12-13-2019

How the Dogface got its color: How genetics and the environment influence color variation within and between species in the Zerene butterfly

Jennifer Fenner

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By

Jennifer Lyn Fenner

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Biological Sciences
in the Department of Biological Sciences

Mississippi State, Mississippi

December 2019

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By

Jennifer Lyn Fenner

Approved:

_____

Brian A. Counterman
(Major Professor)

_____

Matthew W. Brown
(Committee Member)

_____

Ryan C. Range
(Committee Member)

_____

Diana C. Outlaw
(Committee Member)

_____

Justin A. Thornton
(Graduate Coordinator)

_____

Rick Travis
Dean
College of Arts & Sciences

Name: Jennifer Lyn Fenner

Date of Degree: December 13, 2019

Institution: Mississippi State University

Major Field: Biological Sciences

Major Professor: Brian A. Counterman

Title of Study: How the Dogface got its color: How genetics and the environment influence color variation within and between species in the Zerene butterfly

Pages in Study: 103

Candidate for Degree of Doctor of Philosophy

A fundamental question in biology is: How is variation generated? At a basic level, the vast amount of variation and biodiversity is generated through a combination of genetic and environmental processes. Traditionally these processes were treated independently, but recently fields such as evolutionary development have worked to unify our understanding of these mechanisms and to investigate how these processes interact with each other to generate variation. Developmental plasticity provides a fantastic framework for studying how genetic and environmental (GxE) interactions shape and maintain natural variation. Butterflies and their wing color patterns have long been model systems for plasticity. This dissertation seeks to address the gxe mechanisms responsible for generating color variation in the Dogface butterfly, *Zerene. Zerene* is comprised of only two species *Z. cesonia,* the Southern Dogface, and *Z. eurydice,* the California Dogface, that differ in their color patterns. *Z. cesonia* also exhibits a seasonal plastic color pattern, where *Z. eurydice* does not. These features make the *Zerene* system an excellent model for disentangling the gxe processes contributing to variation both within and between species. Using an integrative approach these studies address the role of 1.) larval host plant divergence 2.) seasonal fluctuations and 3.) hybridization on the development of wing coloration

variation. The findings of these studies contribute not only to our understanding of how butterflies generate their colors, but also to the wider knowledge base on how genetics and the environment influence the generation and maintenance of biological variation.

DEDICATION

To Rose and Joe, thank you for your unconditional love and support. You motivate me to be better than I am everyday.

ACKNOWLEDGEMENTS

First and foremost, I have to thank my advisor Brian Counterman, who has made me the scientist I am today. Brian taught me lessons not only about how to think critically and how to approach questions through an evo devo perspective, but, perhaps more importantly, he taught me the art of 'just getting stuff done', to not let anything stand in my way, and to never take 'no' for an answer. Next I need to thank my committee for their support and guidance over the years. Thank you to Matt Brown and Ryan Range for letting me use their labs, reagents, and equipment extensively in my time at MSU and a special thank you to Ryan for helping me to see that all along I was a developmental biologist and I just didn't know it.

Thank you to all the people who have provided training, guidance, and help on these projects: JoVonn Hill and Richard Brown for guidance on the Black belt prairie work; Amanda Lawrence and I^2AT Imaging institute for SEM training; Robert Reed and his lab for teaching me CRISPR; and Elizabeth Evans, Morgan Miller, and Heather Smith for their help rearing butterflies. This work was supported through NSF and Texas Ecolabs.

Lastly, I would like to thank the friends and family who helped me through this long strange journey. I would never have made it through without the love and support of my husband Joe, lab mates Chris Savell and Caleb Benson, and friends Alex Tice, Tyler Schartel, and Justine Moser.

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CHAPTER I

INTRODUCTION

How is diversity and biological variation generated? This has been a classic central question in the field of Biology. At a fundamental level, all of the vast biological variation we see around us can be generated through two processes, either from genetic processes or environmental processes. Historically, these two mechanisms were treated rather independently, with classic evolutionary models focusing on the role of genetics and largely excluding the influences of development and the environment. Over the past few decades major strides have been made to reconcile the fields of evolution and development. With the emergence of the fields such as evolutionary development (Evo Devo), our understanding of biological variation has expanded to not just the examination of genetic and environmental processes independently, but rather, how these genetic by environmental (GxE) influences interact with each other. Developmental plasticity specifically is one such topic that addresses how natural variation is shaped by the complex interplay between genetics and the environment. Phenotypic plasticity is defined as "the ability of individual genotypes to produce different phenotypes, when exposed to different environmental conditions" (Pigliucci *et al*., 2006). The study of how plasticity is generated and maintained can answer specific questions such as how individual variation is produced within a population, but it can also approach broader evolutionary questions of speciation and adaptation. Phenotypic plasticity provides an excellent foundation to examine questions on how gxe interactions shape and drive biological variation.

One of the classic and most charismatic examples of phenotypic plasticity is butterfly wing coloration. The colors of a butterfly can have a huge impact on its fitness and survival. Wing patterns serve multiple roles from mate preference, thermoregulation, aposematism to crypsis (Watt, 1969; Silberglied and Taylor, 1973; Endler, 1984; Mallet and Joron, 1999; Ellers and Boggs, 2004). Examples of adaptive plasticity aiding in these roles can be found throughout the different families of butterflies; from eyespot plasticity driving mate preference changes in *Bicyclus,* to increased melanin production in colder conditions for thermoregulation in Pierid butterflies, to seasonal pupal coloration changes in *Papilio* for crypsis (Hazel et al. 1987; Brakefield and Reitsma, 1991; Ellers and Boggs, 2004; Macias-Munoz et al., 2015). Butterflies and their wing colors provide a fantastic system to study questions such as how gxe interactions drive variation and the role of plasticity on driving and maintaining variation in evolutionary terms of adaptation and speciation.

This dissertation examines the gxe interactions responsible for generating both within and between species variation on the wings of the dogface butterfly, genus *Zerene.* This dissertation uses an integrative Ecological-Evolutionary-Developmental approach to characterize the development of wing color variation. *Zerene* is comprised of only two species *Z. cesonia,* the Southern Dogface, and *Z. eurydice,* the California Dogface, that differ in their color patterns. *Z. cesonia* also exhibits a seasonal plastic color pattern, where *Z. eurydice* does not. These chapters address the major questions of what role genetics and the environment play in maintaining and driving color variation within and between species of *Zerene*. Specifically, these chapters address the role of 1.) larval host plant divergence 2.) seasonal fluctuations and 3.) hybridization on the development of wing coloration variation.

The second chapter, *Lessons from Butterflies of the Black Belt Prairie: The Southern Dogface as an Indicator of Prairie Remnants,* is an ecologically focused chapter addressing the limited distribution of *Z. cesonia* to the highly endangered Black belt prairies. This distribution is driven by a host plant preference for prairie clover, *Dalea.* Although not directly testing questions on color variation, this chapter is included here because it establishes the specific insect-host plant relationship in *Zerene* that is leveraged in Chapter 3, addressing the role of these host plants on coloration. This Chapter (2) provides the groundwork information on the life history of *Zerene* that was integral to our understanding of the role of larval host plants in nutritionally induced color plasticity.

Chapter 3, *Plasticity and divergence in ultraviolet reflecting structures on Dogface butterfly wings,* is an eco-devo chapter that explicitly tests the role of larval host plants on the development of both pigment and structural coloration in *Zerene.* Here we show that ultraviolet (UV) structural coloration as well as pterin pigment granule deposition exhibits a plastic response to alternative host plant consumption. This chapter shows that species level differences in UV coloration cannot be recapitulated by diet changes, and are likely genetically based.

Chapter 4, *Seasonal plasticity in pigment and structural coloration shares the same gxe control in the Dogface butterfly*, is an eco-evo-devo chapter that characterizes two previously un-described seasonal polyphenisms, one in a structural color (UV) and one in a pigmented color (pink pterins). This chapter focuses on characterizing the gxe influences controlling both pink and UV plasticity. The results of this study shows that a polyphenism in pigment and structural coloration share both an environmental trigger and are influenced by the same genetic pathway.

The final chapter, *The Avid Wooer: Hybridization in the Dogface Butterfly,* is an eco-evo chapter that serves as a between-species investigation of the gxe influences generating color

variation. The previous chapters focused on within species variation in the Southern dogface, this chapter addresses the role of hybridization in generating the color variation we observe between species. In 1890 W.G. Wright described a population of intermediately colored *Zerene* butterflies and proposed the existence of a hybrid species in *Zerene*; this chapter tests this through a combination of genomic and morphological evidence. Our results suggest that hybridization occurred historically at low levels with continuous backcrossing to *Z. eurydice,* and that current populations can hybridize and produce fertile offspring in the laboratory. The color patterns generated by the F1 cross does not reflect the variation observed in the wild intermediate population though, suggesting that the intermediates may not carry the *Z. cesonia* color pattern alleles. We propose that the adaptive plasticity for melanin and UV changes may have introgressed into the intermediate population instead.

Collectively, this dissertation seeks to characterize the color variation both within and between species and addresses the roles of gxe processes on generating this variation. I have taken an integrative approach to my research and sought to incorporate natural history and ecological aspects of the organism to the investigation of the genetic and developmental color differences within *Zerene.*

CHAPTER II

LESSONS FROM BUTTERFLIES OF THE BLACKBELT PRAIRIES: THE SOUTHERN DOGFACE AS AN INDICATOR OF PRAIRIE REMNANTS

Previously published as: Fenner, Jennifer, Tyler Schartel, and Brian Counterman. "Lessons from Butterflies of the Black Belt Prairie: The Southern Dogface as an Indicator of Prairie Remnants." *Transactions of the American Entomological Society* 144, no. 2 (2018): 295-309.

2.1 Introduction

In the United States, grasslands are the most imperiled of all terrestrial ecosystems with only ~4% still remaining (Noss, 2013). North American prairies continue to be threatened from urban development, climate change, and lack of ecological management creating a need for proper remnant patch identification. A remnant patch is a fragment of original prairie landscape that contains some component of native vegetation. Since the quality and management of these remnant patches can vary widely, it is important to also assess the functionality of these remnant patches, such as the ability to support multi-trophic relationships between plants and animals (Smith et al., 2010). Butterflies are a charismatic easily observed invertebrate species found in prairie remnants that may serve as a good indicator species. In this study, we propose that the Southern Dogface butterfly, *Zerene cesonia,* driven by host plant preference for prairie clover, *Dalea purpurea,* can serve as an indicator for identifying functional prairie remnant patches. To formally test if *Z. cesonia* can serve as an indicator of prairie remnant patches we focused on the critically imperiled Black Belt prairie as a model system of highly fragmented and threatened North American grasslands.

Across the southern United States, there was once a broad belt of grassland prairies that extended from western Tennessee to Alabama, known as Black Belt prairies. These prairies have historically been hubs of agricultural and cultural development (Peacock and Schauwecker, 2003). Black Belt prairies are characterized by their black, fertile, alkaline soils (pH ranging from 7.5-8.5), which resulted from the receding Atlantic coastline during the Upper Cretaceous period (Leidolf and McDaniel, 1998). Historical surveys from the 1830's suggest that the Black Belt once covered at least 144,000 hectares (Barone, 2005) in crescent shape that extends from McNairy County, TN to Russell County, AL (Leidolf and McDaniel, 1998). Despite its former ecological dominance across the southern U.S. and current peril, southern prairies like the Black Belt, have received little publicity relative to many of the tall grass prairies of the western U.S. (Noss, 2013). This lack of public awareness has resulted in a paucity of studies on the natural history and conservation of Black Belt grasslands.

Today less than 1% of Black Belt prairies remain in discontinuous patches distributed throughout Mississippi and Alabama. They continue to be threatened by problems such as land erosion, over-grazing by cattle, as well as encroachment of eastern red cedar, *Juniperus virginian* due to fire suppression. Black Belt prairies have been categorized as "critically imperiled" by the Mississippi Natural Heritage Program (Mississippi Museum of Natural Science, 2015). Black Belt prairies host a variety of species that are endemic or native to southern grasslands. The Black Belt prairies are recognized as an ecological system within NatureServe's ecological community classification scheme (Comer et al., 2003) and are defined by the presence of the following herbaceous vegetation: *Schizachyrium scoparium* (Little Bluestem), *Sorghastrum nutans* (Yellow Indiangrass), *Dalea candida* (White Prairie-clover), *Liatris squarrosa* (Scaly Blazing Star), and *Silphium terebinthinaceum* (Prairie-dock) (Copyright © 2009 NatureServe).

Although the flora is typically dominated by these species many other plant species, native to the Great Plains and endemic to the southern U.S., are also often found in the Black Belt prairies (DeSelm and Murdock, 1993; Peacock and Schauwecker, 2003, Barone and Hill, 2007).

The Black Belt is on the edge of the range limit for many Great Plains species and these disjunct occurrences of plants and animals between the locations suggest a prehistoric connection between the grasslands (Peacock and Schauwecker, 2003). It has even been proposed that the Black Belt may have served as a refuge for Great Plains species during glacial periods (Brown, 2003). It is estimated climate change is likely to be an increasingly important driver of Great Plains land management, as it continues to impact biodiversity, agriculture, and socioeconomics of the region (Freese *et al*., 2016). The disjunct populations of Great Plains species in the southern Black Belt can provide valuable insights into how these species and communities in the Great Plains may adapt to climate and environmental changes. In this regard, the few Black Belt prairie remnants that still persist may serve as important habitat reservoirs of invertebrate diversity not just for the southern U.S., but also for many prairie ecosystems in North America. The highly fragmented nature of the Black Belt, along with the overlap of flora and fauna between the Black Belt and other North American prairies, suggests the Black Belt could serve as a model of the potential future impacts of agricultural development and prairie conservation across North America.

The Black Belt houses a rich diversity of invertebrate species. This is best exemplified by moth surveys of Black Belt prairies that showed over 50% of the known moth species of Mississippi were present in Black Belt prairies and forests (Brown, 2003). Other invertebrate fauna studies of Black Belt remnants have produced species lists for local grasshoppers, ants, beetles and crayfish (MacGown and Schiefer, 1992; DeSelm and Murdock, 1993; Hill, 2007;

Hill and Brown, 2010; Smith *et al*., 2012;). Invertebrate studies have identified several taxa that are found exclusively in the Black Belt prairies or that are endemic to the disjunct Great Plains and western grasslands (Peacock and Schauwecker, 2003), including the prairie clover bee (*Xenoglossodes albata)* (MacGown and Schiefer, 1992), the prairie mole cricket (*Gryllotalpa major*), 10 species of moths, and four species of longhorn beetles (Schiefer, 1998). However, invertebrate species lists of the Black Belt remain taxonomically limited with several taxa of conservation and ecosystem importance (*e.g.* pollinators, pest species), such as butterflies, still un-described.

Sulphur butterflies, family Pieridae, are often some of the most abundant butterflies found in North American grasslands. These butterflies share a common yellow color pattern with varying amounts of melanization. This color pattern offers a conspicuousness or camouflage that reduces predation risk in the grasslands (Shapiro, 1974; Roland, 2006). These butterflies largely use legumes as larval hosts and can play the roles of both pollinators and pests. For example *Colias eurytheme* are known as major alfalfa pests, while they also serve as minor pollinators for grassland plants (Watt *et al*., 1974). Personal observations of grasslands in northeastern Mississippi from 2011-2013 found a large number of sulphur butterflies (B. Counterman, pers.obs.). Differences in the species compositions of sulphur butterflies among field sites was also observed, and most striking was the high frequency of *Zerene cesonia* at sites recognized as Black Belt prairie remnants.

Zerene cesonia, the Southern dogface butterfly, is the sister genus of *Colias*. *Zerene cesonia* is a non-pest species whose distribution overlaps with both Great Plains grasslands and Black Belt prairies. *Zerene* is composed of only two species: *Z. cesonia* and *Z. eurydice*. *Zerene euydice* has a distribution limited to California and is reported to exclusively use the California

false indigo, *Amorpha californica* as a larval host. *Zerene cesonia* has a range from North America to Argentina and is reported to use a variety of larval host plants, such as alfalfa, soybeans, prairie clovers, false indigo, and clovers (Brock and Kaufman, 2003). The most commonly reported larval hosts are *Dalea* spp. including *Dalea candida* and *Dalea purpurea*, which are defining flora of Black Belt prairies and have a distribution across North America overlapping with the Great Plains (Figure 2.1A). *Dalea* is the only species we have observed *Z. cesonia* to oviposit on in the wild (B. Counterman, pers.obs.). In this study, we propose that the distribution of *Dalea*, a potentially preferred larval host of *Z. cesonia*, influences the high frequencies of *Z. cesonia* observed at several Black Belt prairie sites. Previous surveys of Lepidoptera abundances have proven to be good candidates for indicator status in endangered ecosystems (Kerr *et al*., 2000). Specifically, butterfly communities have been shown to serve as quality indicators for assessing the success of restored Tall-grass prairie remnant patches in Iowa (Shepard and Debinski, 2005). Thus the observed high frequencies of *Z. cesonia* relative to *Dalea* populations could indicate *Z. cesonia* as an indicator species for identifying remnant patches of Black Belt prairie. Given the overlapping distribution of both *Dalea* and *Z. cesonia* with the Great Plains and other North American prairies, *Z. cesonia* could be an indicator of prairie remnants across the United States.

Here, we explore if *Z. cesonia's* affinity for a particular host plant, could drive its association with remnant prairie patches of the Black Belt. To test for an association of *Z. cesonia* with the flora of Black Belt prairies, we surveyed the butterfly communities of grassland sites in the Black Belt region of northeast Mississippi. We were particularly interested in characterizing the composition of sulphur butterfly communities, given the potential importance of sulphur species as both pollinators and agricultural pests (Watt *et al*., 1974). Specifically, we

examined the relationship among sulphur butterflies, host plant distributions, and environmental conditions (soil pH and area) across nine grassland sites. We complemented these field observations with laboratory experiments to assess if oviposition preference of *Z. cesonia* for *Dalea* could be the mechanism driving an association between *Z. cesonia* and prairie remnants.

2.2 Methods

Sampling sites

In this study, nine Northern Mississippi grassland sites were sampled (*see* Table 2.1 for Coordinates). Sites spanned three Mississippi counties and no two sites were further than 115 km from each other (*see* Figure 2.1 for Map). Each grassland site chosen includes a mix of open grassland areas and chalk outcrops bordered by oak, hickory, and pine forests. In addition each site has been historically or currently considered a "Black Belt prairie" remnant. Although, various forms of management are employed across the sites, none of the sites were mowed or fire managed over the course of the study. Five sites, Osborn, Crawford, MSU North Farm, Pulliam, Tombigbee are current remnant prairies. Two sites, Natchez Trace and Morgan Hill, are restored prairie sites, while the last two sites, MSU South Farm and the Black Belt Overlook, were anthropogenic grasslands. These were historical Black Belt grasslands, but are now lacking the characteristic vegetation that defines a Black Belt prairie, such as *S. scoparium* and *S. terebinthinaceum.*

Butterfly Surveys

Each field site was sampled four times during May-July 2014. Sites were only surveyed during peak flight hours (0900 and 1500) and under optimal weather conditions for butterfly flight. All sites were sampled over the course of 2-week intervals: (1) May 13th- 28th, (2) June

4th- 19th, (3) June 23rd-July 3rd, and (4) July 14th-25th. A Pollard walk was performed at each site to minimize impact and to prevent recounting of the same individuals (Pollard, 1970). Pollard transects were standardized to site area (Table 2.1) to account for variation among site sizes. All transects were either 1.5 km or 3.2 km in length. The smallest sites such as North Farm and the Natchez Trace, with an area of 0.004 km^2, were patrolled for 1.5 km and sampled for 1.5 hr. Larger sites, such as Osborn with an area of 0.102 km^2, were sampled for 3.2 km for 1 hr. During the Pollard walks every sulphur butterfly observed within an estimated 5 m visual distance was counted. Each record was identified to species, except for *Colias eurytheme* and *Colias philodice*, which frequently hybridize in the region and were counted as a single species (*see* Figure 2.1). All other butterfly species, excluding skippers, were recorded only in terms of presence or absence in order to produce a species list of butterflies for the Black Belt prairies. It was also noted if a butterfly was seen visiting a plant for nectaring or oviposition purposes. Three prairie sites (Osborn, Crawford, and North Farm) continued to be sampled into the fall (September to November) in order to track seasonal trends in sulphur butterfly counts.

Dalea Measurements

For each study site, standardized quadrat sampling was used to obtain an estimate of prairie clover plants. The average number of *Dalea candida* and *Dalea purpurea* was measured and sampling was conducted along transects, unique from, but partially overlapping with, the Pollard walk transects, established in areas identified as "suitable habitat". Suitable habitat was defined as areas capable of sustaining prairie species; for example, no heavily eroded zones, forested zones or areas covered by debris were included in the transects to estimate *Dalea* densities. Transect length was dependent upon the size of the site. Large sites such as Tombigbee

had transects of 100 m across and 20 m wide, and small sites such as North Farm were 50 m by 10 m. Stems were counted for both *Dalea* species in 1m x 1m quadrat placed at 5 to 10 m intervals and seventeen total quadrats were counted at every site. *Dalea* stem counts were averaged to obtain an estimate of *Dalea* abundance per site.

