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Evolution of Developmental Control Mechanisms

Over-expression of *Ultrabithorax* alters embryonic body plan and wing patterns in the butterfly *Bicyclus anynana*

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ABSTRACT

In insects, forewings and hindwings usually have different shapes, sizes, and color patterns. A variety of RNAi experiments across insect species have shown that the hox gene *Ultrabithorax* (*Ubx*) is necessary to promote hindwing identity. However, it remains unclear whether *Ubx* is sufficient to confer hindwing fate to forewings across insects. Here, we address this question by over-expressing *Ubx* in the butterfly *Bicyclus anynana* using a heat-shock promoter. *Ubx* whole-body over-expression during embryonic and larvae development led to body plan changes in larvae but to mere quantitative changes to adult morphology, respectively. Embryonic heat-shocks led to fused segments, loss of thoracic and abdominal limbs, and transformation of head limbs to larger appendages. Larval heat-shocks led to reduced eyespot size in the expected homeotic direction, but neither additional eyespots nor wing shape changes were observed in forewings as expected of a homeotic transformation. Interestingly, *Ubx* was found to be expressed in a novel, non-characteristic domain – in the hindwing eyespot centers. Furthermore, ectopic expression of *Ubx* on the pupal wing activated the eyespot-associated genes *spalt* and *Distal-less*, known to be directly repressed by *Ubx* in the fly's haltere and leg primordia, respectively, and led to the differentiation of black wing scales. These results suggest that *Ubx* has been co-opted into a novel eyespot gene regulatory network, and that it is capable of activating black pigmentation in butterflies.

Introduction

Insects typically have two pairs of wings that differ in size, shape, or coloration, and these differences are usually attributed to the hox protein Ultrabithorax (Ubx). Ubx is normally expressed in posterior wings during the larval stage (Akam and Martinez-Arias, 1985; Warren et al., 1994), whereas the anterior pair of wings usually does not express any homeotic gene during its development (Carroll et al., 1995). Differences in the morphology of anterior and posterior wings are usually attributed to the way Ubx interacts with multiple wing regulatory network genes to alter network output in the hindwing (Pavlopoulos and Akam, 2011; Tomoyasu et al., 2005; Weatherbee et al., 1999).

Ubx function has been explored to different degrees in a variety of insect species. Removing Ubx function in insect hindwings usually converts the identity of these appendages into that of the forewing (Lewis, 1978; Tomoyasu et al., 2005; Weatherbee et al., 1999) suggesting that Ubx function is necessary to promote hindwing identity. Fewer experiments, however, have tested whether Ubx is sufficient to alter the "hox-free" forewing into hindwing identity. Here the evidence is confined to experiments in *Drosophila* (Castelli-Gair et al., 1990; Pavlopoulos and Akam, 2011), and the butterfly *Junonia coenia* (Lewis et al., 1999). Ectopic expression of Ubx in *Drosophila* forewings led to complete wing-to-haltere transformations (Castelli-Gair et al., 1990; Pavlopoulos and Akam, 2011), suggesting that Ubx expression is sufficient to confer hindwing identity to forewings in flies. In *Junonia*, however, the experiments were less conclusive. Strong ectopic mosaic Ubx expression on the forewing imaginal disc, using a sindbis viral promotor, transformed certain forewing traits, such as scale

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morphology and pigmentation, to hindwing identity in the adult (Lewis et al., 1999), but wing shape was unaltered. However, because only mosaic expression was achieved at ectopic levels that were significantly higher relative to endogenous hindwing Ubx levels, it remains unclear whether uniform Ubx forewing expression, at normal endogenous hindwing levels, is sufficient to produce a complete homeotic transformation of forewing to hindwing identity in *Junonia* or in other butterflies.

Here we test Ubx's sufficiency in transforming a forewing into a hindwing in the butterfly *Bicyclus anynana* using novel transgenic tools. This butterfly, unlike *J. coenia*, has fewer eyespots on the forewing (two) relative to the hindwing (seven), and eyespot number can be used as an extra marker for detecting a homeotic transformation. Unlike *J. coenia*, however, *B. anynana* expresses the hox protein Antennapedia (Antp) in the centers of each eyespot on both forewings and hindwings (Saenko et al., 2011). The forewing is, thus, not strictly speaking a hox-free tissue in this species, and these experiments also allow us to investigate how the two hox genes interact on the wing.

We generated transgenic lines of *B. anynana* carrying the complete coding sequence of *Ubx* from *J. coenia* under the control of a heat-shock promoter (Chen et al., 2011) and over-expressed *Ubx* throughout embryonic and larval development using multiple heat-shocks. We also ectopically expressed *Ubx* on the early pupal wing by means of an infra-red laser beam (to provide a spatially restricted heat-shock) and investigated the response of candidate direct target genes, *Distal-less* and *spalt*. Finally, we cloned and described additional Ubx isoforms that are present in *B. anynana*, but absent in *J. coenia*. These experiments suggest that while the *Ubx* sequence tested is not sufficient to confer hindwing identity to forewings in *B. anynana*, it can activate a gene regulatory network involved in black pigmentation.

Materials and methods

Making the Ubx over-expression transgenic line

A fragment of 762 bp of the *J. coenia Ubx* cDNA (AY074760.1) (a gift from Sean Carroll) containing the entire open reading frame was cloned into the *Pogostick* plasmid (Chen et al., 2011), a *piggyBac* based vector that drives transgene expression via a heat-shock. A mixture of the recombinant plasmid (*Pogostick-JcUbx-up1*) carrying the desired insert and a helper plasmid (pHsp82PBac) carrying a *piggyBac* transposase sequence (Horn et al., 2002) was injected into *B. anynana* embryos within 2 h after egg-laying. Positive offspring were identified by the presence of EGFP fluorescence in their eyes. Integration of *Pogostick-JcUbx-up1* into the genome of *B. anynana* was confirmed by sequencing the genomic flanking regions to the piggyBac insertion using Thermal asymmetric interlaced PCR (TAIL-PCR) (Liu and Whittier, 1995). More details are in S1 Materials and Methods.

Whole-body heat-shocks and laser ectopic heat-shocks

Ubx transgenics and wild-type butterflies were raised in a climate room at 27 °C with a 12:12 h light:dark cycle and 80% relative humidity. In a previous limited characterization of the Ubx transgenic line we discovered that Ubx mRNA levels were significantly reduced 5 h after the end of a single heat-shock (Chen et al., 2011), so multiple heat-shocks were used to assure a high level of ectopic Ubx expression in the following experiments.

Embryonic heat-shocks

Two hours after egg laying (AEL) embryos of the Ubx over-expression line and Wt were collected and given four heat-shock pulses, each consisting of a 1.5 h heat shock at 39 °C, followed by a 6.5 h period at 27 °C. The embryos either completed embryogenesis at 27 °C or were used for immunostainings.

