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SHORT COMMUNICATION

Two sides of the same wing: ventral scales enhance dorsal wing color in the butterfly *Speyeria mormonia*

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ABSTRACT

Biological visual signals are often produced by complex interactions between light-absorbing and light-scattering structures, but for many signals, potential interactions between different light-interacting components have yet to be tested. Butterfly wings, for example, are thin enough that their two sides may not be optically isolated. We tested whether ventral wing scales of the Mormon fritillary, *Speyeria mormonia*, affect the appearance of dorsal orange patches, which are thought to be involved in sexual signaling. Using reflectance spectroscopy, we found that ventral scales, either silvered or non-silvered, make dorsal orange patches significantly brighter, with the silvered scales having the greater effect. Computational modeling indicates that both types of ventral scale enhance the chromatic perceptual signal of dorsal orange patches, with only the silvered scales also enhancing their achromatic perceptual signal. A lack of optical independence between the two sides of the wings of *S. mormonia* implies that the wing surfaces of butterflies have intertwined signaling functions and evolutionary histories.

KEY WORDS: Lepidoptera, Structural color, Spectroscopy, Color perception, Visual modeling

INTRODUCTION

Animals utilize a rich and diverse palette of colors for sexual and aposematic signaling, and they produce these colors in a variety of ways (Johnsen, 2011; Cuthill et al., 2017). Pigmentary colors, for example, result from molecules that absorb certain wavelengths, whereas structural colors arise from nanostructures that scatter light in a wavelength-dependent manner. Although colors are commonly classified as either pigmentary or structural, many animals create vibrant color signals using a combination of absorption and scattering. Cephalopods, for example, produce bright, chromatically tuned color signals through the joint actions of light absorption by pigment-packed chromatophores and light scattering by underlying structures including iridophores and leucophores (Mäthger et al., 2006; DeMartini et al., 2013). Likewise, some birds produce vividly colored feathers through a combination of light absorption by melanin pigment and light scattering by keratin nanostructures (Shawkey et al., 2003; Shawkey and Hill, 2006). To understand the function and evolution of biological color displays, we must consider how light-absorbing pigments and light-scattering nanostructures are used synergistically to enhance the appearance of these signals for potential viewers.

Like the color displays of cephalopods and birds, the diverse color displays on the wings of butterflies are created using multiple types of light-interacting components (Sekimura and Nijhout, 2017). This diversity is commonly attributed to the 'pixel-like' wing scales, which often have distinct optical properties (Stavenga et al., 2004). Some scales are packed with light-absorbing pigments, and butterfly species employ different types of these pigments to achieve different colors (Nijhout, 1991). Other scales have nanostructural modifications, which can give the appearance of white or silver through broadband scattering or specific colors though narrowband scattering (Ren et al., 2020; Lloyd and Nadeau, 2021). By altering the types and locations of these scales, it is thought that butterflies can independently modify the appearances of their dorsal and ventral wing surfaces. For example, in many butterfly species, the dorsal surfaces of the wings are patterned with conspicuous scales for sexual or aposematic signaling and the ventral surfaces are patterned with inconspicuous scales for camouflage (Kemp, 2008; Prudic et al., 2015; Prakash and Monteiro, 2018).

A challenge presented by the two sides of butterfly wings having different signaling functions is that the wings are thin enough that their two sides may not be optically isolated. If so, the appearance of one wing surface could impact the appearance of the other, a potential source of both evolutionary innovation and constraint. For example, the swordtail butterfly, *Graphium sarpedon*, has conspicuous blue-green wing patches whose color is produced through the joint action of dorsal scale pigmentation, wing membrane pigmentation and reflection by the transparent ventral scales (Stavenga et al., 2010). An extreme example is the large transparent wing regions of the clearwing butterfly, *Greta oto*, which require a lack of pigmentation combined with surface modifications of the dorsal wing scales, the wing membrane and the ventral wing scales (Pomerantz et al., 2021). Although wing transparency is not widespread across butterfly species, synergistic interactions between light-absorbing scales on one side of the wing and light-scattering scales on the other side may be more common than previously thought.

