








A genetic switch for male UV iridescence in an incipient species pair of sulphur butterflies

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Edited by Douglas Futuyma, Department of Ecology and Evolution, Stony Brook University, Stony Brook, NY; received May 21, 2021; accepted November 19, 2021

Mating cues evolve rapidly and can contribute to species formation and maintenance. However, little is known about how sexual signals diverge and how this variation integrates with other barrier loci to shape the genomic landscape of reproductive isolation. Here, we elucidate the genetic basis of ultraviolet (UV) iridescence, a courtship signal that differentiates the males of *Colias eurytheme* butterflies from a sister species, allowing females to avoid costly heterospecific matings. Anthropogenic range expansion of the two incipient species established a large zone of secondary contact across the eastern United States with strong signatures of genomic admixtures spanning all autosomes. In contrast, Z chromosomes are highly differentiated between the two species, supporting a disproportionate role of sex chromosomes in speciation known as the large-X (or large-Z) effect. Within this chromosome-wide reproductive barrier, linkage mapping indicates that *cis*-regulatory variation of *bric a brac* (*bab*) underlies the male UV-iridescence polymorphism between the two species. *Bab* is expressed in all non-UV scales, and butterflies of either species or sex acquire widespread ectopic iridescence following its CRISPR knockout, demonstrating that *Bab* functions as a suppressor of UV-scale differentiation that potentiates mating cue divergence. These results highlight how a genetic switch can regulate a pre-mating signal and integrate with other reproductive barriers during intermediate phases of speciation.

evo-devo | speciation | UV iridescence | large-Z effect | genetic coupling

Premating signals such as pheromones, calls, and displays often differ between sexes and species and, by helping animals to tell one another apart, they are integral to the formation of reproductive barriers during speciation itself (1, 2). Mating factors can diverge early in the speciation process due to local adaptation or later due to sexual selection that prevents the generation of unfit hybrids (3). While a coupling of pre-mating and postmating isolation mechanisms is thought to be required for the completion of speciation (4), how mating cue variation actually coincides with other barrier loci to split lineages remains elusive in the empirical literature (5–7).

Previous work on the genetics of hybridization between the sulphur butterflies *Colias eurytheme* and *Colias philodice* highlights their potential for the study of intermediate phases of speciation with gene flow. Initially restricted to the western United States, the range of *C. eurytheme* expanded following both the spread of agricultural alfalfa and the reduction in forest cover in the past 200 y into regions once limited to *C. philodice* (8). As a result, the two species occur in secondary sympatry throughout an anthropogenic contact zone that includes the eastern United States and southern Canada. Both pre- and postzygotic reproductive barriers maintain species status in this system. However, heterospecific matings happen at increased frequency in dense populations (9, 10), partly because males can locate newly emerged females incapable of performing mate rejection behaviors [teneral mating (11)].

Hybrid female sterility forms an intrinsic postzygotic barrier that affects one of the two heterospecific crosses: oogenesis fails in female offspring that inherit a *C. eurytheme* W chromosome and a *C. philodice* Z chromosome (12, 13). This incompatibility is sex-linked and implies that to produce fully fertile progeny, *C. eurytheme* females must select males that are homozygous for a conspecific Z chromosome. An iridescent ultraviolet (UV) pattern acts as a visual mating cue in males and accurately displays their Z-chromosome status to females (9, 14) (Fig. 1A and *SI Appendix*, Fig. S1). UV occurs on the dorsal wing surfaces of *C. eurytheme* males only. The Mendelian *U* locus controls this interspecific variation and was previously mapped to the Z chromosome (15): *C. eurytheme* homozygous recessive males are UV-iridescent (*u/u*), advertising two compatible Z chromosomes to *C. eurytheme* females. Incompatible mates such as *C. philodice* males (*U/U*) and heterozygous hybrids (*U/u*) bear the dominant allele and lack UV. Finally, the female preference trait itself is also linked to the Z chromosome (14). This Z-linked inheritance of genetic incompatibility, mating signal, and mating preference supports an “indicator” model of speciation, which was previously theorized as a system where the mating cue can signal species identity and enable selection against hybrids (3, 16). In this study, we examine the genomic footprint

Significance

Incipient species are at an intermediate stage of speciation where reproductive isolation is counteracted by the homogenizing effects of gene flow. Human activity sometimes leads such species to reunite, as seen in the Orange Sulphur butterfly, which forms large hybridizing populations with the Clouded Sulphur in alfalfa fields. Here we show that sex chromosomes maintain these species as distinct, while the rest of their genome is admixed. Sex chromosomes notably determine which males display to females a bright, iridescent UV signal on their wings. Genetic mapping, antibody stainings, and CRISPR knockouts collectively indicate that the gene *bric a brac* controls whether UV-iridescent nanostructures develop in each species, illustrating how a master switch gene modulates a male courtship signal.

Author contributions: A.H.P., B.A.C., and A.M. designed research; V.F., J.J.H., L.S.L., C.M.F., A.R., A.H.P., and A.M. performed research; K.T. and C.W.W. contributed new reagents/analytic tools; J.J.H., B.A.C., and A.M. analyzed data; and V.F., J.J.H., and A.M. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at <http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2109255118/-/DCSupplemental>.

Published January 10, 2022.

