Ultrabithorax function in butterfly wings and the evolution of insect wing patterns

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Background: The morphological and functional evolution of appendages has played a critical role in animal evolution, but the developmental genetic mechanisms underlying appendage diversity are not understood. Given that homologous appendage development is controlled by the same *Hox* gene in different organisms, and that *Hox* genes are transcription factors, diversity may evolve from changes in the regulation of *Hox* target genes. Two impediments to understanding the role of *Hox* genes in morphological evolution have been the limited number of organisms in which *Hox* gene function can be studied and the paucity of known *Hox*-regulated target genes. We have therefore analyzed a butterfly homeotic mutant '*Hindsight',* in which portions of the ventral hindwing pattern are transformed to ventral forewing identity, and we have compared the regulation of target genes by the *Ultrabithorax* (*Ubx*) gene product in Lepidopteran and Dipteran hindwings.

Results: We show that *Ubx* gene expression is lost from patches of cells in developing *Hindsight* hindwings, correlating with changes in wing pigmentation, color pattern elements, and scale morphology. We use this mutant to study how regulation of target genes by Ubx protein differs between species. We find that several Ubx-regulated genes in the *Drosophila* haltere are not repressed by Ubx in butterfly hindwings, but that *Distal-less* (*Dll*) expression is regulated by Ubx in a unique manner in butterflies.

Conclusions: The morphological diversification of insect hindwings has involved the acquisition of different sets of target genes by Ubx in different lineages. Changes in *Hox*-regulated target gene sets are, in general, likely to underlie the morphological divergence of homologous structures between animals.

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Background

The evolution of arthropods and chordates has been marked by numerous innovations and modifications to their respective body plans over the course of the past half-billion years. Recent progress in understanding the developmental and genetic mechanisms underlying the organization of animal body plans and the formation and patterning of various organs has provided new comparative approaches to understanding morphological evolution. Because many features that differ among arthropods — segment morphology, and appendage number and pattern — or within vertebrates — axial morphology and limb pattern — are regulated by the *Hox* genes, these genes have been implicated at various levels in the evolution of these taxa [1,2].

The first explicit model linking the *Hox* genes to morphological evolution was put forth by Lewis [3], who proposed that the evolution of segmental diversity in the insect lineage involved the evolution of homeotic genes that were not present in primitive arthropods. But the realization that the complement of *Hox* genes has been conserved among insects [4–6], crustacea [7], chelicerates [8–10], myriapods, and even onychophora [11] revealed that the expansion and diversification of homeotic genes preceded the origin and diversification of the entire arthropod clade. The role of *Hox* genes in arthropod evolution must therefore involve changes in their function, regulation, or in the genes that they regulate.

The spatial regulation of some *Hox* genes within the body has been found to differ both between [7,9–12] and within [13,14] certain arthropod taxa. In all of these cases, the various boundaries of *Hox* gene expression demarcate transitions in appendage morphology (or their presence/absence) along the main body axis. These findings indicate that the number and type of appendages, such as a walking leg versus a feeding appendage [14], can evolve through changes in *Hox* gene regulation.

Within certain groups, such as the insects, *Hox* gene expression patterns [1,15] and overall body organization

have remained fairly stable. Yet, there is considerable diversity in some major features of insect body patterns that is most apparent among the body appendages. No modern insect has identical forewings and hindwings but some insect orders, such as Odonata, reflect the primitive condition of wing differentiation, with similar forewings and hindwings, while in other orders (such as Diptera and Lepidoptera) the morphology and function of the different pairs of flight appendages have diverged more noticeably. The differences in morphology between these flight structures in *Drosophila* are due to the regulation of many genes by the Ubx protein in the developing haltere (the dipteran homolog of the hindwing) [16]; by contrast no *Hox* gene acts in the developing forewing [17,18]. We have previously shown that a four-winged insect, the butterfly, also expresses Ubx in the developing hindwings [13]. Differences in hindwing morphology between insects are therefore not due to overt changes in *Ubx* gene expression. Rather, differences between these homologous structures could arise both from concerted changes in gene expression that affect the basic groundplan of both wing pairs and are independent of Ubx control, and from changes in the array of target genes regulated by Ubx. Here, we use both a genetic and a comparative developmental approach to examine how *Ubx* has functioned in the evolution of hindwing morphology.

