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## Resource Allocation and Visual Communication In Lepidoptera

Linda Nicole Fipps

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# RESROUCE ALLOCATION AND VISUAL COMMUNICATION IN LEPIDOPTERA

by

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Bachelor of Science  
University of South Carolina, 2020

Bachelor of Arts  
University of South Carolina, 2020

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Submitted in Partial Fulfillment of the Requirements

For the Degree of Master of Science in

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University of South Carolina

2021

Accepted by:

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## **DEDICATION**

To my parents for always supporting me on my long path to education and to loved ones lost along the way.

## **ACKNOWLEDGEMENTS**

I would first like to thank my major professor, Carol Boggs, for accepting me into her lab as an accelerated Master student. It has been a long ride, but I am beyond grateful for her constant support and encouragement. Thank you to Daniel Speiser for his collaboration on my thesis project, whose extensive knowledge on invertebrate visual ecology has been very valuable. And thank you to Jeffry Dudycha for his willingness to join my committee even though COVID-19 has prevented us from meeting in person. I would also like to acknowledge the work of John paul Currea in Jamie Theobald's lab at Florida International University. His ommatidia detecting algorithm (ODA) helped make cornea measurements much less of a headache than was anticipated.

## ABSTRACT

Visual signals in the form of wing color, size, shape, pattern, and UV reflectance play an important role in the mating and reproductive strategies of diurnal butterflies. Wing-based visual signals are linked to different functions and can be acted on by a variety of selective pressures. In a comprehensive look at the literature on the visual communication strategies of lepidopteran species, I look at how visual signals, most notably color, are used, generated, and perceived. I discuss the costs and benefits of those strategies for the fitness of butterflies and moths and look at lepidopteran visual communication through the lens of life history and resource allocation. Previous dietary restriction studies in other species demonstrated that larval food stress affects adult morphological traits essential for lepidopteran visual communication. Gonzalez (2018) discovered differences in such traits in field collected *Speyeria mormonia* (Lepidoptera: Nymphalidae) across years that differed in drought stress. Here, I asked if the effects of larval nutrient stress on wing and eye morphology could explain the variation Gonzalez found. To address that question, I performed a larval starvation experiment, where *S. mormonia* in the 5th instar of larval development were divided into a control group, fed ad libitum, and a semi-starved, larval food stress treatment group. I measured adult wing length, spectral reflectance on three points of the wing, and facet number, facet area, and eye surface area. Larval food stress significantly decreased wing length and average facet area for both males and females, and only affected the dorsal wing reflectance of females, with treatment females having less red-shifted reflectance curves than control females.

Females had longer wing lengths, while males had larger eye surface areas, more facets, bigger facets, and more red-shifted wing reflectance curves. The results reveal the presence of sex-specific resource allocation strategies and tradeoffs within *Speyeria mormonia*, with males favoring investment into their visual system and wing coloration and females investing more into their overall size. Also, results reveal the possibility of genetic heritability playing a role in the resource allocation strategies of this species.

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## LIST OF ABBREVIATIONS

AIC.....	Akaike information criterion
a.s.l. ....	above sea level
COVID-19.....	coronavirus disease 2019
GLM.....	General Linear Model
LMM.....	Linear Mixed Model
ODA.....	ommatidia detecting algorithm
SMA.....	SubMiniature version A
UV.....	ultraviolet

# **CHAPTER 1: VISUAL COMMUNICATION IN LEPIDOPTERAN SPECIES AS IT RELATES TO MATING AND REPRODUCTIVE SUCCESS**

## **1.1. Introduction**

In the phylum Arthropoda, Lepidoptera is the third largest order of insects, behind Diptera and Coleoptera. With 160,000 described species within over 120 families, this order of insects is estimated to have over 500,000 existing species (Cranston & Gullan, 2009; Regier, 2013; reviewed in Sarto i Monteys, et al, 2016). The order Lepidoptera encompasses all of what we would consider butterflies and moths and are classified as holometabolic insects with four wings covered in scales (Cranston & Gullan, 2009). Members of this group of insects are usually, but not always, characterized by the presence of a proboscis, compound eyes, mandibles for the mouth, segmented antennae, a thorax, an abdomen, and silk-producing glands (Cranston & Gullan, 2009). As a holometabolic insect, Lepidoptera go through four developmental life stages: egg, pupae, larvae, and adult (Miller & Hammond, 2003; growth in butterflies reviewed in Gotthard, 2008).

Genetics and morphological traits, such as the antennae and wings, are frequently used to distinguish butterflies from moths, which comprise roughly 88% of all described Lepidoptera (Zborowski & Edwards, 2007; reviewed in Sarto i Monteys, 2016). Another

characteristic frequently used to classify lepidopteran species is whether they are more active during the daytime or at night. Butterflies, for example, are usually diurnal insects except for the night-flying hedylids, while moths are nocturnal organisms, with exceptions like the day-flying Castiindae moths (Zborowski & Edwards, 2007). For this paper, I will be specifically focusing on traits and behaviors that emerge during the adult life stage of diurnal Lepidoptera.

The primary function of adult butterflies is dispersal and reproduction (Wheeler, 2009). Females carry and manufacture eggs, mate, and must locate a quality oviposition site (Wheeler, 2009). Males are responsible for sperm and spermatophore production and must locate mates. Lepidopterans have evolved multiple mate-seeking strategies: perching, patrolling, lekking, mutual calling, and pupal mating (reviewed in Sarto i Monteys, 2016). Perching and patrolling are the most common strategies. The others are rare in butterflies, although examples in the wild do exist (e.g. lekking in Neotropical butterflies, Srygley & Penz, 1999; explosive mating strategies, Hughes et al, 2000; reviewed in Sarto i Monteys, 2016). For perching, a territorial male sits and waits for a female to fly by, but for patrolling, males actively fly about to seek out females (reviewed in Sarto i Monteys, 2016). In *Pararge aegeria* (Nymphalidae), both behaviors are seen, where males will fight each other over ideal territories and the winners earn perching status, while the losers are forced to patrol (Bergman et al, 2007).

For diurnal species, sunlight allows the use of vision in mate-seeking and locating food or oviposition sites (Bernard & Remington, 1991; reviewed in Rutowski, 2002; reviewed in Sarto i Monteys, 2016). In nocturnal species, reliance on vision is less effective, which is why night-flying insects depend mainly on olfactory senses

(Zborowski & Edwards, 2007; reviewed in Sarto i Monteys, 2016). Research shows that, in diurnal, Castiinae moths, females have lost the ability to excrete long-range sex pheromones, a common strategy in nocturnal moths, supporting the importance of vision over olfaction in diurnal Lepidoptera (reviewed in Sarto i Monteys, 2016). On the other hand, *Theremimima ampelophaga* (Zygaenidae) and *Lymantria dispar* (Erebidae) are examples of mostly nocturnal moths whose vision still plays a vital role in mating (Charlton & Cardé, 1990; Teodora et al, 2007).

For diurnal butterflies, regardless of which mate-seeking behavior they exhibit, once a male has visually detected a female, he will pursue her at close range (reviewed in Sarto i Monteys, 2016). At close range, males will visually assess the female's size, shape, and wing pattern to determine if she is suitable for mating (reviewed in Sarto i Monteys, 2016). It is at close range that females then assess the male, which has been the subject of many lepidopteran studies on female choice and male reproductive success (Wiernasz, 1989; Robertson & Monteiro, 2005; Morehouse & Rutowski, 2010). Males may also release close-range sex pheromones to seduce and court the female, as is seen in *Bicyclus anynana* (Nymphalidae) (Costanzo & Monteiro, 2007), but evidence of this has not been thoroughly studied in most diurnal species (reviewed in Sarto i Monteys, 2016).

This review will focus on the visual communication channel of diurnal Lepidoptera, specifically as it relates to their potential for mating and reproductive success. This paper explores the diverse world of visual signals present in butterflies and moths by looking at how those signals are generated and their associated costs and benefits. The roles of the sender and receiver of such signals are discussed, including any underlying processes associated with each role. The topic of resource allocation and

other related hypotheses are explored through the lens of visual communication before ending with a brief discussion of gaps in the current research literature that need to be addressed to thoroughly understand this mode of lepidopteran communication.

## **1.2. Visual Signals**

### **1.2.1. Types of Visual Signals**

Kawahara et al. (2018) estimated that 15-25% of Lepidoptera can be categorized as diurnal. Restricting activities to the daytime allowed the evolution of a wide range of colorful visual signals that play a major role in sexual selection and intra-specific visual communication (Bernard & Remington, 1991; Burghardt et al, 2000; Robertson & Monteiro, 2005; Oliver et al, 2009). In diurnal Lepidoptera, while males may use their vision to detect and pursue suitable females, it is often females that make the final choice about whether to copulate (reviewed in Sarto i Monteys et al, 2016; Piszter et al, 2016). Because of that male-seducer-female-choice dynamic, sexual dimorphism is very common among butterflies and diurnal moths, where long-range visual signals increase the chances that an individual can identify a suitable mate (Burghardt et al, 2000; Piszter et al, 2016). Sexual dimorphism can manifest itself in differences between the sexes in life history traits, morphology, and behavior (Silberglied & Taylor, 1978; Ziemba & Rutowski, 2000; Robertson & Monteiro, 2005; Lau & Meyer-Rochow, 2007 & 2008; Benítez et al, 2011; Stella et al, 2018; Ehl et al, 2018). For this review paper, I focus on morphology and behavior, where variation in the insect's wing traits and their mate-seeking or courtship rituals play a major role in the visual communication strategies of lepidopteran species as it relates to the organism's potential mating and reproductive success (Silberglied & Taylor, 1978; Ziemba & Rutowski, 2000; Robertson & Monteiro,



2005; Lau & Meyer-Rochow, 2007 & 2008; Benítez et al, 2011; Stella et al, 2018; Ehl et al, 2018).

In terms of shape, size, color, pattern and UV reflectance, wing morphology is a diurnal Lepidoptera's most important visual communication signal, conveying specific messages to conspecifics (Teodora et al, 2007; Parchem et al, 2007; Benítez et al, 2011; Piszter et al, 2016; Ehl et al, 2018; Le Roy et al, 2019). Below, I examine these wing properties in greater detail.

#### **a. Wing shape and size**

Wing shape and size may be acted on by sexual or natural selection and can reflect changes in the environment (Benítez et al, 2011; reviewed in Le Roy et al, 2019). The evolution of wing shape and size has been linked to the optimization of flight ability, and sexual differences in those traits may reflect differences in sex-specific behavior or an individual's potential for reproductive success (Benítez et al, 2011; reviewed in Le Roy et al, 2019). Thus, females may not necessarily discriminate against males solely based on wing size and shape, but a successful courtship may be affected by those characteristics in terms of being able to overcome intra-sexual competition (reviewed in Le Roy et al, 2019). Benítez et al (2011) showed that *Synneuria spp.* (Geometridae) are sexually dimorphic in wing shape, likely due to sex-specific variation in the behavioral use of flight. Flight is vital for males during mate-seeking, territorial disputes, and may also be acted on by sexual selection via female choice. For females, flight is mainly used to locate host plants for feeding and ovipositing (Benítez et al, 2011)

Le Roy et al (2019) looked at the link between wing shape, flight performance and fitness of butterfly species, specifically focusing on what factors have driven wing

shape evolution. With diverse methods of courtship and mate-seeking strategies that often include intricate flight patterns, sexual selection and certain sex-specific behaviors are thought to be a major driver in the evolution of varying wing shapes and sizes in Lepidoptera (reviewed in Le Roy et al, 2019). Females usually have larger wing areas than conspecific males, likely to support a female's larger body mass (Gilchrist, 1990); but for wing shape, studies show that males, at least those in species where males are more active than females, often have larger wing aspect ratios (ratio of wing width to wing height) (reviewed in Le Roy et al, 2019). This relationship between wing aspect ratio and sex corresponds to differences in flight behavior between males and females (Le Roy et al, 2019). For example, male butterflies that patrol rely on an optimal aspect ratio to reduce costs associated with sustained flight, so a male patrolling butterfly with a larger wing width to height ratio compared to a competing male could potentially have higher flight endurance and would be able to search for suitable mates longer (Le Roy et al, 2019). This is seen in *P. aegeria* (Berwaerts et al, 2002), *Tecia solanivora* (Gelechiidae) (Hernandez et al, 2010), and many Neotropical butterflies (DeVries et al, 2010; Cespedes et al, 2015).

Teodora, Mitko, and Tóth (2007) looked at the role of vision in the mating strategies of *T. ampelophaga*, focusing on how the male of the species responded to model females with certain altered characteristics. They used cardboard replicas of the female moth and varied its wing size, shape, color, and posture. When controlling for olfactory stimuli, copulation was initiated significantly more often with models that were twice the normal size of a wild female and with models made with a circular shape as opposed to a square or rhombus shape, cementing the importance of wing size and shape

(and body size) as visual signals for intraspecific communication (Teodora et al, 2007). Preference for females of larger size may be linked to larger females often having higher fecundity and thus the possibility of increased reproductive success (Teodora et al, 2007; Le Roy et al, 2019). This has been documented in numerous lepidopterans (*e.g.*, Boggs & Freeman, 2005; Bauerfeind & Fischer, 2007).

## **b. Wing Color**

Two of the most widely studied visual signals in flying insects are color and color patterns (reviewed in Kemp & Rutowski, 2011; Piszter et al, 2016). Here I discuss how color is used as a visual signal and how it may affect an organism's potential for mating and reproductive success (reviewed in Kemp & Rutowski, 2011). Most butterfly wing color research focuses on how different colors or a simple difference in color saturation on a butterfly's wing may have an attractive or inhibitory effect on potential mates (Teodora et al, 2007; Davis et al, 2007; Davis et al, 2012; Johnson et al, 2014). Color is a well-studied mode of conspecific recognition and communication for diurnal butterflies and moths (Teodora et al, 2007; Davis et al, 2007; reviewed in Kemp & Rutowski, 2011; Davis et al, 2012; Johnson et al, 2014).

Teodora et al (2007) examined the size, shape, and color preferences of *T. ampellophaga* males; the wings of this species are a dark brown, almost black color, with the hindwings being darker than the forewings. Three different color models (black, red, and black with red spots) were tested using female replicas, with no significant difference measured between male responses to the natural black model and the spotted model; however, the red-only model showed an inhibitory effect on male copulation response, supporting the use of color for conspecific recognition and communication in this species

(Teodora et al, 2007). In a similar experiment, *Hyphantria cunea* (Erebidae) males preferred natural white female models over artificial blue models (Hidaka, 1972, cited in Teodora et al, 2007)

In the widely known monarch butterfly, *Danaus plexippus* (Nymphalidae), when a male locates a female and pursues her, he will tackle her to the ground and seemingly force her into mating; upon closer inspection of this mating behavior, females do have the opportunity to exert choice and fight back. Davis et al (2007) linked male wing color to flight performance and mating success by measuring the individual wing size and orange hue of 174 males and observing their mating frequency in a controlled environment. Male *D. plexippus* possessing wings with a deeper shade of orange had significantly increased mating success than males with lighter orange wings. Manipulation of the orange wing color of 93 males yielded no effect on observed mating frequency, suggesting that wing coloration may be linked to another aspect of male mating ability (Davis et al, 2007). Males with larger wings also experienced increased mating success, making evident that wing color and size in *Danaus plexippus* can mark the potential of an individual male's fitness and future mating success. Due to the mating strategies exhibited by *D. plexippus*, male wing color may also play an important role in intrasexual communication during male-male competition.

A related study by Davis et al (2012) showed that *D. plexippus* with darker orange wings, almost red, were able to fly longer distances than those with lighter orange, almost yellow wings (similar results found in Johnson, 2014). Darker wings are a frequently observed characteristic of the migratory generation of monarchs. These findings support a

link between wing coloration and flight performance/endurance as well as an individual's potential for mating and reproductive success (Davis et al, 2012; Johnson, 2014).

### c. **Wing Pattern**

Wing color patterns work well in intra- and intersexual communication within diurnal Lepidoptera and may even be used as warning signals to heterospecifics in territorial or competitive butterfly species (Silberglied & Taylor, 1978; Jiggins et al, 2001; Fordyce et al, 2002; Davis et al, 2012)

Two closely related species, *Lycaeides idas* (Lycaenidae) and *Lycaeides melissa* (Lycaenidae), have very similar, nearly indistinguishable wing patterns (white wings with black, melanic spots and orange aurorae on the wing margin) (Fordyce et al, 2002).

Fordyce et al (2002) used paper models of *L. melissa* females to manipulate their color pattern to see if *L. idas* males could be deceived in initiating copulation with the 'wrong' female. They rationalized that if wing pattern served as a reliable signal for mate recognition, then male *L. idas* should be attracted to the models of *L. melissa* that were altered to look like *L. idas* females. The results confirmed that male *L. idas* were attracted to the altered female *L. melissa* models, suggesting that wing pattern serves as an effective conspecific, mate recognition signal. For these two lycaenid species, the hindwing spots and orange, crescent-shaped aurorae were the two main color pattern elements best used by conspecifics to distinguish between the two Lycaenidae species (Fordyce et al, 2002).

Mimicry, usually controlled by natural and sexual selection, is when a species copies the wing pattern and colors of another to get the same adaptive advantage given to the original species (Jiggins et al, 2001; Bybee et al, 2011). Jiggins et al (2001) looked at

male mate choice in the two recently diverged Müllerian mimetic species: *Heliconius melpomene* (Nymphalidae) and *Heliconius cydno* (Nymphalidae). *Heliconius melpomene* mimics *Heliconius erato*'s black, red, and yellow wing pattern, while *H. cydno* mimics *Heliconius sapho*'s black and white color pattern. In a courtship experiment, each male spent more time courting their conspecific counterpart than the other closely related mimetic species, leading to the conclusion that mimetic wing color patterns can contribute to the process of assortative mating and mate recognition (Jiggins et al, 2001).

#### **d. Wing Melanization**

Much like overall wing pattern, melanization levels are linked to mate preference and an individual's potential for mating and reproductive success (reviewed in Jawor & Breitwisch, 2003); however, where melanization differs from wing color and pattern is that it is often used in thermoregulatory strategies that are vital for flight and hence reproduction of Lepidoptera (Ellers & Boggs 2002; Ellers & Boggs 2003). So, melanin levels can be acted on by both natural and sexual selection, but not always in the same way (Wiernasz, 1989; Ellers & Boggs, 2003).

Diane Wiernasz (1989) demonstrated in a field experiment looking at female mate choice that natural selection and sexual selection are congruent in *Pieris occidentalis* (Pieridae). By manipulating melanin elements on wild caught males using white model enamel, Wiernasz (1989) found that female mate discrimination did not result from the overall effect of wing pattern, but from the pattern of melanization alone. 'Whiting out' the areas of concentrated melanin on the marginal forewing had a significant effect in reducing the mating success of pattern manipulated males when compared to males whose melanin elements were untouched. Increased melanin yielded an individual better

able to thermoregulate in the tested environment, so Wiernasz's results showed natural and sexual selection working in the same direction, favoring higher levels of melanization.

In a field experiment looking at male mate choice, Ellers and Boggs (2003) used female models of varying melanizations and found that natural and sexual selection were actually incongruent in *Colias philodice eriphyle* (Pieridae). Female wing melanization increases with increasing elevation in this species due to its role in thermoregulation (Kingsolver, 1983; Springer & Boggs, 1986; Kingsolver, 1987). While melanization was an effective indicator when determining future female reproductive success, male *C. p. eriphyle* preferred females with lower melanization levels at all elevations (Ellers & Boggs, 2003). At higher elevations, the presence of increased wing melanization is vital for flight ability and survival via thermoregulation but it decreases a female's attractiveness to a male thus decreasing the female's potential for mating success (Kingsolver, 1987; Ellers & Boggs, 2003). This counterintuitive relationship between the effects of natural and sexual selection could be explained by the importance of yellow wing background color for mate recognition in *C. p. eriphyle*. Increased melanization levels would make the yellow coloring less prevalent on the wings, so that conspecifics are harder to identify, thus males preferring less melanized females regardless of elevation.