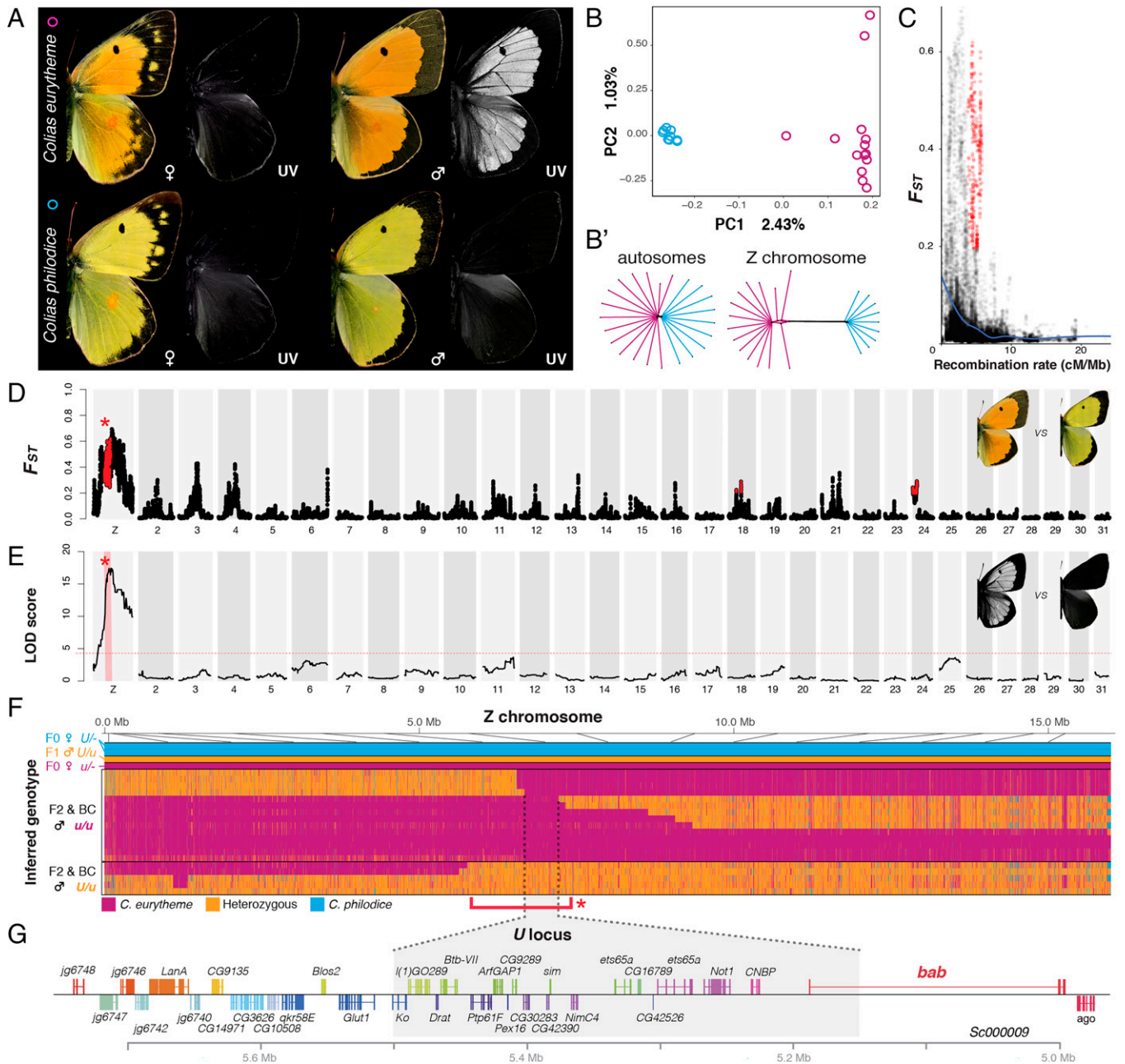


Fig. 1. Large-Z architecture of species differentiation includes the *U*-locus candidate gene *bab*. (A) UV iridescence differentiates males from two incipient species. (B and B') PCA (B) and distance-based phylogenetic network (B') of 22 male whole-genome SNPs from the admixed Maryland population. (C and D) F_{ST} values for *C. philodice* vs. *C. eurytheme* plotted against recombination rate (C), and Manhattan plot (D). Red indicates windows with above-median recombination rate and in the 95th percentile of F_{ST} , including on the Z chromosome (asterisk). (E) Quantitative trait locus (QTL) analysis of the presence/absence of UV in 252 male offspring from F₂ and BCs. (F) Genotype plot for the whole Z chromosome with resequencing data from 23 individuals. Each row is an individual, and each column is a color-coded SNP. The red bracket indicates a 2.5-Mb interval with high F_{ST} and above-median recombination rate. (G) Annotation of the *U*-locus zero-recombinant interval (box) and surrounding region.

of sex-linked reproductive barriers, and fine-map the allelic variation that switches on the male UV signal in *C. eurytheme*.

Results

The Z Chromosomes Define Species Barriers in Secondary Sympatry.

To test a putative role of the Z chromosome as a barrier locus, we conducted a genome scan on 24 males from a sympatric population in Maryland, USA, where *C. eurytheme* settled in 1927 (17). Two individuals were identified as probable recent hybrids and excluded from further analyses. We retained 13

UV, orange males and 9 non-UV, yellow males which formed two discrete clusters based on genome-wide single-nucleotide polymorphism (SNP) clustering by principal-component analysis (PCA) (Fig. 1C and *SI Appendix*, Fig. S2 and Table S1). The Z chromosome showed a large increase in genetic differentiation when compared with autosomes (*SI Appendix*, Figs. S3 and S4 and Table S2), with a Z:A ratio of 12:1, the highest sex chromosome-to-autosome ratio of F_{ST} reported from a whole-genome dataset (7, 18). Heterogeneous landscapes of genomic differentiation can be explained by local barriers to gene flow or by linked selection in regions of low recombination (7, 19).

To parse these two phenomena, we highlighted windows that were both in the top 5% F_{ST} and had an above-median recombination rate, thereby identifying three regions—two narrow autosomal F_{ST} peaks and a 2.5-Mb portion of the Z chromosome (Fig. 1 C and D). These data show that while only a restricted set of autosomal regions is likely under selection in each population, a large fraction of the Z chromosome is refractory to gene flow in a pattern consistent with a causal role in reproductive isolation. In other words, while Z chromosomes can recombine when hybrid F_1 males are produced in the laboratory, this rarely persists in the field, presumably due to the aforementioned isolating mechanisms that select against hybrids.

In addition, nucleotide diversity (π) was depressed on the Z chromosome in both populations and divergence (d_{XY}) was elevated, supporting the inference that the Z chromosome is highly differentiated (SI Appendix, Fig. S3 B and C'). In most scenarios, one expects π on the Z chromosome to be 75% of π on the autosomes (20). For *C. eurytheme*, π_Z/π_A was 0.751 ± 0.118 , matching this expectation (SI Appendix, Fig. S3F). However, for *C. philodice*, π_Z/π_A was 0.532 ± 0.105 , meaning π_Z was lower than expected, which could reflect a recent selective sweep in *C. philodice*.

