

The Visual Ecology of *Speyeria mormonia*

by

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Bachelor of Arts
The University of Pennsylvania, 2016

Submitted in Partial Fulfillment of the Requirements

For the Degree of Master of Science in

Biological Sciences

College of Arts and Sciences

University of South Carolina

2018

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DEDICATION

I would like to dedicate this thesis to my parents, grandparents, and my friends who were my emotional support during the toughest moments of this entire process. I love you all and I couldn't have finished this without your prayers, phone calls, and coffee runs.

ACKNOWLEDGEMENTS

I acknowledge Dr. Soumitra Goushrouy, Dr. Shannon Davis, and the faculty at the IRF facility of the medical school who helped me troubleshoot the protocols for the microscopic imaging of butterfly eyes. I also acknowledge Dr. Nate Morehouse for sharing his template with me which facilitated the completion of the computational model for the spectral sensitivity of *S. mormonia*. I acknowledge Dr. Carol Boggs for her research guidance and her research assistants, especially Lydia Fisher, Emma Wagner, Hannah Whitton, Skylar McDaniel, and Malia Olson for helping me collect and care for butterflies out in the field and at the Rocky Mountain Biological Laboratory. I acknowledge Rebecca Lucia for helping me care for the butterflies and take photographs of them. I acknowledge Rachel Steward for providing a variety of helpful resources for this research project. Finally, I acknowledge Luke Havens, our research specialist, who helped me perform the electroretinography experiments.

ABSTRACT

Variations in environmental factors such as temperature, precipitation, and day length during larval development are known to affect morphological traits in butterflies related to their visual ecology, including eye size and wing color. These vision-related traits are important for the ability of diurnal butterfly species to detect mates, especially at long distances. Thus, changes in environmental conditions may result in phenotypic modifications to butterflies which may alter their visual ecology and subsequently, their reproductive fitness. To study the interaction of phenotypic plasticity and visual ecology in the Mormon Fritillary, *Speyeria mormonia*, I set up a natural-laboratory experiment at the Rocky Mountain Biological Laboratory (RMBL) and collected butterflies from 5 different sites across an elevational gradient, spanning approximately 610 meters during two field seasons. I considered elevation to be a proxy for several shifting microclimate features, including temperature and precipitation. My first goal was to determine whether there was a relationship between elevation and natural variations in the dorsal wing chromaticity, eye surface area, or wing length (a proxy for body size) of male and female adult-stage *S. mormonia* from the study populations. In the case that I did find natural variations in wing chromaticity, my second goal was to use computational models to evaluate whether *S. mormonia* can discriminate between the different “oranges” (quantified using chromaticity values) displayed on the wings of their conspecifics. Across elevations, I found that females tended to be larger than males and that males tended to have larger eyes than females. I also found that butterflies from lower

elevations had longer wings than individuals from higher elevations. Males had greater and more variable wing wear scores than females, and more perceivable variations in dorsal forewing and dorsal hindwing chromaticity, the values of which were linked to wing wear. The results also suggest that *S. mormonia* may have sex-dependent dynamics in the investment of nutrient resources. Females had longer wing lengths and more consistent wing wear scores than males across elevations. Longer wings are useful for female butterflies to maintain more efficient flight maneuverability while carrying heavy egg-loads during oviposition. Males, however, had larger eyes and more variable wing wear scores than females. Males may have larger eyes than females because vision is more important for mate location by males than it is for oviposition site location or mate-recognition by females. This may mean that while females are investing in producing larger bodies to optimize fecundity, males are investing nutritional resources into optimizing mate-seeking ability (i.e. patrolling) to maximize the number of copulations they can engage in. Finally, intersexual trends in wing wear scores suggest that there are different degrees of protandry occurring across the elevational gradient, likely because of sex-dependent reactions to shifts in local environmental factors.

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LIST OF SYMBOLS

A_i	Absorbance of photoreceptor i
e_i	Estimate of noise originating from photoreceptor i
$I(\lambda)$	Irradiance in units of photon flux ($\mu\text{mol}/\text{m}^2\text{s}^{-1}\text{nm}^{-1}$) at a given wavelength
q_i	Photon catch of photoreceptor i
$R^B(\lambda)$	Reflectance of the background stimulus at a given wavelength
$R(\lambda)$	Reflectance of a color stimulus at a given wavelength
ω	Webber fraction, defined as the standard deviation of the noise for a given photoreceptor

LIST OF ABBREVIATIONS

DF chroma	Dorsal forewing chromaticity
DH chroma.....	Dorsal hindwing chromaticity
EESA.....	Estimated Eye Surface Area
RL	Rhabdom length
RMBL	Rocky Mountain Biological Laboratory
ΔS^t	Color discriminability
WL	Wing length
WW	Wing wear

CHAPTER 1

INTRODUCTION

Visual ecology is a field of study which investigates how organisms acquire visual information and how their visual systems change or become specialized in response to selection pressures experienced in their natural environments (Cronin 2014). Butterflies are a classic model in visual ecology because they tend to have excellent color vision and colorful wings. Butterflies have apposition compound eyes, which are composed of thousands of subunits called ommatidia. Each ommatidium is covered by a facet lens and samples light from a small area from the surrounding environment. The integration of information from multiple ommatidia produces a “pixelated” image of the world, where each ommatidium is equivalent to one pixel. Wider facets not only increase sensitivity, but also improve visual resolution by decreasing the blurring effects of diffraction (Land and Nilsson 2012). Male butterflies typically have larger eyes than females do, presumably to detect potential mates more efficiently. Males even have exclusive areas of greater relative visual acuity and sensitivity along the frontal region of their eyes, called acute or “love” zones (Rutowski 2000). These “love” zones are characterized by larger facets, longer rhabdoms, and smaller interommatidial angles. Butterflies also have notoriously colorful wings, the hues of which are produced by the interaction of light with pigments on the wing scales (Stavenga et al. 2014). These wing colors tend to be more vibrant in whichever sex the choosier sex is evaluating during

courtship. For the most part in butterflies, females tend to be the choosier sex and males tend to be the more brightly colored sex (Robertson et al. 2005; Kirkpatrick 1982)

The visual sensory modality is known to be important for mate detection in diurnal butterflies, especially when they need to detect mates from long distances (Hidaka 2010; Li et al. 2017). Given the importance of visual cues to butterflies for conspecific recognition, changes in their wing color or wing or eye morphology may alter their visual ecology by enhancing or diminishing the efficiency of their mating signals (i.e. the wing colors) or the efficiency with which the receiver can perceive such signals (White et al. 2015). One of the ways that butterfly wing and eye morphology might change is through phenotypic plasticity, which is defined as modifications of phenotypes in response to environmental factors which do not come about as a result of genetic changes. There is evidence of phenotypic plasticity in wing morphologies and eye sizes driven by environmental conditions in several species of butterfly, including *Bicyclus anynana* and *Precis coenia*. These butterfly species are known to develop seasonal wing and eye morphologies that are influenced during larval or pupal development periods by environmental factors such as temperature, daylength, and precipitation patterns (Smith 1993; Van Bergen et al. 2017). The quantity or quality of larval food sources, which are directly related to the aforementioned environmental factors, can also promote seasonal variations in morphological aspects of butterfly visual systems, including eye size or brain size (Merry et al. 2011; Snell-Rood 2014; Montgomery et al. 2016).

In this study, I asked if variations in eye size, body size, or wing color occur along an elevational gradient due to differences between the microclimates at each site. To address this question, I set up a natural laboratory experiment at the Rocky Mountain

Biological Laboratory (RMBL) using a study population of local *Speyeria mormonia*. Butterflies were sampled from 5 different elevation sites spanning approximately 610 meters. This species of butterfly is protandrous and belongs to the family Nymphalidae. Protandrous species are characterized by males that emerge from the pupal stage earlier than female conspecifics. *S. mormonia* are typically found along mountainous ranges in open grasslands containing various species of *Compositae*, which is their adult food source, and *Viola spp.* host plant which is their larval food source. The various microclimates within the native range of *S. mormonia* makes it so that their larvae experience different environments during development based on the location at which eggs were laid. My first goal was to quantify inter-elevational differences between traits in *S. mormonia* that are relevant to their visual ecology, including wing color, eye size, and wing size. I obtained wing length and eye measurements by using image analysis software on scaled microscopic images of the butterflies. I used chromaticity, defined as the linear slope in reflectance between the wavelengths of 500 and 600 nm, as a proxy for how “orange” the wing colors were. Based on existing scientific literature, I had reason to believe that the varying microclimates at each elevational site would yield much natural variation in wing color and eye morphology. The second goal of this study was to determine whether the *S. mormonia* of these sample populations can distinguish between the naturally occurring variations of “orange” color on the wings of conspecifics within their natural range using output values from a computational model. Results from the computational model indicate whether differences in wing color effect the efficiency with which members of this species may detect each other visually.