Larval heat-shocks

Forewing and hindwing eyespot-specific gene expression patterns appear during the middle (stage 2.5) of the fifth larval instar (Oliver et al., 2012), so we monitored 4th instar larvae (Ubx and Wt) daily until they molted to the final instar. These larvae were then given four heat-shocks per day at 39 °C, each 1.5 h in duration, separated by 4.5 h intervals at 27 °C, until the pre-pupal stage. Pre-pupae were transferred to 27 °C and reared until adult emergence. Ubx and Wt larvae were treated in parallel throughout these experiments: they were set up in approximate equal numbers in the same heat shock incubator, at the same time. Controls, i.e., non-heat-shocked individuals from the same generation from each line, were reared at 27 °C throughout. Adults were sacrificed by freezing upon emergence.

Laser heat-shocks

We used an infra-red laser heat shock system, similar to the green laser system described in (Ramos et al., 2006), to ectopically express Ubx in a small cluster of cells on the dorsal surface of the pupal forewing. Pupation time was scored by time-lapse photography using a Kodak DC290 digital camera. 14–21 h old pupae were treated with the laser. We used infrared heat pulses of 25 ms, separated by 400 ms intervals, during 20 min. After heat-shock, pupae were placed inside a small cup at room temperature until adult emergence, or until dissected for immunostainings.

Real-time PCR and immunostainings

Transcript levels of ectopic *JcUbx* and endogenous *B. anynana Ubx* (*BaUbx*) before and after heat-shock were quantified by real-time PCR. After 5 days of multiple heat-shocks, the forewing and hindwing discs of 5th instar larvae (Ubx and Wt), as well as non-heat shocked controls, were dissected for RNA isolation 1–2 h after the final heat-shock. Total RNA was extracted using an RNeasy Micro kit (Qiagen) and reverse-transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied biosystems). Real-time q-PCR was performed with TaqMan Universal PCR Master Mix using the Applied Biosystems 7500 Fast Real-Time PCR System. Eukaryotic 18S rRNA was used as the endogenous control. Relative quantification of *Ubx* transcripts was obtained using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). *Ubx* levels were quantified using a *Ubx* (*Ubx*_F: GCG GAG GAG ACG TAT AGA AAT GG, *Ubx*_R: GGC GGT TTT GGA ACC ATA TTT TGA T, *Ubx*_Probe: CAC GCG CTC TGT CTC A) probe, which can amplify both *BaUbx* and *JcUbx* transcripts.

Expression of *Ubx*, *Antp*, *Dll* and *spalt* was assessed using immunostainings in embryos and wings from heat-shocked as well as control animals following the protocol of Brunetti et al. (2001). We used a rabbit anti-*J. coenia* Ubx antibody (at 1:500; a gift from L. Shashidhara), a mouse monoclonal anti-Antp 4C3 (at 1:200; Developmental Studies Hybridoma Bank), a rabbit polyclonal anti-Dll (at 1:200, a gift from Grace Boekhoff-Falk) and Guinea pig polyclonal anti-Spalt (at 1:20,000) antibody (Stoehr et al., 2013). The images were captured on a Nikon 90i microscope with NIS-Elements software (Nikon Instruments, Melville, NY, USA). More details are in S1 Materials and Methods.

Morphological analysis

First instar larvae that survived the heat-shock treatment (and some that were manually “hatched”) were photographed under a stereo microscope (Nikon SMZ1500), and multiple Z-sections were

processed with Helicon Focus. Differences in frequency of observed defects between heat-shocked Wt and Ubx larvae were tested using Chi-square tests. Adult wings were cut from the body and photographed. Wing patterns were scored and measured in Object Image 1.62 (http://www.simon.bio.uva.nl/object-image.html), including forewing eyespot number on both the ventral and dorsal sides, diameters of the white eyespot center, the inner black ring, and the outer gold ring of the M2 and Cu1 eyespots on ventral fore and hindwings, and the distance between the two forewing ventral eyespot centers, as a proxy for wing size. All eyespot diameter measurements were taken along an axis parallel to the wing veins. The data were analyzed in SPSS version 11. Analysis of covariance on eyespot size, using wing size as a covariate, was performed on the data with line (Ubx vs Wt) and treatment (heat-shock vs control) as fixed variables.

Cloning of Ubx from B. anynana

Total RNA was extracted from embryos, larval, and pupal wings at several developmental stages with an RNeasy Micro kit (Qiagen). cDNA was reverse-transcribed from total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied biosystems), and *Ubx* was amplified using degenerate primers 5′-ATG AAC TCC TAT TTC GAG CAG G-3′ and 5′-CTG CGC TTG CGC CTG TTT CTC-3′. Bands were purified using GEL Extraction Kit (Qiagen) and subsequently cloned with the TOPO TA Cloning® Kit (Invitrogen) before sequencing.

Results

Making the Ubx over-expression transgenic line

We used a novel piggyBac construct, *Pogostick-Ubx-up1* (Chen et al., 2011), to introduce an extra copy of *Ubx* into the germ-line of *B. anynana*. The construct carried the *Drosophila* promoter of *hsp70* driving the complete *Ubx* coding sequence from the butterfly *J. coenia* (*JcUbx*), the only butterfly *Ubx* sequence available at the beginning of this study. A single transgenic line was created with a single putative genomic insertion site (Chen et al., 2011). Evidence for construct integration was obtained by the presence of single novel genomic regions flanking the piggyBac arms of *Pogostick* (Supplementary Table 1). Every generation, transgenic offspring were screened on the basis of the EGFP marker protein expressed in the eyes. High levels of EGFP expression could be observed at each developmental stage (Supplementary Fig. 1). Positive individuals were collected over several generations for all the heat shock experiments described below.

Embryonic, larval, and pupal development of B. anynana

Embryonic development in *B. anynana* reared at 27 °C lasts 5 days, larval development takes around 15 days (the last, 5th instar takes around 5–6 days), and pupal development lasts 7 days. Work in *Bombyx mori* suggests that the wing discs are set aside during embryonic development but stay small and in an almost quiescent state during the first four larval stages (Kango-Singh et al., 2001). Wings grow gradually to about 2 mm in diameter during the 5th larval instar until the crawler and pre-pupal stages, during which time wings grow exponentially to attain a large size (7–8 mm in length from base to apex) at pupation. It is unclear when differences in fore and hindwing shape are established, but forewing and hindwing eyespot-specific gene expression patterns appear during the middle of the fifth larval instar, and wing shape is already different at this stage.