The Male Mating Signal Polymorphism Maps to *bab*. The large-Z effect results in extended nonrecombining haplotypes in the natural population that prevent association mapping of trait variation (SI Appendix, Fig. S5). To gain further resolution on the genetic basis of the polymorphic UV signal, we turned to linkage mapping from controlled hybrid crosses. F_2 and backcross (BC) broods showed Mendelian, recessive segregation of the UV state among male offspring (SI Appendix, Fig. S6). We genotyped 484 recombinant males and females using 2b-RAD sequencing, scored UV among the 252 genotyped males, and identified an LOD (logarithm of the odds) interval on the Z chromosome (Fig. 1E and SI Appendix, Fig. S6). We resequenced individuals with recombination events around the *U* locus and refined a 352-kb zero-recombinant window with 18 annotated genes (Fig. 1 F and G and SI Appendix, Tables S4 and S5). This mapping interval includes the 5' intergenic region, promoter, and first exon of the gene *bric a brac* (*bab*), a salient candidate gene as it encodes a transcriptional repressor of male-limited traits in *Drosophila* such as abdominal pigmentation and sex combs (21–27).

Bab Expression Marks Non-UV Scale Cells. To test a role in the regulation of *Colias* male UV, we characterized the expression and developmental functions of *bab* during color scale formation. Butterfly wing scales are macrochaete derivatives that each protrude from a single epidermal cell during pupal development (28). UV scales found in the subfamily Coliadinae are a derived scale type characterized by dense longitudinal ridges; each scale forms a multilayer of chitinous lamellae that selectively reflects UV light by the coherent scattering of incident light (29–32). UV scales are specific to the dorsal wing surface of *C. eurytheme* males, and cover the top of non-UV ground scales (Fig. 2 A and B). Immunofluorescence at the onset of scale emergence reveals Bab expression in the nuclei of all non-UV scale cell precursors (Fig. 2C, SI Appendix, Fig. S7, and Movie S1) regardless of the scale layer (ground, cover), pigment fate (yellow pterins, black melanin), wing surface, and sex. Remarkably, Bab is expressed in ground scales and is absent in the UV cover scales on the dorsal male wing surface. Thus, Bab is negatively correlated with the UV-scale type in *C. eurytheme*: it is consistently expressed in all scale cells fated as non-UV except in the wing cover scales of *C. eurytheme* males. Where it is not expressed, these scales develop layered nanostructures specifically capable of UV-iridescent reflectance. This inactivation in

the sexually dichromatic pattern suggests a repressor function, analogous to the expression of Bab in the *Drosophila* abdominal epithelium (21, 23, 27).

CRISPR Knockouts Yield Ectopic UV Iridescence. To directly test this model, we generated CRISPR-mediated loss-of-function mutations targeting the first exon of the *bab* coding sequence. We collected *C. eurytheme* and *C. philodice* females and micro-injected eggs within 7 h postfertilization. G_0 *bab* crispants showed mosaic phenotypes of high penetrance (51 out of 63 surviving adults), with a widespread gain of UV iridescence in both males and females of both species, including ventral surfaces (Fig. 3 and SI Appendix, Figs. S8–S13). Both pterin and melanin pigment scales and both cover and ground scales differentiated into UV scales following *bab* knockout (KO). Female-specific effects on pigmentation were also noted (SI Appendix, Fig. S11). These loss-of-function assays show that Bab represses the UV identity in all non-UV scale precursors regardless of wing surface, sex, or species. Male UV iridescence is widespread in the *Colias* genus (32), suggesting that the *C. philodice* absence of UV is due to a secondary loss of repression by Bab. The reappearance of UV in *C. philodice bab* crispants (an atavism) implies that the underlying network for producing UV scales is still present in this species.

***bab* Is the Causal Gene for UV-Iridescence Variation in *Colias*.** Linkage mapping of the *U* locus led to a zero-recombination interval of 19 genes (Fig. 1 F and G), and the combined evidence suggests that *bab* is the only gene in this interval that mediates the phenotypic variation. First, the expression pattern of Bab matches the pattern that would be predicted for a repressor of UV scales. This is consistent with the recessivity of the UV-iridescent allele, which would be difficult to explain if the causal gene was an activator of the UV state. Second, mosaic KOs of *bab* result in ectopic UV iridescence, meaning that no other gene in the genome blocks UV iridescence, and thus ruling out the existence of a second repressor at the *U* locus. No fixed amino acid variants of Bab were found between the two species (SI Appendix), suggesting that the mating signal difference involves a noncoding difference. Further experiments will be required to identify *cis*-regulatory elements blocking *bab* in UV-iridescent cover scales, and how it is derepressed in the alternative haplotype.

Discussion

Bab Is a Repressor of Male Dimorphic Traits. Our linkage mapping, expression, and functional assays of UV iridescence show that 1) allelic variation of *bab* causes a male-specific mating signal difference between two incipient species; 2) Bab expression is sexually dimorphic in *C. eurytheme*; and 3) Bab functions as a repressor of the dimorphic trait. This repression of a male-specific feature is analogous to the expression and function of Bab paralogs in *Drosophila* sex-comb formation, abdominal pigmentation, and gonadal stem cell niches (21–23, 26, 27, 33, 34), with a male-specific repression in the presumptive cells forming the male feature, and loss of function resulting in gain or expansion of the male state. Bab is thus a major player in the development of sexually dimorphic features, and might have an ancestral function in the repression of male-specific development at the root of butterflies and flies. It will be critical to study the versatility of its functions comparatively in order to better understand how sexual forms evolve. As knockdowns and knockouts are increasingly amenable in new organisms, testing the repressive nature of Bab should be feasible and may yield a wide range of trait gain or masculinization phenotypes. Further work could also explore how the dimorphic (*C. eurytheme*) vs. monomorphic (*C. philodice*) expression of Bab is achieved. A potential regulator could be the transcription