#### **e. UV Reflectance**

Sexual dimorphism often manifests itself as ultraviolet (UV) reflecting elements on the wings, with males usually, but not always, either being the only sex that reflects ultraviolet light or reflects more UV light compared to their conspecific females

(Silberglied & Taylor, 1978; Robertson & Monteiro, 2005; Stella et al, 2016; Stella et al, 2018). For example, the wings of male *Bicyclus anynana* (Nymphalidae) possess more UV-reflecting marginal wing eyespots than wings of females. Robertson & Monteiro (2005) showed via male eyespot manipulation experiments that female mate preference is a strong driver of male pattern via sexual selection. The size and level of UV reflectance of the central white pupil in the dorsal eyespot of male *B. anynana* was the characteristic of male wings most affected by female choice (Robertson & Monteiro, 2005). Females significantly avoided manipulated males in which the white pupil of the dorsal eyespot was either completely absent or enlarged, showing a female preference for smaller or averaged sized UV-reflecting pupils in the male dorsal wing pattern. This study asserted that visual signals, such as eyespot size and UV reflectance, are the basis of female choice in *Bicyclus anynana* (Robertson & Monteiro, 2005).

In the sympatric sulfur butterflies *Colias eurytheme* (Pieridae) and *Colias philodice* (Pieridae), UV reflectance properties are vital as a sex identifier in *C. eurytheme* and for conspecific recognition between the two species (Silberglied & Taylor, 1978). Silberglied & Taylor (1978) used female butterfly models of both species with manipulated color and UV reflectance to determine the aspect of the wings most effective at eliciting mating behavior in conspecific males. The lab and field mating experiments clearly showed that males of both species rely heavily on visual signals to locate suitable mates (Silberglied & Taylor, 1978; Fordyce, 2002); however, while males pursued and subsequently courted model females of the ‘right’ color, adding UV reflectance significantly reduced male copulation responses. Upon further investigation, male *C. eurytheme* were the UV-reflecting sex of that species with females being completely UV



absorbing. Alternatively, both males and females in *C. philodice* lacked any UV reflecting properties (Silberflied & Taylor, 1978). Thus, females of both species were able to identify conspecific mates and accept or reject their advances, based solely on UV reflectivity, with *C. eurytheme* females being receptive to males with UV reflecting wing patterns and *C. philodice* females rejecting courtship attempts made by UV reflecting males (Silberflied & Taylor, 1978).

In *Battus philenor* (Papilionidae), males possess an iridescent (UV-reflecting) blue coloration on their dorsal hindwing and in mating behavioral experiments where the dorsal hindwing pattern of males was manipulated, the removal of the characteristic UV-reflecting properties significantly reduced their mating and reproductive success (Rutowski & Rajyaguru, 2012). This study supported UV-reflecting, male *B. philenor* color patterns as a visual signal used by females for mate recognition and to assess the quality of a potential mate (Rutowski & Rajyaguru, 2012). *Hypolimnys bolina* (Nymphalidae) females also prefer conspecific males with UV reflecting dorsal wing patterning (Kemp, 2007).

### **1.2.2. What generates color and wing pattern?**

The surface of the wings is comprised of chitin-based scales that are anatomically derived from individual, epidermal wing cells and are structurally made from a lower and upper lamina (Janssen et al, 2001; Parchem et al, 2007; Wijnen et al, 2007; Thayer et al, 2020). Scales are organized into overlapping rows that make up a larger grid of scales containing a series of ridges and cross ribs, the morphology and organization of which varies greatly between species (Figure 1.1) (Freeman, 1998; Janssen et al, 2001; Parchem et al, 2007; Wijnen et al, 2007; Thayer et al, 2020). Scales, and the way they

are organized on the wing's surface, are the structure through which butterflies and moths can express color and color patterns (Janssen et al, 2001; Thayer et al, 2020). Within a scale, color is produced in two ways: through pigment or structural properties (Freeman, 1998; Janssen et al, 2001; Parchem et al, 2007; reviewed in Morehouse & Outomuro, 2019; Thayer et al, 2020).

#### **a. Pigments**

Pigments are color-producing compounds found within scales of a wing that absorb a specific wavelength range of visible light (Wijnen et al, 2007; reviewed in Morehouse & Outomuro, 2019). Most of the variation in wing color and pattern among Lepidoptera is due to differences in the type, amount, and location of embedded wing pigment (Janssen et al, 2001; Parchem et al, 2007; reviewed in Morehouse & Outomuro, 2019). The four most common pigment compounds found in Lepidoptera are melanins, ommatins/ommochromes, pterins, and papiliochromes (Zhang et al, 2017). Melanins are found in all butterfly and moth species, producing black, grey, and brown coloration on the wings (Janssen et al, 2001; reviewed in Jawor & Breitwisch, 2003; Zhang et al, 2017). Ommochromes are most common in the Nymphalidae, pterins are found mainly in the Pieridae, and papiliochromes occur in Papilionidae (Zhang et al, 2017). Those three pigment compounds are responsible for red, yellow, orange and sometimes brown and white coloring (Janssen et al, 2001; Zhang et al, 2017). *Paysandisia archon*'s (Castniidae) wings are characterized by brown forewings, orange hindwings and white spots outlined in black (Stavenga et al, 2018). Using microscopy and spectrophotometry,

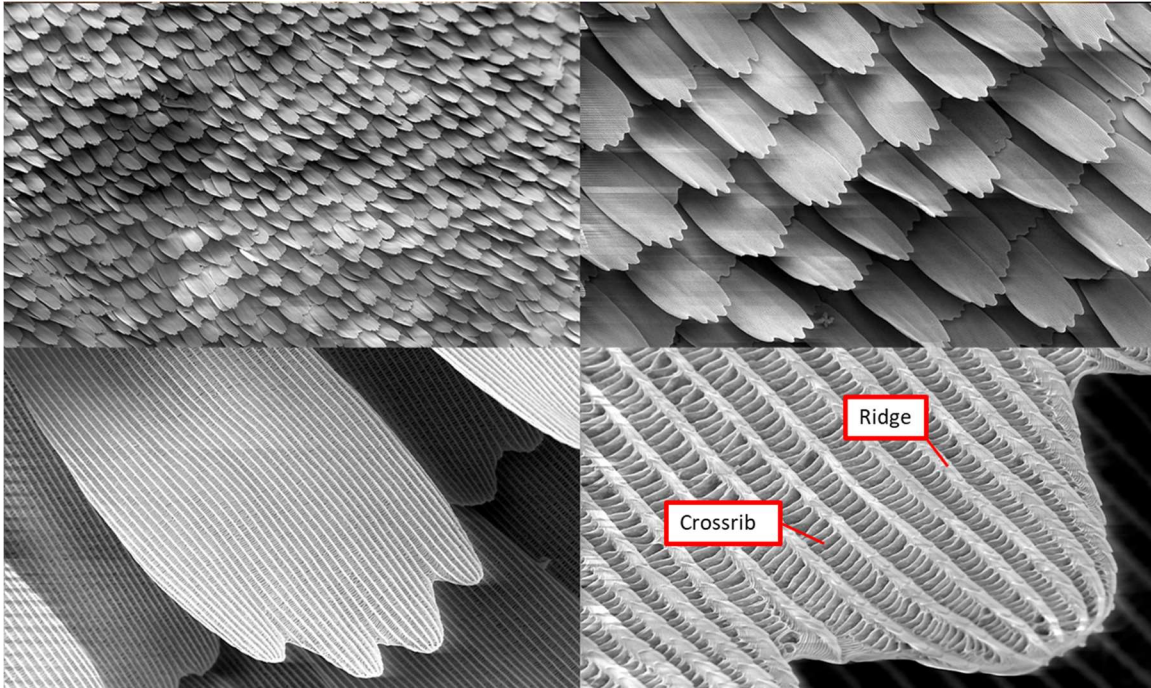


Figure 1.1: Microscopic image of a butterfly's scales. The image was modified to include microscopic images of scale nanostructures and labels were added.

SEM\_image\_of\_a\_Peacock\_wing\_slant\_view\_1.JPG: SecretDisc 11:38, 16 January 2007 (UTC); SEM\_image\_of\_a\_Peacock\_wing\_slant\_view\_2.JPG: SecretDisc 11:38, 16 January 2007 (UTC); SEM\_image\_of\_a\_Peacock\_wing\_slant\_view\_3.JPG: SecretDisc 11:38, 16 January 2007 (UTC); SEM\_image\_of\_a\_Peacock\_wing\_slant\_view\_4.JPG: SecretDisc 11:39, 16 January 2007 (UTC); Microphoto-butterflywing.jpg: ShaddackInachis\_io\_top\_detail\_MichaD.jpg: Michael ApelInachis\_io\_top\_MichaD.jpg: Michael Apel derivative work: howcheng {chat}, CC BY-SA 3.0 <<https://creativecommons.org/licenses/by-sa/3.0/>>, via Wikimedia Commons

Stavenga et al (2018) proved that the white spots on the wings were unpigmented, the black and brown scales had varying densities of melanin, and the pigment ommochrome was responsible for the orange coloration. In *D. plexippus*, variation in the red or orange coloration was due to the variation in the density of pterins and ommochromes in each scale (Janssen et al, 2001; Davis et al, 2012). These and other studies have expanded our knowledge of lepidopteran pigment and color production (pterins, Morehouse et al, 2006; pterins, Wijnen et al, 2007; ommochromes and melanin, Stavenga et al, 2014; papiliochromes and melanin, Stavenga et al, 2014).

Pigment is synthesized during the pupal stage from compounds present within the larval diet, including essential amino acids and other nitrogenous compounds that make up the basic structure of most color-producing wing pigments (melanin production reviewed in Jawor & Breitwisch, 2003; color production and mechanisms reviewed in Kemp & Rutowski, 2011; reviewed in Morehouse & Outomuro, 2019). Aside from melanin, ommochromes, pterins and papiliochromes, other compounds present in the larval diet can affect coloration. For example, pigments such as flavonoids and carotenoids can be sequestered from the larval host plant and cause changes in wing color (Wilson, 1986; Wilson, 2005; Burghardt et al, 2000; Davis et al, 2007). Flavonoids and carotenoids are compounds that produce plants' characteristic color, smell, and taste; it is these compounds that when present may act as oviposition stimulants to attract a butterfly to lay eggs on the plant (Mierziak et al, 2014). When a female butterfly does lay eggs on flavonoid or carotenoid enriched plants, the larvae can accumulate those compounds and use them as pigment during the pupal stage in coloring the wing (Wilson, 1986; Wilson, 2005; Davis et al, 2007; Mierziak et al, 2014). *Melanargia galathea* (Nymphalidae)

wings have a melanic black pattern paired with a background color of either white, cream, or yellow. Via spectrophotometry, Wilson (1986) linked background wing color to the amount of flavonoid present in the wings, with increased flavonoid content corresponding to a more yellow wing. Wilson (1985) reviewed the ability of Papilionids to sequester flavonoids from their larval diet and found that 10 out of the 27 species included in the study did sequester flavonoid compounds into their color producing mechanisms. Burghardt et al (2000) found that male *Polyommatus icarus* (Lycaenidae) were more attracted to females with flavonoid rich wing patterns; and Mizokami et al (2008) demonstrated the presence of flavonoid compounds in the larval diet and adult wings of *Pseudozizeeria maha* (Lycaenidae). They also hypothesized a potential link between flavonoid accumulation and the species' UV-absorbing wing pattern, which could support its role in intra- and interspecific visual communication. Likewise, other studies showed that butterflies with a larval diet rich in carotenoids yield adults with brighter, yellow/orange-colored wings, e.g., in *D. plexippus* (Oberhauser et al, 1996; Davis et al, 2007) and *Helicoverpa zea* (Noctuidae) (Eichenseer et al, 2002).

## **b. Structural Coloring**

Aside from pigment, color can also be produced structurally via the scattering or reflecting of light by “photonic nanoarchitecture” within a specific wavelength range (Freeman, 1998; Wijnen et al, 2007; Piszter et al, 2016; Bálint et al, 2018; reviewed in Morehouse & Outomuro, 2019). The chitin-based top layer of a scale, the cross ridges created by scale organization, and other highly refractive associated structural components usually made of keratin, chitin, or cellulose within the wing can reflect light in a way that produces color (Ghiradella, 1984; Parchem et al, 2007; Bálint et al, 2018;

Thayer et al, 2020). For example, the thickness of the upper scale lamina layer can affect the way light is reflected and thus what color(s) is produced (Parchem et al, 2007; nanostructures in the eyespots of *Caligo memnon* (Nymphalidae), Sackey et al, 2015; Thayer et al, 2020).

Structural color is used adaptively in Lepidoptera to produce colors that may not be easily produced by pigments, such as greens and blues (Parchem et al, 2007; reviewed in Morehouse & Outomuro, 2019; Thayer et al, 2020). Since structural colors are created through reflecting light rather than absorbing it, an iridescent effect is achieved that has frequently been linked to intra- and interspecific communication (Parchem et al, 2007; Tan et al, 2020), thermoregulation (Bosi et al, 2008) as well as crypsis (Tan et al, 2020) strategies in butterflies and moths (Thayer et al, 2007).

In the genus *Apatura* (Nymphalidae), sexual dimorphism is manifested on the dorsal wings of males via structural coloration (Ćurčić et al, 2012). This is similar to the previously discussed male *C. eurytheme* whose UV reflecting wings were used as a sex identifier to conspecific females (Silberglied & Taylor, 1978).

The butterflies most recognized for their use of structural coloration are those in the genus *Morpho* (Nymphalidae), which contain species whose wings are characterized by an iridescent blue, created by an interaction between overlapping scales, the reflectance properties of the upper and lower lamina, and other optical properties caused by scale and wing nanostructures (Giraldo et al, 2016).

A study exploring structural color in the genus *Junonia* (Nymphalidae) focused on what happened to color when the thickness of the lower lamina, a chitinous film within the scales of lepidopteran wings, is altered (Thayer et al, 2020). This study

supported the finding that related butterfly species that exhibit varying structural colors differ in the thickness of the lower lamina and showed that the thickness of the lower lamina determines the color being structurally produced (Thayer et al, 2020). That color may also combine with underlying pigments to express unique color patterns and was important in differentiating between sexes and species (Thayer et al, 2020). Artificial manipulation of the thin lamina film produced almost all spectral colors, even achromatic colors, but was not able to express the colors brown, black and pure red (Thayer et al, 2020). Since those three colors, brown, black, and red, are the dominant pigments in *Junonia* butterflies, pigmentation and structural coloration don't overlap in this genus and may have coevolved (Thayer et al, 2020). Thin lamina films have been observed throughout Lepidoptera and can help explain the wide varieties of wing color and pattern observed in butterflies and moths (*Papilio xuthus* (Papilionidae), Stavenga et al, 2015; *Mimeresia neavei* (Lycaenidae), Bálint et al, 2018; *Sasakia charonda* (Nymphalidae), Stavenga et al, 2020; multiple butterflies including *Anteros formosus* (Riodinidae), *Argyrophorus argenteus* (Nymphalidae), and *Hypochrysops apelles* (Lycaenidae), Ren et al, 2020; Thayer et al, 2020).

### **1.2.3. Overlapping Effects of Pigment and Structural Coloration**

While pigment and structural color may be created in different ways, the two color-producing methods often co-occur in butterfly species and can jointly produce a different color or hue (Freeman, 1998; reviewed in Morehouse & Outomuro, 2019; Thayer et al, 2020). Pierid butterflies are unique in that color pigments are clustered in the cross ribs of butterfly wing scales in structures called granules or beads. Granules are able to both absorb light in the wavelength range of the contained pigment, but also

reflect other wavelengths of light. Thus, pierid butterflies have overlapping origins of color in both structural coloration and pigment (Wijnen et al, 2007). In pierids, the iridescent effect of structural coloration usually manifests on the tips or dorsal wing area of males, while the rest of the wing and the entirety of female wing color is determined mainly through pigmentation (Wijnen et al, 2007). The black regions contain melanin, while other colors characteristic of pierid wings are mostly determined by various pterin pigments (Wijnen et al, 2007). A study looking at the pigmentation of pierid butterfly species found that leucopterin and isoxanthopterin produced white, xanthopterin and dihydroxanthopterin produced yellow and erythropterin produced orange and red (Wijnen et al, 2007).

Similarly, Stavenga et al (2015) found that the orange cream colors produced on the wings of *P. xuthus* were in part due to papiliochrome pigments embedded within the scales, but also due to the combined effect of light scattering nanostructures. Similar conclusions have also been drawn for Nymphalid butterflies such as *Vanessa cardui* (Shelby, 2019) and *Hypolimnys bolina* (Kemp & Macedonia, 2006). The relationship between evolutionary and developmental biology in terms of wing color and pattern production in Lepidoptera is reviewed by Beldade and Brakefield (2002) and again by Beldade and Saenko (2009).

### **1.3. Sender of the Signal**

#### **1.3.1. Who?**

Both males and females can play the role of the sender by using their wings for intraspecific visual communication at different times in the courtship process. In general, the sex being sought out first takes on the task of sending the signal (reviewed in Sarto i



Monteys et al, 2016). So, in a species where the male searches for the female (via perching or patrolling), the female takes on the initial role of the sender (reviewed in Sarto i Monteys et al, 2016). Once a male decides to pursue a female at close range, he now becomes the sender of the cue (reviewed in Sarto i Monteys et al, 2016). On the other hand, if the female is the one that takes on the task of mate seeking, then these roles can be reversed; however, there is not much evidence to support mate seeking behavior in female Lepidoptera.

### **1.3.2. How?**

Earlier, I described the two main methods of mate locating: patrolling and perching (reviewed in Sarto i Monteys et al, 2016). With patrolling, a male actively flies around in search of a female (e.g., *Speyeria mormonia*, Boggs & Freeman, 2005), while perchers sit and wait to spot a female flying by (e.g., *Asterocampa leilia* (Nymphalidae), Rutowski, 2001; reviewed in Sarto i Monteys et al, 2016). In diurnal Lepidoptera, the presence of a visual signal elicits courtship behavior (Fordyce et al, 2002). Regardless of mating strategy, the female's wings act as a long-range visual signal that the male uses in conspecific recognition and, in some cases, to assess the quality of the female (reviewed in Sarto i Monteys et al, 2016). Using that visual signal, location of a potential female mate is followed by male courtship behavior, where a male will pursue the female at close-range (reviewed in Sarto i Monteys et al, 2016). In the perching species *Hipparchia fagi* (Nymphalidae) and *Hipparchia hermione genava* (Nymphalidae), the visual signals of wing size and pattern played a significant role in conspecific recognition and the initiation of male copulation flights at long-range (Pinzari & Sbordon, 2011; e.g., *L. idas* and *L. melissa*, Fordyce et al, 2002; *T. ampellophaga*, Teodora et al, 2007). At

close-range, the male's wings are the focus of the female for the same reasons: mate recognition and to assess the quality of the courting male (reviewed in Sarto i Monteys et al, 2016; e.g., *D. plexippus*, Davis et al, 2012; UV reflecting eyespots in *B. anynana*, Robertson & Monteiro, 2005). At this point, a male may use his wings and other tactics to seduce the female into mating (reviewed in Sarto i Monteys et al, 2016). Male courtship displays can vary greatly among Lepidoptera and can be very elaborate. Male butterflies can employ choreographed flight patterns to perform a sort of 'courtship dance' to get the female's attention and seduce her into mating with him. In *B. anynana*, males pursue conspecific females at an angle and fly around her while quickly opening and closing their wings (Robertson & Monteiro, 2005).

## **1.4. Receiver of Signals**

### **1.4.1. Who?**

Both males and females must be able to visually receive the visual signals discussed in this review and are thus reliant on the components of their visual system to appropriately process those incoming signals (reviewed in Rutowski, 2002). In diurnal Lepidoptera, males usually take on the role of finding a mate, relying on long range visual signals for mate recognition and selection, while females operate primarily on short range visual signals for the same purpose (Rutowski, 2000; reviewed in Sarto i Monteys et al, 2016; Balamural et al, 2019). Studies focusing on the differential use of vision between the sexes reveal the presence of sexually dimorphic visual traits in butterflies (Ziemba & Rutowski, 2000; Rutowski, 2000; Balamurali et al, 2019). Rutowski (2000) found supporting evidence of that when examining eye size in 16 different butterfly species. Males had consistently larger eyes than females and the

regions of the eye specializing in mate location and identification were characterized by special features that increased the visual acuity and sensitivity of that eye region. This supported their claim that vision may be more important for the male's role in mating than a female's (Rutowski, 2000; Land & Nilsson, 2012).

