

1 **Title:** *apterous A* specifies dorsal wing patterns and sexual traits in butterflies

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7

8 **Abstract:** Butterflies have evolved different color patterns on their dorsal and ventral wing  
9 surfaces to serve different signaling functions, yet the developmental mechanisms controlling  
10 surface-specific patterning are still unknown. Here, we mutate both copies of the transcription  
11 factor *apterous* in *Bicyclus anynana* butterflies using CRISPR/Cas9 and show that *apterous A*  
12 functions both as a repressor and modifier of ventral wing color patterns, as well as a promoter of  
13 dorsal sexual ornaments in males. We propose that the surface-specific diversification of wing  
14 patterns in butterflies proceeded via the co-option of *apterous A* into various gene regulatory  
15 networks involved in the differentiation of discrete wing traits. Further, interactions between  
16 *apterous* and sex-specific factors such as *doublesex* may have contributed to the origin of  
17 sexually dimorphic surface-specific patterns. Finally, we discuss the evolution of eyespot pattern  
18 diversity in the family Nymphalidae within the context of developmental constraints due to  
19 *apterous* regulation.

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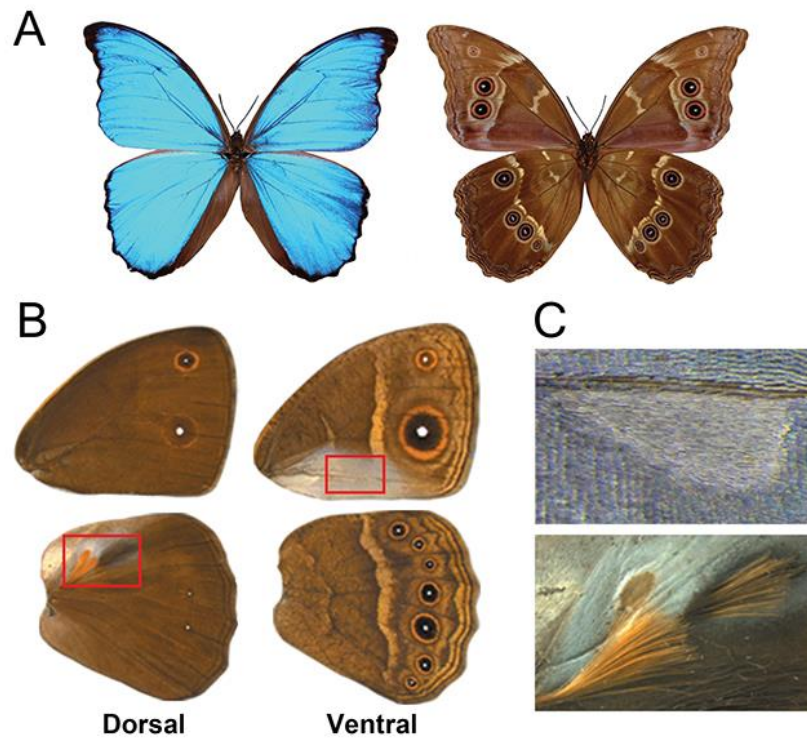
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25 **Main Text:**

26 Butterflies are a group of organisms well known for their diverse and colorful wing patterns. Due  
27 to the dual role these patterns play in survival and mate selection, many butterflies have evolved  
28 a signal partitioning strategy where color patterns appearing on the hidden dorsal surfaces  
29 generally function in sexual signaling, whereas patterns on the exposed ventral surfaces most  
30 commonly serve to ward off predators (1, 2) [Fig 1A]. While the molecular and developmental  
31 basis of individual pattern element differentiation, such as eyespots or transverse bands, has been  
32 previously studied (3, 4), the molecular basis of dorsal and ventral surface-specific color pattern  
33 development remains unknown. Elucidating this process will help us understand the mechanism  
34 of diversification and specialization of wing patterns within the butterfly lineage.