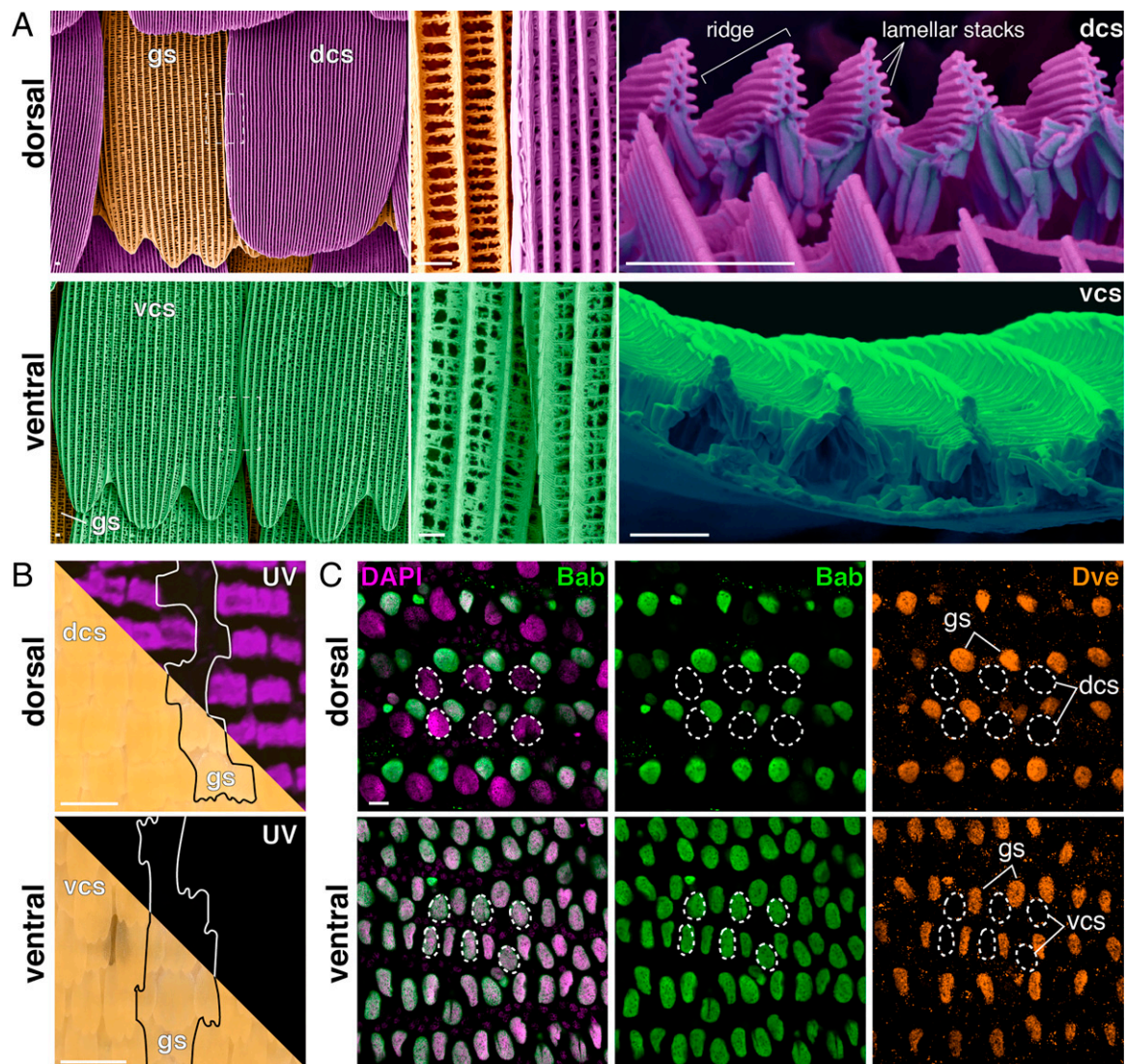


Fig. 2. Bab negatively correlates with UV-scale precursors in *C. eurytheme* male wings. (A) Pseudocolored SEM images highlighting the ultrastructural differentiation of the UV-iridescent dorsal cover scales (dcs; magenta), relative to non-UV ground scales (gs; orange) and non-UV ventral cover scales (vcs; green). (B) Microphotographs of adult *C. eurytheme* male wing surfaces in the visible and UV ranges. Line: damaged areas exposing non-UV ground scales. (C) Immunofluorescence detection of Bab (green) in all UV precursors at 46% pupal development. Magenta: DAPI (nuclei); orange: Dve; circles: cover-scale nuclei. (Scale bars, 2 μ m [A], 100 μ m [B], and 10 μ m [C].)

factor Doublesex, an integrator and master selector gene of somatic sexual identity in insects, including butterflies (35–38). In fruit flies, the DsxM male isoform of Doublesex directly represses Bab via a *cis*-regulatory element in the first intron of *bab1*, thus derepressing abdominal pigmentation, while the DsxF female isoform activates Bab, repressing pigmentation (24, 28). Extrapolating from this precedent, it will be interesting to test if DsxM similarly represses Bab in *C. eurytheme* cover scales, and if its binding sites have mutated in *C. philodice*.

***bab* Is a Genetic Hotspot of Sexual Phenotypic Variation.** There is replicated evidence that *cis*-regulatory evolution of *bab* directly causes sexual trait divergence in flies and Lepidoptera (butterflies and moths), making it a genetic hotspot of phenotypic variation (39). In *Drosophila*, linkage mapping studies have shown that regulatory alleles of the *bab* locus (*bab1/2* recent paralogs) explain natural variation in dimorphic pigmentation and ovariole number (23, 26, 40). In corn borer moths (*Ostrinia nubilalis*), male response to a polymorphic female pheromone blend is driven by a Z-linked regulatory variation in the first intron of

bab (41). This same intron is within the *Colias U*-locus interval. Thus, the 5' portion of the lepidopteran *bab* locus likely underlies variation in a male olfactory preference in recently diverged *Ostrinia* species, as well as in a male visual signal in *Colias*. This leads us to propose that the *bab* locus is a hotspot for the evolution of reproductive isolation, driving species divergence and maintenance across Lepidoptera. Factors related to the function, regulation, and genomic location of *bab* may have predisposed it to the tuning of sexually selected traits. First, repressors have drastic effects on *cis*-regulatory regions and efficiently suppress transcription (42, 43). In this sense, evolving new Bab binding sites in DNA enhancers might be a path of least resistance for optimizing gene expression subtractively, rather than by adding activator binding sites for other selector genes. There is already precedent for the *Drosophila* Bab integrating into a preexisting network and sculpting sexual dichromatism (27, 44) consistent with this idea. Second, the published expression patterns of Bab show it integrates both spatial and sex-determination inputs (21, 24, 25, 34). Due to a hub-like position in gene-regulatory networks, Bab is potentially an input-output

