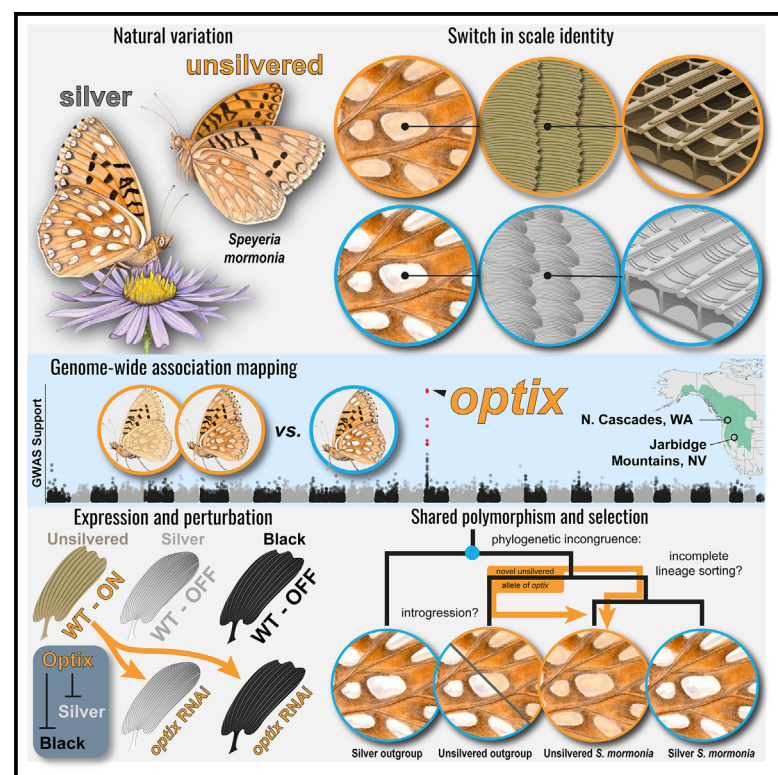


Current Biology

Genetic basis of an adaptive polymorphism controlling butterfly silver iridescence

Graphical abstract



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In brief

Livraghi et al. identify the transcription factor *optix* as a key regulator of silver scale polymorphism in Mormon Fritillary butterflies. Their study reveals selective sweeps and interspecies allelic sharing at *optix* and highlights shared adaptive genetic pathways across distantly related species.

Highlights

- *Speyeria mormonia* butterflies display a polymorphism for iridescent silver scales
- The silvering polymorphism maps to the *optix* transcription factor gene
- *Optix* represses silver scale specification
- *Optix* alleles are shared between *Speyeria* species and are maintained by selection



Report

Genetic basis of an adaptive polymorphism controlling butterfly silver iridescence

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SUMMARY

Identifying the genes and mutations that drive phenotypic variation and which are subject to selection is crucial for understanding evolutionary processes. Mormon Fritillary butterflies (*Speyeria mormonia*) exhibit a striking wing color polymorphism throughout their range: typical morphs bear silver spots on their ventral surfaces and can co-occur with unsilvered morphs displaying a dull coloration.¹ Through genome-wide association studies in two polymorphic populations, we fine-map this difference in silvering to the 3' region of the transcription factor gene *optix*. The expression of *optix* is confined to the unsilvered regions that surround the spots, and these patterns are transformed to a silver identity upon *optix* RNA interference (RNAi) knockdown, implicating *optix* as a repressor of silver scales in this butterfly. We show that the unsilvered *optix* haplotype shows signatures of recent selective sweeps and that this allele is shared with an unsilvered population of *Speyeria hydaspe*, suggesting that introgressions may facilitate the exchange of variants of adaptive potential across species. Remarkably, these findings parallel the role of allelic sharing and *cis*-regulatory modulation of *optix* in shaping the aposematic red patterns of *Heliconius* butterflies,^{2–7} a lineage that separated from *Speyeria* 45 million years ago.⁸ The genetic basis of adaptive variation can thus be more predictable than often presumed, even for traits that appear divergent across large evolutionary distances.

RESULTS

Complex spatial distribution of the silver polymorphism in *Speyeria mormonia*

The ventral hindwings of *Speyeria* (*Argynnis*) *mormonia* display a polymorphism for the presence of iridescent silver scales⁹ (Figures 1A and 1A'). Unsilvered morphs have spots with a light beige color, while the same patterns have a bright metallic look in silver morphs (Figures 1B, 1B', and S1). The difference between silver and unsilvered spots is due to a scale identity shift (Figures 1C–1D'): most notably, unsilvered scales are slightly pigmented and porous (light absorbent), whereas silver scales are unpigmented, with a continuous upper surface (light

reflective).^{10–12} *Speyeria mormonia* occurs across mountainous habitats of western North America, with silver and unsilvered morphs varying in frequencies throughout its range.¹³ However, despite several studies reporting similar polymorphisms in many species of *Speyeria*,^{14,15} none thus far have systematically described their geographic distribution. To accurately map the geographic variation of the silver scale polymorphism in *S. mormonia*, we examined 9,763 records from museum collections across the US and Canada, scoring the prevalence of silver and unsilvered morphs from each locality (Table S1). We observed a complex, heterogeneous landscape of phenotype distributions, with a general decrease in silver morph frequency with increasing latitude (Figures 1E



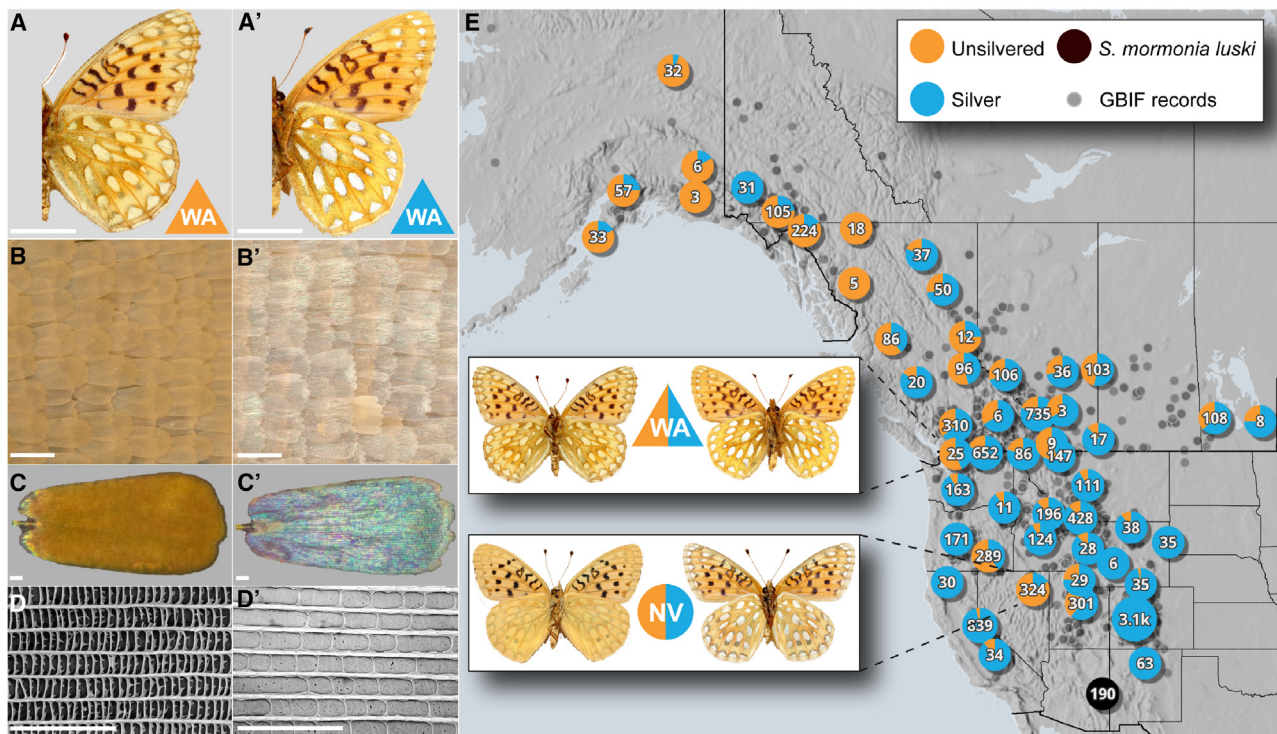


Figure 1. Ultrastructural basis and geographic distribution of the silvering polymorphism in *Speyeria mormonia*

(A and A') Representative *S. mormonia* individuals exhibiting unsilvered and silver ventral phenotypes.

(B and B') Magnified view of unsilvered and silver wing spots.

