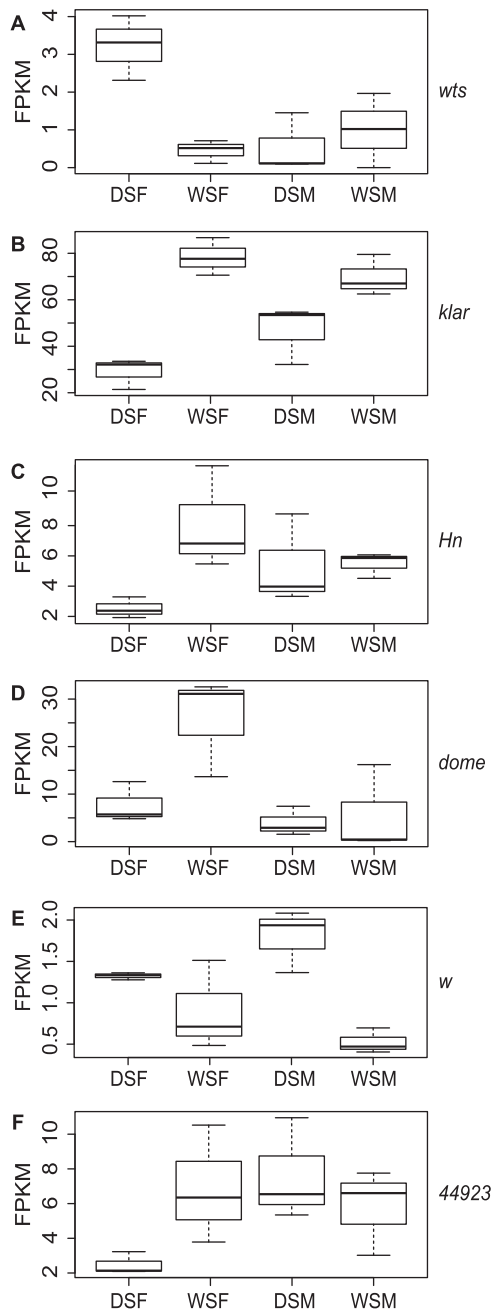


FIG. 3. Expression of DE genes across seasonal forms and sexes. (A) *wts* expression is increased in DSF relative to other forms. (B) *klar* expression is decreased in DSF relative to WSF. (C) *Hn* expression is decreased in DSF relative to WSF. (D) *dome* expression is also decreased in DSF relative to WSF. (E) *w* expression is in DSM relative to WSM. (F) *comp44923* expression is decreased in DSF relative to other forms. Thick lines represent medians. Whiskers represent maximum and minimum values.

analysis between male seasonal forms. In total, 359 contigs were DE within males, corresponding to 96 unique *Drosophila* genes. An enrichment of these homologs resulted in one cluster enriched for phagocytosis (supplementary table S3, Supplementary Material online). Manual inspection of this list uncovered only one vision-related gene, *white* (supplementary table S4, Supplementary Material online). *White*

(*w*) functions in compound eye pigmentation (GO:0048072) and was upregulated in DSM (fig. 3E). In addition, we searched male DE contigs for sex differentiation GO terms and did not find any matches making *Scr* a good candidate for masculinization of eyes in females.

Candidate Gene Approach

Opsins and Eye Pigment-Related Genes Are Downregulated in DSFs

Although Everett et al. (2012) observed differences in eye size between seasonal forms and between sexes, our whole-transcriptome DE analyses yielded only a handful of candidate vision-related genes. This may be because the threshold for detecting a significant log-fold difference using this method is too high. Nonetheless, we were interested in whether our RNA-Seq data could confirm the qPCR results of Everett et al. (2012) for the opsins. Opsin genes are the core component of visual systems because they encode proteins that bind a light-absorbing chromophore and together comprise the visual pigment rhodopsin. Rhodopsin initiates the phototransduction cascade and its absorption spectrum determines photoreceptor cell sensitivity (Briscoe and Chittka 2001). The chromophore in butterflies is 11-*cis* 3-hydroxy retinal. Everett et al. (2012) found that opsin genes have decreased expression in *B. anynana* nonchoosy DSF relative to choosy WSF (table 1). It was previously hypothesized that choosy individuals should have enhanced vision to detect sexual ornament brightness (dorsal eyespot centers) and nonchoosy individuals should have diminished vision due to physiological costs (Everett et al. 2012). We therefore expected to see a higher sensitivity to light, especially in the UV range, for WSF because they choose mates based on the UV-reflectance of their white centers (Robertson and Monteiro 2005). ANOVAs of mRNA expression levels quantified by calculating FPKM, normalized within and between libraries, were used to examine opsin expression levels between treatments.