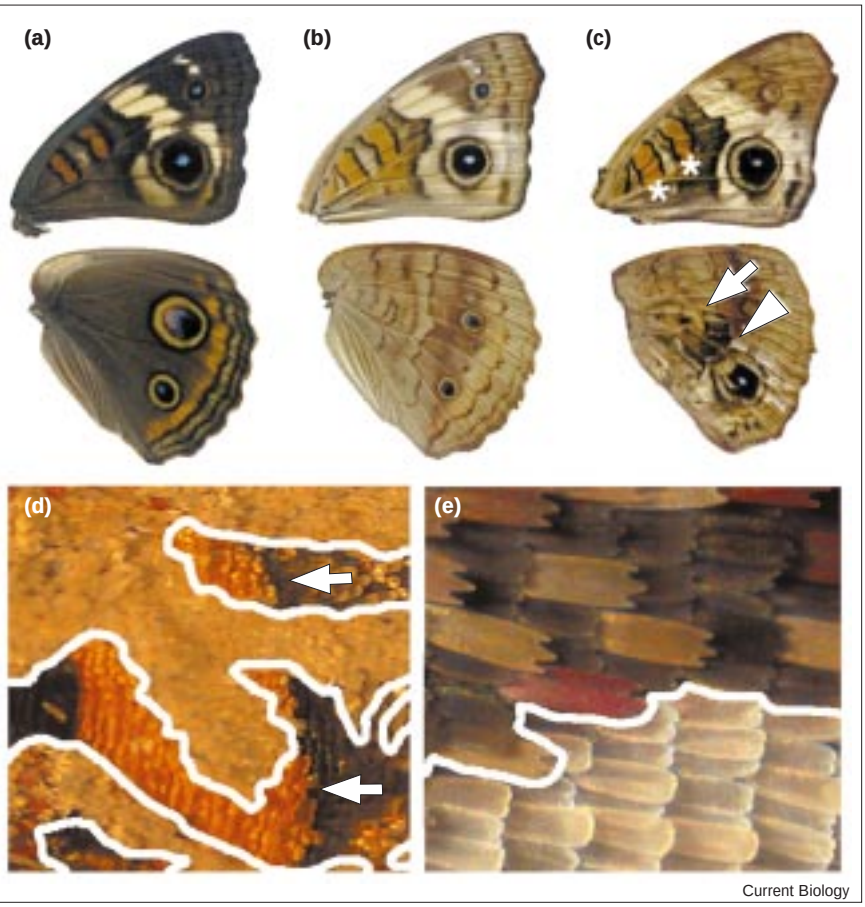
Results

A homeotic mutation causing loss of *Ubx* expression in butterfly hindwings

Sporadic homeotic transformations of individual lepidopteran wings have been reported for over a century [19,20]. We have isolated a dominant mutant stock (now termed *Hindsight*) of the butterfly *Precis coenia* in which the hindwing regularly displays patches of homeotic transformations on its ventral surface (Figure 1b,c) but the dorsal surface appears wild-type (Figure 1a; [21]). These homeotic transformations consist of patches of tissue in which the pigmentation (Figure 1c), organization of color pattern elements (Figure 1d), and scale morphology (Figure 1e) are transformed to that found on the corresponding region of the ventral forewing.

