RESEARCH ARTICLE

Distal-Less Regulates Eyespot Patterns and Melanization in *Bicyclus* Butterflies

JOURNAL OF EXPERIMENTAL ZOOLOGY
PART B
MOLECULAR AND DEVELOPMENTAL EVOLUTION

ANTÓNIA MONTEIRO[1,2]*, BIN CHEN[1,2,3], DIANE M. RAMOS[1], JEFFREY C. OLIVER[2], XIAOLING TONG[2], MIN GUO[1], WEN-KAI WANG[4], LISA FAZZINO[5], AND FIRDOUS KAMAL[6]

[1]Department of Biological Sciences, University at Buffalo, Buffalo, New York
[2]Department of Ecology and Evolutionary Biology, Yale University, New Haven, Connecticut
[3]Institute of Entomology and Molecular Biology, College of Life Sciences, Chongqing Normal University, Shapingba, Chongqing, P.R., China
[4]College of Agriculture, Yangtze University, Hubei Province, Jingzhou, China
[5]Choate Rosemary Hall, Wallingford, Connecticut
[6]Department of Electrical Engineering, University at Buffalo, Buffalo, New York

ABSTRACT

Butterfly eyespots represent novel complex traits that display substantial diversity in number and size within and across species. Correlative gene expression studies have implicated a large suite of transcription factors, including Distal-less (Dll), Engrailed (En), and Spalt (Sal), in eyespot development in butterflies, but direct evidence testing the function of any of these proteins is still missing. Here we show that the characteristic two-eyespot pattern of wildtype *Bicyclus anynana* forewings is correlated with dynamic progression of Dll, En, and Sal expression in larval wings from four spots to two spots, whereas no such decline in gene expression ensues in a four-eyespot mutant. We then conduct transgenic experiments testing whether over-expression of any of these genes in a wild-type genetic background is sufficient to induce eyespot differentiation in these pre-patterned wing compartments. We also produce a Dll-RNAi transgenic line to test how Dll down-regulation affects eyespot development. Finally we test how ectopic expression of these genes during the pupal stages of development alters adults color patters. We show that over-expressing Dll in larvae is sufficient to induce the differentiation of additional eyespots and increase the size of eyespots, whereas down-regulating Dll leads to a decrease in eyespot size. Furthermore, ectopic expression of Dll in the early pupal wing led to the appearance of ectopic patches of black scales. We conclude that Dll is a positive regulator of focal differentiation and eyespot signaling and that this gene is also a possible selector gene for scale melanization in butterflies. *J. Exp. Zool. (Mol. Dev. Evol.) 320B: 321–331, 2013.* © 2013 The Authors. *J. Exp. Zool. (Mol. Dev. Evol.)* published by Wiley Periodicals, Inc.

J. Exp. Zool. (Mol. Dev. Evol.) 320B:321–331, 2013

How to cite this article: Monteiro A, Chen B, Ramos D, Oliver JC, Tong X, Guo M, Wang W-K, Fazzino L, Kamal F. 2013. *Distal-less* regulates eyespot patterns and melanization in *Bicyclus* butterflies. J. Exp. Zool. (Mol. Dev. Evol.) 320B:321–331.

Genetic studies of the evolution of complex morphological novelties have been approached in different ways. From a macro-evolutionary perspective the goal has been to understand how genes become wired into novel developmental networks to produce novel traits (True and Carroll, 2002). From a micro-evolutionary perspective, however, the focus has been to identify the loci, within these novel networks, involved in trait modification across populations and species to better adapt them to their environment (Abzhanov et al., 2004; Shapiro et al., 2006). Butterfly eyespots have been the focus of both macro and micro-evolutionary research programs because they constitute novel complex traits that re-use existing developmental genes in novel networks (Monteiro and Podlaha, 2009) and at the same time display substantial morphological diversity within and across closely related species due to their role in natural and sexual selection (Oliver et al., 2009; Oliver and Monteiro, 2010).

Qualitative and quantitative correlative gene expression studies have implicated proteins such as Distal-less (Dll) (Carroll et al., '95; Brakefield et al., '96; Monteiro et al., 2006), Notch (N) (Reed and Serfas, 2004), Engrailed (En) (Keys et al., '99; Brunetti et al., 2001; Beldade et al., 2005) and Spalt (Sal) (Brunetti et al., 2001; Monteiro et al., 2006) in eyespot development. All four proteins are expressed in the centre of the future eyespots, the focus, during late larval wing disc development (Carroll et al., '94; Keys et al., '99; Monteiro et al., 2006). Wing compartments without eyespots and mutants that vary in eyespot number usually have perfect association with the presence/absence of these proteins (Brakefield et al., '96; Monteiro et al., 2003; Reed and Serfas, 2004; Monteiro et al., 2007). In addition, there is a perfect association during pupal development between the co-expression of Dll and Sal in a disc of cells surrounding the eyespot centre and adult black scales (Brunetti et al., 2001); and the expression of En in a ring of cells and gold scales (Brunetti et al., 2001). *Dll* was also implicated in the regulation of eyespot size by means of a linkage association study (Beldade et al., 2002). These lines of evidence point to Dll, N, En, and Sal's involvement in eyespot focal differentiation, to Dll, En, and Sal's involvement in color scale cell differentiation, and to a role for Dll in the control of eyespot size. Alternatively, loci linked to *Dll* could instead be responsible for eyespot size variation, and expression of all these genes during eyespot development may be merely correlational but not functional. In order to clearly implicate any of these genes in eyespot development it is important to first describe their detailed temporal patterns of expression, and then manipulate the genes directly and ask whether they affect eyespot development. Here we perform such manipulative experiments using recently developed transgenic tools for *Bicyclus anynana* (Marcus et al., 2004; Ramos et al., 2006; Chen et al., 2011).

Grant sponsor: NSF; grant numbers: IBN 0316283, IOB 0653399.
Conflicts of interest: None.
Antónia Monteiro, Bin Chen, Diane Ramos, and Jeffrey C. Oliver contributed equally to this work.
Authors Contributions: See Acknowledgments for details.
Diane Ramos's current address: Department of Natural Sciences, Daemen College, Amherst, NY.
*Correspondence to: Antónia Monteiro, Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT 06511.
E-mail: antonia.monteiro@yale.edu
Received 9 March 2013; Revised 19 March 2013; Accepted 19 March 2013
DOI: 10.1002/jez.b.22503
Published online 30 April 2013 in Wiley Online Library (wileyonlinelibrary.com).

METHODS

Temporal Characterization of Gene Expression in Wild Type and Spotty Larval Wings

We dissected fifth instar larval wing discs from wild type (Wt) and Spotty individuals. Spotty individuals have two additional eyespots on the forewings and we investigated the detailed temporal dynamics of eyespot-associated gene expression in these two lines. Wings were stained for gene products of *Dll*, *N*, *en*, or *sal*, using a rabbit polyclonal anti-Dll (at concentration 1:200), mouse monoclonals anti-N (at 1:20) and anti-En (at 1:5) or Guinea pig polyclonal anti-Sal (at 1:20,000) antibodies. We used goat anti-rabbit (Invitrogen, Carlsbad, CA, USA #T-2767), donkey anti-mouse (Jackson Immunoresearch, West Grove, PA, USA #715-095-150), and goat anti-Guinea pig (Molecular Probes #A11076) secondary antibodies at a concentration of 1:200. Monoclonal antibodies anti-N (C17.9C6-s) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. The Dll and En (4F11) antibodies were a gift from Grace Boekhoff-Falk and Nipam Patel, respectively. The anti-Sal polyclonal antibody (GP66-1) was manufactured by Proteintech Group, Inc. The peptide injected into four Guinea pigs was synthesized by the company and its sequence corresponds to two concatenated *Drosophila spalt major* peptide sequences that, when previously injected in rats and rabbits, produced a successful cross-reactive antibody (de Celis et al., '99). All wings were mounted with ProLong Gold (Invitrogen, Carlsbad, CA, USA) and images captured on a Nikon 90i microscope with NIS-Elements software (Nikon Instruments, Mellville, NY, USA).