#### **1.4.2. Lepidopteran Vision**

All Lepidoptera possess compound eyes. Nocturnal species have superposition compound eyes, meaning that a single light-sensitive rhabdom gets light filtered through multiple facet lenses, ideal for the low light levels at night (Stavenga & Arikawa, 2006). Diurnal species possess apposition compound eyes, meaning that each facet of the eye sends light to its own rhabdom (reviewed in Rutowski, 2002; Land & Nilsson, 2012). From the cross-section image of a butterfly eye seen in Figure 1.2, curved facets or corneal lenses cover the surface of the eye and each facet covers a single ommatidia, which is a structural subunit of a compound eye responsible for visually sampling a small piece of the insect's visual field (Ziemba & Rutowski, 2000; Rutowski, 2002; Stavenga & Arikawa, 2006; Land & Nilsson, 2012). Those ommatidia contain a crystalline cone structure positioned just beneath the facet that concentrates incoming light into the light sensitive rhabdom (Figure 1.2) (Rutowski, 2002; Land & Nilsson, 2012). The rhabdom is surrounded by photoreceptor cells containing visual pigments that specialize in different parts of the light spectrum (Rutowski, 2002; Stavenga & Arikawa, 2006). Lepidoptera usually have nine photoreceptors per ommatidia with three to four different types depending on their capacity for color discrimination, which can vary greatly among

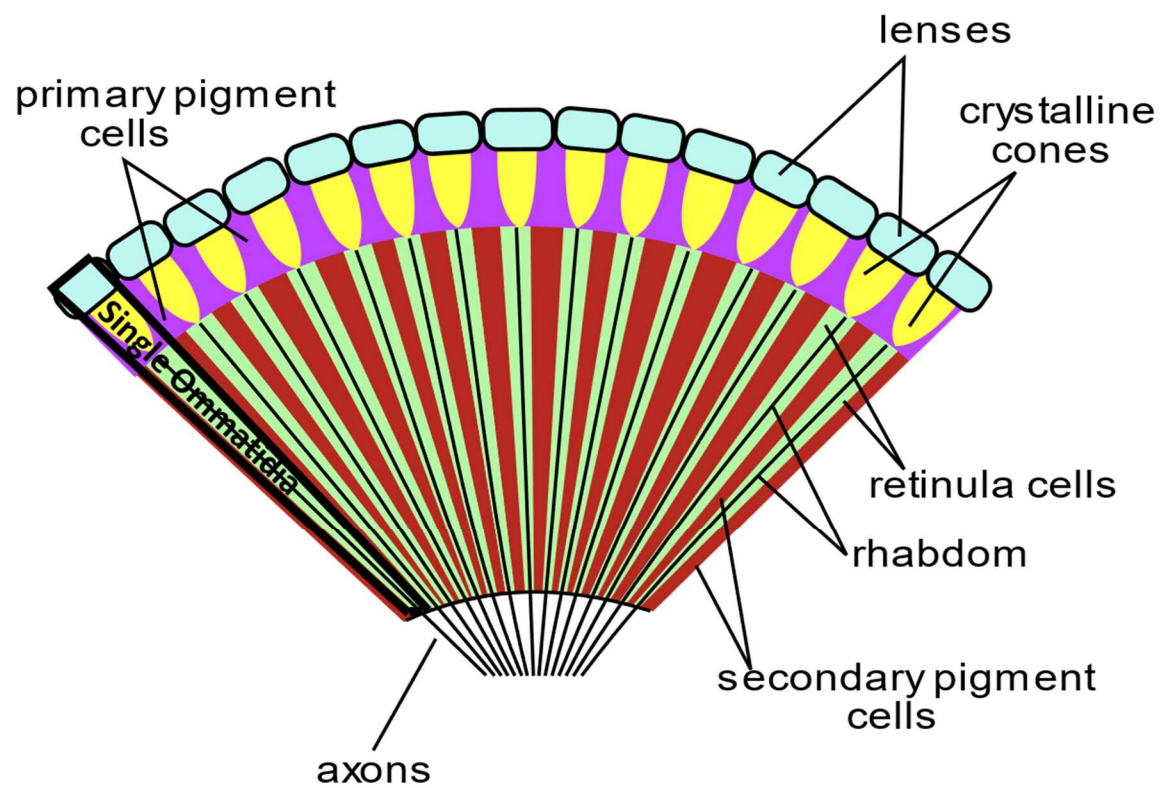


Figure 1.2: A cross-section image of a compound eye. Adding the label for a single ommatidia, this image was adapted from, Bugboy52.40, CC BY-SA 3.0 <<https://creativecommons.org/licenses/by-sa/3.0/>>, via Wikimedia Commons

species based on their habitat and behavioral need for color vision (Stavenga & Arikawa, 2006). Together the rhabdom and surrounding receptors are responsible for translating the incoming image to the underlying neural axons that then send the visual information to the insect's brain for processing (Rutowski, 2002; Land & Nilsson, 2012). Some lepidopteran species may also possess a tapetum next to the rhabdom, which reflects any unabsorbed light out of the eye as eye shine (Rutowski, 2002; Stavenga & Arikawa, 2006). In one compound eye there can be thousands upon thousands of ommatidia, making the study of an appositional compound visual system very complex, with much of the current literature focusing on overall eye and facet size to draw general conclusions about a species' visual ecology (Ziemba & Rutowski, 2000).

#### **1.4.3. Eye morphology and Visual Acuity**

Larger eyes have frequently been linked to increased mate detection and recognition, but bigger eyes are characterized by either more facets, larger facets, or a combination of both (Rutowski, 2000; reviewed in Rutowski, 2002). Larger facets yield more light-sensitive and resolved vision, but more facets result in a larger visual field with not much change in visual resolution and sensitivity (Rutowski, 2000; Ziemba & Rutowski, 2000; reviewed in Rutowski, 2002). Other aspects of eye morphology such as the radius of curvature of the eye and interommatidial angles within the eye play a part in determining visual acuity as well (Rutowski, 2000; Ziemba & Rutowski, 2000).

Interommatidial angles between facets determines visual resolution; so larger facets yield smaller interommatidial angles, which increases visual resolution (Rutowski 2000; Ziemba & Rutowski, 2000; reviewed in Rutowski 2002;). Male butterflies are usually characterized by more acute vision than females and studies supporting that assertion

have shown that males possess a larger radius of curvature, or more flattened eye regions, than their female conspecifics (Rutowski, 2000; Ziemba & Rutowski, 2000; *Boloria eunomia* and *Boloria aquilonaris* (Nymphalidae), Turlure et al, 2016). A study including 80 lepidopteran species showed that larger species, and/or species with larger eyes, had overall larger eye facets and more sensitive visual systems, providing those species with better visual discrimination abilities (Yagi & Koyama 1963, cited in Rutowski, 2000).

#### **1.4.4. Zones of Acuity**

Zones of acuity are regions of an eye that are characterized by larger facets and thus smaller interommatidial angles that increases the visual resolution and sensitivity in that eye region (Rutowski, 2000; Ziemba & Rutowski, 2000). Rutowski (2000) demonstrated that patrolling males in the butterfly *Asterocampa leilia* had the same number of total facets as their conspecific females but had larger facets in the frontal regions of their eyes, which is the eye region most used for mate location and recognition while flying (also shown in Rutowski & Warrant, 2001; *B. eunomia* and *B. aquilonaris*, Turlure et al, 2016). The presence of acute zones in the eyes of Lepidoptera usually corresponds with certain aspects of that insect's behavior and lifestyle, such as the downward-ventral regions favoring foraging behavior and the forward-anterior regions being used for various mating behaviors and for recognizing heterospecific individuals in territorial species (Ziemba & Rutowski, 2000; Marshall & Arikawa, 2014). As a result, each region of the eye contains the photoreceptors and appropriate color channels needed to successfully perform each region-specific task (Marshall & Arikawa, 2014). For example, the tetrachromats *Lycaena heteronea* (Lycaenidae) and *Lycaena rubidus* (Lycaenidae) possess the same four visual pigments (P360, P437, P500, and P568) but

differ in the distribution of those pigments in different regions of their compound eyes (Bernard & Remington, 1991). The ventral region of *L. heteronea* eyes contains all four pigments, but *L. rubidus* individuals lack the blue sensitive pigment (P437) in that same eye region (Bernard & Remington, 1991). *L. heteronea* wings are blue in color and *L. rubidus* wings are reddish-orange, so this difference between the visual ecologies of the two *Lycaena* species may reflect the importance for *L. heteronea* individuals, and the lack of importance for *L. rubidus*, to be more sensitive in the blue range, enhancing conspecific and mate recognition in *L. heteronea* (Bernard & Remington, 1991). Sexually dimorphic traits in the visual system of Lepidoptera in terms of eye structure, size, and pigment distribution patterns have also been observed in *Mycalesis mineus* (Nymphalidae) (Balamurali et al, 2019), *Operophtera brumata* (Geometridae) (Meyer-Rochow & Lau, 2008), and *Orgyia antiqua* (Erebidae) (Lau & Meyer-Rochow, 2007).

#### **1.4.5. Color Vision**

Color vision is vital for the success of diurnal Lepidoptera due to its role in locating oviposition sites, navigating, finding food sources, and for conspecific recognition (Zaccardi et al, 2006; reviewed in Marshall & Arikawa, 2014; reviewed in Sarto i Monteys et al, 2016; Balamurali et al, 2019). Within the context of visual communication and mating success, butterfly color vision and wing coloration may have coevolved, being controlled and influenced by mate choice (reviewed in Stavenga & Arikawa, 2006; evolution of color vision reviewed in Lee, 2008 and Osorio & Vorobyev, 2008; Morehouse and Rutowski, 2010; Lind et al, 2017). If coevolution has occurred, that makes each lepidopteran species not just unique in their wing patterning, but also

unique in their visual ecology (reviewed in Stavenga & Arikawa, 2006; Morehouse and Rutowski, 2010; Piszter et al, 2016; Lind et al, 2017).

Depending on a species' need for color vision, Lepidoptera usually have trichromatic vision with visual pigments sensitive to the ultraviolet, blue, and green spectral range (Zaccardi et al, 2006; Zborowski & Edwards, 2007; reviewed in Marshall & Arikawa, 2014). Within the photoreceptors of a compound eye, the opsin-containing visual pigments determine the spectral sensitivity of that receptor and thus the overall visual sensitivity of the individual (Zaccardi et al, 2006). That range of spectral sensitivity can be modified with the addition of screening or filtering pigments around the rhabdom (reviewed in Stavenga & Arikawa, 2006; Zaccardi et al, 2006). The presence of filtering pigments applied to photoreceptors containing the same opsin protein can produce receptors with varying sensitivities, extending the potential range of color discrimination within those species that lack true tetrachromacy beyond their normal trichromatic range (Zaccardi et al, 2006; reviewed in Stavenga & Arikawa, 2006). Some Lepidoptera have evolved full tetrachromatic color vision, with four unique opsin-containing visual pigments that are sensitive in the UV, green, blue, and red spectral range (Land & Nilsson, 2012; reviewed in Marshall & Arikawa, 2014). Diurnal lepidopteran color vision can range from the 200nm ultraviolet to the 700nm infra-red (Freeman, 1998; Zaccardi et al, 2006; reviewed in Marshall & Arikawa, 2014; Piszter et al, 2016).

#### **1.4.6. Examples:**

In the trichromatic butterfly, *Colias erate* (Pieridae), red screening pigments on their visual receptors increase their sensitivity to red color despite the absence of a red-



sensitive photoreceptor (Marshall & Arikawa, 2014; Jocelyn Liang & Monteiro, 2017). Female *C. erate* contain more red sensitive channels than males, but further research is needed to determine why it may be more advantageous for females than males to see in the red range (Marshall & Arikawa, 2014; Jocelyn Liang & Monteiro, 2017).

In *Pieris rapae* (Pieridae), which also has trichromatic color vision, red filtering pigments are also present, creating peak sensitivities around the 620-640 nm range (Jocelyn Liang & Monteiro, 2017). *P. rapae* displays sexually dimorphic spectral sensitivities, with the males of the species having a photoreceptor that allows them to be sensitive to the blue-violet wavelength range important for recognizing conspecific females (Balamular et al, 2019).

For an extreme example of color vision in Lepidoptera, *Papilio xuthus* have evolved tetrachromatic color vision, allowing them to discriminate red wavelengths without the need for filtering pigments, similar to *L. heteronea* and *L. rubidus* mentioned earlier (Zaccardi et al, 2006; Marshall & Arikawa, 2014). Interestingly, *P. xuthus* have also evolved additional color filtering channels on top of tetrachromacy to enhance their color vision even further (Zaccardi et al, 2006; Marshall & Arikawa, 2014). *P. xuthus* has peak spectral sensitivities in the UV, violet, blue, green, and red range, as well as expresses overall broadband sensitivity in nearly all wavelengths of visible light (Arikawa, 2003).

These examples show just how variable lepidopteran visual systems can be, ranging from plain trichromacy to the incorporation of filtering pigments to true tetrachromacy and beyond. A final example goes a step farther in providing a real-world instance where the coevolution of color vision and wing pattern has enabled closely

related Lepidoptera to visually discriminate within and between species based on even the smallest of color and spectral differences. The lycaenid species *Plebejus argus* and *Polyommatus icarus* have almost identical pigment-produced, blue-colored wing patterns, with slight variation in the scale nanostructures responsible for structural coloration (Piszter et al, 2016). *P. icarus*, whose wing pattern is UV-absorbing, has a peak visual sensitivity in the blue range, while *P. argus*, whose wing pattern is UV reflecting, has a peak sensitivity in the UV range, corresponding to the reflectance maximums of each species' wing pattern (Piszter et al, 2016). These differences are what enable these butterflies to visually discriminate between conspecific and heterospecific individuals (Freeman, 1998; Stavenga & Arikawa, 2006; Piszter et al, 2016).

## **1.5. Hypotheses Related to Lepidopteran Visual Signals**

### **1.5.1. Honest Signal Hypothesis**

An honest signal can be defined as a trait that is a reliable indicator of the quality or condition of the individual who possesses it (Petak, 2019). That signal should thus convey information useful to the individual receiving it (Petak, 2019).

The shape, size, color, or patterning of diurnal lepidopteran wings may act as fitness indicators to potential mates or during inter- and intra-specific competition to warn inferior individuals of their status. Davis et al (2007) showed that *D. plexippus* males with darker, almost red, wings experienced increased mating success than those with lighter, almost yellow, wings. Davis et al (2012) also looked at the relationship between the wing color of *D. plexippus* and flight performance, showing that individuals with darker colored wings were able to fly much longer and farther when compared to those with lighter wings. These findings, taken together, suggest that *Danaus plexippus*

wing color may act as an honest signal in mating for indicating an individual's flight ability (Davis et al, 2012; also *D. plexippus*, Johnson et al, 2014).

Wing color as an honest signal could also be used in territorial species as a warning to conspecifics and sometimes heterospecifics of an individual's fitness. The two locally sympatric species *C. eurytheme* and *C. philodice* have a unique interaction with each other, where the male, UV reflecting, *C. eurytheme* utilizes his wings to warn both conspecific males and *C. philodice* males against interfering during his courtship attempts (Silberglied & Taylor, 1978). Papke et al (2007) studied female mate choice in *C. eurytheme*, finding that females preferred younger mates. That same study found that the brightness of wing UV reflectance was an honest signal of a male's age and was thus correlated to their potential for mating success. There is also preliminary evidence that wing color may be an indicator of underlying condition in *Zerene spp.* (Pieridae) (Fenner et al, 2019) and in *P. rapae* (Tigreros et al, 2014); however, more research needs to be done in other butterfly species exploring the link between visual signals and an individual's fitness before conclusions can be made about wing color and other wing characteristics being an honest signal of underlying fitness in Lepidoptera.

### **1.5.2. Life History Hypothesis – Tradeoffs and Resource Allocation**

Life history can simply be defined as the changes in survival and reproductive patterns that an organism experiences over the course of its life (reviewed in Stearns, 2000). This review specifically looks at how lepidopteran visual communication strategies influence the reproductive and mating success characteristic of a species' life history. A life history strategy is simply a trait related to an organism's life history, such as lifespan, birth rate, reproductive timing, mortality rate, senescence, etc., that theorists

seek to understand via the conceptual framework of evolutionary processes (reviewed in Stearns, 2000). These strategies, however, depend on an organism's resource acquisition and allocation.

Resource allocation is the overall distribution of incoming or stored nutrients to certain life history traits or activities (Boggs, 1981). In adult butterflies, incoming nutrients usually come from the adult diet, while stored nutrients come from the larval diet (Boggs, 1981); there are some butterflies and moths that have evolved a non-feeding adult stage, making those species more heavily reliant on larval nutrient storage for their energetic needs. By studying resource acquisition pattern, allocation to different traits may demonstrate life history tradeoffs.

Tradeoffs are the process by which an increase in one life history trait causes a decrease in another, usually as a resource allocation strategy when an organism or population is faced with limited food availability (Van Noordwick & de Jong, 1986). A good example of this is the slow-fast pace of life/life history continuum, where species classified as slow organisms usually have a long lifespan but invest all reproductive resources and energy into a few offspring to maximize offspring survival (reviewed in Stearns, 2000). Organisms considered fast usually have a shorter lifespan but spread their energy and resources allocated to reproduction to many offspring with only a few surviving (reviewed in Stearns, 2000). Figure 1.3 is adapted from a 1986 paper by Van Noordwick and de Jong that visually models tradeoffs with  $R$  on the x-axis and  $S$  on the y-axis designating two different life history traits (Van Noordwick & de Jong, 1986).  $A$  is the amount of resources available to an individual for investment, and  $B$  is the fraction of  $A$  allocated towards  $R$ , making  $B-1$  the fraction of resources allocated towards  $S$  (Van

Noordwick & de Jong, 1986). Figure 1.3a shows that if the variation in A is large and the variation in B is small, then R is positively correlated with S; however, if the variation in A is small and the variation in B is large, then R is negatively correlated with S as seen in Figure 1.3b. That negative correlation is where we would see the presence of tradeoffs (Van Noordwick & de Jong, 1986).

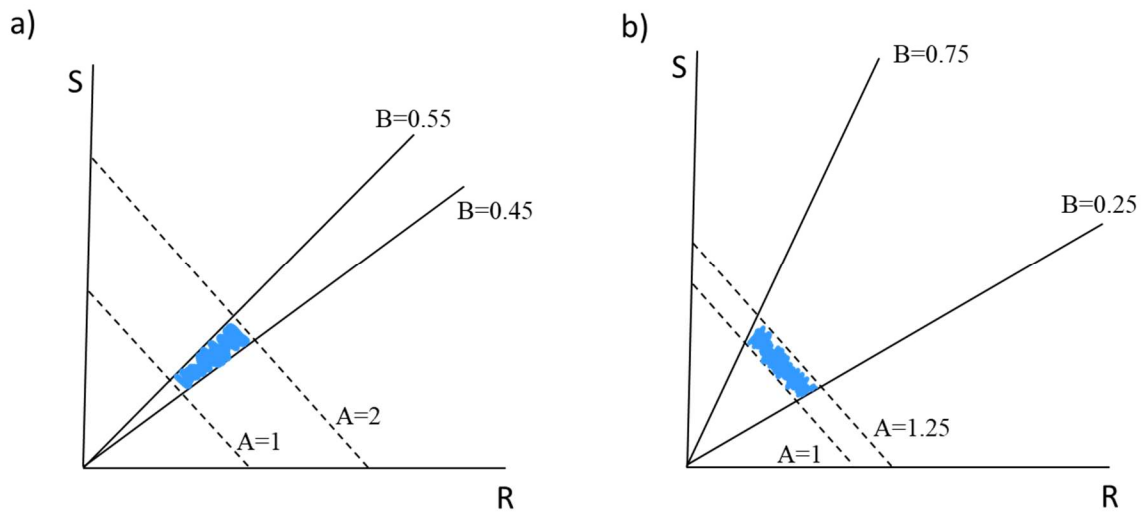


Figure 1.3: Visual explanation of tradeoffs. Adapted from Van Noordwick and de Jong (1986). A = resources available for investment; B = fraction of A allocated to R; B-1 = fraction of A allocated to S.