Soil and pH Measurements

Soil pH measurements for each site were obtained from the USDA Web Soil Survey (http://websoilsurvey.sc.egov.usda.gov/App/HomePage.htm). The weighted pH averages were calculated for each site using the percentage of each soil component present six or more inches below the surface and its respective pH measurements.

The area of each site was calculated by using Google Earth to generate a polygon around the usable habitat for sulphur butterflies at each site, with usable habitat defined as open grassland areas only. All polygons were exported to University of New Hampshire Cooperative Extension KML Tools Project (http://extension.unh.edu/kmlTools/index.cfm) to calculate the area (km^2) of each polygon.

Oviposition Experiments

The oviposition preference of *Z. cesonia* was tested for three suspected host plants that are found at or near the grassland sites: *Dalea purpurea, Glycine Max, and Baptisia australis.* Oviposition experiments were conducted using laboratory-reared individuals and custom oviposition chambers maintained at temperatures of 23-29°C, ~50% relative humidity and 16 hr light cycles. Larvae were fed an artificial diet that contained ~3% *Dalea purpurea,* dried and crushed, that was modified from a *Colias* diet (Taylor *et al*., 1981). Adults were kept in

population cages with small cups of 20% sugar water with no *Dalea* or other host plants available. Females were inspected for spermatophores daily to confirm they had mated. Mated females were used for the oviposition experiments 3-5 day post-mating. The experiments were conducted in oviposition chambers designed following Taylor *et al*., 1981. The chambers consisted of a 21.6 cm tall, and 7.6 cm wide, cylinder of clear acrylic, with one-inch of screen on the top and bottom of the cylinder for air circulation. Plastic petri dish covers were placed on the top and bottom of the chambers.

Inside each chamber ~15 cm cuttings of terminal branch with leaves were placed in 100 ml plastic beaker filled with vermiculite and water, to minimize wilting. Cuttings were obtained immediately before the start of each experiment from healthy, mature plants reared in greenhouses. For the experiments the chambers were placed on a wire shelf ~15 cm below a full spectrum fluorescent light source. Mated females were placed in a chamber for a 6-hr period and then returned to cages with no access to host plants. The number of eggs laid on each cutting was recorded. Since *Z. cesonia* was previously seen to oviposit on *B. australis* in the laboratory, we chose to conduct a series of "choice" and "no choice" trials to assay preference for *D. purpurea* and *B. australis*. For these trials mated females were given two plant cuttings in one of the following combinations: both *D. purpurea* (no choice trial), both *B. australis* (no choice trial), or one cutting of each species (choice trial). Each female was given each of the plant combinations over three consecutive days. In an attempt to minimize the impact of the order that the different hosts were introduced to the individual females, the three plant combinations were rotated for each female's first trial. The no choice experiments provided an opportunity to test for oviposition effects from interactions between potentially suitable hosts. For trials involving *G.*

max, no previous personal observations existed for *Z. cesonia* oviposition or larval feeding and therefore only no-choice trials were conducted to assay the female's willingness to oviposit.

Analysis

A Shannon Diversity Index was calculated from butterfly counts to evaluate butterfly diversity at each site. The Shannon Index characterizes species diversity in a community and accounts for both species abundance and evenness at a site. Principal component analysis (PCA) was conducted to partition the variation in the number of sulphur butterflies observed relative to site characteristics (pH, Area, *Dalea* measurements, and average sulphurs observed). The PCA was a hypothesis free way to investigate potential relationships among the sulphur butterflies and site characteristics. Linear regressions were used to further investigate the relationships between each set of variables that showed an association in the PCA. P values are reported only for significant relationships. A Pearson correlation co- efficient value was calculated from total butterfly counts across all sites for two sulphur species, *Z. cesonia* and *Colias* (*C. eurytheme* and *C. philodice* together), in order to test for a correlative relationship between the two groups. A Pearson correlation co-efficient was also calculated between *Dalea* densities and *Colias* to see if the trend was similar to the correlative relationship observed between *Colias* and *Z. cesonia.* To formally test if *Z. cesonia* could serve as an indicator species for Black Belt prairie sites an Indicator Value Index (IndVal) approach was used to test for an association between site groups (current non-restored Black Belt remnant patches versus anthorpogenic grasslands) and individual sulphur species abundances. The indictor value is designed to assess the predictive value of a species as an indicator for a specific site group (Dufrene and Legendre, 1997). The abundances of each sulphur species within each site group was tested for an association to see if

any one sulphur species had an association with only current Black Belt remnant patches. Analysis was conducted using the Multiplatt function in the R package *indicspecies* (ver1.7.1) (De Caceres and Legendre, 2009; De Caceres and Jansen, 2016).

2.3 Results

Butterfly distributions across grassland sites

We observed a total of 18 macro-butterfly species at the grassland sites (Table 2.2). Each species listed was observed at a Black Belt prairie remnant, however not all species were found at the non-Black Belt sites. The number of species of sulphur butterflies was largely consistent across the grassland sites, except for *Z. cesonia*. Non-restored Black Belt sites generally had the greatest Shannon Diversity Index values; Tombigbee and Osborn had the greatest diversity while the lowest diversity values were from restored prairie sites Morgan Hill and Natchez Trace (Table 2.1).

The five sulphur species found in Mississippi grasslands are: *Zerene cesonia, Colias philodice, Colias eurytheme, Phoebis sennae,* and *Pyrisitia lisa* (Figure 2.1). Of all butterflies observed, *Z. cesonia* showed the greatest variation in observed individuals between sites, ranging from a total of 286 individuals observed at Tombigbee National Forest to zero individuals at two sites (MSU South Farm and Morgan Hill, Figure 2.1). At Black Belt Overlook, only two *Z. cesonia* were observed throughout the study. In contrast at Pulliam prairie there was a total of 198 *Z. cesonia* sightings but only 20 sightings of other sulphur species. A similar trend, where *Z. cesonia* were consistently found in higher numbers than all other sulphur butterflies, was observed across four remaining sites (North Farm, Osborn, Crawford, and the Natchez Trace). A Pearson correlation co- efficient value was calculated and an inverse relationship (r^2 =-0.21) was observed between *Z. cesonia* and *Colias (C. eurytheme* and *C. philodice)* sightings. The site that

had the highest presence of *Z. cesonia*, Tombigbee, was completely absent of all other sulphur species, whereas the site with the highest prevalence of *Colias,* South Farm, had no observations of *Z. cesonia.* Although the difference was not as great at other sites, the inverse trend between observed individuals of *Z. cesonia* and *Colias* was significant ($P = 4.93e^{-2}$) when all sites were considered together.

Indicator species analysis testing for associations between specific species and Black Belt sites suggested *Z. cesonia* can serve as an indicator species for Black Belt prairie. *Z. cesonia* was significantly associated with Black Belt sites ($P = 1.3e^{-2}$). No other sulphur species were significantly associated with Black Belt prairies or non-Black Belt grasslands.

Seasonality of butterfly populations

Throughout the summer, the numbers of each sulphur species sighted per visit were consistent across sites, except for *Z. cesonia.* The abundance of *Z. cesonia* peaked during the third sampling period, at the end of June and beginning of July 2014. Four of the six sites where *Z. cesonia* was present during the study showed the highest counts during this sampling period (Figure 2.2).

Longer-term investigations of three sites (Osborn, Crawford, and North Farm) through the fall months (September – November) revealed *Z. cesonia* numbers remained higher throughout than *Colias* for all sites. Beginning in September, the number of *Colias* observed dropped to no more than one individual per site per visit. Although much reduced from the summer months, *Z. cesonia* were consistently observed at the three sites until November. *Pyrisitia lisa* was the only other sulphur species that was consistently observed at sites through the fall months, until November when, like *Z. cesonia*, their abundances also steeply dropped.

Host plant use and distribution

Adult *Z. cesonia* utilized a wide variety of food plants throughout all sites, whereas *Z. cesonia* larvae were preferential in their host plant feedings. Adult *Z. cesonia* were observed nectaring on a wide range of flowers and did not show any fidelity to a specific plant species at any site. The adults visited common and abundant plant species at each site, such as *Cirsium discolor* and *Coreopsis lanceolata* (Table 2.3). Unlike the adults, the larval stages of *Z. cesonia* were specific to only two plant species. In over 30 hr of field observations throughout this study, adult *Z. cesonia* were commonly found to oviposit on *D. purpurea* and *D. candida;* oviposition was never observed on any other plant species. No other butterfly species was observed to oviposit on either *Dalea* species. No pupae were observed in the wild and only one larva was found. This larva was found on a *D. purpurea,* after watching an adult female oviposit on an adjacent *D. purpurea.* Two other records of *Z. cesonia* larva found at Osborn prairie were located on *D. candida* (B. Counterman, pers. obs.). Two of the previously suggested host plants, *B. australis* and *G. max,* were not found directly in the grassland sites but were observed neighboring the sites via roadsides and agricultural plots adjacent to the grasslands.

Z. cesonia expressed a clear oviposition preference for *D. purpurea* in the laboratory as well as in the field. No choice oviposition experiments of *D. purpurea* and *G. max* (n = 12 females) resulted in a difference of 354 eggs laid on *D. purpurea* to zero laid on *G. max*. In the *B. australis* and *D. purpurea* choice experiments*, Z. cesonia* laid a total of 255 eggs on *D. purpurea,* while only laying 12 on *B. australis* (n = 21 females). Only four females laid the 12 eggs on *B. australis* and ten were laid during "choice" trials with *D. purpurea* present. Of the 12 eggs laid on *B. australis*, five had larvae hatch, but all five died within 24 h. All five showed evidence of feeding on the egg casing and *B. australis* leaves. Across all experiments *Z. cesonia*

females that oviposited laid an average of 43 eggs on *Dalea* and three on the other potential hosts.

The preferred larval host plants, *D. purpurea* and *D. candida,* varied greatly in abundance across the nine sites (Table 2.1), with no *Dalea* observed at three sites: Morgan Hill, MSU South Farm, and the Black Belt Overlook. Two of these three sites, MSU South Farm and Black Belt Overlook were populated almost exclusively by grass species and had very few legume species present. Although Morgan Hill lacked *Dalea,* there were other sulphur host plants present, such as *Chamaecrista fasciculate* (*P. sennae*) and *Mimosa pudica* (*P. lisa*). At the six sites with *Dalea,* average abundance varied from 2.79-17.18 plants/m^2 (Table 2.1). At these sites *Dalea* was often the dominant plant species in eroded areas of lighter colored calciferous soil. In areas with the dark, topsoil characteristic of the Black Belt prairies, *Dalea* was largely distributed in clusters across open areas that received full sunlight throughout most of the day.

Relationship between sites, plants and butterflies

Across all sites the presence of *Dalea* is associated with the presence of *Z. cesonia* (*See* Table 2.1 and Figure 2.1). There were significantly greater numbers of *Z. cesonia* observed at sites with *D. purpurea* and/or *D. candida* present than those without *Dalea*. However, for the other sulphur butterflies there was no significant relationship between the observed numbers at each site with the presence/absence of *Dalea*. *Dalea* averages per site showed a negative correlation (r^2= -0.41) with the average number of *Colias* butterflies per site. In the PCA *P. sennae* and *Colias* clustered with site area in the first two components, the components that explains over 99% of the variation in the data (Figure 2.3A). The average number of *Dalea* and the average number of *Z. cesonia* also formed a cluster through out the first two principal

components. Linear regressions were conducted between variables that grouped together in the PCA to evaluate the relationships between butterfly counts, *Dalea* densities, and other site properties (*i.e*. area, soil pH). Regressions between *Z. cesonia* counts and *Dalea* densities revealed a highly significant positive relationship ($P = 2.62e^{-3}$) (Figure 2.3B). In addition, there were clear positive trends between soil alkalinity, *Dalea,* and *Z. cesonia* counts. The soil pH at sites that contained *Dalea,* ranged from 7.06 to 7.92. The two sites that had no *Dalea* or *Z. cesonia* present also had the most acidic soil pH (5.07 and 6.02). However, these relationships were not significant. The other sulphur butterfly species showed no significant relationship to the variation in *Dalea* and soil pH. However, variation in the numbers of *Colias* and *P. sennae* butterflies observed was significantly affected by site area ($P = 1.25e^{-2}$ and $1.69e^{-2}$).

2.4 Discussion

Sulphur butterflies are one of the more charismatic and identifiable insects that can be found in Black Belt prairies. The broader implications of this study is showing how observations of the sulphur butterfly community assemblies can provide valuable natural history data that can be used to inform future conservation efforts. This study offers insight into pierid butterfly community structure, as well as highlights the potential usefulness of sulphur butterflies in the assessment of threatened grasslands. The broad range of both *Z. cesonia* and *Dalea* lends potential for this indicator relationship to extend to prairie grasslands outside of the Black Belt.

Larval host plant divergence in sulphur butterflies

Pierid butterflies, Whites, and Sulphurs, utilize three host plant groups, legumes, brassicales, and mistletoes. It has been suggested that host-plant shifts are responsible for the rapid diversification among the Pieridae family (Braby and Trueman, 2006). The sulphur

butterflies, *Coliadinae,* specialize only on the nitrogen rich legumes for larval host plants (Braby and Trueman, 2006).The sulphur butterfly genus *Colias* consists of both host plant specialists and generalists, for example *C. philodice* is a generalist legume feeder, while *C. interior* is a specialist feeder on *Vaccinium* (Karowe, 1994).

The sulphur butterfly *Z. cesonia* is commonly reported as a generalist legume feeder (Brock and Kaufman, 2003) but showed a clear preference for *Dalea* in both the laboratory and the wild. Overestimates of *Z. cesonia* host range may be the result of frequent misidentification given that *Zerene* can be strikingly similar in appearance to several *Colias* species. In Black Belt prairies, *Z. cesonia* show a clear preference for a single host plant species, *Dalea purpurea*. This apparent host plant preference in *Z. cesonia* is very similar to host usage in the sister species *Z. eurydice*, the California Dogface, which is reported to feed exclusively on *Amorpha californica* (Brock and Kaufman, 2003). In California and Texas, there are historical records of *Z. cesonia* feeding on *Amorpha fruticosa,* the sister species to *A. californica* (Brock and Kaufman, 2003; Kendall, 1959). Both *Amorpha* and *Dalea* are close relatives and belong to the same subtribe of legumes Amorpheae (Cardoso *et al*., 2012). This relationship suggests there may be more undocumented, local host plant preferences and divergences between the Amorpheae subtribe and *Zerene* that merit further exploration.

Implications for prairie conservation and management

In conservation biology there has been an increased focus on the assessment of ecosystem function in endangered and protected habitats. The goal of these efforts has been to assess the importance and value of the ecosystem as a whole, which are often inferred by investigating the roles of specific, key components of the system (Schwartz *et al*., 2000; Harvey

et al., 2016). Despite the potential importance of Black Belt prairies as refugia for native southern grassland biodiversity and for local pollinator populations (Peacock and Schauwecker, 2003), there is a lack of assessment of the relative functions of different remnant patches. Our results showed large variation in the soil alkalinity and *Dalea* abundance among the remnants, which are both supposed to be diagnostic features of Black Belt prairies (Leidolf and McDaniel, 1998). The relationship observed between Black Belt soil, *Dalea* densities, and number of *Z. cesonia* observed offers a multi-trophic assessment of the health and functionality of remnant Black Belt patches. Butterfly abundances have previously been informative indicators of community diversity in threatened habitats (Blair, 1999; Kocher and Williams, 2000). Here, we propose that *Z. cesonia* can be a key indicator species for identifying and assessing the functionality of Black Belt prairies.

A key first step in conserving and managing the highly threatened Black Belt ecosystem is to properly identify and define remnant patches. The implications of this study on conservation and management efforts are that the observed multi-trophic relationship between soil, plants, and animals can be used as an indicator of a functioning Black Belt prairie remnant. Using this relationship as a measure, two of the study sites (Morgan Hill and Black Belt Overlook) do not appear to be functioning as Black Belt prairies. Interestingly, both of these sites reside in federally protected lands and have been advertised as Black Belt prairies for public outreach. Morgan Hill is a restored prairie located within the Sam D. Hamilton Noxubee National Wildlife Refuge (Hill, 2002). However, the soil pH at Morgan Hill lacks the high alkalinity characteristic of the Black Belt. At least 75% of the soil at Morgan Hill is Falkner or Kipling silt loam, which both have an acidic pH of 3.6 to 6.0 (Websoil survey). Unsurprisingly, Morgan Hill also lacked the presence of *Dalea*, which is a diagnostic flora of the Black Belt prairie. The Black Belt

Overlook is a hayfield along the Natchez Trace National Parkway with highly alkaline soil that is characteristic of the Black Belt prairies. However, despite having the proper soil type, *Dalea* was absent and only two fast-flying *Z. cesonia* were observed throughout the study. It is important to note this site is along a heavily traveled highway that is responsible for introducing many nonnative propagules, and these frequent introductions may increase competitive pressures on native prairie plant species. In addition this site is seasonally mowed for hay, which in turn limits the availability of food plants for adult *Z. cesonia*. Collectively, these results suggests that native flora restoration and management efforts on the Black Belt Overlook would have a high chance of positively impacting local butterfly abundance.

An aspect of this study worth future investigation is if the relationship between *Z. cesonia* and *Dalea* can provide indirect benefits for other species in prairie communities and neighboring agricultural areas. A negative relationship was observed between *Z. cesonia* and *Colias*, which is a common pest of alfalfa. This inverse relationship can be observed best at two sites with Alfalfa agricultural fields, MSU South Farm and MSU North Farm. The numbers of *Colias* at South Farm were higher than at any other site where *Z. cesonia* were absent from, while at North farm, where *Z. cesonia* was present and abundant, *Colias* was found at low numbers, with an average of 1.50 butterflies observed. Further study may be worthwhile to investigate if the *Dalea* and *Z. cesonia* relationship could play a role in reducing the numbers of an agricultural pest. As for butterfly diversity within sites, it was also observed sites that contained the highest abundance of both *Z. cesonia* and *Dalea,* such as Tombigbee, had the highest Shannon Diversity indices, while sites where *Dalea* and *Z. cesonia* were absent, such as Morgan Hill, had the lowest diversity index (Table 2.1). Collectively, our results suggest that grasslands with *Dalea* will not only have a higher abundance of *Z. cesonia,* but will also tend to have higher butterfly diversity.

This study suggests a common sulphur butterfly, *Z. cesonia,* could serve as an indicator species for functional remnants of the North American Black Belt prairies. This butterfly was previously described as a larval host plant generalist, but clearly shows a regional preference for *Dalea purpurea* and *Dalea candida* (Brock and Kaufman, 2003). In contrast, adult *Z. cesonia* were documented visiting a variety of plant species and did not exhibit a clear preference for nectar source. These findings suggest that limited distribution of *Z. cesonia* to the Black Belt prairies in Mississippi may directly result from a strong preference for *Dalea* as a larval host. Importantly, the relationship between the prairie clovers and *Z. cesonia* were largely independent of the prairie size (*i.e*. site area), which suggest sightings of *Z. cesonia* could be a critical indicator of small, cryptic remnant patches (*e.g*. $< 2.5e^{-2}$ km^2).

Conclusions

This study demonstrates how a butterfly community, the Sulphurs, can be used for conservation assessment of critically imperiled grasslands. The presence and abundance of *Z. cesonia* relative to densities of its preferred host plants appears to be a true signal for identifying the few last remaining Black Belt prairie remnant patches throughout the southern United States. Two sites from this study are federally protected lands that have been previously described as being Black Belt prairies, but both sites showed no evidence of the characteristic soil or flora of Black Belt prairies. This was accompanied by a clear lack of *Z. cesonia* butterflies. These results highlight the potential utility of butterfly census counts for the assessment of prairie conservation and restoration efforts. *Z. cesonia's* range extends far beyond Mississippi and encompasses most of the prairie ecosystems of North America. Several *Dalea* species, including *D. pupurea* are also widely distributed across North American prairies. Collectively, our findings suggest that

sulphur butterfly communities may be useful indicator tools for the identification and assessment of prairies, broadly across North America.

Table 2.1 GPS Coordinates and names for each site with biotic (*Dalea* averages) and abiotic (size and soil pH) metrics, as well as Shannon Diversity Index measurements.

Site Number	**Site Name**	**Latitude, Longitude**	**Soil pH**	***Dalea*** **(**plants/m^2)	**Area** (Sq Km)	**Shannon Index**
1	Natchez Trace	34° 9'14.18"N, 88°48'59.49"W	7.5	2.79	$4.18e^{-3}$	1.01
2	Black Belt Overlook	34° 8'57.62"N, 88°49'9.78"W	7.62	0	$3.14e^{-2}$	1.13
3	Tombigbee National Forest	33°55'52.28"N, 88°51'27.18"W	7.32	17.18	$1.55e^{-2}$	3.78
4	Pulliam	33°53'15.92"N, 88°50'0.29"W	7.06	3.03	$1.246e^{-1}$	1.33
5	Osborn	33°30'36.98"N, 88°44'14.57"W	7.84	5.65	$1.02e^{-1}$	2.34
6	MSU South Farm	33°25'11.53"N, 88°47'18.29"W	5.07	0	$4.80e^{-1}$	1.03
7	MSU North Farm	33°27'47.23"N, 88°45'36.24"W	7.55	4.29	$3.89e^{-3}$	1.07
8	Crawford	33°18'2.94"N, 88°36'35.94"W	7.92	4.82	$1.02e^{-1}$	1.51
9	Morgan Hill Noxubee	33°15'11.01"N, 88°46'15.76"W	6.02	0	$1.84e^{-1}$	0.55

Table 2.2 Butterfly species observed at prairie sites.