I expected that males would have larger eyes than females, but that females would have longer wings (a proxy for body size) than males regardless of the elevation from which they were collected. These sexual dimorphisms are common in most protandrous butterfly species (Rutowski et al. 2000). If wing color, as a product of diet-dependent wing pigment production, were primarily affected by the availability of larval food resources (Lindstedt et al. 2010), I expected that low elevation butterflies would be more chromatic, given that precipitation and temperature both generally tend to have negative relationships with elevation, but are directly related to plant yield (Laiolo et al. 2013). However, snow-pack tends to be greatest at higher elevations in this region, which increases the local soil moisture. Therefore, the host plant abundance may be greatest at higher elevations, so there is an alternate possibility that butterflies at higher elevations will be more chromatic instead. I also expected *S. mormonia* to be able to discriminate between a wide variety of “orange” colored wings due to the functional importance of this color as a cue for mating behavior (Carol Boggs, personal communication).

Finally, although the examples I have offered so far involve trait modifications as outcomes of phenotypic plasticity, any observable variations in morphology between these *S. mormonia* populations could be at least partially influenced by local adaptation. No prior work has been done on the population genetics of the *S. mormonia* populations within Gothic County, CO, so I am unsure of the degree of within population variation or gene flow occurring between the butterfly communities. The average traveling dispersal distance of *S. mormonia* is 170 meters (Boggs 1987), but this is subject to change from year to year and includes non-linear (“zig-zag”) displacement. The average linear distance between my collection sites was roughly 3,000 meters, but the higher elevation

sites were generally closer to each other than the sites at lower elevations. The approximate Wrightian neighborhood distance, or geographic space where *S. mormonia* are more likely to be directly related to each other (Watt et al. 1977; Wright 1946), is roughly 875 meters (Dr. Boggs personal communication). Therefore, given the variation in dispersal distances within *S. mormonia* populations and the dearth of scientific literature on the population genetics of *S. mormonia* from this region, any phenotypic variations observed between butterflies cannot be linked to phenotypic plasticity with confidence at this time.

CHAPTER 2

MATERIALS AND METHODS

2.1 STUDY ORGANISMS

2.1.1 Study site and field collection

I collected *S. mormonia*, with the help of research assistants, from 5 different elevations near the Rocky Mountain Biological Laboratory in Crested Butte, CO. Collection took place during two field seasons; the first during mid to late August of 2017 and the second during late July through early August of 2018. The collection sites were located at 2743, 2865, 3048, 3200, and 3353 meters above sea level (Table 2.1). In 2017, I net-captured butterflies and transported them to the lab. In the lab, I fed butterflies 25% sugar water. I photographed all butterflies on graph paper with subdivisions of known length which I used for scale, using a Canon Powershot A4000 camera. I photographed the butterflies within 24 hours of their being captured. I then released the majority of the captured butterflies. Butterflies collected in the 2018 field season were caught using the same protocol, but none were released. They were fed, stored in small envelopes, placed in a box, and shipped overnight to the University of South Carolina. Once they arrived to their destination, I labeled, fed, and moved the butterflies to a moist,

chilled incubator under full spectrum light (20" Aqueon modular LED Aquarium light) and a light:dark cycle of 12h:12h. The temperature schedule was set to 19°C by day and 17°C by night. I photographed the butterflies using a Nikon D5000 camera and measured their wing lengths using electronic calipers. For spectral reflectance measurements, I used a subset of wings from male and female *S. mormonia* collected in 2017 and the wings from all the butterflies collected in 2018.

Table 2.1: List of latitude and longitude coordinates corresponding to the field collection sites visited in 2017 and 2018.

Approximate site elevation (meters above sea level)	Coordinates for 2017 field season	Coordinates for 2018 field season
2743.2	38°51'40.10"N, 106°55'03.03"W	38°53'43" N , 106°53'25"W
2865.12	38°56'54.28"N, 106°59'04.03"W	38°56'51" N, 106°59'04"W
3048	38°58'11.62"N, 106°59'26.65"W	38°58'16.47"N, 106°59'34.40"W
3200.4	38°58'11.02"N, 106°59'00.80"W	38°58'29.12"N, 106°58'40.70" W
3352.8	38°58'29.65"N, 106°58'42.78"W	38°58'16.47"N, 106°59'34.40"W

2.1.2 Lab rearing *S. mormonia*

S. mormonia from RMBL were transferred as first instar larvae to the University of South Carolina in glass 1.5 mL vials in the fall of 2017. They were held at 2°C for 5 months and brought out of diapause in the spring of 2018. After diapause, larvae were raised on host plants, *Viola soraria*, under full spectrum light on a 16h:8h light:dark cycle and a 27°C:15°C temperature cycle. Pupae were weighed, logged into our butterfly records, and placed into individual plastic cups with moistened paper towels and covered

with mesh. Pupae were spritzed with water daily to maintain a rearing environment humid enough to promote successful wing extension upon eclosion as adults. Upon eclosion, each butterfly's emergence date, wing length, and sex were recorded. For 2-4 weeks post-eclosion, adult females were transferred into an incubator with a light:dark cycle of 16h:8h under full spectrum light and temperatures at 27°C:15°C day:night. They were fed 25% sugar water twice a day. Males were placed in groups of 3-4 into separate cylindrical plastic containers with moistened paper towels inside to maintain humidity levels. They were fed 25% sugar water once a day. The eyes of a subset of males and females from this lab-reared population of butterflies were excised and fixed for histology within 24 hours after death.

2.2 EYE AND WING MORPHOLOGY

2.2.1 Wing wear scores

Wing wear scores are categorical scores assigned to butterflies estimating the degree to which their wings have been worn. The score ranges from 1, for freshly emerged butterflies with moist wings without any wear, to a score of 5, for butterflies which have very noticeable scale loss and significant wing tearing that goes beyond the borders (Boggs 1987). A score of 2 is given to adults with dry wings but no wear. Up to 1.5 points, in increments of 0.5, are then added for scale loss (color loss), and an additional 1.5 points are added, in increments of 0.5 for wing tearing/loss. Wing wear can be used as a proxy for age. Wing wear scores were assigned to all field-caught butterflies from 2017 and 2018.

2.2.2 Wing length measurements

Wing length is the linear distance from the basal hinge of the forewing to the tip of the forewing. I measured the wing lengths of field-caught *S. mormonia* collected during the first field season by making linear measurements of wings from photographs of butterflies lying on graph paper with subdivisions of 0.1 cm, which I used for scale. I measured the wing lengths of *S. mormonia* from the second field collection season using electronic calipers.

2.2.3 Estimated eye surface area

I estimated the surface areas of eyes from *S. mormonia* by taking linear measurements of the eye height and 3 different eye radii from scaled images of *S. mormonia* heads. I used the subdivisions on the graph paper background for scale, as explained above. Given that the eye height measurements were consistently less than 2 times the average radius value, I modeled the eyes as oblate spheroids. I plugged the linear measurements into the formula corresponding to the surface area of an oblate spheroid to estimate eye surface area (Rutowski 2000).