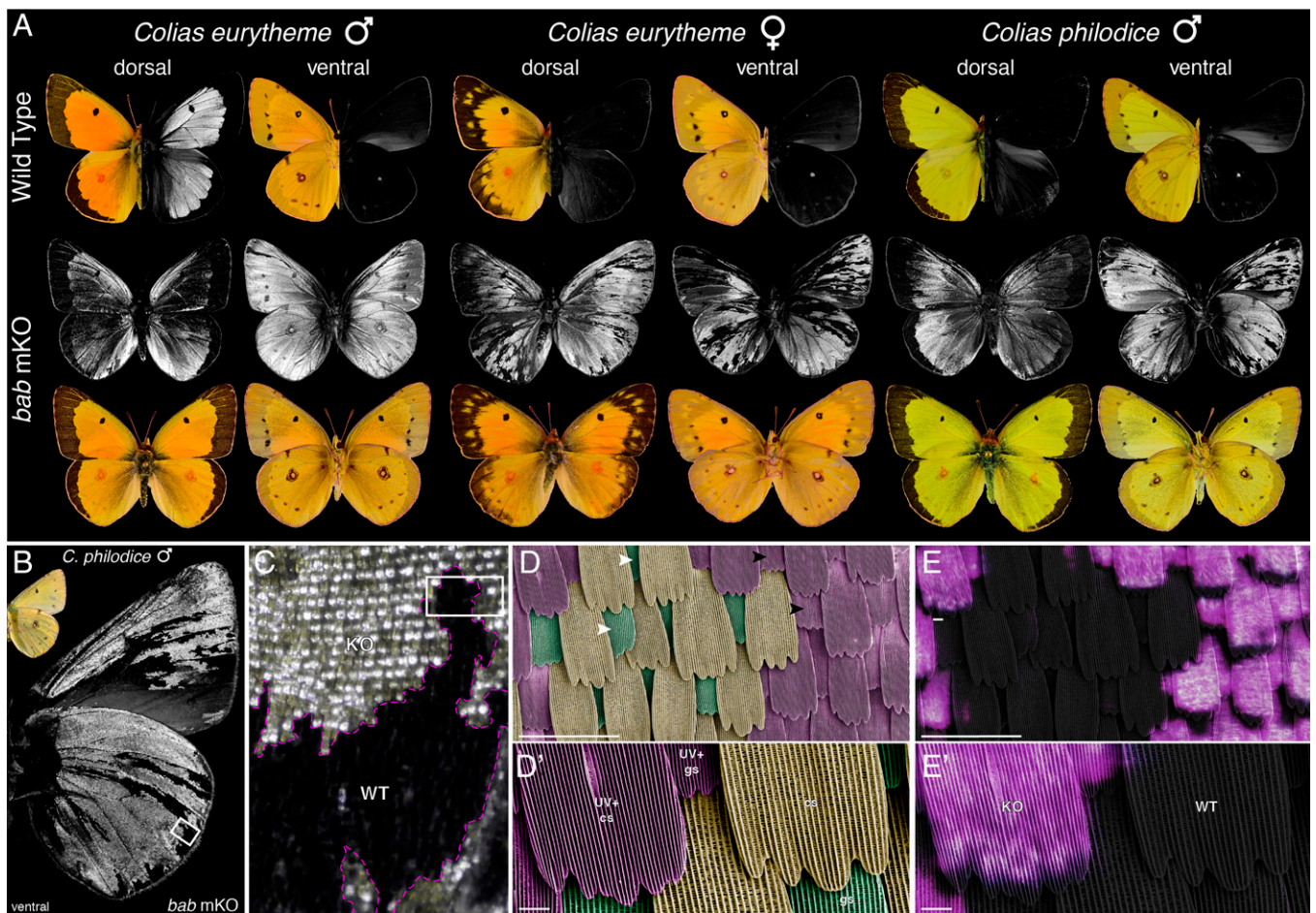


Fig. 3. Bab represses UV-scale fate. (A) G_0 phenotypes resulting from CRISPR mosaic KO (mKO) targeting the first exon of *bab*. Gain of UV iridescence was observed across both species and both sexes, including ventral wing surfaces. (B and C) Magnified views of male *C. philodice* wings featuring extensive ectopic iridescence following *bab* mKO. WT, wild type. (D and D') False-colored SEM views of a mosaic region (box in C), with complete transformation of wild-type cover (cs; orange) and ground scales (green) into UV-iridescent scales with dense longitudinal ridges. Arrowheads indicate examples of ground scales in a wild-type state (white) and in a *bab* mutant clone. (E and E') Correlative light and electron microscopy images featuring a superimposed view of UV-reflected light (magenta) on an SEM view. Both ground and cover scales from *bab* mutant clones are UV-iridescent. (Scale bars, 10 μm .)

gene that facilitates tissue-specific change (45, 46). The large 5' intergenic and intronic regions of *bab* in *Colias* and *Ostrinia*, and of its subfunctionalized duplicates in *Drosophila*, together suggest a complex *cis*-regulatory landscape bearing a multitude of enhancers or silencers (47–49), enabling the evolution of precise changes in specialized tissues (50). Last, the location of Bab on the lepidopteran Z chromosome is relevant to the divergence of premating traits under the assumption that sex chromosomes are more prone to the generation of reproductive isolation than autosomes (20, 51, 52). These possible generative biases will require further investigation among diverged *Drosophila* species, as well as in the *Colias* UV display and *Ostrinia* mate detection systems. Nonetheless, we speculate that the peculiar molecular function, regulation, and genomic location of Bab may collectively explain its propensity to fine-tune variation in sexual traits.