We used the convention of (Reed and Serfas, 2004) to quantify wing ages; this approach provides an internal measure of wing developmental stage, facilitating comparisons among individuals. Gene expression was categorized for each of eight wing compartments, using a scale similar to (Reed et al., 2007). For Dll expression: 0 = no expression, 1 = midline expression extending from wing margin, 2 = midline expression with small focus with a diameter less than twice the width of the midline expression, and 3 = midline expression with a large focus with a diameter greater than twice the width of the midline expression.

For N, En, and Sal expression: 0 = no expression, 1 = midline expression extending from wing margin, 2 = midline and focal expression, and 3 = focal expression alone.

Dll, En, and Sal Vector Preparation

Dll, *en*, and *sal* over-expression piggyBac vectors (Additional File 1A–C) were constructed by cloning the 1.2 kb *Drosophila melanogaster Hsp70 promoter*/EGFP/*Hsp70* polyA fragment from *pBac[3xP3-DsRed, HS-EGFP]* into *pBac[3xP3-EGFPafm]*, cut and inserted both with the *Asc*I restriction enzyme into its single recognition site (both plasmids kindly provided by E. Wimmer). The *EGFP* coding sequence downstream of the *Hsp70* promoter was then replaced with either the 1,077 bp coding sequence of *B. anynana Dll* (AF404825), the 1,160 bp of *B. anynana en*, or the 4,263 bp of *D. melanogaster sal-m* (provided in a plasmid donated by R. Barrio), by using the flanking *Hpa*I and *Not*I restriction sites for the *Dll* plasmid and *Pac*I and *Spe*I restriction sites for the *sal* and *en* plasmids. The new vectors, *pBac [3xP3-EGFPafm, Hsp70-Dll]* (8,882 bp long), *pBac[3xP3-EGF-Pafm, Hsp70-en]* (8,965 bp) and *pBac[3xP3-EGFPafm, Hsp70-sal]* (12,068 bp) direct constitutive expression of *EGFP* in the eyes (the marker for detecting transgenic individuals), and inducible expression of *Dll*, *en*, and *sal* upon heat-shock.

A *Dll* RNAi vector (9,738 bp) was constructed using *Pogostick* (Chen et al., 2011) (Additional File 1D). The reverse compliment *Dll* coding sequence was cloned into the MCS between the *Hsp70* promotor and the intron, and the forward *Dll* coding sequence into the MCS between the intron and *Hsp70* polyA signal. Once this construct is expressed it should induce a double-stranded RNA pin-loop structure that starts the process of RNA interference inside the cells (Chen et al., 2011).

Making of Transgenic Lines and Whole-Body Heat-Shocks

Wild type eggs were injected with each of the plasmid constructs described above and with a *piggyBac* helper plasmid following the same protocol described in (Ramos et al., 2006). Positive individuals were selected based on eye-fluorescence and later confirmed via PCR. We did not establish homozygous lines: the individuals used for the heat-shock experiments were a mix of homozygous, heterozygous, and even potentially Wt if they resulted from the crossing of two heterozygous parents.

We reared one generation of Dll-over-expression and two of wild type animals at 27°C and 80% humidity, and one generation of Dll-over-expression and Wt at 23°C and 80% humidity. We pooled together the data from the two Wt generations reared at 27°C. In the Dll-over-expression generation reared at 27°C, all of the heat-shocks were performed on fifth instar larvae and 0–6 hr old pupae only, whereas more developmental stages were included in the generation reared at 23°C. We reared a single generation at 27°C and 80% humidity for the En and Sal over-expression lines and for the Dll-RNAi line, and performed all of the heat-shocks on this generation for these three lines. Unfortunately, due to the untimely extinction of these lines we were unable to confirm the over-expression and knock-down directly at the mRNA level.

In order to test the effect of gene over-expression and knock-down at precise times during eyespot development, pupation times were obtained by time-lapse photography using a Kodak DC290 digital camera. Pre-pupae were placed inside a plastic container with grid separations in the morning and they usually pupated during the evening and night. A photo was taken every 30 min. Scored pupal ages at the time of the heat-shock represent real pupal ages up to an additional 30 min. Animals were heat-shocked in an incubator oven, inside the plastic container covered with a lid for 2 hr at 39°C at different developmental stages. After the heat-shock they were either placed inside a small net cage with maize plants to finish their larval development, or, if already in the pre-pupal or pupal stage, placed inside a small cubicle mesh hanging cage for adult emergence. Upon emergence the butterflies were sacrificed by freezing.

Morphological Measurements

Forewings were carefully cut from the body and photographed under a microscope (Nikon SMZ1500) attached to a digital camera (Qimaging Micropublisher RTV, Surrey, BC, Canada). Wing measurements were performed in Object Image 1.62 (http://simon.bio.uva.nl/object-image.html). Data were later transferred to MS Excel version X and SPSS version 11 for analysis. The data for each temperature were analyzed separately due to the known effect of rearing temperature on eyespot size in this species (Brakefield, '96).

We measured the following seven characters on all forewings: the diameter of the four black discs of the eyespots present on the ventral and dorsal sides of wings, the diameter of the outer gold ring in the two Cu1 eyespots on the ventral and dorsal sides, and the distance between the two ventral eyespot pupils, as a proxy for wing size. All the eyespot diameter measurements were taken along an axis parallel to the wing veins. For the Cu1 eyespots where we measured both the outer (gold) diameter and the inner (black) diameter of the same eyespot we later calculated the ratio of black to gold diameters to test for differences in color composition in an eyespot in response to the heat-shock.

Because eyespot size is often positively correlated with wing size, in order to detect eyespot size changes independently of wing size we performed all eyespot size analysis using wing size as a covariate. We performed analysis of covariance (GLM analysis) on all eyespot diameters using interpupil distance as the covariate. We used line (Wt vs. Transgenic line), sex, and treatment (heat-shocked vs. control) as fixed variables in the analysis. Our model included all main effects as well as all two-way interactions. If any interaction term was significant, for instance between sex and line, we repeated the analysis for each sex separately and reported it instead of the results of the original analysis. Significant interaction terms for treatment and line are of especial importance and these are primarily reported—these interactions indicate that the different lines

responded differently to the same heat-shock treatment, presumably due to the presence/absence of the transgene product.

We scored eyespot number on the ventral side of one forewing, and on the dorsal side of the other forewing. Eyespot measurements and eyespot scores were done without knowledge of line or treatment identity. Instead we used cage number (for a cluster of animals treated the same way) as the identifiers for our data, and only later, after measurements were taken, did we label the data with treatment and line identifiers.