In this study, we tested whether the highly reflective silver patches found on the ventral sides of wings in certain morphs of the Mormon fritillary, *Speyeria mormonia*, affect the appearance of their orange-and-black patterned dorsal wing surfaces (Fig. 1). The appearance of the orange patches likely plays an important role in sexual signaling, as it does in other fritillaries such as *Argynnis* (Magnus, 1958), but the functional role of the silver patches is unknown. We measured the spectral reflectance of orange patches near the edges of the dorsal surfaces of forewings and hindwings ('marginal patches'), which are backed by silver scales on the

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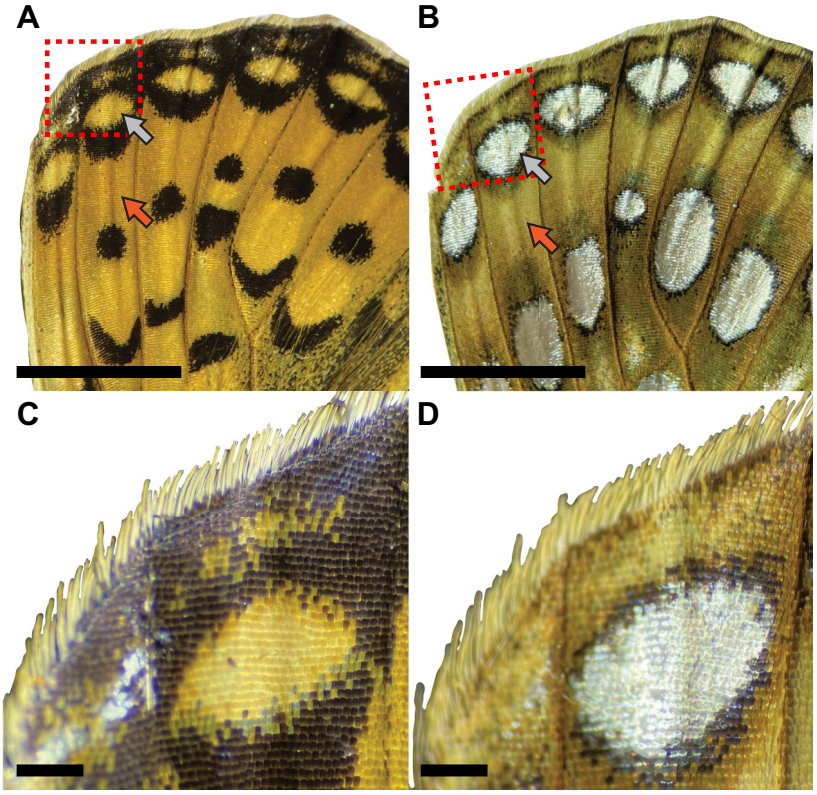

Fig. 1. Wings of the Mormon fritillary *Speyeria mormonia*. The anterior section of a hind wing from a female *Speyeria mormonia* shown from dorsal (A,C) and ventral (B,D) perspectives. The red boxes (A,B) indicate the locations of the magnified inlays (C,D). The grey arrows (A, B) indicate the locations of the marginal wing patch in dorsal (A) and ventral (B) perspectives. The orange arrows (A,B) indicate the locations of the submarginal wing patch in dorsal (A) and ventral (B) perspectives. Scale bars: (A,B) 5.0 mm, (C,D) 0.5 mm.

ventral sides of the wings. We also measured the spectral reflectance of orange patches near the centers of the dorsal sides of forewings and hindwings ('submarginal patches'), which are backed by non-silvered orange scales on the ventral sides of the wings. We measured the reflectance of all dorsal patches first with the ventral scales present and then re-measured dorsal patch reflectance after removing the underlying ventral scales. To quantify the differences in appearance among our wing patch reflectance measurements, we calculated perceptual contrast using a computational model of the *S. mormonia* visual system.

MATERIALS AND METHODS

Specimen collection

We collected females of the silver morph of *Speyeria mormonia* (Boisduval 1869) in 2012 from a population immediately south of the Rocky Mountain Biological Laboratory, Gunnison County, CO, USA (38°57′08″N, 106°58′07″W; 2930 m ASL). We collected eggs from these specimens, transported diapausing first instar larvae to Stanford University (Stanford, CA, USA), and reared these larvae to adults in a greenhouse on potted common blue violets, *Viola soraria*.