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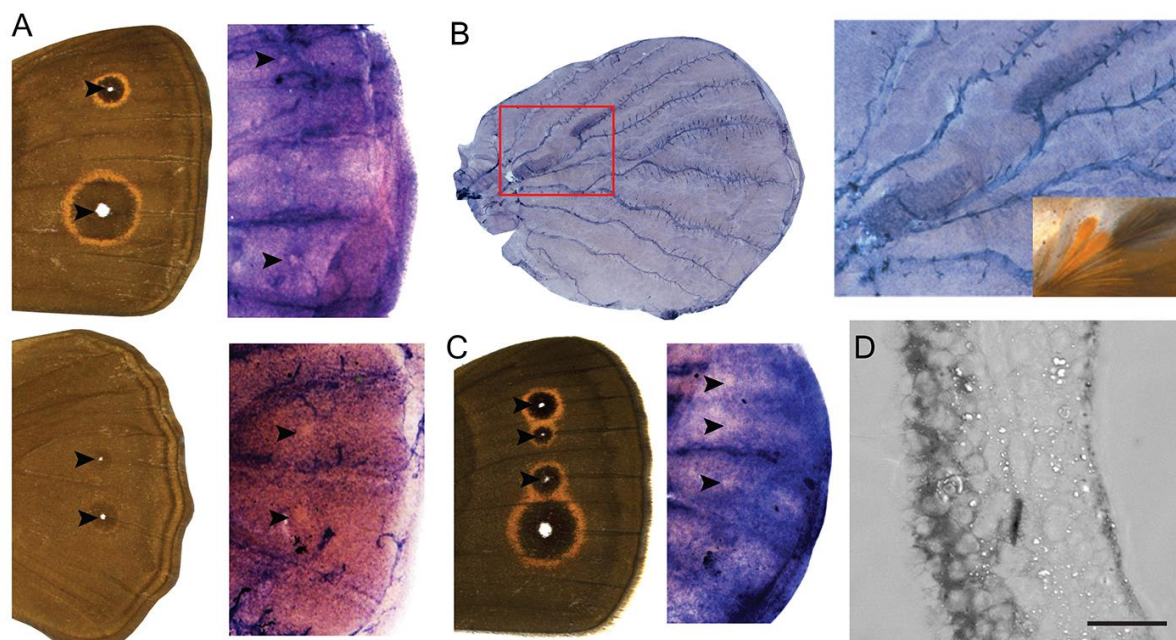
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38 **Figure 1: Dorsal-Ventral surface-specific variation in butterflies** A) Dorsal (left) and ventral  
39 (right) surfaces of *Morpho menelaus* illustrating striking variation in color and patterns between  
40 surfaces. B) Dorsal and ventral surfaces of a male *Bicyclus anynana*. The regions boxed in red  
41 are expanded in C. C) Top: Male-specific forewing ventral androconia with a characteristic  
42 teardrop shape surrounded by silver scales. This is absent from the corresponding dorsal  
43 forewing surface which is instead completely covered with brown scales. Bottom: Male-specific  
44 hindwing dorsal androconia, also surrounded by silver scales, along with two patches of hair-  
45 pencils. These traits are absent from the ventral hindwing.

46

47 We hypothesized that the transcription factor *apterous* (*ap*), a gene expressed in the dorsal wing  
48 surfaces of flies (5), might be implicated in differentiating dorsal from ventral wing patterns in  
49 butterflies. In insects, however, this gene is often present in two copies, *apA* and *apB*, that don't  
50 necessarily share the same expression patterns, and flies are unusual for having lost one of these  
51 copies. In the beetle *Tribolium castaneum*, *apA* is expressed on the dorsal surface whereas *apB* is  
52 expressed on both surfaces (6). In the butterfly *Junonia coenia*, *apA* is expressed on the dorsal  
53 surface of larval wings (7) but, the expression of *apB* and the role of either *apA* or *apB* in wing  
54 development and patterning is not known for this or any butterfly species.

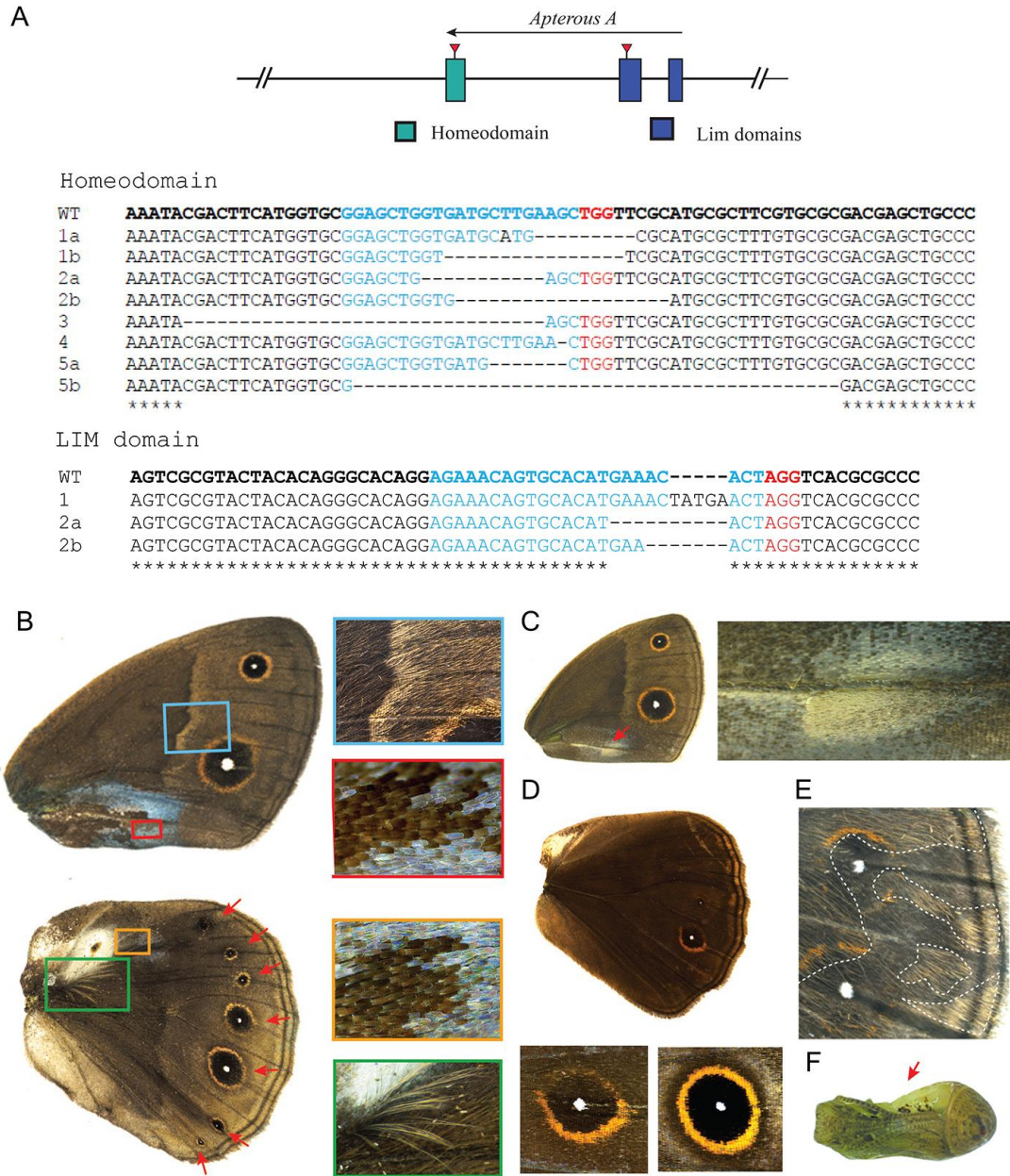
55  
56 To further investigate *ap* expression in butterflies, we cloned both *ap* homologs from the African  
57 squinting bush brown *Bicyclus anynana* [Fig 1B, C], and used *in situ* hybridization to localize  
58 *apA* and *apB* mRNA in developing larval and pupal wing discs. Both homologs of *ap* were  
59 localized to the dorsal surfaces of the wings [Fig 2D, S1B]. In the last larval instar wing discs,  
60 *apA* was expressed uniformly on the wing surface but absent in future dorsal eyespot centers  
61 [Fig2A]. In larval wing discs of the *B. anynana* "Spotty" mutant, which develops two additional  
62 dorsal eyespots, *apA* was absent in the additional centers [Fig 2C]. Furthermore, pupal wing  
63 expression of both *apA* and *apB* was up-regulated in dorsal male-specific cells that give rise to  
64 long and thin modified scales, the hair-pencils, used for dispersing pheromones during courtship  
65 [Fig 2B, S1A]. This pattern of expression was not seen in developing female pupal wings, which  
66 lack hair-pencils [Fig S1A].