The nutritional needs resulting from a given life history reflects an organism's resource allocation strategies (Boggs, 1981). Those allocation strategies can be used to study tradeoffs in a species by looking at what happens to allocation when acquisition is perturbed (Boggs, 1981). Dietary restriction studies show that variation of larval nutrient input can have a noticeable effect on the resources allocated during metamorphosis towards those traits vital for visual communication, specifically eye and wing morphology (*P. aegeria*, Talloen et al, 2007; *D. plexippus*, Johnson et al, 2014). *Pararge aegeria* larvae that were raised on drought stressed plants yielded adults with lighter, less

melanized wings than the control individuals (Talloen et al, 2007). In a similar study done on *D. plexippus* butterflies, applying food stress to the larval stage yielded paler, more yellowish individuals as oppose to the darker orange hues observed in the control group, suggesting color to be an indicator of larval diet quality and/or quantity (Johnson et al, 2014).

For butterfly visual systems, the sexual dimorphic trait of eye size, where males have larger eyes relative to their body size than females, has been observed in *Ochlodes venatus* (Hesperiidae), *Papilio machaon* (Papilionidae), *Celastrina argiolus* (Lycaenidae), *Mellicta athalia* (Nymphalidae), *Issoria lathonia* (Nymphalidae), and more (reviewed in Rutowski, 2000,). This supports the assertion that males invest more resources than females into the development of their visual system, likely due to the importance of male vision in long range mate recognition and location (reviewed in Rutowski, 2000 & 2002; reviewed in Sarto i Monteys et al, 2016). Females may thus allocate more resources than males to their overall wing and body size to optimize egg production and maintenance (Wiklund & Karlsson, 1988) as well as the support of their wings as long-range visual signals to attract conspecific males. This sex-based difference in allocation strategies suggests males and females may experience different effects of limiting nutrients (Boggs, 1981; Karlsson, 1995).

Interestingly, in the nocturnal *H. armigera*, an adult starvation experiment found that nutritional stress applied after adult emergence led to the down regulation of opsin gene expression (Yan et al, 2014). While color vision may be more important or widely used in diurnal Lepidoptera, this study suggests that there is use for it in their nocturnal counterparts, perhaps in foraging and close-range mating behaviors (Yan et al, 2014).

Overall, dietary restriction studies could be used to understand the fitness effects of life history traits and allocation patterns.

## **1.6. Effects on Fitness**

### **1.6.1. Benefits of Visual Communication**

Fitness refers to an individual's ability to contribute offspring to future populations. How wing shape, size, color, and pattern can benefit the fitness of lepidopteran species has already been discussed, mainly focusing on how those wing characteristics can be used as visual signals to increase an organism's potential for mating and reproductive success. Those same wing characteristics that affect short- and long-range sexual visual communication can also affect other life history traits of diurnal Lepidoptera, such as those related to survival and dispersal. This section of the review discusses wing color and pattern in the context of aposematic signaling, predator deterrence strategies, dispersal ability, and thermoregulation as well as how Lepidoptera may adapt to the multifunctional uses of these visual signals (Fordyce et al, 2002; Oliver et al, 2009; Nokelainen et al, 2011; Le Roy et al, 2019; Tan et al, 2020; Rosa & Saastamoinen, 2020).

### **1.6.2. Visual Signals and Predator Deterrence:**

In butterflies that accumulate toxic compounds in their larval diet to assimilate into their adult morphology, bright colors may warn a predator of their unpalatability and toxicity (Sime et al, 2000, Tan et al, 2020), as is seen in the bright orange *D. plexippus* (Davis et al, 2012) and the orange and blue patterning of *B. philenor* (Sime et al, 2000). For species that are not toxic, they may still utilize common warning colors or mimic the warning patterns of poisonous species for the purpose of predator deterrence. This is

particularly seen in the Mullerian mimetics of *Heliconius* butterflies and *Pseudaegeria* spp. moths (Parchem et al, 2007; Zborowski & Edwards, 2007).

In non-poisonous *Delias hyparete* (Pieridae), the presence of common aposematic signaling colors on the wings, red and yellow, significantly lowers an individual's chance of predation (Jocelyn Liang & Monteiro, 2017). By using their wings as a warning signal to predators, *D. hyparete*'s chance of survival increases (Jocelyn Liang & Monteiro, 2017).

In the European populations of *Parasemia plantaginis* (Erebidae), two color morphs are present: white and yellow (Nokelainen et al, 2011). In lab experiments observing the interactions between the moth and one of its natural bird predators, *Parus caeruleus*, the yellow morph shows a higher survival rate than the white morph, supporting yellow as a common warning color (Nokelainen et al, 2011). Interestingly, in a follow-up mating experiment, the male white morphs exhibit higher mating success than its yellow conspecific males, showing a survival-reproduction tradeoff in *P. plantaginis* where natural and sexual selection work against each other (Nokelainen et al, 2011).

A bluish-green colored band plays an active role in deterring predatory birds in the butterfly *Papilio demolion demolion* (Papilionidae) (Tan et al, 2020). The way that the swallowtail's blue-green banding pattern may function to reduce predation is via crypsis (Tan et al, 2020). Tan et al (2020) used paper models of *P. demolion demolion* that manipulated different aspects of the color and pattern of the band to assess how those wing characteristics affects predation risk. The experiments showed that it was not just the blue-green coloration that aided in deterring predators, but it was the confounding



effects of the blue-green color confined in a transversal band, showing that survival significantly decreased in the absence of both color and banding pattern. The authors proposed that the shape of the transversal band may thus disrupt the shape of the butterfly, leading to disruptive coloration; the blue-green color of the band may even allow the butterfly to blend in or camouflage with its green, foliated habitat. The band of this butterfly is also UV reflecting, which may further lend itself to crypsis and the disruption of predator detection (Tan et al, 2020). Similar results of ‘creating a false boundary’ via crypsis coloration patterns to reduce predation risk have also been demonstrated for the butterfly *Anartia fatima* (Nymphalidae), whose wings are also banded (Seymour & Aiello, 2015 cited in Tan et al, 2020).

### **1.6.3. Visual Signals and Dispersal:**

Visual signals have also been linked to an organism’s flight/dispersal ability, most notably wing size and shape (reviewed in Le Roy et al, 2019; *D. plexippus*, Davis et al, 2007 & 2012). Females often have larger wing areas than males (Gilchrist, 1990), while in species where males are more active (such as patrolling for a mate), males have larger wing width-to-height ratios, also called aspect ratio (Le Roy et al, 2019). An optimal wing aspect ratio and area can reduce any cost associated with sustained flying, which can be very beneficial when put in the context of competition or migration (Le Roy et al, 2019). This provides a straightforward relationship linking the wing characteristics of size and shape to the optimization of flight ability/dispersal (Le Roy et al, 2019). Le Roy et al (2019)’s review correlates wing shape and size to butterfly flight performance and fitness, emphasizing the need for studies linking manipulated wing size and shape to its impact on flight performance and mating success.

#### **1.6.4. The Multifunctional Role of Melanin:**

Melanin plays a versatile role in inter- and intra-specific visual communication strategies and thermoregulation as it relates to the life history traits of reproduction, survival, and dispersal (Watt, 1968; reviewed in Kingsolver, 1987; Janssen et al, 2001; Fordyce et al, 2002; Ellers & Boggs 2003; Rosa & Saastamoinen, 2020). Increased melanization yields darker colored wings, which allows an individual to heat up quicker and thus bask in a vulnerable state for less time (Watt, 1968; Kingsolver, 1983; Kingsolver, 1985; Kingsolver, 1987; Ellers & Boggs, 2002; Rosa & Saastamoinen, 2020). Low levels of melanin can leave a butterfly vulnerable to predation, less likely for a male to find a mate or a female to find oviposition sites, and render an individual incapable of nectaring flights, leading to starvation; on the other hand, unnecessarily high melanization levels can lead to death by overheating, cementing the importance of optimal melanin levels essential for survival (Watt, 1968; reviewed in Kingsolver, 1987; Ellers & Boggs, 2002; Rosa & Saastamoinen, 2020). The multifunctional role of melanin has been extensively observed in *Colias* (Kingsolver, 1983; Ellers & Boggs, 2002; Ellers & Boggs 2003) and *Pieris* butterflies (Kingsolver, 1985; Kingsolver 1987; Wiernasz, 1989).

#### **1.6.5. Addressing the Multi-variate Uses of Visual Signals:**

Visual signals have been looked at for the multiple functions they serve in Lepidoptera and examples in this review demonstrate how those functions may work together (Wiernasz, 1989) or in opposite directions of each other (Ellers & Boggs, 2003; Oliver et al, 2009; Nokelainen et al, 2011). A bright color or pattern used to attract mates may make an individual more likely to be detected by predators, but a color or pattern

used in predator deterrence may make that butterfly less attractive as a suitable mate (Oliver et al, 2009). In *Pieris rapae*, females preferred more brightly colored males related to increased amounts of pterin pigments and increased UV wing reflectance (Morehouse & Rutowski, 2010); but *P. rapae* males with characteristically more chromatic and reflective wings experienced an increased risk of predation by predatory birds able to see in the ultraviolet (Morehouse & Rutowski, 2010). Thus, more conspicuous males risk a decrease in survival to gain the added benefit of increased mating success (Morehouse & Rutowski, 2010). From those examples, a question emerges: If a single visual signal, such as wing color or pattern, can serve multiple functions, how could a butterfly evolve to utilize that visual signal in a way that it could still benefit from all of its underlying functions? Oliver et al (2009) addressed that question in a study looking at the butterfly genus *Bicyclus*. They demonstrated the evolution of spatial separation between visual signals that correspond to different functions. Via phylogenetic reconstructions, the authors showed that elements on the dorsal wings of *Bicyclus* species play a major role in mate recognition/signaling and the dorsal forewings were usually characterized by any sexually dimorphic traits typical within a species (Oliver et al, 2009). Visual elements on the ventral wings were important for deceiving or avoiding predators, as in *B. anynana* whose ventral marginal eyespots misdirect predatory attacks towards the wing margin (Robertson & Monteiro, 2005; Parchem et al, 2007; Oliver et al, 2009). When visual signals conflict to serve different functions, spatial separation of those visual signals may be the adaptive answer for lepidopteran species (Oliver et al, 2009). Similar studies on nymphalids have yielded similar results (Jocelyn Liang & Monteiro, 2017); however, *Colias* wing melanization

studies suggest the functions of sexual signaling and thermoregulation are not fully spatially separated. More research needs to be done looking at whether spatial separation of visual signals has occurred in other Lepidoptera.

#### **1.6.6. Additional Costs of Visual Communication**

Generating color and pigment on lepidopteran wings can be energetically costly (Morehouse & Rutowski, 2010; reviewed in Kemp & Rutowski, 2011). Pterin pigments in the wing scales of *Pieris rapae* are responsible for the species' mostly white coloring (Morehouse & Rutowski, 2010). Pterin pigments are rich in nitrogen, which is limiting in the larval diet (Morehouse & Rutowski, 2010). Since larval-derived nutrients determine adult morphology (Boggs, 1981), high levels of pigment in the wings, to become more chromatic and attractive to mates, may be extremely costly for an individual to produce (Morehouse & Rutowski, 2010). Similar to pterins, other pigments such as melanin and ommatins can also be costly to generate from larval accumulated nutrients (Talloen et al, 2007). Not much research has gone into exploring the cost of structural coloration, but a study looking at the costs of generating pigmentation versus structural color could be a great addition to this body of literature (Kemp et al, 2012).

### **1.7. Conclusion**

Lepidoptera are often the focus of studies regarding behavior, evolution, conservation, genetics, the adaptive significance of mimicry, sexual selection, natural selection, and so much more within the field of biology (DeVries, 2001; Boggs et al, 2003). This literature review concentrates on the visual communication strategies of diurnal butterflies and moths, describing the adaptive significance of visual signals such as wing size, shape, color, and pattern. Connections were made between wing

morphological traits and various life history traits, including mating and reproductive success (Wiernasz, 1989; Robertson & Monteiro, 2005; Davis et al, 2007; Benítez et al, 2011), survival (Fordyce et al, 2002; Ellers & Boggs, 2002; Oliver et al, 2009; Jocelyn Liang & Monteiro, 2017), and dispersal (Benítez et al, 2011; Davis et al, 2012; Le Roy et al, 2019; Rosa & Saastamoinen, 2020). Visual signals on the wings were also linked to methods of heterospecific communication (predator avoidance: Nokelainen et al, 2011; Jocelyn Liang & Monteiro, 2017; Tan et al, 2020; heterospecific recognition/competition: Silberglied & Taylor, 1978; Burghardt et al, 2000; Oliver et al, 2009; Davis et al, 2012), and some studies have even looked at the potential for certain aspects of wing morphology to be an honest signal of an organism's underlying condition, which could play a role in mating and competition (Davis et al, 2012; Johnson et al, 2014).

Receiving those visual signals occurs via a butterfly's compound visual system, and the study of lepidopteran visual ecology draws conclusions about a species' visual acuity and sensitivity based on eye morphology such as size, shape, and facet characteristics (reviewed in Rutowski, 2002). Larger eyes increase mate detection and recognition, especially in males who rely on their vision to receive and detect visual signals at long-range (reviewed in Rutowski, 2002). Thus, male butterflies are usually characterized by more acute vision than females, and studies supporting that assertion showed that males possess eye traits corresponding to greater visual resolution when compared to females (Rutowski, 2000; Ziemba & Rutowski, 2000). Zones of acuity usually correspond with an insect's behavior and lifestyle, such as the forward-anterior regions being used for various mating behaviors and recognizing heterospecific individuals (*Asterocampa leilia*, Rutowski, 2000; Ziemba & Rutowski, 2000; reviewed in

Rutowski, 2002; Marshall & Arikawa, 2014). Each region of the eye contains the photoreceptors and appropriate color channels needed to successfully perform each region-specific task, which can vary significantly based on habitat, lifestyle, and behavior between and within species (Bernard & Remington, 1991; Stavenga & Arikawa, 2006; Marshall & Arikawa, 2014).

Crucial for intraspecific communication, females use their wings as long-range visual signals to attract a male and elicit courtship behaviors (reviewed in Sarto i Monteys et al, 2016). At close range, a male's wings act as visual signals to seduce the female into copulation (reviewed in Sarto i Monteys et al, 2016). The mating strategy and extent to which male/female mate choice has an affect varies between species and can change how the visual signals discussed in this review are used and visually received/interpreted. Examples given in this review show that a species' visual sensitivities correlate with the color and reflectance properties of that species' wings (*Papilio xuthus*, Marshall & Arikawa, 2014; *Pieris rapae*, Joceyln Liang & Monteiro, 2017), supporting the notion that wing coloration/morphology and lepidopteran visual ecology may have coevolved (reviewed in Stavenga & Arikawa, 2006; Morehouse & Rutowski, 2010; Lind et al, 2017).

With the vital role that Lepidoptera play in today's world as pollinators, agricultural pests, and important prey (Wagner, 2013; Regier et al, 2013) as well as the diversity of color they add to it, it is important to continue studying their use of visual signals to fully comprehend the visual communication strategies of these complex organisms. More research is needed on how certain wing morphological traits (color, size, shape, pattern, etc.) may function in the visual communication strategies of certain

species, especially looking at how traits such as wing color may act as an honest signal of underlying condition. In terms of color generation, very little research exists looking at how the production of color affects male and female fitness and how resource acquisition and allocation patterns are associated with lepidopteran color producing mechanisms. Additionally, not many studies have looked at the cost of structural coloration or how it may compare to the cost of pigmentation, but further research in that field could shed light on the adaptive significance of color generated via pigments versus scale nanostructures. Oliver et al (2009) studied the spatial separation of signal functions on the wings of *Bicyclus* butterflies and similar studies investigating whether spatial separation has occurred in other Lepidoptera could reveal potential evolutionary solutions to the multivariate uses of visual signals. In terms of butterfly and moth visual systems, not much research has compared traits present in the systems of nocturnal versus diurnal species and virtually no studies have focused on underlying neural processing. Future studies could focus on how food stress or other environmental stressors may alter opsin expression in the visual system of diurnal butterflies and how that compares to its effect in nocturnal Lepidoptera. Regardless, the current research literature on visual communication strategies in diurnal lepidopteran species shows that their wing morphology and visual ecology have a great impact on their potential for mating and reproductive success.

## **CHAPTER 2: THE EFFECTS OF LARVAL FOOD STRESS ON ADULT *SPEYERIA MORMONIA* EYE AND WING MORPHOLOGY AND COLORATION**

### **2.1. Introduction**

Lepidopteran visual communication strategies can be reduced to two components: visual signals on the wings and a compound visual system needed to receive and process those signals. Most butterflies are diurnal, with mate seeking strategies that are mainly based on visual signals prevalent in a sunlit environment (reviewed in Sarto i Monteys, 2016). Both males and females can use vision for mate recognition, but it is often the male that visually detects females at long-range and pursues her as a potential mate; whereas females assess the male at close-range (reviewed in Sarto i Monteys, 2016). The differential use of vision in mate recognition between the sexes supports a comparatively larger role of vision in the mating success of males (Rutowski, 2000). For visual signals, variation in wing morphology and coloration corresponds to changes in life history traits, including mating and reproductive success (e.g., Ellers & Boggs, 2003; Robertson & Monteiro, 2005; Teodora et al, 2007), survival (e.g., Jocelyn Liang & Monteiro, 2017; Tan et al, 2020; Nokelaninen et al, 2011), and dispersal (e.g., Benítez et al, 2011; Davis et al, 2012; Rosa & Saastamoinen, 2020). Wing coloration has also been explored as



potential honest signal of a species' overall fitness (e.g., Davis et al, 2007; Davis et al, 2012).

The development of a Lepidoptera's visual system and wings occurs in the pupal stage and relies on species-specific resource allocation strategies (Graham et al, 1980; reviewed in Miller & Hammond, 2003; Gotthard, 2008). The nutrients used during the pupal stage in creating the adult body plan are accumulated during the larval stage (Boggs, 1981), bringing up the question of what would happen to those eye and wing morphological traits used for visual communication if the larvae experienced limited food availability? In Lepidoptera, larval food stress leads to prolonged larval development time (Bauerfeind & Fischer, 2005; Johnson et al, 2014), reduced investment in flight muscle (Velde et al, 2013), reduced body mass (Bauerfeind & Fischer, 2005; Velde et al, 2013; Johnson et al, 2014), decreased fecundity, decreased reproductive/mating success (Bauerfeind & Fischer, 2005; Moreau et al, 2007; Rosa & Saastamoinen, 2017) and has also been linked to changes in wing morphology such as shape (Pellegrons et al, 2009; Johnson et al, 2014) and reduced wing pigmentation (Talloen et al, 2009; Tigreros et al, 2013; Kemp, 2008; Johnson et al, 2014). Larval dietary restriction studies measuring adult wing size, shape and/or color exist for *Pararge aegeria* (Nymphalidae) (Talloen et al, 2009; Pellegrons et al, 2009; Velde et al, 2013), *Danaus plexippus* (Johnson et al, 2014), *Pieris rapae* (Pieridae) (Tigreros et al, 2013), and *Speyeria mormonia* (Boggs & Freeman, 2005; Boggs & Niitepõld, 2015), but there is a gap in the literature in terms of linking the results of those studies to an adult butterfly's visual ecology.

In *Speyeria mormonia*, larval food stress saw a reduction in pupal mass, adult body mass, wing length, fecundity, and lifespan, but had no effect on resting or flight

metabolic rate (Boggs & Freeman, 2005; Boggs & Niitepõld, 2015; Niitepõld & Boggs, ms). Based on these results, Niitepõld and Boggs (ms) concluded that larval nutrient stress indicated no change in dispersal capacity. Larval dietary restriction also resulted in decreased adult body mass (Boggs & Freeman, 2005; Boggs & Niitepõld, 2015; Niitepõld & Boggs, 2020) and shorter wing lengths (Boggs & Freeman, 2005), characteristics that can affect the visual communication strategies of *S. mormonia*.