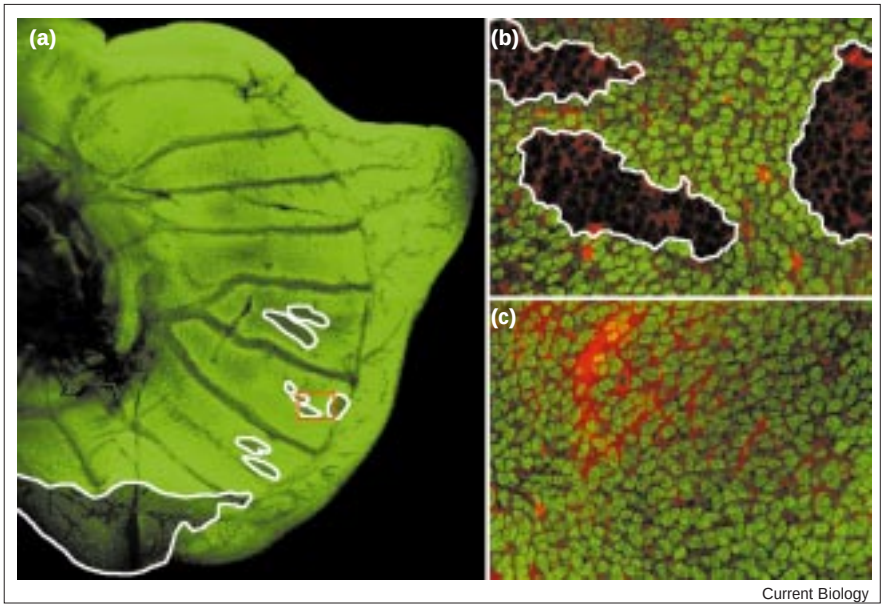
Given that *Ubx* controls hindwing identity in *Drosophila*, and *Ubx* is expressed in all cells of the developing hindwing of *P. coenia* [13], we sought to determine whether Ubx

Figure 1



Transformations of portions of ventral hindwing to ventral forewing pattern in a homeotic butterfly mutant. (a) Dorsal surfaces of a wild-type forewing (top) and hindwing (bottom). Ventral surfaces of forewings and hindwings of (b) wild-type and (c) homeotic *Hindsight* strains reveal changes in pigmentation and color pattern elements in the homeotic hindwing (compare (b) bottom and (c) bottom). The region denoted by the arrow in (c) is magnified in (d), showing that the orange proximal bands, pattern elements found normally in forewings – asterisks in (c) – are now found in a transformed region of the hindwing – arrows in (d). The region denoted by the arrowhead in (c) is magnified in (e), showing that scale morphology is also altered in transformed regions of the hindwing. In *P. coenia*, ventral hindwing scales away from the margin are long and scalloped – the upper part of (e) – while the transformed regions in mutant hindwings have shorter, rounded scales characteristic of the ventral forewing – (e), bottom. The wild-type and transformed regions are separated by a white line (d,e).

Figure 2



Ubx protein expression is lost in patches of ventral cells on the developing hindwings of the *P. coenia* homeotic mutant. Ubx expression is shown in green, and phalloidin-stained cell membranes are shown in red. (a) Ventral view of a mutant hindwing disc showing loss of Ubx expression in several small, and one large, patch (patches are outlined in white). The region denoted by the red box is magnified (×10) in (b), showing that Ubx expression is completely lost from a portion of the hindwing cells. (c) Dorsal surface of the same disc showing that loss of Ubx is restricted to the ventral surface. Magnification as in (b).