Butterfly Species	**Common Name**
Agraulis vanillae	Gulf Fritillary
Callophrys gryneus	Olive Hairstreak
Colias eurytheme	Orange Sulphur
Colias philodice	Clouded Sulphur
Cupido comyntas	Eastern Tailed Blue
Danaus plexippus	Monarch
Junonia coenia	Common Buckeye
Limenitis archippus	Viceroy
Papilio glaucus	Eastern Tiger Swallowtail
Papilio polyxenes	Black Swallowtail
Papilio troilus	Spicebush Swallowtail
Phoebis sennae	Cloudless Sulphur
Phyciodes tharos	Pearl Crescent
Pieris rapae	Cabbage White
Pyrisitia lisa	Little Yellow
Vanessa atalanta	Red Admiral
Vanessa cardui	Painted Lady
Zerene cesonia	Southern Dogface

Table 2.3 Species list of observed nectar plants utilized by adult *Z. cesonia.*

Prairie Plants	**Common Name**
Asclepias spp.	Milkweed
Asclepias tuberosa	Butterfly weed
Blephilia ciliata	Woodmint
Cirsium discolor	Field thistle
Coreopsis lanceolata	Lance-leaved coreopsis
Dalea purpurea	Purple prairie clover
Eupatorium altissimum	Tall boneset
Liatris aspera	Prairie blazing star
Lobelia inflata	Indian tobacco
Prunella vulgaris	Common self-heal
Tribulus terrestris	Puncture vine
Verbena simplex	Narrowleaf vervain

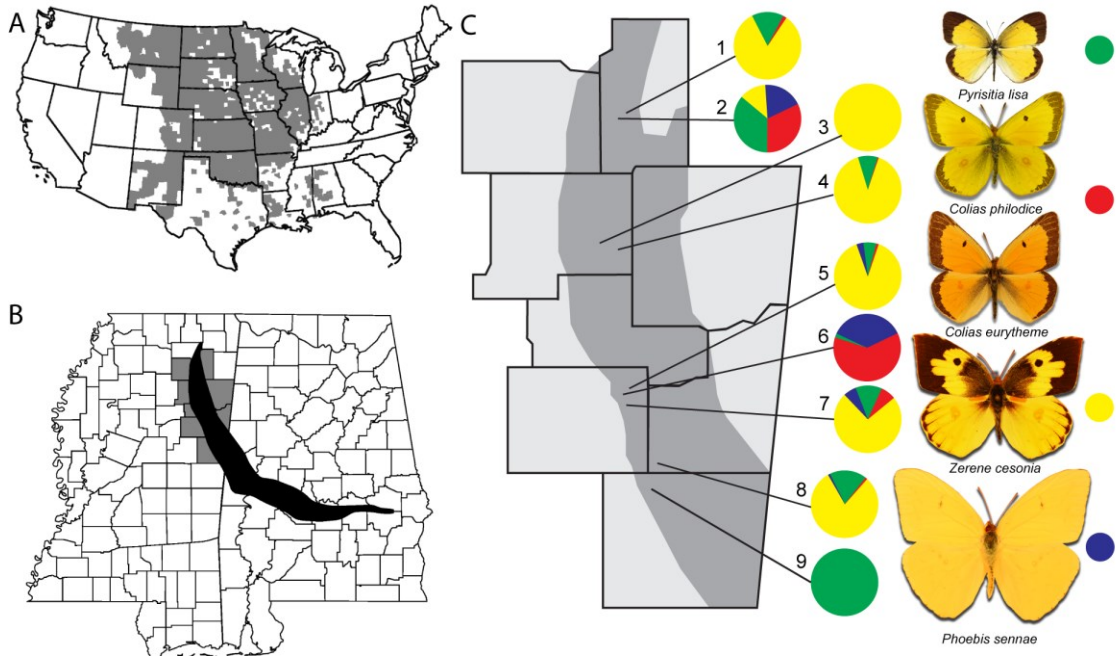


Figure 2.1 Prairie clover and Sulphur butterflies distribution of Black Belt prairies. Map (A) shows the North American distribution of *Dalea purpurea*, generated by data downloaded from the North American Plant Atlas (Kartesz, 2015). Map (B) (modified from Barone, 2005) shows historical distribution of Black belt prairies (black shaded regions), which overlaps with *Dalea purpurea* distributions. The study region counties are highlighted in grey shading and magnified in Map (C) Study site locations are shown on Map (C), numbers correspond to GPS site coordinates found in Table 1. Pie charts show relative frequency of sulphur butterfly species observed at each site.

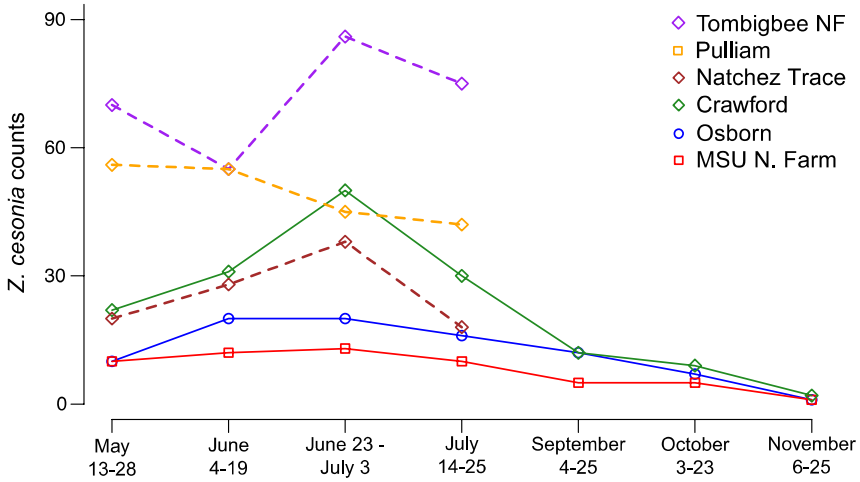


Figure 2.2 Seasonal abundance of Z. cesonia. Data shown for six sites that *Z. cesonia* was observed at for more than one visit. Counts at three sites continued until no Z. cesonia were observed. There is a peak in *Z. cesonia* numbers during the 3rd site visit (June 23rd- July 3rd) for four out of the six sites.

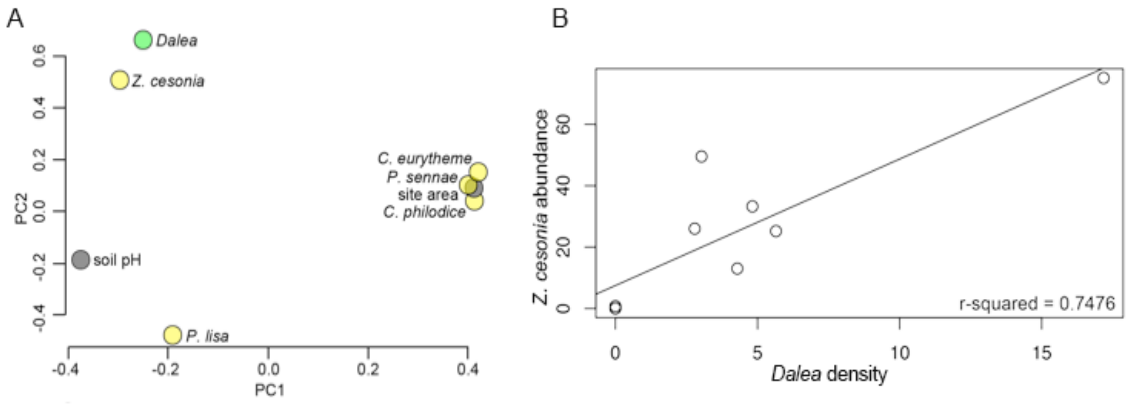


Figure 2.3 Correlations among butterflies, prairie clover and site characteristics. (A) shows the PCA of butterfly counts (sulphur spp.), host plant stem counts (*Dalea*) and site characteristics (site area and soil pH). Principal components one and two account for 99% of the observed variation. PC1 shows two clusters, one of Z. cesonia, *Dalea*, soil pH, and *P. lisa*, and that grouped site areas with the other sulphur species. PC2 shows tight clustering of site area with *Colias* spp. and *P. sennae*, and a separate clustering of *Dalea* and *Z. cesonia* (B) is a Linear Regression showing the significant trend between *Z. cesonia* counts and *Dalea* densities.

CHAPTER III

PLASTICITY AND DIVERGENCE IN ULTRAVIOLET REFLECTING STRUCTURES

Previously published as: Fenner, Jennifer, Luis Rodriguez-Caro, and Brian Counterman. "Plasticity and divergence in ultraviolet reflecting structures on Dogface butterfly wings." *Arthropod structure & development* 51 (2019): 14-22.

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 nanoscale 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, which 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, 1973; 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; Ellers and Boggs, 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 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, 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*, *Z. cesonia* (Southern Dogface) and *Z. 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 *D. 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 *A. 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.

3.1 Methods

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 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.

UV Photography and Wing Size Measurements

High-resolution UV images of wild type *Zerene* (shown in Figure 3.1) 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 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).

Wing Spectrometry

Reflectance of male forewings were 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 (Figure 3.1, 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 (Figure 3.3 A).

Scale Structure Measurements

Electron microscopy was performed to assess the structural differences between differently colored scales. Electron microscopy was performed at The Institute for Imaging & Analytical Technologies (I²AT) at Mississippi State University. 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 using a Ga ion source focused ion beam (FIB) and imaged using a field emission (FE) SEM using a Tescan Lyra3 (Figure 3.2 G-I). The ion beam was directed at a 90^0 angle to the scale and milled perpendicular to the lamella ridges, exposing an ~2 □m x 4 □m region and providing a

cross-section view of the scale. SEM magnifications for imaging ranged from 12.1 – 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 (Figure 3.2 K-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 (Figure 3.2 B-D) (Ghiradella 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 Figure 3.1 A), and (ii) yellow UV reflecting wing regions (closed circles Figure 3.1 A). These wing regions were mounted on aluminum stubs with silver paste and sputter coated with 15 nm Platinum. Stubs were imaged at 400 - 5,000 x 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 cover scales in a 70 mm area from a 400 x-magnified image (Figure 3.4 B). 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 Figure 3.2 F and J). The lamella ridge distances of UV reflecting cover scales were measured from 5,000 x magnified images in a 10 x 10 □m area (Figure 3.4 D). Granule density was measured from wing scales in the non-UV reflecting yellow region of the wing (open circles Figure 3.1 A) at 5,000 x magnification (Figure 3.4 F). 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 □m^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) (Figure 3.4). Discriminant function analysis (DFA) was conducted in R studio with ggord package (Beck, 2017) and MASS package (Venable 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 (Figure 3.3 B). Linear regressions were conducted and plotted in R studio for granule density and scale density (Figure 3.5). Linear regressions were similarly conducted for the scale measurements and UV brightness (rMax).

3.2 Results

UV reflectance differences between species

The UV reflectance and pattern differ between the two species of *Zerene* (Figure 3.1). UV patterns differ between the species in both forewings 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* (Figure 3.1).

UV reflecting structures

In both species of *Zerene,* the UV reflecting scales have similar architectures to those described in *Colias* (Figure 3.1). UV reflecting scales are characterized by tightly-spaced lamella ridges (Figure 3.1). 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 (Figure 3.2 B and 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 trabuculae, 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,

(Figure 3.2 C and 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 (Figure 3.2 K-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 (Figure 3.2 B-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*.

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 (Figure 3.3 A). 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 $p = 1.54E^{-5}$), but when split by larval diet treatments, only individuals fed *D. purpurea* had significantly brighter UV reflectance than wild caught individuals (T-Test $p = 3.31E^{-5}$). 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 (Figure 3.3 A). Upon visual

inspection, no qualitative differences in the shape of the UV pattern were noted among the diet treatments.

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 (Figure 3.3 B). When these scale measurements were analyzed independently, both scale and granule densities showed significant differences between diet treatments (Figure 3.4 A + E), but there were no differences in the lamellar ridge distances of UV reflecting scales (Figure 3.4 C). 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) (Figure 3.4 A-B). The lower density of the UV reflecting scales in the artificial and *A. fruticosa* diets also resulted in 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 (Figure 3.4 B). Pigment granule density was significantly different only between the *D. purpurea* and *A. fruticosa* diet treatments (p = 0.0062) (Figure 3.4 E), with the *D. purpurea* fed showing the highest density of granules. A linear regression between granule density and scale density (Figure 3.5) 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).

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 (Figure 3.6). 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) (Figure 63. A). 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) (Figure 3.6 B).

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 (Figure 3.6 C-D). The artificial diet-reared individuals increased their pupal development time to 12 days and had smaller wings compared to the 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-15).

3.3 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.*

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 *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 *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.

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 prefered 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 prefered 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 suggests, increased pigment granule density does not directly influence UV brightness, but rather may enhance the contrast and perception of the UV signal.

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 *C. 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 was 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 have 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.

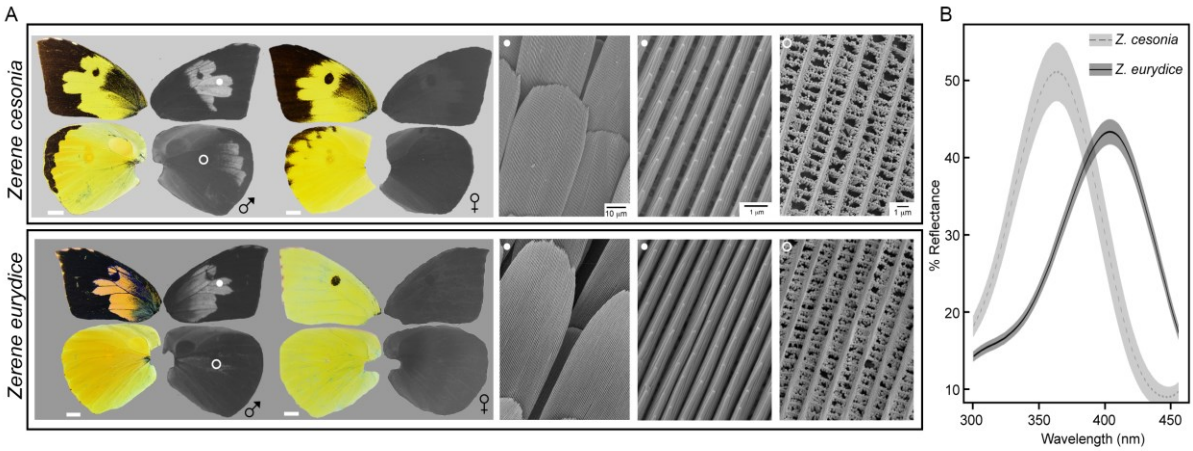


Figure 3.1 Wild Phenotypes. Wild type *Z. cesonia* and *Z. eurydice* showing dorsal wings, both visual color and UV patterns differ between species. (A) Left wings in each panel are color photos and the right wings are photos under UV light. Scale bars equal 0.5 cm. Females of both species do not reflect 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.

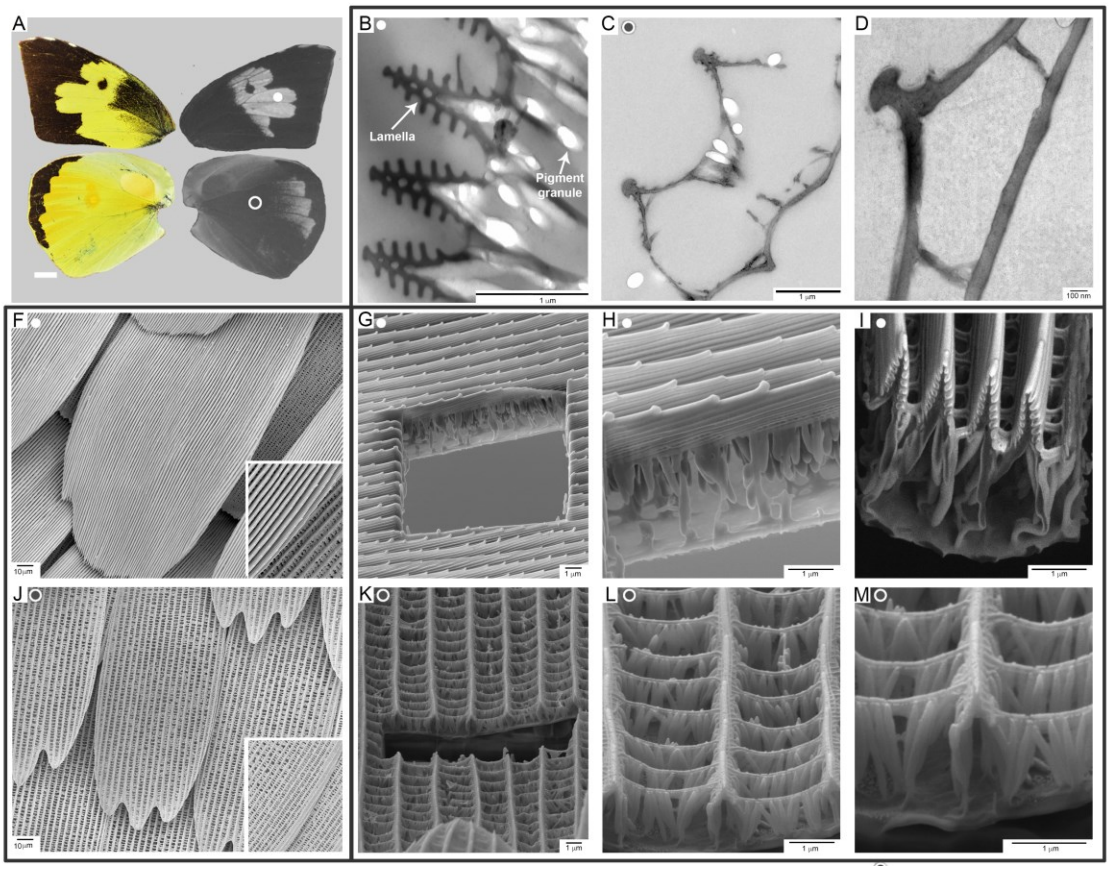


Figure 3.2 Scale Architecture of Z. cesonia. (A) Shows *Z. cesonia* with closed and open circles that indicates the wing regions where the following images were collected. B-D). TEM images of a *Z. cesonia* male. The large lamella ridge stacking in the UV reflecting scales can be observed in (B) while 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 the 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).

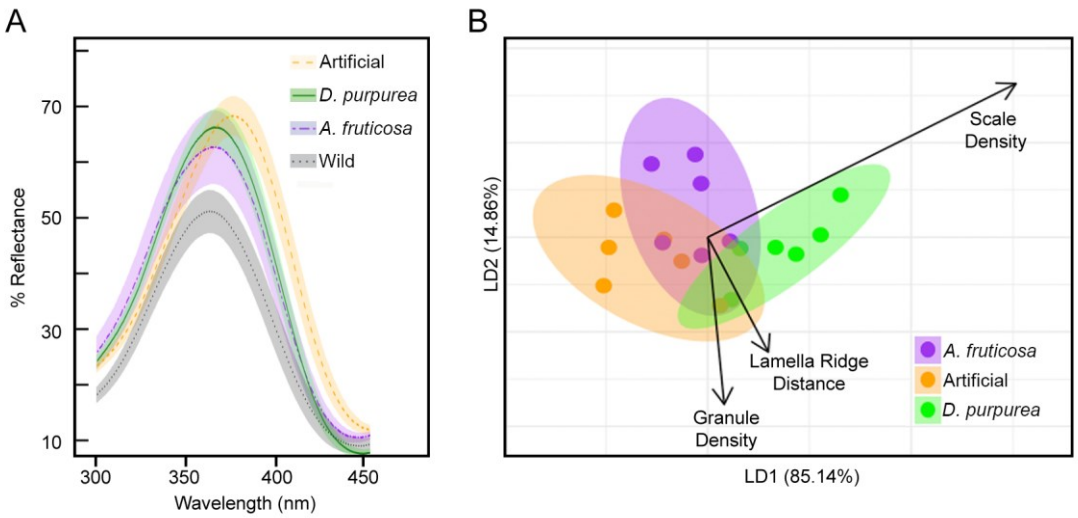


Figure 3.3 Ultraviolet Color Reflectance and Discriminant Function Analysis. (A) shows UV reflectance among the different diet treatments. Laboratory reared individuals had brighter UV reflectance than wild caught *Z. cesonia*. *D. purpurea* fed individuals were significantly brighter than wild individuals ($p = 3.31E-5$). 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.