(C and C') Reflected light micrographs of single scales from each morph; unsilvered scales are brown, whereas silver scales are unpigmented but iridescent.

(D and D') Scanning electron micrographs of the upper surfaces of unsilvered and silver scales. (D) Unsilvered scales have a perforated upper lamina that makes them non-reflective, whereas (D') silver scales have a continuous upper lamina that makes them reflective.

(E) Geographic distribution of the silvering polymorphism (Table S1). Pie charts indicate the observed proportions of silver (blue) and unsilvered (orange) individuals and report the total number of scored individuals at each location. A distinct subspecies, *S. mormonia luskii*, found in Arizona and exhibiting an atypical white-spotted phenotype, is indicated in black. The polymorphic populations sampled in this study for sequencing are highlighted: one from the North Cascades in Washington (WA) and another from the Jarbidge Mountains in Nevada (NV), with representative images of silver and unsilvered individuals shown. Gray points represent 9,717 occurrence records of *S. mormonia* from the Global Biodiversity Information Facility (GBIF) that lack phenotypic information or for which silvering was not scorable from pictures. Scale bars: 1 cm in (A) and (A'), 100 μ m in (B) and (B'), and 10 μ m in (C)–(D').

See also Figures S1–S4 and Tables S1, S2, and S3.

and S2). Local heterogeneity in unsilvered/silver morph frequency is apparent throughout the species range. For example, unsilvered morphs (*S. mormonia artonis*) are prevalent in populations from the Steens, Jarbidge, and Ruby Mountain ranges in southeastern Oregon and northern Nevada, in sharp contrast to surrounding localities where these morphs become rare (Figure S3). Regression analyses between these trait proportions and geoclimatic variables found weak associations ($R^2 = 0.32$ – 0.33) with solar irradiation and isothermality (Figure S2), two variables that are directly determined by latitude. Yet, mid-infrared imaging shows that silver spots do not increase the thermal emissivity of these wing patterns (Figure S4), ruling out a radiative cooling effect¹⁶ that may have explained their higher prevalence at lower latitudes. Unsilvered and silver states may thus function as camouflage strategies, perhaps each specialized for different visual backgrounds. Because none of the tested environmental variables were a strong predictor of observed silvering proportions, we conclude that the observed heterogeneity in prevalence of each morph is influenced by local demographic factors or by

micro-habitat differences that do not scale to the larger distribution range of the species.

The silvering polymorphism maps to *optix*

To determine the inheritance patterns and dominance hierarchy of silvering, we crossed captive-reared *S. mormonia* individuals from a mixed population in Manning Park, British Columbia, generating 931 individuals from 26 broods over 3 generations. Offspring phenotypic frequencies indicate that silver iridescence is inherited as a single-locus, recessive autosomal trait (Table S3), consistent with observations in other *Speyeria* species.^{14,15}

Given the simple genetic basis for the silver/unsilvered switch, we sought to map the underlying locus by genome-wide association (GWA). To achieve this, we resequenced the genomes of 70 individuals sampled from independent admixed populations in the North Cascades (WA) and in the Jarbidge Mountains (NV)—where silver individuals are found at around 40% frequency (Figure S2). Following single nucleotide polymorphism (SNP) calling against an existing genome assembly of *S. mormonia*,⁸ the GWA study (GWAS) identified an ~7-kb

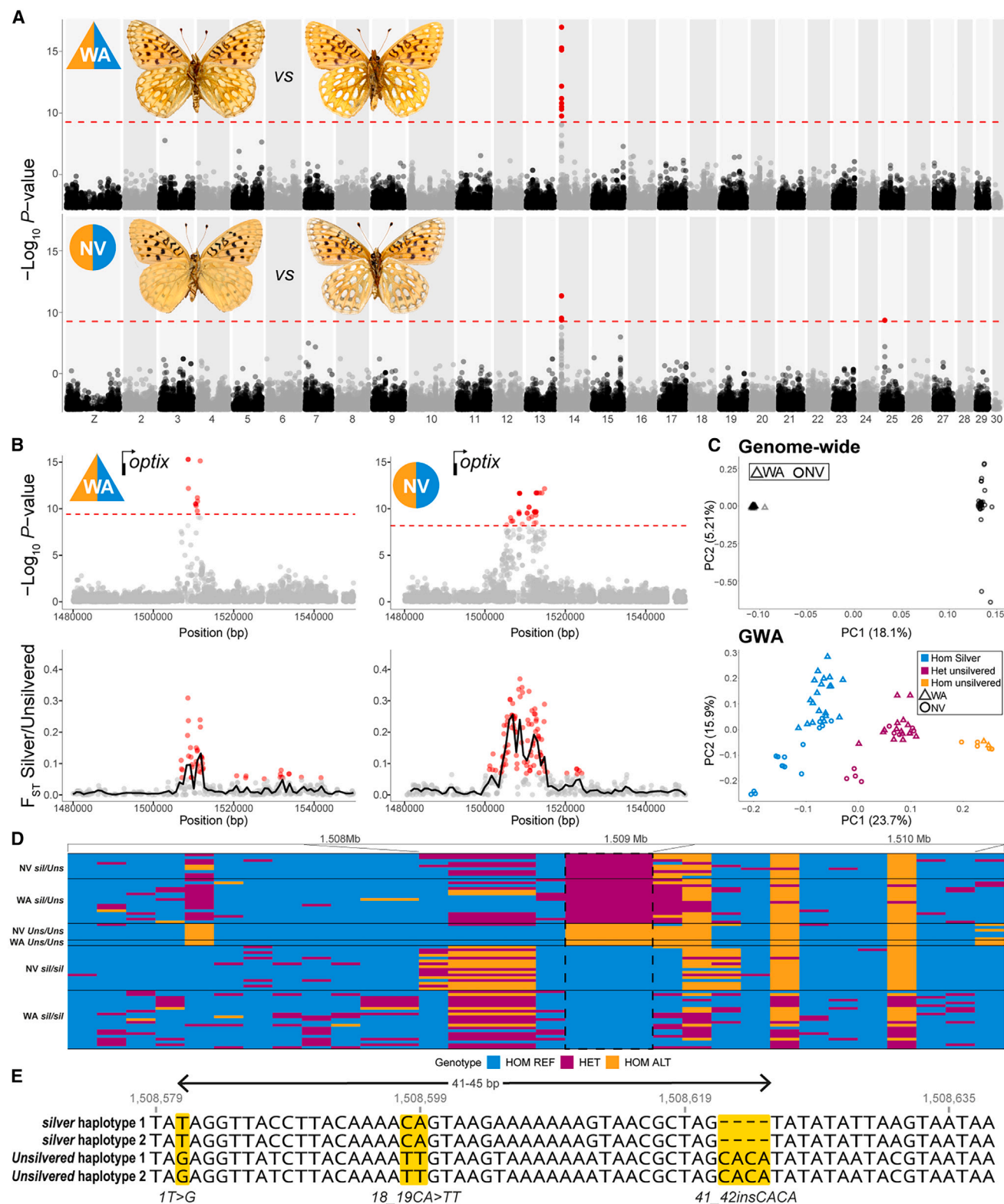


Figure 2. The *S. mormonia* silvering locus maps to the 3' region of the *optix* transcription factor gene

(A) GWAS of the silvering polymorphism reveals a strong association on chromosome 14 in both the WA and NV populations. Chromosome numbers are indicated at the bottom. The y axis shows the Wald test negative $\log_{10} p$ value associated with each variant. The dotted line represents the Bonferroni-corrected genome-wide significance threshold ($p < 0.01$).

(legend continued on next page)

interval on autosome 14 strongly associated with the presence/absence of silver scales (Figure 2A). The associated SNPs map to an intergenic region ~15 kb downstream of the transcription start site of *optix*, a gene with a well-documented and conserved role in butterfly scale pigmentation^{3,17–19} (Figure 2B). The statistical support for the GWA SNPs at the *optix* locus increases in the combined NV and WA datasets when population structure is accounted for (Figures S5A and S5B). A secondary GWA signal found exclusively in the NV population maps to a *DIRS1*-like transposable element insertion site on chromosome 25 (Figures 2A and S5C). Due to the repetitive nature of this 5-kb region (11 copies in the reference genome) and the limitations of short-read genotyping, we cannot confirm the validity of this mapping result and excluded it from further analyses.