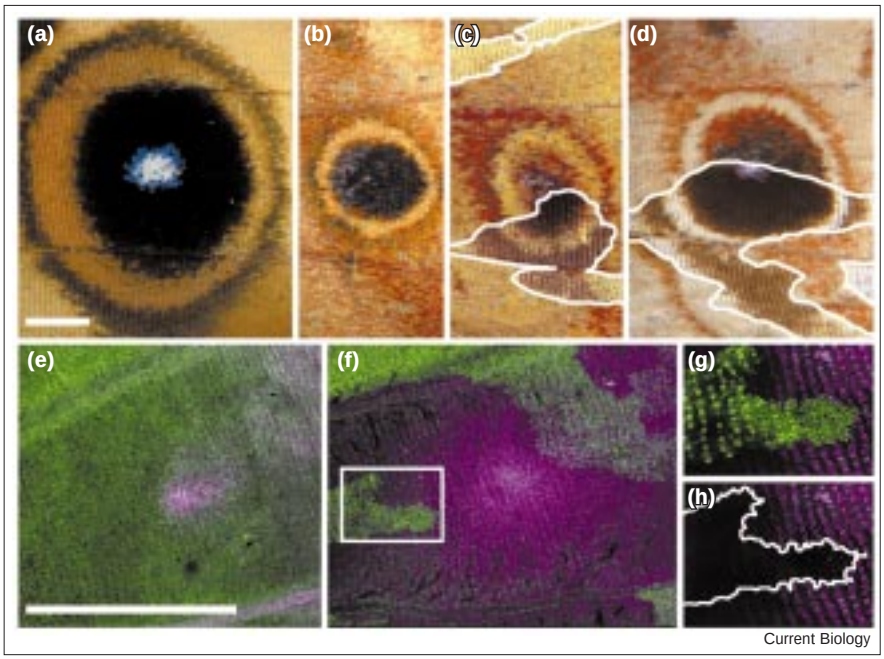
protein expression was altered in *Hindsight* wing discs. We found a variegated loss of Ubx protein expression on the ventral (Figure 2a,b), but not the dorsal (Figure 2c), surface of the developing mutant hindwings. The dominant and complete loss of Ubx expression in groups of cells could be due to a mutation in a regulator of *Ubx* gene expression or a mutation in *Ubx* itself. In either case, it is clear that the alleles of *Ubx* on both chromosomes are unable to produce functional gene products. These data suggest that Ubx normally regulates pigmentation, color pattern elements and scale morphology in *P. coenia* hindwings and that loss of *Ubx* expression results in patterns normally found in the forewing. The ventral restriction of the homeotic patches is intriguing, as it is not paralleled in *Drosophila*. Lepidopteran dorsal and ventral wing surfaces often differ remarkably in pigmentation and color pattern elements but Dipteran wing surfaces do not (Figure 1a,b; [16]); it follows, then, that the genes affecting butterfly wing characters would be differentially regulated between wing surfaces, possibly through ventral-specific or dorsal-specific regulatory elements, and these could include the *Ubx* gene.

The cell-autonomy of homeotic effects on eyespot pattern and gene expression

The homeotic patches on hindwings appear to have cell-autonomous changes in scale pigmentation and morphology, as is apparent from the coincident, sharp discontinuities in these characters at the boundaries between transformed and nontransformed tissue. But these transformations are not cell-autonomous in all wing pattern elements, such as the eyespots [21]. Eyespots are thought to be formed by the diffusion of a signaling molecule(s) from the focus [22,23], a group of cells located at the center of the developing eyespot field. The wild-type ventral posterior forewing eyespot is much larger than the corresponding hindwing eyespot (compare Figure 3a with 3b). If a transformed region of the hindwing includes only peripheral, non-focal portions of an eyespot, there is a change in pigmentation, scale morphology and eyespot size towards that of the corresponding forewing eyespot only within the transformed tissue (Figure 3c). In mutant hindwing eyespots in which the transformed region also includes the focus, however, the overall size of the eyespot is increased towards that of the forewing eyespot (Figure 3d). These larger spots include genetically wild-type cells that do not exhibit transformations of scale morphology (Figure 3d). Taken together, these observations suggest that Ubx is affecting both non-autonomous and cell-autonomous aspects of the development of the eyespot field.