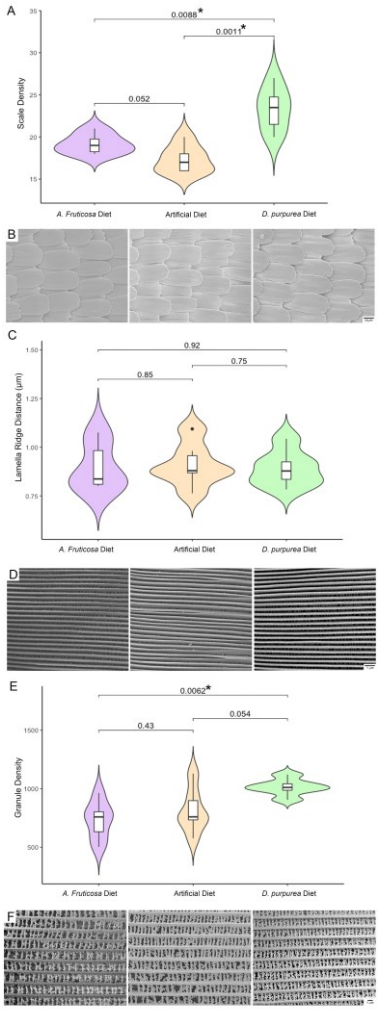


Figure 3.4 Plasticity in Scale Development in Z. cesonia. Differences in the density of UV reflecting scales were observed among the various diet treatments. (A) shows that *D. purpurea* fed individuals have the most UV reflecting scales per area, while the artificial diet individuals have the least. (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 5,000 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 5,000 X of yellow scales shown from left to right: (i) *A. fruticosa* treatment, (ii) artificial diet treatment, and (iii) *D. purpurea* diet treatment.

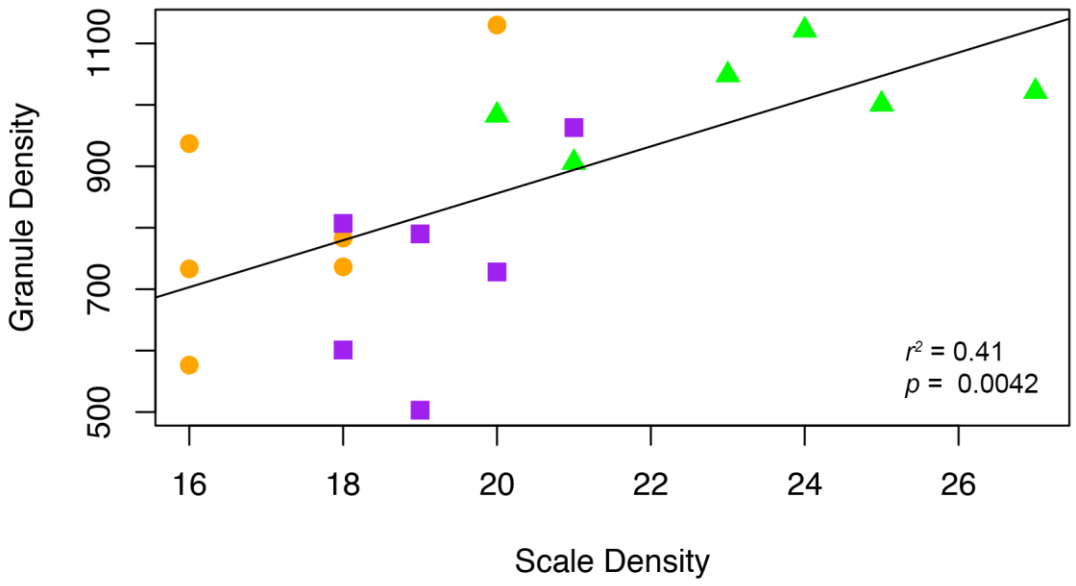


Figure 3.5 Linear Regression Between Granule and Scale Density. A linear regression performed among the all the laboratory reared samples shows the correlation in the decrease of granule density and scale density.

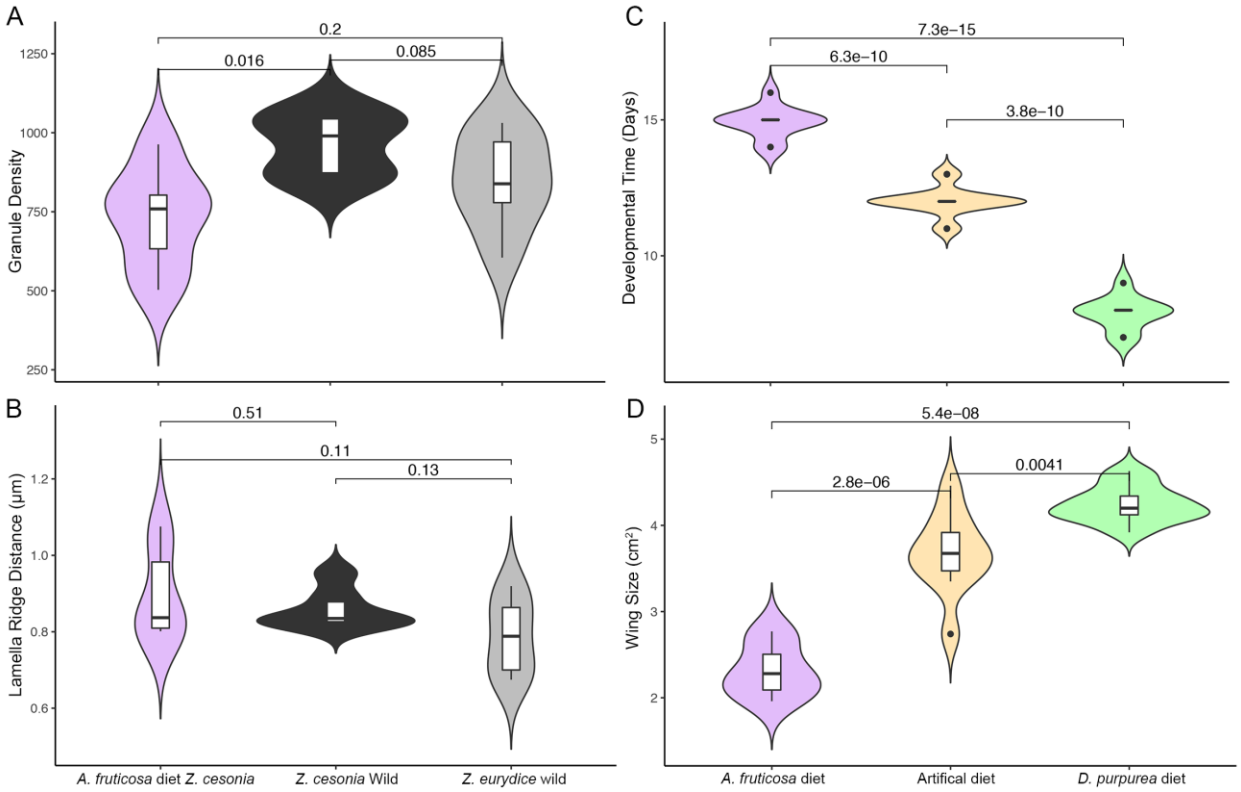


Figure 3.6 Species Comparisons to A. fruticosa Diet and Traits Impacted by A. fruticosa Diet. Scale features of granule density (A) and lamella ridge distance (B) are not significantly different between the two species. *A. fruticosa* reared individuals have larger spacing between lamella ridges than 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.

CHAPTER IV

SEASONAL PLASTICITY IN PIGMENT AND STRUCTURAL COLORATION SHARE THE SAME GXE CONTROL IN THE DOGFACE BUTTERFLY

4.1 Introduction

The vast array of biodiversity and natural variation that we see around us has been generated through a combination of genetic and environmental influences. Phenotypic plasticity, the generation of multiple phenotypes from a single genotype in response to environmental cues, provides a powerful system to study how specific contributions from genetic and environmental interactions impact an organism's development and life history. Seasonal polyphenisms are a type of phenotypic plasticity that yield seasonal morphs in a population and are abundantly expressed throughout the animal kingdom (Mills et al. 2013; Brakefield et al. 2009; Shapiro 1976). Butterflies, and specifically their wing color patterns, have been an attractive and tractable system for studying plasticity and seasonal polyphenisms. Examples of polyphenisms in butterflies can be found across many families and can have many adaptive and selective advantages, from eyespot plasticity driving mate preference changes in *Bicyclus,* to seasonal pupal coloration changes in *Papilio* for crypsis (Brakefield & Reitsma 1991; Macias-Munoz et al. 2016, Hazel et al. 1987).

Perhaps one of the most classic examples of adaptive seasonal plasticity comes from Pierid butterflies. In both *Pieris rapae* and in *Colias eurytheme,* examples of seasonal increases in melanin for thermal regulatory purposes have been studied for decades (Ae 1957; Kingslover

1985; Watt 1967; Watt 1969). The environmental triggers responsible for melanin plasticity have been extensively studied, and these triggers differ between *C. eurytheme* and *P. rapae,* in that photoperiod alone induces the seasonal form in *C. eurytheme,* while in *P. rapae,* both photoperiod and temperature cues are required (Ae 1957; Shapiro 1976; Hoffmann 1972; Stoehr & Wojan 2016). This increase in wing melanin, specifically on the ventral hindwing, during colder-shorter days of the year serves an adaptive role. *C. eurytheme* butterflies with more melanin were found to warm at a faster rate, allowing for more daily flight time and increased egg production (Watt 1968; Watt 1969; Ellers and Boggs, 2002; Ellers and Boggs, 2004). Other wing colors, such as orange pterins and Ultraviolet (UV), have also been shown to exhibit seasonal plasticity in *C. eurytheme* and *P. rapae* (Hoffmann 1974; Obara et al. 2008).

Wing coloration in Pierid butterflies can be generated through either pigment or structure. Pigments are either melanin (black) or pterin (yellow, white, orange, or red), while UV coloration is generated through special nano-structural architecture of pterin scales (Wijnen et al. 2007, Giraldo and Stavenga 2007; Ghiradella et al., 1972; Ghiradella, 1974). The triggers and ecological significance of melanin have been well studied, but this is not necessarily the case for pterin or UV plasticity. *C. eurytheme* also exhibits a reduction in orange pterin pigmentation alongside the melanin increase in response to decreased photoperiods, but unlike melanin, no ecological function for this reduction has been sufficiently tested or explained (Hoffmann 1974). In *P. rapae,* UV reflective females have been shown to lose their UV reflection in response to seasonal changes (Obara et al 2008). This change may have implications for mate preference, given that UV is a sexually selected signal in many Pierid species. In *C. eurytheme* it has been shown that UV brightness is the number one indicator of male mating success (Papke et al., 2007). UV brightness has not been shown to be seasonally plastic in *C. eurytheme,* though.

Although melanin plasticity on its own has been well studied, none of these studies have investigated both the gxe interaction responsible for the generation of structural and pigment plasticity together. In this study, we characterize the shared gxe interactions responsible for generating seasonal plasticity in both a pigment and a structural color.

Here, we describe two seasonally plastic colors, one in pink pigmentation and one in UV structural coloration, in the Southern Dogface butterfly, *Zerene cesonia. Zerene* is the sister genus to *Colias* butterflies and exhibits similar pterin, melanin, and UV wing coloration. *Z. cesonia* exhibits a completely unique color morph from those of *C. eurytheme* though. This morph described as the '*rosa'* form is a morph with pink ventral wing coloration (Figure 4.1A). The hindwing pattern is similar to the hindwing melanin pattern in *C. eurytheme.* Pink coloration is also expressed in the discal hindwing spots that, in *C. eurytheme,* are patterned by the melanin patterning *spalt,* which happens to be one of the few wing patterning genes where expression localization has been studied (Stoehr et al., 2013). Given the similarities to melanin patterning in *C. eurytheme,* we test if the pink coloration in *Z. cesonia* serves a similar role to that of melanin in *C. eurytheme*. Specifically, we examine four questions: 1.) Is the *rosa* form a seasonal polyphenism? If so, 2.) what is the environmental trigger? 3.) Is pink coloration influenced by a melanin patterning gene? And 4.) Is pink involved in thermoregulation? Collectively, the answers to these questions provide an examination of both the genetic and environmental interactions that influence plastic color development on a butterfly wing.

4.2 Methods

Butterfly collection

Adult *Z. cesonia* were collected from February through October from Osborn prairie (33°30'36.98"N, 88°44'14.57"W). In the laboratory, both sexes were released into flight cages,

allowed to mate, and females were allowed to oviposit on *Dalea purpurea.* After death, the wings of both sexes were preserved to track the phenotypic changes in wing color patterns throughout the season.

Larval rearing and treatment conditions

Eggs laid from wild caught females were collected daily and placed into a designated environmental treatment chamber. After hatching, larvae were reared on an artificial diet, and after eclosion, 30 individuals from each treatment were sacrificed and preserved for imaging. Batches of larvae were first reared in one of three different temperature and photoperiod treatments to observe if the seasonal polyphenism could be recreated in a laboratory setting. The Summer and Winter treatments were designed based on the average local daytime temperatures that the local Mississippi *Z. cesonia* would encounter in the Summer months (Summer Treatment 27°C, 16:8 hour photoperiod) and in the spring or fall (denoted as Winter Treatment 21°C, 8:16 hours). The Coldshock treatments were created to more closely mimic the natural daily temperature shifts experienced by *Z. cesonia* in the wild (10hr daylight and 24 hour temperature cycle of 25°C for 10hr; 16°C for 6hr; 10°C for 2hr; 16°C for 6hr). Subsequently, two more batches of larvae were reared in either the Daylight (Summer temperature with Winter photoperiod: 27°C 8:16hr) and Temperature (Winter temperature with Summer photoperiod: 21°C, 16:8hr) treatments, which were designed to test if the seasonal polyphenism could be induced by photoperiod or temperature alone or if both environmental cues are required for the induction of the winter phenotype.

Imaging

Wings were photographed using a Nikon D7000 camera with an AF-S Micro Nikkor 105 mm Lens for visual color images. Ultraviolet color images used the same lens and camera but with the addition of a BAADER U-Filter, 60 nm IR and VIS filter. Light microscopy images of scales on the wing were taken on a Leica M205C dissecting scope with a Cannon 650D camera. Images of individual scales removed from the wing were taken with light microscopy on an Acioskop 2 plus (Carl Zeiss) microscope with a Cannon 5DS camera. Scales were removed from the wing using a tungsten needle and submerged in clove oil, which has the same refractive index as insect cuticle (Wasik et al, 2015).

Scanning Electron Microscopy (SEM) images were taken with a JEOL JSM-6500F FE-SEM at 5 kVolts. Samples were prepared by cutting the wing into regions isolated by color (pink, yellow, or black) and wing regions were adhered to aluminum stubs with silver paste. Stubs were coated with15 nm platinum. Images were taken from one Summer treatment female and one Winter treatment female in order to qualitatively observe scale morphology between the pink scales and the yellow scales.

Landmark Based Geometric Morphometrics and Pink Quantification

Landmark based geometric morphometrics were used in order to quantitatively measure changes in the UV patterns between the laboratory and wild males. Twelve landmarks were chosen and placed on the hindwing to encompass the entire UV pattern. Landmarks consist of Type I, placed at wing veins, Type II, placed at the maximum points of the UV pattern, and Type III, anterior and posterior points of the structure (Bookstein 1991). These landmarks were placed using the ImageJ (Rasband, 1997) package Pointpicker (Thévenaz, 2003). The landmarks were

exported from ImageJ into MorphoJ (Klingenberg, 2011). A procrustes fit, aligning by the principle axis, was performed on the data set and a covariance matrix was produced using the procrustes coordinates. A canonical variant analysis was performed from the covariance matrix and plotted in a scatterplot with 95% confidence ellipses.

Hindwing pink was quantified by transforming color photos into binary black and white images in ImageJ; pink elements appeared as black on a white hindwing background. The pink elements were measured and then standardized by total hindwing area to obtain percent area of hindwing pink. Plots and student's t-tests comparing hindwing pink were conducted in Rstudio.

Thermal Measurements

Rate of warming was quantified for 40 butterflies ranging from completely yellow (0% pink) to maximally pink (80% of the hindwing was pink). Our experimental design for measuring the rate of warming followed the methods of Watt 1968, using a thermistor with probes placed on the body of a butterfly. Butterflies from the field and laboratory experiments were preserved in glassine envelopes and allowed to desiccate for 16-24 months. Individual butterflies were placed in a black 12-inch cube chamber, made of one-inch thick black foam. The cube chamber had no top, allowing for an overhead heat lamp. The bottom of the cube chamber was lined with white poster board. Eight thermocouples were inserted through the foam, and the wires were taped to the white poster board, approximately one inch from the end of each thermocouple. To calibrate before each set of readings, the chamber was cooled and maintained at 25°C for three minutes. After three minutes, the heat lamp was turned on, and the ambient temperatures in the chamber were recorded. The setup was then calibrated by adjusting the heat lamp position and distance to achieve an increase from 25 to 37°C in 3 minutes. This calibration

was chosen to match the temperature range and rate of warming observed in *Colias* butterflies. Once the setup was calibrated, sets of six preserved butterflies were placed on the white poster board with their right ventral wing on the surface. Thermocouple probes were inserted between the closed wings, firmly touching the upper thorax. Two probes were left without butterflies to record the ambient warming rate. The 40 butterflies were randomly sampled for initial readings. Each butterfly had readings taken two additional times, each time with a different thermocouple probe. For each reading, the chamber was recalibrated and the temperature was held at 25°C for three minutes before warming was initiated. The heat lamp was then turned on and the temperature from each probe was recorded every three seconds, for three minutes. To estimate the rate of warming for each reading, a linear regression was fit to the increase in temperature over the three-minute period and a slope was determined. The warming rate for each individual is an average of the slopes from the three readings.

CRISPR/cas9 Knockouts

Single Guide RNAs (sgRNAs) were designed from a *spalt* transcript pulled from *Z. cesonia* transcriptome (Rodriguez Caro et al., *in prep*). Guides were designed by manually scanning for areas of the transcript that are comprised of 18-22 base pairs beginning with G and ending with NGG. Guide sequence was ordered from Integrated DNA Technologies as Alt-R® crRNA in Alt-R® CRISPR-cas9 system. The guide sequence was 5'-GCAAATGTTTTGTAGCAGATGG-3'. Guide complexes were formed by mixing Alt-R® crRNA with Alt-R® tracerRNA to form the sgRNA. Alt-R® S.p. Cas9 Nuclease V3 and the sgRNA were combined and injected into developing embryos. Embryo injections occurred between 3-5 hours after eggs were laid and injected with a concentration of 1ug of Cas9 to100 ng

of Guide. Borosilicate glass needles were used for injections and eggs were affixed to glass slides with double-sided sticky tape. After injections, eggs were kept in a petri dish in rearing chamber with 50% humidity, 16:8 hour photoperiod, and 27°C temperature. After three days, host plant cuttings were laid across the slide allowing larvae to immediately climb onto host plants once hatching occurred on day four. Larvae were reared on unlimited *D. purpurea* till pupation. Upon, emergence, adults were frozen, pinned, and either a leg or wing snips were removed for genotyping. DNA was extracted with a Qiagen DNeasy Kit from legs flash frozen in liquid nitrogen. Wing snips were immersed overnight at room temperature in 20ul TE and homogenized with a pestle. PCR using Q5 high-fidelity Taq polymerase was conducted using primers that flank the guide sequence. Forward primer 5'-GAACGGACGTTCTTTGGTGT-3' and reverse primer 5'-GAAGGCGATGGTGGTGAG-3'. PCRs were cloned using pGEM®-T Easy Vector System and sequenced with capillary Sanger sequencing using BigDye Terminators.

4.3 Results

Z. cesonia is seasonally polyphenetic in both pigment and structural colors

Seasonal collections revealed that pink *rosa* morphs followed seasonal trends, these pink *rosa* morphs were also found to lack hindwing UV patterns that are found in the yellow morph. Plasticity in UV patterning has not been described in *Z. cesonia* before, and was also tracked alongside pink coloration to examine if lack of hindwing UV patterning also followed seasonal trends. Collections of *Z. cesonia* from February through October 2017 tracked the presence and absence of the color morphs. From February through March, only the *rosa* morph was present, in April both morphs were observed and by the end of May *rosa* morphs were completely absent. *Rosa* morphs do not appear in the collection again until September, and by October, the yellow summer morph is completely absent. The amount of pink on an individual butterfly was found to

increase with average colder temperatures. The average amount of pink coloration on the hindwing of an individual was higher during the colder months of February and October (average temperatures of 12°C and 10°C) than in warmer months of April and September (average temperatures of 20°C and 24°C) (Figure 4.1B). It was also observed that *rosa* morphs lack hindwing UV patterns, and plasticity in UV coloration shows a similar seasonal trend as pink. Hindwing UV patterns were completely lost on wild caught *rosa* form males (figure 4.1B). Males with at least 6% of their hindwings expressing pink coloration lost hindwing UV reflectance. Males collected from the spring and fall that had less than 6% pink coloration on their hindwings retained some hindwing UV pattern. Yellow summer butterflies (May, June, July, and August) expressed no pink coloration and all had hindwing UV patterns. This suggests that both hindwing UV coloration and pink pigmentation are seasonally polyphenetic.