For *S. mormonia*, females have larger wings and bodies than males (Boggs & Ross, 1993; Boggs & Niitepõld, 2015), males have larger eyes than females, and males are usually the more chromatic (Gonzalez, 2018). Gonzalez (2018) used *Speyeria mormonia* adults caught in the field during the years 2017 and 2018 from the populations found near Rocky Mountain Biological Lab, Gunnison County, Colorado, USA (38°57'N, 106°58'W, 2,900 m a.s.l.), to study how the wing and eye morphology of these butterflies varied between years. Compared to 2018 individuals, 2017 butterflies had greater eye surface areas, longer wing lengths and decreased wing chromaticity values, calculated based on the slope of measured spectral reflectance curves (Gonzalez, 2018). Since 2018 was a year characterized by an early snow melt date and drought stress in Gunnison County, CO (gothicwx.org), larval resources in the environment may have been more limited in 2018 than 2017. If *S. mormonia* larvae are experimentally subjected to food stress, the variation in wing and eye morphology of the resulting adult butterflies may explain the variation Gonzalez (2018) found between butterflies caught in different years. I tested this hypothesis via an intermittent larval starvation experiment. I expected adults from larvae fed ad libitum to have larger eyes, more facets, a larger average facet area, longer wing lengths, and reflectance curves that are more red-shifted than adults

from larvae that were intermittently starved. Likewise, I expect males to have larger eyes, increased visual sensitivity, and more chromatic wings. If these expectations are correct, they will show that the resource allocation strategies associated with larval-derived nutrients in *Speyeria mormonia* strongly affects characteristics related to their system of visual communication. The results may also reveal the evolution of a sex-dependent resource allocation strategy within this butterfly species.

## **2.2. Methods**

### **2.2.1. Study Organism**

*Speyeria mormonia* butterflies are day-flying, nonmigratory and produce only one generation per year (Boggs, 2002). The larvae used in this experiment originated from the study population in Colorado in open montane meadows near the Rocky Mountain Biological Laboratory (38°57'N, 106°58'W, 2,900 m a.s.l.). Like most holometabolic insects, *S. mormonia* has four developmental stages: egg, larva, pupa, and adult. Females oviposit their eggs in leaf debris (Boggs, 2002). The eggs hatch and the unfed, first instar larvae then overwinter in a diapause state (Boggs, 2002). The following spring the larvae break diapause and feed on leaves of *Viola spp* (Violaceae) (Boggs, 2002). Larvae grow exponentially and go through 5 growth molts, or instars, with most of the nutrients allocated towards the pupal and adult stage coming from the 5<sup>th</sup> instar (Boggs, 2002; Boggs & Niitepõld, 2015). After diapause is broken, the larval stage lasts a minimum of approximately 35 days in laboratory growth chambers (18:6 light:dark cycle at 27°C:15°C), at which point the larvae attach to the underside of a leaf by silk-like threads and pupate. Pupation usually lasts 10-12 days until the butterfly emerges as an adult (Boggs, unpubl. Data). Adult *S. mormonia* nectar from Compositae species (Boggs,

1988). Females usually only mate once, while males can mate multiple times (Boggs 1986). Their flight season is from late June to early September depending on when the snow melts and their adult lifespan ranges from 0-50 days in the field (Boggs, 1987).

### **2.2.2. Growing Violets**

Violets were grown in a growth chamber at the University of South Carolina, on a 18:6 light:dark cycle at 27°C:15°C. The violets were fertilized as needed and watered twice per week. Violets experienced occasional mild drought stress mostly due to low humidity levels the week(s) prior to larval rearing. The growth chambers where the violets were grown were the same growth chambers used in the following rearing process.

### **2.2.3. Larval Rearing**

In the summer of 2019, field-collected females laid eggs in the lab at the Rocky Mountain Biological Laboratory and those eggs hatched in late August 2019. The resulting larvae were stored by brood in glass vials labelled with their mother's number (hereafter, brood number) and placed with a wet paper towel in a partially sealed Ziploc bag in a refrigerator. They were transported back to Dr. Carol Boggs' lab at the University of South Carolina, Columbia, SC in an ice chest in mid-September 2019. Upon arrival, the larvae were stored in a growth chamber set to a 8:16 light:dark cycle with a constant temperature of 1°C to maintain diapause. On January 25<sup>th</sup>, 2020, the larvae from 10 different broods were transferred to a growth chamber kept at a 16:8 light:dark cycle at 11°C:5°C. This transfer was meant to ease the larvae out of diapause; however, only 4 broods survived that method, so 6 additional broods were taken

immediately out of diapause and into the larval rearing stage, skipping that transition period.

Approximately 50 larvae from 10 different broods were used (totaling 500 larvae to start). The larvae from each brood were placed onto a damp paper towel and, using a magnifying visor and a paint brush to handle the larvae more easily, 50 healthy larvae were chosen from each brood (larvae were considered healthy if they were elongated, moving, plump, not shrunken) and placed together in an organdy bag. There was one organdy bag per brood, giving a total of 10 bags. Once each bag was filled with larvae, two healthy violet leaves were carefully placed into the organdy bags and thread was wrapped around the base of the bags to fasten the bag in place on the violet plant and prevent any possible escapes. A piece of tape was placed on part of the bag to label each bag by the brood number of the larvae it contained. Once the bags were secure on the violets, they were sprayed twice with a squirt water bottle to hydrate the larvae and sprayed again the following day.

After roughly two weeks, the larvae were transferred to new leaves to replenish their food source. Each bag was removed from the violets by pinching the leaves contained in each bag by their stems at the base of the bag. This was meant to ensure no organisms were lost or could escape. The thread holding the bag together was unwound and the leaves and caterpillars were pulled out to lay on a white piece of paper, which made it easier to see the dark-colored larvae. A paint brush was used to remove all remaining larvae and frass from the bag, making sure no larvae escaped. All living larvae were counted, recorded, and placed back in their respective organdy bags over three new violet leaves for further rearing. Once one brood had been counted, the debris

on the white paper was discarded and the next brood was done until all larvae had been transferred. The larvae were monitored and were given more leaves as needed as they progressed through their first four larval instars. As they grew larger, some broods (i.e., those with higher survival rates that could no longer fit in the same bag) were separated and the bags were designated by brood number plus a letter (A, B, C, etc.). Out of the 500 larvae used, 116 survived the entire rearing process. That mortality rate was expected based on previous laboratory rearing experiments performed on *Speyeria mormonia*.

When a larva reached critical size (2.5 cm in length, Boggs & Niitepõld, 2015), indicating its fifth and final instar stage of development, it was separated into one of two groups. Keeping no more than three larvae per bag at this stage, half of them were placed in a control group (58 larvae) and the other half were placed in a treatment group (58 larvae). Each control group was paired with a treatment group within the same brood. The control group was given two leaves per individual and the leaves were replenished as needed for *ad libitum* access to food. Each treatment group was given one leaf per individual and their leaves were replaced only when the paired control group's leaves were replenished. This resulted in the treatment larvae experiencing two to three bouts of starvation from being left without any food source. The larvae were checked twice a day, recording the date, time, number of larvae in each bag, and the number of leaves added to each bag anytime the bags needed changing. This process continued until each of the 116 larvae had successfully pupated.

Pupae were moved to individual small cups lined with a damp paper towel. The purpose of the wet paper towel was to replicate a humid environment characteristic of

*Speyeria mormonia*'s native environment. Once the pupa had been properly placed, the cup was covered with cheese cloth or equivalent material to allow the monitoring of the individual for adult eclosion and to prevent any damage to the wings, which are very soft and delicate upon emergence. A rubber band was used to secure the cheese cloth and paper towel in place, making sure the butterfly would not be able to get underneath the paper towel, which could also lead to wing damage. The cup was labeled with the individual's number (assigned by order of pupation), brood, group (1 for control or 2 for treatment), and date of pupation. The pupae were expected to eclose within approximately two weeks. Pupae were kept in the same growth chamber that they were reared in and sprayed with water daily to maintain local humidity.

Upon eclosion, the 107 butterflies that survived to adulthood (54 control vs. 53 treatment) were frozen at -20°C within 24 hours after clearing the meconium, allowing time for the wings to harden. The butterflies were handled with spade-tip forceps and stored in glassine envelopes to avoid damage or scale removal from the wings. The envelope was labeled with the butterfly's date of eclosion, assigned number, brood number, and sex.

#### **2.2.4. Alterations due to COVID-19**

About 3/4ths of the way through the rearing process, the COVID-19 pandemic led to changes in normal laboratory operations in the Biological Science Department at the University of South Carolina. Accommodations had to be made and the larvae were kept at roughly a 14:10 light:dark cycle and at around 25°C:18°C. At that point, 21 larvae had already pupated, 17 males (6 control, 11 treatment) and 4 females (all control). To test whether the change in rearing environment had a significant effect on the results of this

experiment, a General Linear Model (Systat 13.2; Systat Software, San Jose, CA, USA) was run for all dependent variables described below (see data analysis), with “pre-post-covid” as a category variable. The tests showed no significant effect of changes brought on by the pandemic, so this variable was ignored in the final analyses.

#### **2.2.5. Wing lengths**

Wing length was included in this experiment to determine whether larval treatment was effective (Boggs & Freeman, 2005). As a proxy for body size, the wing lengths of 100 *S. mormonia* (51 control vs. 49 treatment) were measured from the wing hinge at the base of the wing to the farthest point at the wing tip using a caliper ruler.

#### **2.2.6. Corneal Peels**

The methods for corneal peels were adapted from Ziemba and Rutowski (2000). Using fine dissecting scissors and forceps, the head of each butterfly was cut off and the dorsal region of each eye was carefully marked using white out (total: 90 *S. mormonia*; 48 control vs. 42 treatment). The eyes were cut away from the head and soaked in a 10% sodium hydroxide solution for one to two hours to soften the underlying tissues attached to the cornea. After soaking, the eyes were removed from the solution bath and placed under a microscope. Fine forceps were used to carefully separate the cornea from the remaining emulsified tissue and fine scissors were used to make small cuts around the perimeter of the cornea to lay it flat on a microscope slide. Once placed on a slide, the cornea was mounted in 20% glycerol to keep it hydrated and it was sealed using a cover slip and lab-grade nail polish. The microscope slide was labeled with the orientation (dorsal, ventral, posterior, anterior) of the cornea, and the butterfly’s number, sex, and group (control = 1; treatment = 2).



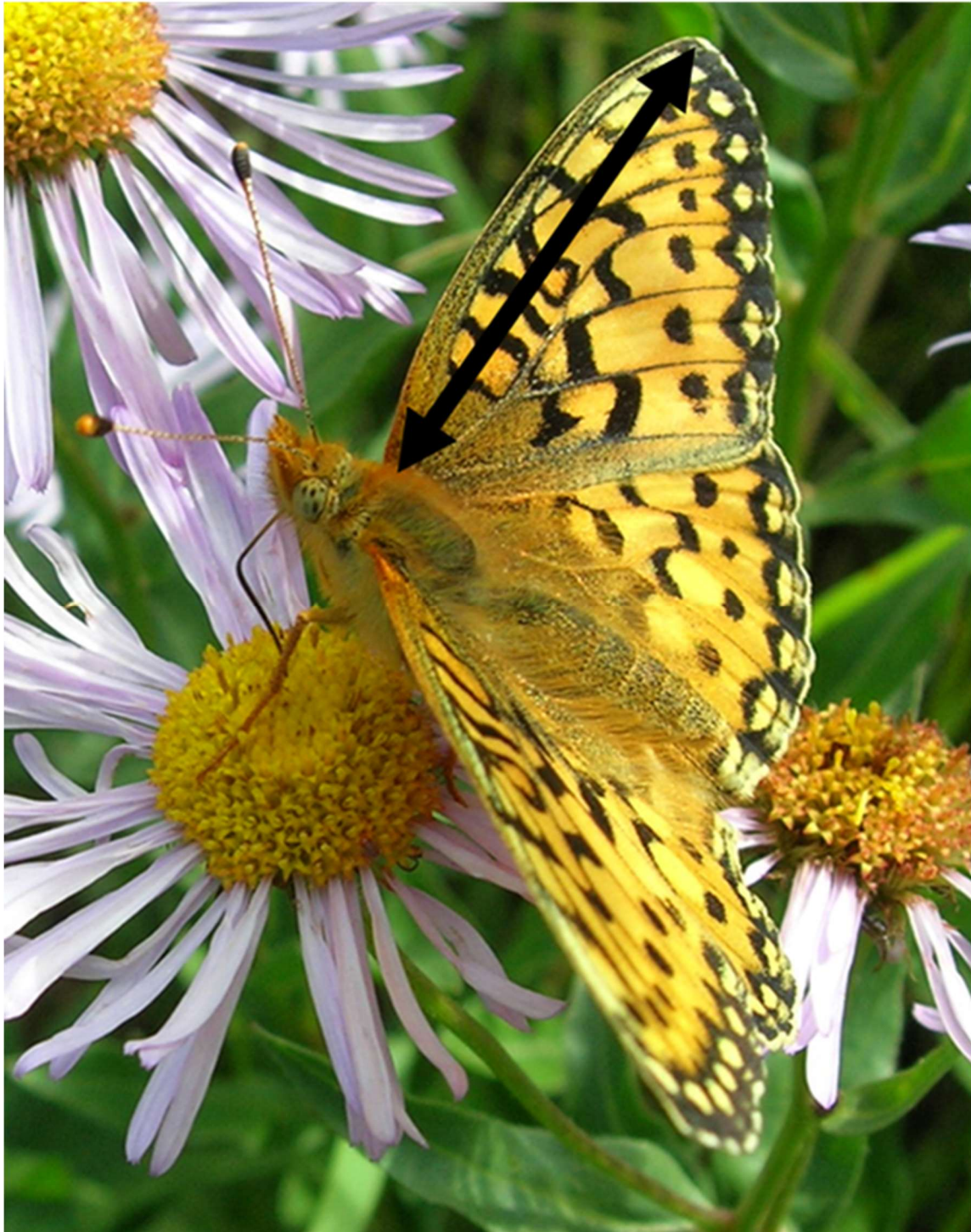


Figure 2.1: Wing Length. The double arrow indicates how wing length was measured on *Speyeria mormonia* adults. Photo by Carol Boggs.

Pictures of the mounted corneas were taken using an M165FC stereomicroscope, a DFC295 digital microscope camera, and Application Suite V4.4.0 (Leica Microsystems, Buffalo Grove, IL, USA). Those resulting images were used to create corresponding masked images in Inkscape 1.0.2.0 (<https://inkscape.org/release/inkscape-1.0.2/>). Using the Spyder (Python 3.8.5) application, the masks and original cornea images were used as input files in the ommatidia detecting algorithm (ODA) (Currea et al, 2021). The program output yielded measurements of eye surface area, facet counts, and average facet diameter, which were used to calculate average facet area.

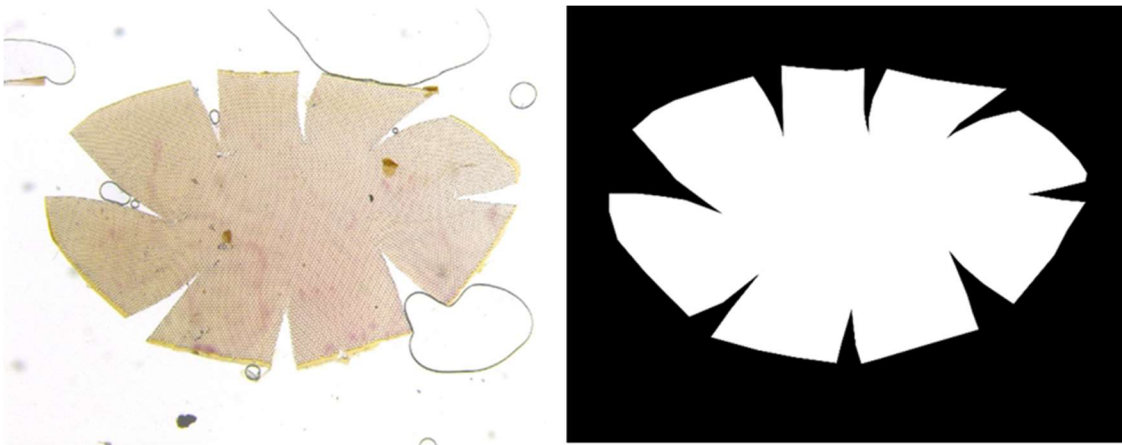


Figure 2.2: Corneas; Image of a cornea mount (left) and an example of a cornea mask made in inkscape (right). The masks were used to guide the measurement software.

### 2.2.7. Wing Reflectance Measurements

Spectral reflectance measurements were taken for three spots on the wings of 101 adult *S. mormonia* in the 400-700nm visible light wavelength range. Figure 2.3 shows that spot A corresponds to the dorsal forewing, spot B to the dorsal hindwing, and spot C to the ventral hindwing. Measurements were taken using a modified Olympus CX-31 microscope (Center Valley, PA, USA). To do so, wings were removed from specimens,

placed on a microscope slide painted matte black, and then the slide was positioned on the stage of the microscope. To illuminate samples, the right eyepiece of the microscope was replaced with a custom-made SMA adapter to which an optical fiber (QP400-1-UV-VIS; Ocean Optics, Dunedin, FL, USA) was attached that carried light from a 20 W tungsten-halogen lamp (HL-2000-HP-FHSA; Ocean Optics). Light was focused from the optical fiber on to samples using an Olympus 4X PlanC N UIS2 objective. The focused beam from the objective covered approximately four scales on the wings of butterflies. To measure the light reflected by samples, the left eyepiece of the microscope was replaced with a custom-made SMA adapter also attached to an optical fiber (QP400-1-UV-VIS; Ocean Optics). The optical fiber led to a Flame-S-VIS-NIR-ES spectrometer (Ocean Optics) that was operated using Ocean View software (Ocean Optics). A metal disc coated with Spectralon was used as the reflectance standard for wing reflectance measurements.

The spectral reflectance data was uploaded into a segmented regression analysis software (Segreg: <https://www.waterlog.info/segreg.htm>) to calculate the wavelength at which the reflectance curve plateaus (95% minimum confidence interval). For points A and B, only the data for 540-670 nm wavelengths were input into the Segreg software, and for point C, only the data from 500-670nm was used. This helped the software to more accurately analyze the data. The wavelengths at which the reflectance curves for each point on the wing stopped increasing, or plateaued, was used to draw conclusions





Figure 2.3: Wing spots for reflectance measurements. Locations on the wings of an adult *Speyeria mormonia* where the three reflectance measurements were taken. Spot A is on the dorsal forewing. Spot B is on the dorsal hindwing; and spot C is on the ventral hindwing. These spots were chosen based on what parts of the wing are visible to potential mates when the individual is basking or not basking. Dorsal wing photo by Carol Boggs.

about the amount of ommochrome pigment present. Ommochromes are the wing pigments responsible for the red-orange coloration in Nymphalidae (Nijhout, 1991 cited in Reed & Nagy, 2005), so since a reflectance curve is just the inverse of an absorption curve, the more red-wavelength-shifted the reflectance curve is, the more ommochrome pigment is present in that spot on the wing.

#### **2.2.8. Data Analysis**

All data analyses were done using Systat 13.2 (Systat Software, San Jose, CA, USA). All data was subjected to a General Linear Model (GLM) and via backwards selection, the best model was chosen based on the lowest Akaike information criterion (AIC) value. If brood was significant in the initial GLM analysis, the dependent variable and its associated independent variables were re-analyzed using a Linear Mixed Model (LMM) with brood assigned as a random effect.

For wing length, the effects of treatment, sex, and sex\*treatment were used as the independent variables.

For the corneal measurements, there were three models each with different dependent variables. The first model's dependent variable was eye surface area, the second model looked at average facet area, and the third model focused on facet count. The independent variables for all three models included treatment, sex, sex\*treatment, brood, and wing length. Wing length was included in the models for eye measurement to determine whether the effects of treatment on eye surface area, facet counts, and facet area were independent of adult body size.