In general, nonchoosy DSF have decreased expression relative to the other three groups, which validates prior qPCR results from Everett et al. (2012). The long-wavelength (*LWRh*) opsin gene was the third most highly expressed gene across libraries; however, *LWRh* opsin expression was not significantly different between sexes or seasonal forms nor was the interaction between these factors significant (fig. 4A, sex: $F = 0.203$, $P = 0.664$; seasonal form: $F = 0.010$, $P = 0.992$; sex \times seasonal form: $F = 3.888$, $P = 0.084$). The blue (*BRh*) opsin gene was on average the 18th most highly expressed gene and was DE between seasonal forms (fig. 4B, sex: $F = 1.570$, $P = 0.246$; seasonal form: $F = 10.211$, $P = 0.013$; sex \times seasonal form: $F = 3.416$, $P = 0.102$). A TukeyHSD test showed that this difference came from comparing DSF with WSF and WSM. One-way ANOVAs confirmed this trend in DSF relative to WSF ($P = 0.029$) and WSM ($P = 0.027$), but not DSM ($P = 0.112$). The ultraviolet (*UVRh*) opsin was the 55th most expressed gene across libraries, and did not show differential expression between groups using a two-way ANOVA (fig. 4C, sex: $F = 0.041$, $P = 0.845$; seasonal form: $F = 3.014$, $P = 0.121$; sex \times seasonal form: $F = 3.945$, $P = 0.082$).

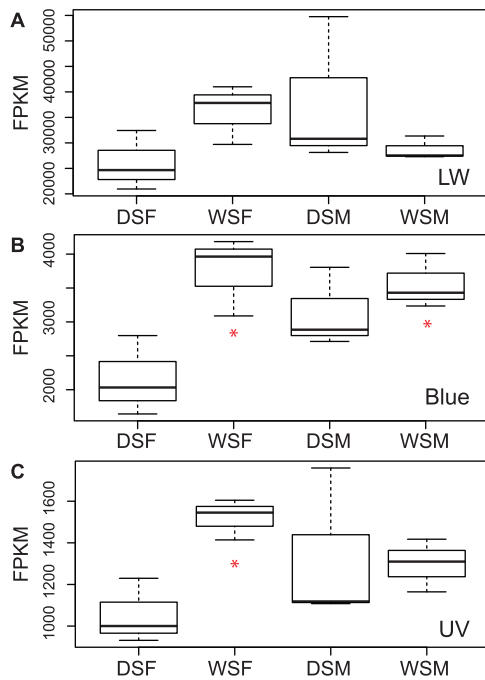


Fig. 4. Opsin expression in *Bicyclus anynana* seasonal forms and sexes. Box plots show FPKM values. Thick lines represent medians. Whiskers represent maximum and minimum values. Asterisks denote differential expression relative to DSF using one-way ANOVAs. (A) Long-wavelength (*LWRh*) opsin expression. (B) Blue (*BRh*) opsin expression. (C) Ultraviolet (*UVRh*) opsin expression.

However, one-way ANOVAs showed that DSF had decreased expression relative to WSF ($P = 0.012$), but not relative to males in either season (DSM $P = 0.305$, WSM $P = 0.1042$). Furthermore, WSM and DSM had similar levels of expression in all three opsin genes (LW $P = 0.345$, blue $P = 0.361$, UV $P = 0.897$). Our results showed that nonchoosy DSF do indeed downregulate *BRh* and *UVRh* mRNA relative to WSF, suggesting a decreased sensitivity. Downregulation of opsin genes was not observed in nonchoosy males, which may be due to differences in energetic demands between the sexes. Females have the additional metabolic burden of producing eggs so may be under greater selective pressure to reduce nonessential physiological functions.

We hypothesized that additional phototransduction or eye pigmentation genes might be regulated in a similar manner to the opsins. As similar expression patterns provide insight into functional categories (Eisen et al. 1998), we searched our female-specific DE genes for contigs with opsin-like patterns of expression and explored their putative functions. We found 102 contigs with log fold change (logFC) patterns of expression that were similar to the opsins, but eliminated 80 that were too variable after plotting their FPKM values and visually inspecting them. We used BLASTx against NCBI to determine the functions of the remaining 22 contigs and found functional descriptions for 16 of these genes through comparisons to other insects (table 3). Using this approach, we identified two vision-related contigs in particular that had expression patterns similar to the opsins. One contig potentially involved in phototransduction encoded a