2.2.4 Hematoxylin and Eosine Staining and imaging of *S. mormonia* rhabdoms

I obtained average rhabdom lengths by performing light microscopy on images of cryosections of eyes from lab-reared *S. mormonia*. Within 24 hours after death, I excised butterfly heads, cut them in half, and fixed them in 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M Phosphate-buffered saline (PBS) overnight. After fixation, I washed the eyes with PBS three times. To cryoprotect the samples, I washed them with

10%, 20%, and 30% sucrose in 0.1M PBS, for 1 hour per wash. After this, I placed the eyes individually into plastic molds, embedded them in OCT gel (Sakura Tissue Tek

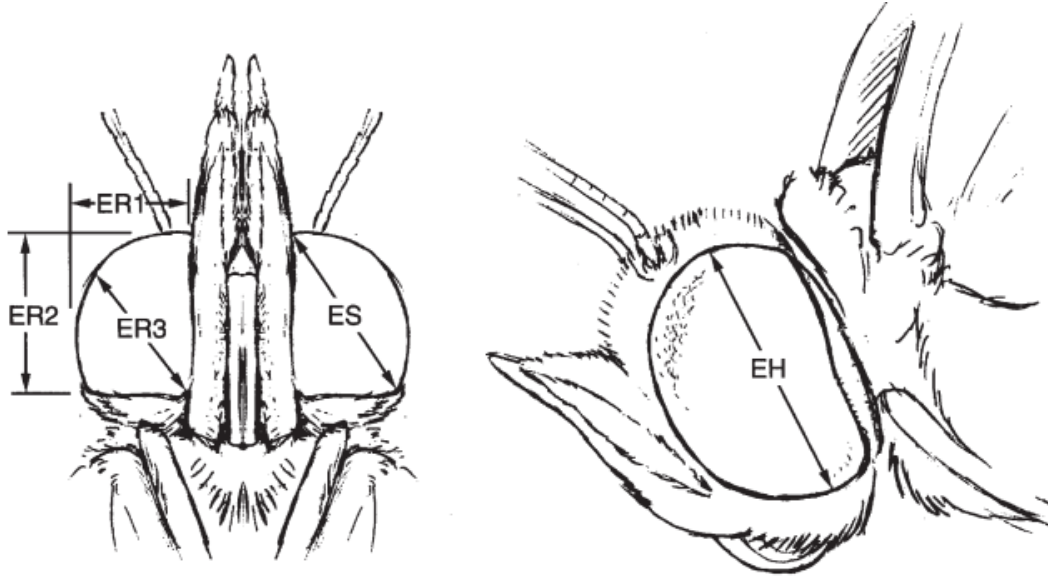


Figure 2.1: A diagram depicting the various measurements of radius (ER1-ER3) and eye height (EH) that I obtained from individual *S. mormonia*, using image analysis software, to estimate eye surface area. Figure reproduced from Rutowski (2000).

Tokyo) and placed them into a -20° freezer until the gel became completely opaque. Once frozen, I used a Leica CM1850 cryostat (Buffalo Grove, IL) to cut the molds into 16-20-micron thick sections. To improve the contrast of the microscope images, I labeled them with Hematoxylin and Eosine (H&E), which combine to stain nucleic acids violet and proteins, membranes, and tissues different shades of pink. I put my cryosections through a chemical treatment schedule, described on table 2.2, on a Leica Autostainer XL (model: ST5010, Buffalo Grove, IL). After staining, I imaged the cryosections under an Olympus CX31 light microscope. I took photographs of the microscope images using a Nikon D5000 camera which attached to a NDPL-1(2x) lens via a Ø23.2 - Ø30.5mm lens holder.

Table 2.2 Routine H&E Staining Protocol and chemical wash schedule for the Leica Autostainer XL.

Treatment	Duration/Notes
95% Alcohol	5 min
Water Wash	Rinse
Hematoxylin stain	7 minutes
Water wash	Rinse
Acid alcohol	1 dip
Water wash	Rinse
Ammonia water	30 sec
Water wash	Rinse
Eosin (made up fresh)	5 drops, 2 minutes
95% alcohol	3x. 5 minutes/wash
100% alcohol	2 washes, 5 minutes/wash
Xylene	3 washes, 5 min/wash

2.2.5 Corneal extractions

I froze the excised heads of 49 *S. mormonia* ($n_f=31$, $n_m=18$) in a -20° freezer to prepare them for corneal extractions. I adapted this technique from Ziemba and Rutowski (2000) to obtain eye surface area and facet area measurements for different regions of the eyes of *S. mormonia*. To soften the corneal layer, I removed the eyes from the freezer and placed them into 10% aqueous NaOH solution for 20 to 25 minutes.

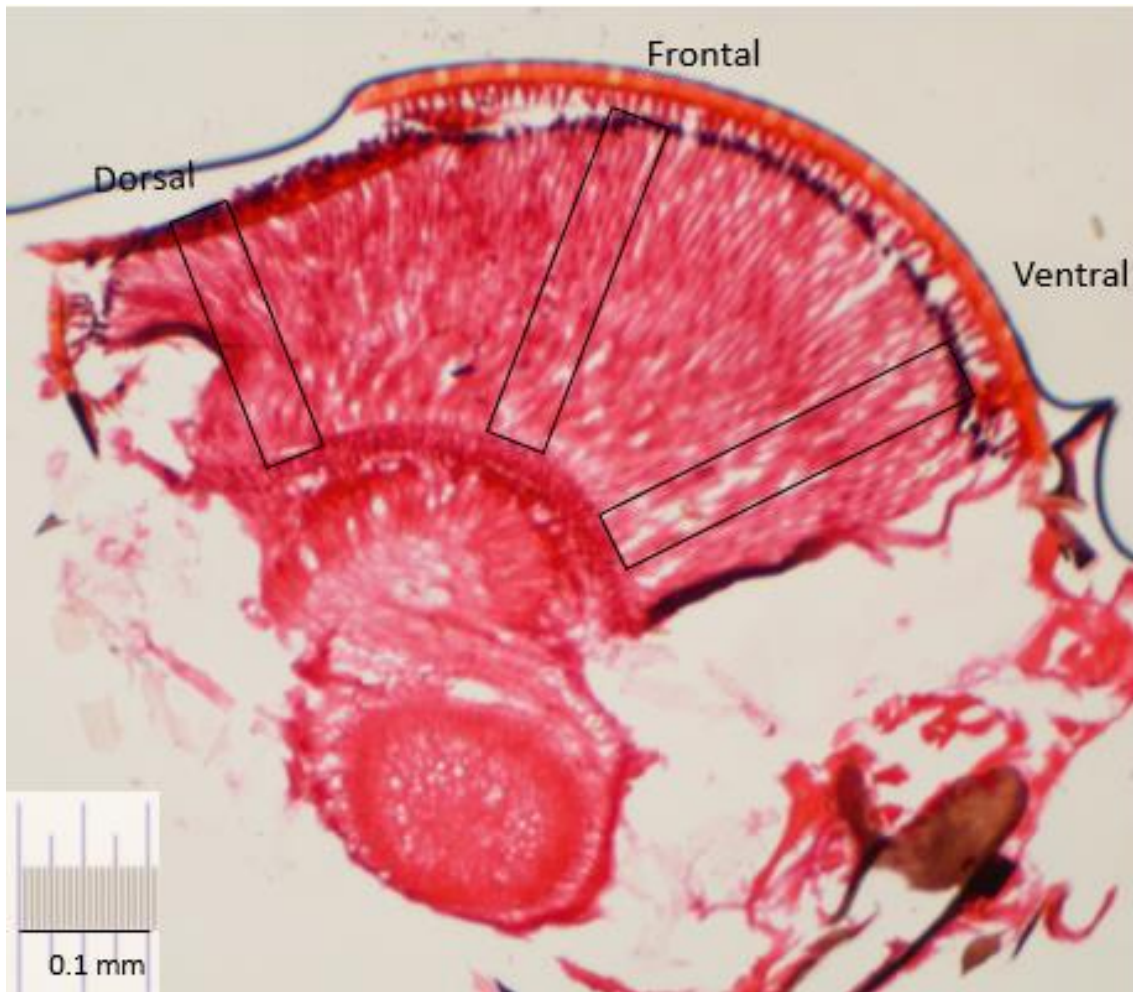


Figure 2.2: A cryosection of an eye from *S. mormonia* stained with Hematoxylin and Eosine.