Next, we more closely examined patterns of SNP sharing within the chromosome 14 GWA interval in each sampled population. The association signal at *optix* is accompanied by an increased but not fixed F_{ST} signal, driven by high frequencies of heterozygous unsilvered individuals (Figures 2C and 2D). Genome-wide principal-component analysis (PCA) largely clusters individuals by geography, whereas local PCA in the GWA interval clusters individuals by phenotype along PC1, with PC2 recovering some geographic signal (Figure 2C). Scoring variants by zygosity state within the GWA interval identified 3 SNP positions as well as a 4-bp indel in perfect association with phenotypes (Figures 2D, S6, and S7), forming a 41- to 45-bp haplotypic block of 3 linked variants (1T>G, 18_19CA>TT, and 41_42insCACA). We then performed a PacBio HiFi assembly of an *Uns/Uns* individual that confirms the presence of the associated SNPs as well as the short 4-bp indel in both assembled haplogenomes (Figure 2E). A short-read coverage analysis shows that flanking large structural variants in the region are not in perfect linkage disequilibrium with these variants (Figure S8). In conclusion, GWAS mapping of silvering variation narrowed down a small haplotype associated with phenotypes in the 3' region of *optix*. These sites could be involved in the *cis*-regulatory control of *optix* transcription or in the post-transcriptional regulation of its transcript.

Optix represses silver scale specification

To test the developmental role of *optix* in silver pattern formation, we reared silver individuals collected from Colorado, a location where silver alleles are nearly fixed,²⁰ and examined *optix* expression by hybridization chain reaction (HCR). In early pupal wings (28% stage), *optix* expression is restricted to two small patches of presumptive “coupling scales” (Figure 3A)—the future zone of contact between forewings and hindwings—

consistent with previous studies in other butterflies.^{11,19} At 54% of pupal development (Figure 3B), *optix* shows widespread expression in developing scales but is markedly absent from the presumptive silver hindwing spots as well as from various presumptive black pattern elements. These results suggest that *optix* is repressed in patterns with either silver or melanic scales and may thus act as a repressor of these differentiated states in *S. mormonia*.

Next, we sought to perturb *optix* to test this hypothesis. To achieve this, we used pupal wing electroporation of Dicer-substrate short interfering RNAs (DsiRNAs)²¹ instead of CRISPR mutagenesis, bypassing the need for a breeding colony by allowing experiments in field-collected larvae that survived their obligatory diapause. Remarkably, RNA interference (RNAi) knockdowns of *optix* resulted in a shift from non-reflective scale states to silver, showing that *optix* is necessary to prevent silver scale specification (Figures 3C–3E, red arrows). We also observed position-dependent effects, where orange areas transformed to black rather than silver (white arrows), thus implying that *optix* combines with other unknown factors to determine color scale identity. In summary, *optix* is downregulated in silver spots and represses silver scale identity, confirming its function as a switch gene that causes the silvering polymorphism in natural populations.

The unsilvered allele shows signatures of selective sweeps

The observed variation in polymorphism frequencies across populations hints at the influence of non-neutral evolutionary forces maintaining this genetic diversity. The recessive silver state is nearly fixed in the Cascade and Klamath mountain ranges (OR, North CA) and in the Eastern Rockies (CO, NM, SD). In contrast, the populations we sampled in WA and NV show intermediate and high frequencies of the dominant unsilvered allele (Figure S3). To better understand these dynamics, we carried out differentiation and selection scans of the *optix* silvering locus. Using the three associated SNPs identified in the GWAS as diagnostic of either unsilvered or silver haplotypes (Figure 2D), we generated a set of phased SNPs on chromosome 14 and coded each haplotype as associated to unsilvered or silver states (i.e., as containing *Uns* and *sil* alleles, respectively). Recalculating F_{ST} in sliding windows on these phased haplotypes shows an elevated signal, reflective of the higher differentiation between individual alleles (Figures 4A and 4A'). Allele divergence is also higher at the *optix* locus, as shown by elevated Dxy (Figures 4B and 4B'), and nucleotide diversity (π) is reduced within the elevated F_{ST} window for unsilvered haplotypes in both

(B) The associated SNPs are located ~15 kb downstream of the *optix* transcription start site (black arrow) and are accompanied by moderate differentiation between silver and unsilvered individuals as measured by F_{ST} , calculated in 250-bp sliding windows. F_{ST} windows in the 99th percentile of the chromosome-wide distribution are shown in red.

(C) PCA, based on genome-wide SNPs, clusters samples by geography (PC1). Performing a PCA using the GWA interval clusters the samples by phenotype along PC1 and by geography on PC2.

(D) Genotype plot for the GWA region. Each row is an individual and each column is a SNP color-coded for its state relative to the reference haploid genome assembly (a silver individual from WA). Three fixed SNPs are found between silver and unsilvered individuals in both populations (highlighted by black dashed box).

(E) Multiple sequence alignment from assembled PacBio haplogenomes from both a [*sil/sil*] silver and an [*Uns/Uns*] unsilvered individual. Perfectly associated variants within a 41- to 45-bp region are highlighted in yellow.

See also Figures S5–S8 and Table S3.

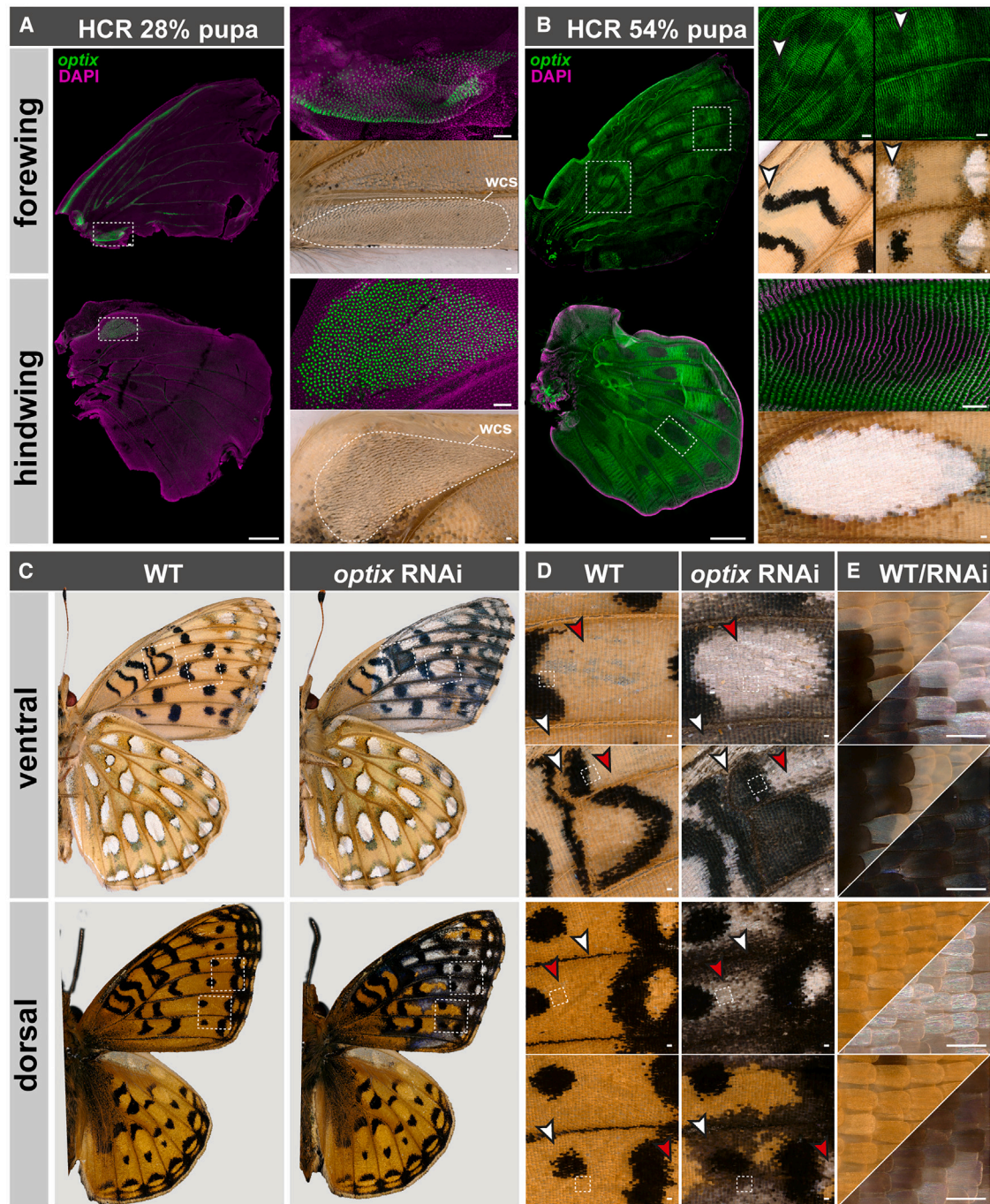


Figure 3. *Optix* represses silver and black scale specification

(A) HCR stainings in 28% pupal wings show *optix* expression in the presumptive wing-coupling scales (wcs), a patch of tooth-shaped scales that create friction in a zone of contact between the forewing and hindwing.