Real-Time PCR

Four fourth instar larvae of each line (Dll-overexpression and Wt) were collected and kept in RNA*later* (Ambion, Foster City, California, USA) both before and at several time points after a 3 hr heat-shock treatment at 39°C. Total RNA was isolated from the larvae using a RNeasy Mini kit (Qiagen, Valencia, CA, USA), and subsequently treated with RNase-free DNase I (Qiagen) to eliminate genomic DNA. cDNA was reverse-transcribed from total RNA using random nanomers using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA). Real-Time-PCR was performed with TaqMan Universal PCR Master Mix and Custom TaqMan Gene Expression Assays in STANDARD mode using Applied Biosystems 7500 Fast Real-Time PCR Systems. Eukaryotic 18S rRNA was used as the endogenous control. Relative quantification of *Dll* transcript was obtained using the 2-$\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) normalizing levels across samples using 18S rRNA levels.

Confirming Elevated Protein Levels in Larval Wings Following Heat-Shock Using an *Ultrabithorax* Over-Expression Transgenic Line

Because our transgenic lines went extinct before we could confirm that protein levels were elevated in wing discs upon a single 2 hr heat-shock we used a different *B. anynana Ultrabithorax* over-expression transgenic line, whose creation was previously described (Chen et al., 2011), for this purpose. Wt and Ubx 5th instar larvae were heat-shocked for 2 hr (as described above). Forewings from both heat-shocked and control individuals were dissected in alternating order 7–11 hr after the beginning of the heat-shock. Wings were stained with a rabbit anti-*Junonia coenia* Ubx antibody (1:500; a gift from L. Shashidhara). We used a secondary goat anti-rabbit at 1:200 (Molecular Probes #T-2767; as described above). Wings were photographed with the same exposure time under a fluorescent scope. Wing "brightness" was used as a measure of protein expression levels. We used a threshold tool (in Photoshop) to first remove auto-fluorescent trachea from all wing images simultaneously. Then we averaged the color of each wing using the Blur/Average Filter tool. Wing darkness was obtained using the *K*-value of the Histogram tool in Photoshop (using a color picker), and brightness was obtained by subtracting this value from 100. Forewings of Wt individuals do not express Ubx and, thus, brightness levels indicate background levels of labeled secondary antibody.

Laser Heat-shocks

We used an infrared laser system, similar to a previously described green-laser system (Ramos et al., 2006), to heat small areas of pupal wing epidermis (~0.5 mm^2) in whole live pupae in order to ectopically activate *Dll*, *en*, and *sal* on the wing. The laser system has a continuous infrared beam shining on the specimen that is interrupted with an electronically controlled shutter. We optimized the heat-shocking conditions by controlling the shutter. We varied the duration of the heat pulses, but kept the interval between pulses and the total heat-shock duration constant at 1 sec, and 20 min, respectively. We applied variable heat-shocking conditions to pupae of the Dll, Sal, En, and Wt lines, shortly after pupation and compared the adult wing phenotypes between them. Pupation time was scored for each animal using time-lapse photography and used to calculate pupal age at the time the laser heat-shock was applied. Pupae were between 0.5 and 24 hr old, but with an average age of 10 hr at the time of the laser heat-shock. This average age was the same across lines.

RESULTS AND DISCUSSION

Dll, *En*, *Sal*, and *N* Protein Expression in Eyespot Centers is Aborted in Some Forewing Compartments in Wild Type *B. anynana* But Not in the Mutant Spotty

B. anynana does not develop eyespots in all its forewing compartments. In order to explore the mechanism of focal differentiation that controls eyespot number we began by performing a detailed temporal characterization of gene expression in *B. anynana* wild-type (Wt) and mutant (Spotty) individuals carrying two additional forewing eyespots. We assessed the temporal dynamics of Dll, En, Sal, and N protein expression throughout fifth instar wing disc development. In early discs, expression of these proteins was similar between Wt and Spotty individuals (Fig. 1A,B and Additional Files 2 and 3). In particular, small foci of protein expression were visible in the four middle compartments (M1, M2, M3, and Cu1) of early wing discs whereas no focal expression was visible in the flanking anterior and posterior compartments. In late stage wing discs, however, expression of the four proteins decreased in two middle compartments (M2 and M3) while it continued in the M1 and Cu1 compartments of Wt individuals. In Spotty individuals, expression of the four proteins was maintained in the original four middle compartments throughout larval wing development. We conclude that natural decreasing levels of Dll, En, Sal, and N in a subset of wing compartments during larval development correlates with fewer forewing eyespots in Wt wings.

Over-Expression of *Dll* During the Larval Stage Leads to the Differentiation of Extra Eyespots

In order to test whether over-expression of Dll, Sal, or En during the larval stage is sufficient to complete focal differentiation in the

