A female alternative life-history strategy arose via novel recruitment of homeobox gene, *BarH-1*

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Colias butterflies (the “clouded sulphurs”) often occur in mixed populations where females exhibit two color morphs, yellow/orange or white. White females, known as Alba [A-], reallocate resources from colored pigment synthesis to reproductive and somatic development. Due to this tradeoff Alba females develop faster and have higher fecundity than orange females. However while colored pigments are costly to produce, males preferentially mate with orange females and transfer nutrient rich spermatophores during mating. Thus the wing color morphs represent alternative life history strategies (ALHS) that are female-limited, wherein tradeoffs, due to divergent resource investment, result in distinct phenotypes with associated fitness consequences. Here we map the genetic basis of Alba in *Colias croceus* to a transposable element insertion downstream of the *Colias* homolog of *BarH-1.* We use CRISPR/Cas9 to validate *BarH-1*’s functional role in the wing color switch and antibody staining confirms expression differences in the scale building cells of pupal wings. We use scanning electron microscopy to determine that *BarH-1* expression in the wings causes a reduction in pigment granules within wing scales, and thereby gives rise to the white color. Further lipid and transcriptome analyses reveal additional physiological differences that arise due to Alba,

suggesting pleiotropic effects beyond wing color. While male-limited ALHS are well documented, comparatively few examples of female-limited ALHS are known. This is either due to biological reality or our lack of understanding of how ALHS manifest in females, highlighting the need for mechanistic insights, as none currently exist. These findings provide, to our knowledge, the first known mechanism for a female ALHS and support an alternative view of color polymorphism as indicative of pleiotropic effects with life history consequences.

Evolutionary theory predicts that positive selection will remove variation from natural populations, as genotypes with the highest fitness go to fixation [1]. However, across diverse taxa ALHS are maintained within populations at intermediate frequencies due to balancing selection[2]. Theoretical modelling and mechanistic insights have advanced our understanding of ALHS evolution and maintenance (e.g. negative - frequency dependent selection)[3]. However, the majority of studies, and consequently our insights, are biased toward male strategies that are morphologically dramatic (e.g. ruff [4,5] and side-blotched lizards [6]). Whether this bias reflects true differences in the frequency of alternative strategies between the sexes or is simply an artifact is unknown [7]. As trade-offs and selection regimes are often sex specific, the lack of female insights severely limits our understanding of the mechanisms, maintenance, evolution, and co-evolution of alternative strategies in general [7]. Yet despite calls for further investigation, to our knowledge no genetic mechanism for a female limited ALHS has been identified. Here we identify one such mechanism in the butterfly *Colias croceus* (Pieridae).

Approximately a third of the nearly 90 species within the butterfly genus *Colias* exhibit a female-limited ALHS known as Alba [8]. The switch between strategies is controlled by a single, autosomal locus that causes Alba females to reallocate guanosine triphosphate (GTP), amounting to several percent of their nitrogen budget, from the synthesis of pteridine pigments to other areas of development [9]. Consequently, Alba females have white wings, while non-Alba females are orange/yellow (Figure 1A). As a result of this trade-off, Alba females gain fitness advantages over orange females due to faster development as pupa, larger fat body stores, and significantly more mature eggs at eclosion [10]. Despite these developmental advantages and the dominance of the A- allele, Alba remains polymorphic due to tradeoffs in abiotic and biotic factors [10-14]. For example, Alba's development rate advantage is higher only in cold temperatures and as a result of density-dependent, interference competition with other white Pierid species and sexual selection, males preferentially mate with orange females [10-13]. The

mating bias likely has significant fitness costs for Alba because males transfer essential nutrients during mating and multiply mated females have increased fecundity and longevity [15,16]. Field studies confirm Alba frequency and fitness increases in species that inhabit cold and nutrient poor habitats, where the occurrence of other white Pierid butterflies is low, while in warm environments with nutrient rich host plants and a high co-occurrence with other white species, orange females exhibit increased fitness and frequency [12-14].