I transferred the eyes to a dissecting scope for the removal of the cornea. I marked the dorsal regions of the eyes with whiteout. This allowed me to determine the orientation of the different eye regions of interest on the corneal surface. I used fine dissecting scissors to cut around the perimeter of the eyes and fine forceps to carefully pull the cornea away from underlying soft structures. I made cuts at the ventral, dorsal, medial, lateral, and diagonal regions of the extracted corneal layer to spread it flat on the slides. If the cornea was rigid, I added more 10% NaOH to soften the tissue. Once the cornea was extracted and flattened, I added 2-3 drops of glycerol to the corneal spreads and placed a cover slip over them. They were sealed using EM grade clear nail polish. I imaged the slides under

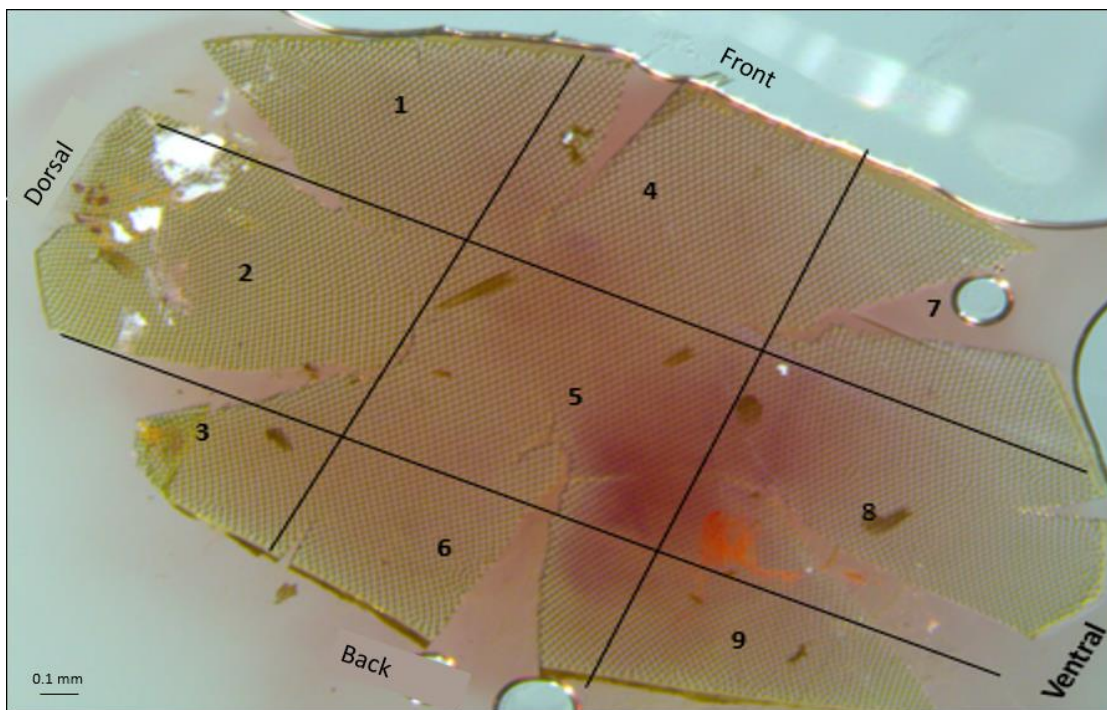


Figure 2.3: A diagram of a corneal spread with the nine quadrants of the eye labeled (Ziemba and Rutowski 2000): dorsal-anterior (quadrant 1), dorsal-central (quadrant 2), dorsal-posterior (quadrant 3), equatorial-anterior (quadrant 4), equatorial-central (quadrant 5), equatorial-posterior (quadrant 6), ventral-anterior (quadrant 7), ventral-central (quadrant 8), and ventral-posterior (quadrant 9). The front of the eye is also referred to as the anterior region, and the back of the eye is also referred to as the posterior region.

a Leica M165FC fluorescent stereo microscope with a DFC295 camera attachment (Buffalo Grove, IL) at 3.2x and 8x magnification with an ocular micrometer for scale. I then processed scaled images with ImageJ. To obtain average facet area measurements, I focused on areas within 9 different quadrants of the corneal surface, corresponding to the dorsal-medial (quadrant 1), -central (quadrant 2), and -lateral (quadrant 3), equatorial-medial (quadrant 4), -central (quadrant 5), and -lateral (quadrant 6), and ventral-medial (quadrant 7), -central (quadrant 8), and -lateral (quadrant 9), regions of the eye (Ziemba and Rutowski 2000). Within each quadrant, I measured the area of the selected region of space and counted the facets within this region. I calculated average facet area by dividing the area value by the number of facets counted in units of $\mu\text{m}^2/\text{facet}$.

2.3 SPECTROSCOPY

I took reflectance measurements from wings of 29 field-caught butterflies (15 females, 14 males) collected in 2017 from sites which were 2743, 3048, 3200, and 3353 meters above sea level in elevation. I also took reflectance measurements from a total of 94 wings (56 females and 37 males) of butterflies collected in 2018, from all five of the elevations sampled. I took measurements from the dorsal forewings and dorsal hindwing of each butterfly. I used an Ocean Optics Flame spectrometer with a QR400-7-uv-vis fiber optic cable (Dunedin, FL) and a spectralon, certified reflectance standard by Labsphere (North Sutton, NH) to get spectral reflectance recordings. I placed samples onto a spectrally flat, black background with near 0% reflectance. Then, using a probe holder, I oriented the collection probe at 45° below the zenith, and then placed the probe holder on top of the wing sample. I calculated the chromaticity values from spectral reflectance data. Chromaticity here is defined as the linear slope of the spectral

reflectance curve between 500 and 600 nm. The chromaticity value indicates how pure the specific wavelength of visible light or color is to the viewer. A higher slope value indicates greater chromaticity and corresponds to a wing which would look more “orange” to humans. Lower chromaticity values correspond to more “yellow” or “less saturated orange” looking wings.



Figure 2.4: A diagram of the wing regions of *S. mormonia* from which I collected dorsal forewing (A) and dorsal hindwing (B) chromaticity reflectance measurements. These wing regions were chosen because they tended to have less wing wear.

2.4 COMPUTATIONAL MODELLING OF VISUAL SENSITIVITY

2.4.1 Electrophysiology

I assisted Luke Havens in performing electrophysiology on 3 male and 3 female *S. mormonia*, all of which were lab-reared, aged between 2-4 weeks old (Caves et al. 2016). We began by anesthetizing the butterflies on ice for 15-30 minutes until immobile, and then excised their protruding appendages (antenna, legs, etc.). We then affixed a plastic rod onto the dorsal side of the butterfly using hot wax, and then oriented the insect so that its anterior eye region faced the light source and objective lens. We used Tungsten microelectrodes, made by electrolytic sharpening. We pushed one electrode into the retina of the right eye to record electrical responses to light stimuli. We placed a reference electrode in the left eye of the butterfly and a ground electrode in the head or abdomen of the insect. The doors of the light-tight Faraday cage, lined with black felt, were closed. We dark adapted butterflies for 15 minutes before exposing them to light. The light was produced by a 150 W tungsten-halogen lamp (tungsten-halogen lamp, Spectral Products, ASBN-W150-PV). Light intensity was controlled by a neutral density filter wheel (Edmund Optics, model #54-082). We randomly exposed butterflies to wavelengths of light ranging from 400-700 nm (separated by intervals of 10 nm) using a monochromator (Monochromator: Spectral Products, CM110). A computer-controlled shutter (Uniblitz LS3) controlled the duration of light exposure. The different wavelengths we used as stimuli were presented in random order, each lasting for 1 second, with 9 second time lapses separating the flashes of light throughout the experimental runs. We ran the same procedure two times for each insect. DC recordings of electrical responses from the butterfly eyes were amplified (A-M Systems model 3000

AC/DC amplifier), digitized, and stored in LabView with a custom program developed by Luke Havens. I accessed and analyzed the ERG data using ADInstruments PowerLab (8/35). To analyze spectral sensitivity information obtained from the ERG data, I calculated the magnitude of response (MoR), defined as the absolute value of the difference between the peak and trough of the voltage response, for each wavelength used in the light trials. I normalized the MoR values to 1 and averaged them across trials for each insect. I then pooled the averages from all 3 males and females, averaged them, and plotted them against wavelength. The wavelengths of maximum absorbance for the short, medium, and long wavelength sensitive visual pigments most applicable to my models were 385, 470, and 550 nm, respectively (Yuan et al. 2010). These wavelengths were plugged into a visual pigment sensitivity template developed by Stavenga et al. (1993) to get the alpha absorbance curves corresponding to each visual pigment type. I used Microsoft Excel to plot the alpha absorbance curves corresponding to the three visual pigments against the MoR curve obtained from ERG data analysis. This allowed us to determine which wavelengths of maximum sensitivity in the long-wavelength range was most likely to produce the sensitivity peaks on the MoR curve, by inspection of the graph. This ultimately gave us an idea of what the spectral sensitivity curve looks like for *S. mormonia*.