Over-expressing Ubx alters the embryonic body plan

B. anynana late embryos and larvae have three pairs of thoracic legs (T1–T3), no leg-like structures in abdominal segments A1 and A2, and four pairs of prolegs in the A3–A6 segments. Around 50% of Ubx heterozygotes (derived from crosses between Ubx individuals and Wt animals) and 90% of Wt embryos treated with multiple heat-shock pulses developed to hatch as first instar larvae. Around 13% of transgenic Ubx larvae exhibited profound morphological alterations after heat induction, with defects in the appendages and body wall of the head, thorax, and the abdomen (Fig. 1A–J). The largest frequency of defects (82%, $N=47$) ($\chi^2=38.83$, $p<0.001$) was seen in the thoracic segments, where fewer and smaller legs (Fig. 1B–D), abnormal placement of legs (Fig. 1E), fused segments (Fig. 1C–E) or fused limbs (Fig. 1D) developed. The next largest frequency of defects was seen in the head (11%, $N=6$) ($\chi^2=4.69$, $p=0.030$), where leg-like structures appeared in place of the mouth parts (Fig. 1G), head parts were missing or were unrecognizable (Fig. 1H), or the groove between the head and thorax was absent (Fig. 1G and H). We also observed fewer prolegs and abnormal placement of prolegs in the abdomen (7%, $N=4$) (Fig. 1J). In contrast, only 3 (out of 340, or $<1\%$) WT individuals exposed to HS showed any morphological changes. These changes consisted of fused segments in the abdominal region. This low frequency of abdomen fusions was identical between the two lines and is likely caused by the heat-shock ($\chi^2=0.002$, $p=0.934$).

We next confirmed that the defects primarily observed in the Ubx line were due to increased Ubx protein levels expressed across the embryo. Wt heat-shocked embryos showed the same Ubx expression pattern as non-heat shocked Ubx embryos: strong Ubx expression in the first segment of the abdomen (A1), weak expression in A2 and the posterior of the T3 segment (Fig. 1K). By contrast, in the Ubx line, ectopic Ubx was observed across the whole embryo at high levels (Fig. 1K). The above results indicate that the presence of the Ubx protein throughout the embryo may be leading to the homeotic-like transformations in the mouthparts, and changes to the number and position of limbs in the thorax and the abdomen.

Embryonic ectopic Ubx alters Distal-less expression

In order to explore how limb defects were produced we examined the expression of the limb selector protein, Distal-less (Dll). Dll was expressed in a typical pattern in the appendages of the head and thorax, and in vestigial embryonic abdominal appendages, the pleuropodia in abdominal segment A1, of control individuals (Fig. 1M and N), whereas abnormal Dll expression was observed in heat-shocked Ubx embryos. Dll was expressed at low levels in some limb primordiae (arrows in Fig. 1L, $n=3$) as compared with Wt (Fig. 1M), and this low expression was also visible during later limb extension stages (Fig. 1P, arrows, $n=7$). In some animals Dll was absent in presumptive thoracic limb-forming regions (arrowhead in Fig. 1O) and the limb was also absent.

Over-expression of Ubx reduces eyespots but does not transform forewings into hindwings

We next tested whether whole-body heat-shocks during the last instar of larval development were sufficient to produce a homeotic transformation of forewings into hindwings.

We first tested whether ectopic expression levels of *Ubx* mRNA and protein on the forewing were comparable to endogenous *Ubx* hindwing levels. The fore and hindwing discs of repeatedly heat-treated late 5th instar Ubx larvae expressed several fold higher *Ubx* mRNA levels than similarly treated Wt controls (Fig. 2B).