67  
68 **Figure 2: *apA* mRNA localization in developing wing discs of *Bicyclus anynana*** A) *apA*  
69 expression is uniform across the epidermis but absent in future dorsal eyespot centers of  
70 forewings (top) and hindwings (bottom). B) Male wings (28 hours after pupation) showing *apA*  
71 mRNA localization. The boxed area is expanded to the right highlighting up-regulated *apA*  
72 expression in the hair-pencil regions. Inset shows the hair-pencils in adult male *B. anynana*. C)  
73 *apA* expression is absent in the two additional eyespot centers of forewings in the *B. anynana*  
74 “Spotty” mutant. D) Cross-sectional view of a developing wing disc showing dorsal-specific *apA*  
75 expression. Scale bar is 20 $\mu$ m.

76

77 To functionally test the role of *ap*, we used the CRISPR/Cas9 system to disrupt the  
78 homeodomain and LIM domain of *apA* [Fig 3A] and the LIM domain of *apB* [Fig S2A] [Table  
79 S2]. A range of mosaic phenotypes were observed in both types of *apA* mutant individuals [Fig  
80 3B-F]. A few of these lacked wings, whose absence was visible upon pupation [Fig 3F], and  
81 some adults had mosaic patches of ventral-like scales appearing on the dorsal surface [Fig 3E].  
82 In other mutants, the sex pheromone producing organ, the androconial organ, of the ventral  
83 forewing appeared on the dorsal surface in males with its associated silver scales [Fig 3B,C].  
84 Males also had modified hair-pencils with loss of characteristic ultrastructure and coloration and  
85 absence of silver scales associated with the dorsal androconial organ of the hindwing [Fig3B]. In  
86 addition, in some males and females, extreme mutant individuals showed improper wing hinge  
87 formation, the appearance of the ventral white band on the dorsal surface [Fig 3B], and in one  
88 case, all seven eyespots on the dorsal hindwing [Fig 3B], a surface that normally exhibits, on  
89 average, zero to one eyespot in males and one to two eyespots in females. *apA* clones also led to  
90 an enlarged outer perimeter to the gold ring in dorsal hindwing and forewing eyespots [Fig 3D].  
91 CRISPR/Cas9 disruption effects on the target sequence were verified in a few individuals, which  
92 showed the presence of deletions in the targeted regions [Fig 3A].



93

94 **Figure 3: CRISPR/Cas9 mosaic wing pattern phenotypes of *apA* knockdown** A) Top:

95 Regions of the *apA* gene in *B. anynana* targeted using the CRISPR/Cas9 system. Bottom:

96 Sequences of the homeodomain and LIM domain regions of mutant individuals compared with

97 the wildtype sequence in bold. Blue is the region targeted and the PAM sequence is in red.