Both pink and UV plasticity share an environmental trigger

Both summer and winter morphs were successfully recapitulated in the laboratory; with Summer conditions produced only yellow morphs, while, Winter and Coldshock conditions yeilded *rosa* form butterflies (Figure 4.2). Although both Winter and Coldshock conditions produced *rosa* individuals, the phenotype was more dramatic in the Coldshock treatment. Similar to what was observed in the natural population, not all males in the Winter and Coldshock conditions completely lost hindwing UV patterns, but those that retained UV patterns did have a reduction in hindwing UV. Geometric morphometrics show that *rosa* form males from the laboratory and wild *rosa* males have a different pattern than summer form individuals. This suggests that laboratory raised *rosa* males are not different in their coloration from wild caught *rosa* males. Likewise, for the hindwing pink quantification laboratory *rosa* males were not

significantly different than wild *rosa* males, and both wild and laboratory *rosa* males are different from summer form males (Figure 4.3). A sexually dimorphic response was observed within the Winter and Coldshock treatments. Females in both treatments exhibited the *rosa* phenotype, but the pink patterns were only clearly observable in Coldshock males. Winter males reduced or lost hindwing UV patterns but did not strongly exhibit the full pink ventral patterns associated with the *rosa* form (Figure 4.2). This suggests females may have a lower temperature threshold for inducing the pink polyphenism than males.

Both pink and loss of hindwing UV were induced in the Winter and Coldshock conditions and both share the same environmental trigger of reduced photoperiod and temperature. Individual treatments with either decreased day length or decreased temperature alone were not enough to induce the *rosa* phenotype, suggesting both photoperiod and temperature are triggers for these polyphenisms. Although, temperature may play a stronger role, especially in UV loss, given that 25% of males lost hindwing UV despite neither males nor females expressed the pink ventral patterns (Figure 4.2).

Pink butterflies warm faster than yellow butterflies

Thermal measurements show that butterflies with ventral pink coloration warm faster than yellow butterflies. Figure 4.4 is a linear regression showing that butterflies with more pink coloration warm at a faster rate than butterflies with less or no pink coloration. Males and females did not exhibit any different patterns in rate of warming, suggesting that presence or absence of UV coloration, a male-only trait, does not have an impact of warming rate.

Scale development differs between seasonal forms

Investigations of scale level differences between yellow and *rosa* form butterflies revealed organizational changes as well as the development of unique scale types. *Rosa* form butterflies did not simply replace yellow-pigmented scales with pink-pigmented scales, but, rather, added ~~both~~ unique pink and white scales to a background of yellow scales, altering the overall organization (figure 4.5). Both pink and white scales possess pigment granules, suggesting that white and pink coloration are pterin pigments because pigment granules are unique to pterin scales (Ghiradella 1972, Ghiradella and Radigan 1976, Stavenga et al. 2004). In the laboratory treatments, both Winter and Coldshock females produced white scales, but males did not. Both laboratory and wild *rosa* males produced non-UV reflecting scales on the hindwing as well as black melanin scales with a completely different nano-structure than the melanin scales found in the summer form male *Z. cesonia* butterflies (Figure 4.5). The melanin scales found in *rosa* form butterflies have the same morphology as melanin scales in both yellow and *rosa* form female *Z. cesonia.*

A melanin-patterning gene influences both pink and UV plasticity

CRISPR/Cas9 knockouts of the gene *spalt* produced *rosa* form features in areas where *spalt* is expected to be expressed. Knockout individuals developed pink scales on the ventral discal cell area as well as pink marginal spots, which appear black in yellow summer morphs. White scales were produced across the hindwing on 2 individuals. Hindwing UV was also absent in 3 of the 7 male individuals. Scale level investigation showed that these males, even though they lacked UV patterning, produced UV and non-UV reflecting scales as well as both black scale types in the UV reflecting region, where summer form butterflies would only express UV reflecting cover scales in this region (Figure 4.6). Genotyping showed small deletions of 1 to 13

base pairs in KO individuals. CRISPR knockouts had 9% survivorship with 64% of the surviving adults expressing a *rosa* phenotype.

4.4 Discussion

Characterization of novel plastic phenotypes

Plasticity in melanin has been well characterized in Pierid butterflies. Plasticity in pterin coloration has only briefly been examined in *C. eurytheme* (Hoffmann 1974). In this study, we have examined a previously uncharacterized pink plasticity. Pink coloration on the ventral hindwing appears in the same areas as black melanin in *C. eurytheme.* Pterin pigments, rather than melanin, likely cause pink coloration, though, given that pink scales contain pigment granules, which are a unique feature of pterin scales (Ghiradella 1972, Ghiradella and Radigan 1976, Stavenga et al. 2004). The *rosa* individuals not only replaced black scales with pink scales in the discal spots on the hindwing, but also added pink and white scales all across the hindwing on a yellow background. This creates a very different organizational pattern between the yellow and pink butterflies. Yellow individuals have neatly organized rows of yellow cover scales overlaying yellow ground scales, but in the *rosa* individuals, we find pink and white scales scattered among the yellow scales with multiple cover scales overlapping each other and thus losing this neat, straight organization (Figure 4.5). Plasticity in scale organization has also been shown to occur in diet stressed individuals in *Z. cesonia* when fed a non-optimal diet individuals produced less UV reflecting scales per unit area and cover scales became more visible (Fenner et al. 2019). In diet stressed individuals, less cover scales were produced, where here we have overlapping cover scales and different types of cover scales produced. These studies show that *Z. cesonia* has a degree of plastic control, not just in the physical structures and pigments of the scales produced, but also in the overall scale organization across the wing.

This study also presents a previously un-described polyphenism in *Z. cesonia,* UV patterning changes. In *P. rapae* there is a loss in female ventral UV reflectance in response to seasonal changes (Obara et al. 2008). In *C. eurytheme* and *Z. cesonia,* UV is a male only trait that is expressed on the dorsal side of the wings, and in *C. eurytheme* UV is an honest signal of male quality and the number one determinate of male mating success (Papke et al., 2007; Rutowski, 1977; Silberglied and Taylor, 1978). This plasticity in UV patterning likely has ramifications for mate preference that should be explored. This may lead to changes in female preference between the seasonal forms. For example, this phenomenon has been shown in *Bicyclus* butterflies, where a seasonal change in number and size of UV reflective eyespots drives a switch from female-to-male lead mate choice (Robertson and Monteiro, 2005; Prudic et al., 2011). The UV patterning differences between the two forms of *Z. cesonia* are driven by scales changes in both UV and melanin scales. UV reflecting scales have tight, close spacing of their lamella ridges, while non-UV reflecting pterin scales do not, and pigment granules are clearly visible (Fenner et al. 2019). Light microscopy and SEM show that *rosa* form individuals do not produce UV reflective scales on their hindwings but produced standard yellow pterin scales (Figure 4.5). These individuals also produce different melanin scales in the hindwing black margin. In yellow individuals, the hindwing black margin borders the UV pattern, these melanin scales have a different nano-structure to the black melanin scales in *rosa* individuals that lack the hindwing UV (Figure 4.5). It is important to note that the melanin scales of the forewing that border the UV pattern in both *rosa* and yellow form individuals resemble the hindwing melanin scales of yellow morphs. In females, where no UV scales are produced, all melanin scales resemble the hindwing scales of the *rosa* morph. This suggests that there may be a relationship between melanin scales and UV scales. The difference in melanin scale morphology

may impact the amount of light absorbed and may serve as a method for enhancing the brightness of the UV signal, similar to the function of pterin pigment granules inside UV scales which absorb extraneous UV light (Fenner et al. 2019; Wijnen et al. 2007, Wilts et al. 2011). The connection between melanin scale morphology and UV patterning needs to be further explored.

Pink pterin coloration functions like melanin in Colias

Although the placement of pink on the hindwing of *Z. cesonia* is in the same location as the melanin in *C. eurytheme*, pink is not a melanin, but a pterin (Watt 1968). This pterin pigment seems to function similarly to the melanin in *C. eurytheme,* though. Thermal measurements show that pink *Z. cesonia* warm at a faster rate than yellow individuals, and that the more pink on the wing the faster the rate of warming (Figure 4.4). For Pierid butterflies to fly, body temperature must reach at least 30°C, to reach this body temperature, sulfur butterflies exhibit a basking behavior known as a lateral basking posture (Kingslover 1985). Personal observations of *Z. cesonia* in the laboratory and in the wild found that butterflies exhibit this basking posture, orienting wings closed and perpendicularly to solar radiation or light source. This position allows solar radiation to be absorbed by the basal part of the ventral hindwing and conducted to the body (Watt 1968). In *C. eurytheme,* individuals with more melanin on their ventral hindwings absorb more solar radiation and warm faster than individuals with less melanin (Watt 1968, Watt 1969). In *Z. cesonia,* the increase in pink coloration serves the same role. Here we are showing that *Z. cesonia* increases pterin coloration to achieve the same functions as melanin increases in *C. eurytheme.* Interestingly, this is directly opposite to what is observed in *C. eurytheme,* where individuals that increase in melanin decrease their pterin pigmentation (Hoffmann 1974).

This use of a pigment other than melanin for thermoregulation is unique to *Z. cesonia* as compared to other Pierids, but the use of other pigments, such as ommochromes, has been shown in butterflies of the Nymphalid family. In *Junonia* butterflies, ommochromes pigments change from tan to red during colder conditions, and red butterflies warm faster and reach higher temperatures than tan butterflies (Brakefield and Larsen, 1984; Daniels et al., 2014, Jarvi et al. 2019). Here we provide an example in *Z. cesonia* of pink versus yellow pterins functioning similarly to the tan and red ommochromes.

Both pigment and structural colors share same gxe controls

In this study, we not only characterize two different novel plastic colors, but we also present an example of how a pigment and a structural color are controlled by the same gxe mechanisms. Here we show that the trigger for the two polyphenisms is temperature and photoperiod changes together. In *C. eurytheme,* the trigger is day length, and temperature changes cannot induce the seasonal form (AE 1957; Hoffmann 1972). It has been hypothesized that only using photoperiod as a trigger, which is a stable and true representation of the season, prevents *C. eurytheme* individuals from inducing the melanized form under minor weather fluctuations. Concerns have recently been raised though about climate change causing a mismatch between seasonal forms and actual daily temperatures. Individuals with increased melanin suffer from overheating in hotter conditions, which has a negative impact on survival and egg production (Ellers and Boggs, 2002; Ellers and Boggs, 2004). With temperature shifts and more extreme temperature swings, it has been suggested that melanization in *Colias* may face selective pressure under a changing temperature environment (Kingslover et al 2016). *Z. cesonia,* on the other hand, is influenced by decreased photoperiod and temperature together.

This need for both cues may help buffer *Z. cesonia* from the effects of color morph to environment mismatch that can be expected in the photoperiod-only driven seasonal form of *Colias.* This need for a stable trigger is likely even more important to *Z. cesonia* because of the shared trigger by the pink and UV plasticity. With UV as a sexual ornament, it could be devastating to the fitness of an individual to express the wrong UV pattern in the wrong time of year, which could lead to the development of a more complex environmental trigger in *Z. cesonia* than in *Colias.* It was not unexpected to find the same environmental trigger for the pigment and structural color changes in *Z. cesonia,* but it was surprising to find that both UV and pink are manipulated by the same genetic influences.

The expression and localization of color pattern genes have not been well studied in Pierids. The melanin patterning eyespot gene, *spalt,* is a transcription factor that maps to both Nymphalid eyespots and to melanic wing pattern elements in Pierids (Stoehr et al 2013). Expression of *spalt* in *Colias* occurs in the dense black margins on the dorsal forewing as well as in the primitive eyespot and discal spots on the ventral hindwing. It is important to note that *spalt* was not shown to control melanin across all aspects on the wing, but was expressed in only areas that also happen to be influenced by environmental stimuli (Stoehr et al 2013). Given the location of *spalt* expression in *Colias,* as well as the shared locations of melanin and pink between *C. eurytheme* and *Z. cesonia, spalt* provided an excellent candidate to test questions about a shared genetic control between pink and UV plasticity. Knockouts (KO) of *spalt* suggest that both pink and UV plasticity share gxe control. In *spalt* KO areas where *spalt* is expected to be expressed, the *rosa* phenotype was recapitulated. Pink scales developed at the basal section of the hindwing, and black melanin discal spot scales were replaced with pink pterin scales (Figure 4.6). On the dorsal side of the wing, the melanin scales developed the *rosa* form nanostructures,

and UV scales failed to develop. Although no UV pattern could be observed in the *spalt* KO, periodically, UV scales and yellow morph black scales could be observed amongst the *rosa* form black and non-UV yellow scales. This is likely due to the mosaic nature of the CRISPR/ cas9 KO system in first generation KOs, which fails to impact every single cell in the organism.

Collectively, these results improve our understanding of the gxe influences that control seasonal polyphenisms. This study provides evidence for how both a pigment and a structural color, two fundamentally different methods for producing colors, can be controlled by the same mechanisms.

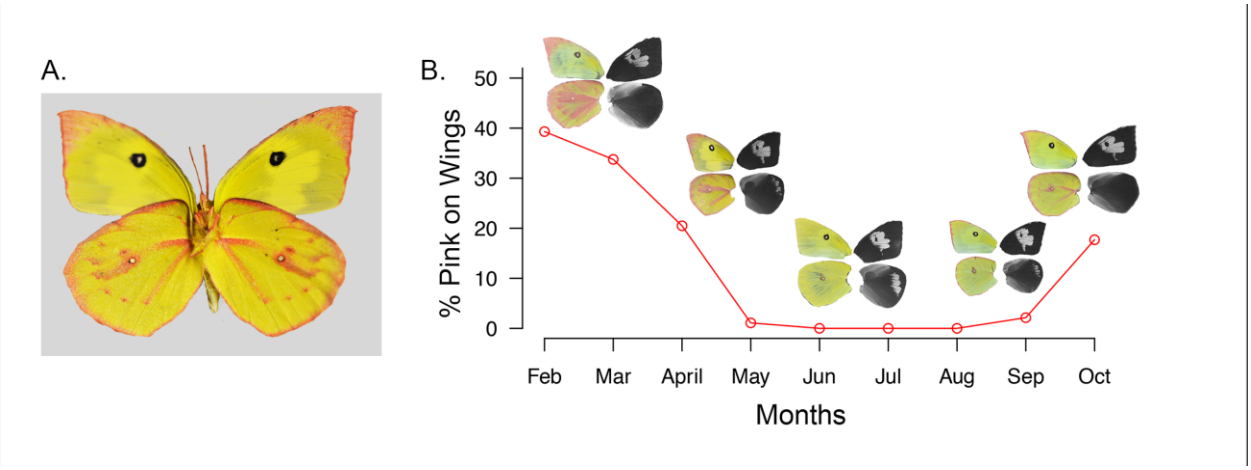


Figure 4.1 The pink and UV polyphenism in Z. cesonia. *Z. cesonia* is seasonally polyphenetic in both pink pigmentation and UV structural coloration. (A) rosa morph *Z. cesonia* specimen contains pink pigmentation on the ventral hindwing in the same location as melanin in *C. eurytheme*. (B) *Z. cesonia* exhibits pink pigmentation and no hindwing UV in the early and later months of the year, yellow pigmentation and strong hindwing UV in the summer, and intermediate amounts of pink and UV reflection in the intervening months.

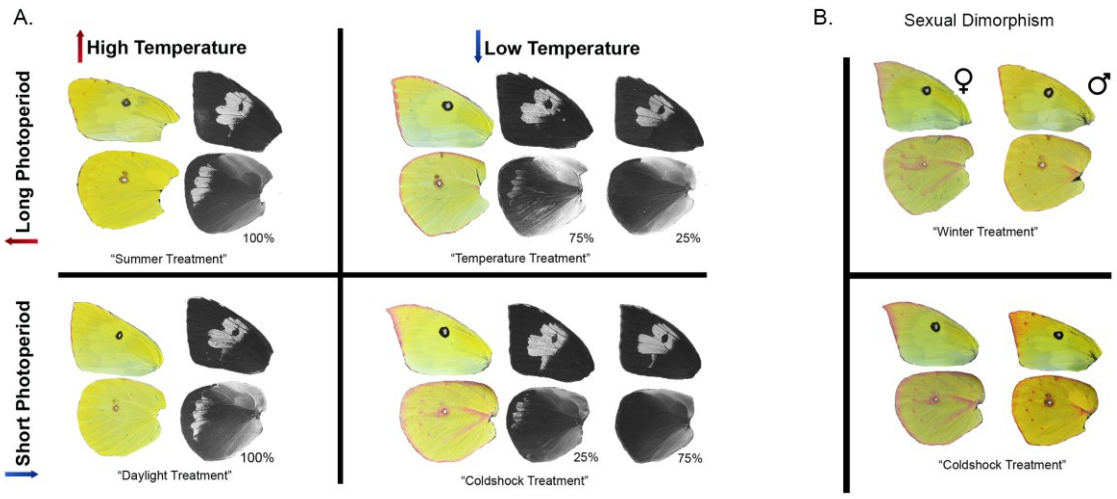


Figure 4.2 Conditional Rearing Results. Both seasonal forms were successfully recreated in the laboratory through conditional rearing. (A) Only decreases in photoperiod and temperature together were enough to induce the *rosa* form in both pink and UV. (B) *Z. cesonia* exhibited a sexually dimorphic response, with females exhibiting the pink polyphensim more strongly at a lower temperature threshold than males. Pink phenotypes were only strongly induced in males under Coldshock conditions, while under Winter conditions, pink was only expressed at the basal section of the wing and the discal spots.

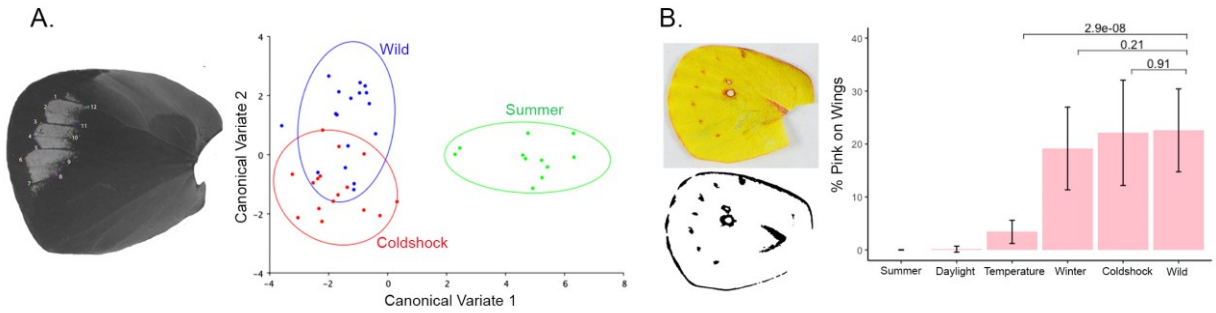


Figure 4.3 Quantification of color differences. The *rosa* form was quantifiably reproduced in the lab with both UV and pink coloration on the Coldshock reared individuals were significantly different from the summer forms, but not significantly from wild caught *rosa* individuals. (A) Shows the hindwing of a summer form male with the placement of the landmarks and a canonical PCA showing that the variation in wild and Coldshock males overlap and are both different from the summer form males. (B) Shows the binary files of the pink elements on the wing that were measured and a bar plot of average percent pink coloration on the hindwings.

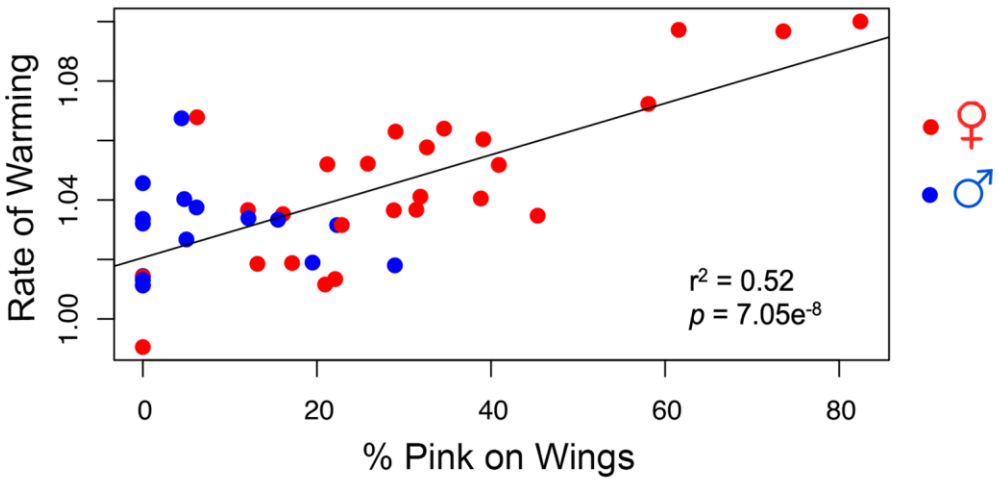


Figure 4.4 Linear Regression of pink and rate of warming. A linear regression showing that pink butterflies warm at a faster rate than yellow butterflies, and specifically the more pink on the wing the faster the rate of warming. Males tend to have less pink than females and warm slower than females, which can have upwards of 80% of their hindwings covered in pink pigmentation.