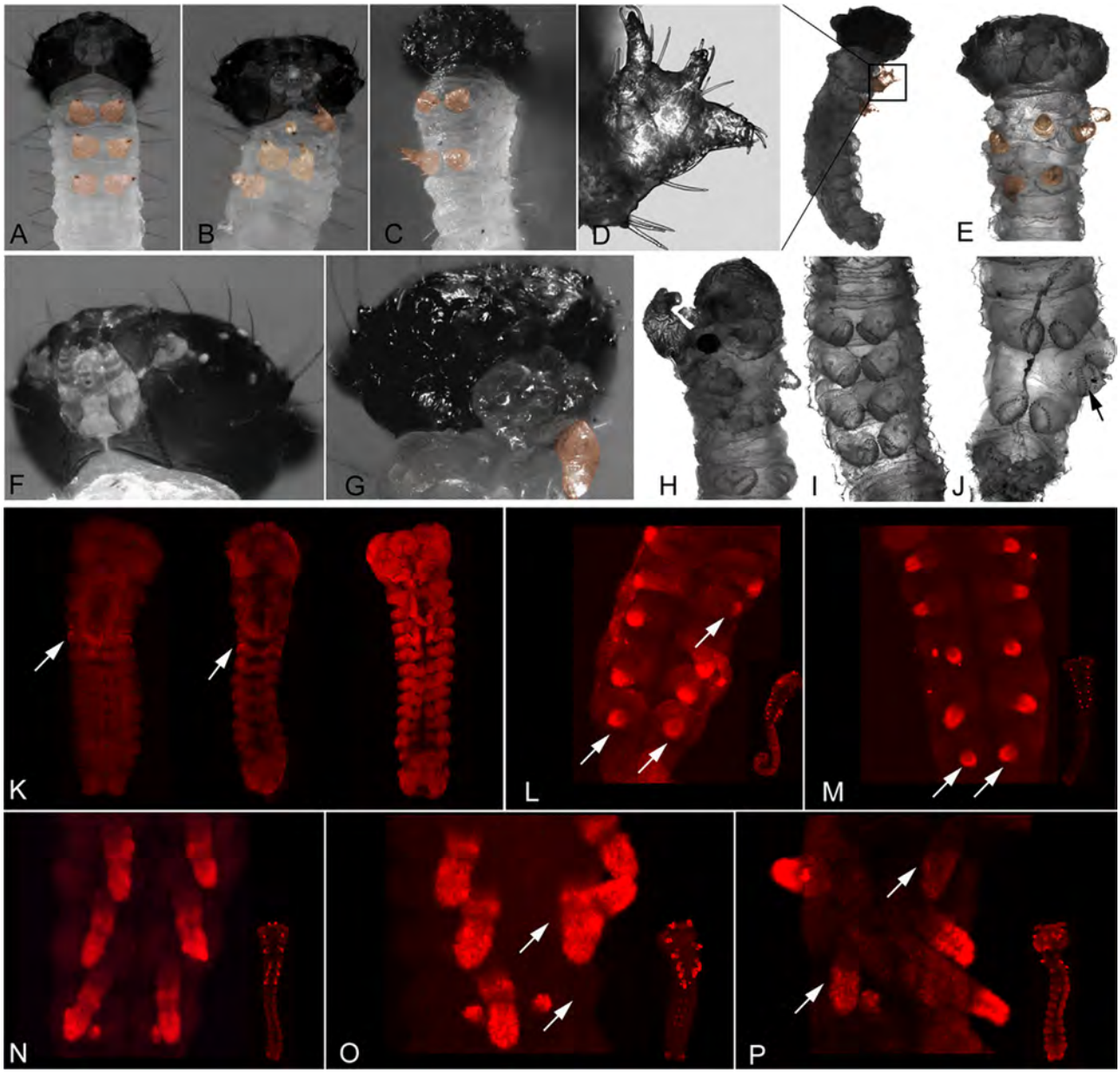

Fig. 1. Ubx over-expression during embryonic development causes a variety of phenotypes. (A) Wild type 1st instar larva shows normal thoracic leg patterning (pink): three pairs of thoracic legs with hooks. (B–E) Following heat-shock Ubx transgenic individuals display fewer legs, abnormal placement of legs, fused segments or fused limbs. (B) A single limb develops on the T3 segment and the other 4 limbs are disordered. (C and D) The embryo develops only two thoracic segments and two pairs of limbs, and one limb (D) has three claw-like structures at the end. (E) Ubx Embryo exhibits fused thorax segments and disordered limbs. Wild type mouth parts (F) are replaced by leg-like structure (pink) in the head (G). (H) Head parts were missing or were unrecognizable and the groove between the head and thorax was absent. (I) The Wt pattern of four pairs of abdominal prolegs is disordered and fewer prolegs develop in Ubx treated individual (J). (K) Wt treated embryos (middle) showed same Ubx expression pattern (arrows) as control embryos (left), whereas, high levels of ectopic Ubx was observed across the whole embryo in the treated Ubx line (right). (M–P) Dll expression patterns in Wt (M, N) and Ubx lines (L, O and P) after heat-shock (whole embryos images are shown in the right corner). Before the limbs have extended, lower level of Dll expression in some presumptive appendage-forming regions (arrows) could be detected in the Ubx line (L) compared with Wt (M). (O) Dll expression was missing from the right T3 limb (arrowhead) and abnormal Dll expression was observed in branched T2 limbs (arrow). (P) Ubx embryo with only five limbs showed both normal and lower levels of Dll expression in limbs (arrows).

Heat-shocked Ubx wings also had elevated Ubx protein relative to similarly treated Wt wings (Fig. 2C; forewing $F_{1,\ 22}=40.92$, $p<0.001$; hindwings $F_{1,\ 17}=23.45$, $p<0.001$). Expression levels in heat-treated Ubx forewings (mean wing brightness=57.8) were comparable to untreated hindwing controls (mean wing brightness=58.6) (Fig. 2B). Heat-shocks lowered Ubx protein levels in Wt hindwings relative to untreated Wt hindwings ($F_{1,\ 9}=8.449$, $p=0.023$) presumably because of the known repressive effects of heat-shock on ongoing gene transcription and translation (Maldonado-Codina et al., 1993; Pauli et al., 1992).

In addition to its uniform expression throughout the hindwing (of either Ubx or Wt animals), Ubx was also co-expressed with Antp in the hindwing eyespot foci in *B. anynana* during the late larval (5–6 days of the 5th instar) and early pupal stages (Fig. 3). Stainings using the FP6.87 monoclonal antibody, that targets both Ubx and abdominal-A, exhibited the same hindwing-restricted eyespot center expression pattern (Supplementary Fig. 2). We confirmed that this eyespot-specific expression was absent in *J. coenia*, as previously reported (Warren et al., 1994; Weatherbee et al., 1999) (Fig. 3).

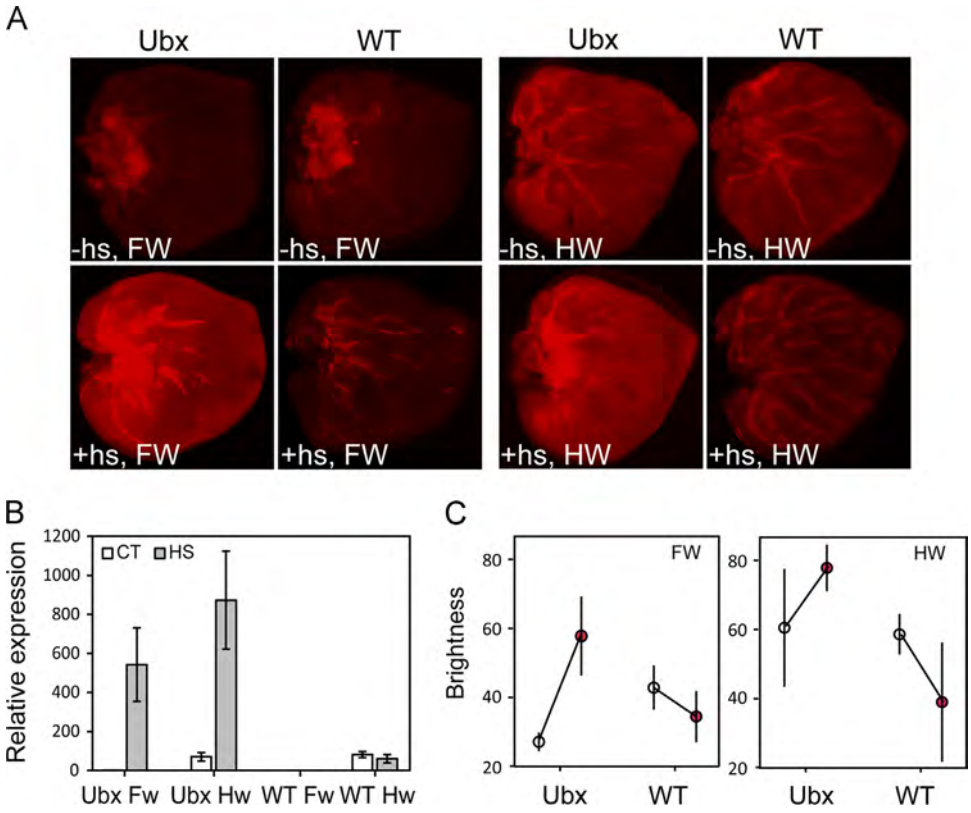

Fig. 2. Ubx is expressed at high levels in larval wings after whole-body heat shocks. (A) Forewings and hindwings photographed with the same exposure time. (B) Quantification of Ubx protein levels via measurements of wing brightness (heat-shocked data points correspond to red symbols). Error bars represent 95% CI. Note that Ubx levels in forewings (FW) in the Ubx line after heat-shock (+hs) are comparable to wildtype (WT) hindwing (HW) levels before a heat-shock (−hs). (C) Quantification of *Ubx* transcript levels in wings via Real-time PCR after 5 days of multiple heat-shocks.

Heat-shocks administered throughout the 5th larval instar produced no detectable changes in wing shape or eyespot number in Ubx transgenics relative to Wt controls. The heat-shock had a significant effect on reducing eyespot size in both Ubx and Wt controls, but there was also a significant interaction effect between treatment and line (Supplementary Table 2), indicating that eyespots of transgenic individuals, became disproportionately smaller relative to heat-shocked Wt individuals (Fig. 4). Hindwing ventral eyespots are on average smaller than their homologous eyespots on the forewing (Fig. 4D, left). These results indicate that Ubx has a repressive effect on eyespot development and Ubx over-expression further reduces eyespot size on both wing surfaces (Fig. 4D, right). We conclude that Ubx over-expression during the last larval stage alters eyespot size, but is not sufficient to alter forewing shape and eyespot number into hindwing identity.