In order to determine when Ubx acts upon the eyespot developmental pathway in hindwings, we analyzed the expression of the *Dll* gene which marks the developing eyespot field [24,25]. In late fifth instar larval wing discs, circular clusters of cells expressing high levels of *Dll* mark the eyespot foci. There is no apparent difference in the number of cells expressing *Dll* in the posterior ventral eyespot foci of forewings and hindwings, indicating that Ubx does not repress the establishment of hindwing foci (data not shown). In pupae 24 hours old, high levels of *Dll* expression continue in the epithelial cells of the focus but expand to include the surrounding scale cells, apparently in response to signaling from the focus. At this stage, the forewing ventral eyespot field is larger than the hindwing eyespot field, as measured by the area of *Dll*-expressing epithelial cells in the focus and the surrounding scale cells

Figure 3



The *Distal-less* (*Dll*) gene, which is deployed in a novel spot pattern in butterfly wings, is regulated by Ubx. (a,b) Adult ventral posterior eyespots on wild-type (a) forewings and (b) hindwings, illustrate the differences in the size and color of this pattern element between the two flight appendages. (c) A ventral homeotic hindwing eyespot, in which the transformed region does not include the focus. There is an increase in size of the circular eyespot field and changes in pigmentation towards that of the corresponding forewing eyespot. These effects are restricted to the transformed region (outlined in white). (d) An eyespot in the same position as that shown in (c) but in which the transformed region includes the eyespot focus. In addition to the increase in size and pigmentation changes in the transformed region (outlined in white), the non-transformed tissue (top) also shows an increase to a size more similar to that of the forewing eyespot (compare with (a) and (b) [21]). (e–h) Ventral view of 24 h pupal hindwing discs showing Ubx (green) and Dll (magenta) protein expression patterns. In homeotic mutant hindwings (f), there is an increase in the area of cells expressing Dll in the focus and surrounding scale cells compared with wild type (e). A higher magnification of the boxed region of (f), in (g), shows that, in regions where Ubx is expressed, Dll expression is markedly decreased. (h) The same image as in (g), showing only Dll expression, and the region containing Ubx-expressing cells is outlined in white. Scale bars = 1 mm.

(data not shown). These observations suggest that Ubx may be regulating the signal from the focus.

To examine this possibility, we examined *Hindsight* mutant pupal hindwings, in which patches of cells that lack Ubx protein expression encompass a portion of the focus. We found that the expression of *Dll* clearly increases compared with that found in wild-type hindwings (compare Figure 3e with 3f). Outside of these patches, where Ubx expression is 'normal' in the eyespot field, *Dll* is expressed at very low levels in a cell- autonomous fashion (Figure 3f–h). Our results suggest that hindwing eyespot size may be controlled by Ubx at two steps in the eyespot developmental pathway. First, Ubx depresses the production of the focal signal, which is relieved when a portion of the focus loses Ubx expression. And second, Ubx affects the response of genes that are downstream of the focal signal — for example, *Dll*. Because the eyespot pattern element has no counterpart in other insect orders, we deduce that Ubx regulation of eyespot patterning genes must have evolved within the Lepidoptera.

The divergence of Ubx-regulated target gene sets between Lepidoptera and Diptera

We have shown that the differences in pigmentation, scale morphology and color pattern elements between *P. coenia* forewings and hindwings are regulated by Ubx. Recall that in Diptera (such as *Drosophila*) it is the differences in size, shape and pattern between the highly modified hindwings (halteres) and forewings that are regulated by Ubx. These observations suggest that the hindwings of the common four-winged ancestor of both orders also expressed Ubx, and over the course of evolution different sets of genes expressed in the ancestral hindwing may have become Ubx-regulated in the two lineages. One prediction of the above scenario is that some of the Ubx target genes in the *Drosophila* haltere would not be regulated by Ubx in the hindwings of four-winged insects.

In order to examine the effects of Ubx on gene regulation in butterfly hindwings, we cloned *P. coenia* homologs of three genes we have recently shown to be repressed by Ubx in portions of the developing *Drosophila* haltere [16]: *Drosophila serum response factor (DSRF)*; an *Achaete–Scute complex (AS-C)* gene [26]; and *wingless (wg)* [24]; and we have examined their expression in developing wings. These genes have largely similar expression patterns in *Drosophila* wing discs and in *P. coenia* forewing discs. Butterfly *wg* is expressed along the dorsal–ventral boundary (Figure 4a). The *DSRF* homolog is expressed in all intervein regions (Figure 4b), and the *AS-C* homolog is expressed in a double row of cells straddling the

dorsal–ventral boundary (Figure 4c). This demonstrates that aspects of the wing groundplan are shared between dipterans and lepidopterans.