Using a *de novo* reference genome for *C. croceus* that we generated via Illumina and PacBio sequencing (supplementary materials and supplementary table ST1), and three rounds of bulk segregant analyses (BSA) using whole genome sequencing of a female and two male informative crosses for Alba, we mapped the Alba locus to a ~3.7 Mbp region (supplementary materials, supplementary figure SF1, & supplementary table ST2). Then, with whole genome re-sequencing data from 15 Alba and 15 orange females from diverse population backgrounds, a SNP association study was able to fine map the Alba locus to a single ~430 kb contig that fell within the ~3.7 Mbp BSA locus (Figure 1B)(supplementary materials & supplementary table ST3). The majority of SNPs significantly associated with Alba (70 of 72) were within or flanking a *Jockey-like* transposable element (TE) (Figure 1B&C). We determined the TE insertion is unique to the Alba morph in *C. croceus* by assembling the orange and Alba haplotypes for this region, then quantifying differences in read depth between morphs within and flanking the insertion, and comparing the region to other butterfly genomes (*Danaus plexippus* & *Heliconius melpomene*) (supplementary materials and supplementary materials & supplementary figure SF2, SF3, & SF4)

The Alba specific TE insertion was located ~30 kb upstream of a *DEAD-box helicase*, and ~6kb downstream of *BarH-1*, a homeobox transcription factor (Figure 1C). *BarH-1* is an intriguing find as its knockout in *Drosophila melanogaster* causes a dramatic decrease in pigment granules within the eye, changing eye color from red (wild type) to white [17]. To validate the functional role of *BarH-1* in the Alba phenotype we generated CRISPR/Cas9-mediated deletions of exons 1 and 2 in a mosaic knockout approach (supplementary materials, supplementary tables ST4 & ST5, and supplementary figure SF5). *BarH-1* deletions gave rise to a mosaic lack of pigmentation in the eyes of males and females of both morphs, consistent with *BarH-1*'s expected role in insect eye development (Figure 1D). Additionally, on the dorsal side of the wings, females with an Alba genotype exhibited a white/orange color mosaic, while males and

orange females displayed no wing KO phenotypes, despite those individuals exhibiting mosaic phenotypes in the eye. (Figure 1E).

To further investigate the role of BarH-1 in developing wing scales, we used *in situ* hybridization of BarH-1 on wings from 2 day old pupae of orange and Alba females of *C. croceus*, as well as *Vanessa cardui*. The BarH-1 protein is highly expressed in the scale building cells of both species (Fig. 2), suggesting a previously undescribed role of BarH-1 in the developing wing scales of butterflies. Comparison between orange and Alba females of *C. croceus* further documents Alba as a gain of BarH-1 function, as scale building cells in the developing wing show a BarH-1 expression pattern that is Alba limited (Figure 2).

In butterflies both pigments and scale morphology can affect wing color [18] and while Alba females exhibit large reductions in colored pteridine pigments compared to orange [9], whether morphs differed in wing scale morphology was unknown. Using scanning electron microscopy (supplementary materials) we found Alba scales exhibited significantly less pigment granules, the structures that store pteridine pigments in Pierid butterflies [19], compared to orange (t_3 = -4.55, P = 0.016), suggesting reduced granule formation as the basis of the Alba color change (Figure 3A,B). As expected, pigment granules were also significantly reduced ($t_{5.45}$ = 10.78, $p < 0.001$) in the white regions of the CRISPR/Cas9 *BarH-1* KO individuals (Figure 3C,D), demonstrating that *BarH-1* is affecting pigment granule formation to give rise to Alba. To further test whether pigment granule reduction alone was sufficient for the orange/white color change, we chemically removed the pigment granules from the wing of an orange *C. croceus* female, resulting in formerly orange regions turning white likely due to the scattering of light from remaining non-lamellar microsctructures on the wing (Figure 3 E)[20]. Thus, the white wings of Alba *C. croceus* (Figure 1A, 3A) differ from other white Pierid species, as the latter exhibit abundant pigment granules in their scales (Figure 3F and supplementary figure SF6), documenting that there are multiple routes to white wing color in Pieridae.