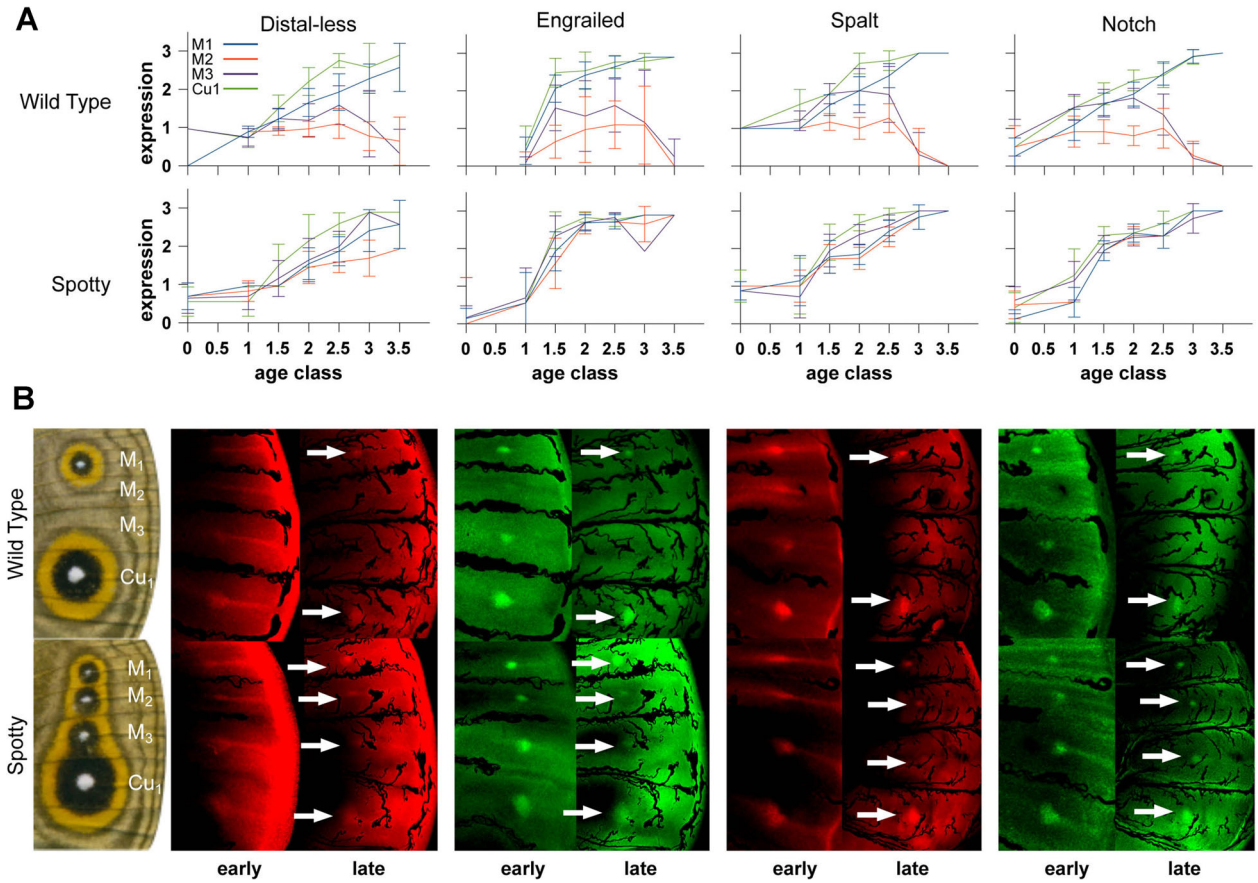

Figure 1. Protein expression differences in the eyespot field correlate with eyespot number variation between Wild type and Spotty mutants. (A) Temporal expression dynamics of four proteins in Wt (top row) and Spotty (bottom row) larval wing discs. Each line corresponds to a specific wing compartment; eyespots develop in adult wings of Wt and Spotty individuals in the M1 and Cu1 wing compartments, while M2 and M3 compartments only develop eyespots in adult Spotty individuals. Error bars are 95% CI of expression level classes as described in the Methods Section. (B) Representative early and late larval wings depicting the four gene products in Wt (top row) and Spotty (bottom row) individuals. All four gene products show similar expression between Wt and Spotty individuals in early stages, but expression in M2 and M3 is down-regulated in late stage wild type individuals. Arrows indicate focal expression in late stage fifth instar wing discs (see also Additional Files 2 and 3).

M2 and M3 wing compartments, we developed novel *piggyBac* vectors and over-expression transgenic lines containing the complete coding sequences of *B. anynana Dll* and *en*, and the complete coding sequence of *Drosophila sal* driven by the *Drosophila* heat-shock promoter of *hsp70* (Ramos et al., 2006) (Additional Files 1A–C).

There are special challenges posed by activating transgenes by means of a heat-shock in a phenotypically plastic butterfly where both wing size and eyespot size is influenced by rearing temperature (Brakefield and Reitsma, '91; Windig, '94; Brakefield et al., '96). The heat-shock promoter from *hsp70* from *Drosophila*, however, is currently the only tested (inducible) promoter in *B. anynana* (Ramos et al., 2006; Chen et al., 2011), so these challenges were overcome with an appropriate experimental design. We performed a full-factorial design of four groups total: transgenic heat-shocked, wildtype heat-shocked, transgenic control (no heat-shock) and wildtype control, where heat-shocked and control individuals are reared in the same generation (see Additional File 4 for total number of animals analyzed). We looked for patterns where the effect of the heat-shock was different in transgenic versus wild-type individuals in wings of comparable size, for example, either exaggerating or reducing eyespot phenotypes (and producing a significant treatment by line interaction in statistical terms). This would indicate that the transgene had an activator or repressor effect on eyespot development, respectively, beyond any heat-shock effect.

When larvae were reared at 27°C, heat-shocks during the late larval stage led to the appearance of small extra eyespots in the Dll transgenic line, but not in Wt individuals nor in treated En or Sal transgenics (Fig. 2A,B; interaction of line × treatment: Dll $F_{1,\ 207} = 5.166$, $P = 0.024$; En $F_{1,\ 359} = 0.125$, $P = 0.724$; Sal $F_{1,\ 363} = 0.294$, $P = 0.588$). When Dll larvae were reared at 23°C, however, heat-shocks no longer led to a differential increase in eyespot number in Dll transgenics relative to Wt controls (interaction of line × treatment: $F_{1,\ 309} = 0.007$, $P = 0.936$). These results suggest that raising Dll levels in larvae reared at high temperature, but not those reared at lower temperature, is sufficient to differentiate additional eyespots in the M2 and M3 pre-patterned wing compartments.

Dll Over-Expression Increases Eyespot Size and changes Eyespot Color Composition

It was previously shown that polymorphism at *Dll* correlated with changes in eyespot size (Beldade et al., 2002), so here we tested whether changes in *Dll* expression levels during the larval and/or pupal developmental stage caused changes in eyespot size. Heat-shocking larvae led to relatively larger dorsal eyespots in Dll individuals but not in Wt individuals. In particular, heat-shocks had significant opposite effects on the size of the dorsal Cu1 eyespot. Eyespot size increased in the Dll line, whereas it decreased in Wt individuals (Fig. 3A; line × treatment $F_{1,\ 399} = 4.005$, $P = 0.046$). Heat-shocks also had opposite effects on the black disc of the dorsal M1 eyespot, increasing it in Dll males, but reducing it in Wt males (Fig. 3B; line × treatment $F_{1,\ 172} = 4.957$, $P = 0.027$). Zero to 6 hr pupal heat-shocks produced no changes in line by treatment interactions regarding eyespot size. These results suggest that the heat-shock, on its own, has a negative effect on relative eyespot size, but Dll over-expression overcomes this effect by increasing eyespot size.

When Wt and Dll lines were reared at 23°C there were also significant changes in relative eyespot size and eyespot color composition between the lines. Pre-pupal heat-shocks had significant opposite effects on the relative size of the Cu1 ventral eyespot: increasing it in Dll but decreasing it in Wt individuals (Fig. 3C; line × treatment $F_{1,\ 317} = 6.429$, $P = 0.012$). Six to 12 hr pupal heat-shocks produced more golden eyespots in wild-type individuals, but it did not change eyespot color composition in *Dll* individuals (Fig. 3D; line × treatment $F_{1,\ 199,} = 17.830$, $P < 0.001$).

In summary, Dll over-expression during the pre-pupal stage at 23°C and during the larval stage at 27°C increased relative eyespot size. These size shifts were primarily observed on the ventral Cu1 eyespot at 23°C and on the dorsal Cu1 eyespot at 27°C. The increase in eyespot size on opposite surfaces at different temperatures suggests a complex interaction between rearing temperature and gene regulation in this seasonally plastic species (Brakefield, '96), which we cannot fully explain. Dll over-expression later in development (6–12 hr pp) prevented eyespots from becoming "more golden" as observed in Wt. Because *Dll* is secondarily expressed in the area of the black scales around this time during pupal development, over-expression on the gene everywhere on the wing may have altered the balance of activators versus repressors at the transition point between *Dll* expression (black scales) and *en* expression (gold scales), resulting in a "blacker" eyespot.