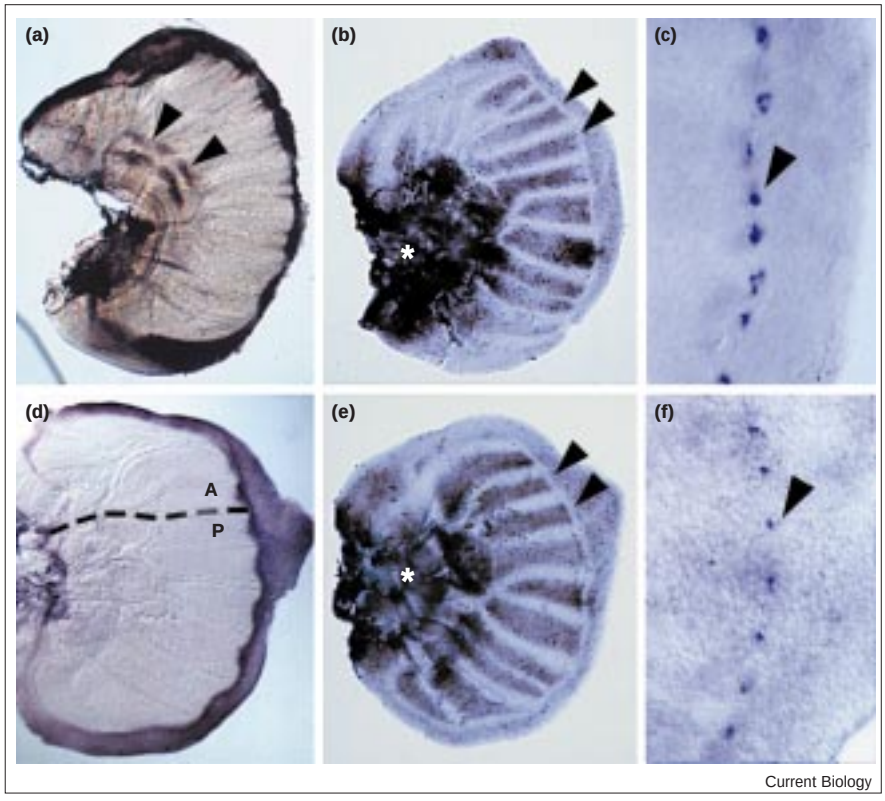
But portions of the expression patterns of *DSRF*, *AS-C* and *wg* that are repressed by Ubx in *Drosophila* halteres are not repressed in *P. coenia* hindwings. Unlike the expression patterns of the homologous genes in halteres, butterfly *wg* is not repressed along the posterior margin in the hindwing (Figure 4d), nor is *P. coenia SRF* repressed in intervein regions (Figure 4e), and the *AS-C* homologue is not repressed in cells flanking the dorsal–ventral boundary (Figure 4f). These differences in the regulation of *wg*, *SRF* and *AS-C* between *Drosophila* halteres and butterfly hindwings suggest that these genes became repressed by Ubx when an ancestral hindwing evolved into a haltere in the dipteran lineage, with a concomitant reduction of appendage size, loss of margin bristles, and change in shape.

We have found two additional examples of Ubx-regulated differences in gene expression between fly and butterfly flight appendages. First, we note that *wg* is expressed in two stripes in butterfly forewings (Figure 4a) that roughly correspond to the future location of the proximal band elements (Figure 1c). This portion of the *wg* pattern is absent from butterfly hindwings and has no counterpart in flies and represents a novel feature regulated by Ubx in butterflies. Secondly, *Dll* is expressed along the margin of both butterfly wings [25] and the *Drosophila* forewing, but this expression is modified in halteres [27] and may be regulated by Ubx.