2.4.2 Computational Model of Color Discriminability

In order to determine whether *S. mormonia* are able to distinguish between inter-sexual and inter-elevational variances in wing chromaticity, I used a photoreceptor-noise limited visual sensitivity model developed by Vorobyev and Osorio (1998). I completed the calculations for color discriminability values corresponding to the model on a

template provided by Dr. Nate Morehouse (personal communication). From this model, I was able to obtain color discriminability (ΔS^t) output values. A ΔS^t value is a quantitative unit of measure corresponding to color discriminability. It indicates how discriminable the colors of two different stimuli are from each other. A ΔS^t value that is greater than 1 indicates that the butterflies can discriminate between the “colors” of two stimuli based on their individual reflectance spectra; if the ΔS^t value is less than 1, the two stimuli are indistinguishable. The greater the value of ΔS^t , the better they can discriminate between the two stimuli. Given our dearth of equipment to conduct epimicrospectrophotometry, I used existing scientific literature about the phylogenetic relatedness between the short-, medium-, and long- wavelength sensitive rhodopsin proteins across several butterfly species to have an idea of the absorbance maxima which are most applicable to the *S. mormonia* visual system and of the retina-wide relative abundances of different photoreceptor types. This information was used to determine the input values for the model. Most butterflies in the family *Nymphalidae* are trichromatic. Therefore, I used a template pertaining to the visual system of *Hymenoptera*, which are also trichromatic and like nymphalid, possess three major visual opsin types with peak sensitivities in the UV (300-400 nm), Blue (400-500 nm), and Green (500-600 nm) wavelength ranges of visible light. A typical ommatidium of a nymphalid contains 9 photoreceptors distributed throughout the distal, proximal, and basal end of the rhabdom. The blue- and UV-sensitive visual opsins are distributed in the distal R1 and R2 photoreceptors, but the green-wavelength sensitive opsins are in the proximal R3-8 photoreceptors. Long wavelength sensitive opsins have also been identified in the 9th (basal) photoreceptor of *Vanessa cardui*. The rhabdom length of *V. cardui*, also a

nymphalid butterfly, is 420 micrometers (Briscoe et al. 2003), which is close to the average rhabdom length I calculated for male *S. mormonia*, which was 437 μm . The wavelengths corresponding to the visual pigment sensitivity peaks of *S. mormonia*'s closest relatives, including *Dryas iulia* and *Speyeria leto*, are 385, 470, and 550 nm (Yuan et al. 2010). I set the relative abundance of UV-:Blue-:Green- sensitive photoreceptors to 4.78:1:17.33 in this model, based on the photoreceptor distribution discovered in *Vanessa cardui* (Briscoe et al. 2003). This species has the same basic ommatidia morphology as most other nymphalid butterflies. I plugged these values into Vorobyev and Osorio's (1998) receptor noise limited model of spectral sensitivity, to obtain a measure of color discriminability in units of ΔS^t (Morehouse and Rutowski 2010). The following formulas were used to calculate the color discriminability between different pairs of stimuli.

$i = 1, 2, \dots, n$; q_i is the quantum catch of photoreceptor i ,

$$q_i = \ln \left(\frac{Q_i}{Q_i^B} \right)$$

$$Q_i = \int R(\lambda) I(\lambda) A_i(\lambda) d\lambda,$$

$$Q_i^B = \int R^B(\lambda) I(\lambda) A_i(\lambda) d\lambda,$$

$$e_i = \omega \sqrt{\frac{n_j}{n_i}}$$

$$\Delta q_i = q_i(\text{focal stimuli 1}) - q_i(\text{focal stimuli 2})$$

$$(\Delta S^t)^2 = \frac{(e_1^2)(\Delta q_3 - \Delta q_2)^2 + (e_2^2)(\Delta q_3 - \Delta q_1)^2 + (e_3^2)(\Delta q_1 - \Delta q_2)^2}{(e_1 e_2)^2 + (e_1 e_3)^2 + (e_2 e_3)^2}$$

Here, q_i represents the photon catch value for each photoreceptor given adaptation to the background stimulus, where λ is the wavelength, R is the reflectance of the stimulus, R^B is the reflectance of the background, I is the irradiance ($\mu\text{mol}/\text{m}^2\text{s}^{-1}\text{nm}^{-1}$), and A_i is the absorbance of photoreceptor i (Vorobyev and Osorio 1998). Absorbance curve corresponding to the three photoreceptor types were obtained by plugging the wavelengths of maximum sensitivity for each photoreceptor into a formula that produces pigment absorbance curves (Stavenga et al. 1993). The factor, e_i , is an estimate of noise produced from the receptors. I set the webber fraction (ω), which is the standard deviation of the noise in a given photoreceptor, to 0.01. This value has been used to model the spectral sensitivity of the visual systems of other diurnal butterflies, including *Papilio xuthus* and *Pieris rapae* (Morehouse and Rutowski 2010; Koshitaka et al. 2008). Since I used retina-wide relative abundances of photoreceptors as opposed to ommatidia-wide receptor abundances, “ η_j/η_i ” represents the value of the abundance of the most common receptor type over receptor type i . This assigns the least amount of noise to the most common receptor type. In this case, the different stimuli corresponded to the reflectance data from wing regions of butterflies belonging to different elevation/sex groups. I used reflectance data from the butterflies collected at 2743 meters above sea level (the lowest elevation) and from butterflies collected at 3352 meters above sea level (the highest elevation) as representative data for “low” and “high” elevation groups, respectively. *S. mormonia* can discriminate between the “color” of a pair of wings if the

model's output ΔS^t value is greater than 1. Given my interest in mating cues and detection of such cues, special attention was placed on the dorsal hindwing and forewing stimuli because these regions are known to be important for mate recognition. Since the ventral regions of the wings, which have silver washed speckles, are instead important for camouflage or predator avoidance (Wilts et al. 2013), I did not use reflectance data corresponding to this wing region in the model.

From this computational model I calculated ΔS^t output values. The ΔS^t values corresponding to the models which compared the reflectance data from the wings of butterflies collected in 2017 and 2018 were plotted and analyzed independently because there was an effect of collection year on wing chromaticity values. The wing wear scores of the wing samples from which I obtained reflectance measurements varied and ranged between values of 1 to 4.5. I performed computational models using long-wavelength sensitive pigment absorbance data with different sensitivity peaks because when I used 570 nm as the wavelength of peak absorbance for this pigment, I produced an absorbance curve which fit the MoR curve much closer than the absorbance curve with the peak at 550 nm. *S. mormonia* adults are diurnal and are usually found in open meadows. Therefore, I plugged in irradiance data corresponding to an open sky at midday, which Dr. Nate Morehouse shared with me. In the model, I also used reflectance data corresponding to different types of vegetation, including less chromatic green vegetation, more chromatic green vegetation, and brown vegetation, to account for the different backgrounds against which *S. mormonia* may view conspecifics in their natural environments.

2.5 STATISTICAL ANALYSIS

I used Systat version 13.1 to perform general linearized models and post-hoc tests.