Extreme Large-Z Effect in an Anthropogenic Contact Zone. Our genomic scan of differentiation focused on two incipient species that recently reunited due to human activity (alfalfa agriculture). We caught a remarkable signature of a large-Z effect (analogous to large-X) with widespread admixture across autosomes, and strong differentiation of the entire Z chromosome. This heterogeneous landscape of differentiation is the most pronounced identified so far from genome-wide data (18),

supporting a role for sex chromosomes as key drivers of reproductive isolation (20, 51). Collectively with the evidence that both premating and postmating isolating mechanisms are sex-linked in this system (12, 14, 15), these data highlight that species status in this system is determined by Z chromosomes, while autosomes are largely exchangeable (though some divergence on autosomes was also observed). While linked selection (*syn*. divergence hitchhiking) in regions of low recombination can sometimes explain such extensive blocks of divergence (7, 19), our linkage map did not indicate that this is the case on the Z chromosome for the *Colias* sympatric pair. The *U* locus in particular overlapped with a 2.5-Mb region with above-median recombination in between-species crosses (red bracket in Fig. 1F). High F_{ST} indicates this region is refractory to gene flow in wild populations, suggesting it acts as a genomic barrier to gene flow rather than as a block of divergence hitchhiking, and might include further Z-linked barrier loci in addition to the male color signal variation (12–15). Overall, these data establish the anthropogenic contact zones of *Colias* butterflies as a promising system for the study of large-Z(X) effects in speciation with gene flow.

Coupling of Reproductive Barriers. The data in hand suggest the *U*-locus variation causes premating isolation by accurately displaying sex-chromosome compatibility of courting males to

C. eurytheme females. In summary, the *Colias* UV mating signal is polymorphic and recessive in areas of secondary contact, with both heterospecific and hybrid males lacking UV iridescence because they carry the dominant *U* allele of *C. philodice*. This allele drives uniform expression of Bab, preventing UV-scale development with a dominant effect. Conversely, the recessive *C. eurytheme u* allele represses expression of Bab in dorsal male cover scales, leading to UV fated scales. This Z-linked UV signal allows *C. eurytheme* females to choose Z-compatible mates, thereby prezygotically selecting against Z-linked hybrid sterility (3, 9, 10, 12–14). The recessivity of the trait allows the rejection of Z-heterozygous males and prevents a 25% cost in reproductive fitness (SI Appendix, Fig. S1), an effect that would not be possible if Bab were an activator of UV, which would likely be dominant. In this way, a large-Z effect that couples pre- and postmating barriers likely drives reproductive isolation in these butterflies (5, 18), akin to the genetic architectures of speciation in other species (16, 53), and previously conceptualized as an “indicator” mechanism that enables assortative mating (3). Further work is needed to decipher other putative barrier loci on this chromosome. In any case, these findings highlight how a simple genetic switch for a mating cue can influence the origin of species and the maintenance of biodiversity.

Experimental Procedures

Genomic Scans of Differentiation in Sympatric Males. Samples originated from a large syntopic population at an alfalfa farm in Buckeystown, MD. Whole genomes from 24 males were resequenced at 14.3× mean coverage, aligned to the *C. eurytheme* reference genome assembly (54), and used in a population scan analysis pipeline (55).

Linkage Map. Interspecific crosses consisting of an F₂ and two BC broods generated 528 recombinant individuals of known pedigree, sex, and UV phenotype. 2b-RAD sequencing was used to genotype 484 males and females in a HiSeq 4000 SE50 run, using the *Bcl*I enzyme and adapters, yielding a 16-fold representation reduction (56). Genotypes were used to build a linkage map as

described elsewhere (54). Select individuals were resequenced at a 15× mean coverage to narrow the *U*-locus interval.

Bab Expression and Loss-of-Function Assays. A custom rabbit polyclonal antibody was generated against the N-terminal 1 to 365 residues of the *C. eurytheme* Bab protein, and used with a guinea pig anti-Dve (57) for whole-mount immunofluorescence in pupal wings. Heteroduplex mixes of Cas9 recombinant protein and two equimolar sgRNAs (500:125:125 ng/μL) were injected into syncytial embryos 1 to 7 h after egg laying (AEL) for targeted mutagenesis of the first exon of *bab*.

CRISPR-Mediated KO of *bab*. Two overlapping gRNAs were designed targeting the first exon of *bab* within the *U* locus. Heteroduplex mixes of Cas9:sgRNA1:sgRNA2 (500:125:125 ng/μL) were prepared and microinjected into butterfly syncytial embryos 1–7 h AEL. Eggs incubated for 2 d at 28°C were then placed on vetch sprouts aged 7 to 14 d at an average greenhouse temperature of 24°C. Two rounds of injections were successfully performed under these conditions resulting in 51 of 63 adults displaying crispant phenotypes.

UV Photography. UV photography was performed using a full-spectrum converted Lumix G3 camera, mounted with Baader U-Venus filters and UV-transmitting lenses, under the illumination of blacklight bulbs or 365 nm light-emitting diodes.

Data Availability. Whole-genome sequencing data reported in this article have been deposited in the Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>) under BioProjects PRJNA663300, PRJNA719421, and PRJNA723900. SNP calling, genotyping data, and computer code have been deposited in the Dryad digital repository (10.5061/dryad.4b8gthtcc) (55).

ACKNOWLEDGMENTS. We thank B. Wang, S. Barao, W. Watt, and R. Canalicchio for butterfly rearing, access, and expertise; R. Rogers, S. Van Belleghem, and P. Rastas for bioinformatics assistance; C. Brantner and C. Day for scanning electron microscopy (SEM); M. Matz for 2b-RAD protocols; L. Livraghi for vector graphics; and M. Perry for sharing the Dve antibody. We also thank the core facilities at The George Washington University (GWU), University of Texas at Austin, and University of Maryland, Baltimore, for generating sequence data, and the GWU high-performance computing team for providing computing infrastructure (58). This work was supported by NSF Awards IOS-2108227 and IOS-1656553 and Swedish Research Council Award 2017-04386.

