Arthropod Structure & Development 51 (2019) 14–22

Contents lists available at ScienceDirect

Arthropod Structure & Development

journal homepage: www.elsevier.com/locate/asd

Plasticity and divergence in ultraviolet reflecting structures on Dogface butterfly wings

Jennifer Fenner[*], Luis Rodriguez-Caro, Brian Counterman

Department of Biological Sciences, Mississippi State University, MS, 39762, United States

ARTICLE INFO

Article history:
Received 11 January 2019
Received in revised form
24 May 2019
Accepted 3 June 2019

Keywords:
Lepidoptera
Ultraviolet signal
Wing pattern

ABSTRACT

The vast diversity of animal coloration is generated through a combination of pigment and structural colors. These colors can greatly influence the fitness and life history of an organism. Butterflies and their wing colors are an excellent model to study how these colors can impact the development and success of an organism. In this study, we explore species differences in structurally-based ultraviolet coloration in the *Zerene* butterfly. We show clear species differences in ultraviolet (UV) pattern and reflectance spectra. By varying larval diet, we show evidence for developmental plasticity in the structure and organization of UV reflecting scales in *Zerene cesonia.* We further show that feeding the larval host plant of *Zerene eurydice* to *Z. cesonia* does not result in greater similarity in scale structure or UV coloration to the sister species. These results not only demonstrate a connection between plasticity in a male ornamentation, UV wing pattern, and larval resource acquisition, but also identify candidate structural and organizational changes in wing scales responsible for the trait variation.

1. Introduction

The colors of an organism can have a tremendous impact on their lives. The amazing diversity of colors found in nature largely results from a combination of pigmentation and structural features. Pigment based color patterns are the result of the acquisition, production and deposition of pigments during development. Structurally-based coloration, such as the iridescence of adult beetles and butterfly wings, are the results of light reflecting chitin structures that begin developing during pupal life stages. Together, these pigments and structures can produce complex, multi-functional color patterns that not only have major impacts on an individual's survivorship and reproductive output, but also provide biologically inspired designs for material manufacturing (Gan et al., 2016; Guan et al., 2018). Studying factors that influence variation in color pattern in nature offers great promise in advancing our understanding of basic biological processes, such as pattern development and adaptive evolution, that can have broad implications.

The diversity of form and function of butterfly wing patterns offers an excellent model to study the development and evolution of animal coloration. Butterfly wing color patterns serve multiple roles in nature that vary from mate preference, thermoregulation, aposematism to crypsis (Watt, 1969; Silberglied and Taylor, 1978; Endler, 1984; Mallet and Joron, 1999; Ellers and Boggs, 2004). In butterflies, and many other organisms, adult color pattern variations are often the result of plastic responses during development to varying environmental conditions. The trait differences produced by the plastic response can be an important source of adaptive variation that increases an individual's survivorship and chance of mating. Through butterfly wings, we can explore the causes of phenotypic plasticity through development for traits that are well-known to be important for survivorship and mating success.

Environmentally induced pigment variation has been well documented on butterfly wings. For example, among many species of Pierid butterflies, melanization patterns on adult wings vary in response to larval photoperiod and temperature (Kingslover and Wiernasz, 1991; Stoehr and Wojan, 2016). Studies of *Colias* butterflies have shown that much of this variation is adaptive: greater wing melanization leads to faster warming rates, which in-turn increases daily flight time and numbers of eggs laid (Ellers and Boggs, 2002, 2004). Similar observations of pigment based color pattern plasticity have been made for several nymphalid butterflies as well. In *Junonia* butterflies, red ommochrome pigment based wing patterns differ with seasons which, similar to the melanic

---

[*] Corresponding author. 295 Lee Boulevard, Department of Biological Sciences, MS 39762, United States.
E-mail address: Jls1393@msstate.edu (J. Fenner).

https://doi.org/10.1016/j.asd.2019.06.001
1467-8039/

plasticity in *Colias*, likely facilitates thermoregulation (Brakefield and Larsen, 1984; Daniels et al., 2014). In *Bicyclus* butterflies, not only does wing eyespot patterns differ between wet and dry seasons, but remarkably, so does color pattern based mate preference (Robertson and Monteiro, 2005; Prudic et al., 2011). Collectively, there are numerous examples on butterfly wings of plasticity in pigmentation providing the variation for rapid adaptation to changing environments.

Evidence has also been shown for plasticity in structurally based color patterns in butterflies, such as the iridescent ultraviolet (UV) reflection present on the male dorsal wings of many species of Pierid sulphur butterflies. These males possess bright and iridescent UV patterns that result from a highly organized nanoscale architecture that reflects light in the UV spectrum (Ghiradella et al., 1972; Ghiradella, 1974). In *Colias* butterflies, UV brightness is the greatest indicator of male mating success, as variation in UV brightness appears to serve as an honest signal of male quality (Rutowski, 1977; Silberglied and Taylor, 1978; Papke et al., 2007). The development of the UV reflecting architecture on scales is temporally restricted to the pupal stage and is therefore limited by resources acquired during the larval stages before pupation. It has been demonstrated that larval nutrients and thermal stresses can significantly reduce UV brightness (Kemp and Rutowski, 2007). In *Pieris* butterflies, it has been shown that pterin pigment granules absorb UV light and that the UV signal can be amplified by the removal of pigment granule structures within the wing scales (Wijnen et al., 2007; Giraldo and Stavenga, 2007). These pigment granules are oblong chitin structures that traverse between the upper and lower lamellar scale surfaces and contain nitrogen rich pterin pigments. Nitrogen is a key resource for butterfly development and is limited by larval stage acquisition (Morehouse et al., 2007; Morehouse and Rutowski, 2010; Tigreros, 2013). Thus, it may be possible for limited larval resources to drive changes in the development of scale structures and organization that impact UV brightness on adult wings.

Here, we explore how larval diet impacts the development of scale structures and colors on the wings of *Zerene* butterflies. Specifically, we (1) characterize within and between species variation in UV reflectance, (2) test the role of larval host use in driving species differences in UV reflectance, and (3) test how larval diet impacts the development of wing scale structures and organization that influence UV reflectance. *Zerene* is the sister genus to *Colias* and shares the characteristic yellow-orange and black wing colorations of the Coliadinae. Like *Colias*, *Zerene* males have bright UV patterns on their wings. There are only two species of *Zerene*, *Zerene cesonia* (Southern Dogface) and *Zerene eurydice* (California Dogface), and they differ in their UV patterns and larval host plant use. *Z. cesonia* has bright UV patterns on its dorsal fore and hindwings, and shows a strong oviposition preference for *Dalea purpurea* and *Dalea candida* in parts of its distribution (Fenner et al., 2018). In contrast, *Z. eurydice*, only has UV patterns on its dorsal forewing and appears to feed exclusively on *Amorpha californica* and *Amorpha fruticosa* (Riddell, 1941). Here, we leverage this difference in host plant preference and UV signals to test how larval diets impact the development of scale structures and features known to influence structural coloration on butterfly wings.

2. Methods & materials

2.1. Larval rearing and diet treatments

Z. eurydice butterflies were collected near Middle Lion campground in Los Padres National Forest California (34°33′07.65″N, 119°09′55.41″W) in August of 2015 and 6 male samples were preserved for phenotypic analysis. *Z. cesonia* butterflies were collected from Osborn prairie Mississippi (33°30′36.98″N, 88°44′14.57″W) in September 2013 and released in cages at Mississippi State University to establish a laboratory colony. Ten wild male *Z. cesonia* were preserved and assayed to represent wild type phenotypes. Multiple wild caught *Z. cesonia* females were placed in cages with mature *D. purpurea* for oviposition. Hatched *Z. cesonia* larvae were allowed to feed on *D. purpurea* leaves during 1st instar.