protein homologous to alpha-tocopherol transport protein (fig. 3F, *comp44923*), which may be important for vision if it has a similar function to the gene *pinta* (*prolonged depolarization afterpotential is not apparent*). The *Drosophila* gene *pinta* is found in retinal pigment cells and preferentially binds all-trans-retinol in vitro (Wang and Montell 2005). The contig we identified, similar to *pinta*, has a CRAL/TRIO domain and is orthologous to *Manduca sexta* Msex010502, which is part of a large protein family that has undergone expansion and lineage-specific duplications in lepidopterans (Smith and Briscoe 2015). Another contig was a *henna*-like transcript (*Hn*) that regulates eye pigment biosynthesis (Bel et al. 1992); this contig was also DE using the whole-transcriptome two-factor and one-factor comparisons. Many, though not all butterfly photoreceptors have filtering pigments that affect light sensitivity and color vision (Briscoe 2008). Loss of eye filtering pigments has been reported in some butterflies that may rely more on chemosensory modalities, specifically olfaction and gustation for foraging and mate choice, rather than on vision (Briscoe and Bernard 2005). It is possible that the observed downregulation of an eye pigmentation gene in our phenotypically plastic species may, under more extreme environments, be followed by loss of entire pigmentation-related pathways.

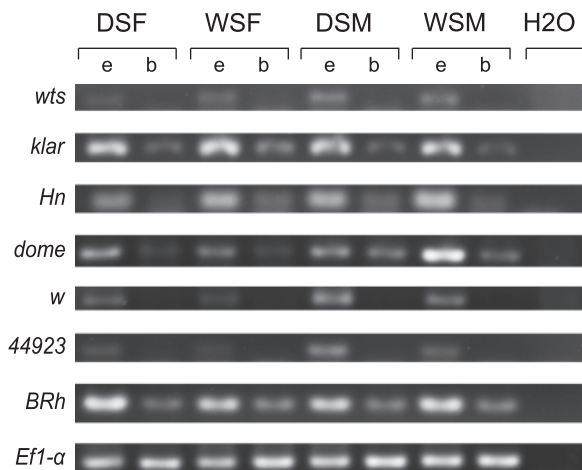
To validate the vision-related function of these DE genes in *Bicyclus*, we examined their expression in eye tissue. We did reverse transcription PCRs (RT-PCRs) for *wts*, *klar*, *Hn*, *dome*, *w*, and *comp44923* using eye (retina + optic lobe) and brain (without optic lobe) tissue. We found that *wts*, *w*, and *comp44923* are only expressed in eye tissue (fig. 5). *Klar*, *Hn*, *dome*, and positive control *BRh* are expressed in both eye and brain but have seemingly higher expression in eyes (fig. 5).

Butterfly Eye Development, Phototransduction, and Eye Pigment Genes

As butterfly head tissue is primarily composed of eyes (retina + optic lobe), we expected to find a large number of vision-related genes in our assembly, even if few were identified as being significantly DE in the transcriptome-wide analysis. As this study also represents, to our knowledge, the first transcriptome-wide characterization of candidate vision genes in butterflies, we undertook a manual search of the Trinity assembly for additional candidate genes involved in eye development, phototransduction, and pigmentation (Hardie 2001; Kumar 2001; Jeffery 2005; Friedrich et al. 2011). We found 203 genes and tested each gene for differential expression using two-way ANOVAs on FPKM values. FDR corrections to P values from sex, season, and interaction effects were applied, after which only one gene was found to be significantly DE (supplementary table S5, Supplementary Material online). Although most of these vision-related genes were not significantly DE after multiple testing corrections, it is interesting to note the top significant genes based on uncorrected P values (table 4). We found 34 genes involved in eye development with $P < 0.05$ across effects (e.g., *csw*, *dac*, *Egfr*, *eya*, *klar*, *toe*, and *toy*). Thirteen of these genes were DE across sex, 27 across seasonal form, and 5 showed a significant interaction. We found eight phototransduction genes with $P < 0.05$ for sex, seasonal form, and/or interaction. Three

Table 3. BLAST Results for Contigs with Opsin-Like Expression Patterns.

Contig ID	Description	Top Hit
comp33544_c0	Centromere protein 1 and 1-like	(<i>Danaus plexippus</i>) hypothetical protein KGM_06860
comp33612_c0	Sugar transporter	(<i>Bombyx mori</i>) sugar transporter 4
comp42445_c0	FAM50 homolog; neurogenesis	(<i>Danaus plexippus</i>) hypothetical protein KGM_09648
comp42682_c0	Pigment binding; small molecule binding	(<i>Danaus plexippus</i>) Bombyrin
comp43633_c0	Chemosensory binding; odorant binding; serine/threonine kinase	(<i>Bombyx mori</i>) uncharacterized protein LOC101743765
comp44249_c0	Serine protease inhibitor; antitrypsin isoform; peptidase activity	(<i>Manduca sexta</i>) serpin 1
comp44923_c0	Transporter activity; alpha-tocopherol transfer	(<i>Danaus plexippus</i>) putative CRAL/TRIO domain-containing protein
comp46683_c0	Secreted protein; salivary cys-rich secreted protein	(<i>Danaus plexippus</i>) hypothetical protein KGM_05173
comp47781_c0	Heparin sulfate O-sulfotransferase-like	(<i>Bombyx mori</i>) heparin sulfate O-sulfotransferase-like
comp48361_c0	BTB/POZ domain-containing protein	(<i>Bombyx mori</i>) BTB/POZ domain-containing protein 2-like
comp48541_c0	Nicotinamide riboside kinase	(<i>Bombyx mori</i>) nicotinamide riboside kinase 2-like isoform X1
comp49621_c0	L-asparaginase and like; lysophospholipase	(<i>Danaus plexippus</i>) lyso
comp49695_c0	Glucose dehydrogenase; glucose dehydrogenase precursor and acceptor	(<i>Danaus plexippus</i>) hypothetical protein KGM_15606
comp50819_c1	Hematological and neurological expressed 1-like protein	(<i>Danaus plexippus</i>) hypothetical protein KGM_13882
comp52506_c0	Inter-alpha-trypsin inhibitor heavy chain H4	(<i>Bombyx mori</i>) inter-alpha-trypsin inhibitor heavy chain H4-like
comp115040_c0	Phenylalanine hydroxylase; protein henna-like	(<i>Danaus plexippus</i>) phenylalanine hydroxylase