We next tested whether the physiological tradeoffs of Alba reported for New World species [9,10], which were discussed in the introduction, were also seen in *C. croceus*, an Old World species, as shared tradeoffs would suggest the Alba mechanism is conserved genus wide. To compare abdominal lipid stores between morphs, we conducted high performance thin layer chromatography on 2 day old adult females reared under two temperature treatments (Hot: 27^{o}C vs. Cold: 15^{o}C during pupal development, supplementary materials). We found Alba

females had larger abdominal lipid stores than orange in both temperature treatments, though the difference was only significant in the cold treatment (cold: $t_{28.7} = 3.30$, $P = 0.003$, hot: $t_{22.5} = 0.45$, $P = 0.66$) (Figure 4A), consistent with the known effects of temperature on Alba fitness in New World *Colias* species [9].

We then conducted RNASeq on pupal wing and abdomen tissue, at the time of pteridine synthesis (i.e. when allocation tradeoffs are likely realized) to identify genes that exhibited differential expression between morphs (supplementary materials & supplementary tables ST6, ST7). We found that *vitellogenin 1*, which encodes an egg yolk precursor protein synthesized in the fat bodies of insects [21], was significantly upregulated within Alba abdomen tissue (logFC of 4.8) (Figure 4B). Additionally, consistent with previous reports of GTP reallocation in Alba females [9], *RIM*, a Rab GTPase effector [22], was one of the most highly differentially expressed (DE) genes in both tissues (log Fold Change (FC) increase in Alba of 3.4 in the abdomen and 5.1 in the wings) (Figure 4B,C). RIM acts as a molecular switch by converting guanosine diphosphate to GTP, thereby activating its associated Rab GTPase, which is in turn involved in synaptic vesicle exocytosis and secretory pathways [23]. These results are consistent with previous qualitative findings of Alba females in the North American species *C. eurytheme* (Alba females have larger fat body stores, emerge from the pupa with significantly more mature eggs, and reallocate GTP from pigment synthesis to somatic development [9,10]). Our findings thus quantitatively demonstrate that the trade-offs associated with the Alba ALHS are likely consistent across the *Colias* genus, suggesting that Alba may be due to the same genetic mechanism and corroborating previous work that proposed Alba is homologous across *Colias* [8].

Gene set enrichment analyses identified 85 functional categories that were significantly enriched in the abdomen tissue of Alba females (supplementary materials, supplementary table ST8 & ST9, supplementary figures SF7 & SF8), notably downregulation of 'positive regulation of GTPase activity' (adjusted p value < 0.0001), 'regulation of Notch signalling pathway' (adjusted p value = 0.03), and 'canonical Wnt signalling' (adjusted p value < 0.01). While the Wnt pathway is known to regulate wings patterns in several butterfly species [24,25], these findings are curious as they are observed in abdomen tissue rather than wing, suggesting potential unexplored pleiotropic effects of these pathways outside of the wing. In wing tissue, 35 functional categories were significantly enriched and downregulated in Alba including 'regulation of transcription' (adjusted p value < 0.0001) and 'positive regulation of GTPase activity' (adjusted p value < 0.0001), while 'protein catabolic process' (adjusted p value < 0.0001) was upregulated

(supplementary figures SF7 & SF8). *BarH-1* was not DE between morphs in our RNASeq data, suggesting that morph specific expression differences are temporal and likely occur earlier in development. Further functional studies of candidate genes are needed to better understand their mechanistic roles in the trade-off.