Using the data calculated from the reflectance curves (the wavelengths at which each reflectance curve stops increasing or plateaus), three different models were created

based on which spot on the wing the measurement came from A) dorsal forewing, B) dorsal hindwing, or C) ventral hindwing. Visual inspection of the data revealed the potential of a sexually dimorphic response to the larval starvation treatment, so the three reflectance GLM models were run separately by sex. The independent variables tested were treatment and brood. Further analysis also tested the hypothesis that the means for each of the three wing spots, separated by treatment and sex, were different from each other using a paired t-test in Systat 13.2. If brood was found significant for any of the three wing spots measured for reflectance, indicating a potential heritable effect or an effect of common rearing conditions, a Wilcoxon signed rank test was used to determine if the allocation patterns across broods within a sex were similar across the wing.

## **2.3. Results**

### **2.3.1. Wing Length**

Wing length was used in this experiment as a proxy for determining whether the larval starvation experiment was effective. Larval food stress did have an effect, with treatment butterflies having reduced wing lengths when compared to control individuals ( $F_{1,97} = 36.829$ ,  $p < 0.001$ ; Figure 2.4).

### **2.3.2. Eye Surface Area**

Males had larger eyes than females ( $F_{1,80} = 249.394$ ,  $p < 0.001$ ; Fig 2.5). Larval starvation did not influence eye surface area (Figure 2.5). The effects of sex\*treatment, treatment, and wing length on eye surface area were also insignificant, while brood ( $F_{8,80} = 2.705$ ,  $p < 0.011$ ) did have a significant effect. Using brood as a random variable in an LMM analysis, sex ( $F_{1,80} = 248.053$ ,  $p < 0.001$ ) was still the only significant variable.

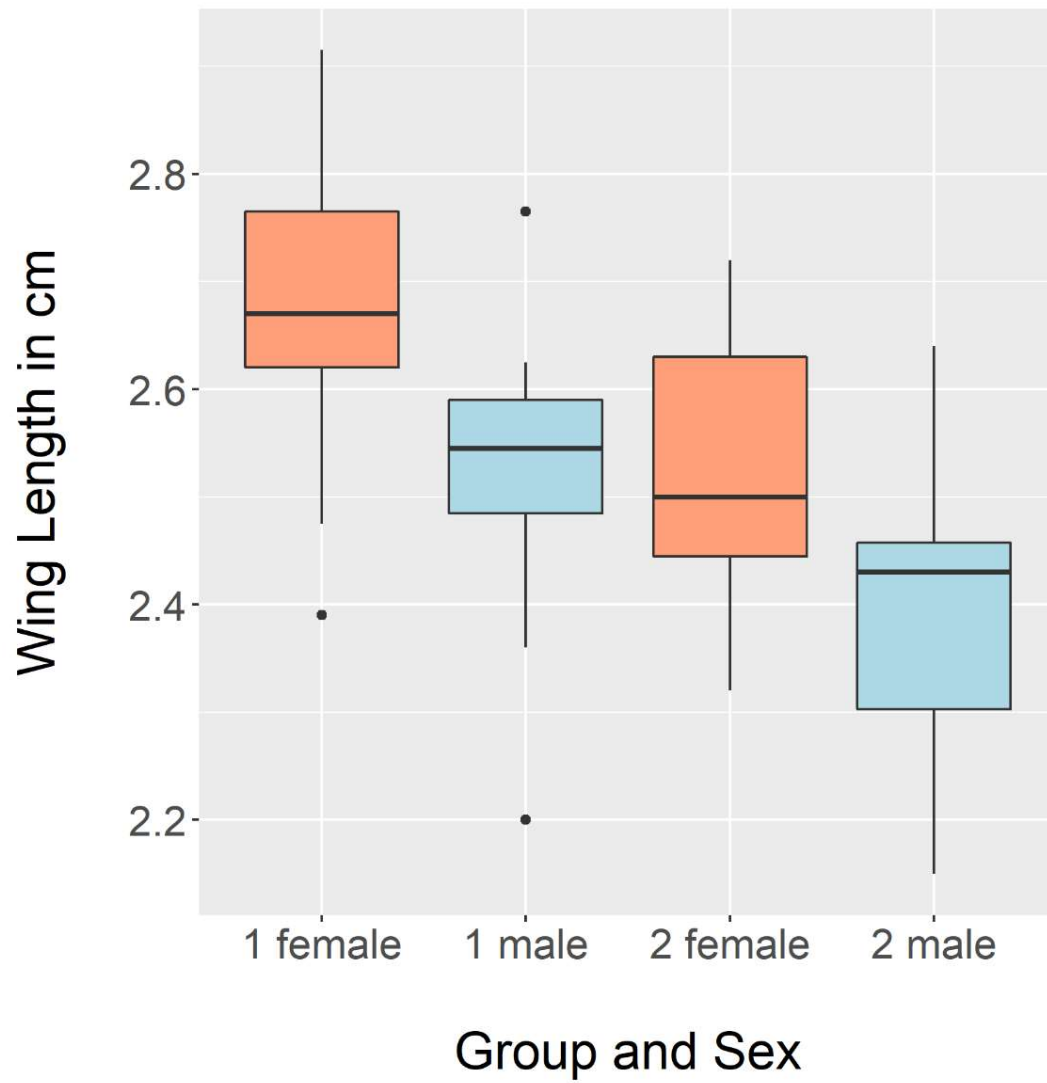


Figure 2.4: The effect of larval food stress treatment and sex on wing length. 1 = control, 2 = larval food stress treatment. Both sex ( $F_{1,97} = 36.829$ ,  $p < 0.001$ ) and treatment significantly affected wing length (see text).

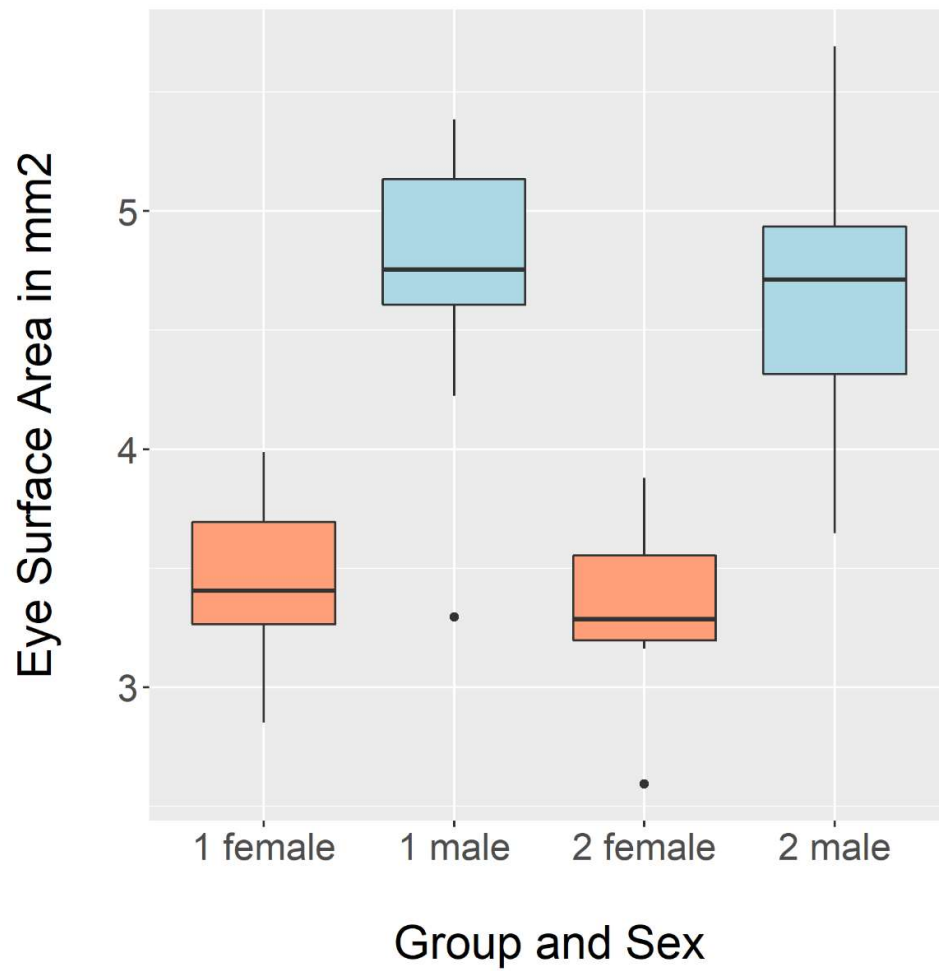


Figure 2.5: The effect of larval food stress treatment and sex on eye surface area. 1 = control, 2 = larval food stress treatment. Sex significantly affected eye surface area, but treatment did not ( $F_{1,79} = 2.104$ ,  $p > 0.050$ ) (see text).



### 2.3.3. Facet Counts

Males have significantly more facets overall than females ( $F_{1,80} = 193.121$ ,  $p < 0.001$ ; Figure 2.6). Sex\*treatment, wing length and treatment did not affect total facet counts, but brood ( $F_{8,80} = 6.129$ ,  $p < 0.001$ ) was significant. An LMM using brood as a random variable confirmed these results, still finding sex\*treatment, wing length and treatment insignificant and sex ( $F_{1,80} = 191.361$ ,  $p < 0.001$ ) significant.

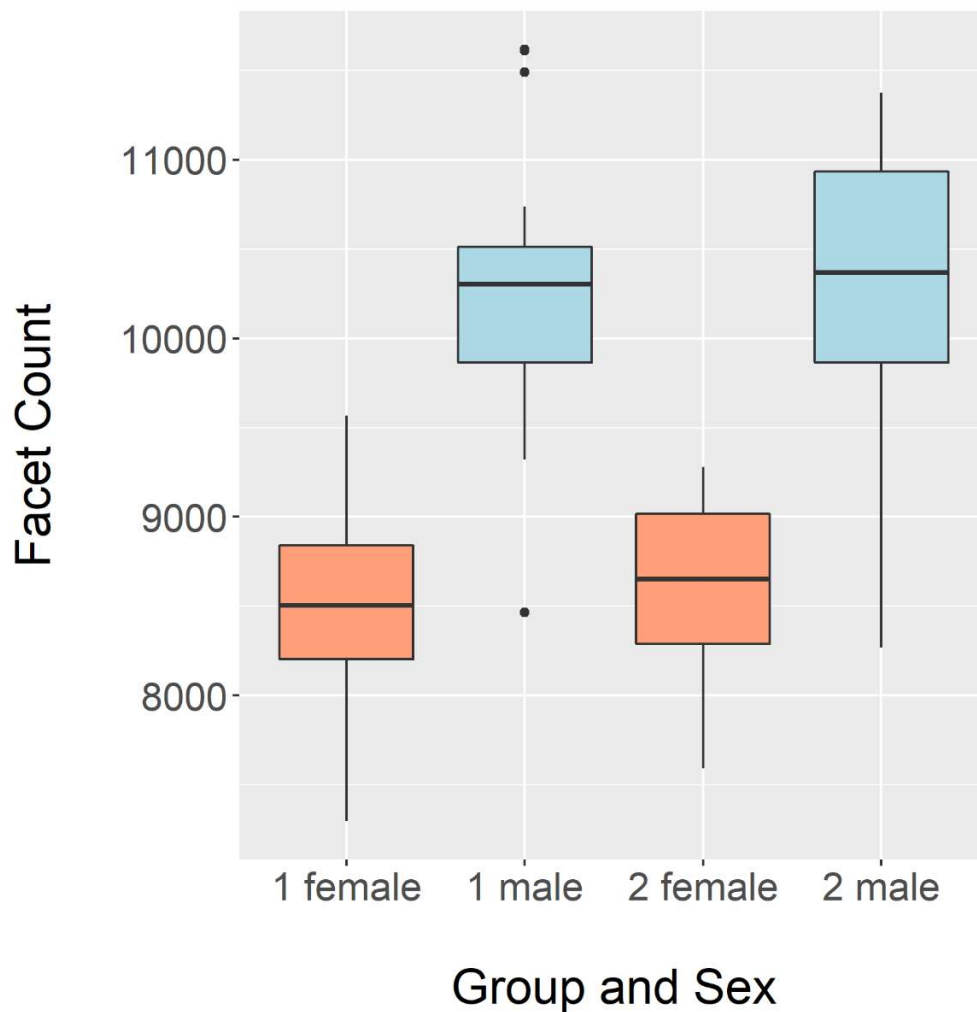


Figure 2.6: The effect of larval food stress treatment and sex on total eye facet count. 1 = control, 2 = larval food stress treatment. Sex significantly affected facet number, but treatment did not ( $F_{1,78} = 0.197$ ,  $p > 0.050$ ) (see text).

#### 2.3.4. Average Facet Area

The best fit model based on backwards selection included treatment ( $F_{1,86} = 4.375$ ,  $p = 0.039$ ), sex ( $F_{1,86} = 100.426$ ,  $p < 0.001$ ), and wing length ( $F_{1,86} = 3.854$ ,  $p = 0.053$ ) (Figure 2.7). Control butterflies had larger facet areas than treatment butterflies and males had larger facet areas than females. The effect of treatment was independent of wing length, meaning facet area did not decrease with treatment solely because treatment yielded smaller butterflies. Sex\*treatment and brood were insignificant.

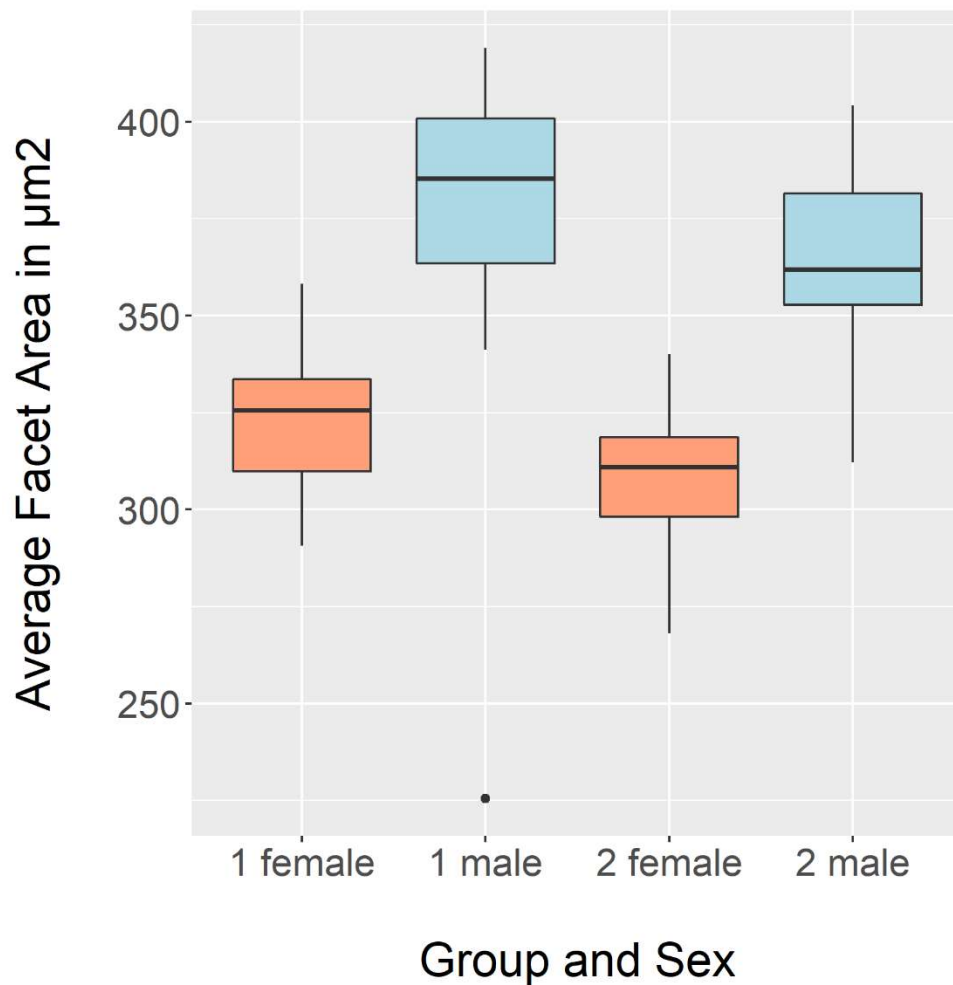


Figure 2.7: The effect of larval food stress treatment and sex on average facet area. 1 = control, 2 = larval food stress treatment. Both sex and treatment significantly affected facet area (see text).

### 2.3.5. Reflectance

Visual inspection of the data suggested a sexual difference in reflectance measurements, so the General Linear Models used to analyze the reflectance data were done separately for each sex looking at the independent variables of brood and treatment.

#### Spot A

On the dorsal forewing, the reflectance curves of males were more red-shifted than females ( $F_{1,87} = 16.946$ ,  $p < 0.001$ ) and treatment females were significantly more red-shifted than those of control females ( $F_{1,24} = 6.9655$ ,  $p = 0.014$ ) (Figure 2.8). Brood was significant for both males ( $F_{9,43} = 3.1339$ ,  $p < 0.006$ ) and females ( $F_{8,24} = 3.2375$ ,  $p = 0.012$ ), and an LMM found that treatment only had a significant effect in females ( $F_{1,24} = 6.9655$ ,  $p = 0.014$ ), not males ( $F_{1,43} = 0.4025$ ,  $p > 0.050$ ).

#### Spot B

For the dorsal hindwing, results show that control females had reflectance curves that were more red-shifted than treatment females ( $F_{1,30} = 14.1306$ ,  $p < 0.001$ ) and that males were more red-shifted than females overall ( $F_{1,80} = 26.791$ ,  $p < 0.001$ ) (Figure 2.9). Brood had a significant effect on wing spot B reflectance for males ( $F_{9,41} = 2.1099$ ,  $p = 0.051$ ) and females ( $F_{8,30} = 6.2529$ ,  $p < 0.001$ ). When brood was included as a random variable, treatment was only significant for females ( $F_{1,30} = 14.1306$ ,  $p < 0.001$ ), not males ( $F_{1,41} = 0.0509$ ,  $p > 0.050$ ).

#### Spot C

Larval food stress had no effect on ventral hindwing reflectance, but, in general, males had reflectance curves that were more red-shifted than females ( $F_{1,78} = 11.857$ ,  $p = 0.001$ ) (Figure 2.10). Brood only had a significant effect on the ventral hindwing spot for

males ( $F_{9,39} = 4.5184$ ,  $p < 0.001$ ), not females ( $F_{8,29} = 1.3191$ ,  $p > 0.005$ ), but when controlling for brood in males, an LMM still showed treatment as insignificant.