(B) By 54% of pupal development, *optix* is localized throughout the wing, except in the presumptive silver and black pattern elements (examples indicated with arrowheads).

(C–E) Electroporated RNAi knockdowns of *optix* result in homeotic scale transformations from orange to silver (red arrowheads) or black (white arrowheads) on both wing surfaces. Wild-type (WT) and RNAi-treated images correspond to the left and right side of the same individuals, with the WT horizontally flipped to facilitate comparisons here. Additional RNAi knockdown replicates are shown in Figure S9. Scale bars: 1 mm in (A) and (B) (left), 100 μ m in (A) and (B) (right), and 100 μ m in (D) and (E).

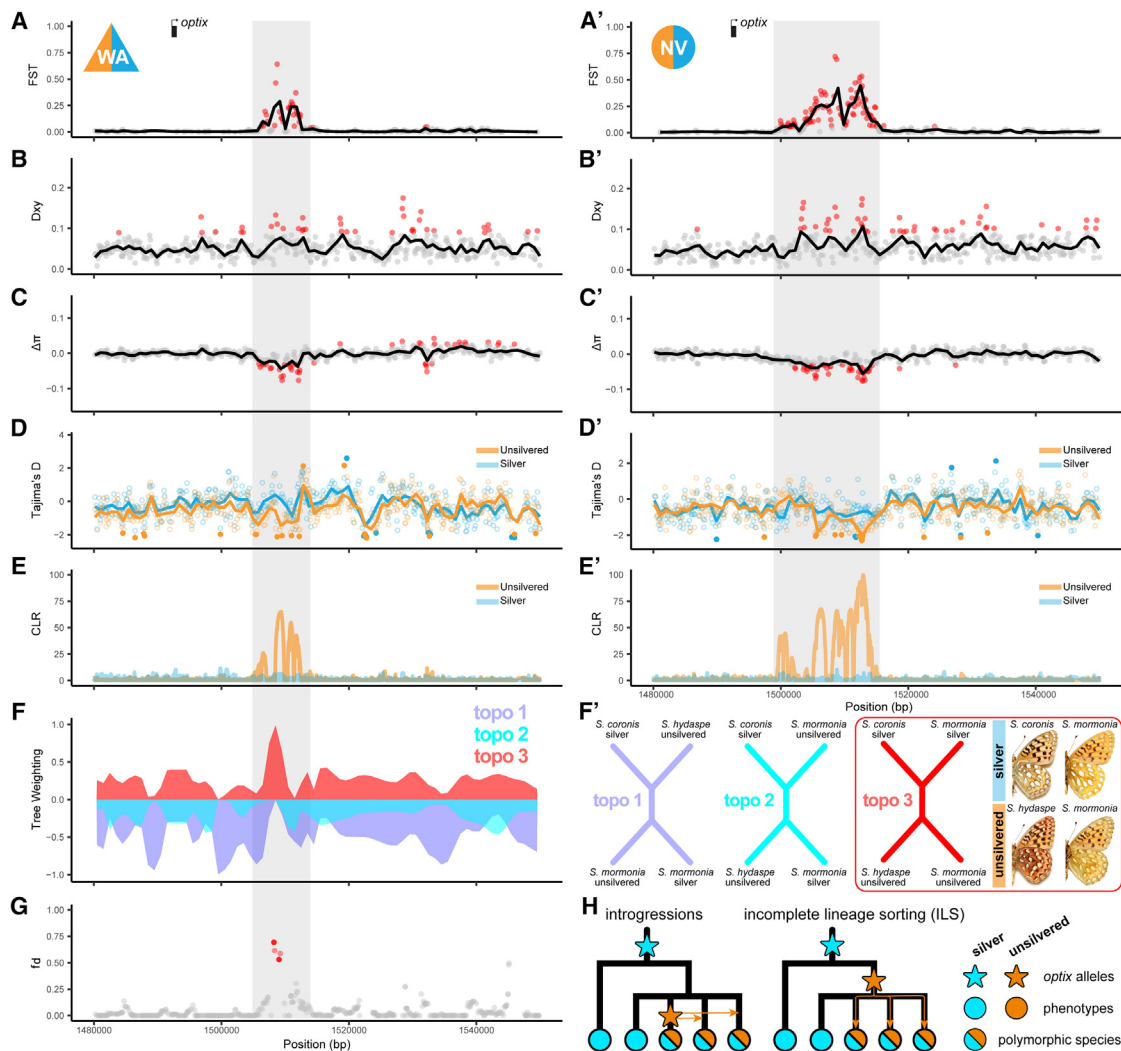


Figure 4. Evidence of selection and allele sharing at the *optix* locus

(A and A') Strong differentiation as measured by F_{ST} between individually phased unsilvered and silver alleles measured in fixed windows of 50 informative sites. This signal is observed at the 3' end of *optix* in both WA (A) and NV (A') populations.
(B–C') (B and B') The same genomic interval is accompanied by locally elevated D_{xy} , and (C and C') a reduction in nucleotide diversity ($\Delta\pi = \pi_{\text{unsilvered}} - \pi_{\text{silver}}$) whereby unsilvered alleles show a reduction in diversity compared with silver alleles.
(D and D') A reduction in Tajima's D can also be observed for the unsilvered haplotype concurrent with the F_{ST} and $\Delta\pi$ signals, indicating the presence of non-neutrally evolving sites.
(E and E') Haplotype-based selection statistics show elevated CLR at the locus for unsilvered alleles only (Figure S10).
(F and F') Phylogenetic weighting of phenotypes shows strong support for shared alleles between *S. hydaspe* and unsilvered *S. mormonia* as measured by TWISST.²² Topo1: expected species-level topology with monophyletic *S. mormonia*²³; Topo3: topology grouping lineages by silencing state.
(G) Proportion of introgression or gene flow between *S. hydaspe* and unsilvered *S. mormonia* (red arrow in F'), as measured by f_d ,²⁴ shows an excess of shared sites between them, as compared with the silver *S. coronis*.²³ Red dots (A–C' and G) and filled dots (D and D') highlight absolute values in the 99th percentile of their chromosome-wide distribution.
(H) Possible evolutionary scenarios explaining the sharing of unsilvered alleles between polymorphic *Speyeria* species (Figure S11). Introgressions involve the gene flow of unsilvered alleles between differentiated lineages by hybridization, whereas a scenario strictly based on ILS implies the pre-existence of both silver and unsilvered alleles in an ancestral population and their coalescence in differentiating lineages without gene flow.

populations (Figures 4C and 4C'). Lastly, Tajima's D is also reduced over this genomic region for the unsilvered haplotypes only, indicating the presence of sites deviating from neutral evolution (Figures 4D and 4D').

We next scanned the *optix* locus for selective sweeps using the composite-likelihood ratio (CLR) statistics implemented in

SweepD²⁵ and Sweepfinder2.²⁶ These scans show selective sweeps at the unsilvered allele in both the WA and NV polymorphic populations (Figures 4E, 4E', and S10), matching the position of the differentiated haplotypes, which span the 3 perfectly associated SNPs and blocks of hitchhiking variants around them (Figure S6). No signatures of selective sweeps were found

at the silver haplotypes. Because selection scans can only detect recent episodes of positive selection,^{27,28} silver alleles may have been fixed at these locations in a more distant past and thus accumulated genetic diversity that would obscure a potential ancient sweep signal.

Unsilvered alleles are shared between species

The genus *Speyeria* is relatively large and rapidly radiating, with several occurrences of sympatric species harboring monomorphic populations with either unsilvered or silver scales (Figure S11). Most interspecific crosses of *Speyeria* spp. can produce fertile offspring in laboratory conditions,^{14,15} and hybridization in the wild likely explains pervasive phylogenetic incongruence among Western North-American species.^{29,30} We therefore asked whether shared alleles are present between the locally monomorphic unsilvered *S. hydaspe* and unsilvered *S. mormonia*. To test for allelic sharing between the two species, we applied a topology weighting test (TWISST²²), with 4 defined groups all sampled from the same WA North Cascade location: a silver outgroup (*Speyeria coronis*), unsilvered *S. hydaspe*, and unsilvered and silver phased alleles of *S. mormonia*. TWISST scans group the samples by phenotypic state rather than by species phylogeny at the GWA interval (Figures 4F and 4F'), suggesting that the unsilvered alleles from *S. hydaspe* and *S. mormonia* are identical by descent. We then refined which sites are shared between unsilvered *S. mormonia* and *S. hydaspe* by calculating the f_d statistic in sliding windows²⁹ and found a strong signal of phylogenetic discordance concurring with elevated F_{ST} and CLR (Figure 4G). These results suggest that the unsilvered alleles of *S. mormonia* may have originated by adaptive introgression from an unsilvered, diverged species (Figure 4H). Alternatively, the unsilvered *optix* allele could have evolved in a population ancestral to several *Speyeria* species and then coalesced into its lineages without requiring gene flow, a phenomenon known as incomplete lineage sorting or ILS.³¹

DISCUSSION

Genetic repeatability of adaptation in diverged traits

Is the genetic basis of adaptation predictable?^{32,33} Empirical studies of phenotypic convergence suggest it is to some extent, as the same genes are often found to drive the repeated evolution of traits with simple genetic architecture or show parallel signatures of differentiation across populations that have adapted to similar ecological challenges.^{34,35} This evolutionary repeatability or “gene reuse”³⁴ can arise through independent mutations at the same hotspot loci, the introgression of adapted alleles across diverged gene pools, or the ILS of standing genetic variation. Here, we found that the *optix* locus drives color pattern variation at levels of replication that span the micro- and macroevolutionary scales, depicting a nuanced view of how the genotype-phenotype map shapes phenotypic diversity.