It is unclear why the larger of the two eyespots on the forewing (Cu1) responded to the heat-shocks more readily than the smaller anterior eyespot. It appears that altering eyespot size is easier to

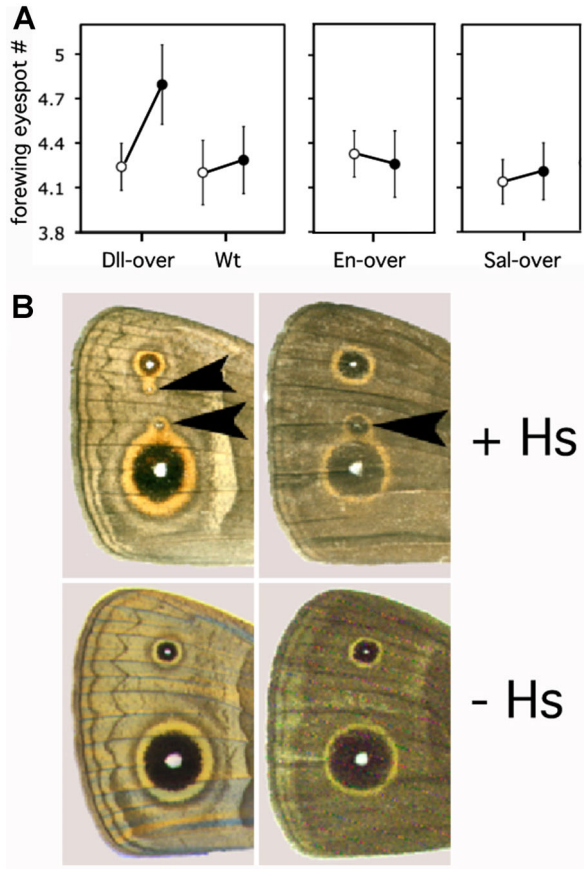

Figure 2. Over-expression of Dll leads to extra eyespots. (A) Total number of eyespots scored on the right dorsal and left ventral side of the forewings for Wildtype (Wt), Distal-less (Dll-over), Engrailed (En-over), and Spalt (Sal-over) over-expression transgenic butterflies, with and without application of a heat-shock (hs) treatment during the 5th larval instar (heat-shock is represented by a filled circle). Error bars represent 95% confidence intervals. (B) Typical positions (M2 and where extra eyespots appeared in Dll individuals after hs (top, black arrows), relative to a non-heat-shocked Dll animal (bottom; see Additional File 1 for vector design and Additional File 4 for number of butterflies analyzed).

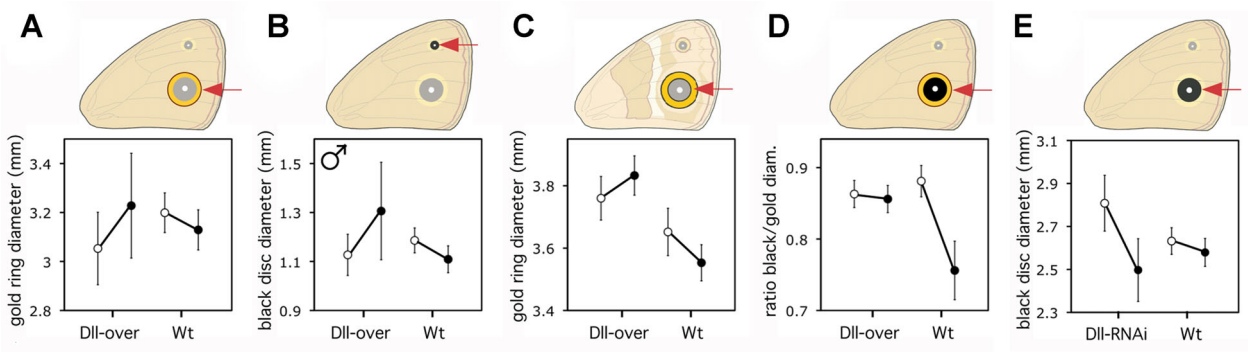

Figure 3. *Dll* over-expression and down-regulation affects the size of the eyespots. Size of eyespot measurements performed in adults after heat-shock (filled symbol) and in controls (open symbols). (A–D) Dll over-expression line versus Wt. (E) Dll-RNAi line versus Wt. (A,B) Wing measurements taken in adults reared at 27°C. (C,D) Wing measurements taken in adults reared at 23°C. (A) Diameter of the dorsal Cu1 eyespot gold ring. (B) Diameter of the dorsal M1 eyespot black disc. (C) Diameter of the ventral Cu1 eyespot gold ring. (D) Ratio of the black to gold diameters of the dorsal Cu1 eyespot. (E) Diameter of the dorsal Cu1 eyespot black disc. *Y*-axis in A–C, and E represents corrected means for a particular wing size, obtained from a linear regression of eyespot size on wing size. Heat-shocks were performed during the 5th larval stage except in C and D (pre-pupal and 6–12 hr post pupation, respectively). Error bars represent 95% confidence intervals (see Additional File 1 for vector design and Additional File 4 for number of butterflies analyzed).

achieve by overall gene over-expression when the central signaling cells are already fated to produce a large eyespot (see Discussion Section).

Direct Quantification of *Dll* mRNA Levels Show That Heat-Shocks Increase *Dll* Transcription

In order to directly verify that the phenotypes we observed stemmed from increases of *Dll* mRNA levels in response to the heat-shock, we quantified *Dll* mRNA levels in Dll and Wt animals before and at several hours after the heat-shock using quantitative real-time PCR. We found that *Dll* mRNA levels were substantially raised in Dll transgenic individuals, but not Wt individuals, as early as 9 hr after the heat-shock, and that subsequently *Dll* levels dropped gradually to more normal levels (Fig. 4). We conclude that the heat-shocks are producing a significant elevation of *Dll* mRNA levels that is later likely to translate into additional protein being expressed on the larval wings, and into additional and larger eyespots in adults. Unfortunately, due to the extinction of this line, we were not able to confirm that Dll protein levels were elevated in heat-shocked individuals in the larval wing discs. However, we used a separately generated *B. anynana* transgenic line that drove a different transcription factor (Ultrabithorax) under the same heat-shock promoter (Chen et al., 2011) to confirm that a single 2-hr heat-shock is able to induce a heat-shock in larval wing tissue and elevate protein levels of a transgene in this tissue (Additional File 5). While different proteins have different stabilities, and Ultrabithorax protein levels do not necessarily indicate what Dll protein level would have looked like, these data show that the heat-shock treatment applied is adequate to elevate protein levels of transgenes in larval wing tissue. The data also support the inference that changes in eyespot size and number observed in the Dll line are likely due to increased levels of Dll protein in larval wings.

Down-Regulating Dll Leads to Smaller Eyespots

Given the significant effects of over-expressing *Dll* during larval development on increasing eyespot number and size, we predicted that down-regulating this gene, via transgenic RNAi, would impact these phenotypes in opposite ways. Heat-shocking Dll-RNAi larvae reared at 27°C led to no changes in eyespot number

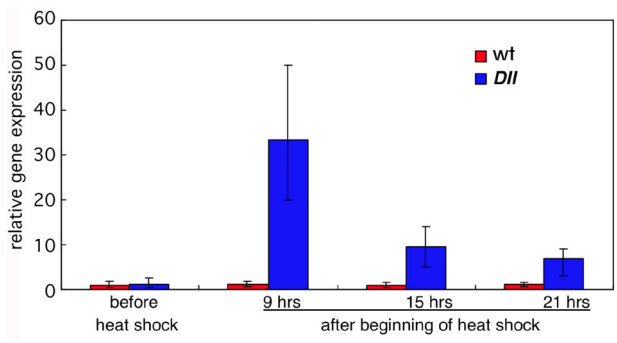

Figure 4. *Dll* mRNA levels are raised in Dll transgenic larvae, but not in Wt larvae, after a heat-shock. Real-time PCR quantification of *Dll* levels in both Dll and Wt larvae, before and at several time points after the beginning of a 3 hr heat-shock at 39°C. Relative quantification in $2^{-\Delta\Delta C_T}$ indicates the levels of *Dll* transcript normalized to the internal standard 18S rRNA. The error bars indicate the range of minimum and maximum of four biological replicates.

relative to non-heat-shocked animals from the same line ($F_{1,\ 55} < 0.001$, $P = 0.991$). Larval heat shocks, however, decreased the relative size of the dorsal Cu1 eyespot in the Dll-RNAi line but did not alter eyespot size in Wt individuals (Fig. 3E; line × treatment: $F_{1,\ 366} = 5.429$, $P = 0.020$). Dll knock-downs, thus, led to reductions in eyespot size, as predicted, but contrary to predictions they did not alter eyespot number. We conclude that these experiments support a role for *Dll* as a positive regulator of eyespot size during the larval stages, but *Dll*'s necessity in regulating eyespot presence or absence on the wing may only be uncovered once stronger reductions in Dll levels through multiple heat-shocks are achieved. These future experiments also need to document complete elimination of Dll protein levels, something we did not confirm due to the untimely extinction of this line.