98 Deletions are indicated with '-'. B) The dorsal forewing and hindwing of a mutant male

99 highlighting some of the ventral-like phenotypes and defects. C) *apA* knockdown phenotype with

100 the ventral teardrop shape androconial organ appearing on the dorsal surface (red arrow). D) A  
101 dorsal hindwing of a mutant with the width of the gold ring (bottom left) resembling that of  
102 corresponding ventral eyespot (bottom right). E) Mosaic phenotype seen on the dorsal surface  
103 with a greater density of ventral-like light colored scales. Clones are indicated with a dashed  
104 white line. F) Pupa with wings missing from one side (red arrow).

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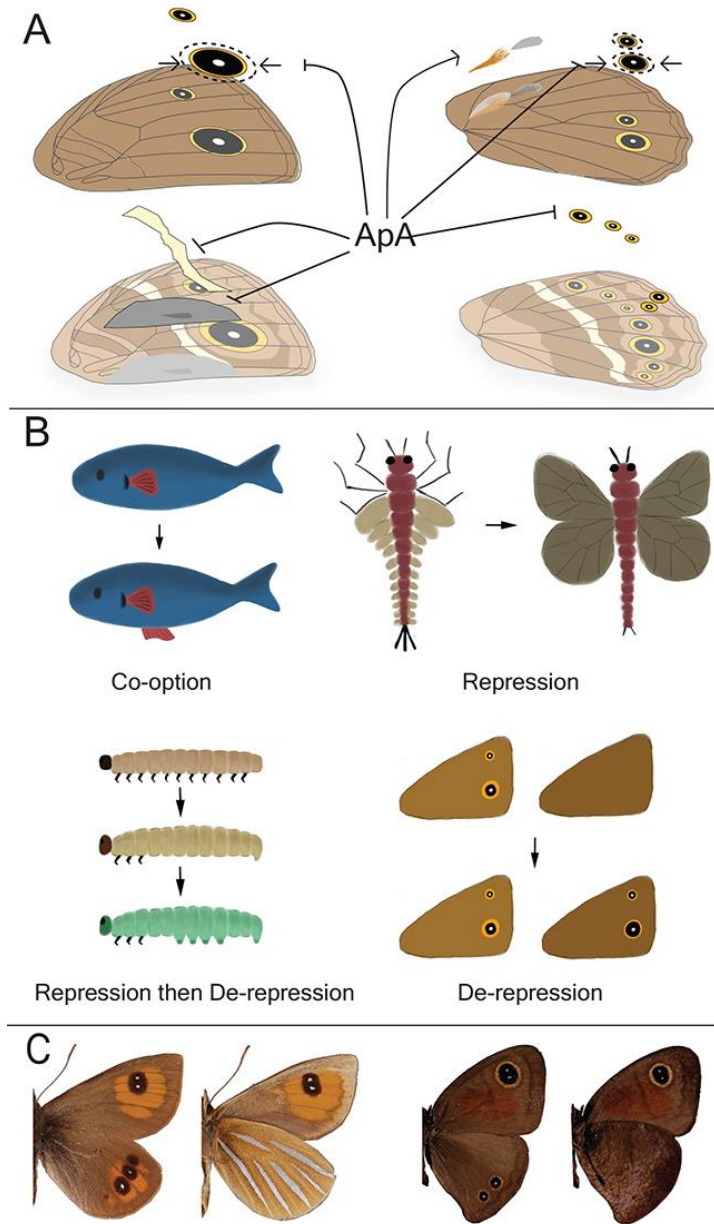


106 No striking transformations of dorsal to ventral identity were observed in *apB* mutants. Some of  
107 the *apB* knockout phenotypes included wing hinge defects, a clone of ventral-like scales on the  
108 dorsal surface [Fig S2E], affected hair pencils [Fig S2D], a missing hindwing in one case,  
109 improperly developed wings [Fig S2C] and notched margin development [Fig S2B]. Sequencing  
110 showed the presence of mutations in the targeted region [Fig S2A].

111  
112 Knockdown of *apA* in a variety of insects from different lineages indicates that *apA* is necessary  
113 for wing growth and development and its function in this process seems to be highly conserved  
114 (5, 6, 8). However, our experiments, in agreement with others, also indicate a varying degree of  
115 co-option of this transcription factor into late wing development processes such as wing  
116 patterning and exoskeletalization. In *T.castaneum*, knockdown of *apA* and *apB* individually  
117 shows almost no phenotypic effects while their simultaneous knockdown leads to more dramatic  
118 phenotypes such as elytral exoskeletalization defects, depending on the developmental stage.  
119 Therefore, both *apA* and *apB* in beetles are important for wing developmental processes (6). In  
120 *B. anynana*, both *ap* copies appear to function in margin specification and overall wing  
121 development but only *apA* appears to have a dominant role in the control of dorsal-surface  
122 specific wing patterning.