At the microevolutionary level, unsilvered alleles are identical across two distinct *S. mormonia* populations and are shared with an unsilvered species of the same genus. At the macroevolutionary level, our findings in *S. mormonia* parallel the repeated involvement of *optix* alleles in shaping adaptive variation in *Heliconius* and *Hypolimnys*,^{2,3,28,36–38} two lineages that diverged from *S. mormonia* 45–70 mya.^{8,39} In these two other lineages,

optix variation does not control silvering and camouflage strategies but instead switches the phenotypic expression of black to red and orange color patterns used in mimicry and aposematic signaling. It is noteworthy that the *optix* locus has already revealed multiple cases of adaptive introgression within the *Heliconius* radiation, as well as evidence that independently evolved *cis*-regulatory modules recombined to make new red patterns.^{4,7,40,41} We have now found a second lineage where *optix* alleles are shared between diverged species and that can then potentially be maintained by selection. Thus, patterns of silvering diversity within Nearctic species of the *Speyeria* genus, within and between species, may be explained by the repeated selection of the alleles that exist as ancient standing genetic variation (ILS) or via hybridization between inter-fertile lineages. Incomplete reproductive isolation and genome-wide phylogenetic discordance in *Speyeria* spp.^{14,29,30,42} warrant further studies to clarify the extent to which this variant is spread across other polymorphic *Speyeria* lineages.¹ In addition, silvering polymorphisms exist in Palearctic species of the *Argynnis*/*Fabriciana* genus⁴³ and may have derived from ancestral polymorphisms at the *optix* alleles as well or have evolved by parallel evolution using *de novo* mutations.

Remarkably, the reuse of *optix* across macroevolutionary time invokes convergent genetics but without phenotypic convergence: how does the same locus drive two distinct effects, red-orange pattern variation in *Heliconius*/*Hypolimnys* and silver dimorphism in *Speyeria*? Elements of an answer come from perturbation studies that examined the phenotypic effects of *optix* loss of function with CRISPR knockouts (KOs) and RNAi.^{11,17,37,44} In short, *optix* acts as a master selector gene that can switch both the pigmentary and ultrastructural identity of wing scales. First, all species examined so far show that *optix* is necessary for activating ommochrome pigmentation (red-orange scales) while also repressing dark melanin outputs in these same scales. In other words, *optix* deficiency results in a conversion of red-orange patterns to black in at least some parts of the wings,^{17,37,44} including in *S. mormonia* (Figures 3C and 3D). This function of *optix* in upregulating ommochromes while repressing melanic states thus appears conserved but varies in its spatial distribution across species. Second, *optix* also modulates scale ultrastructures, as it is required for the specification of wing-coupling scales in several species.^{11,17} In *Junonia coenia*, *optix* KOs also result in a gain of blue iridescence by thickening the scale's lower lamina.^{17,45} In *S. mormonia*, we found that wild-type (WT) silver scales lack *optix* and that buff and orange scales convert to reflective silver upon *optix* knockdown, implying an increase in upper lamina coverage that explains their high reflectance (the reverse from its effect in wing-coupling scales). The collective evidence shows that *optix* controls the deposition of chitin in the lower or upper laminae, resulting in changes in scale reflectance and iridescence, in both taxon- and position-dependent manners. Future research is needed to identify the co-factors that determine the localized phenotypic effects of *optix*, such as its role in repressing either silver or melanic fates in *S. mormonia*.

These data establish the *optix* transcription factor gene as a versatile genetic tool for the evolutionary diversification of wing patterns, including distinct adaptive polymorphisms involved in camouflage and mimicry across three lineages separated by 45–70 Ma of divergence. More broadly, selector genes like *optix* are thought to act as regulatory hubs that integrate positional

information and orchestrate the regulation of cell phenotypes. These so-called “input-output” genes may be privileged mutational targets of morphological evolution by combining evolvable expression with coordinated downstream effects on cell identity.³³ We extrapolate that similar genes can drive the repeated evolution of both convergent and divergent phenotypes by combining a *cis*-regulatory architecture that facilitates their spatial refinement (as seen in the diverse expression patterns of *optix* across butterflies) and an inherent capacity to switch cell responses in a given tissue (e.g., dual roles in pigmentary and ultrastructural outputs).

Limitations of the study

Although we could not procure the developmental samples to test this, we predict that unsilvered morphs express *optix* in the unsilvered spots. Testing this would clarify whether GWA SNPs control *optix* recruitment in the spots in a spatial or temporal manner. Another expectation from this working model is that RNAi knockdowns should have strictly equivalent effects between the two morphs.

Importantly, the ecological functions of the silver and unsilvered wing spots remain to be formally studied. A role in thermoregulation is unlikely because *S. mormonia* silver spots do not show noticeable heat-dissipating properties (Figure S4). Wing spots may be involved in intraspecific signaling (courtship, male competition, or species recognition), but a differential effect of the two forms may be difficult to detect. The exposure of the polymorphic pattern at the tip of the forewing (i.e., following Oudemans’ principle⁴⁶) suggests a function when the butterfly is at rest (Figure S1). Unsilvered morphs are likely cryptic, with beige and brown features potentially matching visual backgrounds of dirt and dry vegetation (Figure S1C). Silver spots, although more conspicuous, might make edge recognition difficult and represent a form of disruptive camouflage against vertebrate predators^{47,48} or might be used to deter or confuse invertebrate predators.^{49,50}

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to, and will be fulfilled by, the lead contact, Luca Livraghi (miles.livraghi@gmail.com).

Materials availability

This study did not generate new materials.

Data and code availability

- The data and code used in this study were made available on the NCBI short-read archive (SRA) under BioProjects: PRJNA841853, PRJNA1017100, PRJNA1052080.
- All original code has been deposited at Zenodo Data: <https://doi.org/10.5281/zenodo.15007413> and is publicly available as of the date of publication.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, L.L., J.J.H., W.M.R., C.L.B., and A.M.; methodology, L.L., V.M.S., C.C.T., and N.Y.; investigation, L.L., J.J.H., L.S.L., V.M.S., C.C.T., N.Y., S.M.V.B., A.H., C.M.T.K., W.M.R., C.L.B., and A.M.; writing, L.L., J.J.H., and A.M.; funding acquisition and resources, L.L. and A.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dimethyl Sulfoxide (DMSO)	Any	N/A
EDTA, 0.5 M, pH 8.0	Bioland Scientific	EDTA01
EGTA, 0.5 M, pH 8.0	Research Products Intl.	E14100-50.0
Formaldehyde, 37%	Sigma Aldrich	8187081000
Glycine, 10 g/L	Any	N/A
Methanol	Any	N/A
NaCl, 5 M	Any	N/A
PBS, 10x	UFC Bio	BPBS74-10X
Proteinase K	Promega	MC5005
SSC, 20x	Any	N/A
Tris buffer, pH 8.0, Low EDTA (0.1 M)	Quality Biological	351-324-721
Tris-HCl, 1 M, pH 7.5	Quality Biological	351-006-101
Tween 20	Bioworld	42030016-1
Critical commercial assays		
Zymo Quick-DNA Tissue/Insect 96 Kit	Zymo Research	D6017
Zymo Quick-DNA Tissue/Insect 96 Kit	Zymo Research	D6017
Ball mill for tissue lysis	Retsch	MM400
3.5mm stainless steel (SS316) bearing balls	Any	N/A
High Molecular Weight DNA Genomic-tip 100/G extraction kit	Qiagen	10223
Collection Microtubes (racked, 10 x 96)	Qiagen	19560
Collection Microtube Caps (120 x 8)	Qiagen	19566
TissueLyser Adapter Set (2 x 96)	Qiagen	69984
Hybridization Buffer, Probe Wash Buffer, Amplification Buffer	Molecular Instruments	N/A
HCR Alexa Fluor 488 Amplifier (B1)	Molecular Instruments	N/A
Deposited data		
<i>Speyeria mormonia</i> reference genome assemblies	Cicconardi et al. ⁸ ; this study	NCBI:PRJNA686707; PRJNA1195957
<i>Speyeria mormonia</i> whole genome sequences from wild-caught individuals in the North Cascades (WA) and the Rockies (CO)	This study	NCBI:PRJNA841853
<i>Speyeria mormonia</i> and <i>Speyeria</i> spp., whole genome sequences from wild-caught individuals, Sep 19 '23	This study	NCBI:PRJNA1017100
PacBio HiFi Revio Sequencing Long-reads from <i>Speyeria mormonia</i> (WA, USA, unsilvered phenotype)	This study	NCBI:PRJNA1052080
Phased assemblies from unsilvered WA individual	This study	NCBI: PRJNA1052080; PRJNA841853
Experimental models: Organisms/strains		
<i>Speyeria mormonia</i> , wild-caught, WA	Meadows Campground in North Cascades (2020)	NCBI:txid2026161
<i>Speyeria mormonia</i> , wild-caught, NV	Bear Creek Meadow, Jarbidge Mountains (2022)	NCBI:txid2026161