Discussion

The diversity of insect hindwing patterns illustrates the broad range of possible morphologies that can evolve in homologous structures that are regulated by the same *Hox* gene. Although some hindwing differences are due to concerted changes in the wing groundplan that occur in both pairs of wings, our analysis reveals clear changes in the regulation of genes downstream of Ubx between two lineages that diverged at least 200 million years ago (Figure 5). Our recent analysis of haltere development in *Drosophila* [16] revealed that a substantial fraction of the wing patterning genes we surveyed were selectively and

Figure 4



Butterfly *wg*, *DSRF* and *AS-C* homologs are expressed in hindwings in the presence of Ubx. *In situ* hybridizations on **(a–c)** forewing discs and **(d–f)** hindwing discs with riboprobes complementary to *P. coenia wg* (a,d), *SRF* (b,e) and an *achaete-scute* homolog (c,f) transcripts. A forewing (a) and a hindwing (d) imaginal disc reveal that *wg* transcripts are expressed along the entire wing margin of both discs, whereas in the *Drosophila* haltere *wg* is not expressed in the posterior compartment [16]. The dotted line in (d) marks the anterior–posterior compartment boundary. Note that the posterior compartment (P) in butterflies is much larger than the anterior compartment (A; deduced from *engrailed/invected* expression). This is the inverse from the situation observed in the *Drosophila* haltere [16]. Also note that in the forewing disc, *wg* expression is observed in a position corresponding to the future proximal bands (arrowheads in (a)) which are absent from the hindwing, indicating that Ubx may also regulate this novel portion of the *wg* expression pattern in the *P. coenia* hindwing. A forewing (b) and a hindwing (e) imaginal disc show that *P. coenia SRF* transcripts are localized to intervein cells in both discs (arrowheads). The base of the discs shows high background staining (asterisks), as is observed with many other riboprobes. A forewing (c) and a hindwing (f) disc show *P. coenia achaete-scute* transcription in cells along the future wing margin of both discs (arrowheads); (c,f) are shown at four times the magnification of specimens in (a,b,d,e).

independently regulated by Ubx. This survey included genes at various levels of regulatory hierarchies that guide the formation and patterning of several traits. From this survey, it seems reasonable to infer that Ubx may regulate the expression of dozens of genes in the haltere [28]. We have observed that, in *Precis*, Ubx regulates traits (scale morphology, pigmentation and eyespot pattern) that are not found on halteres, and Ubx does not regulate in butterfly hindwings the few genes we surveyed that are repressed by Ubx in halteres. It is likely then that the regulation of a considerably larger number of genes has diverged between the two structures.

The comparison of gene expression in the developing haltere and hindwing is informative as to what differences have evolved, but it does not reveal how changes in gene regulation evolve. One of the potential mechanisms for the evolution of gene repression by Ubx in the dipteran lineage would be the evolution of Ubx binding sites in the *cis*-regulatory elements that control expression of these genes in flight appendages (Figure 5). This would provide the means by which hindwing morphology could evolve while conserving forewing morphology and Ubx protein function, the latter being constrained by its many roles in other structures. This view is bolstered by our observation that Ubx does not regulate certain aspects of *wg* and *Dll* expression that are Ubx-regulated in *Drosophila*, but *Ubx* does regulate novel aspects of *wg* and *Dll* expression in butterflies. Evaluation of this hypothesis will require functional comparisons of homologous *cis*-regulatory elements from butterflies, flies, and more primitive insects, to determine how they have evolved in each lineage.

Conclusions

The Ubx protein regulates detailed aspects of scale morphology, pigmentation and eyespot pattern in the hindwing of the butterfly *P. coenia* and regulates a different set of characters in the homologous haltere of the fruit fly *Drosophila*. The differences in hindwing morphology between these two species is due in part to the divergence of the target gene sets regulated by Ubx during the evolution of butterflies and flies from a common four-winged ancestor. Changes in *Hox*-regulated target gene sets are likely to underlie the morphological divergence of homologous structures in other animals.