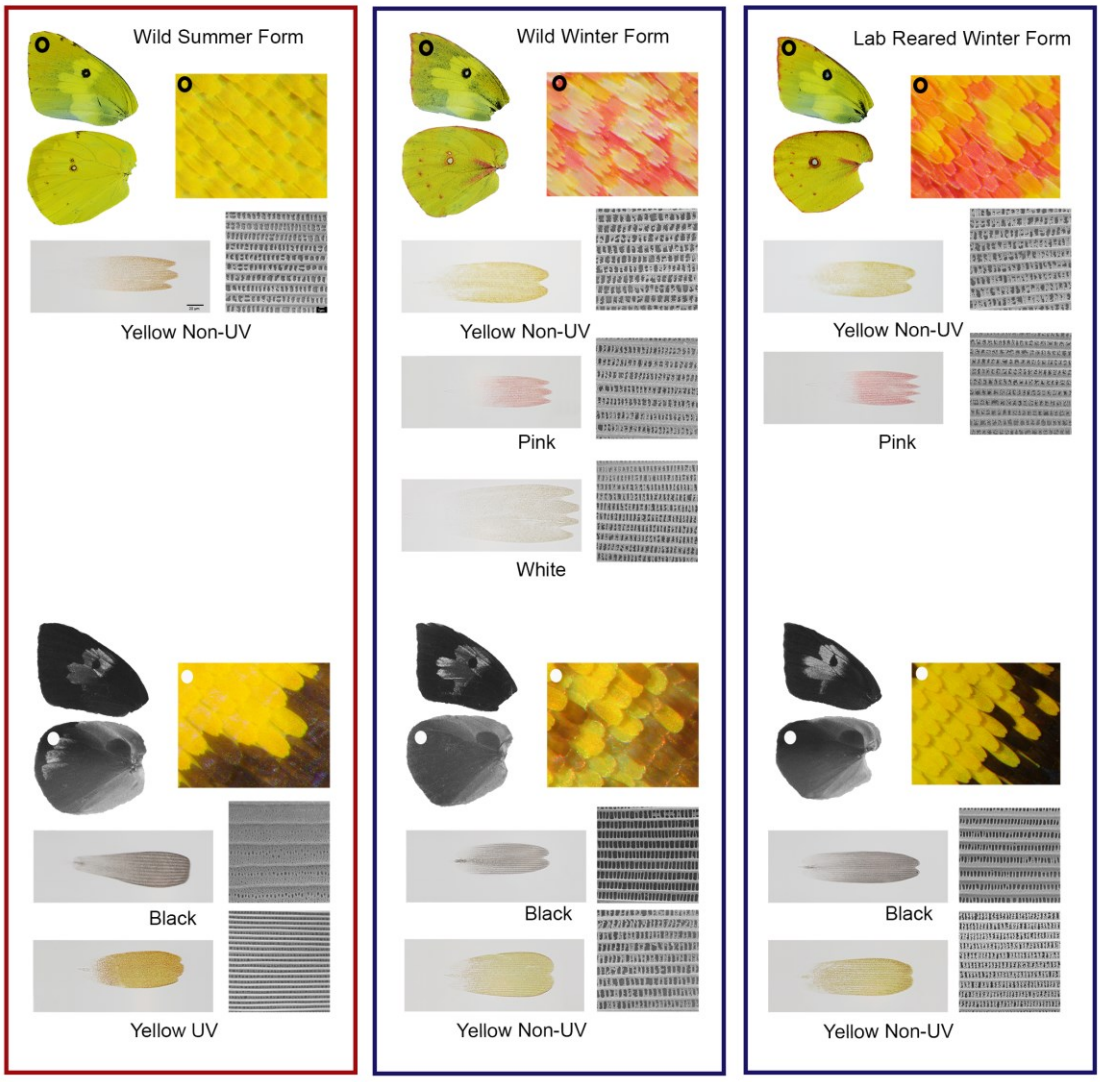
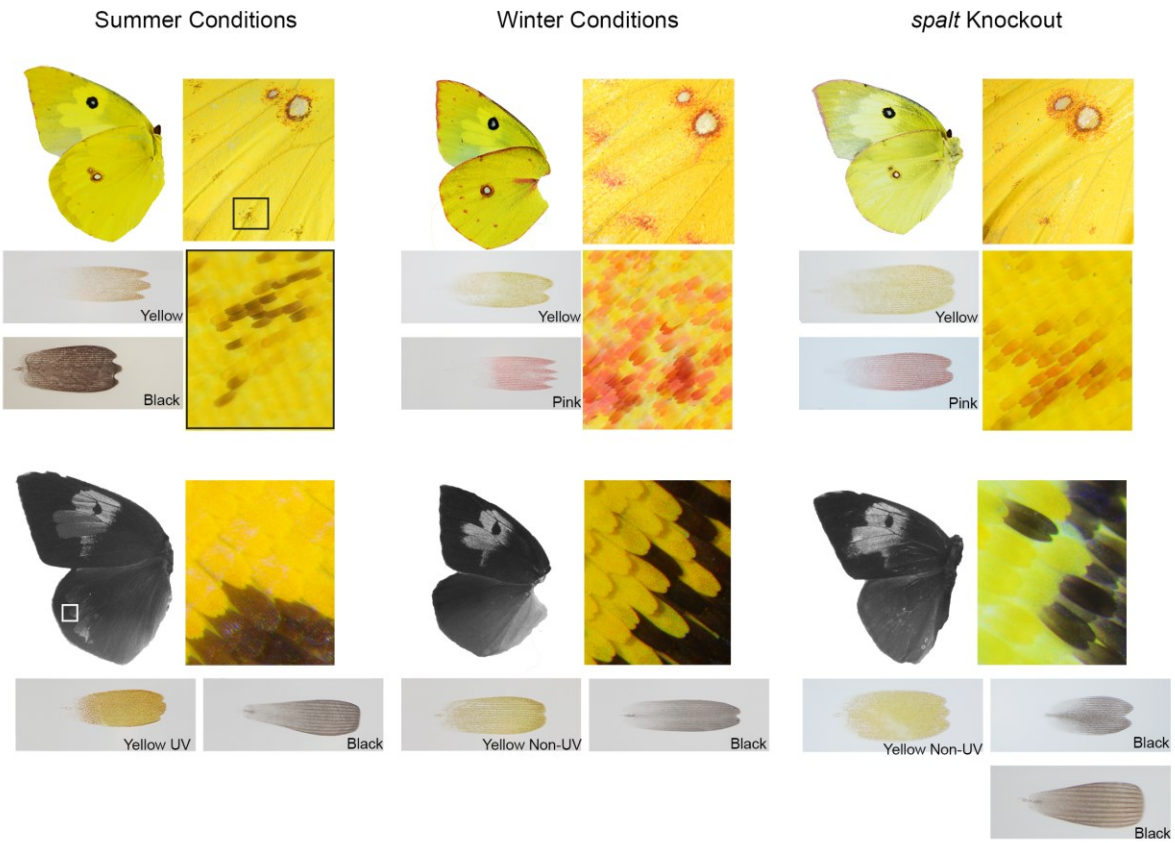


Figure 4.5 Scale differences between forms. Scales differed both on the pink ventral hindwing and in the UV reflective dorsal hindwing between the morphs. Laboratory reared *rosa* form individuals produced the same pink and yellow scales as wild caught *rosa* individuals, although, males in the laboratory did not produce the white scales that both wild caught males and females and laboratory caught females produced. Rosa form males failed to produce UV reflecting scales on the hindwing and produced black melanin scales with different nano-morphologies from the black scales in UV reflecting summer males.



Wild Type: AGGCAAATGTTTTGTAGCAGATGGTACACGTCGTATTACCGCGCA
***spalt* KO**: AGGCAAATGTTTTGTAGCAGATGG---------- ACCGCGCA Δ 13
***spalt* KO**: AGGCAAATGTTTTGTAGCAGATGGTACACGTCGTATT−CCGCGCA Δ 1
***spalt* KO**: −GGCAAATGTTTTGTAGCAGGTGGTACACGTCGTATTACCGCGCA Δ 1

Figure 4.6 spalt KO Results. *spalt* knockout individuals produced pink scales and failed to produce enough UV reflecting yellow scales on the hindwing to produce a UV pattern. *spalt* males also produced the black melanin scales that are found in the *rosa* form. The deletions to the gene were 1-13 base pair deletions.

CHAPTER V

THE AVID WOOER: HYBRIDIZATION IN THE DOGFACE BUTTERFLY

5.1 Introduction

How does a new species arise? This is a central question in evolution. The speciation process was traditionally described as the process of a single lineage splitting into two, and it was thought that hybrid speciation, the process of two distinct lineages reproducing and contributing to the formation of a separate daughter species, was a relatively rare phenomenon. Despite this, examples of hybrid speciation can be found across the plant and animal kingdoms, from *Helianthus* sunflowers, to Darwin's finches, to *Xiphophorus* swordtail fish (Rieseberg, 2000; Lamichhaney et al. 2018; Schumer et al. 2016). Butterflies are a well-studied systems in terms of hybrid speciation, with hypothesizes of hybrid speciation dating back to the early 1900's (Gerolud 1914). *Heliconius* butterflies have become a model for studying questions on speciation in the face of gene flow. A hybrid species *Heliconius heurippa* is an excellent example of how selection on a hybrid trait, sexual selection on color patterns, can lead to the rapid isolation of a hybrid from its parental lineages (Mavárez et al., 2006). Examples of hybrid speciation can also be found in *Lycaeides* butterflies, with an isolated hybrid lineage occupying a habitat unique from the parental lineages (Nice et al., 2012). With *L. melissa* occurring in Great Basin habitats and *L. anna* occurring in wet mid-elevation meadows, the hybrid species occupies a novel alpine habitat. This hybrid lineage also exhibits intermediate wing color patterns between the two parental species and strong natal host plant preference which likely contributes to

isolation from its parental lineages (Gompert et al., 2006). Butterfly color patterns are driven by both sexual and natural selection and can provide a fantastic system to address questions about the genetic and environmental influences contributing to the origins of a hybrid species. In this study, we test the claims of a putative hybrid species in the Pierid butterfly, *Zerene.*

In a 1914 paper by Gerould titled "Species building by hybridization and Mutation" a description of a putative hybrid species in the *Zerene* butterfly is described. *Zerene* is comprised of only two species, the Southern Dogface, *Z. cesonia* and the California Dogface, *Z. eurydice.* The males of the two species differ in color patterns, host plant preferences, and range. *Z. cesonia* has a yellow forewing and has melanin and UV patterning on the hindwing, while *Z. eurydice* is orange with iridescence on the forewing and lacks both melanin and UV patterning on the hindwing (Figure 5.1). *Z. cesonia* and *Z. eurydice* both feed on host plants in the Amorphea subtribe, but *Z. cesonia* prefers prairie clover, *Dalea spp.,* while *Z. eurydice* prefers false indigos, *Amorpha californica and A. fruticosa* (Riddell, 1941; Fenner et al., 2018). *Z. cesonia* ranges from the southeastern United States through central America down to Argentina, while *Z. eurydice* is strictly limited to California and Baja California (Brock and Kaufman, 2003). The two species only overlap briefly in the late spring to early summer when *Z. cesonia* expands its range out to California. This overlap occurs in the San Bernardino Mountains and here a population of *Zerene* butterflies can be found that express a wing phenotype that is intermediate to the two species. Given the color pattern, host plants differences, and habitat differences *Zerene* provides a great system to test for hybrid speciation.

First described by W.G. Wright in 1890 intermediates possess a color pattern that resembles the hindwing of *Z. cesonia* and the forewing of *Z. eurydice.* W.G. Wright goes on to describe that his hypothesis of hybridization is supported by the behavior of *Z. eurydice* because

"*as a wooer, Eurydice is exceptionally energetic and persistent, not hesitating to ignore all rules of propriety, of species, and of genera"* (Wright, 1890). Wright continues to conclude "*this possibility of a hybrid form will be a delightful study for some future student"* (Wright, 1890). In this study we sought to test the claims of hybrid speciation in *Zerene.* We use a combination of genomic and morphological evidence to test this claim of hybridization that has been unanswered for over 100 years.

5.2 Methods

Collections

Populations from both pure species as well as intermediates were collected from California. 20 pure *Z. eurydice* were collected from Middle Lion Campground (34°33'04.95"N: 119°09'57.62"W), 178 intermediates were collected from the San Bernardino Mountains (34°10'47.63"N: 116°55'36.57"W), and 5 pure *Z. cesonia* were collected from Palm Springs (33°46'59.86"N: 116°32'46.26"W). Additionally, 25 *Z. cesonia* were collected from Osborn prairie MS (33°30'35.19"N: 88°43'39.12"W) to increase the sample size for the morphological analysis.

Landmark Based Geometric Morphometrics

Morphometics were performed on both melanin and UV patterns on the forewing with a minimum of 20 individuals from each group, *Z. cesonia, Z. eurydice,* and intermediates. Landmarks for the forewing were chosen in order to cover the entire UV pattern and the basal melanic region. For UV 18 landmarks were used and 7 were used for the melanin pattern. Landmarks are of Type I, placed at wing veins, Type II, placed at the maximum points of the UV pattern, and Type III, anterior and posterior points of the structure (Bookstein 1991). These

landmarks were manually placed using the ImageJ package Pointpicker. The landmarks were exported from ImageJ into MorphoJ. A Procrustes Fit, aligning by Principle axis, was preformed on the data set and a covariance matrix was produced using the Procrustes coordinates. A canonical variant analysis was preformed on the data in MorphoJ.

Spectroscopy

UV spectroscopy was preformed on 20 samples from each group using a HR 2000+ ES Ocean Optics spectrometer with a Halogen - Deuterium light source (DH-2000). UV reflectance was measured for each individual in triplicate from the same wing regions (cell M2). Measurements were standardized with a magnesium oxide standard and for each measurement UV probe was positioned at a stationary 45^0 angle above the specimen with a 3 mm spot size diameter. Raw spectra files were trimmed to only focus on the UV wavelength, average UV spectra for each treatment was calculated and graphed in R studio.

Sequencing and Analysis

Whole genome sequencing was conducted on five pure *Z. cesonia,* five pure *Z. eurydice,* and ten intermediate individuals. All individuals chosen were male and DNA was extracted using a Qiagen Dneasy kit and whole genomes were sequenced at 20x coverage with 150 bp paired end Illumina reads. Sequences were mapped to the *Z. cesonia* genome (Rodriguez-Caro et al., *in prep*) and genotypes were called for population genetic analysis. Population genomic estimates of relative Fst, absolute genetic divergence (dxy), nucleotide diversity (π) were calculated between populations in sliding windows following the methods and python scripts from Van Belleghem et al., 2017. Patterson's D was also calculated following Van Belleghem et al., 2017

and was conducted to test for introgression between the pure *Z. cesonia* population and the intermediates. *Colias eurytheme* was used as an out group and Patternson's D was calculated for the three populations as ((*Z.eurydice*, Intermediate),*Z. cesonia*),*Colias eurytheme*. This test examines if there is an excess of *Z. cesonia* shared alleles in either the *Z. eurydice* or the Intermediate population. If more alleles are shared between *Z. cesonia* and the intermediate population this would suggest introgression of those shared alleles, rather then the shared alleles coming from an ancestral source.

Crosses

Crosses were set up in the laboratory between a *Z. cesonia* female and a *Z. eurydice* male as well as a reciprocal cross between *Z. eurydice* female and a *Z. cesonia* male. An F1 cross was conducted on a single mating pair to achieve an F2 generation. Wings were preserved for UV and visual color imaging.

5.3 Results

Morphological evidence does not support hybridization

Of the 143 individuals collected in 2013 from the San Bernardino Mountains no males expressed hindwing UV. Approximately half of the individuals, 73, had hindwing black margins, while 70 males did not. Geometric morphometrics on both UV pattern and black melanin pattern on the forewing showed that the males from the intermediate population strongly overlap with the *Z. eurydice* pattern and do not overlap with the *Z. cesonia* (Figure 5.2). Although there is overlap, both UV and melanin patterns contain variation that is not encompassed by the variation observed in the pure *Z. eurydice.* This suggests that the individuals are not of an F1 generation.

The morphometrics does not support the hypothesis of a hybrid species, or frequent and current hybridization given the lack of overlap with *Z. cesonia*.

The spectroscopy data shows that intermediates have the same UV spectra as *Z. eurydice* (Figure 5.3). The UV spectra in *Z. eurydice* is shifted towards the 400 nm blue wavelength as compared to *Z. cesonia,* intermediates also show this shift. This shows that the intermediates have a UV color reflectance that is the same as *Z. eurydice* and is not intermediate between the two species or overlap at all with *Z. cesonia.*

F1generation differs from wild intermediates

A *Z. cesonia* female and a *Z. eurydice* male were crossed and produced 178 F1 offspring. An F2 cross of a single mating pair was achieved; no other individuals mated or produced offspring. The *Z. cesonia* male and *Z. eurydice* female cross produced 171 larvae that died at pupation. No offspring from this cross survived to adulthood. These results show that hybridization between the two species is possible though and can lead to viable and fertile offspring.

The patterns observed amongst the F1 individuals do not reflect what is observed in the wild intermediates. F1 individuals have a UV and melanic patterns that span the variation found in both *Z. cesonia* and *Z. eurydice* as well as intermediates phenotypes between the two (Figure 5.4). F1 individuals can have patterns that appear as pure of each species, while others have patterns that present as intermediate between the species. Most, 73, of the male individuals have forewing UV patterns that resemble pure *Z. cesonia,* while only 19 have pure *Z. eurydice* like patterns, and 33 have a forewing pattern that is intermediate between the two pure species. Most of the hybrids, 90 individuals, have hindwing UV patterns and only 35 do not. This data further

supports the lack of frequent and current hybridization in the wild because no wild intermediates expressed hindwing UV patterns, while in F1 individuals approximately half of the males expressed hindwing UV.

Genomic evidence suggest historical hybridization events

Genomic analysis suggests low divergence between the two pure populations. Fst, a measure of population differentiation, between pure populations show very low divergence on the autosomes with occasional peaks of divergence, with most of the divergence between species driven by the Z chromosome (Figure 5.5). The fst patterns observed between the intermediates and *Z. cesonia* is very similar to what was observed between pure species, while fst between *Z. eurydice* and the intermediates is low with only a few sites showing differences (Figure 5.5). Dxy, a measure of absolute differences or segregating sites between two populations, shows absolutely no differences on the Z chromosome between the intermediates and *Z. eurydice* (Figure 5.6). Patternson's D, a statistic that estimates allele sharing between populations, which compared the intermediates to both populations shows allele sharing occurring between both pure populations (Figure 5.7A). These alleles will provide candidates for adaptive introgression. Lastly Figure 5.7B shows that as Fst increases admixture drops, which highlights the importance of gene flow to contributing to the observed patterns of divergence between the species rather than allopatric divergence with selection on multiple loci. Collectively these results suggest that hybridization occurred historically and the two species have had low levels of gene flow. The intermediate population though is not a hybrid species because they did not show any signs of a mosaic genome and have very few differences from the pure *Z. eurydice* populations. Most likely, the wild intermediate populations are multi-generational backcrosses to *Z. eurydice.* These

patterns suggest that the San Bernardino populations have had infrequent hybridization events and continued backcrosses to pure *Z. eurydice.*

5.4 Discussion

Hybrid speciation was once thought to be very rare, but examples of hybrid species can be found across the many families of butterflies (Mavárez et al., 2006; Nice et al., 2013). Some of the criteria for identifying a hybrid species are 1.) a mosaic genome 2.) reproductive isolation from the parental population and 3.) evidence that the isolation is a result of hybridization (Schumer et al., 2014). In this study we tested the first criteria and found that *Zerene* did not meet this criteria. Suggesting that hybrid speciation is not occurring in *Zerene,* rather the evidence shows that hybridization between pure species can and most likely happened historically at low frequency.

Historical hybridization with multigenerational backcrossing

Both the genomics and morphometrics support that the wild intermediates are not F1 hybrids and that their color patterns maybe derived from multigenerational backcrossing to pure *Z. eurydice.* The variation observed in the wild intermediates does not reflect what was observed in the F1 generation. The variation in the wild intermediates strongly overlaps with *Z. eurydice* (Figure 5.2). The wing areas that differ from pure *Z. eurydice* is an increase in melanin, both in the presence of a hindwing melanin margin and increased melanin on the forewing. In turn this increase in forewing melanin leads to a slight decrease in forewing UV, i.e. an area that is UV reflective in pure *Z. eurydice* is not in the San Bernardino intermediate population because melanin scales have replaced UV scales. The F1s do not show this pattern, with most of the

individuals expressing both *Z. cesonia* hindwing and forewing UV patterns, suggesting that F1's can overlap in variation more with *Z. cesonia* than *Z. eurydice*.

The genomics also suggests that the wild intermediates are not F1 hybrids, where one would expect to see an equal sharing of alleles between the two parental species, instead there is no observable differences in the Z chromosome between pure *Z. eurydice* and the intermediates and very little autosomal differences (Figure 5.5 and 5.6). The Patternson's D statistic does show that alleles are shared between *Z. cesonia* and the intermediates though (Figure 5.7A). This suggests that small amounts of introgression have been occurring between *Z. cesonia* and the intermediate population.