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Speyeria mormonia</i> , wild-caught, CO	Rocky Mountain Biological Laboratory (summers 2022 and 2023)	NCBI:txid2026161
<i>Speyeria coronis</i> , wild-caught, WA	Meadows Campground in North Cascades, WA (2020)	NCBI:txid2793757
<i>Speyeria hydaspe</i> , wild-caught, WA	Meadows Campground in North Cascades, WA (2020)	NCBI:txid1666726
Oligonucleotides		
DsiRNA sequence, <i>Smor_optix_E1</i> : 5'-GGAACGUUGUGAAGCAGUAUUACG-3'	IDT DNA Technologies	N/A
DsiRNA sequence, <i>Smor_optix_E1</i> : 3'-GACCUUGCAACACUUCGUCAUAAUGCU -5'	IDT DNA Technologies	N/A
Software and algorithms		
ArcGIS Online	ArcGIS Online	URL: https://maps.arcgis.com
Geneious Prime	Geneious	URL: https://www.geneious.com/
R / <i>colorspace</i> package		DOI: https://doi.org/10.18637/jss.v096.i01
R / <i>cowplot</i> package		URL: https://CRAN.R-project.org/package=cowplot
R / <i>dismo</i> package		URL: https://CRAN.R-project.org/package=dismo
R / <i>ENMeval</i> package		DOI: https://doi.org/10.1111/2041-210X.13628
R / <i>ggplot2</i> package v.3.4.2	Tidyverse/Hadley Wickham	URL: https://ggplot2.tidyverse.org
R / <i>gridExtra</i> package v.2.3	CRAN/Baptiste Auguie	URL: https://cran.r-project.org/web/packages/gridExtra
R / <i>spThin</i> package		URL: https://onlinelibrary.wiley.com/doi/10.1111/ecog.01132
R / <i>tidyverse</i> package v.2.0.0	Tidyverse/Hadley Wickham et al.	URL: https://www.tidyverse.org/
R / <i>GenotypePlot</i> package v.0.2.1	GitHub/Jim Whiting	URL: https://github.com/JimWhiting91/genotype_plot
Java / GATK (Genome Analysis Toolkit) v.4.1.4.1	GATK/Broad institute	URL: https://gatk.broadinstitute.org/
Java / Picard v.2.20.7	Picard/Broad institute	URL: https://broadinstitute.github.io/picard/
C / Samtools v.1.9	Github/Samtools	URL: https://github.com/samtools/samtools
C / BWA (Burrows-Wheeler Aligner) v.0.7.17-r1188	GitHub/lh3/bwa	URL: https://github.com/lh3/bwa
Rust / Mosdepth v.0.3.3	Github/Brent Pedersen	URL: https://github.com/brentp/mosdepth
Java / Beagle v.5.4	Washington.edu/Brian Browning	URL: https://faculty.washington.edu/browning/beagle/beagle.html
C/C++ / Plink v.1.9	Cog-genomics/Shawn Purcell and Christopher Chang	URL: www.cog-genomics.org/plink/1.9/
C/C++ / GEMMA v.0.98.3	Github/Pjotr Prins	URL: https://github.com/genetics-statistics/GEMMA
C/C++ / Hifiasm v.0.1.17	Github/Haoyu Cheng	URL: https://github.com/chhylp123/hifiasm
Python / Insitu Probe Generator v.0.3.2	Github/Ryan Null	URL: https://github.com/rwnull/insitu_probe_generator
C++ / Vcftools v.0.1.17	Sourceforge/Petr Danecek et al.	URL: https://vcftools.sourceforge.net/
C++ / Bcftools v.1.15.1	Github/Petr Danecek et al.	URL: https://github.com/samtools/bcftools
C++ / SweeD v.3.0	Excelis Lab/Pavlos Pavlidis et al.	URL: https://cme.h-its.org/exelixis/web/software/sweed/
C++ / SweepFinder2	GitHub/Michael de Giorgio	URL: https://github.com/mdegiorgio/SweepFinder2

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Python / <i>genomics_general</i>	GitHub/Simon Martin	URL: https://github.com/simonhmartin/genomics_general
Python / <i>twisst</i>	GitHub/Simon Martin	URL: https://github.com/simonhmartin/twiss
Other		
32-watt T8 Gro-Lux wide-spectrum grow tubes	Sylvania Lighting	22362
Butterfly habitat pop-up cage, 15.7" x 15.7" x 23.6"	Restcloud	B074ZHXF48
Small Clear Plastic Souffle / Cup Lid	Solo	PL100N
Borosilicate capillaries with filament	WorldPrecision Instruments	18100F-3
Gravity needle puller	Narishige International	PC-10
Three-axis MM33 right-handed manipulator	Drummond Scientific	3-000-024-R
Single pressure micro-injectors with footswitch	Tritech Research Inc.	MINJ-1
Pulse-length control module	Tritech Research Inc.	MINJ-2
Needle holder	Tritech Research Inc.	MINJ-4
Compressed air faucet adapter	Tritech Research Inc.	MINJ-38NPT14QC
Polyurethane tubing 1/4" OD, 1/8" ID	Tritech Research Inc.	TT-1-4OD
Binocular stereomicroscopes with 10–25× magnification	Any	N/A
Timelapse Camera	Technaxx	Technaxx TX-164
Square-wave electroporator	BTX	ECM 830
ECM 830 foot pedal	BTX	45-0211 or later model
Genetropes, L-shape, bent gold tip 5mm	Harvard Apparatus	450115

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Speyeria mormonia (synonym: *Argynnis mormonia*) are the smallest of the North American fritillaries, occupying alpine meadows throughout the Western US and Canada. They are a univoltine species, diapausing as first instar larvae close to egg laying sites comprising several *Viola* species. Two populations were sampled for genome resequencing in this study, both polymorphic for the silvering trait. The first population was sampled in August 2020 from the Meadows Campground in the North Cascades, Washington. The outgroup species used in the study (*S. hydaspe* and *S. coronis*), were sampled from this same population. A second population of *S. mormonia* was sampled in August 2022 from Bear Creek Meadow in the Jarbidge Mountains, Nevada. Finally, a third population was sampled for *in vivo* electroporation and *in situ* hybridization experiments from meadows located around The Rocky Mountain Biological Laboratory in Crested Butte, Colorado, between June–August of 2023 and 2024. Females were allowed to lay eggs on plant species of the genus *viola*, after which eggs were collected and monitored for hatching. First instar larvae were allowed to undergo their obligate diapause by placing larvae in loosely capped scintillation vials within a ziplock bag with a moist paper towel at 4°C. Larvae were removed from diapause by transferring them from moist glass scintillation vials to Tupperware containers with *V. sororia* leaves and exposing them to continuous light at 25°C for 72 hours. They were regularly stimulated with a fine paintbrush or a gentle breath of air while being monitored for feeding behavior. Once feeding was observed, they were moved to a 18L:6D photoperiod and maintained at 25°C. Larvae were reared in ventilated Tupperware containers lined with a folded Kimwipe, with *V. sororia* leaves securely placed in 1.5-mL tubes filled with shipping refrigerant gel to minimize water loss.