After 1st instar, larvae were split into three diet treatments: an artificial diet, an *A. fruticosa* diet, and a *D. purpurea* diet. The *A. fruticosa* and *D. purpurea* diets were used (1) to assess the impact of divergent host plant use on UV coloration and (2) with an artificial diet to assess the impact the larval resource limitation on wing scale development and UV brightness. Individuals given the *A. fruticosa* diet or *D. purpurea* diet were provided an ample amount of fresh plant tissue, to prevent resource limitation. Larval densities were limited to no more than 3 individuals per cage (0.3 m^3), in attempt to reduce opportunities for resource competition as much as possible, within the rearing incubators. Each cage was fitted with a 1-gallon pot that contained a single *A. fruticosa* or *D. purpurea* plant, grown from seed in a greenhouse with controlled environmental conditions. Host plants in larval cages were replaced as needed from the greenhouse plant stock, to assure there was always leafage available (every 4–5 days in early larval stages and every other day during last instar). Based on a 17-day larval cycle, this resulted in individual larvae feeding on ~ 7–8 individual plants. This schema prevented larvae from experiencing a limited availability of host plant, and limited opportunity for resource competition among larvae. The artificial diet was modified from a lima bean based *Colias* diet recipe that was supplemented with 3% wet *D. purpurea* tissue as a feeding stimulant (Taylor et al., 1981). Hatched larvae given artificial diet were placed individually into 74 ml polystyrene cups with scoops of artificial diet 2–3 times the size of the larvae. Cups and artificial diet were replaced every other day, or sooner, if there were signs of microbial contamination. This feeding schema allowed all larvae to have continuous, unabated access to food resources. To investigate if diet had an effect on larval development time, the growth rate of laboratory-reared individuals was measured as the number of days from 2nd larval instar to pupation. Tracking began at 2nd larval instar rather than at hatching, due to the small size of newly hatched larvae and the high death rate before 2nd instar (~30% survival of hatchlings) (Shelby and Counterman, *in prep*.). After pupal eclosion, at least 6 adult male specimens from each treatment were preserved for data collection.

2.2. UV photography and wing size measurements

High-resolution UV images of wild type *Zerene* (shown in Fig. 1) were taken with a Nikon D7000 camera and AF-S Micro Nikkor 105 mm Lens with the addition of a 2″ Baader U-Filter (350 nm). This filter has a transmission peak at 350 nm, with a bandwidth 60 nm (320–380 nm), and blocks the rest of the spectral range from 200 nm to 1120 nm. Wings were removed with microscissors and placed flat on a camera stand with solid non-UV reflective background. The camera and stand were placed in a closed chamber and illuminated with two 13-Watt compact fluorescent black light bulbs (GE CFL 78957). This imaging setup was designed to have similar conditions as Rutowski et al. (2007), to facilitate the comparison of images across studies. For wing size measurements forewings and hindwings were photographed with a color and size standard using the same camera and lens described above, excluding the Baader U-Filter. Wing sizes were measured only for forewings, as most forewings are fully intact after removal, unlike hindwings that often had missing or torn proximal regions due to the removal process. Color photographs of male forewings had 1 mm scale bars embedded and were transformed into binary (black and white) files using ImageJ (Rasband, 1997). Next, the

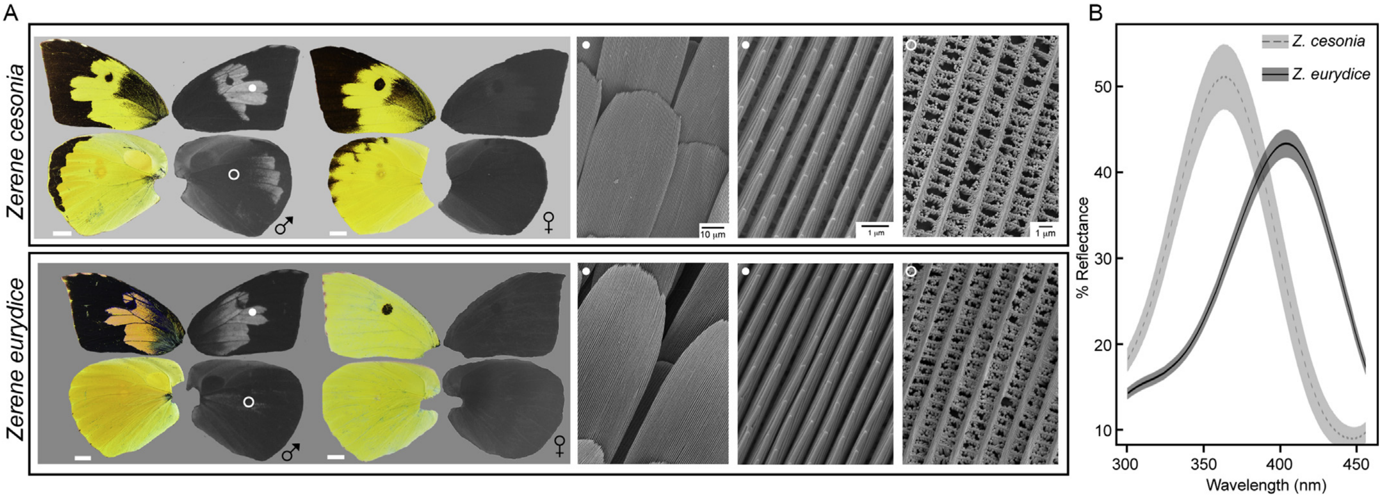

Fig. 1. Wild Phenotypes. Wild type *Z. cesonia* and *Z. eurydice* showing dorsal wings, both visual color and UV patterns differ between species. (A) Left wings in each panel are color photos and the right wings are photos under UV light. Scale bars equal 0.5 cm. Females of both species do not reflect in the UV. SEM images from the two species show similar scale structures between the two species. Images are from a male of each species and panels from left to right show i) a UV reflecting scale at 1,000X, ii) the lamella ridge structures of a UV reflecting scale at 5,000X, and iii) the visibility of pigment granules in Non-UV reflecting yellow scale at 5,000X. The top row is *Z. cesonia* and the bottom row is *Z. eurydice*. The white circles (open and closed) show wing locations for SEM images. (B) UV reflectance peaks differ between the two species with *Z. eurydice* reflecting closer towards the blue with an rMax at 403 nm, while *Z. cesonia* has a reflectance peak at 363 nm. Shaded regions show 95% confidence intervals of reflectance for each species. UV reflectance was measured in the wing region indicated by the closed white circle. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

white background was removed from the image and area of the remaining black region (e.g. forewing) was recorded. The wing sizes for each diet treatment were averaged across all individuals. Violin plots, linear regressions and Student's T-tests were conducted in R studio with the R package ggplot2 (Wickham, 2016).