123  
124 Interestingly, our work shows that *apA* has multiple different, often antagonistic functions in  
125 surface- and sex-specific development between the fore- and hindwings. For example, *apA* acts  
126 as a repressor of male androconial organs and silver scale development in forewings, while it  
127 promotes hair-pencil formation and silver scale development on the dorsal hindwings of males  
128 [Fig 4A]. These effects point to the likely interaction between *apA* and other factors such as sex-

129 specific (*doublesex*) or wing-specific (*Ultrabithorax*) factors that together can specify sex- and  
130 surface-specific pattern development. We previously showed that *Ultrabithorax* (*Ubx*) is  
131 expressed in the hindwings but not forewings of *B. anynana* (9). To verify whether genes from  
132 the sex determination pathway were present in androconial organs of *B. anynana* we localized  
133 the expression of *doublesex* (*dsx*) in both male and female pupal wings using *in situ*  
134 hybridization. We found that *dsx* is highly expressed in male androconial organs and hair-  
135 pencils, but not in corresponding locations on female wings [Fig S1D]. These data support a  
136 likely combinatorial function reminiscent of the interactions between the hox gene *Scr* and *dsx* in  
137 the determination of the male-specific sex combs in the legs of *D. melanogaster* (10). The  
138 presence or absence of *Ubx*, type of *dsx* splice variant and *apA* may be sufficient to give each sex  
139 and wing surface a unique identity. Given the fact that the proteins of the LIM-homeodomain  
140 subfamily, to which *ap* belongs, are unique in their ability to bind other proteins via their LIM  
141 domain (11), their involvement in such a large range of developmental processes, as repressors  
142 and activators, is likely.



143

144 **Figure 4: The role of *apterous* in surface-specific wing patterning in *B. anynana* and**

145 **evolution of serial homologs in butterflies.** A) A schematic of the different functions of *apA* on

146 the dorsal surface of *B. anynana*. *apA* acts as a repressor of ventral traits such as the white

147 transversal band, forewing androconia, hindwing eyespots, and the outer perimeter of the gold

148 ring, and acts as an activator of hindwing hair-pencils and silver scales. B) Different modes of

149 serial homolog evolution involving the co-option of a (*fin*) gene network to a novel body

150 location (23), repression of the ancestrally repeated (wing) network in a subset of body segments  
151 (modified from (24)), repression followed by de-repression of the (limb) network in certain body  
152 segments (20), and de-repression of a never expressed (eyespot) network on a novel body  
153 location. C) *Argyrophenga antipodium* (left) and *Cassionympha cassius* (right) males with dorsal  
154 eyespots lacking ventral counterparts. Dorsal is to the left for each species.

155

156

157

158 Mutations in *apA* point to this gene functioning as a dorsal surface selector in *Bicyclus*  
159 butterflies. Selector genes comprise a small set of developmental genes that are critical for  
160 specifying cell, tissue, segment, or organ identities in organisms (12). The wing selector hox gene  
161 *Ubx* allows hindwings to have a different identity from forewings. For example, the restricted  
162 expression of *Ubx* in hindwings of most insects examined so far, is required for membranous  
163 wing formation in beetles and bugs (13), haltere formation in flies (14) and hindwing specific  
164 color patterns in butterflies (15). When *Ubx* is mutated, in all the examples described above,  
165 hindwings acquire the identity of forewings. In *B. anynana*, *apA* functions in similar ways along  
166 the dorsal-ventral axis of each wing – mutations in this gene make dorsal wing surfaces acquire a  
167 ventral identity. This type of homeotic mutation was also observed in a limited way, in bristles  
168 along the margin of the wings of *D. melanogaster*, where *ap* mutant clones developed bristles  
169 with a ventral identity (16). *B. anynana*, however, appears to have made inordinate use of *apA*  
170 for surface-specific color patterning and sexual trait development across the entire wing.

171  
172 Further, this work highlights the possible role of *apA* in the development and evolution of serial  
173 homologs such as eyespots in butterflies of the family Nymphalidae. The appearance of  
174 additional eyespots on the dorsal surface of hindwings in *apA* knockdown mutants, and the  
175 absence of *apA* mRNA at the precise position where a few dorsal eyespots develop in both fore-  
176 and hindwings at the stage of eyespot center differentiation, implicates *apA* as a repressor of  
177 eyespot development in *B. anynana*. The additional gaps in *apA* expression observed in Spotty  
178 mutants further suggests that genetic mechanisms of eyespot number evolution on the dorsal  
179 surface proceeded via local repression of *apA*.

180

181 We propose that the ancestral presence of a repressor (*apA*) of a gene regulatory network in a  
182 specific body location, followed by repression of the repressor represents a novel mode of serial  
183 homolog diversification [Fig 4B]. Broad comparative work across 400 genera of butterflies  
184 indicated that eyespots originated around 90 MYA within Nymphalidae on the ventral hindwing  
185 surface, and appeared ~40MY later on the dorsal surfaces (17–19). We propose that the original  
186 ventral restriction of eyespots was due to the ancestral presence of *apA* on dorsal wing surfaces,  
187 and that eyespots' later appearance on these surfaces was due to local *apA* repression. This mode  
188 of serial homolog diversification is similar but also distinct from the mechanism previously  
189 proposed to lead to the re-appearance of abdominal appendages in lepidopteran larvae - via local  
190 repression of the limb repressor hox protein, *Abdominal-A* (*Abd-A*) (20, 21). In contrast to  
191 eyespots, when arthropod appendages first originated they were likely present in every segment  
192 of the body (22). Limbs were later repressed in abdominal segments, and finally they were de-  
193 repressed in some of these segments in some insect lineages (20). So, while the last steps of  
194 abdominal appendage and eyespot number diversification are similar (de-repression of a  
195 repressed limb/eyespot network), the early stages are different.