I used RStudio 3.4.3 to perform multifactor ANOVA and two-sample t-tests to identify significant differences in morphometric and colorimetric parameters like wing wear, chromaticity wing length, estimated eye surface area and facet area between sexes or elevation groups. I also used RStudio to perform linear regression models. I performed F-tests for variance using the data analysis toolkit plug-in of Microsoft excel 2003 prior to performing t-tests on RStudio

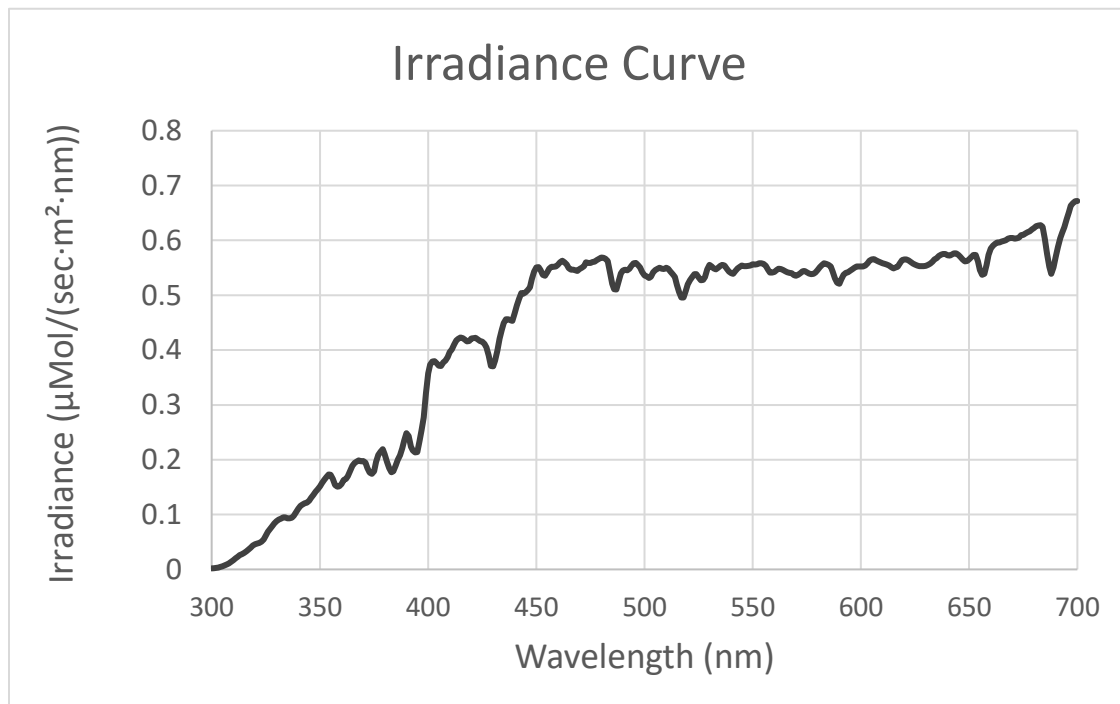


Figure 2.5: A scatterplot showing the irradiance data I used for the computational model of visual sensitivity. The Irradiance data was collected at mid-day in Pittsburgh, PA by Dr. Nate Morehouse and is representative of an open sky environment.

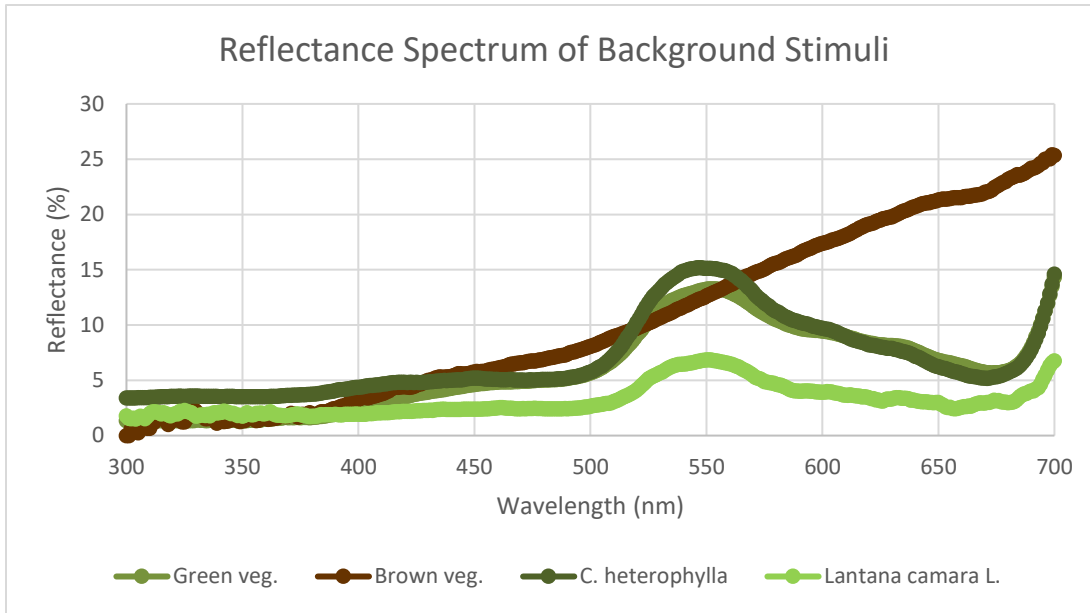


Figure 2.6: The reflectance spectra of the background stimuli which I used for the computational model for spectral sensitivity. Reflectance data from *Collinsia heterophylla* and *Lantana camara* were obtained from Dr. Nate Morehouse while reflectance data from the brown and green vegetation were obtained from open source data provided by Dr. Miriam Henze.

CHAPTER 3

RESULTS

3.1 EYE AND WING MORPHOLOGY

3.1.1 Wing Wear Scores

Elevation, sex, and the interaction of elevation by sex were plugged into a multifactor ANOVA as co-factors and were determined to have significant effects on average wing wear scores. Average wing wear scores were greater at lower elevations compared to higher elevations (Fig. 3.1) This was particularly true when I compared the average scores corresponding to butterflies collected at 2865 meters to those collected at 3353 meters ($p=0.0017$). Average wing wear scores were also greater in males compared to females (Fig 3.2). The differences between the wing wear scores of males and females at the three lowest elevations were significantly greater than the corresponding differences observed at the two highest elevations (Fig 3.3), meaning that there was an effect by the interaction of sex and elevation on average wing wear scores ($F_{4,172}=2.604$, $p=0.038$).

3.1.2 Wing length

The interaction of sex by elevation, wing wear, and collection year were plugged into a multifactor ANOVA as co-factors and were determined to have significant effects on average wing length values. Females had greater average wing lengths compared to males (Fig 3.4), but the degree to which I saw this female-biased sexual dimorphism

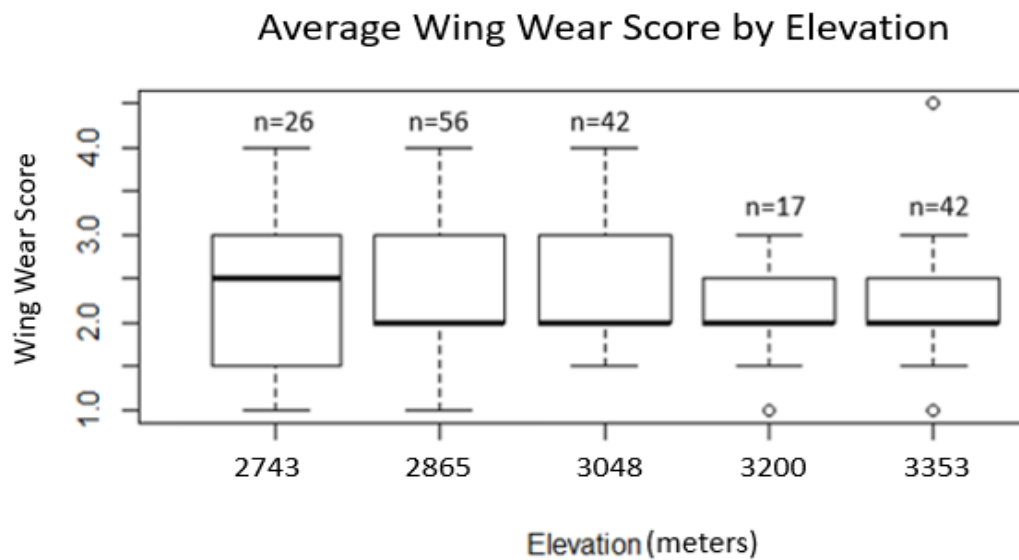


Figure 3.1: A box plot of average wing wear score as a function of elevation. Data was pooled from 2017 and 2018. Wing wear was most different between the 2865-meter (2.51 ± 0.75) and 3353-meter (2.06 ± 0.59) elevation groups. ($F_{4,172}=3.988$, $p=0.004$)