2.3. Wing spectrometry

Reflectance of male forewings was measured to assay UV brightness and Chroma hue. UV brightness (rMax) is defined as the point of maximum reflectance in the UV wavelengths (300–450 nm). UV brightness for each treatment was determined by averaging rMax across all samples in the treatment group. The average Chroma hue, defined as the position (nm) of rMax along the wavelength, was also calculated for each treatment. Wing reflectance was measured on an HR 2000 + ES Ocean Optics spectrometer with a Halogen - Deuterium light source (DH-2000). All reflectance measurements were standardized with a “white” magnesium oxide standard (Ocean Optics). Wing reflectance measurements were taken from the same forewing region (Fig. 1, closed circles) where UV reflectance is highest across the forewing. Measurements were replicated in triplicate for each individual. For each measurement the probe was positioned at a stationary 45^0 angle above the specimen, and measurements were taken from a 3 mm diameter region of the wing. Raw spectra files were trimmed to only the UV wavelength and average UV spectra for each diet treatment was calculated and graphed with 95% confidence intervals (Fig. 3 A).

2.4. Scale structure measurements

Electron microscopy was performed to assess the structural differences between differently colored scales. Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM) were first performed to ascertain the differences between UV and non-UV reflecting yellow scales on *Z. cesonia* wings. A single cover scale from the UV reflecting region of a male *Z. cesonia* forewing was removed and milled used a Ga ion source focus ion beam (FIB) and imaged using a field emission (FE) SEM using a Tescan Lyra3 (Fig. 2G–I). The ion beam was directed at a 90^0 angle to the scale and milled perpendicular to the lamella ridges, exposing an ~2 μm × 4 μm region and providing a cross-section view of the scale. SEM magnifications for imaging ranged from 12.1 to 42.8 kx at 5 kVolts. The process was repeated for a non-UV reflecting scale from the same region of a female *Z. cesonia* forewing (Fig. 2K–M). TEM was performed on a single male *Z. cesonia* sample to compare cross sections of yellow non-UV, yellow UV, and black cover scales to confirm that pigment granules are present in both UV and non-UV reflecting yellow scales. The wing was cut and separated into black, UV, and yellow wing regions and treated with Karnovsky's fixative (2.5% glutaraldehyde, 3.7% formaldehyde in 0.1 M of sodium cacodylate with 0.1 M of sucrose at pH 7.2), osmication was performed in 1% osmium tetroxide in the same buffer, and dehydration was through ethanol. Wing regions were embedded in Spurr's resin (Sigma–Aldrich) and sections were stained with Uranyl acetate and Lead citrate and imaged on JOEL 2100 JEM. Chitin structures are stained and visible, while pigment granules do not stain but appear as white hollow beads in scales possessing pigment granules (Fig. 2B–D) (Ghiradella et al., 1972; Ghiradella and Radigan, 1976; Stavenga et al., 2004).

SEM images of *Z. cesonia* and *Z. eurydice* wing scales were collected to assess morphological differences between species, as well as variation among *Z. cesonia* diet treatments. Ten *Z. cesonia* samples were prepared for the SEM from the wild and *D. purpurea* diet treatments and six samples were used for artificial and *A. fruticosa* diet treatments, due to limited availability. For *Z. eurydice*, six wild caught males had wing scales prepped for SEM. Wings were sectioned with a razor blade into the following regions: (i) melanic regions, (ii) yellow non- UV reflecting regions (open circle Fig. 1A), and (ii) yellow UV reflecting wing regions (closed circles Fig. 1A). These wing regions were mounted on aluminum stubs with silver paste and sputter coated with 15 nm Platinum. Stubs were imaged at 400–5000× magnification on a JEOL JSM-6500F FE-SEM at 5 kVolts.

Analysis of SEM images was performed using ImageJ (Rasband, 1997). Scale density was determined by counting the numbers of

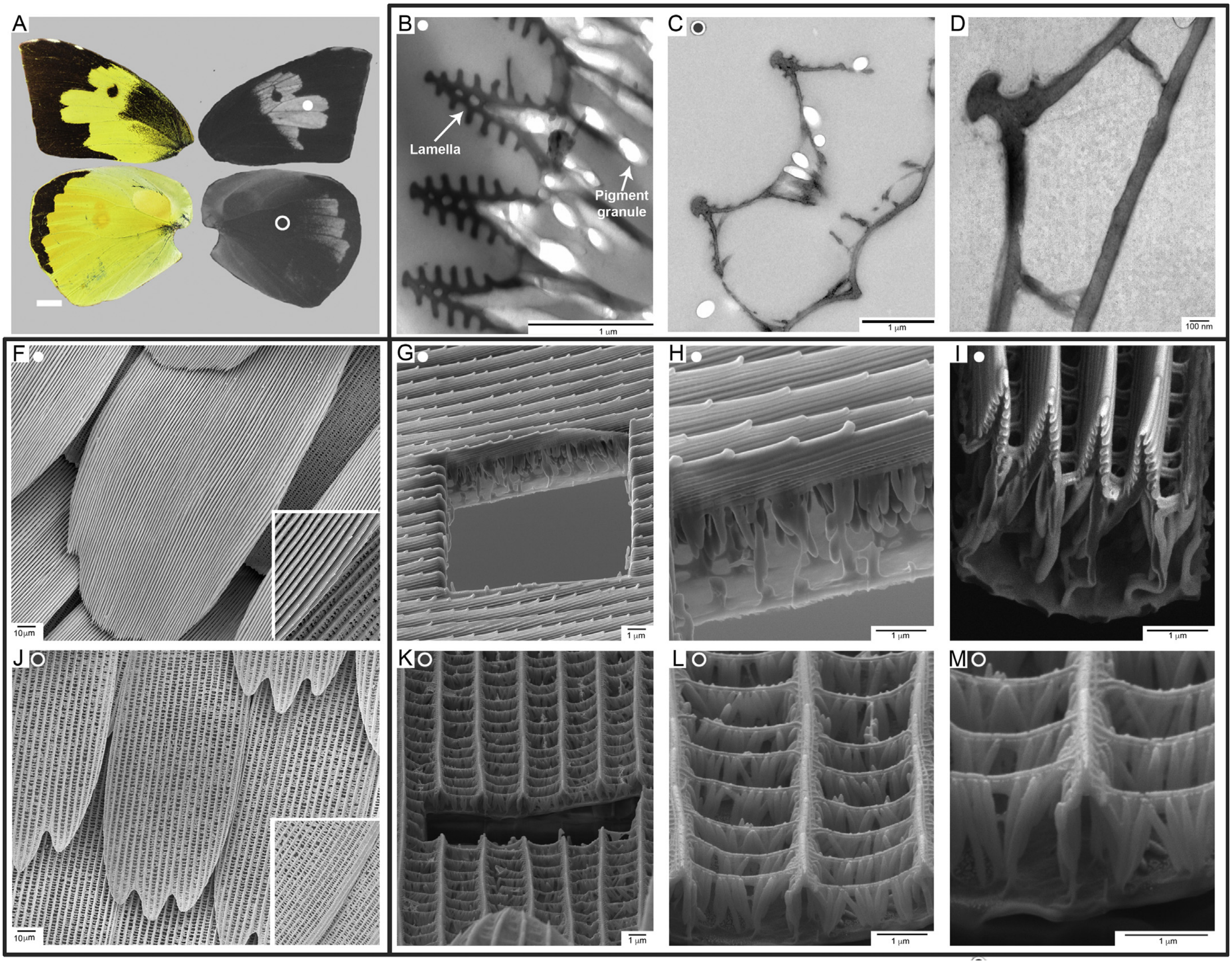

Fig. 2. Scale Architecture of *Z. cesonia*. (A) Shows *Z. cesonia* with closed and open circles that indicates the wing regions where the following images were collected. (B–D) TEM images of a *Z. cesonia* male. The large lamella ridge stacking in the UV reflecting scales can be observed in (B) while lamella are smaller and more condensed in the non-UV yellow scale (C) and black melanin scale (D). Pterin pigment granules appear as hollow white oblong structures in both (B) and (C). (F–I) SEM and FIB cross sections (G–I) of UV reflecting scale structures from a male *Z. cesonia* forewing. (J–M) SEM and FIB (K–M) cross sections of non-UV reflecting yellow scales from a female *Z. cesonia* forewing. In (F) and (J) both cover and ground scales are visible and inserts show magnifications of the morphological differences of cover scales and ground scales. (F) Shows that cover scales have the UV reflecting morphology, while the ground scales have a morphology more similar to the yellow non-UV reflecting cover and grounds scales shown in (J). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