Ectopic Expression of *Dll* Via Localized Laser Heat-Shocks Led to Ectopic Black Scales and Changes in Eyespot Shape

In the pupal stages of wing development Dll and Sal protein expression domains map to the black scales in the adult eyespot, whereas the En domain maps to the gold scales (Brunetti et al., 2001). We decided to test whether ectopic expression of any of these genes in a small patch of cells on the pupal wing was sufficient to activate the differentiation of black and/or gold scales. We subjected young pupae to local pulses of heat induced by an infrared laser. Pulses of 500–100 msec led to a clot of denatured proteins in the pupal wing visible immediately after treatment or upon pupal wing dissections (six individuals; Fig. 5A, B). Pulses of 50 msec led to visible cell mortality in the developing pupal wing, corresponding approximately to the shape of the laser beam (15 out of 16 treated wings showed rectangular-shaped patches of dead auto-fluorescent cells 24 hr after laser treatment; Fig. 5C). Pulses of 25 msec showed no evidence of cell mortality in the pupal wing 24 hr after laser treatment (46 out of 46 dissected individuals; not shown). We used this pulse duration (25 msec) for the rest of our experiments.

From the 88 Wt pupae treated that were reared to adulthood, one displayed a small patch of around 10 ectopic gold scales, and one had a smaller eyespot relative to the size of the corresponding eyespot on the untreated wing (not shown). From the 40 En animals treated none showed any wing pattern alteration. From the 67 Sal animals treated, one displayed a very large pattern aberration on the treated wing that we assumed was not due to the laser due to the extent of the affected region, and one displayed some minor wing damage with no ectopic scales. From the 182 Dll animals treated, five displayed patches of ectopic black scales (Fig. 5D,E), five displayed changes in eyespot shape where the eyespot area was enlarged and bulging in a particular dimension relative to no such enlargement in the untreated wing (Fig. 5F), two displayed enlarged eyespots in a symmetrical radial way (relative to the untreated wing), one had an extra eyespot on the M3 wing compartment (the untreated wing had none), one had ectopic gold and black scales around a laser-damaged wing area, and two displayed slight wing laser damage with no ectopic scales (Fig. 5E). The appearance of gold or gold and black scales simultaneously is consistent with the hypothesis that the laser damaged the wing epidermis in that particular individual resulting in the appearance of ectopic gold and black scales (Nijhout, '85; Brakefield and French, '95; Monteiro et al., 2006). This phenomenon is still not completely understood but the hypothesis is that the same growth factors that are also candidate morphogens for eyespot signaling are being produced around a site of epidermal damage (Monteiro et al., 2006). The appearance

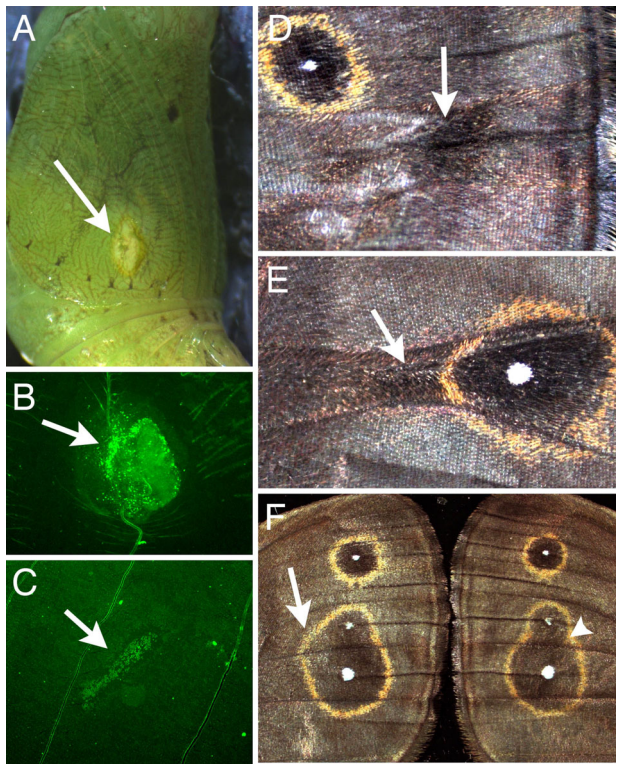

Figure 5. Ectopic activation of *Distal-less* via localized laser heat-shocks leads to ectopic black scales and eyespot deformations. Pulses of 500 msec duration produce a visible wing clot immediately after the 20 min heat-shock (A). Pulses of 100 msec also produce a clot visible upon wing dissection, 24 hr after the hs, and epidermal cell death (B) (cells auto-fluoresce upon blue light excitation). Pulses of 50 msec led to cell death in the pupal epidermis (C). Pulses of 25 msec led to no observable pupal wing damage (not shown) nor to adult wing damage in Wt (not shown) but led to patches of ectopic black scales (D, E) or to "bulges" (arrow) in the eyespots in the Dll-over expression line (F) relative to untreated eyespots on the control wing (arrow head). Individual F is a unusual Dll transgenic individual in that it also contains an extra eyespot on the dorsal forewing.

of black scales, without the simultaneous appearance of gold scales, is not commonly observed in "damage" experiments and suggests that *Dll*, but not the other two genes, is sufficient to activate the developmental program that produces black scales.

Our experiments show that *Dll* is a positive regulator of the eyespot developmental network. Raising *Dll* levels during the larval stage allows focal differentiation to be completed in compartments that normally arrest this process in Wt wings, and thereby alters eyespot number. Eyespot size also increases and decreases when *Dll* levels are elevated and depressed, respectively, during the larval stage. This suggests that genetic variation at *Dll*, previously implicated in the control of eyespot size via a linkage association study, is indeed likely partly responsible for eyespot size variation in *B. anynana* (Monteiro et al., '94; Beldade et al., 2002).

Effects of Dll manipulations via whole-body heat-shock on eyespot number and size were subtle and did not affect all wing compartments in the same degree. There may be several explanations for these results. It is possible that the single 2-hr heat-shock approach adopted in this study was insufficient to produce larger phenotypic effects. Although there is a 30-fold change in Dll mRNA levels (across the whole larvae) immediately following the heat-shock, levels fall rapidly within a 24-hr period. Elevating or knocking-down Dll levels at a single time point during the 5th instar (that lasts around 8 days) may or may not have coincided with a critical period for Dll function in focal differentiation. In addition, Dll expression varies naturally across wing compartments in Wt wings in the area of the focus (Additional File 2). Elevating Dll levels by means of a global heat-shock primarily affected eyespot focus differentiation and eyespot size in those wing compartments that already had the highest levels of Dll expression (M1, M2, M3, and Cu1). It is likely that focal differentiation and eyespot size determination are part of the same process: focal differentiation requiring passing an initial threshold level of gene expression, and eyespot size determination correlating with levels of gene expression beyond this threshold. For instance, artificial selection experiments for eyespot size inevitably lead to increases in eyespot number (Monteiro et al., '94; Beldade and Brakefield, 2003) where the extra eyespots occur primarily in wing compartments M2 and M3 (Beldade and Brakefield, 2003). It is unclear at this point, however, whether Dll over-expression, in higher levels or applied in a more continuous fashion, would be sufficient to initiate the differentiation of a complete eyespot outside of the pre-patterned focal areas of the wing. Our results, so far, suggest that a combination of genes need to be expressed simultaneously for focal differentiation to take place, but raising Dll levels in a pre-patterned area of the wing, containing already significant amounts of the other three genes studied here and/or expression of genes not yet discovered, is sufficient to differentiate additional foci and to increase the size of eyespots.