Materials and methods

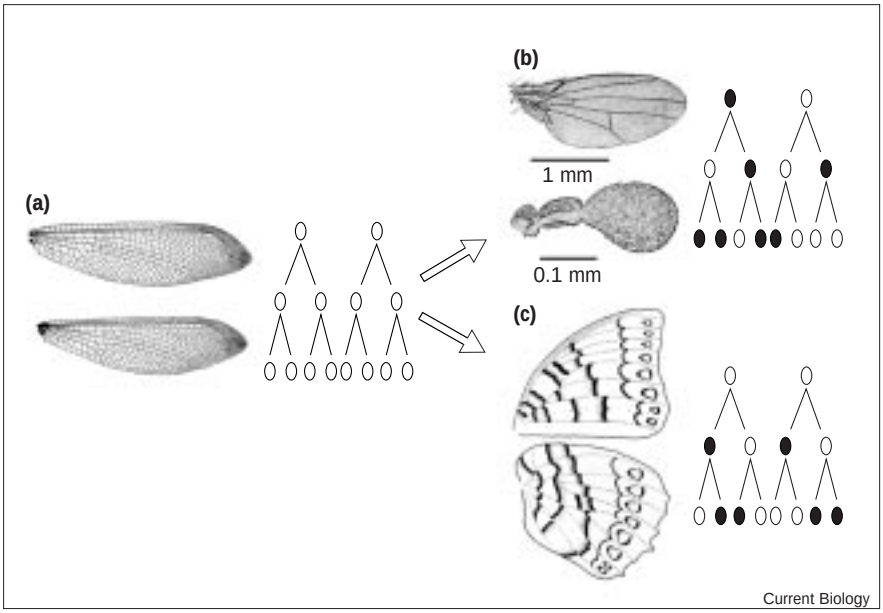
Stocks

The *P. coenia* homeotic mutant *Hindsight* stock was derived from spontaneous mutants [21] arising in a Rosa [29] background. Transmission of these mutant phenotypes appears to be dominant with low penetrance and variable expressivity, but with selection can become fully penetrant. We examined Ubx protein expression in 16 specimens from a fully penetrant *Hindsight* strain. All of the discs taken from specimens displayed patches in which Ubx expression was lost. We also examined 29 specimens from an incompletely penetrant stock and found that 76% of these displayed patches in which Ubx expression was lost. This correlates well with the frequency of adult homeosis observed in siblings (75%).

Immunolocalizations, cloning and analysis of butterfly genes

Immunohistochemistry on butterfly larval wing discs for Ubx [13] and *Dll* [25] expression was performed as previously described [13,25]. The *P. coenia SRF* gene was cloned by low stringency screening of an embryonic cDNA library [24]. A PCR fragment containing the MADS box of the *Drosophila SRF* gene [30] was used to prepare a radiolabeled probe. *P. coenia* SRF and *Drosophila* SRF share an identical MADS domain (80% identity at nucleotide level) and also show long stretches of identity outside the MADS domain. *P. coenia SRF*

Figure 5



The evolution of insect hindwing patterns and the divergence of Ubx-regulated target gene sets. A schematized view of the course of the evolution of the dipteran (b) and lepidopteran (c) lineages from a common four-winged ancestor (a) which had similar forewings and hindwings. On the left of each panel are drawings of wing pairs and on the right are schematics representing genetic regulatory hierarchies for wing development. In this scenario, Ubx, although expressed in the ancestral hindwing (a), did not yet regulate genes in the wing patterning hierarchy to differentiate hindwing from forewing morphology. Subsequently, many genes (represented by black ovals) fell under the control of Ubx and these sets of Ubx-regulated genes differed between the (b) dipteran (*wg*, *AS-C*, *SRF* and so on) and (c) lepidopteran (*Dll*, scale morphology genes and so on), and presumably other, insect lineages. The drawings are adapted from [31,32].

sequence data have been submitted to EMBL/GenBank (Accession number AF120007). Hybridization *in situ* to butterfly wing discs was performed as previously described using specific butterfly *wg* [24], *achaete-scute* [26] and *SRF* homolog cDNAs.

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