cover scales in a 70 mm area from a 400×-magnified image (Fig. 4B). Only whole cover scales were counted, scales that could not be observed from scale edge to scale edge were omitted for consistency. Ground scales were omitted, because they lack the UV reflecting structures of the cover scales and have similar morphology to a yellow non-UV reflecting scale (see Fig. 2F, J). The lamella ridge distances of UV reflecting cover scales were measured from 5000× magnified images in a 10× 10 μm area (Fig. 4D). Granule density was measured from wing scales in the non-UV reflecting yellow region of the wing (open circles Fig. 1A) at 5000× magnification (Fig. 4F). This region of the wing is composed of yellow pterin pigmented scales that do not have the nanostructures responsible for UV reflection, and individual pigment granules are clearly visible between lamellae. Individual granules were counted within 10 μm^2 area for three separate images from this wing region, for each individual. Averages were estimated across individuals for all diet treatments for scale density, granule density, and lamella ridge distance, Student's T-Tests were performed among the three diet treatments for the scale measurements and violin plots were generated in R studio with ggplot 2 (Wickham, 2016) (Fig. 4). Discriminant function analysis (DFA) was conducted in R studio with ggord package (Beck, 2017) and MASS package (Venables and Ripley, 2002) to assess how different variables (scale density, granule density, and lamella ridge distance) discriminates the diet treatments (artificial, *D. purpurea*, *A. fruticosa*) from each other (Fig. 3B). Linear regressions were conducted and plotted in R studio for granule density and scale density (Fig. 5). Linear regressions were similarly conducted for the scale measurements and UV brightness (rMax).

3. Results

3.1. UV reflectance differences between species

The UV reflectance and pattern differs between the two species of *Zerene* (Fig. 1). UV patterns differ between the species in

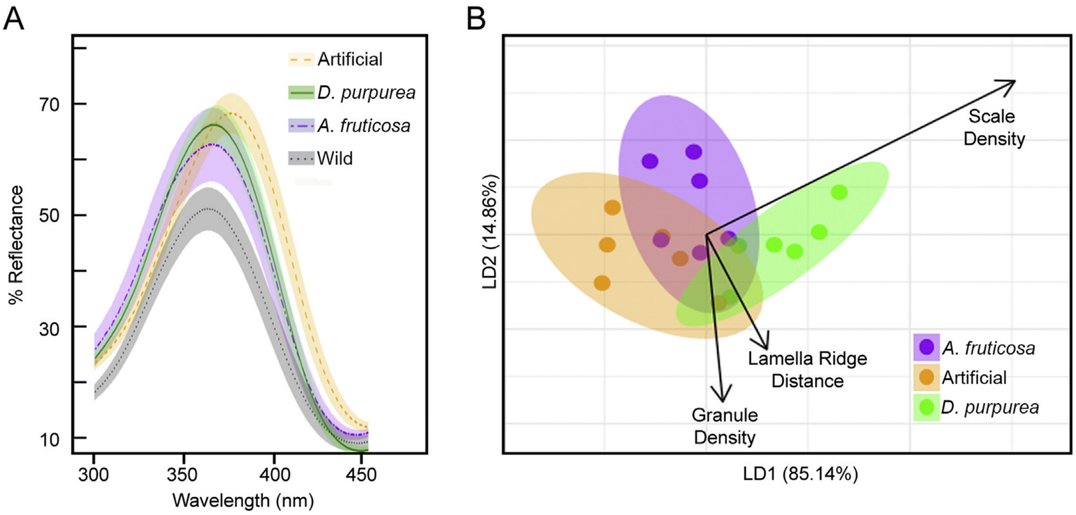

Fig. 3. Ultraviolet Color Reflectance and Discriminant Function Analysis. (A) Shows UV reflectance among the different diet treatments. Laboratory reared individuals had brighter UV reflectance than wild caught *Z. cesonia. D. purpurea* fed individuals were significantly brighter than wild individuals ($p = 3.31$ E^{-5}). A chroma shift is observed between individuals raised on the artificial diet with the peak shifting towards 377 nm. (B) Discriminant function analysis shows that scale density best discriminates between the three diet treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

both fore and hindwings. On the *Z. eurydice* forewing, all yellow scales are UV reflective, in contrast *Z. cesonia* has a narrow patch of yellow scales that are not UV reflective, resulting in distinctive UV patterns between the species. On the hindwing, *Z. eurydice* lacks the UV reflective patch that is present on the hindwing margins of *Z. cesonia.* In both species UV patterns are sexually dimorphic, with only males reflecting UV. Unexpectedly, there was also a clear and consistent difference in the wavelength of the peak UV reflectance (rMax) between species, with an average rMax at 363 nm in *Z. cesonia,* versus an average at 403 nm in *Z. eurydice* (Fig. 1).

3.2. *UV reflecting structures*

In both species of *Zerene,* the UV reflecting scales have similar architectures to those described in *Colias* (Fig. 1). UV reflecting scales are characterized by tightly-spaced lamella ridges (Fig. 1). The lamellae ridges on these scales are highly ornate structures with a “Christmas tree”-like shape created by the stacking of lamella during scale development, which are clearly visible in the cross section images from UV reflecting scales in (Fig. 2B, I). These ridges on the upper scale surface are connected by narrow cross ribs, that create open windows that allow light to enter inside the scale. These upper surface structures are connected to the lower scale surface by trabeculae, often observed below the cross ribs. Inside the scales, pigment granules can be observed that, in some instances, appear to traverse between the upper and lower surfaces. Scales from non-UV reflecting yellow regions have similar structures, (Fig. 2C, K–M), however the lamella ridges are more widely spaced and lack the highly stacked lamella. In these yellow scales, pigment granules are clearly visible (Fig. 2K–M). In TEM images of yellow cover scales from both UV reflecting and non-UV reflecting wing regions, pigment granules are clearly present as hollow unstained ellipsoids and are absent from the black melanin scale (Fig. 2B–D). Collectively, these results show that the UV reflecting scales in both *Zerene* species have very similar structures and organizations and that the structural differences expected between UV and non-UV reflecting scales are visible and quantifiable in *Z. cesonia.*

3.3. *Larval diet does not change UV brightness*

Changes were not observed in UV brightness on the wings of *Z. cesonia* individuals that fed on different species of larval host plant (Fig. 3A). However, changes in UV brightness were observed between laboratory reared individuals and wild caught. Overall, laboratory reared individuals had brighter UV reflectance than wild caught individuals (T-Test $p = 1.54$ E^{-5}), but when split by larval diet treatments, only individuals fed *D. purpurea* had significantly brighter UV reflectance than wild caught individuals (T-Test $p = 3.31$ E^{-5}). The wings of *A. fruticosa* fed individuals did show a lower average UV brightness than the *D. purpurea* fed; however, the variance was much larger and there was no significant difference in UV brightness (T-Test $p = 0.32$).