Reflectance spectrometry

To measure the spectral reflectance of dorsal patches on the wings of *S. mormonia*, we first removed the left forewing and left hindwing from each individual. We clamped wings between two metal slides that each had a circular cutout in their middle. By using this setup, we held wings stable while preventing unwanted reflections from materials in front of or behind the wings. The metal slides held wings flat, and we measured reflectance from patch areas with surfaces approximately normal to the viewing axis of the microscope objective.

We measured spectral reflectance using a setup described previously that included a modified Olympus CX-31 microscope (Center Valley, PA, USA) with a 10X PlanC N UIS2 objective, a 20W tungsten halogen lamp (HL-2000-HP-FHSA; Ocean Optics), and a Flame-S-VIS-NIR-ES spectrometer (Ocean Optics) (Kingston et al., 2019). Our reflectance setup did not allow us to take reliable UV reflectance measurements, so we limited our reflectance analysis to the wavelength range 400–700 nm. We standardized our measurements using Spectralon (WS-1-SL; Ocean Optics). We also performed a negative control measurement, in which we confirmed that the reflectance of an empty slide was zero. This ensured that light transmitted through the wing did not appreciably reflect off background surfaces and contribute to the measured wing reflectance.

We first measured the reflectance of each dorsal wing patch with the ventral scales underlying it present. We then removed the slide holding the wing from the microscope stage without changing the stage position, used a scalpel to gently remove all of the ventral scales directly underlying the dorsal patch of interest, placed the slide back onto the microscope stage, and then re-measured the reflectance of the dorsal patch. By conserving both the stage position and the position of a wing within the metal slide encasing it, we were confident that we took repeated measurements from wings at identical locations and surface angles. We repeated this process for one marginal orange patch and one submarginal orange patch for one forewing and one hindwing from each of 20 individual female *S. mormonia* (Fig. 1).

Reflectance data analysis and computational modeling

We used the R program 'pavo' (Maia et al., 2013) to analyze all spectral reflectance measurements. Briefly, we imported reflectance data, applied a locally weighted smoothing

Journal of Experimental Biology (2023) 226, jeb246396. doi:10.1242/jeb.246396

algorithm to remove electrical noise, and zeroed spurious negative values. The 'pavo' visual models require reflectance data from 300 to 700 nm and our UV reflectance data were unreliable, so we tested models with (1) the reflectance values for wavelengths less than 400 nm set to zero and (2) the reflectance values for wavelengths less than 400 nm set to the reflectance value measured at 400 nm. Both modeling scenarios had equivalent results for all of our analyses. We chose to display results for the second approach because wing pigments from *S. mormonia* have <10% reflectance in the UV wavelengths (300–400 nm) (Briscoe et al., 2010).

Using pavo, we compared the brightness of each dorsal orange patch with its underlying ventral scales present with the brightness of the same dorsal patch with its ventral scales removed. We also compared the brightness of dorsal orange marginal patches (backed by silvered scales) with the brightness of dorsal orange submarginal patches (backed by non-silvered scales). We calculated the brightness of individual reflectance spectra using the total brightness metric (B1), which is the sum of the relative reflectance from 300 to 700 nm. We then used a Shapiro–Wilk test for each of our comparisons to assess the normality of the data. If data were normally distributed, we followed up with a paired *t*-test. If data were not normally distributed, we followed up with a paired Wilcoxon signed-rank test.

We used a receptor-noise limited model (Vorobyev and Osorio, 1998; Vorobyev et al., 2001) to analyze whether the presence and type of ventral scales had significant effects on the perception of orange dorsal wing patches by other *S. mormonia*. We first constructed a computational model of the *S. mormonia* visual system using the 'vismodel' function in pavo. As previously mentioned, we were unable to reliably measure UV reflectance (300–400 nm), so we compared visual models either including or excluding a UV-sensitive spectral class of photoreceptor (peak absorbance of 350 nm). The results were not significantly different between the modeling scenarios. We are presenting results that exclude UV-sensitive photoreceptors. We estimated photoreceptor peak absorbances of 450, 550 and 620 nm, and receptor densities of 2, 6 and 1, respectively, based on published data for the fritillary *Speyeria aglaja* (Belušič et al., 2021). We assumed Weber chromatic and achromatic fractions of 0.05 as per other butterfly visual studies (McCulloch et al., 2017). We then used our visual model in a bootstrapping approach to calculate noise-corrected chromatic and achromatic perceptual distances among patches. This approach allows us to measure the perceptual contrast among patches and provides a 95% confidence interval, which can be inspected to see whether it exceeds the theoretical discrimination threshold of one noise-weighted Euclidean distance (N-WEB) (Maia et al., 2013).