Here we report that the genetic basis of a female-limited ALHS arises from the co-option of the homeobox transcription factor *BarH-1*, primarily known for its role in the morphogenesis of the insect eye, neurons and leg segments [26]. We document that *BarH-1* has a similar function in eye morphogenesis of butterflies, and also find it is expressed during wing scale development in butterflies from the families Pieridae and Nymphalidae, which last shared a common ancestor > 70 million years ago. This novel finding suggests a conserved function of *BarH-1* in scale morphogenesis that warrants further study and suggests a parsimonious route to *BarH-1*'s gain of function in the ALHS Alba phenotype, with co-option from a role in wing scale development rather than its previously described functions. *BarH-1*'s well characterized role in determining cell fate through gene repression [27] suggests it is involved in the repression of pigment granule formation, providing an explanation for the Alba allele being dominant and a gain of function that results in the absence of a phenotype (i.e. orange wing color). To what extent *BarH-1* has an active pleiotropic role in other tissues or developmental stages remains to be determined, as the extensive physiological responses we document could easily arise from a simple reallocation following the absence of pigment granule formation. Given the emergence of "toolkit" genes for butterfly wing patterning, wherein specific genes have been found to be repeatedly involved in wing color variation across distant species (e.g. *cortex* [28]), determining to what extent BarH-1 is involved in other wing phenotypes and ALHS is of interest, especially given the pleiotropic effects on life history documented here. Finally, our results and others (e.g. side-blotched lizards [29] & damselflies [30]) suggest that investigating to what extent ALHS are associated with color variation in other systems is warranted, especially in cases where such variation is female limited.

Author Contributions

AW conducted butterfly rearings and lab work, analysed the data, conducted the electron microscopy, and wrote the manuscript with CWW and input from the coauthors. MWP, KT, CWW, and AW conducted the CRISPR/Cas9 knockout experiment. RN and JH assisted with bioinformatics. PL and RK conducted HPTLC and PL and AW analyzed the data. AW, CS,

CWW and OB conducted fieldwork. MC conducted lab work. CWW supervised the work at all stages.

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Figure 1. Color variation in *Colias croceus* and the genetic mechanism of Alba. (A) *Colias croceus* male, orange female, and Alba female (left to right). (B) SNPs significantly associated with the Alba phenotype (red) within the ~3.7 Mbp Alba locus identified via 3 rounds of bulk segregant analysis. Contigs in this region shown as alternating dark and light blue. (C) The location of Alba associated SNPs (red) on the ~430 kb outlier contig identified in the GWAS. Gene models for the DEAD-box helicase, the Jockey-like transposable element, and *BarH-1* shown at the top of the panel. (D) Loss of green color in the *C. croceus* eye following *BarH-1* mosaic KO. KO regions are black. (E) Orange color is seen on the dorsal forewing (upper) and hindwing (lower) of an Alba female following *BarH-1* mosaic KO.