**FIG. 5.** RT-PCR of eye and brain tissue. RT-PCR in DSF, WSF, DSM, and WSM eye (e) and brain (b) tissue show that *wts*, *w*, and *comp44923* are only expressed in eye tissue. *klar*, *Hn*, and *dome* are expressed in both eye and brain tissue. *Blue rhodopsin* (*BRh*) and *elongation factor 1-α* (*EF1-α*) are positive controls and are both found in eye and brain tissue.

phototransduction genes were DE across sex (*cry*, *rdgA*, and *shakB*), 4 across seasonal form (*Pld*, *rdgC*, *BRh*, and *shakB*), and 3 showed a significant interaction (*Arr2*, *CG11426*, and *shakB*). Finally, eight eye pigment genes had $P < 0.05$ for sex, seasonal form, and/or interaction. Three genes were DE across sexes (*p*, *st*, and *w*), five across seasonal form (*dor*, *Dysb*, *lt*, *or*, and *w*), and one showed a significant interaction (*Pu*).

Eye Development Gene Networks in Butterflies

To relate our candidate vision genes in butterflies to known eye development networks in other arthropods, we inspected them for homologs of *Drosophila* genes involved in

visual system specification, retinal determination, and photoreceptor differentiation. We first examined a gene network controlling visual system specification, *wingless*/Armadillo (*wg/Arm*) (Rivera et al. 2010). We identified homologs of *split ends* (*spen*), *wingless* (*wg*), and *armadillo* (*arm*) in our assembly but not *eyeless* (*ey*), *spitz* (*spi*), *rhomboid* (*rho*) or *Cyclin E* (*CycE*). *Spen* is a positive regulator of the *wg/Arm* signaling pathway that controls a variety of cellular processes during development (Chang et al. 2008). *Spen* expression in the developing eye stimulates *wg/Arm* signaling and causes a small eye phenotype in *Drosophila* (Chang et al. 2008). We explored the expression of these genes and found no difference in expression between different sexes and seasonal forms (fig. 6A). One reason for the absence of several genes in this network from our transcriptome as well as this lack of difference in expression could be that we sampled gene expression after eye development and growth were complete—in early emerging adults (see Das Gupta et al. 2015).

The next gene network we explored was one controlling retinal determination (Rivera et al. 2010). *Hedgehog* (*hh*) signaling acts upstream of *decapentaplegic* (*dpp*), which affects regulatory genes *ey*, *eyes absent* (*eya*), *sine oculis* (*so*), and *dachshund* (*dac*; Pappu et al. 2003). The regulatory proteins encoded by *ey*, *eya*, *so*, and *dac* are critical for retinal determination and eye development (Pappu et al. 2003). *Hh* and *dpp* initiate eye morphogenesis and, together with *eya*, are required for the progression of the morphogenetic furrow; *dac* is required for the initiation of the furrow, but not its progression, and induced *dac* expression can cause ectopic eye development (Chen et al. 1997). We found homologs of *dpp*, *eya*, *so*, and *dac* in our *Bicyclus* de novo assembly but we did not find an *ey* ortholog (fig. 6B), given that this gene stops being expressed in adult heads of *B. anynana* (Das Gupta et al. 2015). Two-way ANOVAs of these genes show that *dac*

Table 4. Significant *P* Values of Two-Factor ANOVAs for Eye Development, Phototransduction, and Eye Pigment Genes.