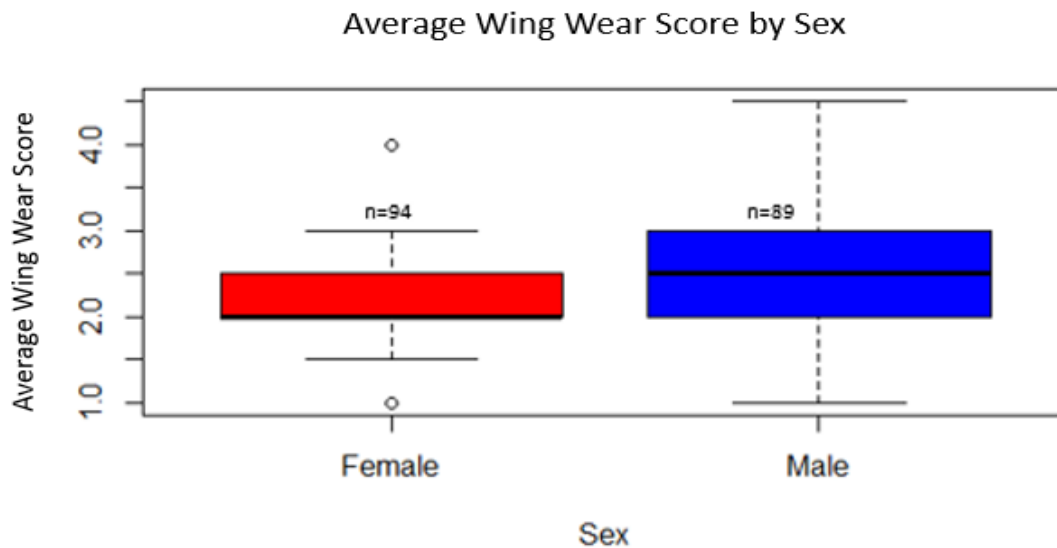


Figure 3.2: A box plot of the average wing wear scores assigned to male versus female butterflies. Data was pooled from 2017 and 2018. Males have significantly more wing wear (2.54 ± 0.77) than females (2.07 ± 0.58). ($F_{1,172}= 24.496$, $p=1.76e^{-6}$)

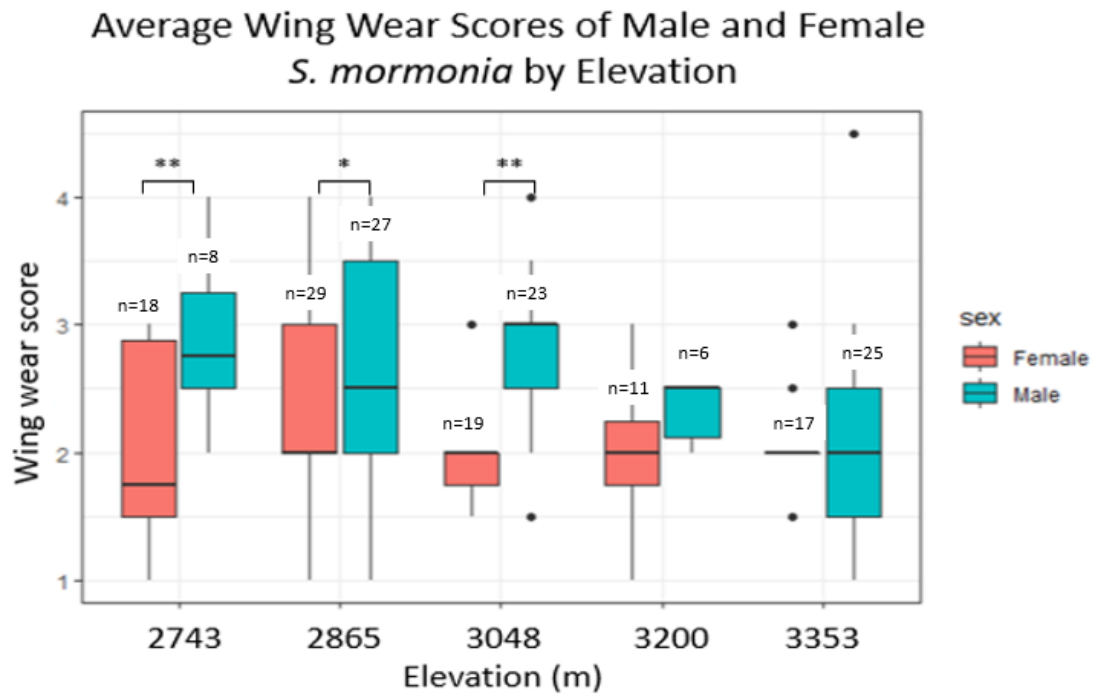


Figure 3.3: A box plot of average wing wear scores for male and female *S. mormonia* as a function of elevation. Butterflies collected from the elevations of 2743, 2865, and 3048 meters had the greatest disparities in average wing wear scores between males and females ($p=0.007$, $p=0.015$, and $p=9.99\text{E-}06$, respectively). Data was pooled from 2017 and 2018.

varied across elevations (Fig 3.5), with the greatest disparity occurring at the lowest elevation ($F_{9,171}=7.907$, $p=9.94\text{e-}10$). Average wing length also varied between elevations (Fig 3.6). As wing wear increased wing length decreased (Fig 3.8; $F_{1,171}=53.58$, $p=9.24\text{e-}12$), and the butterflies collected in 2018 had smaller average wing lengths than those collected in 2017 (Fig 3.7; $F_{1,171}=4.98$, $p=0.027$). A separate ANOVA model, with only the interaction between collection year and sex as co-factors, indicated that the sexual dimorphism between female and male average wing length was greater in 2017 than in 2018 (Fig 3.9).

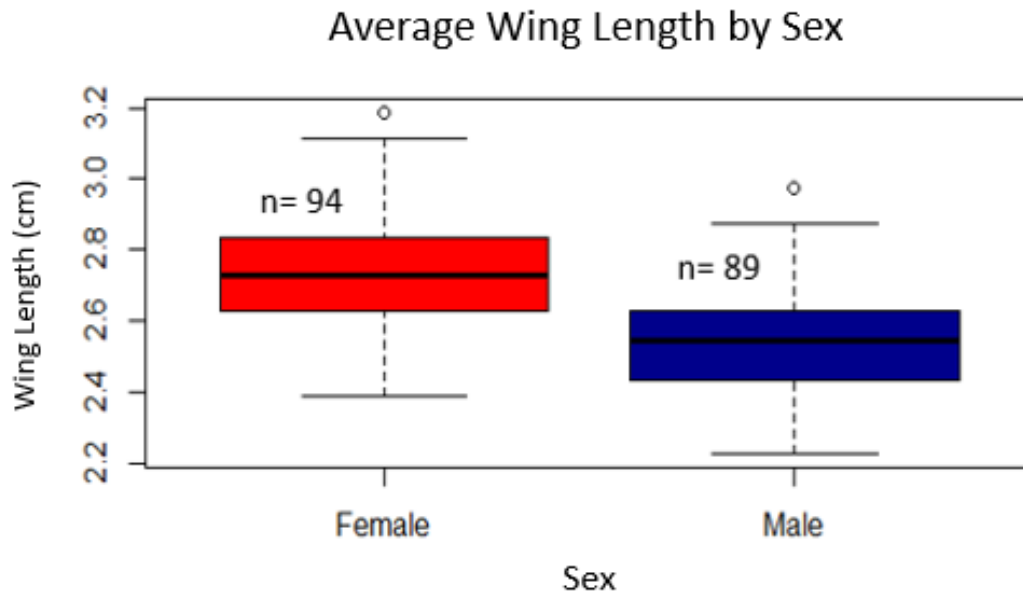


Figure 3.4: A box plot of the average wing length in cm of female versus male *S. mormonia*. Wing length data was pooled from both collecting seasons. The average wing length for females (2.73 ± 0.15 cm) was significantly larger than that of males (2.54 ± 0.16 cm).