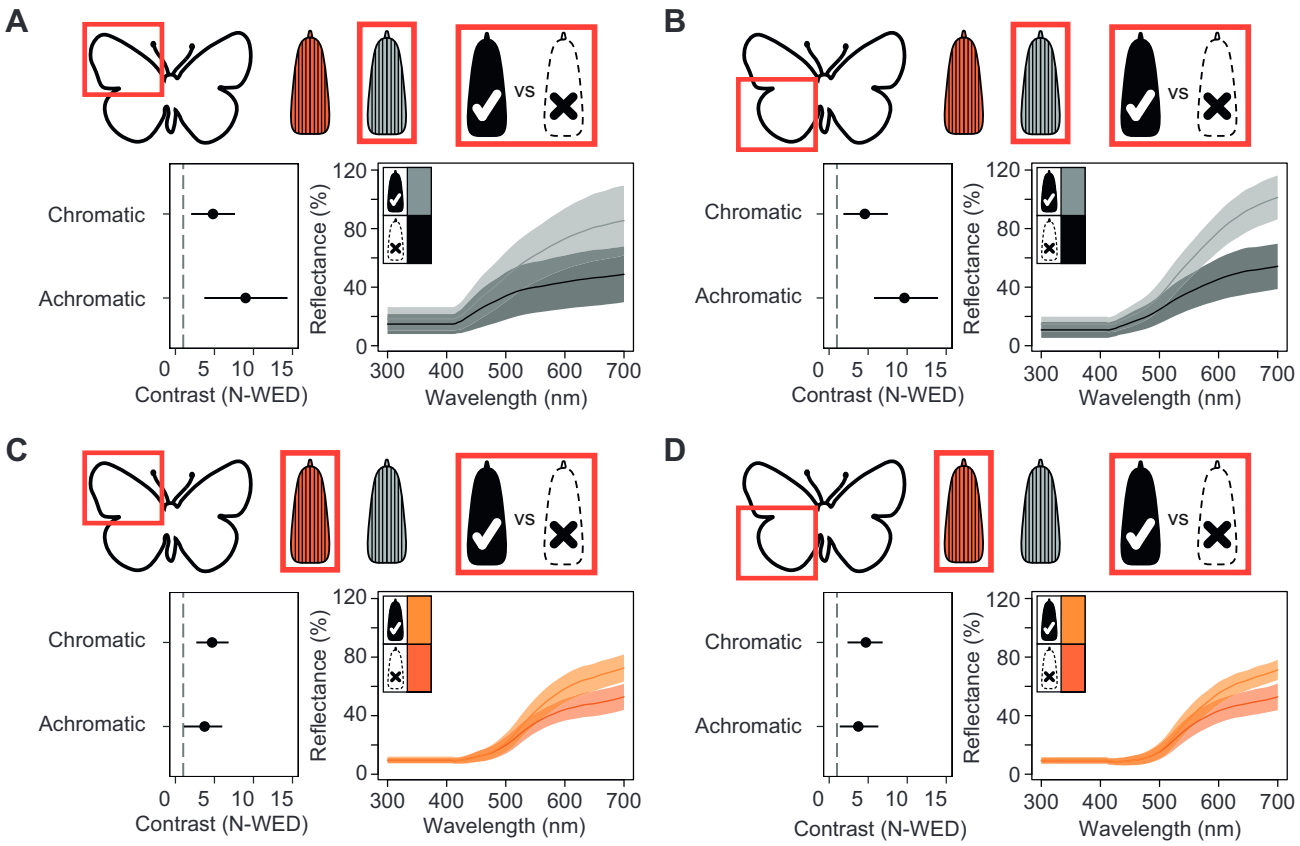

Fig. 2. Reflectance spectra and perceptual contrast whisker plots comparing dorsal orange patches from unaltered (ventral scales present) and altered (ventral scales removed) wings from *Speyeria mormonia* (*N*=20). Comparisons between ventral scales present and absent conditions for marginal patches on the forewing (A), marginal patches on the hindwing (B), submarginal patches on the forewing (C), and submarginal patches on the hindwing (D). For each of the comparisons, the upper small diagrams indicate whether the compared patches are on the forewing or hindwing, whether the patches are marginal (silver) or submarginal (orange), and whether ventral scales are present or absent. Each reflectance plot shows mean reflectance spectra for the comparison indicated at the top of the panel as well as 95% confidence intervals. Each reflectance plot is paired with a whisker plot showing the mean chromatic and achromatic perceptual contrast (black dots) of the dorsal orange patches investigated as well as 95% confidence intervals (black lines). The dashed vertical line represents 1.0 noise-weighted Euclidean distance (N-WEB) and reflectance comparisons with contrast confidence intervals encompassing this value are not significantly noticeably different to our computational model of the *S. mormonia* visual system.

Journal of Experimental Biology (2023) 226, jeb246396. doi:10.1242/jeb.246396

RESULTS AND DISCUSSION

Results

Scales on the ventral sides of the wings make orange patches on the dorsal sides of wings brighter. Marginal patches on the dorsal forewings (Fig. 2A) and hindwings (Fig. 2B) were significantly brighter (58% and 65%, respectively; $P<0.05$) when they were backed by silvered scales than when they were not. Likewise, submarginal patches on the dorsal forewings (Fig. 2C) and hindwings (Fig. 2D) were significantly brighter (27% and 25%, respectively; $P<0.05$) when they were backed by non-silvered scales than when they were not.