Function	Gene	Sex	Season	Interaction
Eye development	<i>a</i>	0.358	0.017	0.418
	<i>Afti</i>	0.546	0.003	0.549
	<i>aop</i>	0.032	0.014	0.017
	<i>AP-1sigma</i>	0.001	0.035	0.527
	<i>bab2</i>	0.840	0.001	0.888
	<i>boi</i>	0.655	0.036	0.306
	<i>Bx42</i>	0.289	0.013	0.195
	<i>csw</i>	0.483	0.006	0.800
	<i>dac</i>	0.457	0.004	0.401
	<i>Dad</i>	0.317	0.020	0.538
	<i>Doa</i>	0.540	0.020	0.890
	<i>E(spl)mbeta-HLH</i>	0.170	0.017	0.510
	<i>E(spl)mgamma-HLH</i>	0.025	0.010	0.230
	<i>Egfr</i>	0.268	0.044	0.098
	<i>eya</i>	0.040	0.062	0.900
	<i>gl</i>	0.036	0.011	0.134
	<i>Gp150</i>	0.006	0.031	0.920
	<i>holn1</i>	0.015	0.919	0.720
	<i>hth</i>	0.005	0.137	0.916
	<i>kay</i>	0.001	0.015	0.110
	<i>klar</i>	0.423	0.000	0.040
	<i>lin-52</i>	0.076	0.012	0.250
	<i>PDZ-GEF</i>	0.570	0.015	0.380
	<i>peb</i>	0.114	0.014	0.119
	<i>pelo</i>	0.258	0.013	0.320
	<i>rst</i>	0.011	0.646	0.976
	<i>sca</i>	0.378	0.012	0.312
	<i>scrib</i>	0.179	0.081	0.043
	<i>skd</i>	0.070	0.005	0.667
	<i>ssh</i>	0.049	0.032	0.090
	<i>Tak1</i>	0.830	0.188	0.007
	<i>tio</i>	0.045	0.004	0.035
	<i>toe</i>	0.008	0.750	0.826
<i>toy</i>	0.709	0.011	0.828	
Phototransduction	<i>Arr2</i>	0.422	0.250	0.039
	<i>CG11426</i>	0.931	0.385	0.009
	<i>cry</i>	0.011	0.389	0.975
	<i>Pld</i>	0.134	0.045	0.112
	<i>rdgA</i>	0.030	0.857	0.876
	<i>rdgC</i>	0.667	0.038	0.422
	<i>BRh</i>	0.246	0.013	0.102
<i>shakB</i>	0.009	0.001	0.047	
Eye pigment	<i>dor</i>	0.066	0.003	0.387
	<i>Dysb</i>	0.657	0.012	0.761
	<i>lt</i>	0.960	0.014	0.057
	<i>or</i>	0.131	0.023	0.680
	<i>p</i>	0.009	0.191	0.553
	<i>Pu</i>	0.642	0.652	0.036
	<i>st</i>	0.002	0.337	0.219
<i>w</i>	0.022	0.010	0.560	

NOTE.—Italics indicated *P* values < 0.05. However, these values were not significant after FDR correction.

expression varies ($P < 0.05$) between seasonal forms and *eya* varies by sex ($P < 0.05$) (table 4), whereas there is no difference in expression for *so* nor *dpp*. Because *dac* and *eya* function to induce eye development, we predicted that their expression would be upregulated in specimens with larger eyes. We found that *eya* fits this predicted pattern (higher expression in males with larger eyes) whereas the opposite trend was true for *dac*, which had higher expression in DS forms that have smaller eyes relative to WS forms.

Finally, we explored genes involved in photoreceptor differentiation which involves several interacting developmental pathways. We explored genes involved in the signaling of the homeobox, Notch, JAK/STAT, and EGFR (epidermal growth factor receptor) signaling pathways. First, we found a *BarH1* (*B-H1*) homolog in our *Bicyclus* transcriptome. In *Drosophila*, *B-H1* is a homeobox gene necessary for R1 and R6 photoreceptor progenitor differentiation and primary pigment cell development (Higashijima et al. 1992). For the Notch pathway, we found a *Notch* homolog that was not DE (fig. 6C, supplementary table S5, Supplementary Material online); however, *Aftiphilin* (*Afti*) expression varied ($P < 0.05$) between seasonal forms (table 4) and had higher expression in WS forms with larger eyes. *Afti* modulates the Notch pathway and a knockdown of this gene in *Drosophila* results in irregular ommatidial size and neuronal disruption (Kametaka et al. 2012). Although JAK/STAT genes *hopscotch* (*hop*) and *Signal-transducer and activator of transcription protein at 92E* (*Stat92E*) were not significantly DE, we found their target *dome* upregulated in WSF (fig. 6D). Furthermore, in *Drosophila*, *Egfr* plays a critical role in R8 spacing (Baonza et al. 2001) during the morphogenetic furrow which stimulates the differentiation of R8 cells (Freeman 1997). An *Egfr* homolog had higher expression in WS forms ($P < 0.05$; table 4, fig. 6E).