196

197 The comparative work also showed that the origin of dorsal eyespots was dependent on the  
198 presence of corresponding ventral eyespots in ancestral lineages (19). This implies that the extant  
199 diversity of eyespot patterns is biased/limited due to developmental constraints imposed by *apA*.  
200 Interestingly, while ~99% of the species in our database display such constraints i.e dorsal  
201 eyespots always having ventral counterparts, a few butterflies display dorsal eyespots that lack  
202 ventral counterparts [Fig 4C]. The molecular basis for these rare patterns remains to be explored.

203

204 In summary, we uncover a key transcription factor that due to its restricted expression on dorsal  
205 wing surfaces may have allowed butterflies to develop and evolve their strikingly different dorsal  
206 and ventral wing patterns under natural and sexual selection. Future comparative work across  
207 species is necessary to further test this hypothesis.

208

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273

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279

## **Supplementary Materials:**

### **Materials and Methods**

#### Animals

*Bicyclus anynana* butterflies were reared in a temperature controlled room at 27°C with a 12:12 hour light:dark cycle and 65% humidity. The larvae were fed on corn plants while the adults were fed on banana.

#### Cloning and probe synthesis

*apA* sequence was obtained from (25) and *apB* and *dsx* sequences were identified from the *B.anynana* genome (26). The sequences were amplified with primers specified in Table S1, sequenced and then cloned into a PGEM-T Easy vector (Promega). Sense and anti-sense digoxigenin-labelled (DIG) riboprobes were synthesized *in vitro* using T7 and SP6 polymerases (Roche) and purified by ethanol precipitation. The product was hydrolysed to ~150bp using carbonate buffer at 60°C for 40-50 minutes followed by ethanol precipitation and resuspension in 1:1 volume of DEPC treated water:formamide.

#### In-situ hybridization

The protocol was modified slightly from (27). Briefly, larval or pupal wings were dissected from the last instar caterpillars or around 24-28 hrs after pupation respectively in PBS and transferred to glass well plates containing PBST (PBS+0.1% Tween20) at room temperature. The PBST was then immediately removed and the tissues fixed in 5% formaldehyde for 45 (larval) or 60 min (pupal) on ice, followed by 5 washes with cold PBST. The tissues were then incubated with 25µg/ml proteinase K in cold PBST for 3 (larval) or 5 minutes (pupal), washed twice with 2mg/ml glycine in cold PBST, 5 washes with cold PBST and gradually transferred to a prehybridization buffer (5X Saline sodium citrate pH 4.5, 50% formamide, 0.1% Tween20 and 100µg/ml denatured salmon sperm DNA). A post-fixation step with 5% formaldehyde was done only for larval wings followed by removal of peripodial membrane on ice (just for larval wings). The wings were incubated in prehybridization buffer at 60-65°C for 1 hour and then in hybridization buffer (prehybridization buffer with 1g/L glycine and 70 to 140 ng/ml riboprobe) for 24 hours, followed by 6 to 10 washes in prehybridization buffer at 60-65°C. The tissues were then gradually transferred back to PBST at room temperature, washed 5 times in PBST and blocked overnight at 4°C (PBST+1% BSA). The DIG-labelled probes were then detected by incubating the tissues with 1:3000 Anti-DIG Alkaline Phosphatase (Roche) in block buffer for two hours, washed 5 times with block buffer, incubated in alkaline phosphatase buffer (100mM Tris pH 9.5, 100mM NaCl, 5mM MgCl<sub>2</sub>, 0.1% Tween) and finally stained with NBT/BCIP (Promega) solution at room temperature till colour developed. The reaction was stopped by washing in 2mM EDTA in PBST and again with PBST. The sections were either mounted on slides with ImmunoHistoMount medium (Abcam) or post-fixed with 5% formaldehyde before wax embedding and sectioning (Advanced Molecular Pathology Lab, IMCB, Singapore).

#### Preparation of Cas9 mRNA and guide RNA

pT3TS-nCas9n was a gift from Wenbiao Chen (Addgene plasmid #46757). The plasmid was linearized with XbaI digestion and purified using a GeneJET PCR Purification Kit (Thermo Scientific). Cas9 mRNA was obtained by *in vitro* transcription using the mMESSAGING