As viewed by our computational model of the *S. mormonia* visual system, silvered scales on the ventral sides of wings significantly affected the chromatic and achromatic perceptual signals of marginal orange patches on dorsal forewings (Fig. 2A) and hindwings (Fig. 2B). In comparison, non-silvered scales on the ventral sides of wings significantly altered the chromatic perceptual signal of submarginal orange patches on dorsal forewings (Fig. 2C) and hindwings (Fig. 2D), but did not significantly change their achromatic perceptual signal.

When wings are unaltered, orange marginal patches on the dorsal forewings (Fig. 3A) and hindwings (Fig. 3B), which are both backed by silvered scales, were significantly brighter than the neighboring orange submarginal patches (42% and 52%, respectively; $P<0.05$), which are backed by non-silvered scales. After we removed scales from the ventral sides of wings, the marginal patches on the dorsal forewings (Fig. 3C) and hindwings (Fig. 3D) were no longer significantly brighter than the neighboring submarginal patches (15% and 15%, respectively; $P>0.05$).

As viewed by our computational model of the *S. mormonia* visual system, unaltered orange marginal patches on the dorsal forewings (Fig. 3A) and hindwings (Fig. 3B) were significantly contrasting from neighboring orange submarginal patches using both chromatic and achromatic perception. In the absence of ventral scales, the marginal and submarginal patches remained significantly contrasting using chromatic perception, but were not significantly contrasting using achromatic perception (Fig. 3C,D).

Discussion

The dorsal and ventral sides of the wings of *S. mormonia* are not optically independent. Ventral scales, whether silvered or non-silvered, alter the appearance of dorsal orange patches. Overall, silvered ventral scales have a greater impact than non-silvered ventral scales on the appearance of orange patches on the dorsal sides of wings. Silvered ventral scales do more to increase the brightness of these orange patches than non-silvered ventral scales. Further, both types of ventral scale enhance the chromatic contrast of dorsal orange patches, but only the silvered ventral scales enhance their achromatic contrast as well.