Individuals reared on the artificial diet did not significantly differ in UV brightness from the *D. purpurea* or *A. fruticosa* fed individuals (T-Test $p = 0.62$ and 0.34). Artificial diet reared individuals did show a slight shift in peak UV reflectance (Chroma hue) with an rMax of 377 nm, compared to an Rmax of 366 nm for the *D. purpurea* fed (Fig. 3A). Upon visual inspection, no qualitative differences in the shape of the UV pattern were noted among the diet treatments.

3.4. *Plasticity in the development of UV scale architecture and organization*

Among the different diet treatments, changes in both UV scale organization and ultrastructure were observed. Discriminant function analysis revealed that scale density differences best discriminated between the larval diet treatments, followed by granule density, then lamella ridge distance (Fig. 3B). When these scale measurements were analyzed independently, both scale and granule densities showed significant differences between diet treatments (Fig. 4A + E), but there were no differences in the lamellar ridge distances of UV reflecting scales (Fig. 4C). Scale density, measured as the number of UV reflecting scales per unit area (70 mm^2), was significantly lower in the artificial diet compared to the *D. purpurea and A. fruticosa* diets ($p = 0.0011$ and 0.0088, respectively) (Fig. 4A–B). The lower density of the UV reflecting scales in the artificial and *A. fruticosa* diets also resulted in

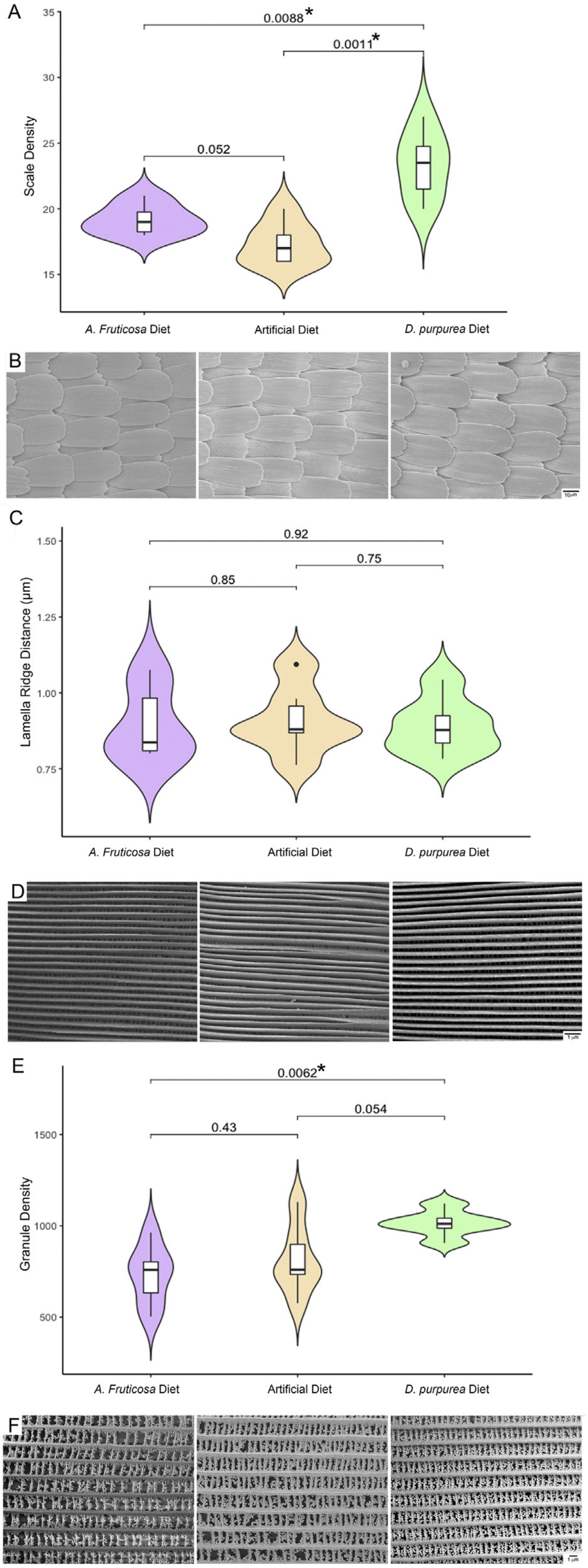

Fig. 4. Plasticity in Scale Development in *Z. cesonia*. Differences in the density of UV reflecting scales were observed among the various diet treatments. (A) Shows that *D. purpurea* fed individuals have the most UV reflecting scales per area, while the artificial diet individuals have the least. (B) SEM images at 400 X shown from left to right: i) *A. fruticosa* treatment, ii) artificial diet treatment, and iii) *D. purpurea* diet treatment. (C) Lamella ridge distance differed among individuals, but not significantly between treatments. (D) SEM images of a UV reflecting scale at 5000 X shown left to right: i) *A. fruticosa* treatment, ii) artificial diet treatment, and (iii) *D. purpurea* diet treatment. (E) Shows that pigment granule densities were impacted by diet treatments, with *D. purpurea* fed individuals have significantly more pigment granules than *A. fruticosa* and artificial diet reared individuals. (F) SEM images at 5000 X of yellow scales shown from left to right: i) *A. fruticosa* treatment, ii) artificial diet treatment, and iii) *D. purpurea* diet treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

an increased exposure of ground scales that lack the UV reflecting lamellae ridge structures. This contrasted with the *D. purpurea* fed individuals, which showed the highest density of UV reflecting scales and minimal ground scale exposure (Fig. 4B). Pigment granule density was significantly different only between the *D. purpurea* and *A. fruticosa* diet treatments ($p = 0.0062$) (Fig. 4E), with the *D. purpurea* fed showing the highest density of granules. A linear regression between granule density and scale density (Fig. 5) revealed a significant correlation in the decrease of granule and scale density across treatments ($r^2 = 0.41$; $p = 0.0042$). Linear regressions of granule density, scale density and lamella ridge distance using individuals from all diet treatments, showed no significant relationships with UV brightness ($r^2 = 0.29, 0.18, 0.37$; $p = 0.19, 0.44, 0.076$, respectively).

3.5. *Inter and intra-specific variation in UV scale architecture*

Scale architectural structures of lamella ridge distance and pigment granule densities did not significantly differ between the two species of *Zerene* ($p = 0.13$ and $p = 0.085$ (Fig. 6). The *Z. cesonia* larvae reared on *A. fruticosa* developed pigment granule densities that were significantly less than wild *Z. cesonia* ($p = 0.016$), but not different from the densities found in *Z. eurydice* ($p = 0.19$) (Fig. 6A). Lamella ridge densities were not significantly different between the *A. fruticosa* reared *Z. cesonia* and the wild *Z. cesonia* or wild *Z. eurydice* ($p = 0.51$ and $p = 0.11$) (Fig. 6B).

3.6. *Additional traits impacted by alternative host plant consumption*

Individuals reared on different diets developed at different rates and sizes. Larvae that fed only on *D. purpurea* developed the fastest and had the largest wings (Fig. 6C–D). The artificial diet reared individuals increased their pupal development time to 12 days and had smaller wings compared to the *D. purpurea* reared individuals that had only a 7 day pupation period. In contrast, the *A. fruticosa* reared individuals doubled the pupal development time (14 days) and as a group had significantly smaller wings than *D. purpurea* fed individuals ($p = 7.32e\text{-}15$).