We expected to find eye developmental genes DE between males and females and between seasonal forms because males have larger eyes relative to females and WS forms have larger eyes relative to DS forms (Everett et al. 2012). The expression pattern for these genes should match their effect on eye size phenotype (i.e., genes that cause “small eye phenotypes” when induced should be upregulated in small eye individuals). We found that some developmental genes followed expected expression patterns (e.g., *eya*, *dome*, and *Egfr*), whereas others did not (e.g., *dac*, *wg*, *hh*, *N*, *hop*, and *Stat92E*). However, we note that many genes within a developmental pathway directly affect or modulate a cell's response to another pathway (fig. 6). For example, *spen* (*wg*/arm regulator) stimulates EGFR signaling, and EGFR and JAK/STAT pathways are antagonistic to Notch (Frankfort and Mardon 2004; Doroquez et al. 2007; Flaherty et al. 2009). Although our transcriptome-wide analysis identified just a handful of DE genes, our ANOVA results suggest that key eye developmental pathway genes vary in expression between seasonal forms and sexes and could be driving divergent phenotypes, especially differences in eye morphology. Upregulation of eye photoreceptor differentiation genes in individuals with larger eyes may coincide with a higher facet number in these individuals (Everett et al. 2012).

Eye Loss and the Evolution of Phenotypic Plasticity

The possible role of developmental phenotypic plasticity in shaping vision is not well documented. Previous studies of cave-adapted animals examined presumably fixed genetic differences that contributed to eye reduction or eye loss. We expect loss of vision to be accompanied by consistent downregulation of phototransduction genes or their absence

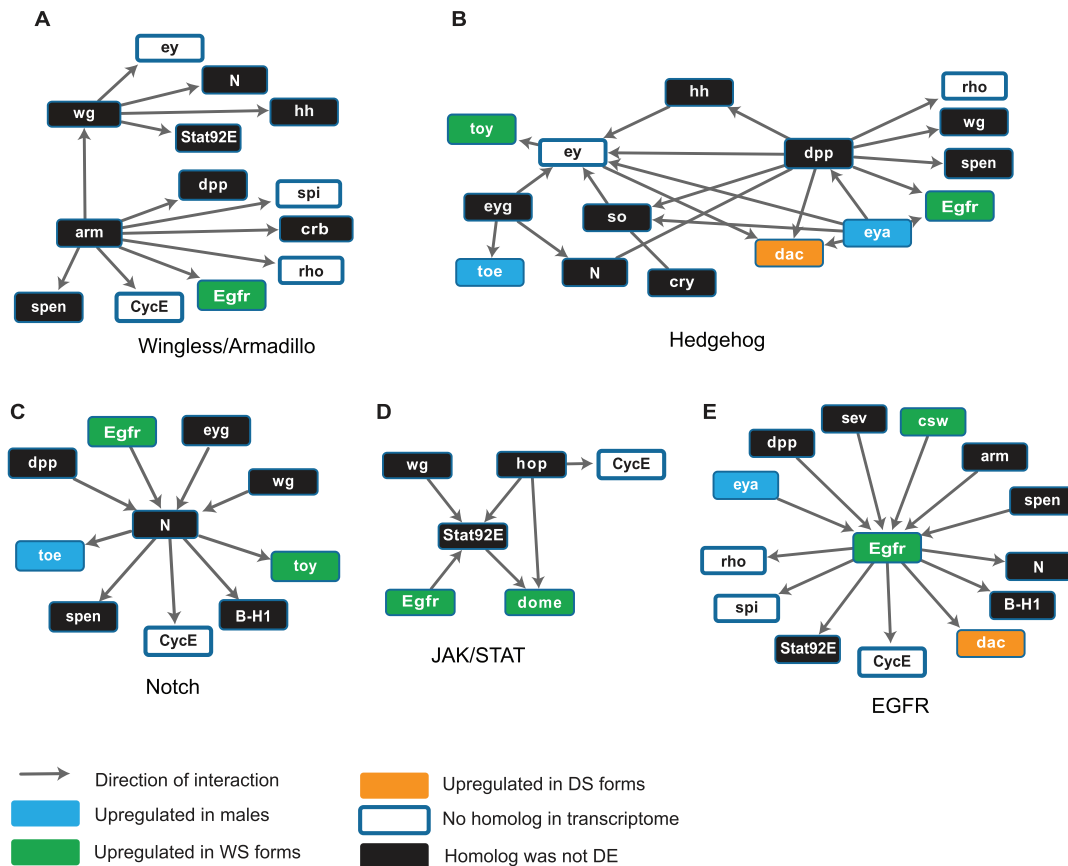


FIG. 6. Eye development gene networks. (A) Wingless/Armadillo (*wg/arm*) signaling pathway. (B) Hedgehog (*hh*) signaling pathway. (C) Notch (*N*) signaling pathway. (D) JAK/STAT signaling pathway. (E) Epidermal growth factor receptor (EGFR) signaling pathway. Arrows reflect direction of interaction from Flybase data for Cytoscape (Shannon et al. 2003). Blue nodes represent genes upregulated in males. Orange nodes represent genes upregulated in DS forms. Green nodes represent genes upregulated in WS forms. Black nodes represent genes with a homolog to the *Drosophila* gene in our transcriptome but which are not DE and white nodes indicate genes where no homolog was found in our assembly.