Our laser-heat shock results suggest, in addition, that *Dll* is a strong candidate for being a selector gene for wing melanization. Isolated black patches of scales as well as pattern bulges associated

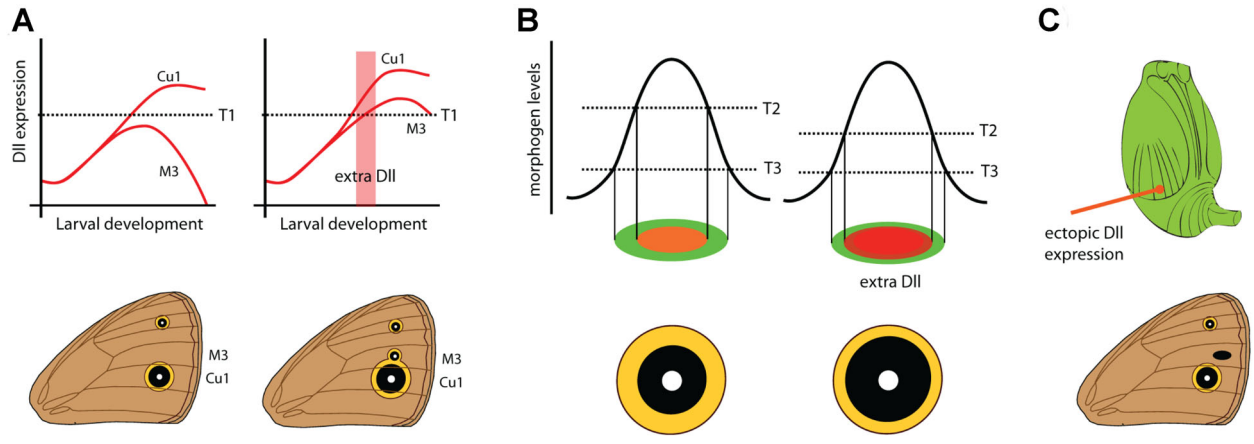

Figure 6. Summary of results and hypothetical mechanisms of Distal-less over- and ectopic expression on larval and pupal wings. (A) In Wt larval wings (left), Dll protein expression in the foci is dynamic over time and varies with wing compartment (e.g., Cu1 and M3). Eyespot centers (foci) become established when Dll levels reach a certain threshold (T1). Foci that don't reach that threshold (e.g., M3 foci) don't complete differentiation, and no eyespot develops in those compartments (bottom row). In the Dll over-expression line (right), increased Dll levels during larval development allow the M3 foci to complete differentiation and potentially make the Cu1 foci more "potent", leading to additional and larger eyespots on the adult wing. (B) In the early pupal stages of development, during focal signaling, additional Dll protein provided via a heat-shock may shift the threshold that determines the boundary between Dll-expressing central cells (red disc) and Engrailed-expressing outer cells (green disc), leading to "blacker" eyespots. (C) Dll ectopic expression during the early pupal stage, via a laser heat-shock, is able to induce black scales on the adult wing, irrespective of focal signaling.

to preexistent eyespots formed in a small percentage of Dll transgenic individuals treated with the laser. While our data are too preliminary to propose a mechanism for these pattern bulges, the isolated black patches do suggest that Dll expression during the pupal stage is sufficient to initiate black scale differentiation. The lack of consistent effects in all animals treated could result from non-optimized laser heat-shocking conditions, the precise timing of the 20-min treatment, or from the mix of Dll heterozygotes and homozygotes used for these experiments, among other factors. Recently, the morphogen Wingless was shown to be sufficient to initiate black pigment production at pre-patterned areas of a *Drosophila* wing (Werner et al., 2010). Wingless is also expressed in the center of *B. anynana* eyespots (Monteiro et al., 2006), before Dll is expressed in the area that maps to the black scales (Monteiro et al., 2006). In the future it will be exciting to discover whether both Dll and Wg are involved in the same pigmentation network in each of these species and, if yes, whether these genes regulate each other.

We were unable to confirm with either qPCR or immunohistochemistry that either En or Sal were being properly expressed following a heat-shock due to the untimely extinction of these transgenic lines. Therefore we cannot conclusively dismiss these genes in playing a role in eyespot development or scale color differentiation. Future replication of these experiments needs to be performed, whereupon the data presented here can be reexamined. Multiple heat-shocks can be attempted in an effort to test whether insufficient levels, or insufficient duration of the over-expression lead to an absence of phenotypes with these genes. In addition, once the Sal gene is sequenced from *Bicyclus*, this copy should be tested instead of the current *Drosophila* copy in the event that functional sequence differences between the two copies may have led to the absence of phenotypic effects in *Bicyclus*.

CONCLUSIONS

Transgenic work in butterflies is in its infancy, and here we report the first functional data obtained for an emerging model butterfly species. The phenotypic and gene expression data obtained for Dll support a functional role for this gene in eyespot development, in promoting focal differentiation, eyespot size, and eyespot color composition (Fig. 6). More limited ectopic expression data also suggest a role for this gene in promoting the differentiation of black scales (Fig. 6). Future replicate experiments with this gene, and other genes associated with eyespots will be necessary to continue to elucidate the role that these genes play in the development and origin of these novel traits. In particular, direct visualization of Dll, Sal, and En proteins after a single or multiple whole body heat-shock, and/or local laser heat-shock, would help elucidate the precise mechanisms that give rise to the phenotypes presented in this study.

We conclude that Dll appears to have been recruited from its ancestral roles in central nervous system and ventral limb development (Panganiban and Rubenstein, 2002) to two new roles in eyespot patterning and in scale melanization on butterfly wings. Phenotypic data and qPCR data for Dll over-expression (but not down-regulation) support these conclusions. Future experiments with novel transgenic lines and direct measurements of transgenic protein levels in developing wings, would be welcome to confirm these results.

ACKNOWLEDGMENTS

We thank Dennis Pietras and Robert Rak for growing corn for hungry larvae. This work was supported by NSF IBN 0316283 and IOB 0653399 awards to A.M. Each author contributed to this study as follows: A.M. designed and coordinated the study, produced the Dll over-expression and Dll-RNAi lines, collected and analyzed the Dll heat-shock data and the laser heat-shock data for all three genes, and drafted the manuscript. B.C. designed and constructed the Dll plasmid vectors, helped produce the Dll lines, collected and analyzed the qPCR data, and helped draft the manuscript. D.R. produced the En and Sal plasmid vectors and the correspondent transgenic lines, collected and analyzed the data for these lines, and helped draft the manuscript. J.C.O. collected all the Spotty and Wt comparative gene expression data and helped draft the manuscript. X.T. helped collect the qPCR data. M.G. helped collect the Dll over-expression data. L.F. helped collect the En data. W.K. W. helped collect the Dll-RNAi data and some of the laser heat-shock data. F.K. designed the laser-heat-shock system. All authors read and approved the final manuscript.