In order to test whether an earlier onset of Ubx expression on the forewings would be sufficient to produce a more complete forewing to hindwing homeotic transformation, we subjected first instar larvae to three heat-shocks per day (four heat-shocks per day led to almost complete mortality) until the middle of the pupal stage. These experiments induced high levels of Ubx protein expression on the forewings of late 5th instar (even slightly above control hindwing levels, not shown), and led to no overt changes in wing shape or eyespot number (not shown). Finally, we subjected early embryos to two heat-shocks per day until larval hatching and observed the same lack of effect in the adult wing patterns. We conclude that Ubx ectopic expression either initiated at the earliest possible time during development (but sustained only during embryonic development), or initiated during the 1st instar (and maintained throughout larval development), is not sufficient to confer hindwing identity to forewings.

Ectopic Ubx induced by laser heat shocks drove ectopic Distal-less and Spalt expression on the pupal wing and led to scale melanization

Infra-red laser induced heat-shocks (Ramos et al., 2006) were applied to the forewings of young pupae in order to explore the effect of ectopic clusters of Ubx expression on the adult wing. Wings expressed Ubx protein 3–4 h after the end of the heat-shock (Fig. 5B), but no such expression was observed in Wt-treated wings (Fig. 5A). None of the 38 Wt-treated animals displayed any wing pattern alteration aside from some missing scales on the wing (Fig. 5C). Conversely, three types of abnormal phenotypes appeared in the 46 Ubx-treated pupae. These included the appearance of an enlarged eyespot following targeting of non-focal (central signaling) cells (1 instance) (Fig. 5D); the appearance of patches of ectopic black scales around the lasered line of cells (7 instances) (Fig. 5E and F); and the appearance of a cluster of hair-like scales along the laser line, changing the identity of these scales into that of hindwing scales (1 instance) (Fig. 5G and H). In summary, ectopic expression of Ubx induced both homeotic transformations (hair scales), as well as novel phenotypes (black scales and enlarged eyespots) that were unexpected.

To examine how ectopic Ubx might have led to the differentiation of black scales, the most prevalent phenotype, we analyzed the expression of Dll and Spalt, proteins normally associated with black scales in *B. anynana* at this stage of wing development (Brunetti et al., 2001; Monteiro et al., 2006). Seven to ten hours following the laser heat-shock, Spalt protein was detected in cells along the laser line (Fig. 5I and J) whereas no ectopic Ubx protein could be detected at the same time (not shown). This suggests that the early Ubx expression is short-lived but is sufficient to activate *spalt*. Double staining with anti-Spalt and anti-Dll antibodies

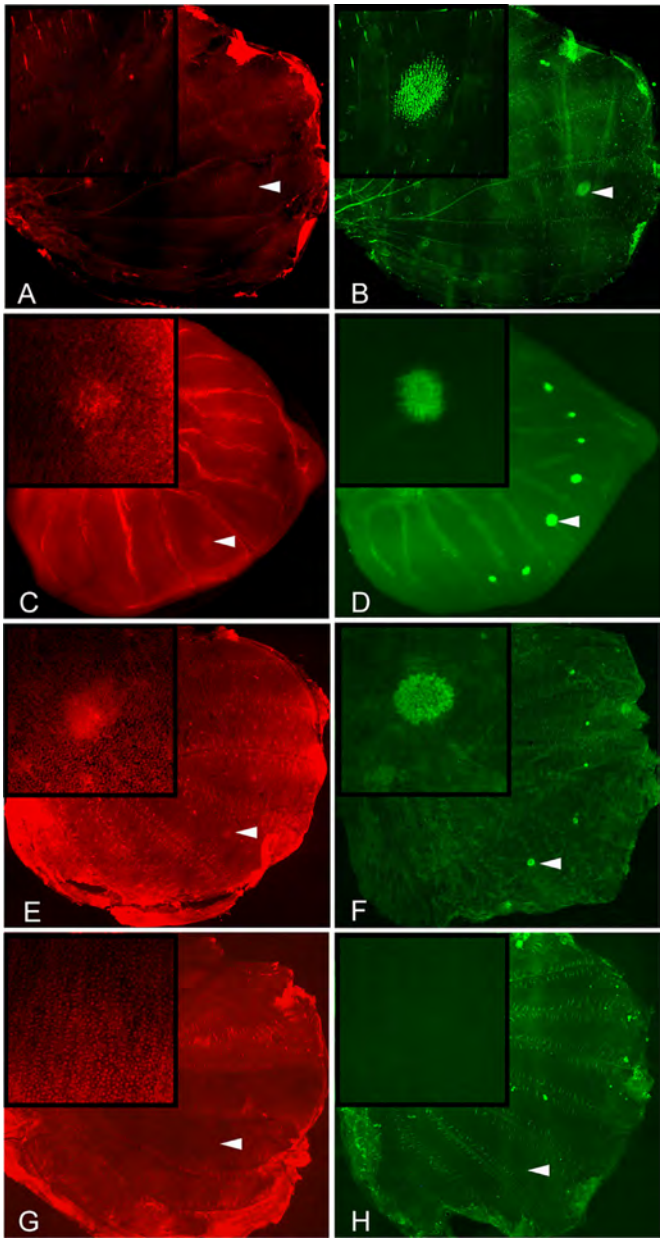

Fig. 3. Ubx and Antp are expressed in the hindwing eyespot centers of *B. anynana* but not *J. coenia*. Ubx (red) is absent from the eyespots centers in the pupal (and larval, not shown) forewings of *B. anynana* (A), while Antp (green) is present in those cells (B). Ubx and Antp are co-expressed in the eyespot centers of *B. anynana* hindwings in the late larval (C and D) and pupal (E and F) stages, but not in *J. coenia* pupal (and larval, not shown) hindwings (G and H). Arrowhead points to the Cu1 eyespot center shown at higher magnification in the left inset.

showed that Spalt and Dll were both present in approximately the same cells along the laser line ($n=3$) (Fig. 5K–M). From these results, we conclude that ectopic Ubx in the early pupal wing of *B. anynana* activates the targets *spalt* and *Dll* and leads to the differentiation of black scales.

Ubx has multiple post-embryonic isoforms in B. anynana

The above experiments indicate that ectopic JcUbx produces stronger phenotypes during embryonic development relative to post-embryonic development in *Bicyclus*. We wondered whether the lack of homeotic phenotypes post-embryonically could be due to the presence of multiple *Ubx* isoforms, as shown in *Drosophila* (Kornfeld et al., 1989; O'Connor et al., 1988), and to the insufficiency of a single isoform to transform forewings into hindwings in butterflies (but not in flies). In order to gather evidence for this hypothesis, we cloned *B. anynana Ubx* (*BaUbx*) from a collection of different tissues and developmental stages. We identified two *BaUbx* isoforms, *BaUbx-a* and *BaUbx-b*. The 703 bp fragment from *BaUbx-a* encodes 234 amino acids and shares 97% amino acid identity with JcUbx (Supplementary Fig. 3). The shorter *BaUbx-b* fragment has a 288 bp deletion but shares the same sequence with *BaUbx-a* outside the deleted region, suggesting that these two isoforms may be splice variants of the same gene.