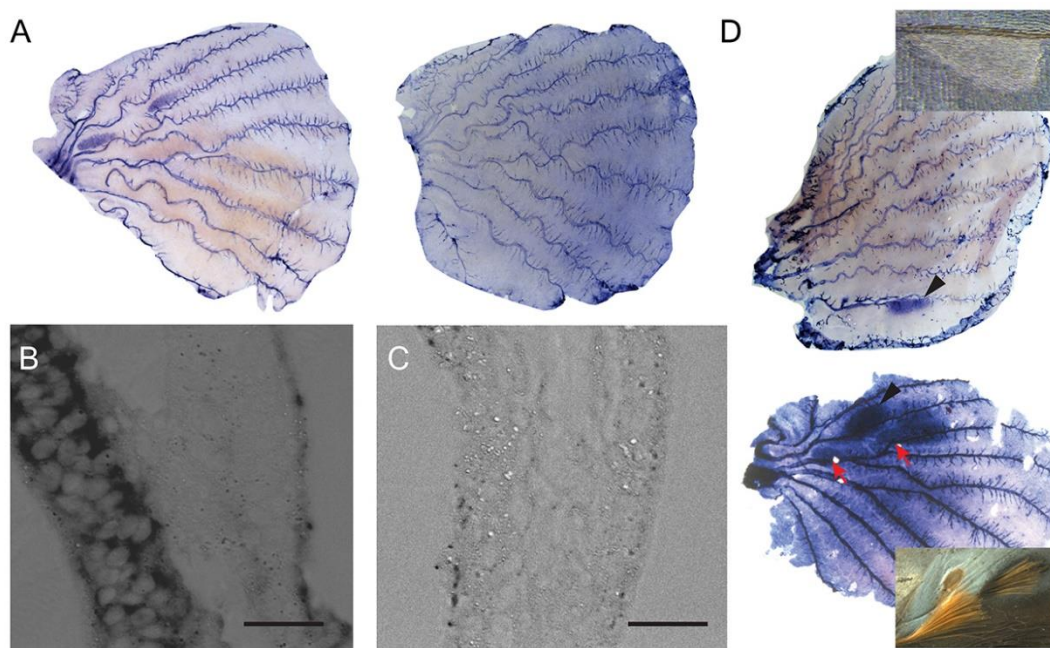
mMACHINE T3 kit (Ambion), tailed using the Poly(A) Tailing Kit (Ambion) and purified by lithium chloride precipitation. The guide RNA templates were prepared using a PCR based method according to (28). The candidate targets were manually designed by searching for a GGN<sub>18</sub>NGG sequence on the sense or anti-sense strand of *apA* and *apB*, preferably targeting the LIM and homeobox domains of the transcription factor (Table S1). They were blasted against the *B. anynana* genome on LepBase.org to check for off-target effects. The template DNA sequence was used to perform an *in vitro* transcription using T7 RNA polymerase (Roche) at 37°C overnight, purified by ethanol precipitation and re-suspended in DEPC treated water.

### Microinjections

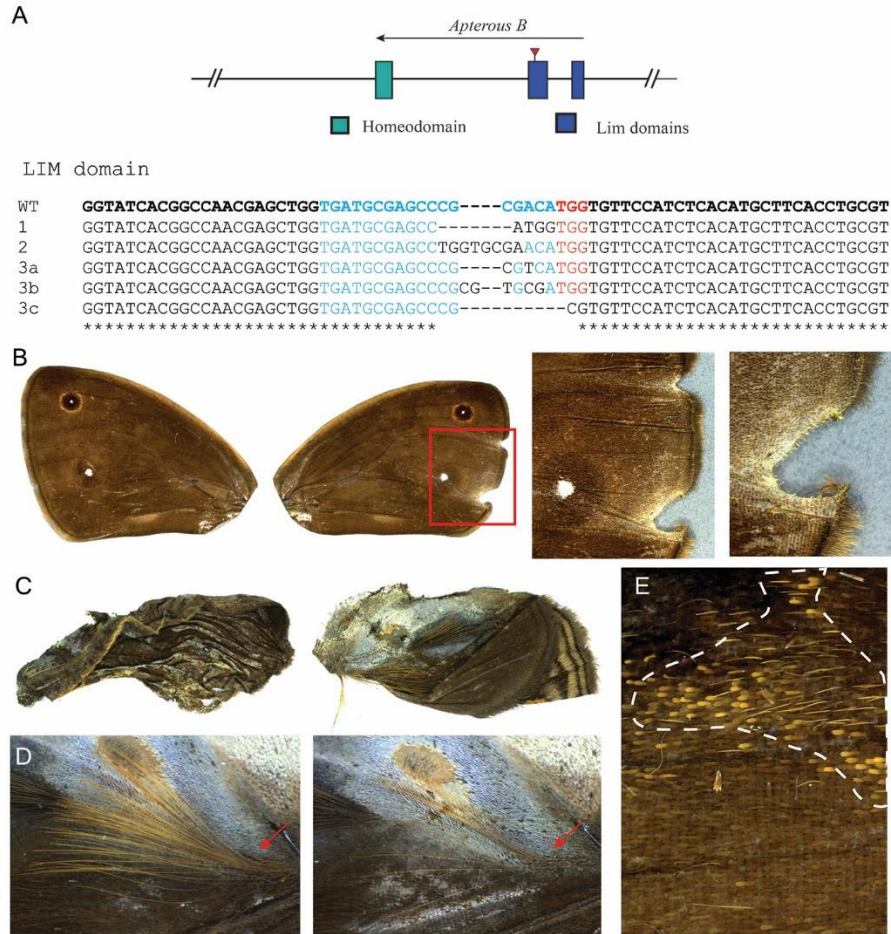
Eggs were collected on corn leaves within one to two hours of egg laying and were arranged on thin strips of double-sided tape on a petri dish. Cas9 mRNA and guide RNAs were mixed along with green food dye (1:80) (Table S2) and injected into the eggs with a Borosil glass capillary (World Precision Instruments, 1B100F-3) using a Picospritzer II (Parker Hannifin). A piece of wet cotton was placed in the petri dish and the eggs were allowed to develop in an incubator at 27°C and high (~80%) humidity. Hatched caterpillars were placed on young corn plants using a brush. Adults that emerged were scored for their phenotypes (Table S2).