Introgression of adaptive plasticity

In order for the intermediates to be expressing the increased melaninic color patterns of *Z. cesonia* we would expect to find places where the intermediates have fixed differences from *Z. eurydice* that have introgressed from *Z. cesonia.* Nowhere though do we find peaks of divergences between the species disappearing in the intermediates, or a complete sharing of *Z. cesonia* alleles and not *Z. eurydice* alleles. This suggests that the color pattern differences are not due to a single locus of major effect as observed in other butterflies such as *Heliconius* and *Papilio* (Saenko et al., 2019; Iijima et al., 2019). This leads to the question of how exactly do the San Bernardino population produce this increased melaninc form. Here we propose an alternative hypothesis of introgression of adaptive plasticity. Previous work has shown that *Z. cesonia* is a plastic species that increases pterins and changes their UV patterns for thermoregulation purposes (Fenner et al., *in prep*). *Z. eurydice* is not known to be plastic. The color patterns expressed by the San Bernardino population is an increase in melanin, which is

known in *Colias,* the sister genus to *Zerene,* as a plastic response to colder conditions. Throughout the Pierid family examples of plasticity relating to decreased temperatures and photoperiods can be found (Ae 1957; Kingslover 1985; Watt 1968; Ellers and Boggs, 2002). The intermediate population occurs in a very different habitat from that experienced by pure *Z. eurydice.* The San Bernardino Mountains reaches 10,000 ft elevation and temperatures can be much colder than what is experienced at the base of the mountain. Ecologically, increased melanin in the San Bernardino populations could serve an adaptive purpose and could allow these individuals to warm faster. Since the color patterns observed between the intermediate populations and *Z. cesonia* do not reflect a simple introgression of coloration or a recapitulation of the alternative winter morph observed in *Z. cesonia,* we propose that the loci introgressed into the intermediate population are loci controlling the ability to be plastic, that the intermediates represent hybrid phenocopies. We propose that the increased melanin phenotype of the intermediates is a plastic response to the colder environment of the San Bernardino Mountains and that this plasticity was introgressed into the population from hybridization with the plastic *Z. cesonia*. Norm of reaction experiments should be conducted in *Z. eurydice* populations to test this hypothesis. Which in the words of W. G. Wright 'should be a delightful study for some future student'.

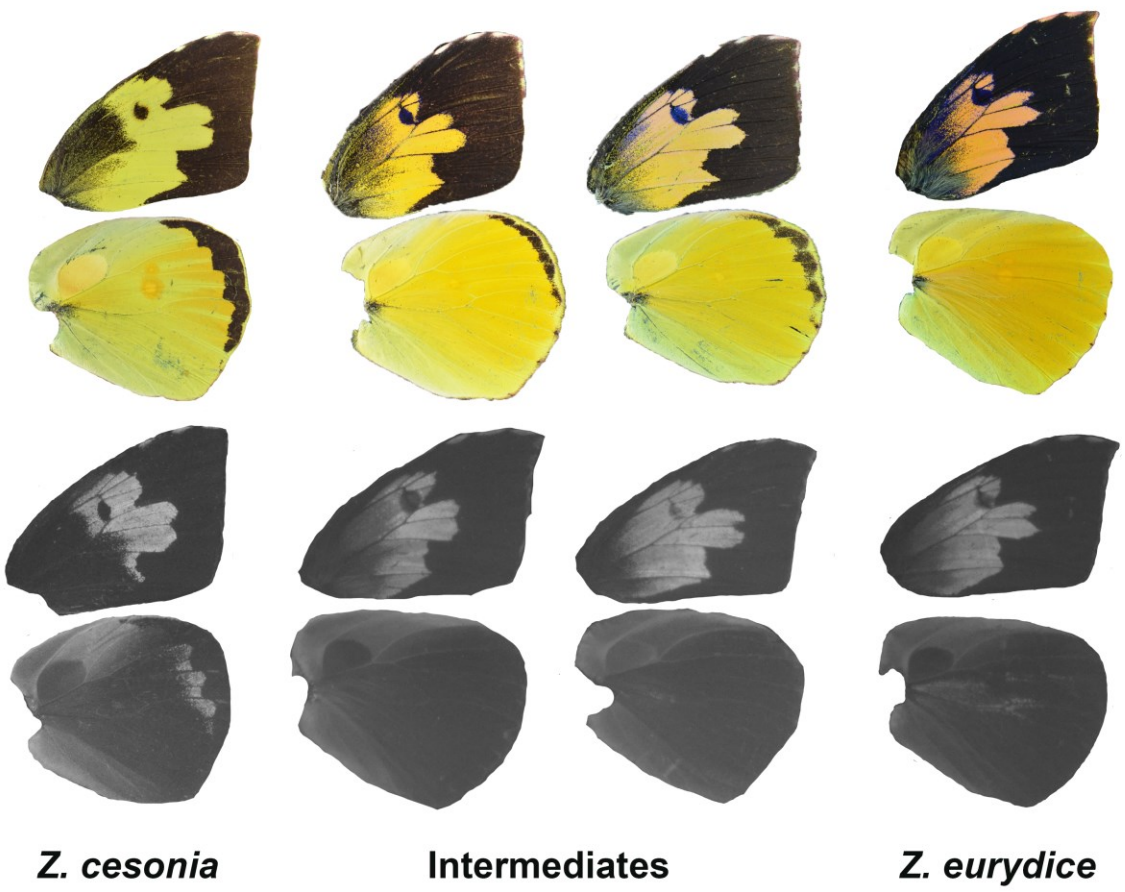


Figure 5.1 UV and Visual color in Zerene. Left side shows *Z. cesonia* and right side shows *Z. eurydice* color patterns, in the middle two examples of intermediate individuals are shown.

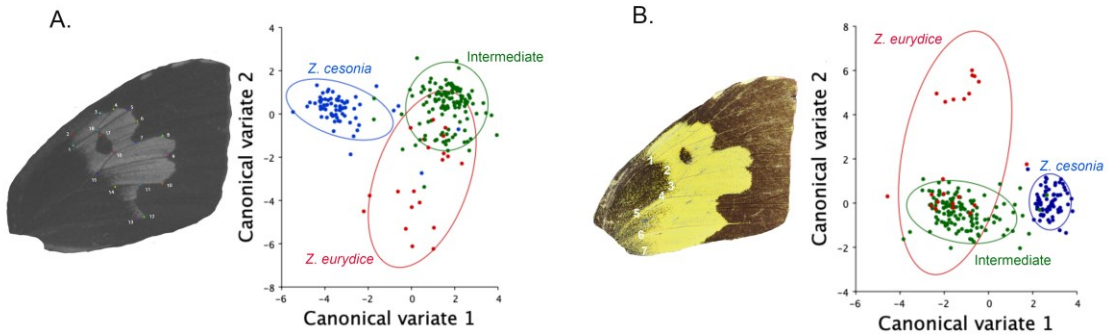


Figure 5.2 UV and Forewing Melanin Morphometrics. Morphometrics show intermediates only overlap in color patterns with *Z. eurydice*. (A) shows the UV color patterns and (B) shows the melanin pattern on the forewing. Examples of landmarks on a *Z. cesonia* is shown next to each CVA plot. CVA plots show that the variation in the intermediates overlap with *Z. eurydice,* but do not overlap with *Z. cesonia*, suggesting the forewing patterns are not intermediate between the species.

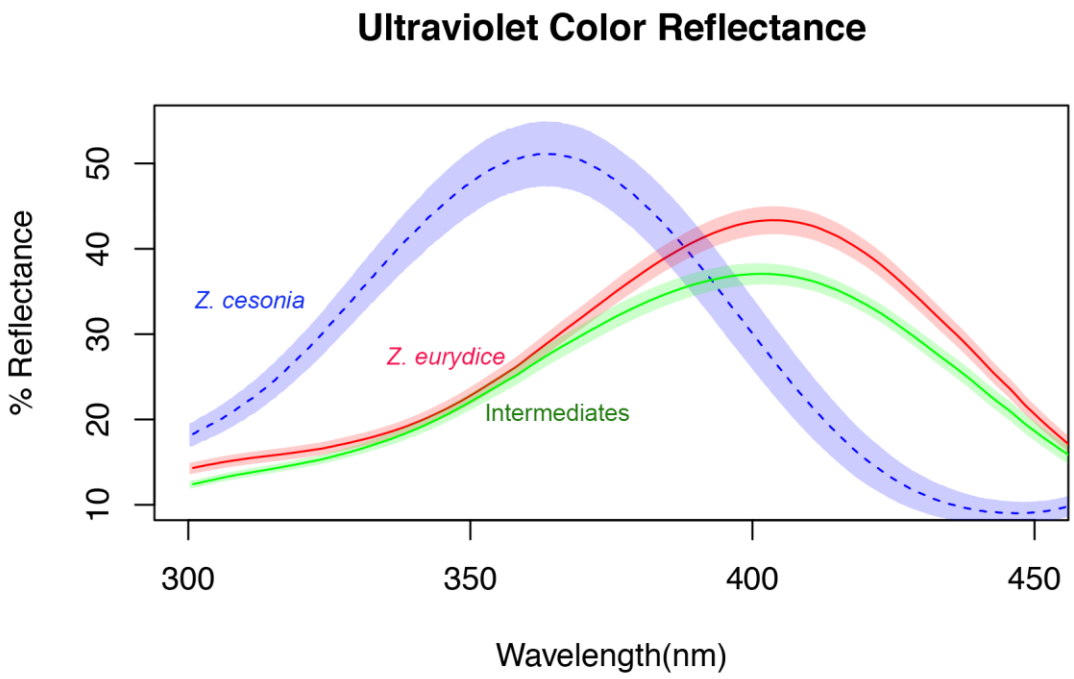


Figure 5.3 UV Spectra. The UV spectra of the intermediates is identical to that of the *Z. eurydice*.

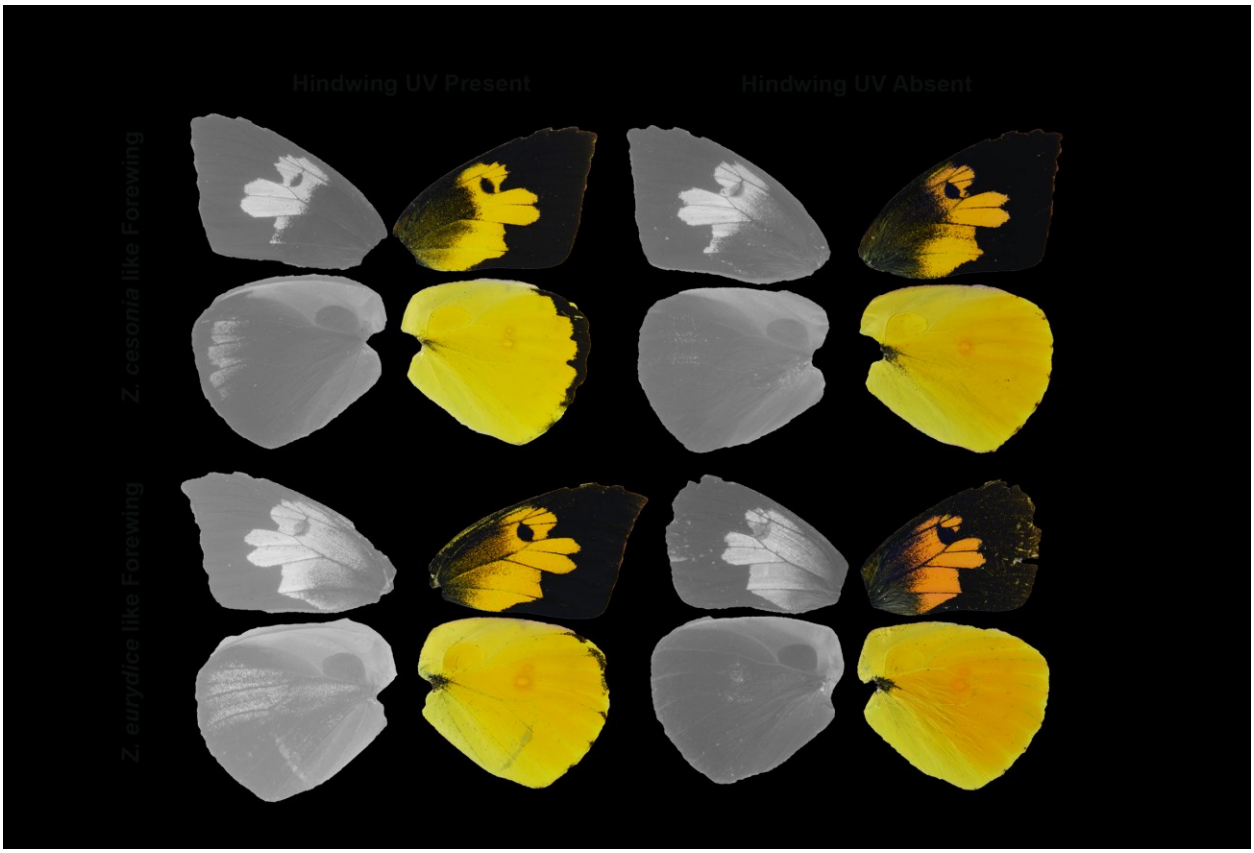


Figure 5.4 Examples of the F1 color patterns. Pure patterns as well as intermediate patterns were observed among the F1 individuals.

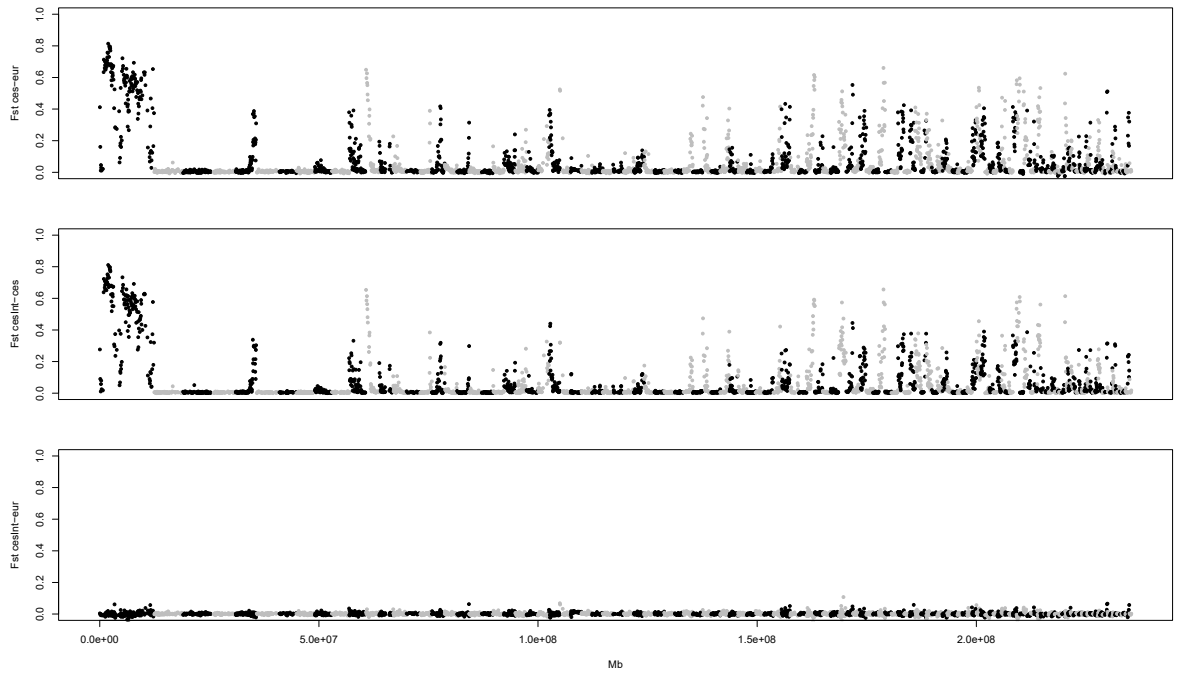


Figure 5.5 Fst between populations. Top row is *Z. eurydice* to *Z. cesonia*. Middle row is *Z. cesonia* to intermediates. Bottom row is *Z. eurydice* to intermediates. Fst is very low on the autosome between the two pure populations except on the Z chromosome. Fst is similar between the intermediates and *Z.cesonia* as between the two pure populations. Fst is extremely low between the intermediates and *Z. eurydice* with only a few loci differing.

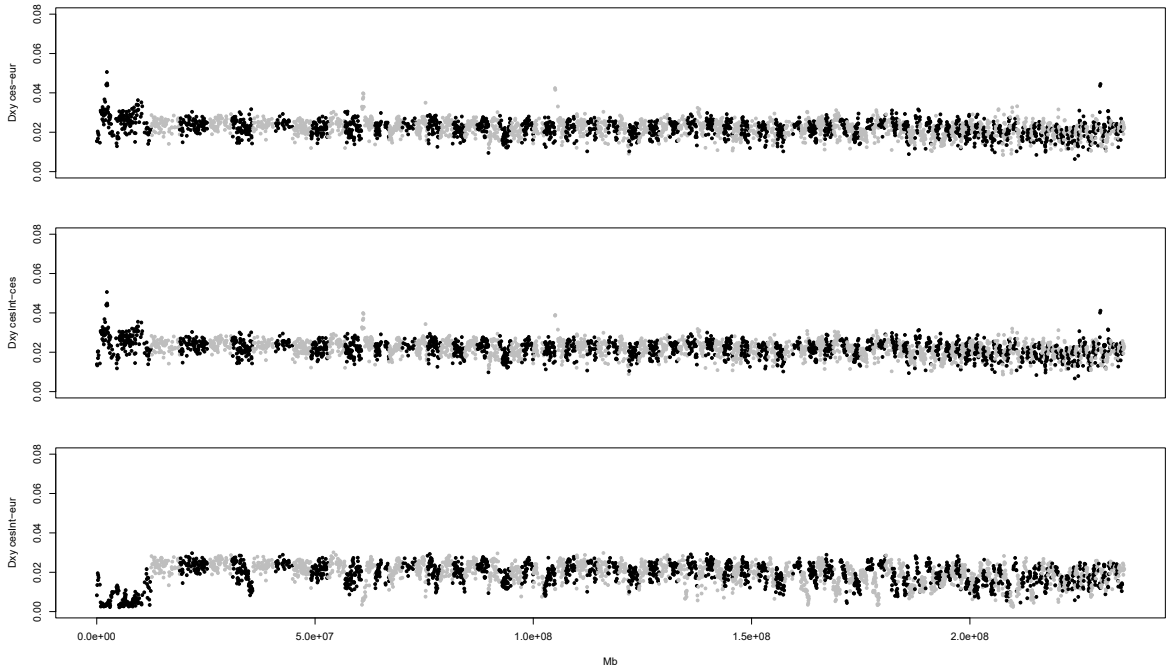


Figure 5.6 Dxy between populations. Top row is *Z. eurydice* to *Z. cesonia*. Middle row is *Z. cesonia to* intermediates. Bottom row is *Z. eurydice* to intermediates. Dxy show no differences on the Z chromosome between the intermediates and the *Z. eurydice* populations.

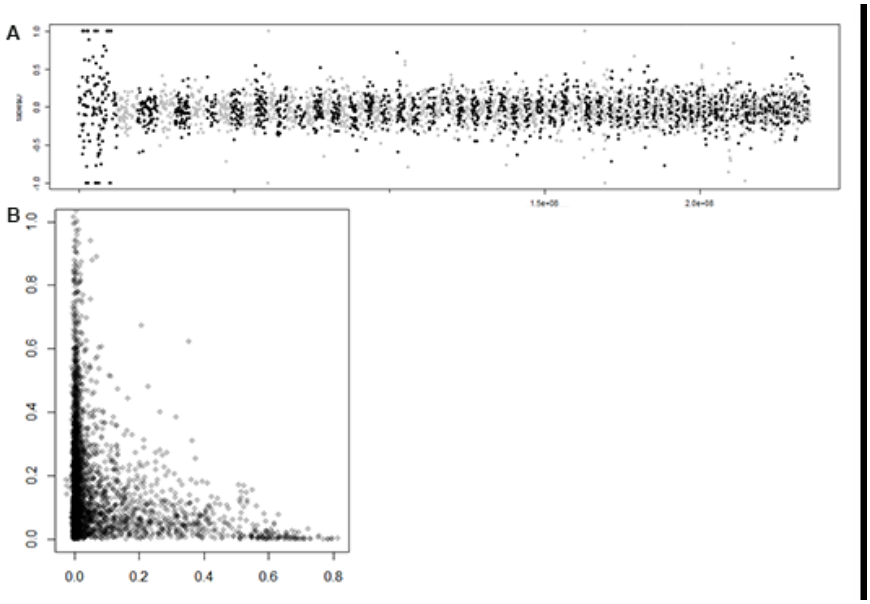


Figure 5.7 Patterson's D and Admixture plot. (A) shows Patterson's D which shows alleles sharing of the intermediates with both pure populations, but an excesses of alleles are shared with *Z. eurydice* than *Z. cesonia*. (B) is a plot showing that admixture decreases as Fst increases.

REFERENCES

Ae, Shigeru Albert “Effects of photoperiod on Colias eurytheme” 1957. *The Lepidopterist’s News* 11, no. 6. 207- 214.

Barone, John A. "Historical presence and distribution of prairies in the Black Belt of Mississippi and Alabama." *Castanea* (2005): 170-183.

Barone, John A., and Jovonn G. Hill. "Herbaceous flora of blackland prairie remnants in Mississippi and western Alabama." *Castanea* 72, no. 4 (2007): 226-235.

Beck, M.W., 2017. ggord: Ordination Plots with ggplot2. R package version 1.0.0. https://zenodo.org/badge/latestdoi/35334615.

Blair, Robert B. "Birds and butterflies along an urban gradient: surrogate taxa for assessing biodiversity?" *Ecological applications* 9, no. 1 (1999): 164-170.