To obtain expression profiles for each of the two *Ubx* isoforms, we performed RT-PCR on separate mRNA extracts from embryos, late larval wing discs, and pupal wing discs. *BaUbx-a* was expressed in both embryos and wing discs, whereas *BaUbx-b* was only expressed in wing discs (Fig. 6). To be able to compare our results with those obtained previously in *J. coenia*, where mosaic and strong ectopic expression of Ubx in the late larval forewing resulted in partial homeotic transformation into hindwings (Lewis et al., 1999), we examined whether multiple splice variants were also present in *J. coenia*. In contrast to *B. anynana*, only one isoform, homologous to *BaUbx-a*, was amplified in embryos and in larval and pupal wing discs covering various developmental stages.

Discussion

Ectopic expression of JcUbx, sharing 97% amino acid identity with BaUbx-a, in *B. anynana* produced a series of body plan transformations in both the larvae and adults. The larval body plan transformations, induced during embryonic development, were stronger and more numerous than those observed in the adult body, induced post-embryonically.

Embryonic phenotypes

Some of the embryonic/larval phenotypes we observed are similar to those observed in *Drosophila* (in similar heat-shock experiments) whereas others are different. Shared phenotypes include fused segments with fewer limbs in the thorax, ectopic limbs on the head, a deformed head, and abdominal defects (Gonzalez-Reyes and Morata, 1990; Lamka et al., 1992; Vachon et al., 1992). Distinct phenotypes include the expression of the limb selector gene Dll in the presence of ectopic Ubx in the thorax and abdomen, albeit at reduced levels in some limbs. We begin by discussing these distinct phenotypes.

The over-expression of Ubx in *Drosophila* using heat-shocks leads to the repression of *Dll* in the thorax via direct binding of Ubx to an early limb enhancer of *Dll* (Castelli-Gair and Akam, 1995; Vachon et al., 1992). Later in development, however, *Dll* expression is independent of Ubx. *Dll*'s own product binds a separate enhancer to maintain *Dll* expression (Castelli-Gair and Akam, 1995). So, in order for Ubx to repress limbs in *Drosophila*, Ubx ectopic expression has to occur early in development, before the beginning of *Dll*'s autocatalytic activity. This means heat-shocks have to be performed before the embryo is 3 h old (Castelli-Gair and Akam, 1995).

At present it is still unclear whether Ubx can repress thoracic limbs in lepidoptera, as it does in *Drosophila*. We begun heat-shocks 2 h after egg laying, 1 h earlier than similar experiments in *Drosophila*, which led to complete *Dll* repression in *Drosophila* (Castelli-Gair and Akam, 1995). We observed some embryos with reduced Dll expression in limb primordial, before limb extension, and some limbs missing altogether from thoracic segments. The loss of thoracic limbs in Ubx over-expression embryos may be due to a direct repressive effect of Ubx on *Dll* expression but also due to

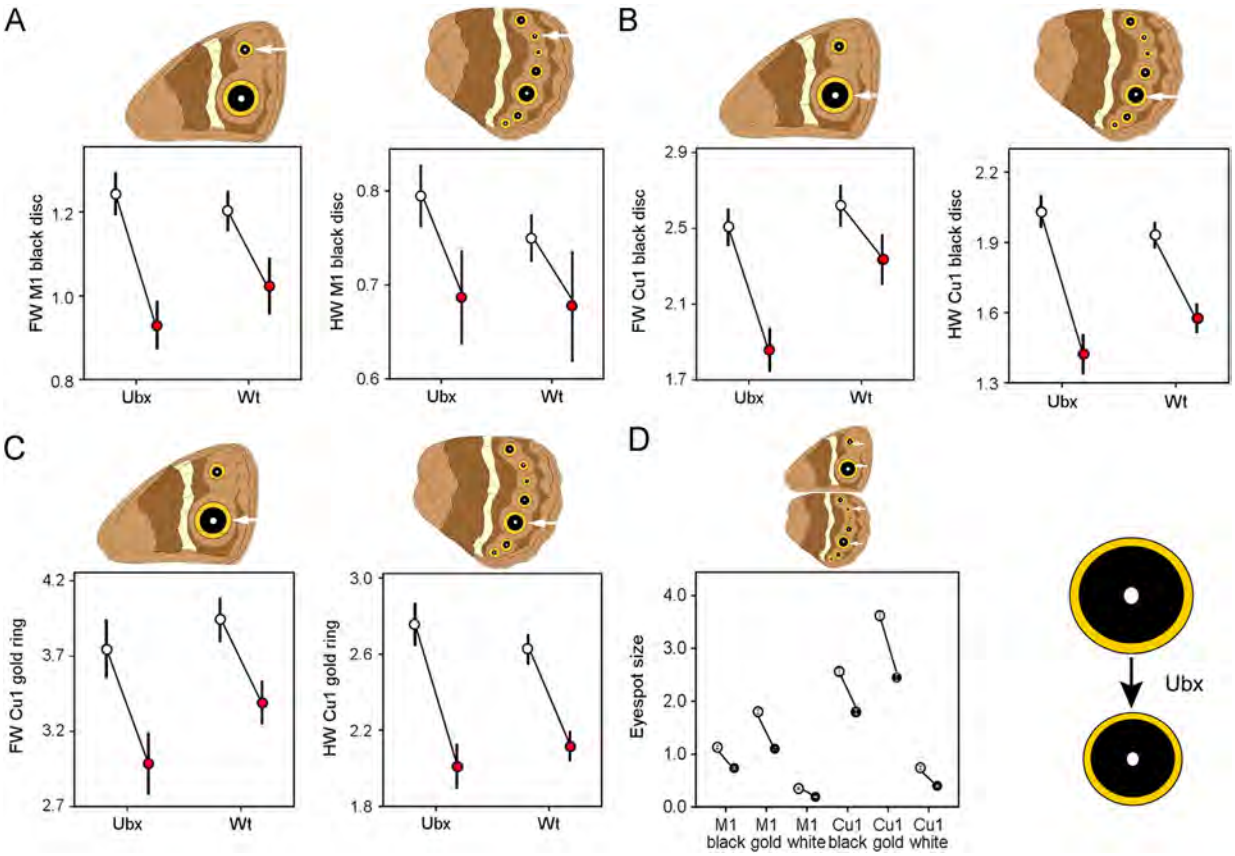

Fig. 4. Eyespot phenotypes of control and heat-shocked Ubx and Wt individuals during the 5th larval instar. Heat-shocked (red symbols) Ubx individuals showed significantly larger reductions in size relative to Wt heat-shocked or control non-heat-shocked individuals (open symbols). Diameter of the ventral M1 (A) eyespot black disc, Cu1 eyespot black disc (B) and gold ring (C) on the forewing (left) and hindwing (right), respectively. (D) Size comparison of orthologous eyespots on forewings and hindwings in Wt (open symbols: forewing; filled symbols: hindwing). Ubx has a repressive effect on eyespot size (D right). *Y*-axis represents corrected means for a particular wing size (from analyses of covariance on eyespot size using wing size as the covariate). Error bars represent 95% confidence intervals. *F* statistics, and *P*-values given in Supplementary Table 2.

the indirect effect of Ubx on segment fusions. The indirect effect is supported by the results in Fig. 1O, where fused segments/limbs are expressing Dll at high levels. In a small number ($n=3$) of Wt animals, the heat-shock alone led to the fusion of abdominal segments. This phenomenon was also observed in the locust (*Schistocerca gregaria*) and in *Drosophila* when the heat-shock was administered before the blastoderm stage (Mee and French, 1986; Welte et al., 1995). While it is possible that some of the fused segmentation patterns we observed in Ubx individuals could be due to the heat-shock alone, which was administered before the blastoderm stage, the large frequency of observed abnormalities seen in this line, as apposed to the Wt line, are likely due to the effect of Ubx over-expression.