A revision of the *Speyeria* genus has been proposed, making it a sub-genus of *Argynnis*, and the *Speyeria* (*Argynnis*) *mormonia* species is undergoing a potential taxonomic revision, with a proposed restoration of most of its subspecies into the species *Argynnis bischoffii* W. H. Edwards, 1869.⁵¹ Given the widespread evidence of phylogenetic discordance in this reticulated lineage, we are awaiting further phylogenomic studies to clarify its taxonomic qualifiers before adopting these names.

METHOD DETAILS

Phenotypic data collection

A total of 9,763 occurrence records for *S. mormonia* were obtained from a combination of museum records, private collections, and online repositories (GBIF, iNaturalist), as summarized in Table S1. In brief, *S. mormonia* individuals were examined from museum and

private collections, scoring the presence of the silvering trait for each locality. To supplement the silvering trait distribution, research-grade GBIF records including from iNaturalist entries featuring scorable pictures of ventral wing surfaces were added to the dataset. Unusual cases where ambiguous silvering traits existed were left unscored. Each individual and corresponding latitude and longitude, as well as silvering states where available, were plotted on a basemap in ArcGIS.

Speyeria mormonia crosses

Thirty-one silver and 23 unsilvered wild-caught females were transported in individual glassine envelopes to the lab for egg laying. Females were housed in plastic containers lined with filter paper. Infrared heat lamps supplied high temperature and light, which stimulate egg-laying.⁵² For the original wild-caught females, lamps were operated by an automatic timer on a 17L:7D photoperiod. For later generations, the light period was adjusted to 14L:10D. Temperature ranged from 22 to 30°C and relative humidity ranged from 74 to 89%. All adults were fed with a 20% sucrose solution in an artificial feeder. Females were provided with either *Viola sororia* or *V. papilionacea* leaves for egg laying, which were briefly soaked in a mild bleach solution and placed in a water-pik. Upon hatching, first instar larvae were transferred at 2–7°C for the diapause period. Larval diapause was interrupted by transferring the larvae to a moist filter paper, and subsequently transferring single broods to individual rearing containers with *V. papilionacea* leaves. Larvae were allowed to pupate, given a unique code, and identified to a brood and larval cohort. Silvering trait was recorded and used to establish crosses over two subsequent generations. Phenotypic proportions and inferred genotypes for broods with more than 3 individuals are reported in Table S3. All the genetic crosses were generated at Princeton University from individuals captured in the summer of 1998 by WMR in Manning Park, BC. The resulting butterflies were not stored in conditions favoring DNA preservation.

Wild population sampling and DNA extraction

Individuals sampled for genome re-sequencing included 19 unsilvered and 20 silver individuals from the Meadows Campground in the North Cascades, WA and 15 unsilvered and 16 silver individuals from the Bear Creek Meadow in the Jarbidge Mountains, Nevada. Wild-caught samples were collected, wings stored in glassine envelopes and bodies kept in DESS buffer (20% dimethyl sulfoxide, 0.25M ethylenediaminetetraacetic acid, saturated sodium chloride, pH 8.0). Samples were subsequently stored at -20°C. DNA was extracted from adult thoraxes using a Zymo Quick-DNA Tissue/Insect 96 Kit and concentrations measured with a Qubit spectrophotometer.

Genome sequencing and genotype calling

Sequencing libraries (PE 150) were generated and sequenced in an Illumina NovaSeq6000 S4 Flow cell at the Institute for Genome Sciences (University of Maryland - Baltimore) generating resequencing data at 19.7x mean coverage, as measured with the *mos-depth* package.⁵³ Each sample was then aligned to the *S. mormonia* reference assembly⁸ using *bwa-mem* with default parameters.⁵⁴ PCR duplicates were marked and removed using *picard*.⁵⁵ Genotypes were called using the Genome Analysis Tool Kit (GATK) *HaplotypeCaller*⁵⁶ using parameters optimized for butterfly systems.⁵ Briefly, joint genotyping of individual genomic VCF records (gVCF) was performed with GATK's *GenotypeGVCFs*, using default parameters. Genotype calls were included in downstream analysis only if they met the following criteria: quality (QUAL) ≥ 30 , minimum depth ≥ 10 , maximum depth ≤ 100 (to avoid false SNPs due to mapping in repetitive regions), overall depth $\leq 100 \times$ number of samples, strand bias (FS) < 200 , quality by depth (QD) ≥ 5 , and for variant calls, genotype quality (GQ) ≥ 30 . The resulting VCF files were split into indel and SNP files, and subsequent analyses were performed only on SNPs, except for the GWAS which was also performed on the indel VCF file. Further filtering was performed using *bcftools*,⁵⁷ removing minor allele frequencies (MAF) of > 0.02 and missing genotypes (F_MISSING) of < 0.1 .

Principal component analysis and SNP visualization

To perform a principal component analysis on the whole genome dataset, SNPs were first linkage-pruned from a VCF file containing samples from both populations, then a PCA was performed on both a VCF file containing SNPs along the whole genome, as well as local sets of SNPs confined to the Genome Wide Association interval. PCA analyses were performed using *PLINK* v1.9.⁵⁸ The package *genotypeplot*⁵⁹ was used to inspect the GWA interval for trait associated SNPs and small indels, plotting each SNP coded in the VCF file as either homozygous for the reference allele, homozygous for the alternate allele, or heterozygous.

Long-read assembly of an unsilvered morph

High-molecular-weight DNA was extracted from an unsilvered individual from the WA population, homozygous for the fixed associated SNPs within the GWA interval (Figure 2D), using the QIAGEN Genomic-tip 100/G kit. DNA was sequenced on a PacBio Revio system at the Institute for Genome Sciences (University of Maryland - Baltimore), producing 28.2 Gb of sequence data. Genome assembly was performed using *HiFiasm* v0.15.1⁶⁰ using default parameters, resulting in two phased haploid assemblies with lengths of 447.9 Mb (N50 = 15.4 Mb; 201 scaffolds) and 442.8 Mb (N50 = 14.4 Mb; 121 scaffolds).

Hybridization chain reaction

To ensure accurate developmental timing for dissections, total pupation time was recorded from onset of pupa formation to eclosion using a TX-164 timelapse camera (Technaxx). Total development time was used to calculate pupal % development. Ten HCR⁶¹ probes were designed against the *optix* coding exon (Table S4), using the *insitu_probe_generator* tool,⁶² permitting a GC content in the 35–75% range, and allowing limiting runs of poly-GC and poly-AT to a maximum of 3 bp. Pupal wings were dissected from

the pupal case in cold 1X PBS as previously described in other butterflies,³ transferred to a fixative solution (750 μ L PBS 2 mM EGTA, 250 μ L 37% formaldehyde) containing 9.25% formaldehyde at room temperature for 30 min and washed four times in PBS containing 0.01% Tween20 (PBT). For pupae at earlier stages of development (28%), samples were permeabilized in 1 μ g/ μ L of ProteinaseK diluted in PBT solution for 5 min. Later-stage pupae (54%) were first permeabilized using detergent solution (10% SDS, 20% Tween, 1 M Tris-HCl, pH 7.5, 0.5 M EDTA, pH 8.0, 5 M NaCl) for 30 min at room temperature and then further digested in 1 μ g/ μ L of Proteinase K in PBT for 7 min at 55°C. Samples were then washed with a stop solution containing PBT and 2-mg/mL glycine and followed by two additional PBT washes. After transferring wings to a post-fix solution (850 μ L PBT, 150 μ L 37% formaldehyde) containing 5.55% formaldehyde for 20 min, wings were washed four times with PBT before following the rest of the protocol as in previously published procedures.⁶³

Pupal wing electroporation and RNAi

Dicer-substrate siRNAs (DsiRNAs) were designed against the *optix* coding exon (5'-GGAACGUUGUGAAGCAGUAUUACG-3'), ordered as Custom DsiRNAs with standard purification (IDT DNA Technologies), resuspended at 100 μ M in 1X *Bombyx* injection buffer (pH 7.2, 0.5 mM NaH₂PO₄, 0.5 mM Na₂HPO₄, 5 mM KCl), and stored as frozen aliquots at -70°C until use. Electroporation procedures followed a previously established protocol.²¹ To target the dorsal pupal wings, the negative electrode was placed in contact with a droplet of phosphate buffered saline (PBS) positioned on the ventral side of the peeled wing, while the positive electrode was placed on the agarose pad on the dorsal side (poles are reversed when targeting the ventral wing surface). Electroporation was then carried out with 5 square pulses of 280 ms at 10 V, separated by 100-ms intervals.