- R. J. Safran, E. S. Scordato, L. B. Symes, R. L. Rodriguez, T. C. Mendelson, Contributions of natural and sexual selection to the evolution of premating reproductive isolation: A research agenda. *Trends Ecol. Evol.* **28**, 643–650 (2013).
- M. R. Servedio, J. W. Boughman, The role of sexual selection in local adaptation and speciation. *Annu. Rev. Ecol. Evol. Syst.* **48**, 85–109 (2017).
- M. R. Servedio, The evolution of premating isolation: Local adaptation and natural and sexual selection against hybrids. *Evolution* **58**, 913–924 (2004).
- J. Kulmuni, R. K. Butlin, K. Lucek, V. Savolainen, A. M. Westram, Towards the completion of speciation: The evolution of reproductive isolation beyond the first barriers. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **375**, 20190528 (2020).
- R. K. Butlin, C. M. Smadja, Coupling, reinforcement, and speciation. *Am. Nat.* **191**, 155–172 (2018).
- D. Ortiz-Barrientos, A. Greal, P. Nosil, The genetics and ecology of reinforcement: Implications for the evolution of prezygotic isolation in sympatry and beyond. *Ann. N. Y. Acad. Sci.* **1168**, 156–182 (2009).
- M. Ravinet *et al.*, Interpreting the genomic landscape of speciation: A road map for finding barriers to gene flow. *J. Evol. Biol.* **30**, 1450–1477 (2017).
- W. Hovanitz, The ecological significance of the color phases of *Colias chrysotheme* in North America. *Ecology* **25**, 45–60 (1944).
- R. E. Silberglied, O. R. Taylor, Ultraviolet reflection and its behavioral role in the courtship of the sulfur butterflies *Colias eurytheme* and *C. philodice* (Lepidoptera, Pieridae). *Behav. Ecol. Sociobiol.* **3**, 203–243 (1978).
- O. R. Taylor Jr., Random vs. non-random mating in the sulfur butterflies, *Colias eurytheme* and *Colias philodice* (Lepidoptera: Pieridae). *Evolution* **26**, 344–356 (1972).
- T. J. Thurman, E. Brodie, E. Evans, W. O. McMillan, Facultative pupal mating in *Heliconius erato*: Implications for mate choice, female preference, and speciation. *Ecol. Evol.* **8**, 1882–1889 (2018).
- J. W. Grula, O. R. Taylor Jr., Some characteristics of hybrids derived from the sulfur butterflies, *Colias eurytheme* and *C. philodice*: Phenotypic effects of the X-chromosome. *Evolution* **34**, 673–687 (1980).
- B. Wang, “Introgression and genomic differentiation in sympatric, hybridizing *Colias* butterflies,” PhD thesis, University of Massachusetts Amherst, Amherst, MA (2005).
- J. W. Grula, O. R. Taylor Jr., The effect of X-chromosome inheritance on mate-selection behavior in the sulfur butterflies, *Colias eurytheme* and *C. philodice*. *Evolution* **34**, 688–695 (1980).
- R. E. Silberglied, O. R. Taylor, Ultraviolet differences between the sulphur butterflies, *Colias eurytheme* and *C. philodice*, and a possible isolating mechanism. *Nature* **241**, 406–408 (1973).
- S. A. Saether *et al.*, Sex chromosome-linked species recognition and evolution of reproductive isolation in flycatchers. *Science* **318**, 95–97 (2007).
- A. H. Clark, “The butterflies of the District of Columbia and vicinity” (Bulletin of the US National Museum, No. 157, US Government Printing Office, 1932).
- D. C. Presgraves, Evaluating genomic signatures of “the large X-effect” during complex speciation. *Mol. Ecol.* **27**, 3822–3830 (2018).
- R. Burri *et al.*, Linked selection and recombination rate variation drive the evolution of the genomic landscape of differentiation across the speciation continuum of *Ficedula* flycatchers. *Genome Res.* **25**, 1656–1665 (2015).
- M. A. Wilson Sayres, Genetic diversity on the sex chromosomes. *Genome Biol. Evol.* **10**, 1064–1078 (2018).
- A. Kopp, I. Duncan, D. Godt, S. B. Carroll, Genetic control and evolution of sexually dimorphic characters in *Drosophila*. *Nature* **408**, 553–559 (2000).
- J.-L. Couderc *et al.*, The *bric à brac* locus consists of two paralogous genes encoding BTB/POZ domain proteins and acts as a homeotic and morphogenetic regulator of imaginal development in *Drosophila*. *Development* **129**, 2419–2433 (2002).
- T. M. Williams *et al.*, The regulation and evolution of a genetic switch controlling sexually dimorphic traits in *Drosophila*. *Cell* **134**, 610–623 (2008).
- W. A. Rogers *et al.*, Recurrent modification of a conserved *cis*-regulatory element underlies fruit fly pigmentation diversity. *PLoS Genet.* **9**, e1003740 (2013).
- J. R. Salomone, W. A. Rogers, M. Rebeiz, T. M. Williams, The evolution of *Bab* paralog expression and abdominal pigmentation among *Sophophora* fruit fly species. *Evol. Dev.* **15**, 442–457 (2013).
- S. De Castro, F. Peronnet, J.-F. Gilles, E. Mouchel-Vielh, J.-M. Gibert, *bric à brac* (*bab*), a central player in the gene regulatory network that mediates thermal plasticity of pigmentation in *Drosophila melanogaster*. *PLoS Genet.* **14**, e1007573 (2018).
- M. J. Roessel, E. M. Camino, S. Grover, M. Rebeiz, T. M. Williams, *cis*-regulatory evolution integrated the *Bric-à-brac* transcription factors into a novel fruit fly gene regulatory network. *eLife* **7**, e32273 (2018).
- A. Dinwiddie *et al.*, Dynamics of F-actin prefigure the structure of butterfly wing scales. *Dev. Biol.* **392**, 404–418 (2014).
- H. Ghiradella, Development of ultraviolet-reflecting butterfly scales: How to make an interference filter. *J. Morphol.* **142**, 395–409 (1974).