4. Discussion

Ultraviolet coloration among *Colias* butterflies serves as a signal for species recognition and is an honest indicator of male quality (Kemp and Rutowski, 2007). It has been shown that nutrient stress can affect scale architecture, such as lamella ridge distance, which then impacts the quality of an individual's UV signal (Rutowski et al., 2005; Kemp et al., 2006; Morehouse et al., 2007). Here, we have characterized species level differences between the only two species in the genus *Zerene* and investigated how larval diets can impact UV coloration and scale development within the species *Z. cesonia.*

4.1. *Larval host plant divergence does not explain UV differences between species*

Species differences in peak UV spectra cannot be explained by host plant usage. *Z. cesonia* has an oviposition preference for

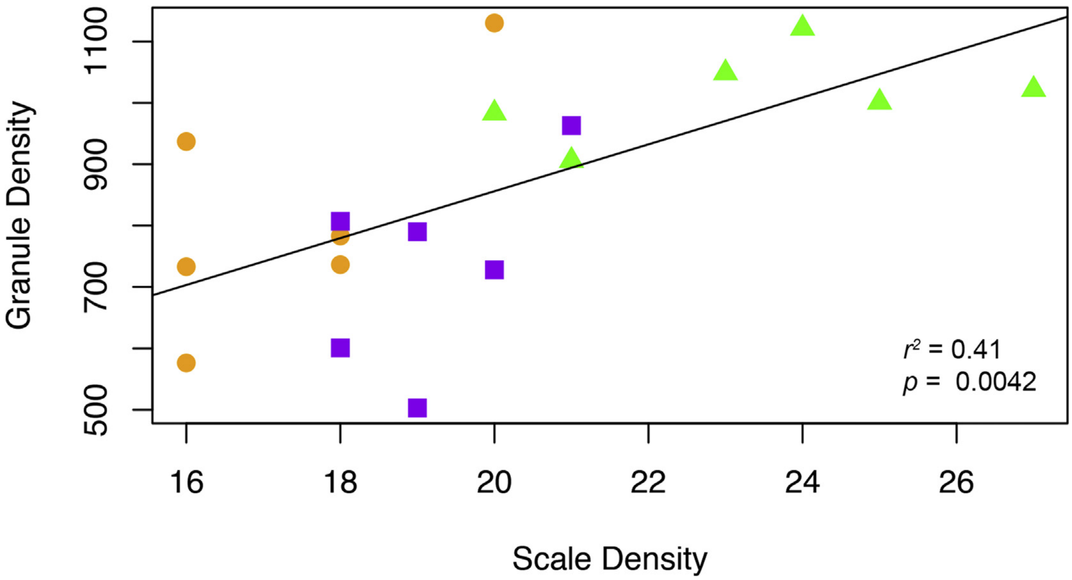

Fig. 5. Linear Regression Between Granule and Scale Density. A linear regression performed among all the laboratory reared samples shows the correlation in the decrease of granule density and scale density.

D. purpurea, and has been reported to utilize *A. fruticosa* as a larval host, when it is present (Fenner et al., 2018). In contrast, *Z. eurydice* specializes on the sister species *A. californica* and *A. fruticosa* (Riddell, 1941). We predicted that feeding *Z. cesonia* larva *A. fruticosa*, may result in adult wing patterns that were more similar to *Z. eurydice*, if the species differences in wing color were due to larval host plant use. We found no evidence that *A. fruticosa* reared *Z. cesonia* had more similar UV patterns or reflectance to

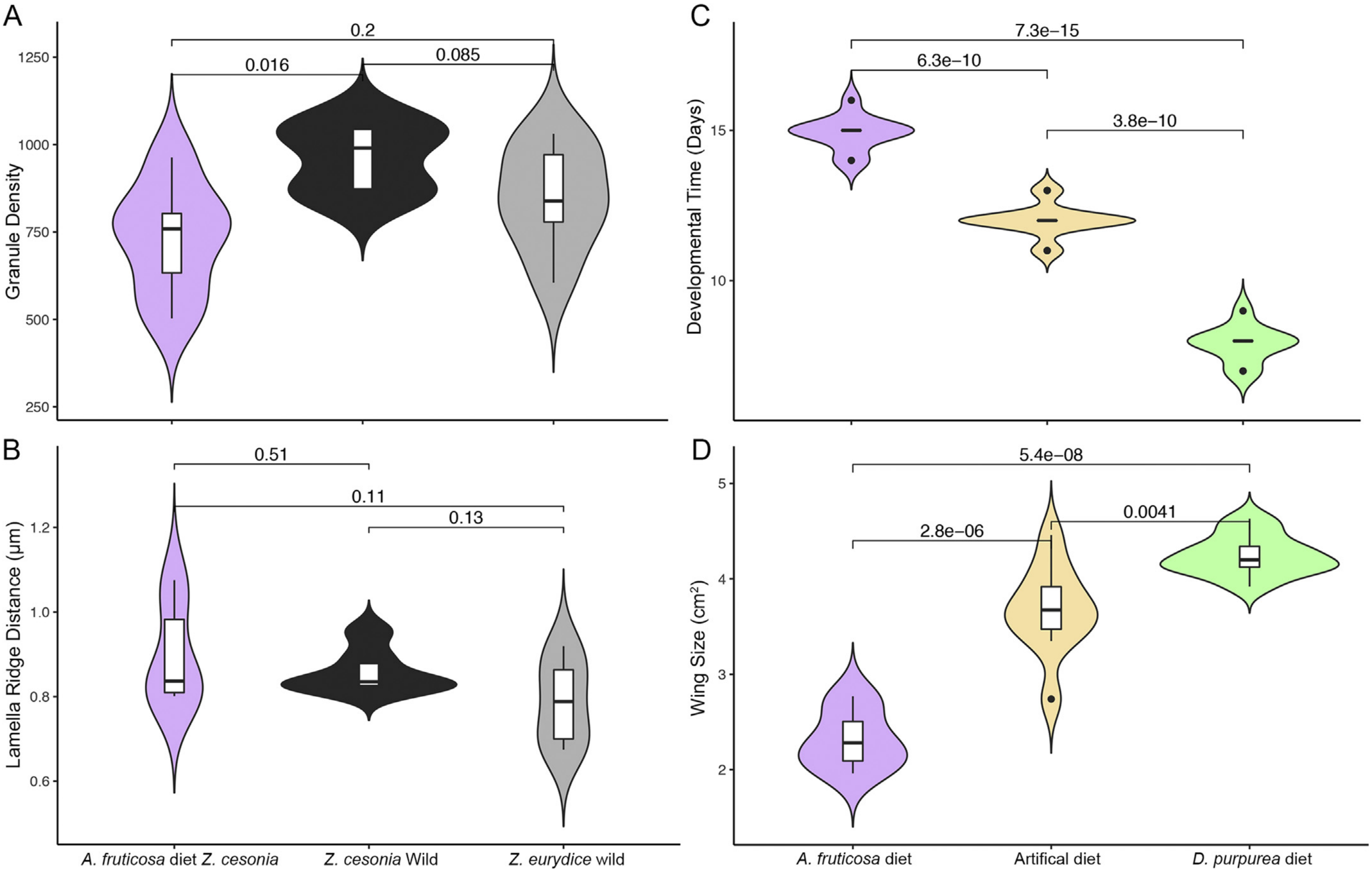

Fig. 6. Species Comparisons to *A. fruticosa* Diet and Traits Impacted by *A. fruticosa* Diet. Scale features of granule density (A) and lamella ridge distance (B) are not significantly different between the two species. *A. fruticosa* reared individuals have larger spacing between lamella ridges than either wild species, but the spacing is not significantly different from wild *Z. cesonia* or wild *Z. eurydice*. The *A. fruticosa* reared individuals also have less pigment granules than either species and are significantly different from wild *Z. cesonia*, but not significantly different from wild *Z. eurydice*. (C and D) Shows other traits negatively impacted by alternative host plant consumption, including that *A. fruticosa* and artificial diet reared individuals both have smaller wings and longer developmental times than *D. purpurea* fed individuals.