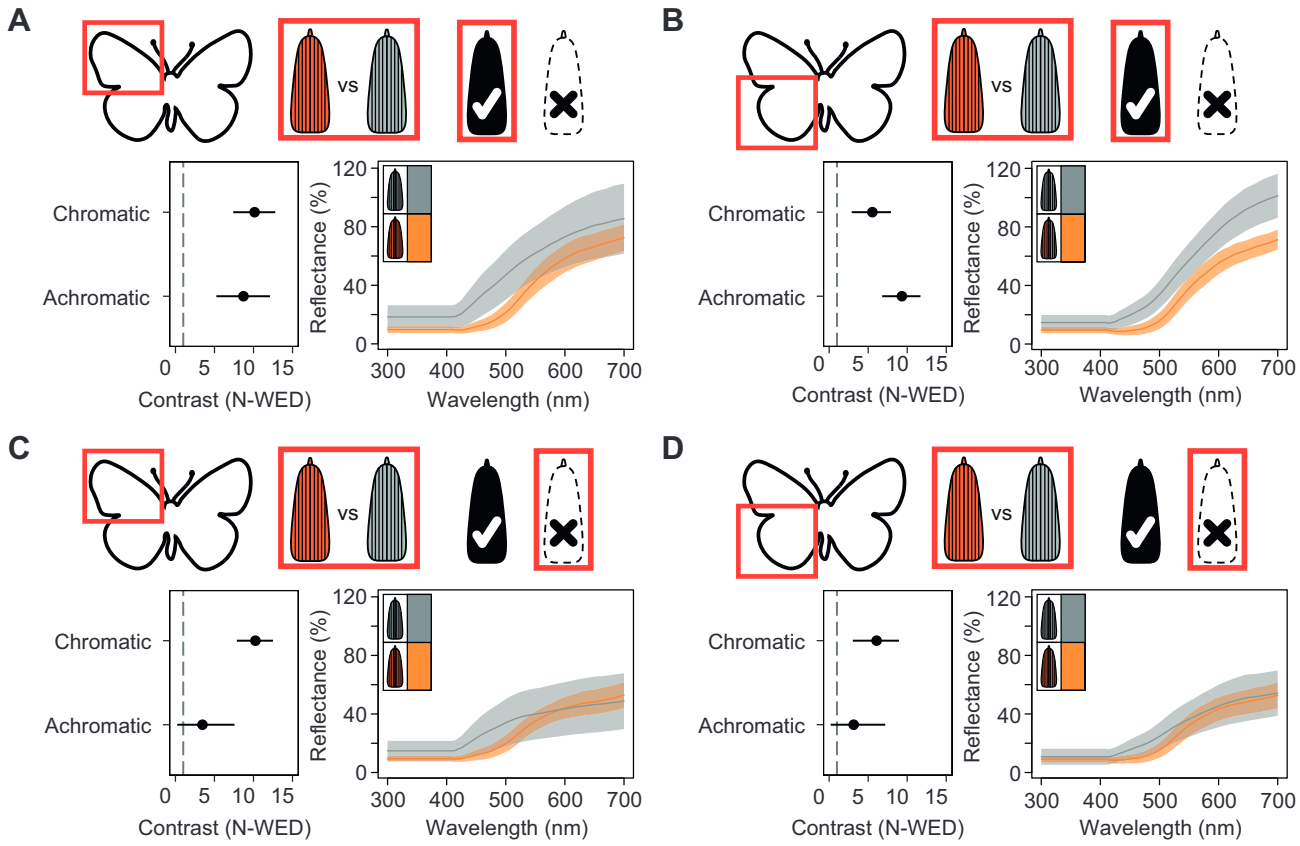

Fig. 3. Reflectance spectra and perceptual contrast whisker plots comparing dorsal orange patches from marginal and submarginal regions of wings from *Speyeria mormonia* (*N*=20). Comparisons between marginal and submarginal patches on the forewing with ventral scales present (A), on the hindwing with ventral scales present (B), on the forewing with ventral scales absent (C), and on the hindwing with ventral scales absent (D). For each of the comparisons, the upper small diagrams indicate whether the compared patches are on the forewing or hindwing, whether the patches are marginal (silver) or submarginal (orange), and whether ventral scales are present or absent. Each reflectance plot shows mean reflectance spectra for the comparison indicated at the top of the panel as well as 95% confidence intervals. Each reflectance plot is paired with a whisker plot showing the mean chromatic and achromatic perceptual contrast (black dots) of the dorsal orange patches investigated as well as 95% confidence intervals (black lines). The dashed vertical line represents 1.0 noise-weighted Euclidean distance (N-WEB) and reflectance comparisons with contrast confidence intervals encompassing this value are not significantly noticeably different to our computational model of the *S. mormonia* visual system.