Similar segment fusions to those observed in *B. anynana* following Ubx ectopic expression have been observed in studies that looked at the effect of mutations in two other hox genes. In silkworms, for instance, mutant embryos for *antennapedia* displayed fused thoracic segments (Chen et al., 2013), whereas *Tribolium castaneum*, *sex comb reduced* mutants exhibited fusions of the first thoracic segment (T1) with the head (Shippy et al., 2006). It appears, thus, that the correct expression of thoracic hox genes is necessary for proper segment development. The mechanism by which disruption of these genes leads to the segment fusions, however, is still unclear.

Adult phenotypes

The large differences in mRNA levels observed between heat-shocked and control larval wing discs were not paralleled at the level of Ubx protein expression (Fig. 2C). As ribosome number often limits how much protein can be produced irrespective of number of mRNA molecules in a cell, a linear relationship is not expected between mRNA levels and protein levels in cells (Concin et al., 2003; Maier et al., 2009). It was especially important, thus, that the heat-shock treatment applied led to comparable levels of Ubx protein in Wt hindwings and Ubx-line forewings since Ubx protein molecules are the functional entities that are involved in regulating down-stream targets. This allowed us to test whether the presence of Ubx protein in forewings was sufficient to produce a forewing to hindwing homeotic transformation.

Over-expression experiments performed during the larval stages produced different results from those obtained in *Drosophila* and *J. coenia*. These results may be related to the different number of *Ubx* isoforms detected in each of these species, the degree of functional equivalence between isoforms, the spatial domain of ectopic Ubx induction (uniform expression throughout the wing versus expression limited to cell clusters), the use of heterologous versus endogenous *Ubx* sequences, differences in overall levels of expression, or the requirement for presence of Ubx in forewings from the earliest embryonic stages all the way to adult emergence.

Drosophila has six different Ubx protein isoforms caused by alterative splicing (Ia, Ib, IIa, IIb, IVa and IVb) (O'Connor et al., 1988). Isoforms I and II are abundant in haltere discs, and a small amount of isoforms IV can also be detected in these discs. However, it is not clear what the relative expression levels of forms a and b are in these discs (de Navas et al., 2011). However, larval over-expression of a single isoform encoded by all exons, at

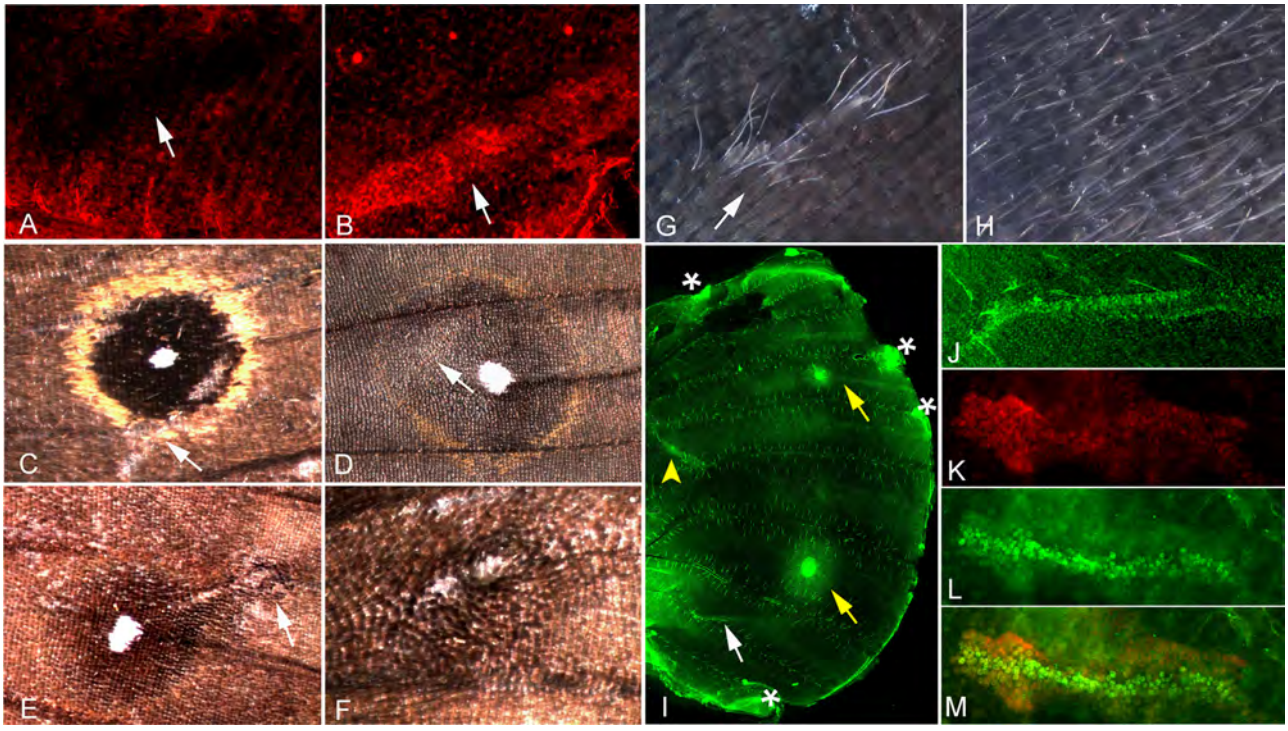

Fig. 5. Laser-induced ectopic expression of Ubx on the pupal forewing leads to various phenotypes. (A) No ectopic Ubx expression was observed in heat-shocked (hs) Wt wings, whereas ectopic Ubx protein was detected in Ubx transgenics, 3–4 h after the end of the hs (B). (C) Laser hs produced minor wing damage across an eyespot field in Wt individuals, whereas it led to an enlarged eyespot in Ubx transgenics (D). (E, F) Laser hs led to patches of ectopic black scales in the Ubx over-expression line, or to (G) a cluster of hair-like scales, characteristic of dorsal hindwings (H). (I–J) 7–10 h following the laser hs, Spalt was expressed in cells along the laser line (arrow) whereas no ectopic Ubx expression could be detected at the same time (data no shown). Yellow arrows mark additional Spalt expression domains in the eyespot centers, scale-building cells around the centers, and along the distal intervein regions. Yellow arrowhead marks the discal-cell cross-vein, whereas asterisks mark staining artefacts. (K–M) A different wing shows co-expression of Dll (K) and Spalt (L) along the laser line, observed only in Ubx transgenics, but not Wt wings. (M) Merged channels.