Population genetics analyses

To calculate haplotype-specific statistics, we first phased a VCF file containing all populations using *beagle* v5.4.^{64,65} A custom script (<https://doi.org/10.5281/zenodo.15007413>) was then used to separate each haplotype into a new entry in the VCF, recoding individual haplotypes as either "_A" or "_B" based on the three diagnostic SNPs identified in the GWAS. Fixation index (F_{st}), absolute divergence (D_{xy}), nucleotide diversity (π), Tajima's D and proportion of introgression (f_d) statistics were then calculated on individual alleles using the *genomics_general* toolkit (https://github.com/simonhmartin/genomics_general) and specifying the "-sites" option for windows of 50 fixed informative sites. The same phased and recoded VCF was used to generate selective sweep statistics using the software package *SweepD*²⁵ as well as *SweepFinder2*.²⁶ Support for phylogenetic hypotheses consistent with shared haplotypes of unsilvered alleles were evaluated using *Twisst*²² (topology weighting by iterative sampling of subtrees; <https://github.com/simonhmartin/twisst>).

QUANTIFICATION AND STATISTICAL ANALYSIS

Species distribution modeling

We used the occurrence records featured in Table S2 to construct a suite of species distribution models (SDMs) for the species using WorldClim data and elevation as predictors.⁶⁶ Prior to modeling, we removed dubious records and those with erroneous coordinate data (e.g. occurring over bodies of water). We further thinned records by using a spatial buffer of 5 kilometers through the R package *spThin*.⁶⁷ Background points were randomly sampled across the extent of the study region. Using the package *ENMeval* we constructed MaxNet species distribution models using all combinations of linear, quadratic, and product predictors across regularization values of 0.5, 1, 2, and 3.⁶⁸ We partitioned the data using a checkerboard approach.

We assessed SDM performance by calculating the Akaike Information Criterion (AIC) score and selected the top performant model based on this metric. We also compared area under curve (AUC) and continuous Boyce index (CBI) scores, ensuring that these values were sufficiently robust in our top model. The top model achieved an AUC score of 0.92 and a CBI of 0.99 on the validation partition. We created a binary range map from this top model by thresholding such that occurrence probability values greater than 20% were represented as a potential range map. Finally, we clipped this binary map to an expert-drawn range map for the species.⁶⁹

Geospatial and bioclimatic regression analyses

Using the geographic phenotype data, we used beta-regression analysis to detect associations between local silvering frequency and environmental variables. To calculate silvering frequencies, phenotypic records were binned into 20-km radius clusters by calculating the distance between occurrence records from latitude and longitude coordinates, and applying a complete hierarchical clustering approach to these pairwise distances. Clusters of 20 km were identified by "cutting" the resulting dendrogram by this distance. Elevation data were obtained from the SRTM 90m Digital Elevation Database.⁷⁰ BioClim variables were obtained from WorldClim.⁶⁶ We obtained three additional variables—downward surface solar radiation, total precipitation, and leaf area index—from the Copernicus Climate Store ERA5-Land Monthly Dataset across the years 1970–2024,⁷¹ for the adult flight period of the butterfly (June–September).

Thermal emissivity analyses

To evaluate the impact of the silvering polymorphism on the thermodynamics of *Speyeria mormonia*'s wings, we used thermal imaging to measure increase in wing temperature under sunlight illumination. Figure S4A illustrates the measurement setup modeling the thermodynamic system of a butterfly wing, as previously described.¹⁶ Briefly, wings were suspended above the

temperature-controlled cold plate which represents the cold sky, using a thermo-radiatively transparent polyethylene thin film. A xenon lamp (Thorlabs HPLS-30-03, color temperature = 6,500K) simulating the solar radiation illuminates the wing at an angle of 15° to the vertical. Although in the real world, the sun and sky are on the same side of the wing, which is different from our artificial system where the sun and sky are on opposite sides of the wing, the two thermodynamic systems are equivalent considering that the wing is extremely thin. Because of the thinness, the conduction through the wing thickness occurs instantly, resulting in an insignificant temperature gradient across the wing thickness. A thermal camera (FLIR T640) was positioned above the wing to monitor the thermal radiance from the wing along the normal axis. We first used the thermal camera to measure the optical properties of the wings. Since a butterfly wing is thin enough to be semi-transparent at the responsive wavelengths of the thermal camera, the irradiance perceived by the camera, Φ_{cam} , comprises three components as shown in Figure S4A: (1) the thermal radiance from the wing, Φ_w , (2) the thermal radiance of the ambient environment, Φ_t , reflected by the wing and (3) the thermal radiance of cold plate underneath, transmitting through the wing, Φ_r .¹⁶ The perceived irradiance can be expressed as:

$$\Phi_{cam} = \Phi_w + \Phi_t + \Phi_r,$$

where:

$$\Phi_w(T_w) = 2\pi \int_0^{\theta_{cam}} d\theta \cos \theta \sin \theta \int_{\lambda_{cam}} d\lambda \left[\eta(\lambda) \varepsilon_o(\lambda, \theta) \frac{2hc^2}{\lambda^5} \frac{1}{\exp \exp \left(\frac{hc}{k_b T_w} \right) - 1} \right],$$

$$\Phi_t(T_{bg}) = 2\pi \int_0^{\theta_{cam}} d\theta \cos \theta \sin \theta \int_{\lambda_{cam}} d\lambda \left[\eta(\lambda) t_o(\lambda, \theta) \frac{2hc^2}{\lambda^5} \frac{1}{\exp \exp \left(\frac{hc}{k_b T_{bg}} \right) - 1} \right],$$

$$\Phi_r(T_a) = 2\pi \int_0^{\theta_{cam}} d\theta \cos \theta \sin \theta \int_{\lambda_{cam}} d\lambda \left[\eta(\lambda) r_o(\lambda, \theta) \frac{2hc^2}{\lambda^5} \frac{1}{\exp \exp \left(\frac{hc}{k_b T_a} \right) - 1} \right],$$

and where ε_w , t_w , r_w are the emissivity, transmittance, and reflectance of the wing respectively. T_w , T_a , and T_{bg} are the temperature of the wing, ambient environment, and cold plate. The resulting perceived irradiance is:

$$\Phi_{cam}(T_{cam}) = 2\pi \int_0^{\theta_{cam}} d\theta \cos \theta \sin \theta \int_{\lambda_{cam}} d\lambda \left[\frac{2hc^2}{\lambda^5} \frac{1}{\exp \exp \left(\frac{hc}{k_b T_{cam}} \right) - 1} \right].$$

By applying a temperature difference between the ambient environment and the cold plate, the temperature of the wing measured by the camera, T_{cam} , would range from T_{bg} to T_a , dependent on thickness. To simplify these relations, we made a few approximations. First, we assumed the wings' reflectance to be small and nearly constant, using a reflectance value of 0.05 in calculations. The transmission was then calculated as:

$$t_w = 1 - \varepsilon_w - 0.05$$

Secondly, when the xenon lamp was off, the true wing temperature, T_w , was approximated as the ambient temperature of the ambient temperature, given the wing was sufficiently distant from the cold plate. These assumptions simplified the equations to:

$$\int_{\lambda_{cam}} d\lambda \eta(\lambda) I(T_{cam}, \lambda) = \int_{\lambda_{cam}} d\lambda \eta(\lambda) \varepsilon_w(\lambda) I(T_w, \lambda) + \int_{\lambda_{cam}} d\lambda \eta(\lambda) t_w(\lambda) I(T_{bg}, \lambda) + \int_{\lambda_{cam}} d\lambda \eta(\lambda) \varepsilon_w(\lambda) I(T_a, \lambda).$$

By capturing a single snapshot of the wing with the xenon lamp off, we can derive the optical properties of interest using the known temperatures of the ambience and cold plate. After acquiring the approximated emissivities of the wings, we turned on the lamp and recorded the rise of wing temperature. The measured temperatures were then converted into the real wing temperatures following the same equation with the previously measured optical properties. The illumination of the xenon lamp had an intensity of 60mW/cm², which is about 2/3 full sun. We measured three pairs of unsilvered/silver morphs of *Speyeria mormonia* to compare the temperature rises of the patches at the same locations of their hindwings (Figure S4B) induced by the illumination.

Genome-wide association study

To perform the GWAS, VCF files were further filtered by removing non-variant sites and keeping only bi-allelic SNPs. Missing genotypes were imputed and phased using *beagle* v5.4.^{64,65} A binary filesset was generated using *PLINK* v1.9⁶⁸ for input into *GEMMA* v0.98.3,⁷² which was used to compute site-wise Wald χ^2 test *P*-values, accounting for population structure by supplying a relatedness matrix as a covariate. Genome-wide associations were performed both separately for each population (WA and NV) as well as combining both datasets. SNPs were considered statistically significant if passing a Bonferroni corrected genome-wide significance threshold ($p < 0.01$).