in transcriptomes due to accumulated mutations or pseudogenization (Lahti et al. 2009; Friedrich et al. 2011). In visual plasticity, we expect vision-related genes to maintain their coding sequences but vary in expression in predictable patterns determined by environmental conditions. Vision loss studies have compared the transcriptomes of different species (Friedrich et al. 2011; Meng et al. 2013) or different populations (Aspiras et al. 2012) which may have substantially diverged at the genomic level, yet these studies do provide a starting list of candidate genes to explore in the context of visual plasticity. In this study, we compared transcripts from individuals from the same stock population merely reared at different temperatures after egg laying. Although we did not create inbred lines to ensure genetic similarity between the individuals used because of the difficulty of doing so in butterflies, the observed differences are nonetheless likely due to phenotypic plasticity because our results—at least for the opsins—have now been replicated twice in the current study and in Everett et al. (2012).

Conclusions

Previous studies have correlated vision gene presence or absence or expression differences with extreme eye phenotypes, whereas the molecular basis of phenotypically plastic changes in eye morphology and physiology remained obscure. In our

study, we combined analysis of whole transcriptomes with a candidate gene approach to identify DE genes potentially driving divergent phenotypes in a polyphenic butterfly. We found that opsin genes (*BRh* and *UVRh*), a pigment biosynthesis (*Hn*), and a possible eye pigment transport gene (*comp44923*) were downregulated in nonchoosy, DSF relative to WSF. Moreover, we found three eye development genes (*klar*, *wts*, and *dome*) DE between DSF and WSF that might contribute to smaller eyes in DSF. Finally, we identified genes in developmental signaling pathways that varied in expression between sexes and seasonal forms. We propose that genes regulating developmental pathways are good candidates for driving divergent eye phenotypes; however, future studies should sample transcriptomes earlier during development to better capture differences during important differentiation stages. Our results suggest that plasticity of vision-related gene expression, particularly in females, may underlie eye phenotypic variation and this plasticity in visual systems is likely to be of evolutionary importance.

Materials and Methods

Animals and RNA Extraction

Butterflies were reared at Yale University and at the National University of Singapore at 17 and 27 °C to produce the DS

and WS forms, respectively, see Everett et al. (2012) for full husbandry details. Adults were frozen at -80°C on the morning of emergence when only approximately 0–3 h old. The butterflies were shipped to UC Irvine on dry ice and stored at -80°C until RNA extraction. RNA was extracted using TRIzol (Life Technologies, Grand Island, NY) from the heads of 12 individual animals; 3 DSF, 3 WSF, 3 DSM, and 3 WSM. RNA was DNase-treated and purified using a NucleoSpin RNA II kit (Macherey-Nagel, Bethlehem, PA). Purified RNA was quantified using a Qubit 2.0 Fluorometer (Life Technologies) and quality checked using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). A TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA) was used to make 12 double-stranded cDNA libraries from our polyadenylated RNA. A Qubit Fluorometer and an Agilent Bioanalyzer were used to quantify and quality check the libraries after preparation. Libraries were then normalized and pooled according to their concentrations. Pooled libraries were run on a 2% agarose gel and size selected for DNA at approximately 280–340 bp. A GeneClean III kit (MP Biomedical, Santa Ana, CA) was used to recover and purify DNA from the gel, and Agencourt AMPure XP (Beckman Coulter, Brea, CA) beads were used for a second purification. Libraries were sequenced in the UCI Genomics High-Throughput Facility using a HiSeq 2500 (Illumina), paired end 100-cycle sequence run.

Assembly and Read Mapping

Raw sequenced reads were low-quality trimmed and parsed using custom perl and python scripts. De novo transcriptome assemblies were constructed using Trinity (Grabherr et al. 2011; Haas et al. 2013). Several different assembly protocols were considered, such as using trimmed or untrimmed libraries and varying the number of genotypes used to construct the assembly. The final “reference assembly” chosen, based on low contig number and longest N50, was constructed using four trimmed libraries from one of each treatment type (DSF, WSF, DSM, and WSM). Each sequenced library was then mapped back to the reference assembly using RSEM (Li and Dewey 2011) from which we extracted raw read count data and FPKM. FPKM was further normalized between libraries using NOISeq (Tarazona et al. 2011) to compare expression of candidate vision genes.