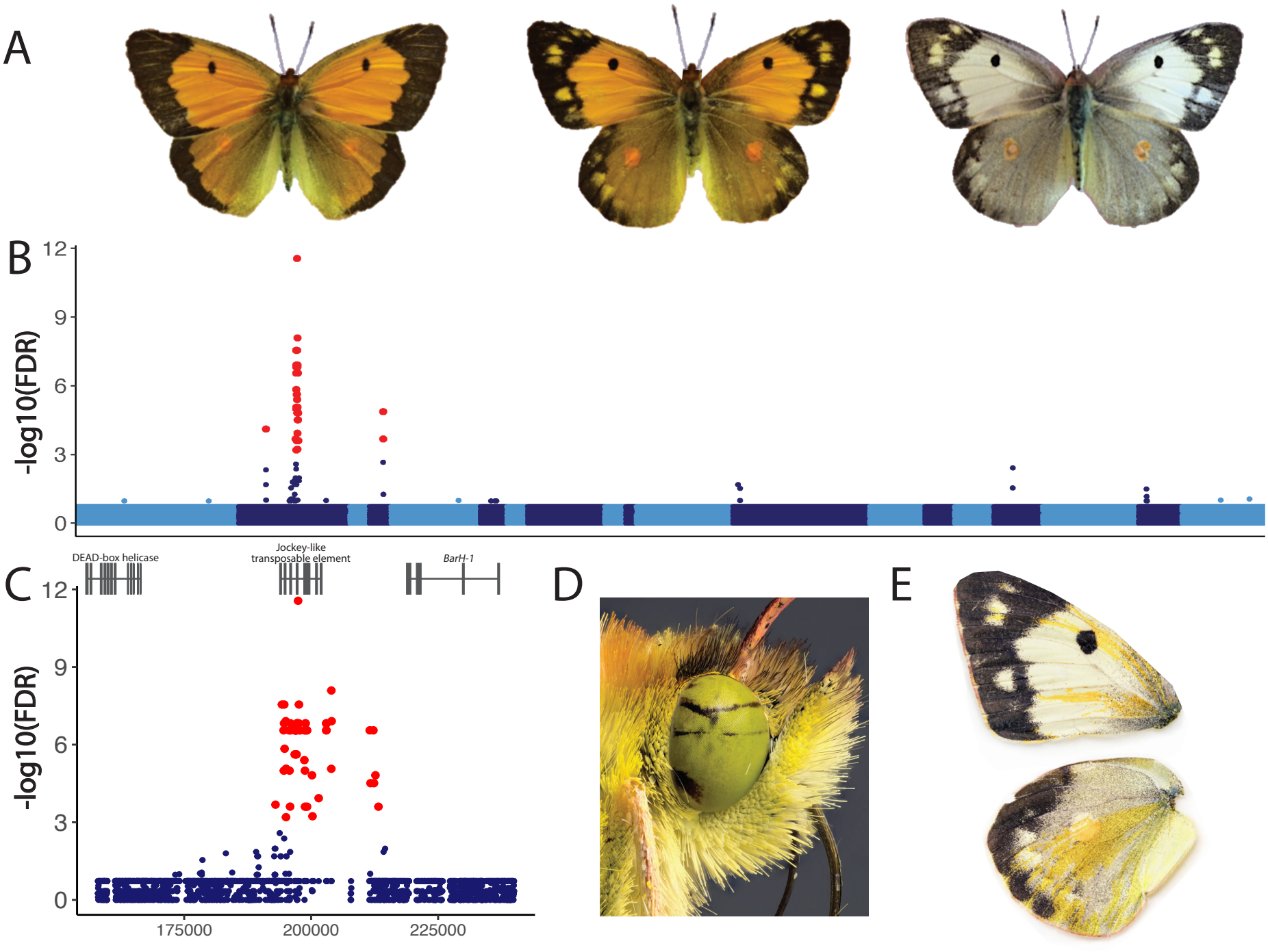
Figure 2. BarH-1 antibody staining in *C. croeus* and *V. cardui* pupal wings. (A) Depiction of approximate location of antibody images on the *C. croceus* Alba forewing and the scales within the region. (B) DAPI (nuclei) and BarH-1 staining within the scale building cells within the black margin region of the Alba forewing. BarH-1 is expressed in melanic Alba scale building cells. (C) DAPI (nuclei) and BarH-1 staining of white regions of the Alba forewing. Large nuclei are scale building cells while small nuclei are epithelial cells. Antibody staining shows BarH-1 expression in the white Alba scale building cells. (D) Depiction of approximate location of antibody images on the *C. croceus* orange forewing and the scales within the region. (E) DAPI (nuclei) and BarH-1 staining of orange regions of the orange forewing. Large nuclei are scale building cells while small nuclei are epithelial cells. Antibody staining shows a lack of BarH-1 expression in the orange scale building cells. (F) Depiction of approximate location of antibody images on the *C. croceus* orange hindwing and an illustration of the scale heterogeneity found within the region. (G) DAPI (nuclei) and BarH-1 staining of the orange hindwing. Large nuclei are scale building cells while small nuclei are epithelial cells. Antibody staining shows heterogenity in BarH-1 expression in the scale building cells within this region presumably corresponding to the variation in scale color, where melanic scale building cells express BarH-1, but orange do not. (H) Hind and fore wing of *V. cardui.* (I) DAPI (nuclei) and BarH-1 staining of *V. cardui* . Both the scale building and socket cells can be observed and express BarH-1.