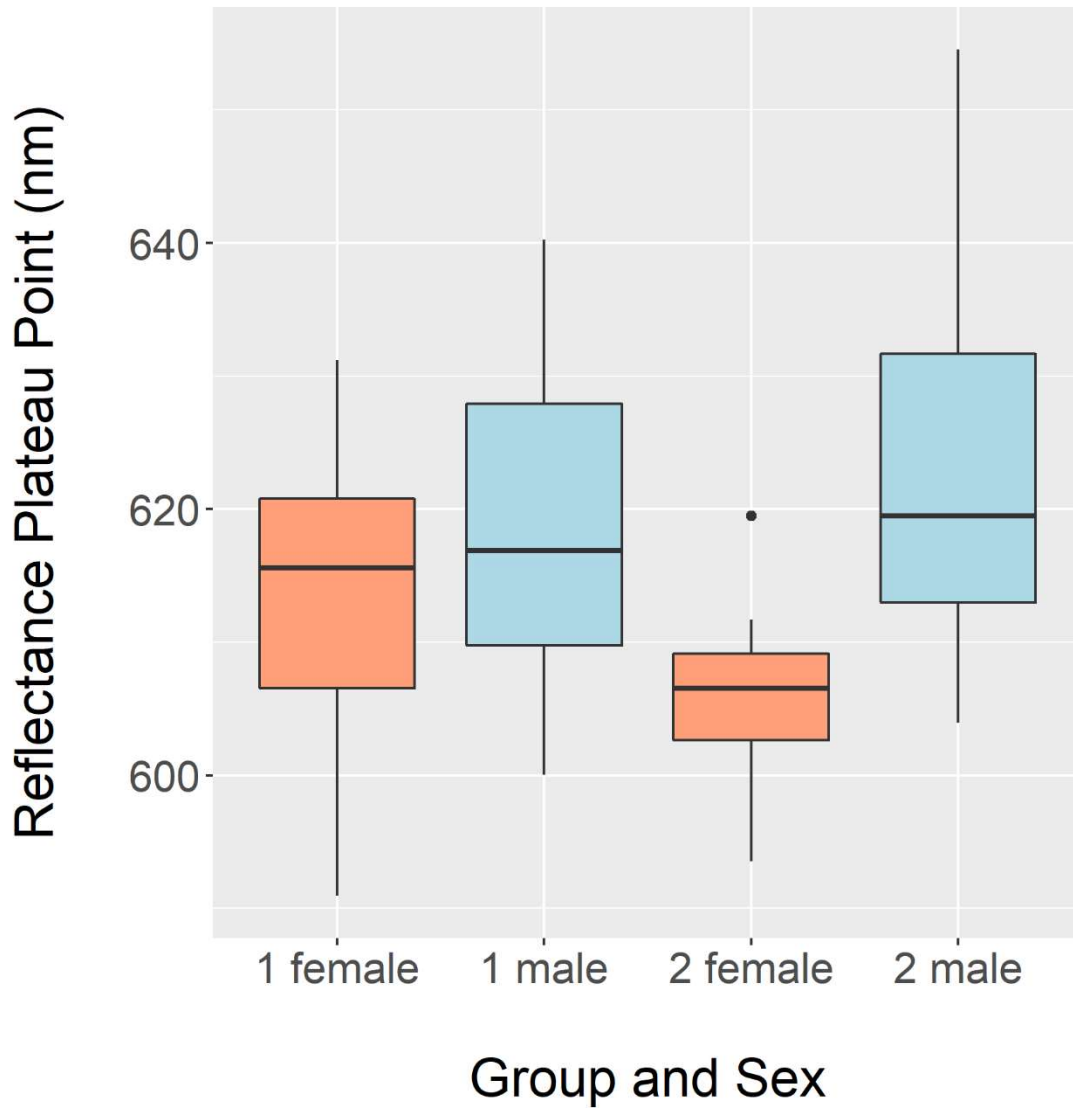


Figure 2.8: The effect of larval food stress treatment and sex on dorsal forewing reflectance (wing spot A). Reflectance is presented here as the wavelength at which the reflectance curves plateau. 1 = control, 2 = larval food stress treatment. Females were significantly affected by larval food stress treatment, but males were not (see text).

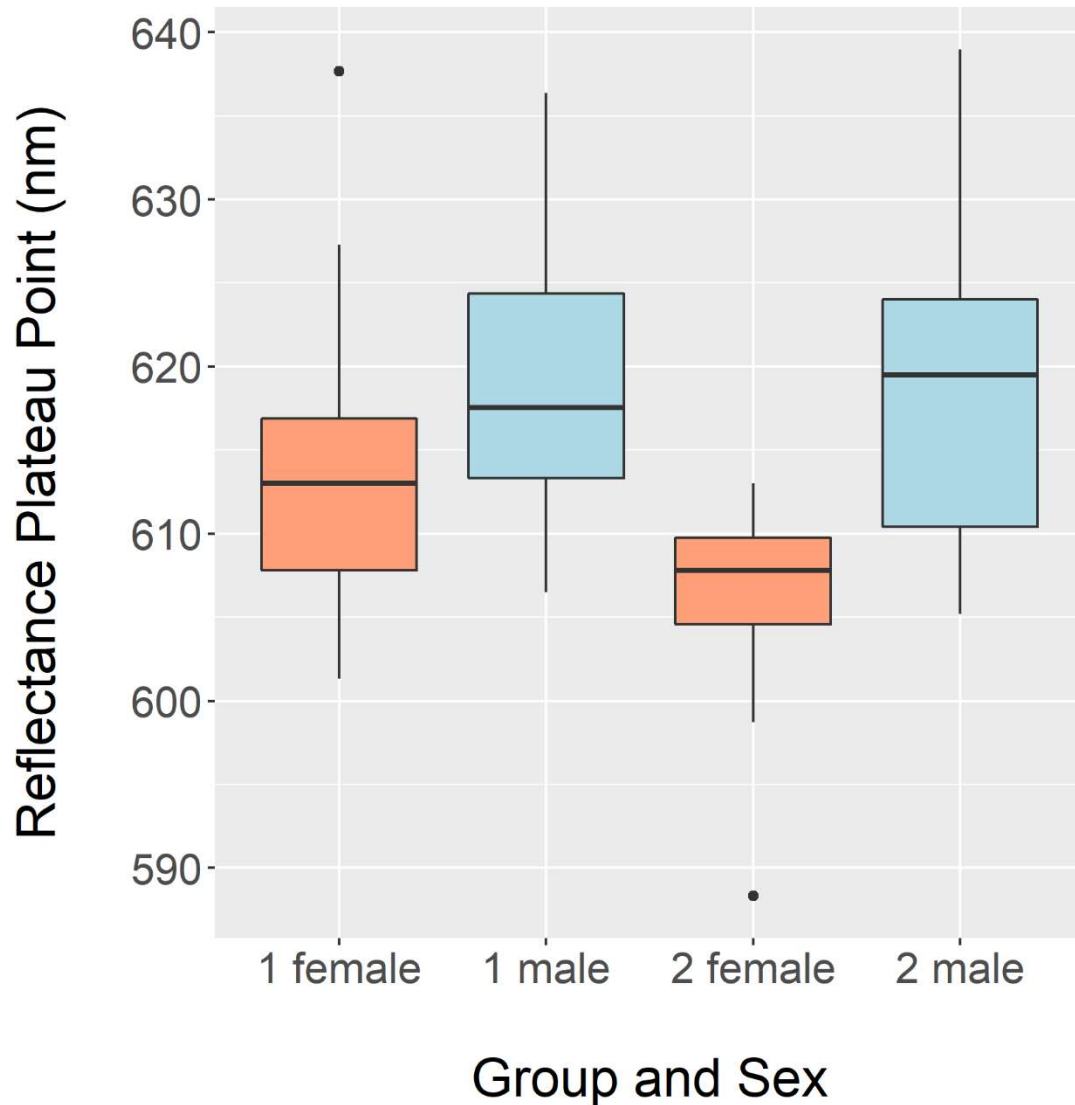


Figure 2.9: The effect of larval food stress treatment and sex on the dorsal hindwing reflectance (wing spot B). Reflectance is presented here as the wavelength at which the reflectance curves plateau. 1 = control, 2 = larval food stress treatment. Larval food stress significantly affected female reflectance curves but had no significant effect on male reflectance (see text).

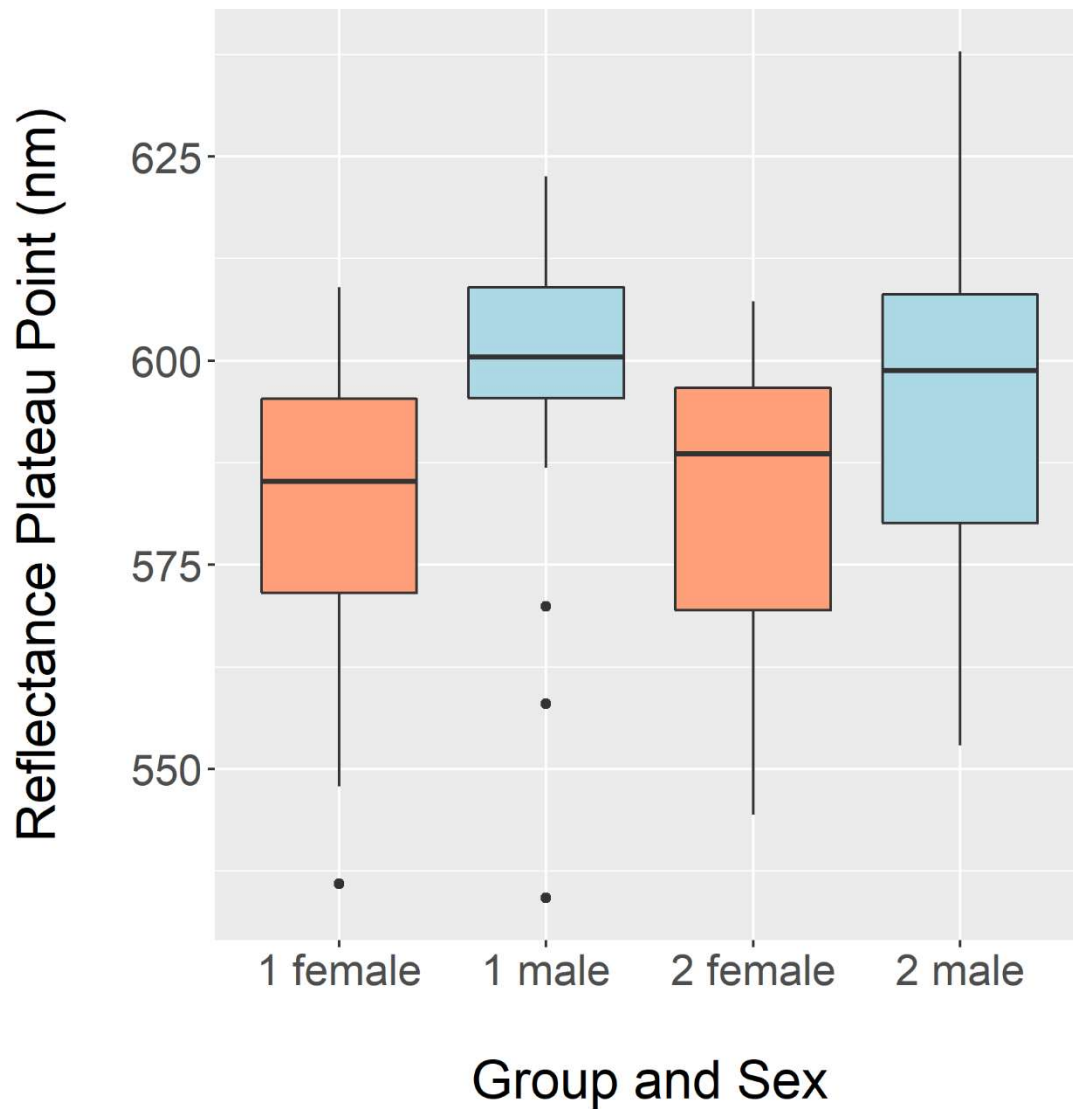


Figure 2.10: The effect of larval food stress treatment and sex on ventral hindwing reflectance (wing spot C). Reflectance is presented here as the wavelength at which the reflectance curves plateau. 1 = control, 2 = larval food stress treatment. Treatment did not have a significant effect on neither males ( $F_{1,39} = 0.7949$ ,  $p > 0.050$ ) nor females ( $F_{1,29} = 0.0372$ ,  $p > 0.050$ ) (see text).

## Reflectance Patterns Across Wing Spots

Paired t-tests showed that reflectance patterns did not differ significantly between spot A and spot B for any combination of sex and treatment. However, reflectance patterns did vary significantly when comparing spot C to both spot A and B (Table 2.1). Results show that dorsal and ventral reflectance patterns significantly differed, whereas dorsal fore- and hindwing patterns did not. A Bonferroni correction was used to adjust for multiple comparisons, and therefore the critical value for p is 0.017.

Table 2.1: Results of a paired t-test for comparisons among the three wing spots within sex and treatment. The wing spots are shown visually in Figure 2.3. Spot A is on the dorsal forewing, B is on the dorsal hindwing, and C is on the ventral hindwing.

Male						
Control				Treatment		
Variable	t	df	p-value	t	df	p-value
A vs. B	0.433	23	0.669	-0.251	31	0.803
A vs. C	4.595	23	0.000	5.219	31	0.000
B vs. C	4.954	23	0.000	5.504	32	0.000
Female						
Control				Treatment		
Variable	t	df	p-value	t	df	p-value
A vs. B	-1.664	25	0.109	0.517	14	0.613
A vs. C	6.056	25	0.000	2.739	14	0.016
B vs. C	7.963	26	0.000	3.162	14	0.007

In a Wilcoxon signed rank test, regardless of sex or spot on the wing, all comparisons performed were found insignificant (Table 2.2).

Table 2.2: Results of a Wilcoxon signed rank test for comparisons among the three wing spots within and between sexes. The wing spots are shown visually in Figure 2.3. Spot A is on the dorsal forewing, B is on the dorsal hindwing, and C is on the ventral hindwing.

	<i>Wing Spots Compared</i>	<i>W-value</i>	<i>p-value</i>
<i>Male vs. Male</i>	A vs. B	11	0.306
	A vs. C	24	0.361
	B vs. C	20	0.384
<i>Female vs. Female</i>	A vs. B	13.5	0.466
	A vs. C	21.5	0.453
	B vs. C	22	0.476
<i>Male vs. Female</i>	A vs. A	17.5	0.472
	B vs. B	10	0.458
	C vs. C	18	0.500



## 2.4. Discussion

In this study, larval food stress resulted in shorter wing lengths when compared to control butterflies, confirming that the methods used in this experiment were successful at applying food stress to treatment larvae (previously demonstrated in Boggs & Freeman, 2005). This result parallels Gonzalez's (2018) results, who found that field-collected 2018 butterflies had smaller wing lengths, with 2018 being a year of early snow melt and drought compared to 2017.

Larval food stress treatment did not affect overall facet counts and eye surface area but did have a significant effect on average facet area, yielding a decreased average facet area in stressed individuals. These results are not consistent with Gonzalez (2018) who demonstrated that 2018 *Speyeria mormonia* had smaller eye surface areas than 2017 butterflies. Gonzalez did not compare facet area or facet count between years, nor did she separate the effect of year by sex, leaving the rest of the data on eye morphology incomparable.

For wing reflectance, spot A and spot B, both of which are on the dorsal side of the wings, only showed a significant effect of larval food stress treatment in females, with control females yielding more red-shifted reflectance curves than stressed females. Larval food stress treatment was insignificant for both males and females on the ventral wing spot (C). In Gonzalez's (2018) experiment, she found that 2018 butterflies had higher dorsal reflectance values overall when compared to 2017 individuals; however, she did not measure ventral reflectance. While the two dorsal wing spots measured were consistent between each study, Gonzalez (2018) measured reflectance using a different method and interpreted the reflectance curves using slope rather than the wavelength at

which the curves plateaued. This leaves the reflectance data of the two studies incomparable.

This experiment tested whether the variation Gonzalez (2018) measured between 2017 and 2018 field collected *Speyeria mormonia* could be explained by variation in larval resources. This study concludes that the differences Gonzalez (2018) found between the two years could potentially be due to 2018 being characterized by limited larval nutrients in the environment; however, the results of this experiment do not provide adequate evidence to support or refute that assertion. Below, I outline other implications for the results of this study.

#### **2.4.1. Eye morphology**

In an apposition compound eye, facets make up the surface of the cornea and each facet covers a single ommatidia (reviewed in Rutowski, 2002; Land & Nilsson, 2012). Ommatidia are structural subunits of a compound eye responsible for visually sampling a piece of a butterfly's visual field (Land & Nilsson, 2012; Ziemba & Rutowski, 2000; Stavenga & Arikawa, 2006). The number and area of facets corresponds to the number and size of ommatidia, so larger or wider facets equal bigger ommatidia and thus increased visual resolution and sensitivity (reviewed in Rutowski, 2002; Land & Nilsson, 2012). In general, a larger eye surface area has been linked to increased mate detection and recognition, but larger eyes must be characterized by more facets, larger facets, or some combination of the two (Rutowski, 2000). Larger facets yield more light-sensitive and resolved vision, but more facets result in a larger visual field with no change in visual resolution (reviewed in Rutowski, 2002; Rutowski, 2000; Ziemba & Rutowski, 2000).

Larval food stress in *Speyeria mormonia* only showed a significant effect on average facet area, with treatment butterflies showing a decrease in average facet area, indicating a decrease in visual acuity relative to control individuals. A change in facet area should have also been accompanied by either a change in eye surface area, facet count, or both, but data analysis returned the effect of larval food stress treatment as insignificant on those two eye characteristics. This discrepancy could be explained by a change in eye surface area and facet count not large enough to be found significant. Figure 2.5 shows that eye surface area in larval stressed treatment butterflies was slightly smaller than control butterflies and Figure 2.6 shows that facet count increased with larval food stress compared to control individuals. A significant decrease in average facet area for stressed butterflies paired with an insignificant increase in facet count could explain the small, insignificant change observed in eye surface area, as well as why average facet area was the only eye characteristic considered significantly affected by larval food stress treatment.

#### **2.4.2. Wing Reflectance**

The spectral reflectance data was interpreted using the wavelengths at which the reflectance curves stopped increasing or plateaued. Those values were compared by treatment and sex, allowing me to draw conclusions about the amount of ommochrome pigment present in the wings relative to the other butterflies. Since a reflectance curve is just the inverse of an absorption curve, the more red-wavelength-shifted the reflectance curve is, the more ommochrome pigment is present in that spot on the wing. For example, males consistently had reflectance curves that plateaued at higher wavelengths than females, reflecting a more red-shifted curve and suggesting a higher relative amount

of ommochrome pigment in male *S. mormonia* wings. To the human eye, more pigmented wings may look ‘more orange’ in appearance (Appendix A).

Larval food stress treatment only affected female dorsal wing reflectance (spot A and spot B), with control females possessing more red-shifted reflectance curves and thus more pigmented wings than stressed treatment females. This differential response to treatment by males and females on wing reflectance reveals a sexual difference in resource allocation strategies related to investment in wing pigmentation.

Based on a paired t-test, reflectance measurements on the dorsal wings of *S. mormonia* significantly differed from ventral reflectance measurements. Data analysis also supports the dorsal side as more chromatic and resource-demanding to produce compared to the ventral side. Results of this experiment indicate that wing pattern development is independent on ventral and dorsal wing surfaces (Darwin, 1871 and Wallace, 1889 cited in Oliver et al, 2009), and the same may be true for fore- and hindwing pattern development (*Delias hyparete*, Jocelyn Liang & Monteiro, 2017; *Bicyclus anynana*, Oliver et al, 2009). Oliver et al (2009) suggests that dorsal wing characteristics are vital for mate recognition and signaling, while ventral wing characteristics are used primarily for predator avoidance. On the dorsal wing surface of *Bicyclus anynana*, forewing pattern showed a differentially more important role in mating than hindwing pattern and was more likely to show sex-based differences than the dorsal hindwing, suggesting the spatial separation of visual signals (Oliver et al, 2009). Independent wing pattern development and separate resource allocation strategies partitioned by dorsal/ventral and forewing/hindwing surfaces could explain why there

was no similar allocation pattern between any of the three wing points and may also support the spatial separation of visual signals in *S. mormonia*.

#### **2.4.3. Brood Effects**

Brood had significant effects on eye surface area, facet counts, and all three wing spots measured for reflectance, suggesting that variation in these measurements could be heritable or due to common rearing conditions, since larvae were separated by broods and thus raised in the same environment. The leaves on the violets used for larval rearing undoubtedly varied in size and quality. A Wilcoxon signed rank test showed no evidence to support similar allocation patterns to wing pigment on the dorsal forewing, dorsal hindwing, and ventral hindwing for a given brood within or between sexes. This implies that the variation in reflectance between the three wing spots does not have a genetic or common environment effect. However, since this study only looked at one dorsal forewing spot, one dorsal hindwing spot, and one ventral hindwing spot, resource allocation patterns may differ not just between dorsal and ventral wing, but between fore- and hindwing as well. Such differences could still be influenced by brood (maternal environmental effects, genetic variation, and/or larval rearing environment), but more evidence is needed to support that hypothesis.