Braby, M. F., and J. W. H. Trueman. "Evolution of larval host plant associations and adaptive radiation in pierid butterflies." *Journal of evolutionary biology* 19, no. 5 (2006): 1677-1690.

Brakefield, Paul M., and TORBEN B. LARSEN. "The evolutionary significance of dry and wet season forms in some tropical butterflies." *Biological journal of the Linnean society* 22, no. 1 (1984): 1-12.

Brakefield, Paul M., and Nico Reitsma. "Phenotypic plasticity, seasonal climate and the population biology of Bicyclus butterflies (Satyridae) in Malawi." *Ecological Entomology* 16, no. 3 (1991): 291-303.

Brakefield, Paul M., and W. Anthony Frankino. "Polyphenisms in Lepidoptera: multidisciplinary approaches to studies of evolution and development." *Phenotypic plasticity of insects: mechanisms and consequences* (2009): 337-368.

Brock, Jim P., and Kenn Kaufman. *Kaufman field guide to butterflies of North America*. Houghton Mifflin Harcourt, 2006.

Brown, Richard L. "Paleoenvironment and biogeography of the Mississippi Black Belt: evidence from insects." *Blackland Prairies of the Gulf Coastal Plain. Peacock E, and T. Schauwecker (eds). The University of Alabama Press, Tuscaloosa* (2003): 11-26.

Briscoe, Adriana D. "Reconstructing the ancestral butterfly eye: focus on the opsins." *Journal of Experimental Biology*211, no. 11 (2008): 1805-1813.

Cáceres, Miquel De, and Pierre Legendre. "Associations between species and groups of sites: indices and statistical inference." *Ecology* 90, no. 12 (2009): 3566-3574.

Cardoso, Domingos, Luciano P. De Queiroz, R. Toby Pennington, Haroldo C. De Lima, Émile Fonty, Martin F. Wojciechowski, and Matt Lavin. "Revisiting the phylogeny of papilionoid legumes: New insights from comprehensively sampled early-branching lineages." *American Journal of Botany* 99, no. 12 (2012): 1991-2013.

Comer, Patrick, Don Faber-Langendoen, Rob Evans, Sue Gawler, Carmen Josse, Gwen Kittel, Shannon Menard et al. "Ecological systems of the United States: a working classification of US terrestrial systems." *NatureServe, Arlington, VA* 75 (2003).

Daniels, Emily V., Rabi Murad, Ali Mortazavi, and Robert D. Reed. "Extensive transcriptional response associated with seasonal plasticity of butterfly wing patterns." *Molecular ecology* 23, no. 24 (2014): 6123-6134.

De Cáceres, M., F. Jansen, and M. M. De Caceres. "Package 'indicspecies'. R package version 1.7. 5." (2015).

DeSelm, H. R., and Nora Murdock. "Grass-dominated communities." *Biodiversity of the southeastern United States: Upland terrestrial communities. John Wiley and Sons, New York* (1993): 87-141.

Dufrêne, Marc, and Pierre Legendre. "Species assemblages and indicator species: the need for a flexible asymmetrical approach." *Ecological monographs* 67, no. 3 (1997): 345-366.

Ellers, Jacintha, and Carol L. Boggs. "The evolution of wing color in Colias butterflies: heritability, sex linkage, and population divergence." *Evolution* 56, no. 4 (2002): 836-840.

Ellers, Jacintha, and Carol L. Boggs. "Functional ecological implications of intraspecific differences in wing melanization in Colias butterflies." *Biological Journal of the Linnean Society* 82, no. 1 (2004): 79-87.

Endler, John A. "Progressive background in moths, and a quantitative measure of crypsis." *Biological Journal of the Linnean Society* 22, no. 3 (1984): 187-231.

Fenner, Jennifer, Tyler Schartel, and Brian Counterman. "Lessons from Butterflies of the Black Belt Prairie: The Southern Dogface as an Indicator of Prairie Remnants." *Transactions of the American Entomological Society* 144, no. 2 (2018): 295-309.

Fenner, Jennifer, Luis Rodriguez-Caro, and Brian Counterman. "Plasticity and divergence in ultraviolet reflecting structures on Dogface butterfly wings." *Arthropod structure & development* 51 (2019): 14-22.

Freese, Curtis, Kyran Kunkel, and Mark Sommer. "Climate change mitigation and adaptation through large-scale grassland conservation." *American Prairie Reserve*, Bozeman, MT. 2016.

Gan, Zongsong, Mark D. Turner, and Min Gu. "Biomimetic gyroid nanostructures exceeding their natural origins." *Science advances* 2, no. 5 (2016): e1600084.

Gerould J. H. 1914. Species-building by hybridization and mutation. *The American Naturalist* **48**: 321-338.

Giraldo, M. A., and D. G. Stavenga. "Sexual dichroism and pigment localization in the wing scales of Pieris rapae butterflies." *Proceedings of the Royal Society B: Biological Sciences* 274, no. 1606 (2006): 97-102.

Ghiradella, Helen, Daniel Aneshansley, Thomas Eisner, Robert E. Silberglied, and Howard E. Hinton. "Ultraviolet reflection of a male butterfly: interference color caused by thin-layer elaboration of wing scales." *Science* 178, no. 4066 (1972): 1214-1217.

Ghiradella, Helen. "Development of ultraviolet-reflecting butterfly scales: How to make an interference filter." *Journal of Morphology* 142, no. 4 (1974): 395-409.

Ghiradella, H., and W. Radigan. "Development of butterfly scales. II. Struts, lattices and surface tension." *Journal of morphology* 150, no. 2 (1976): 279-297.

Guan, Yu, Huilan Su, Chengzhi Yang, Lingling Wu, Shikun Chen, Jiajun Gu, Wang Zhang, and Di Zhang. "Ordering of Hollow Ag-Au Nanospheres with Butterfly Wings as a Bio-template." *Scientific reports* 8, no. 1 (2018): 9261.

Harvey, Eric, Isabelle Gounand, Colette L. Ward, and Florian Altermatt. "Bridging ecology and conservation: from ecological networks to ecosystem function." *Journal of Applied Ecology*54, no. 2 (2017): 371-379.

Hazel, Wade, Ruth Brandt, and Todd Grantham. "Genetic variability and phenotypic plasticity in pupal colour and its adaptive significance in the swallowtail butterfly Papilio polyxenes." *Heredity* 59, no. 3 (1987): 449.

Hill, J. G. "A note on a continuing Black Belt prairie restoration effort at Noxubee National Wildlife Refuge." *Journal of the Mississippi Acad*

Hill, JoVonn G. "The grasshopper (Orthoptera: Romaleidae: Acrididae) fauna of Black Belt Prairie remnants in Alabama and Mississippi." *Journal of Orthoptera Research* 16, no. 2 (2007): 139-145.

Hill, JoVonn G., and Richard L. Brown. "The ant (Hymenoptera: Formicidae) fauna of Black Belt prairie remnants in Alabama and Mississippi." *Southeastern Naturalist* 9, no. 1 (2010): 73-85.

Hoffmann, Richard J. "Environmental control of seasonal variation in the butterfly Colias eurytheme. I. Adaptive aspects of a photoperiodic response." *Evolution* (1973): 387-397.

Hoffmann, Richard J. "Environmental control of seasonal variation in the butterfly Colias eurytheme: effects of photoperiod and temperature on pteridine pigmentation." *Journal of insect physiology* 20, no. 10 (1974): 1913-1924.

Iijima, Takuro, Shinichi Yoda, and Haruhiko Fujiwara. "The mimetic wing pattern of Papilio polytes butterflies is regulated by a doublesex-orchestrated gene network." *Communications biology* 2, no. 1 (2019): 257.

Järvi, Vilja V., Karin RL Van Der Burg, and Robert D. Reed. "Seasonal Plasticity in Junonia coenia (Nymphalidae): Linking Wing Color, Temperature Dynamics, and Behavior." *The Journal of the Lepidopterists' Society* 73, no. 1 (2019): 34-42.

Kartesz, J. T. "North American plant atlas." *North American Plant Atlas.* (2015).

Kemp, Darrell Jon, P. Vukusic, and R. L. Rutowski. "Stress-mediated covariance between nano-structural architecture and ultraviolet butterfly coloration." *Functional Ecology* 20, no. 2 (2006): 282-289.

Kemp, Darrell J., and Ronald L. Rutowski. "Condition dependence, quantitative genetics, and the potential signal content of iridescent ultraviolet butterfly coloration." *Evolution*61, no. 1 (2007): 168-183.

Kendall, Roy O. "More larval foodplants from Texas." *J. Lepid. Soc* 13 (1959): 221Ð228.

Kerr, Jeremy T., Alissa Sugar, and Laurence Packer. "Indicator taxa, rapid biodiversity assessment, and nestedness in an endangered ecosystem." *Conservation biology* 14, no. 6 (2000): 1726-1734.

Kingsolver, Joel G. "Thermoregulatory significance of wing melanization in Pieris butterflies (Lepidoptera: Pieridae): physics, posture, and pattern." *Oecologia* 66, no. 4 (1985): 546-553.

Kingsolver, Joel G., and Diane C. Wiernasz. "Seasonal polyphenism in wing-melanin pattern and thermoregulatory adaptation in Pieris butterflies." *The American Naturalist* 137, no. 6 (1991): 816-830.

Kingsolver, Joel G., and Lauren B. Buckley. "Climate variability slows evolutionary responses of Colias butterflies to recent climate change." *Proceedings of the Royal Society B: Biological Sciences* 282, no. 1802 (2015): 20142470.

Kocher, Scott D., and Ernest H. Williams. "The diversity and abundance of North American butterflies vary with habitat disturbance and geography." *Journal of Biogeography* 27, no. 4 (2000): 785-794.

Lamichhaney, Sangeet, Fan Han, Matthew T. Webster, Leif Andersson, B. Rosemary Grant, and Peter R. Grant. "Rapid hybrid speciation in Darwin's finches." *Science* 359, no. 6372 (2018): 224-228.

Leidolf, Andreas, and Sidney McDaniel. "A floristic study of black prairie plant communities at sixteen section prairie, Oktibbeha County, Mississippi." *Castanea* (1998): 51-62.

MacGown, M. W., and T. L. Schiefer. "Disjunct distribution and a new record for an anthophorid bee, Xenoglossodes albata (Hymenoptera: Anthophoridae), in southeastern United States." *Entomological news (USA)* (1992).

Macias-Muñoz, Aide, Gilbert Smith, Antónia Monteiro, and Adriana D. Briscoe. "Transcriptome-wide differential gene expression in Bicyclus anynana butterflies: female vision-related genes are more plastic." *Molecular biology and Evolution* 33, no. 1 (2015): 79-92.

Mallet, James. "Hybrid speciation." *Nature* 446, no. 7133 (2007): 279.

Mallet, James, and Mathieu Joron. "Evolution of diversity in warning color and mimicry: polymorphisms, shifting balance, and speciation." *Annual review of ecology and systematics* 30, no. 1 (1999): 201-233.

Mavárez, Jesús, Camilo A. Salazar, Eldredge Bermingham, Christian Salcedo, Chris D. Jiggins, and Mauricio Linares. "Speciation by hybridization in Heliconius butterflies." *Nature*441, no. 7095 (2006): 868.

Mills, L. Scott, Marketa Zimova, Jared Oyler, Steven Running, John T. Abatzoglou, and Paul M. Lukacs. "Camouflage mismatch in seasonal coat color due to decreased snow duration." *Proceedings of the National Academy of Sciences*110, no. 18 (2013): 7360-7365.

Mississippi Museum of Natural Science. 2015. Mississippi State Wildlife Actions Plan 2015-2025. Mississippi Department of Wildlife Fisheries and Parks, Jackson, MS.

Mississippi Natural Heritage Program. 2002. Special plant tracking list Jackson Mississippi. Mississippi Department of Wildlife, Fisheries and Parks, Mississippi Museum of Natural Science, MS.

Morehouse, Nathan I., Peter Vukusic, and Ron Rutowski. "Pterin pigment granules are responsible for both broadband light scattering and wavelength selective absorption in the wing scales of pierid butterflies." *Proceedings of the Royal Society B: Biological Sciences* 274, no. 1608 (2006): 359-366.

Morehouse, Nathan I., and Ronald L. Rutowski. "In the eyes of the beholders: female choice and avian predation risk associated with an exaggerated male butterfly color." *The American Naturalist* 176, no. 6 (2010): 768-784.

Nice, Chris C., Zachariah Gompert, James A. Fordyce, Matthew L. Forister, Lauren K. Lucas, and C. Alex Buerkle. "Hybrid speciation and independent evolution in lineages of alpine butterflies." *Evolution: International Journal of Organic Evolution* 67, no. 4 (2013): 1055-1068.

Noss, Reed F. *Forgotten grasslands of the South: natural history and conservation*. Island Press, 2012.

Noss, Reed F., Edward Terhune LaRoe, and J. Michael Scott. *Endangered ecosystems of the United States: a preliminary assessment of loss and degradation*. Vol. 28. Washington, DC, USA: US Department of the Interior, National Biological Service, 1995.

Obara, Yoshiaki, Gaku Ozawa, Yuya Fukano, Kenta Watanabe, and Toshiyuki Satoh. "Mate preference in males of the cabbage butterfly, Pieris rapae crucivora, changes seasonally with the change in female UV color." *Zoological science* 25, no. 1 (2008): 1-6.

Papke, Randi S., Darrell J. Kemp, and Ronald L. Rutowski. "Multimodal signalling: structural ultraviolet reflectance predicts male mating success better than pheromones in the butterfly Colias eurytheme L.(Pieridae)." *Animal Behaviour* 73, no. 1 (2007): 47-54.

Parnell, Andrew J., James E. Bradford, Emma V. Curran, Adam L. Washington, Gracie Adams, Melanie N. Brien, Stephanie L. Burg et al. "Wing scale ultrastructure underlying convergent and divergent iridescent colours in mimetic Heliconius butterflies." *Journal of The Royal Society Interface*15, no. 141 (2018): 20170948.

Peacock, Evan, Timothy Schauwecker, Scott Simon, and Frank F. Schambach. *Blackland prairies of the gulf coastal plain: nature, culture, and sustainability*. The University of Alabama Press, 2003.

Pigliucci, Massimo, Courtney J. Murren, and Carl D. Schlichting. "Phenotypic plasticity and evolution by genetic assimilation." *Journal of Experimental Biology* 209, no. 12 (2006): 2362-2367.

Prudic, Kathleen L., Cheonha Jeon, Hui Cao, and Antónia Monteiro. "Developmental plasticity in sexual roles of butterfly species drives mutual sexual ornamentation." *science* 331, no. 6013 (2011): 73-75.

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

Riddell, Janet. "Some remarkable forms and aberrations in the subgenus Zerene Hübner (Lepidoptera, Pieridae)." *Transactions of the Royal Entomological Society of London*91, no. 9 (1941): 447-457.

Rieseberg, L. H. Crossing relationships among ancient and experimental sunflower hybrid lineages. *Evolution Int. J. Org. Evolution* **54**, 859–865 (2000)

Robertson, Kendra A., and Antonia Monteiro. "Female Bicyclus anynana butterflies choose males on the basis of their dorsal UV-reflective eyespot pupils." *Proceedings of the Royal Society B: Biological Sciences* 272, no. 1572 (2005): 1541-1546.

Roland, Jens. "Effect of melanism of alpine Colias nastes butterflies (Lepidoptera: Pieridae) on activity and predation." *The Canadian Entomologist* 138, no. 1 (2006): 52-58.

Rutowski, Ronald L. "The use of visual cues in sexual and species discrimination by males of the small sulphur butterflyEurema lisa (Lepidoptera, Pieridae)." *Journal of Comparative Physiology* 115, no. 1 (1977): 61-74.

Rutowski, R. L., J. M. Macedonia, N. Morehouse, and L. Taylor-Taft. "Pterin pigments amplify iridescent ultraviolet signal in males of the orange sulphur butterfly, Colias eurytheme." *Proceedings of the Royal Society B: Biological Sciences* 272, no. 1578 (2005): 2329-2335.

Rutowski, Ronald L., Joseph M. Macedonia, Darrell J. Kemp, and Laura Taylor-Taft. "Diversity in structural ultraviolet coloration among female sulphur butterflies (Coliadinae, Pieridae)." *Arthropod structure & development* 36, no. 3 (2007): 280-290.

Saenko, Suzanne V., Mathieu Chouteau, Florence Piron-Prunier, Corinne Blugeon, Mathieu Joron, and Violaine Llaurens. "Unravelling the genes forming the wing pattern supergene in the polymorphic butterfly Heliconius numata." *EvoDevo* 10, no. 1 (2019): 1-12.

Schiefer, Terence L. "Disjunct distribution of Cerambycidae (Coleoptera) in the black belt prairie and Jackson prairie in Mississippi and Alabama." *The Coleopterists' Bulletin* (1998): 278-284.

Schumer, Molly, Gil G. Rosenthal, and Peter Andolfatto. "How common is homoploid hybrid speciation?." *Evolution* 68, no. 6 (2014): 1553-1560.

Schwartz, M. W., C. A. Brigham, J. D. Hoeksema, K. G. Lyons, M. H. Mills, and P. J. Van Mantgem. "Linking biodiversity to ecosystem function: implications for conservation ecology." *Oecologia* 122, no. 3 (2000): 297-305.

Shapiro, Arthur M. "Beak-mark frequency as an index of seasonal predation intensity on common butterflies." *The American Naturalist* 108, no. 960 (1974): 229-232.

Shapiro, Arthur M. "Seasonal polyphenism." In *Evolutionary biology*, pp. 259-333. Springer, Boston, MA, 1976.

Shepherd, Stephanie, and Diane M. Debinski. "Evaluation of isolated and integrated prairie reconstructions as habitat for prairie butterflies." *Biological Conservation* 126, no. 1 (2005): 51-61.

Smith, Daryl. *The Tallgrass Prairie Center guide to prairie restoration in the Upper Midwest*. University of Iowa Press, 2010.

Smith, Beverly A., Richard L. Brown, Wallace Laberge, and Terry Griswold. "A faunistic survey of bees (Hymenoptera: Apoidea) in the Black Belt Prairie of Mississippi." *Journal of the Kansas Entomological Society* 85, no. 1 (2012): 32-48.

Silberglied, Robert E., and Orley R. Taylor. "Ultraviolet reflection and its behavioral role in the courtship of the sulfur butterflies Colias eurytheme and C. philodice (Lepidoptera, Pieridae)." *Behavioral Ecology and Sociobiology* 3, no. 3 (1978): 203-243.

Stavenga, D. G., Sally Stowe, Katharina Siebke, Jochen Zeil, and K. Arikawa. "Butterfly wing colours: scale beads make white pierid wings brighter." *Proceedings of the Royal Society of London. Series B: Biological Sciences* 271, no. 1548 (2004): 1577-1584.

Stoehr, Andrew M., Joseph F. Walker, and Antónia Monteiro. "Spalt expression and the development of melanic color patterns in pierid butterflies." *EvoDevo* 4, no. 1 (2013): 6.

Stoehr, Andrew M., and Erin M. Wojan. "Multiple cues influence multiple traits in the phenotypically plastic melanization of the cabbage white butterfly." *Oecologia* 182, no. 3 (2016): 691-701.

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

Tigreros, Natasha. "Linking nutrition and sexual selection across life stages in a model butterfly system." *Functional Ecology* 27, no. 1 (2013): 145-154.

United States National Vegetation Classification. 2016. USNVC Database. Version 2.0. Federal Geographic Data Committee, Vegetation Subcommittee, Washington, DC usnvc.org. (accessed 12.09.2017).

Van Belleghem, Steven M., Pasi Rastas, Alexie Papanicolaou, Simon H. Martin, Carlos F. Arias, Megan A. Supple, Joseph J. Hanly et al. "Complex modular architecture around a simple toolkit of wing pattern genes." *Nature ecology & evolution* 1, no. 3 (2017): 0052.

Venables, W. N., and B. D. Ripley. "Modern applied statistics (Fourth S., editor) New York." (2002).

Watt, Ward B. "Adaptive significance of pigment polymorphisms in Colias butterflies. I. Variation of melanin pigment in relation to thermoregulation." *Evolution* 22, no. 3 (1968): 437-458.

Watt, Ward B. "Adaptive significance of pigment polymorphisms in Colias butterflies, II. Thermoregulation and photoperiodically controlled melanin variation in Colias eurytheme." *Proceedings of the National Academy of Sciences* 63, no. 3 (1969): 767-774.

Watt, Ward B., Peter C. Hoch, and Susan G. Mills. "Nectar resource use by Colias butterflies." *Oecologia* 14, no. 4 (1974): 353-374.

Wickham, H., 2016. ggplot2: elegant graphics for data analysis. Springer.

Wijnen, B., H. L. Leertouwer, and D. G. Stavenga. "Colors and pterin pigmentation of pierid butterfly wings." *Journal of Insect Physiology* 53, no. 12 (2007): 1206-1217.

Wilts, Bodo D., Primož Pirih, and Doekele G. Stavenga. "Spectral reflectance properties of iridescent pierid butterfly wings." *Journal of Comparative Physiology A* 197, no. 6 (2011): 693-702.

Wright, William Greenwood. "butterflies of the west coast of the United States." (1908).