Z. eurydice, than their conspecifics. However, those *Z. cesonia* that were fed *A. fruticosa* did develop pigment granule densities that were more similar to *Z. eurydice*, than *Z. cesonia*. Interestingly, the *Z. cesonia* that were fed *A. fruticosa* had lower pigment granule densities than wild caught individuals of both species, also had brighter UV than both wild caught species. However, across our diet treatments we saw no significant relationship between UV brightness and pigment granule densities or the other measures of scale architecture and organization. These findings suggest the lower UV brightness of the wild caught individuals likely results from developmental differences that remain unresolved.

These UV spectra differences suggest there could also be concordant divergence in photoreceptors of the two species. The UV receptor absorbance peaks in *Pieris* are at 360 nm, very near the peak UV reflectance of 366 nm in *Z. cesonia*. Interestingly, pierid butterflies appear to have experienced a duplication of opsin receptors in blue wavelength, with the duplicated receptor's peak absorbance at 425 nm in *Pieris rapae* (Briscoe, 2008; Wilts et al., 2011). The peak UV reflectance of 403 nm in *Z. eurydice* is shifted much further in to the blue spectrum, which likely drives a blue iridescence found on male *Z. eurydice* wings. This UV/blue peak in *Z. eurydice* may be absorbed more by the duplicated blue receptor in Pierids, instead of the UV receptor. Further work on the photoreceptors and opsin genes of *Zerene* and other Pierids is needed to determine if these UV signals may have different receptors in the sister *Zerene* species.

4.2. *Impacts of utilizing an alternative larval host plant*

Although larval diet did not cause changes in UV brightness, differences in the scale structures and organization that influence UV brightness were observed. Most notably, individuals fed the artificial diet or less preferred host plant, had fewer cover scales with UV reflecting structures and more visible ground scales with UV absorbing structures on their wings. This was coupled with the observation that the density of UV absorbing structures (pigment granules in non-UV reflecting scales) was positively correlated with the density of scales with UV reflecting structures. Therefore, it appears that the individuals that fed on the preferred host (*D. purpurea*), had the highest density of UV reflecting scales and the highest density of UV absorbing structures. However, the pigment granules inside the UV reflecting scales are largely blocked from absorbing UV, due to the tightly spaced UV reflective lamella ridges on the cover scales, and thus *D. purpurea* fed individuals produce a bright UV signal. It is important to note that among the artificial diet and *A. fruticosa* fed butterflies, despite having relatively less UV reflecting scales and greater exposure of the underlying ground scales butterflies did not have a weaker UV signal. This is likely caused by the exposed ground scales containing less UV absorbing pigment granules. Overall these changes in the UV reflecting and absorbing structures appear to counter each other and result in no detectable differences in UV brightness among treatments.

We propose an alternative perspective of how the changes in UV scale structure, particularly pigment granule density, may influence UV signal. The density of UV absorbing pigment granules has the potential to influence the contrast of UV signal across the wing. The pigment granules of non-UV reflective cover scales (scales lacking the highly stacked lamella ridge structures) may dampen extraneous UV light (Rutowski et al., 2005; Wijnen et al., 2007; Wilts et al., 2011). This is supported by observations that UV reflectance has been shown to increase on some Pierid wings, after pigment granules were experimentally removed (Wijnen et al., 2007; Wilts et al., 2011). We suggest that pigment granules may enhance the contrast of the UV pattern and that this contrast may influence mating success. In this regard, consider the difference in the perception of a strobe light in a room with extraneous light coming through an open window, versus in a room with a closed or tinted window. In the dark room, the flash of the strobe light may appear “brighter”, simply due to the increased contrast, even though the actual brightness of the light may not have changed. We propose that as our data suggest, increased pigment granule density does not directly influence UV brightness, but rather may enhance the contrast and perception of the UV signal.

4.3. *Developmental plasticity in scale structure and organization*

Here we present evidence of diet induced plasticity in scale structures and organization. Laboratory reared *Z. cesonia* did not show UV reflectance differences, but did show differences in UV scale organization and granule densities. In *Colias eurytheme* lamella ridge distance and UV brightness co-vary, with more closely spaced ridges producing brighter UV reflectance (Kemp et al., 2006). A similar relationship was recently observed in Nymphalid butterflies with blue iridescent structural coloration, where lamella ridge distance correlated with peak color reflectance in *Heliconius* butterflies (Parnell et al., 2018). Although not significantly different, there were tighter spaced lamella ridges in *D. purpurea* fed individuals than in *A. fruticosa* and artificial diet individuals. Given the lack of differences between species and treatments, lamella ridge spacing may be under tighter developmental control and be less plastic than other scale features, such as granule density and scale organization. Differences in UV cover scale densities was significant with *D. purpurea* fed individuals having significantly more UV reflecting scales per unit area than the alternative diets. Although variation in scale density may seem an obvious mechanism influencing color brightness on the wing, this relationship has not been previously reported for other species, such as *C. eurytheme*. Differences in pterin pigment granules in non-UV reflecting scales was also significant with the *D.purpurea* fed individuals having the most pterin pigment granules and the alternative diets having significantly less pterin granules. In *P. rapae* pterin pigment granules absorb UV light and females which lack pigment granules have higher UV reflectance than males with granules (Giraldo and Stavenga, 2007). The linear regression between pigment granule density and UV cover scale density suggests these scale features have a correlated decrease when under dietary stress.

Given the lack of UV reflection differences between diet treatments, but clear scale differences, we hypothesize that in resource poor environments *Z. cesonia* may buffer the environmental effects by decreasing both UV reflecting and UV absorbing scale features. Nutrient stress environments may cause males to produce less UV reflecting scales and in order to maintain a bright and attractive UV signal, the number of pigment granules that absorb UV light must also be reduced. This plasticity suggests there may be a balancing effect between scale structures that allows males to compensate in a resource poor environment.

Collectively, these findings suggest that the development of scale architecture and organization of the scales responsible for producing a UV signal is plastic. Individuals reared with unlimited food, whether it is the preferred or alternative host plant, developed wings that were significantly brighter than either species of the wild caught individuals, but not different from each other. These results suggest that plasticity in UV scale development can allow males to compensate for a poor nutrient environment and still produce a bright, high quality, UV signal. This suggests a tentative connection between the development of a male ornamentation (UV scales) and resource acquisition. Further tests of the resource limitations and the UV scale structural variation that follow individual fitness will be able to determine if the UV brightness may be an “honest signal” of fitness to potential mates.

Funding

This work was supported by Texas EcoLab (Braun & Gresham, PLLC) and NSF award 1755329 to BAC.

Acknowledgments

We would like to thank Friends of the Blackbelt prairie for providing guidance on sample collecting. We thank Nate Morehouse for guidance and initial measurements for spectrometry work. We thank Giselle Thibaudeau and Amanda Lawrence at I^2AT Imaging Institute for FIB Image and for SEM training. We thank Elizabeth Evans, Heather Smith, and Morgan Miller for help with butterfly rearing and spectrometry measurements.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.asd.2019.06.001.

References

Beck, M.W., 2017. Ggord: Ordination Plots with Ggplot2. R Package Version 1.0.0. https://zenodo.org/badge/latestdoi/35334615.