### Sequencing and genotyping mutants

Genomic DNA was extracted from leg tissues of mutant individuals using the E.Z.N.A Tissue DNA Kit (Omega Bio-tek). The region surrounding the target sequence was amplified by PCR, purified by ethanol precipitation, and used to check for presence of mutations using the T7 endonuclease I (T7EI) assay. Sequences from individuals with disruptions at the targeted regions were cloned into a PGEM-T Easy vector (Promega) and sequenced.



**Figure S1: *apB* and *dsx* mRNA localization in developing wing discs of *Bicyclus anynana*** A) Male (left) and female (right) hindwing discs (28 hours after pupation) showing *apB* mRNA localization. Up-regulated *apB* expression in the hair-pencil regions is seen only in male wings. B) Cross-sectional view of a developing wing disc showing dorsal-specific *apB* expression. C) Cross-sectional view of a developing wing disc stained with *apB* sense probe as control. Scale bar for B and C is 20 $\mu$ m. D) *dsx* mRNA localization in a male forewing and hindwing (~30 hours after pupation) with upregulation in future androconial (arrowheads) and hair-pencil regions (red arrows). Insets show the androconia and hair-pencils in adults.



**Figure S2: CRISPR/Cas9 mosaic wing pattern phenotypes of *apB* knockout** A) Top: Region of the *apB* gene in *B.anymana* targeted using the CRISPR/Cas9 system Bottom: Sequences of the LIM domain region of mutant individuals compared with the wildtype sequence in bold. Blue is the region targeted and the PAM sequence is in red. Deletions are indicated with '-'. B) The forewings of a mutant individual showing differences in shape and marginal defects of the right wing as compared to the left. The boxed area is expanded to the right. C) *apB* knockout mosaic phenotype with overall wing development affected. D) A mutant with reduced number of hair-pencils (right) as compared to a control (left). Red arrows indicate the base of the hair-pencils. E) Mosaic phenotype seen on the dorsal surface with a greater density of ventral-like light colored scales. The clone is indicated with a dashed white line.

**Table S1:** List of primers and guide RNA sequences used in this study

Gene	Primer Name	Primer Sequence
<i>Apterous A</i> ( <i>ApA</i> )	AM 31	Forward 5' CGGGAGGCCTGTCTTCTGGC 3'
	AM 32	Reverse 5' CGTCCGAGCTGGTGATGAGGG 3'
<i>Apterous B</i> ( <i>ApB</i> )	AM 136	Forward 5' CGAACAGTTGAATGCGTATTG 3'
	AM 137	Reverse 5' GGCCACTTTTCTCTTTCTTGG 3'
<i>Doublesex</i> ( <i>dsx</i> )	AM 322	Forward 5' CAGAGCATAGCACAGCACACGTC 3'
	AM 323	Reverse 5' CCACTATTCGTGGGAGATGATGCC 3'
<i>ApA</i> Homeodomain CRISPR Guide	AM 158	5'GAAATTAATACGACTCACTATAGGAGCTGGTGATGCTT GAAGCGTTTTAGAGCTAGAAATAGC 3'
<i>ApA</i> LIM domain CRISPR guide	AM 235	5'GAAATTAATACGACTCACTATAGGAGAAACAGTGCACA TGAAACACGTTTTAGAGCTAGAAATAGC3'
<i>ApB</i> LIM domain CRISPR guide	AM 145	5'GAAATTAATACGACTCACTATAGGTGATGCGAGCCCGC GACAGTTTTAGAGCTAGAAATAGC3'
<i>ApA</i> Homeodomain Genotyping	AM 194	Forward 5' CATTTTTGCGACACGAGACGTC 3'
	AM 167	Reverse 5' CTAAGTGTCTCGACTATATG 3'
<i>ApA</i> LIM domain CRISPR Genotyping	AM 257	Forward 5' GTACAGTAATTAGTTCATCAAAC 3'
	AM 258	Reverse 5' CTTTTAGTTGTGTGCATTTTAAG 3'
<i>ApB</i> LIM domain CRISPR Genotyping	AM 385	Forward 5' CACTAGATTAGCCTAAGGTC 3'
	AM 386	Reverse 5' CTGTTTTGTAGGAGAAATATGG 3'

**Table S2: CRISPR/Cas9 injection concentrations and mutation frequencies**

Guide	Guide RNA Conc (ng/ul)	Cas9 mRNA Conc (ng/ul)	Number of eggs injected	Number of adults	Mutation Rate (%) <sup>+</sup>
<i>ApA</i>	360	600	631	9	44 (4/9)
Homeodomain	450	900	882	35	42.8 (15/35)*
<i>ApA</i>					
LIM Domain	400	900	424	17	47 (8/17)
<i>ApB</i>					
LIM Domain	400	900	228	45	26 (12/45)

+ The number within brackets is individuals with mutant wing phenotypes/total number of adults

\* 4 of the 15 mutant individuals were pupae with wings missing from one side as shown in Figure 3F