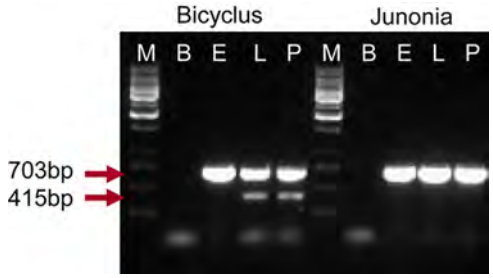

Fig. 6. Two *Ubx* isoforms are present in *B. anynana* but only one isoform was found in *J. coenia*. Gel-separation of multiple *Ubx* isoforms in larval (L) and pupal (P) wings of *B. anynana* contrasts with a single isoform in embryos (E) and comparable life stages/tissues in *J. coenia.* Bands are the products of semi-quantitative RT-PCR. B=negative control using ddH_2O as the PCR template. The two isoforms are marked with arrows.

levels almost equivalent to those normally observed in the haltere, and only during the last larval instar, was sufficient to produce a nearly complete homeotic transformation of the adult wing into a haltere (Pavlopoulos and Akam, 2011). We discovered that *B. anynana* has at least two *Ubx* isoforms expressed in larval and pupal wings, whereas a single isoform is expressed in embryos. Whole-body over-expression of a JcUbx protein sharing high similarity with the largest *B. anynana* Ubx isoform changed eyespot size, but did not alter eyespot number nor wing shape in predicted ways. This remained the case despite variable ectopic initiation times, as early as the embryonic stage. The most likely explanation for these results is that a single Ubx isoform is not sufficient to confer hindwing identity to forewings in *B. anynana*, and that additional factors are playing a role. One possibility is that the second Ubx isoform is also required for hindwing specification. An alternative explanation is that, similar to what is observed in *Oncopeltus* (Chesebro et al., 2009), *Ubx* needs to be expressed constantly from the embryonic stage until the adult for a complete homeotic transformation to take place in *B. anynana*. Given that late larval expression in *Drosophila* is sufficient to transform wings into halters (Pavlopoulos and Akam, 2011), we tend to favor the former explanation for our results in *Bicyclus*.

Despite the lack of homeotic transformations in wing shape and eyespot number, eyespot size was altered in a homeotic-like fashion: the larger forewing eyespots became smaller and more hindwing-like in size. These data support previous results from the "*Hindsight*" homeotic mutant in *J. coenia*. Ubx absence from eyespot central signaling cells, the foci, produced larger hindwing eyespots in these mutants (Weatherbee et al., 1999). Both these experiments suggest that Ubx is sufficient to repress focal cell activity, leading to smaller eyespots on the hindwing, relative to the corresponding forewing eyespots in both species. In *B. anynana*, it appears that the single large isoform is able to perform this function. However, the smaller eyespots observed naturally on the hindwing in this species could be due to the wildtype expression of Ubx in the eyespot centers (above background levels) instead of the ubiquitous Ubx expression on the hindwing. If due to the first type of expression domain (eyespot center expression), then, the transformations observed in eyespot size do not qualify as homeotic in the traditional sense.

Ectopic Ubx over-expression in clusters of cells in the pupal forewing, by means of a laser heat-shock, led to a single appearance of a discrete cluster of hair-like scales. These scales are more commonly found in hindwings, but are not exclusive to hindwings. This type of transformation was not observed in forewings that

expressed Ubx homogeneously during comparable stages of pupal development. Similar homeotic transformations were observed in *J. coenia* using recombinant sindbis viruses that transformed isolated clusters of cells into hindwing identity. This suggests that the single large Ubx isoform in *B. anynana* is able to modify scale morphology, as long as Ubx is expressed in a non-homogenous fashion on the wing or at transient (high or low) levels. We cannot know which of these factors is responsible for the phenotype because it is difficult to compare laser induced Ubx levels with those on the wildtype hindwing since laser-induced expression is transient. It is known, however, that wing epidermal cells have fundamentally distinct behaviors at boundaries with cells not expressing the same selector genes versus against cells that do (Baena-Lopez and Garcia-Bellido, 2006). Because, homogeneous ectopic expression in *J. coenia* was not achieved, it is unclear whether it would have led to distinct results in this species, just like it did in clustered versus homogeneous Ubx expression in *B. anynana*.

One surprising result in our study was the appearance of black scales following laser-ectopic expression of Ubx in pupal forewings. Black scales were also observed more frequently than ectopic hair-like scales, following similar laser perturbations. The differentiation of different scale types following ectopic expression of the same gene may be due to induction of the gene at different levels or at slightly different critical periods during development in the different individuals. Ectopic expression of the selector gene *eyeless* in *Drosophila*, for example, led to the differentiation of different traits (a complete eye or just red pigment) depending on *eyeless* expression levels (Halder et al., 1995). In *B. anynana*, black scales are normally associated with Spalt and Dll proteins in the pupal stage of eyespot development, but not with Ubx (Brunetti et al., 2001). Ubx is naturally (but weakly) expressed in eyespot centers of hindwing eyespots in both larval and pupal stages of development in *B. anynana*, but not in *J. coenia*. Dll and Spalt are also expressed in these centers, together with additional transcription factors, ligands, and receptors (Monteiro and Prudic, 2010). Moreover, Antennapedia is strongly expressed in eyespot centers in both forewing and hindwing eyespots (Saenko et al., 2011). We propose that Ubx ectopically activates *Dll* and *spalt* in forewings because it serves this same role in the hindwing eyespot centers or, because it substitutes for the activity of Antp, the earliest transcription factor expressed in forewing and hindwing eyespot centers, before the expression of *Dll* and *spalt* (Oliver et al., 2012; Saenko et al., 2011). In the latter scenario, Antp would be the natural activator of *Dll* and *spalt*, not Ubx. The absence of other eyespot-centrally-expressed-genes at these ectopic locations, would lead to black scale differentiation, instead of the normally white scales found in the eyespot centers. Recently we showed that ectopic expression of Dll on the forewing, using the same laser heat-shock system, led to black scale differentiation (Monteiro et al., 2013). This same pigmentation-inducing role for Dll was recently also demonstrated in *Drosophila biarmipes* (Arnoult et al., 2013). This suggests that Ubx's role in differentiating black scales is probably due to Ubx's ability to up-regulate *Dll*. The role of Sal in the process of black scale differentiation remains unclear.

The laser ectopic expression data shows that Ubx plays different roles in regulating the same target genes in embryos versus pupal wings in *B. anynana*, and also in *Drosophila* versus *Bicyclus*. *Dll* is repressed by Ubx in the embryonic legs of flies (Vachon et al., 1992; Weatherbee et al., 1998), whereas *spalt* is repressed by Ubx in the fly's haltere (Vachon et al., 1992; Weatherbee et al., 1998). In *Bicyclus*, we provide the first experimental evidence of Ubx being able to promote *Dll* and *spalt* expression in any system, coinciding with Ubx's (and/or Antp's) co-option into the novel eyespot gene regulatory network.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.08.020.

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