#### **2.4.4. Conclusion**

Lepidopteran visual communication strategies are based on eye and wing morphology. A butterfly's wings act as visual signals to conspecifics and a butterfly's visual system receives and processes those signals. Adult *Speyeria mormonia* males patrol when looking for a mate, meaning they rely on their vision heavily to spot potential mates as they are flying. Once a male detects a female at long-range and pursues her, a

female will assess the male at close range and exercise mate choice (reviewed in Sarto i Monteys et al, 2016; Rutowski, 2000). Patrolling male butterflies are usually the sex characterized by more acute vision, and this experiment shows that, regardless of treatment, male *S. mormonia* have larger eyes, more facets, and larger facets than females, indicating a bigger visual field and more acute vision vital for long-range mate detection. For females, vision is still important for locating oviposition sites, finding food, and recognizing mates, but since most of their need for vision occurs at close range they do not require as acute or sensitive vision as do males (Rutowski, 2000; reviewed in Sarto i Monteys et al, 2016). The relationship seen here between sex and the visual traits measured display a clear sexual difference in how resources are allocated to the visual system of *S. mormonia*, with males investing more resources into the development of their visual system than females (Rutowski, 2000).

For wings, sexual dichromatism is common in many Lepidoptera where males are often the more chromatic or brightly colored sex, such as with *Favonius taxila* (Imafuku & Hirose, 2016), *Maniola jurtina* (Nicoli Aldini, 2019), butterflies in the genus *Junonia* (Oliver & Monteiro, 2010), and as demonstrated here in *Speyeria mormonia*. This study demonstrates that male reflectance curves are more red-shifted when compared to females, suggesting that males have relatively higher levels of ommochrome pigment deposited in the measured wing spots, but females, based on wing length measurements, are consistently larger than males. Many studies link increased female body size to increased fecundity, suggesting a female's investment into their overall size may be an indicator of potential reproductive success to males (Boggs & Freeman, 2004; Berger et al, 2008; Bauerfeind & Fischer, 2007). These findings suggest that males invest

comparatively more resources than females in wing pigmentation, while females invest more in their overall wing and body size, important for the maintenance and production of eggs (Boggs & Niitepõld, 2015). Both wing size and color are considered vital visual signals during courtship (e.g., Robertson & Monteiro, 2005; Davis et al, 2007; Teodora et al, 2007; Pinzari & Sbordonì, 2011)

Under larval food stress treatment, *Speyeria mormonia* females seem to differentially sacrifice pigment, while males sacrifice visual acuity. Larval-derived nitrogen is a limiting nutrient in the diet of many Lepidoptera and is vital in both the generation of pigment and the production of eggs (color production and mechanisms reviewed in Kemp & Rutowski, 2011; Boggs, 1997). These findings thus reveal the potential of a female-specific tradeoff, where females faced with limited larval nutrients may sacrifice wing pigmentation to maintain allocation to reproduction (i.e., maintenance and production of eggs), as well as a male-specific tradeoff, where larval stressed males may sacrifice increased visual acuity to maintain the wing ornamentation needed for successful mating and courtship.

Larval dietary restriction studies can reveal how larval-derived nutrients are allocated to adult morphology and life history traits (Boggs & Freeman, 2005; Boggs & Niitepõld, 2015; Johnson et al, 2014; Bauerfeind & Fischer, 2005; Reim et al, 2019; Lebeau et al, 2018). The next step in this line of research would be to reanalyze the corneas included in this study to specifically look at the average facet area in each region of the eye to determine if zones of acuity are present in *S. mormonia*, as Gonzalez (2018) suggests. Zones of acuity are regions of an eye characterized by larger facets and thus increased visual resolution in that specific eye region, usually corresponding to some

aspect of an insect's behavior and lifestyle (reviewed in Rutowski, 2002; Rutowski, 2000; Ziemba & Rutowski, 2000; Marshall & Arikawa, 2014). If larval food stress treatment results in a differential effect on eye regions, conclusions could be drawn about how limited larval nutrients affects certain life history traits based on how the eye regions corresponding to behaviors related to those life history traits respond to food stress. The cornea images from the Gonzalez (2018) study should also be remeasured using the methods outlined in this thesis, simply for comparative purposes between the two experiments. For the wings, ommochrome pigment extractions from the control and treatment *S. mormonia* wings included in this study could validate how pigment quantity and composition compares to the reflectance data. Furthermore, a study looking at the possibility of spatial separation of visual signals on the wings of *S. mormonia* could shed more light on the resource allocation patterns and tradeoffs revealed in this thesis.



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## **APPENDIX A: DIGITAL WING IMAGING**

We acquired digital images of butterfly wings using an M165FC stereomicroscope, a DFC295 digital microscope camera, and Application Suite V4.4.0 (Leica Microsystems, Buffalo Grove, IL, USA).

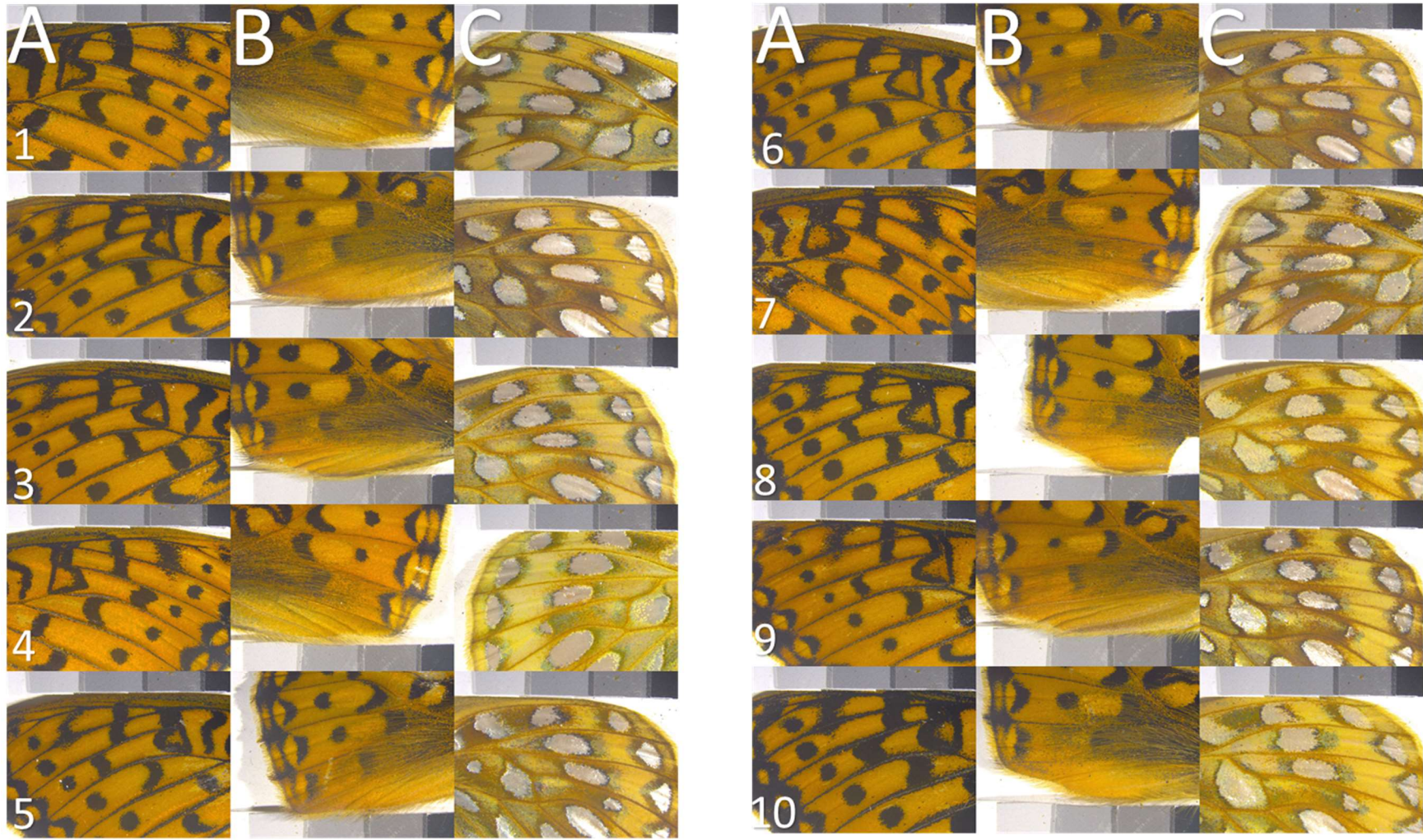


Figure A.1: Control Male Wings. Column A = dorsal forewing, B = dorsal hindwing, and C = ventral hindwing. Each row (1-10) signifies a different individual male butterfly. Greyscale bars are included in each picture.



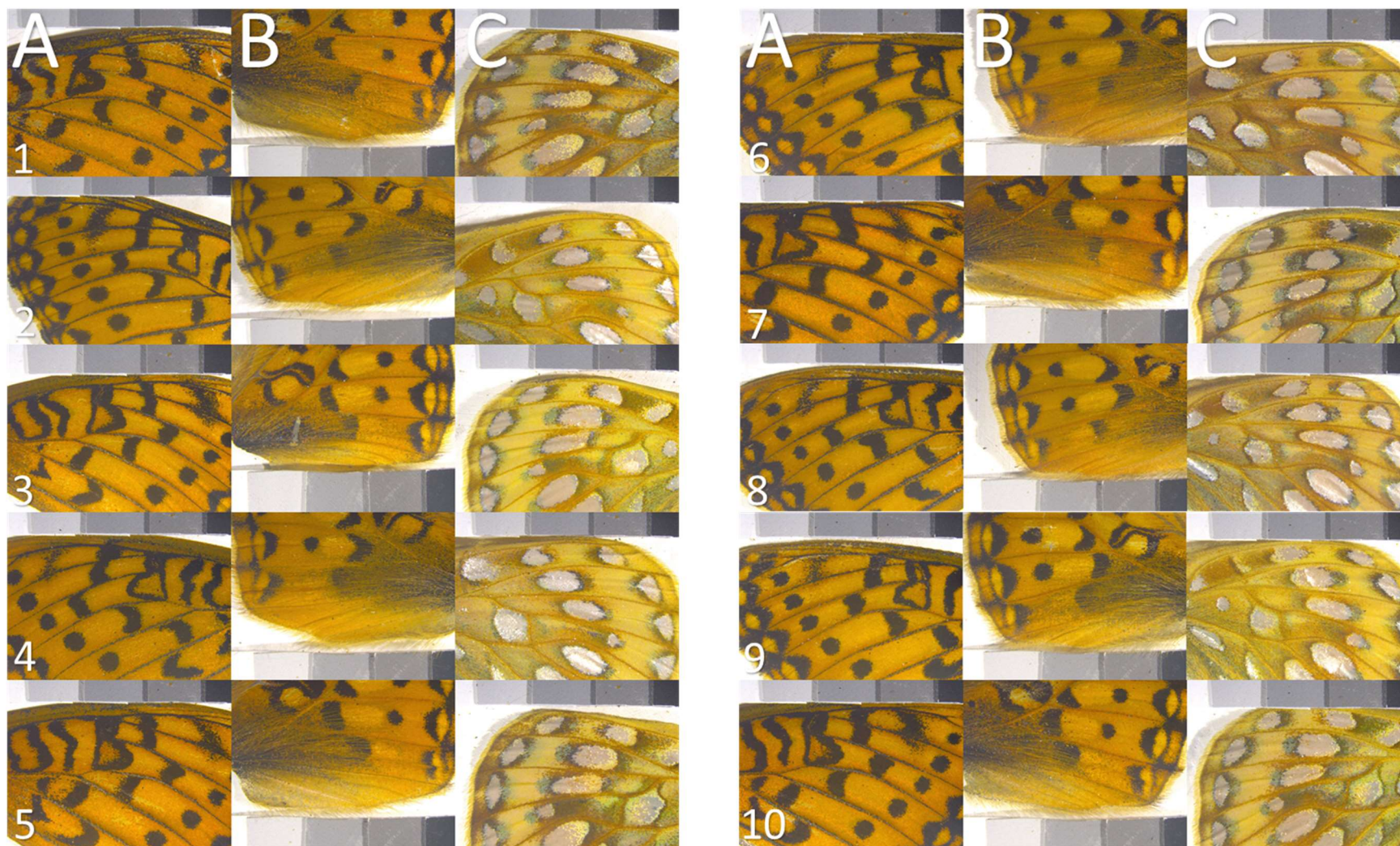


Figure A.2: Treatment Male Wings. Column A = dorsal forewing, B = dorsal hindwing, and C = ventral hindwing. Each row (1-10) signifies a different individual male butterfly. Greyscale bars are included in each picture.



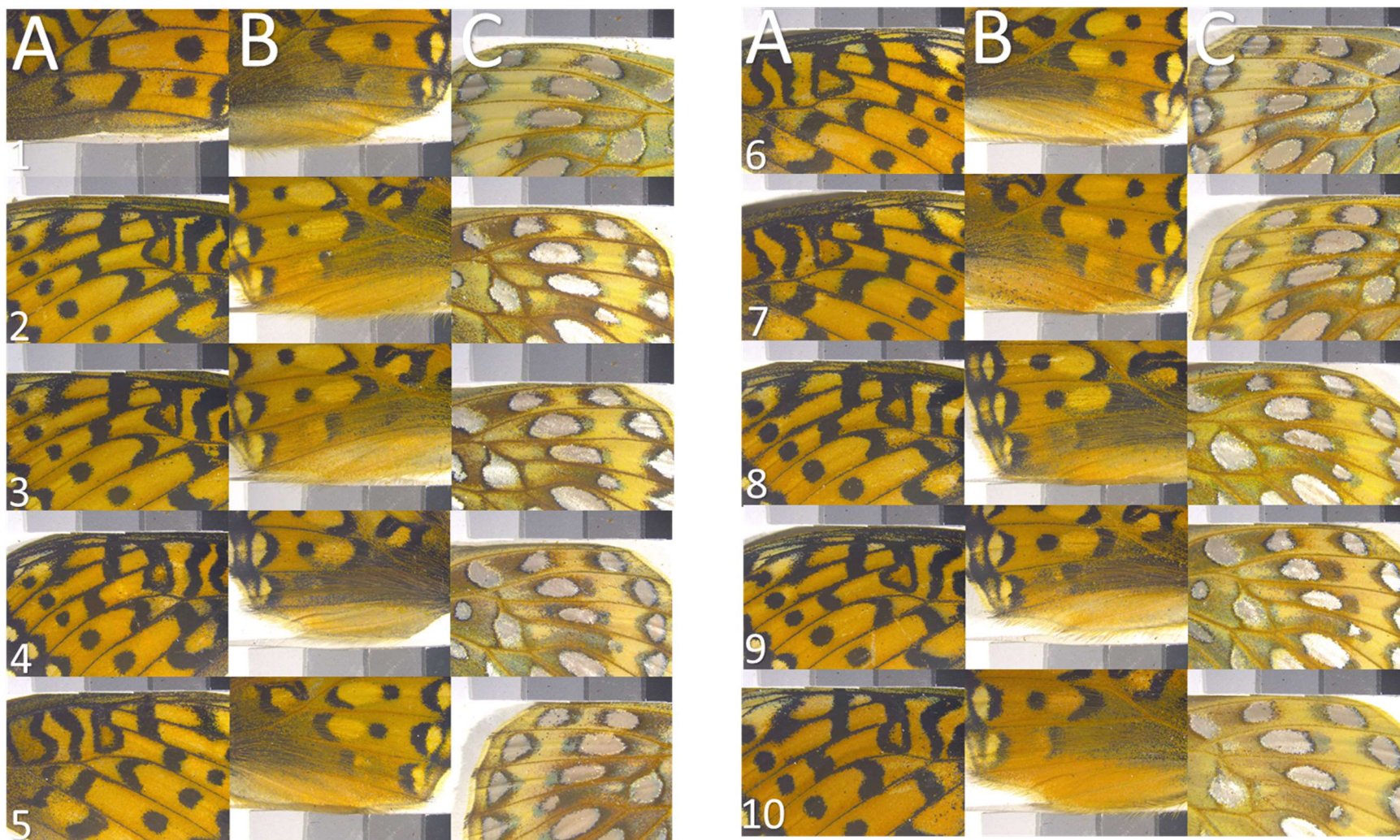


Figure A.3: Control Female Wings. Column A = dorsal forewing, B = dorsal hindwing, and C = ventral hindwing. Each row (1-10) signifies a different individual female butterfly. Greyscale bars are included in each picture.



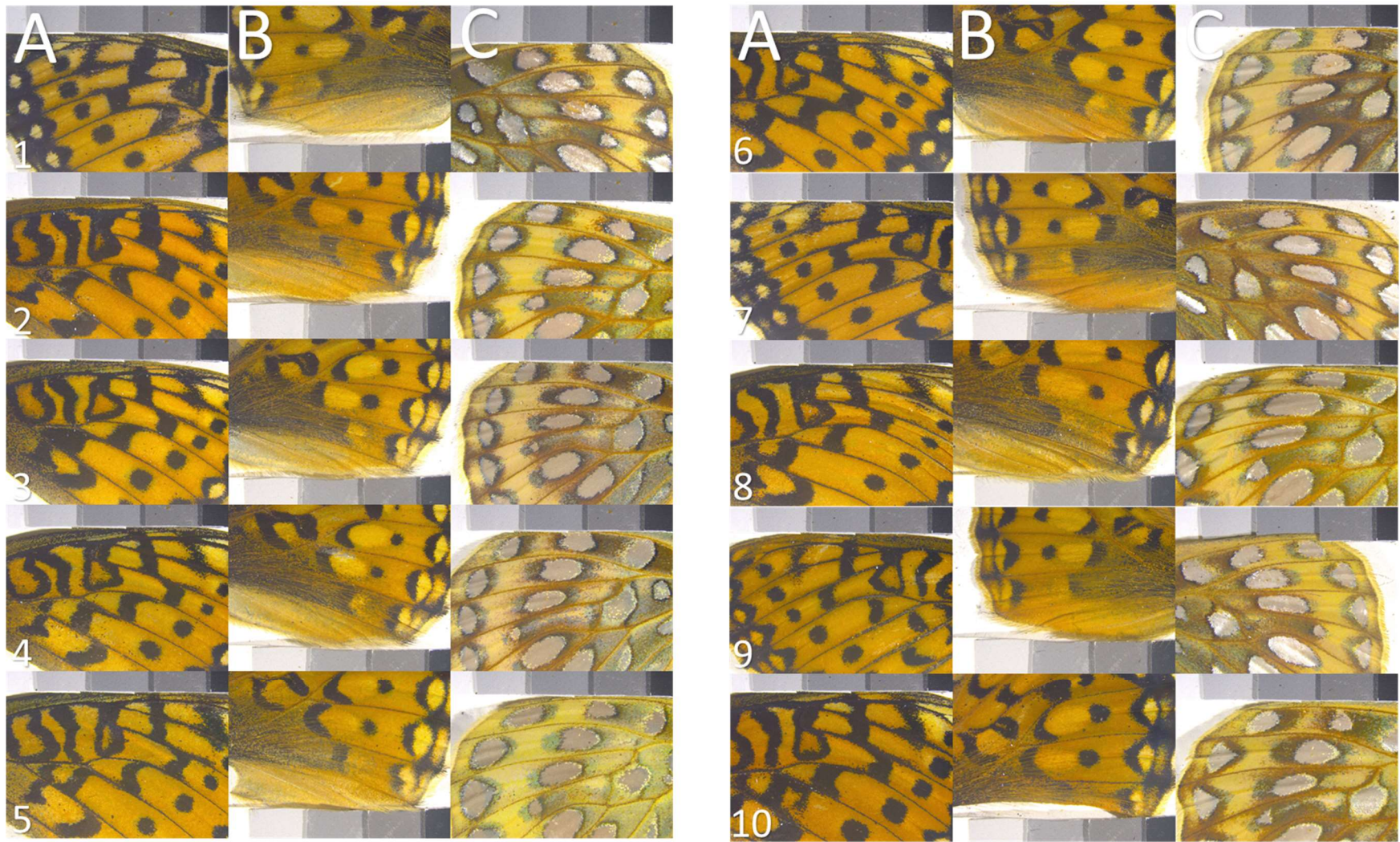


Figure A.4: Treatment Female Wings. Column A = dorsal forewing, B = dorsal hindwing, and C = ventral hindwing. Each row (1-10) signifies a different individual female butterfly. Greyscale bars are included in each picture.