Our results demonstrate that the reflectivity and patterning of the ventral sides of the wings of *S. mormonia* can have significant effects on how color characteristics of the dorsal sides of the wings are perceived by conspecific viewers, which suggests that the dorsal and ventral wing surfaces are not functionally isolated from one another.

The lack of optical independence between the two sides of butterfly wings has evolutionary and functional implications. The dorsal and ventral wing surfaces are usually considered functionally distinct signaling structures, with the dorsal surfaces often bearing bright patterns for intraspecific or aposematic signaling and the ventral surfaces often bearing dull patterns for camouflage and sometimes deimatic displays such as eyespots (Prakash and Monteiro, 2018). Our results deviate from this story by showing that the ventral scales on the wings of *S. mormonia* have a significant effect on the reflectivity of the dorsal wing patches and on how these color patches are perceived by other butterflies. Overall, this implies that the two wing surfaces of butterflies may have evolutionary fates for visual signaling that are more intertwined than previously thought.

The orange patches on the wings of *S. mormonia* (and closely related species) are thought to be used by females for sexual signaling because males are attracted towards orange swatches of similar chroma to the wings of females (Magnus, 1958). Males search for mates during mid-day under open skies by patrolling above the dense vegetation (McCulloch et al., 2017). The bright orange of females' dorsal wing surfaces may aid in mate detection and be a sexually selected trait. To attract males for mating, females would benefit from wings with large, bright, orange dorsal surfaces. Further, males of closely related species (e.g. *Argynnis*) seem to choose females based on the size, chromaticity and fluttering rate of their wings, so the visual signal of a female's wings is presumably an important sexually selected trait (Magnus, 1958). It is likely that females producing brighter and more saturated orange signals will be more quickly detected by patrolling males. Reflections from the silvered scales of butterflies are largely viewing angle independent (Ren et al., 2020; Dolinko et al., 2021), so our findings are relevant to the varied angles at which silver scales will be illuminated and viewed during mating interactions under natural conditions.

The broadband reflective silver scales on the ventral wing surfaces of *S. mormonia* may be a metabolically inexpensive way for nutrient-limited females to boost their sexual signal. Although the functional role of the silvered patches in *S. mormonia* has been debated (e.g. they may function as thermal insulators or primary sexual signals; see Roberts, 2000; Boggs and Freeman, 2005), our results demonstrate that the ventral silvered patches undeniably increase the achromatic and chromatic visual signals produced by the dorsal orange patches. While changes in the type and quantity of pigment embedded in the dorsal orange scales could alter the chromatic contrast of the orange reflectance signal, these changes would likely decrease the brightness of the signal. Adding an underlying broadband reflector, however, may be an efficient way to not only boost the achromatic contrast of a dorsal orange patch but also increase the chromatic contrast of the patch as the light reflected by the underlying reflector passes through the patch twice. The increase in chromatic contrast achieved via this method would have the added benefit of not requiring more orange pigment (i.e. ommochrome), which is metabolically expensive to produce for an insect that obtains the requisite nitrogen only in the larval stage (Roberts, 2000; Stavenga et al., 2014; Figon and Casas, 2019). By considering the synergistic interactions of light-absorbing and light-scattering structures, we may better understand the function and evolution of complex biological color displays seen in butterflies and a diversity of other taxa.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: D.R.C., C.L.B., D.I.S.; Methodology: D.R.C.; Software: D.R.C.; Validation: D.R.C., C.L.B., D.I.S.; Formal analysis: D.R.C.; Investigation: D.R.C.; Resources: C.L.B., D.I.S.; Data curation: D.R.C.; Writing - original draft: D.R.C., D.I.S.; Writing - review & editing: D.R.C., C.L.B., D.I.S.; Visualization: D.R.C.; Supervision: D.I.S.; Project administration: D.R.C., D.I.S.; Funding acquisition: C.L.B.

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Data availability
All relevant data can be found within the article.

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