LITERATURE CITED

Abzhanov A, Protas M, Grant BR, Grant PR, Tabin CJ. 2004. Bmp4 and morphological variation of beaks in Darwin's finches. Science 305:1462–1465.

Beldade P, Brakefield PM. 2003. Concerted evolution and developmental integration in modular butterfly wing patterns. Evol Dev 5:169–179.

Beldade P, Brakefield PM, Long AD. 2002. Contribution of Distal-less to quantitative variation in butterfly eyespots. Nature 415:315–317.

Beldade P, Brakefield PM, Long AD. 2005. Generating phenotypic variation: prospects from "evo-devo" research on *Bicyclus anynana* wing patterns. Evol Dev 7:101–107.

Brakefield PM. 1996. Seasonal polyphenism in butterflies and natural selection. Trends Ecol Evol 11:275–277.

Brakefield PM, French V. 1995. Eyespot development on butterfly wings: the epidermal response to damage. Dev Biol 168:98–111.

Brakefield PM, Reitsma N. 1991. Phenotypic plasticity, seasonal climate and the population biology of *Bicyclus* butterflies (Satyridae) in Malawi. Ecol Entomol 16:291–303.

Brakefield PM, Gates J, Keys D, et al. 1996. Development, plasticity and evolution of butterfly eyespot patterns. Nature 384:236–242.

Brunetti CR, Selegue JE, Monteiro A, et al. 2001. The generation and diversification of butterfly eyespot color patterns. Curr Biol 11:1578–1585.

Carroll SB, Gates J, Keys DN, et al. 1994. Pattern formation and eyespot determination in butterfly wings. Science 265:109–114.

Carroll SB, D WS, A LJ. 1995. Homeotic genes and the regulation and evolution of insect wing number. Nature 375:58–61.

Chen B, Hrycaj S, Schinko JB, et al. 2011. Pogostick: a new versatile piggyBac vector for inducible gene over-expression and down-regulation in emerging model systems. PLoS ONE 6:e18659.

de Celis JF, Barrio R, Kafatos FC. 1999. Regulation of spalt/splat-related gene complex and its function during sensory organ development in the Drosophila thorax. Development 126:2653–2662.

Keys DN, Lewis DL, Selegue JE, et al. 1999. Recruitment of a hedgehog regulatory circuit in butterfly eyespot evolution. Science 283:532–534.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408.

Marcus JM, Ramos DM, Monteiro A. 2004. Germ line transformation of the butterfly *Bicyclus anynana*. Proc R Soc B 271:S263–S265.

Monteiro A, Podlaha O. 2009. Wings, horns, and butterfly eyespots: how do complex traits evolve? PLoS Biol 7(2):e1000037.

Monteiro AF, Brakefield PM, French V. 1994. The evolutionary genetics and developmental basis of wing pattern variation in the butterfly *Bicyclus anynana*. Evolution 48:1147–1157.

Monteiro A, Prijs J, Bax M, Hakkaart T, Brakefield PM. 2003. Mutants highlight the modular control of butterfly eyespot patterns. Evol Dev 5:180–187.

Monteiro A, Glaser G, Stockslagger S, Glansdorp N, Ramos DM. 2006. Comparative insights into questions of lepidopteran wing pattern homology. BMC Dev Biol 6:52.

Monteiro A, Chen B, Scott L, et al. 2007. The combined effect of two mutations that alter serially homologous color pattern elements on the fore and hindwings of a butterfly. BMC Dev Biol 8:22.

Nijhout HF. 1985. Cautery-induced colour patterns in *Precis coenia* (Lepidoptera: Nymphalidae). J Embryol Exp Morphol 86:191–203.

Oliver JC, Monteiro A. 2010. On the evolution of sexual dimorphism in butterflies. Proc R Soc B 278:1981–1988.

Oliver JC, Robertson KA, Monteiro A. 2009. Accomodating natural and sexual selection in butterfly wing pattern evolution. Proc R Soc B 276:2369–2375.

Panganiban G, Rubenstein JLR. 2002. Developmental functions of the Distal-less/Dlx homeobox genes. Development 129:4371–4386.

Ramos DM, Kamal F, Wimmer EA, Cartwright AN, Monteiro A. 2006. Temporal and spatial control of transgene expresson using laser induction of the *hsp70* promoter. BMC Dev Biol 6:55.

Reed RD, Serfas MS. 2004. Butterfly wing pattern evolution is associated with changes in a Notch/Distal-less temporal pattern formation process. Curr Biol 14:1159–1166.

Reed RD, Chen P-H, Nijhout HF. 2007. Cryptic variation in butterfly eyespot development: the importance of sample size in gene expression studies. Evol Dev 9:2–9.

Shapiro MD, Marks ME, Peichel CL, et al. 2006. Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. Nature 428:717–723.

True JR, Carroll SB. 2002. Gene co-option in physiological and morphological evolution. Annu Rev Cell Dev Biol 18:53–80.

Werner T, Koshikawa S, Williams TM, Carroll SB. 2010. Generation of a novel wing colour pattern by the Wingless morphogen. Nature 464:1143–1148.

Windig JJ. 1994. Reaction norms and the genetic-basis of phenotypic plasticity in the wing pattern of the butterfly *Bicyclus anynana*. J Evol Biol 7:665–695.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Additional File 1. Schematic of vectors used in this study. All vectors are based on the transposable element *piggyBac* and include the *3xP3-EGFP* marker cassette, and the heat-shock inducible promoter from *Drosophila Hsp70* placed 5' of the inducible transgene. (A) Dll over-expression vector; (B) En-overexpression vector; (C) Sal-overexpression vector; (D) Dll-RNAi vector. The ampicillin resistance gene (amp) and plasmid origin of replication (ori), as well as the *Drosophila* white intron (in) are also included inside the piggyBac left and right arms in this last vector.

Additional File 2. Temporal dynamics of gene expression in Wild type and Spotty *B. anynana* forewings. Each row corresponds to one of eight wing compartments. In the M1 and Cu1 compartments, eyespots develop in both Wt and Spotty genotypes (solid rectangles); in the M2 and M3 compartments, eyespots only develop in the Spotty genotype (dashed rectangles).

Additional File 3. Adult wild type and Spotty forewings. Nomenclature for the eight wing compartments analyzed in Additional file 2.

Additional File 4. Number of transgenic and Wild type individuals used in the heat-shock experiments at different stages of development. Temp, temperature (in °C) the larvae were reared at; Stage, stage of development when the heat-shock was applied; Hs, heat-shocked animals; C, control animals; mal, males; fem, females.

Additional File 5. A 2 hr heat-shock raises protein levels in *B. anynana* larval forewings. Larval forewings of wild type (Wt) and Ultrabithorax (Ubx) over-expression transgenic lines are stained for Ubx protein expression (normally no protein is found in forewings) in control and heat-shocked individuals. (A) Wt control. (B) Wt wings following a heat-shock. (C) Ubx control. (D) Ubx wings following a heat-shock. (E) Ubx heat-shocked (hs) individuals have the brightest wings. Error bars are 95% CI of the mean brightness values. n = numbers of wings analyzed.