Figure 3. Butterfly forewings and scanning electron microscope (SEM) images of their wing scale microstructures. (A) A WT Alba female wing and SEM of one of its wing scales, showing the near absence of pigment granules. (B) A WT orange female wing and SEM, showing an abundance of pigment granules. (C) An Alba female that is a *BarH-1* mosaic KO, SEM images illustrate significant differences in the number of pigment granules in Alba (C) and orange (D) mosaic regions. These differences are consistent with those observed in WT animals. (E) Dorsal forewing of an orange female where pigment granules were chemically removed from distal ½ of wing. SEM inset showing absence of pigments in the white region. (H) A *Pieris brassicae* female

forewing, with SEM showing abundance of pigment granules despite its white color, indicating there are multiple routes to white wing color within Pieridae.

Figure 4. Physiological differences between female morphs. A) The mass corrected total neutral lipid content for female morphs in two temperature treatments. Alba females, on average, have larger neutral lipid stores, however there is an interaction between morph and temperature as the difference is only significant in the cold treatment. B) Volcano plots visualize gene expression differences between female morphs in abdomen tissue. Each point is a gene. Grey circles indicate not significantly DE between morphs, while blue circles are significantly DE. The black circle is *vitellogenin1* and the black triangle is *RIM*. The X-axis is the log of the fold change (FC), positive log(FC) indicates the gene is upregulated in Alba individuals. C) Volcano plots visualize gene expression differences between female morphs in wing tissue. Color coding, shapes, and axes are described above.

Figure 1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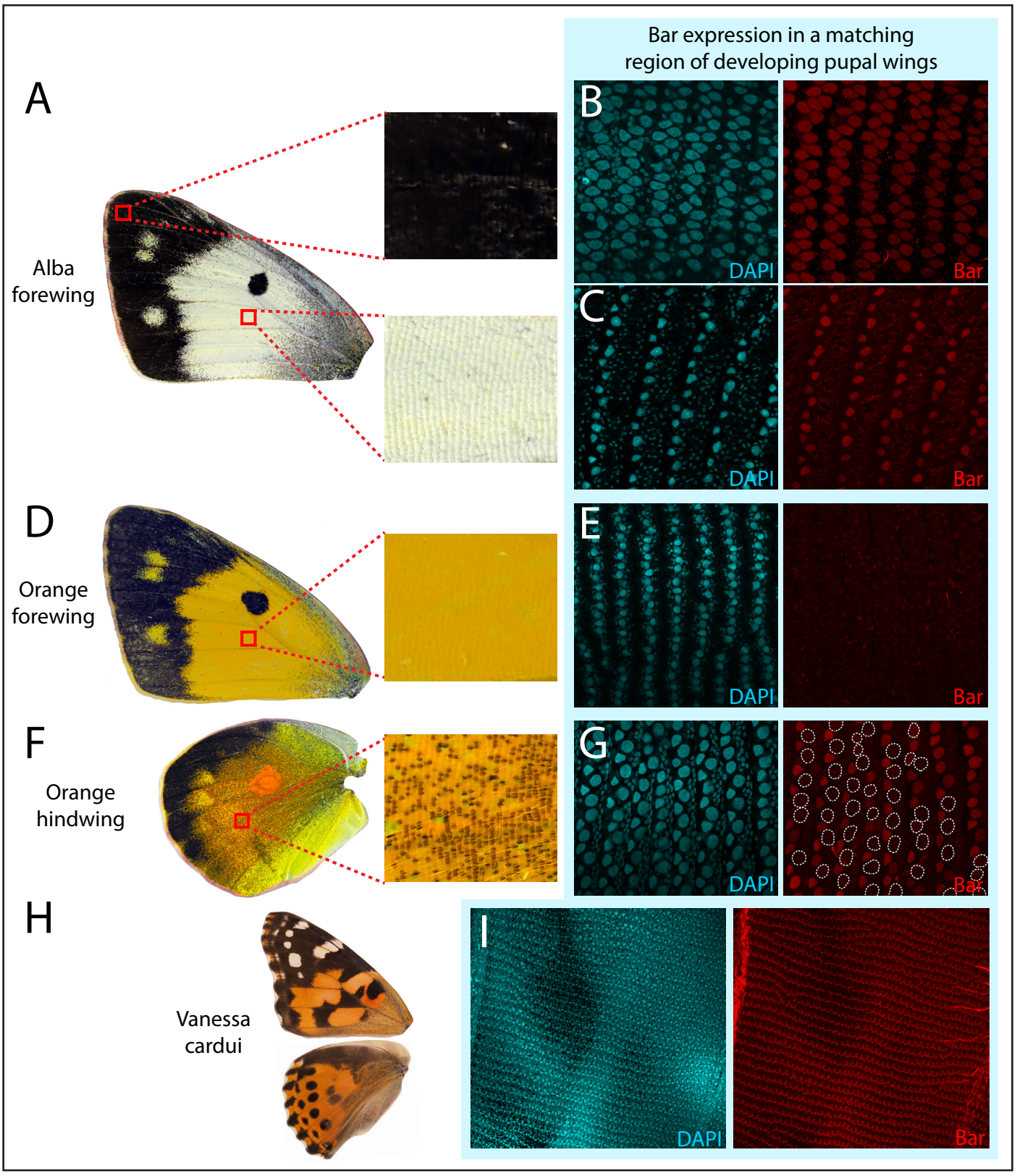