30. R. L. Rutowski, J. M. Macedonia, N. Morehouse, L. Taylor-Taft, Pterin pigments amplify iridescent ultraviolet signal in males of the orange sulphur butterfly, *Colias eurytheme*. *Proc. Biol. Sci.* **272**, 2329–2335 (2005).
31. D. J. Kemp, P. Vukusic, R. L. Rutowski, Stress-mediated covariance between nanostructural architecture and ultraviolet butterfly coloration. *Funct. Ecol.* **20**, 282–289 (2006).
32. D. Stella, Z. Faltýnek Fric, M. Rindoš, K. Kleisner, P. Pecháček, Distribution of ultraviolet ornaments in *Colias* butterflies (Lepidoptera: Pieridae). *Environ. Entomol.* **47**, 1344–1354 (2018).
33. N. Camara, C. Whitworth, A. Dove, M. Van Doren, Doublesex controls specification and maintenance of the gonad stem cell niches in *Drosophila*. *Development* **146**, dev170001 (2019).
34. H.-M. G. Bourbon *et al.*, Tissue-specific versus pleiotropic enhancers within the *bric-a-brac* tandem gene duplicates display differential regulatory activity and evolutionary conservation. *bioRxiv* [Preprint] (2021). <https://doi.org/10.1101/2021.03.25.436949> (Accessed 20 December 2021).
35. K. Kunte *et al.*, *doublesex* is a mimicry supergene. *Nature* **507**, 229–232 (2014).
36. H. Nishikawa *et al.*, A genetic mechanism for female-limited Batesian mimicry in *Papilio* butterfly. *Nat. Genet.* **47**, 405–409 (2015).
37. F. Rodriguez-Caro *et al.*, Novel *doublesex* duplication associated with sexually dimorphic development of dogface butterfly wings. *Mol. Biol. Evol.* **38**, 5021–5033 (2021).
38. A. Prakash, A. Monteiro, *Doublesex* mediates the development of sex-specific pheromone organs in *Bicyclus* butterflies via multiple mechanisms. *Mol. Biol. Evol.* **37**, 1694–1707 (2020).
39. A. Martin, V. Orgogozo, The loci of repeated evolution: A catalog of genetic hotspots of phenotypic variation. *Evolution* **67**, 1235–1250 (2013).
40. D. A. Green II, C. G. Extavour, Convergent evolution of a reproductive trait through distinct developmental mechanisms in *Drosophila*. *Dev. Biol.* **372**, 120–130 (2012).
41. M. Unbehend *et al.*, *bric à brac* controls sex pheromone choice by male European corn borer moths. *Nat. Commun.* **12**, 2818 (2021).
42. E. Preger-Ben Noon, F. P. Davis, D. L. Stern, Evolved repression overcomes enhancer robustness. *Dev. Cell* **39**, 572–584 (2016).
43. S. S. Gisselbrecht *et al.*, Transcriptional silencers in *Drosophila* serve a dual role as transcriptional enhancers in alternate cellular contexts. *Mol. Cell* **77**, 324–337.e8 (2020).
44. J. T. Hughes, M. E. Williams, M. Rebeiz, T. M. Williams, Widespread *cis*- and *trans*-regulatory evolution underlies the origin, diversification, and loss of a sexually dimorphic fruit fly pigmentation trait. *J. Exp. Zool. B Mol. Dev. Evol.*, 10.1002/jez.b.23068 (2021).
45. D. L. Stern, V. Orgogozo, Is genetic evolution predictable? *Science* **323**, 746–751 (2009).
46. A. Orteu, C. D. Jiggins, The genomics of coloration provides insights into adaptive evolution. *Nat. Rev. Genet.* **21**, 461–475 (2020).
47. L. Baudouin-Gonzalez *et al.*, Diverse *cis*-regulatory mechanisms contribute to expression evolution of tandem gene duplicates. *Mol. Biol. Evol.* **34**, 3132–3147 (2017).
48. G. Kalay, J. Lachowiec, U. Rosas, M. R. Dome, P. Wittkopp, Redundant and cryptic enhancer activities of the *Drosophila yellow* gene. *Genetics* **212**, 343–360 (2019).
49. B. Borsari *et al.*, Enhancers with tissue-specific activity are enriched in intronic regions. *Gen. Res.* **31**, 1325–1336 (2021).
50. S. B. Carroll, Evo-devo and an expanding evolutionary synthesis: A genetic theory of morphological evolution. *Cell* **134**, 25–36 (2008).
51. M. M. Patten, Selfish X chromosomes and speciation. *Mol. Ecol.* **27**, 3772–3782 (2018).
52. C. Fraïsse, H. Sachdeva, The rates of introgression and barriers to genetic exchange between hybridizing species: Sex chromosomes vs autosomes. *Genetics* **217**, iyaa025 (2021).
53. D. Ortiz-Barrientos, B. A. Counterman, M. A. Noor, The genetics of speciation by reinforcement. *PLoS Biol.* **2**, e416 (2004).
54. K. Tunström *et al.*, A complex interplay between balancing selection and introgression maintains a genus-wide alternative life-history strategy. *bioRxiv* [Preprint] (2021). <https://doi.org/10.1101/2021.05.20.445023> (Accessed 20 December 2021).
55. V. Ficarrota *et al.*, Data from: A genetic switch for male UV-iridescence in an incipient species pair of sulphur butterflies (2021). Dryad. <https://doi.org/10.5061/dryad.4b8gthtcc>. Deposited 16 December 2021.
56. S. Wang, E. Meyer, J. K. McKay, M. V. Matz, 2b-RAD: A simple and flexible method for genome-wide genotyping. *Nat. Methods* **9**, 808–810 (2012).
57. M. Perry *et al.*, Expanded color vision in butterflies: Molecular logic behind the three-way stochastic choices that expand butterfly colour vision. *Nature* **535**, 280–284 (2016).
58. G. MacLachlan *et al.*, Building a shared resource HPC center across university schools and institutes: A case study. *arXiv* [Preprint] (2020). <https://arxiv.org/abs/2003.13629> (Accessed 20 December 2021).