Brakefield, P.M., Larsen, T.B., 1984. The evolutionary significance of dry and wet season forms in some tropical butterflies. Biol. J. Linn. Soc. 22, 1–12.

Briscoe, A.D., 2008. Reconstructing the ancestral butterfly eye: focus on the opsins. J. Exp. Biol. 211, 1805–1813.

Daniels, E.V., Murad, R., Mortazavi, A., Reed, R.D., 2014. Extensive transcriptional response associated with seasonal plasticity of butterfly wing patterns. Mol. Ecol. 23, 6123–6134.

Ellers, J., Boggs, C.L., 2002. The evolution of wing color in *Colias* butterflies: heritability, sex linkage, and population divergence. Evolution 56, 836–840.

Ellers, J., Boggs, C.L., 2004. Functional ecological implications of intraspecific differences in wing melanization in *Colias* butterflies. Biol. J. Linn. Soc. 82, 79–87.

Endler, J.A., 1984. Progressive background in moths, and a quantitative measure of crypsis. Biol. J. Linn. Soc. 22, 187–231.

Fenner, J., Schartel, T., Counterman, B., 2018. Lessons from butterflies of the black belt prairie: the southern Dogface as an indicator of prairie remnants. Trans. Am. Entomol. Soc. 144, 295–308.

Gan, Z., Turner, M.D., Gu, M., 2016. Biomimetic gyroid nanostructures exceeding their natural origins. Sci. Adv. 2, e1600084.

Ghiradella, H., 1974. Development of ultraviolet-reflecting butterfly scales: how to make an interference filter. J. Morphol. 142, 395–409.

Ghiradella, H., Radigan, W., 1976. Development of butterfly scales. II. Struts, lattices and surface tension. J. Morphol. 150, 279–297.

Ghiradella, H., Aneshansley, D., Eisner, T., Silberglied, R.E., Hinton, H.E., 1972. Ultraviolet reflection of a male butterfly: interference color caused by thin-layer elaboration of wing scales. Science 178, 1214–1217.

Giraldo, M.A., Stavenga, D.G., 2007. Sexual dichroism and pigment localization in the wing scales of *Pieris rapae* butterflies. Proc. R. Soc. B 274, 97–102.

Guan, Y., Su, H., Yang, C., Wu, L., Chen, S., Gu, J., Zhang, W., Zhang, D., 2018. Ordering of hollow Ag-Au nanospheres with butterfly wings as a bio-template. Sci. Rep. 8, 9261.

Kemp, D.J., Rutowski, R.L., 2007. Condition dependence, quantitative genetics, and the potential signal content of iridescent ultraviolet butterfly coloration. Evolution 61, 168–183.

Kemp, D.J., Vukusic, P., Rutowski, R.L., 2006. Stress-mediated covariance between nano-structural architecture and ultraviolet butterfly coloration. Funct. Ecol. 20, 282–289.

Kingsolver, J.G., Wiernasz, D.C., 1991. Seasonal polyphenism in wing-melanin pattern and thermoregulatory adaptation in *Pieris* butterflies. Am. Nat. 137, 816–830.

Mallet, J., Joron, M., 1999. Evolution of diversity in warning color and mimicry: polymorphisms, shifting balance, and speciation. Annu. Rev. Ecol. Evol. Syst. 30, 201–233.

Morehouse, N.I., Rutowski, R.L., 2010. In the eyes of the beholders: female choice and avian predation risk associated with an exaggerated male butterfly color. Am. Nat. 176, 768–784.

Morehouse, N.I., Vukusic, P., Rutowski, R., 2007. Pterin pigment granules are responsible for both broadband light scattering and wavelength selective absorption in the wing scales of pierid butterflies. Proc. Biol. Sci. 274, 359–366.

Papke, R.S., Kemp, D.J., Rutowski, R.L., 2007. Multimodal signalling: structural ultraviolet reflectance predicts male mating success better than pheromones in the butterfly *Colias eurytheme* L.(Pieridae). Anim. Behav. 73, 47–54.

Parnell, A.J., Bradford, J.E., Curran, E.V., Washington, A.L., Adams, G., Brien, M.N., Burg, S.L., Morochz, C., Fairclough, J.P.A., Vukusic, P., Martin, S.J., 2018. Wing scale ultrastructure underlying convergent and divergent iridescent colours in mimetic *Heliconius* butterflies. J. R. Soc. Interface 15, 20170948.

Prudic, K.L., Jeon, C., Cao, H., Monteiro, A., 2011. Developmental plasticity in sexual roles of butterfly species drives mutual sexual ornamentation. Science 331, 73–75.

Rasband, W.S., 1997. ImageJ. US National Institutes of Health, Bethesda, MD, USA.

Riddell, J., 1941. Some remarkable forms and aberrations in the subgenus *Zerene* Hübner (Lepidoptera, Pieridae). Trans. R. Entomol. Soc. Lond. 91, 447–457.

Robertson, K.A., Monteiro, A., 2005. Female *Bicyclus anynana* butterflies choose males on the basis of their dorsal UV-reflective eyespot pupils. Proc. Biol. Sci. 272, 1541–1546.

Rutowski, R.L., 1977. The use of visual cues in sexual and species discrimination by males of the small sulphur butterfly *Eurema lisa* (Lepidoptera, Pieridae). J. Comp. Physiol. 115, 61–74.

Rutowski, R.L., Macedonia, J.M., Morehouse, N., Taylor-Taft, L., 2005. Pterin pigments amplify iridescent ultraviolet signal in males of the orange sulphur butterfly, *Colias eurytheme*. Proc. Biol. Sci. 272, 2329–2335.

Rutowski, R.L., Macedonia, J.M., Kemp, D.J., Taylor-Taft, L., 2007. Diversity in structural ultraviolet coloration among female sulphur butterflies (Colinadinae, Pieridae). Arthropod Struct. Dev. 36, 280–290.

Silberglied, R.E., Taylor, O.R., 1978. 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.

Stavenga, D.G., Stowe, S., Siebke, K., Zeil, J., Arikawa, K., 2004. Butterfly wing colours: scale beads make white pierid wings brighter. Proc. Biol. Sci. 271, 1577–1584.

Stoehr, A.M., Wojan, E.M., 2016. Multiple cues influence multiple traits in the phenotypically plastic melanization of the cabbage white butterfly. Oecol 182, 691–701.

Taylor Jr., O.R., Grula, J.W., Hayes, J.L., 1981. Artificial diets and continuous rearing methods for the sulfur butterflies Colias eurytheme and *C. philodice* (Pieridae). J. Lepid. Soc. 35, 281–289.

Tigreros, N., 2013. Linking nutrition and sexual selection across life stages in a model butterfly system. Funct. Ecol. 27, 145–154.

Venables, W.N., Ripley, B.D., 2002. Modern Applied Statistics with S, fourth ed. Springer, New York. ISBN 0-387-95457-0.

Watt, W.B., 1969. Adaptive significance of pigment polymorphisms in *Colias* butterflies, II. Thermoregulation and photoperiodically controlled melanin variation in *Colias eurytheme*. Proc. Natl. Acad. Sci. Unit. States Am. 63, 767–774.

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer.

Wijnen, B., Leertouwer, H.L., Stavenga, D.G., 2007. Colors and pterin pigmentation of pierid butterfly wings. J. Insect Physiol. 53, 1206–1217.

Wilts, B.D., Pirih, P., Stavenga, D.G., 2011. Spectral reflectance properties of iridescent pierid butterfly wings. J. Comp. Physiol. 197, 693–702.