Figure 2

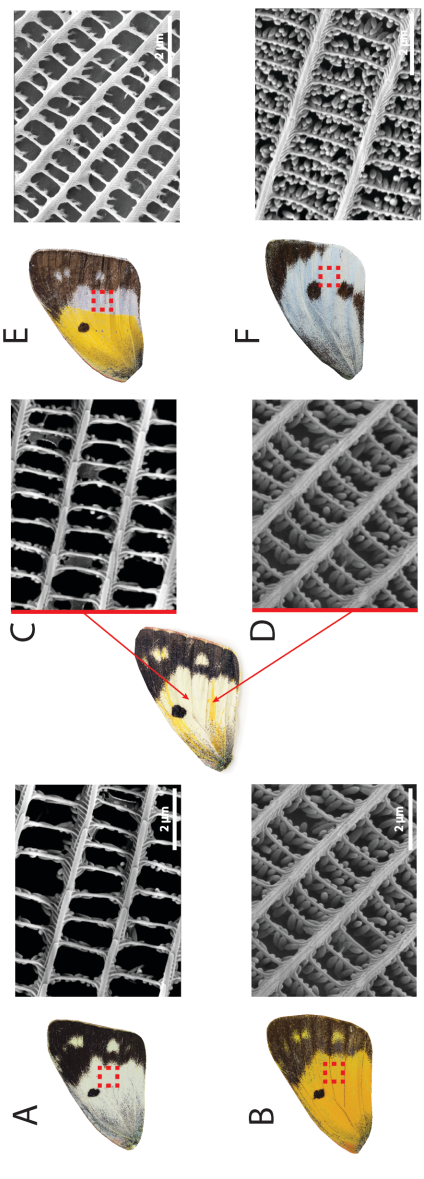

Figure 3

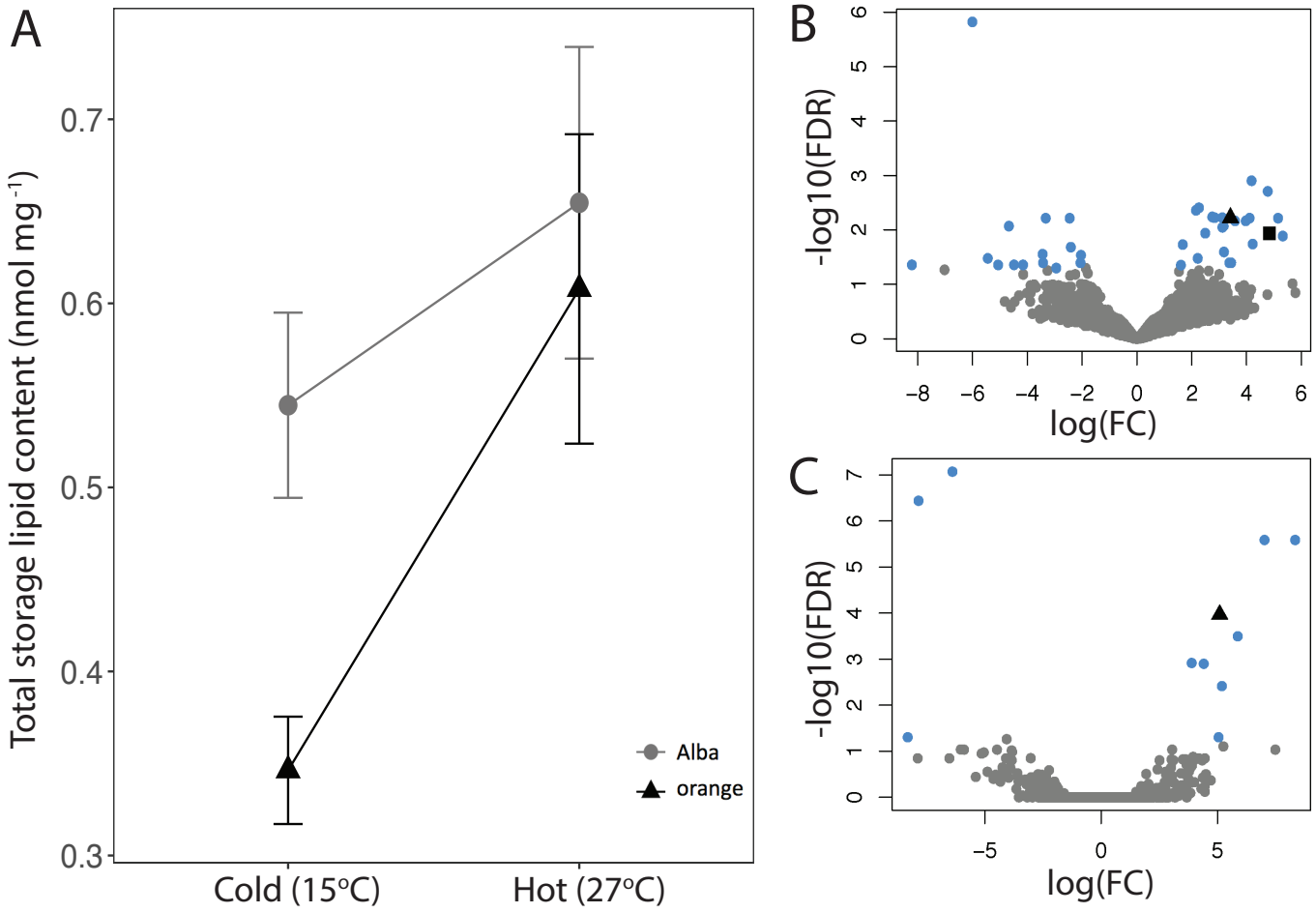
A
Total storage lipid content (nmol mg-1)
0.7
0.6
0.5
0.4
0.3
Cold (15°C)
Hot (27°C)
Alba
orange
B
-log10(FDR)
log(FC)
C
-log10(FDR)
log(FC)

Figure 4