Whole-Transcriptome Analysis

We performed differential gene expression analysis for all Trinity-assembled contigs using edgeR, a Bioconductor package that uses a variety of statistical models to analyze read count data (Robinson and Smyth 2007, 2008; Robinson et al. 2010; McCarthy et al. 2012). A generalized linear model was fit to raw count data from all 12 libraries; this model included terms for sex, seasonal form, and sex \times seasonal form interaction. We also fit a model to each of the sexes (six libraries) comparing seasonal effects to examine season-dependent gene expression within the two sexes separately. These analyses included filtering to remove contigs expressed at less than 1 count per million for at least three groups, and

between sample normalization using a trimmed mean of the log expression ratios (trimmed mean of M values [TMM]) (Robinson and Oshlack 2010). Contigs were considered significantly DE when the FDR was less than 0.05 (Storey and Tibshirani 2003; Dabney and Storey 2013). Results were visualized by creating heatmaps of DE genes using the Heatplus R package (Ploner 2012).

Opsin Genes

For preliminary analysis, we used CLC Genomics Workbench (CLC bio 2012) to create a de novo assembly of our libraries. We used long-wavelength, blue, and ultraviolet (*LWRh*, *BRh*, and *UVRh*) opsin sequences from *Danaus plexippus* (monarch) and *Bombyx mori* (silk moth) to extract matching *B. anynana* sequences. We used MEGA 5 (Tamura et al. 2011) alignments to determine consensus sequences for each opsin coding gene. Sequences for the three opsin genes were aligned to our Trinity assembly using command-line Basic Local Alignment Search Tool (BLAST+; Camacho et al. 2009) to identify the contig ID of the top match. We plotted normalized FPKM for these contigs in R (R Core Team 2013) to visualize expression levels. For each contig, we performed two-way ANOVAs and one-way ANOVAs to compare treatment groups.

Opsin-Like Patterns of Contig Expression

To identify contigs with similar expression patterns as the opsins, we searched our female-specific DE contig list for contigs downregulated in DSF. We retained contigs with a positive logFC, similar to that of the opsin genes. As opsin genes had small logFC between male seasonal forms, we reduced our list further by eliminating contigs with large logFC between male seasonal forms. We confirmed expression pattern by plotting FPKM and eliminating contigs whose expression did not resemble that of the opsins through visual inspection. Functions of remaining contigs were determined by a nucleotide BLAST, BLASTx, against the NCBI database (UniProt Consortium 2013).

GO Terms

We used TransDecoder in the Trinity suite to extract protein coding transcripts and amino acid sequences from our Trinity assembly. BLAST+ was used to align peptide sequences to *Drosophila* Flybase (Marygold et al. 2013)-translated sequences. The top best hit for each sequence was retained when the *E* value was less than 1×10^{-5} . GO terms for homologous proteins were obtained from Flybase. Functional enrichment analyses of DE contigs were performed using a DAVID (Huang et al. 2009) v6.7, which grouped genes with similar functions into functional clusters.

Candidate Genes

We searched *Drosophila* homologs for genes involved in eye development, adaptation to dark conditions, and phototransduction (Hardie 2001; Kumar 2001; Jeffery 2005; Friedrich et al. 2011). We also searched GO terms for vision-related terms such as: “eye,” “photoreceptor,” “phototransduction,” “R7,”

“R8,” and “pigment.” Two-way ANOVAs were performed to examine the effects of sex, seasonal form, and sex × seasonal form using all 12 libraries for 203 contigs. To correct for multiple tests, FDR was calculated using the qvalue R package (Storey and Tibshirani 2003; Dabney and Storey 2013).

Reverse Transcription PCR

Twelve animals (4 DSF, 4 WSF, 4 DSM, and 4 WSM) were sacrificed 0–3 h after eclosing. Eyes (retina + optic lobe) and brains (without optic lobe) were dissected, placed in RNAlater, and shipped to UC Irvine. Upon arrival, samples were placed in a freezer at -80°C . RNA was extracted using TRIzol (Life Technologies) and purified using the Nucleospin RNA II kit (Macherey-Nagel), which includes a DNase-treatment step. For RT-PCR, each 25 ml reaction had 2.5 μl Advantage 2 PCR buffer (Life Technologies), 2.5 ml dNTPs (2 mM), 0.5 μl Choice-Taq Blue (Denville Scientific, South Plainfield, NJ), 0.5 ml (1:20 diluted) SuperScript II Reverse Transcriptase (Life Technologies), 1 μl 20× primer mix (supplementary table S6, Supplementary Material online, Integrated DNA Technologies, Coralville, IA), 17 μl H_2O , and 1 μl RNA. The PCR reaction consisted of 45 cycles of 95°C for 30 s, 55°C for 30 s, and 68°C for 55 s. We visualized amplification by running the PCR products on a 2% agarose gel.

Supplementary Material

Supplementary figures S1–S3 and tables S1–S6 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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