3.1.3 Estimated Eye Surface Area

According to a multi-factor ANOVA with sex, year, and wing length as cofactors, sex and year significantly affected average estimated eye surface area values. Males had larger average eye surface areas than females (Fig. 3.9; $F_{1,104}=52.09$, $p=8.98e-11$). Also, the average eye surface area for all butterflies was smaller in 2018 than in 2017 ($F_{1,104}=18.8$, $p=3.37e-05$). A separate ANOVA demonstrated the significant effect of the interaction between elevation and sex on average estimated eye surface area data from 2017. Females collected in 2017 tended to have larger eyes at higher elevations, but male counterparts tended to have smaller eyes at higher elevations (Fig. 3.12).

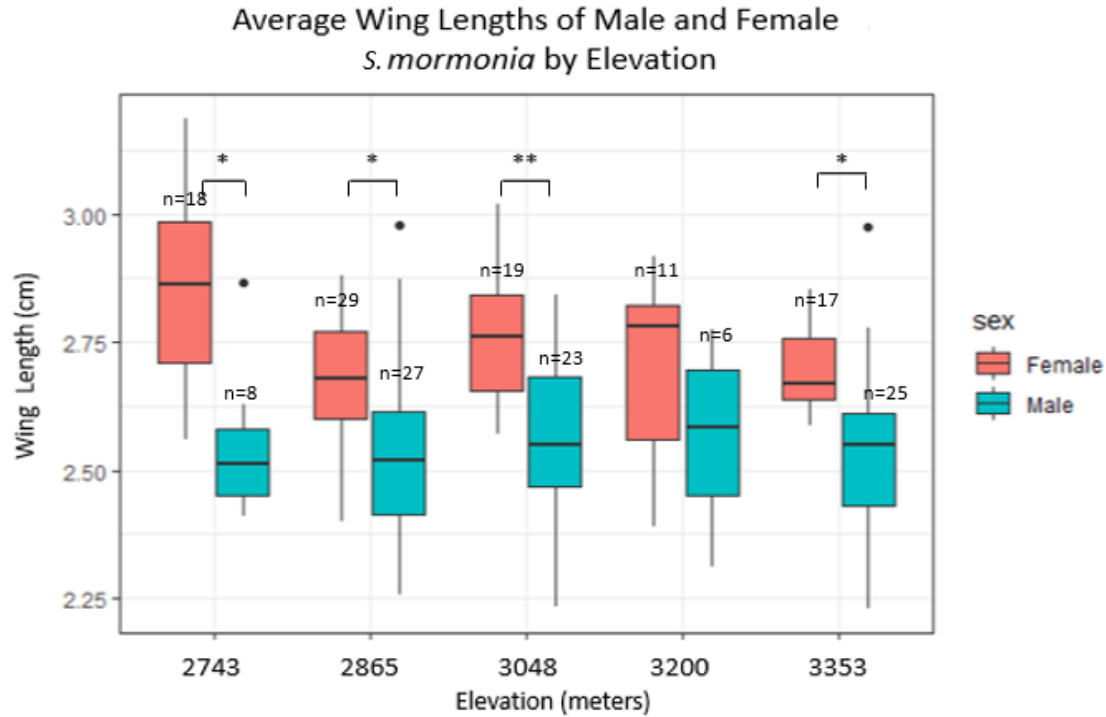


Figure 3.5: A box plot representing the average wing lengths of male and female *S. mormonia* at different elevations. The data came from the 2017 and 2018 field collection seasons. Wing length is more variable in females than males. There are different degrees of female-biased sexual dimorphism in wing length at different elevations, with the greatest dimorphism occurring at the lowest elevation.

3.1.4 Average facet areas

A two-sample t-test revealed that there was an affect by sex on the average facet areas within particular regions of the eye (Fig 3.11). Males had larger average facet areas compared to females within regions 1 ($t_{45,0.05}=-4.92$, $p=1.185e-05$), 4 ($t_{24,19,0.05}=-6.5521$, $p=8.578e-07$), 5($t_{45,0.05}=-8.2226$, $p=1.639e-10$), 7($t_{45,0.05}=-8.49$, $p=6.773e-11$), and 8($t_{19,063,0.05}=-2.6789$, $p=0.01482$) of the eye. This suggests that males have larger eyes than females because they have larger facets packed into regions corresponding to the medio-dorsal, frontal, and ventral parts of their eyes.

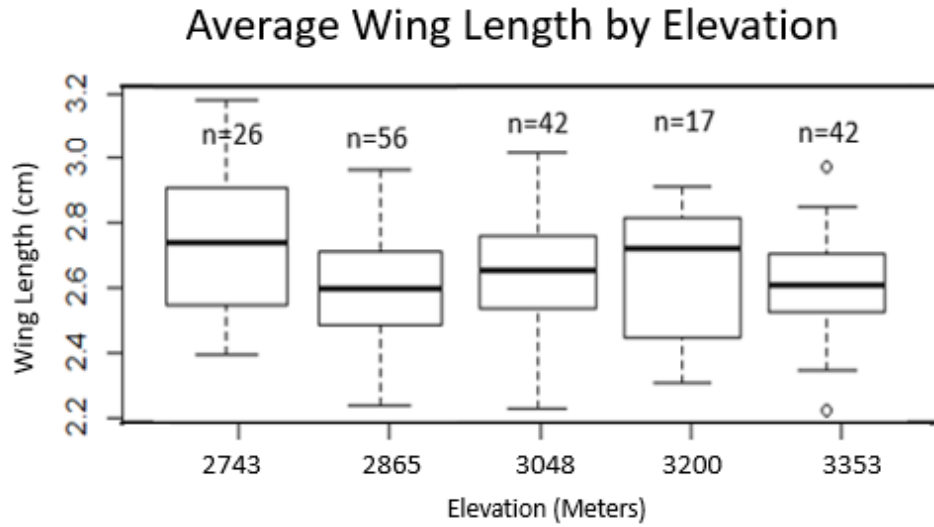


Figure 3.6: A box plot of the average wing length of *S. mormonia* from different elevations from pooled 2017 and 2018 data. A post-hoc test indicated that the most significant difference in average wing length occurs between butterflies collected at the 2743-meter elevation (2.76 ± 0.22) and those collected at the 3353-meter elevation (2.61 ± 0.15) ($p \approx 0.01$).

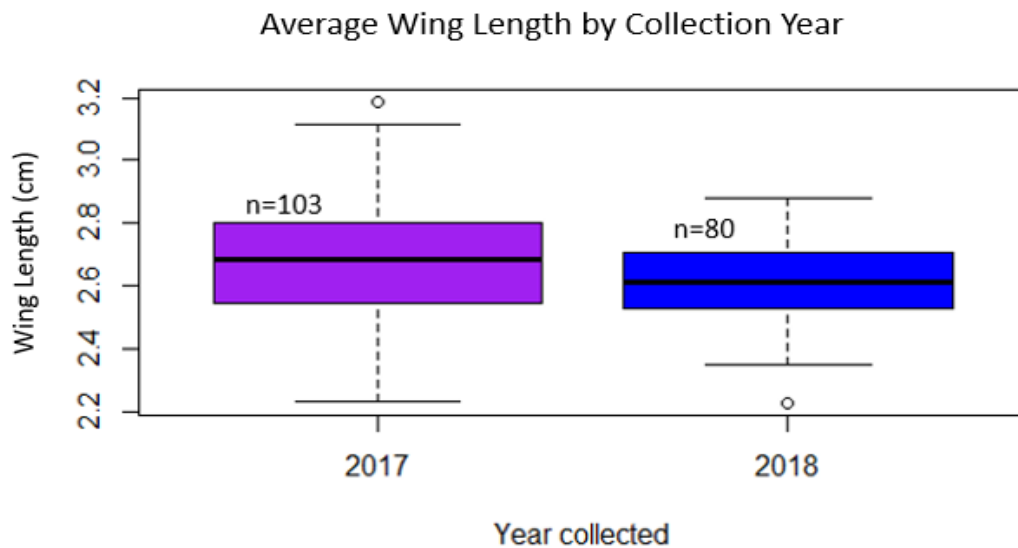


Figure 3.7: A box plot of the pooled average wing length for each collection year. The average wing length of butterflies collected in 2017 was significantly larger (2.66 ± 0.21 cm) than that of butterflies collected in 2018 (2.61 ± 0.14 cm).

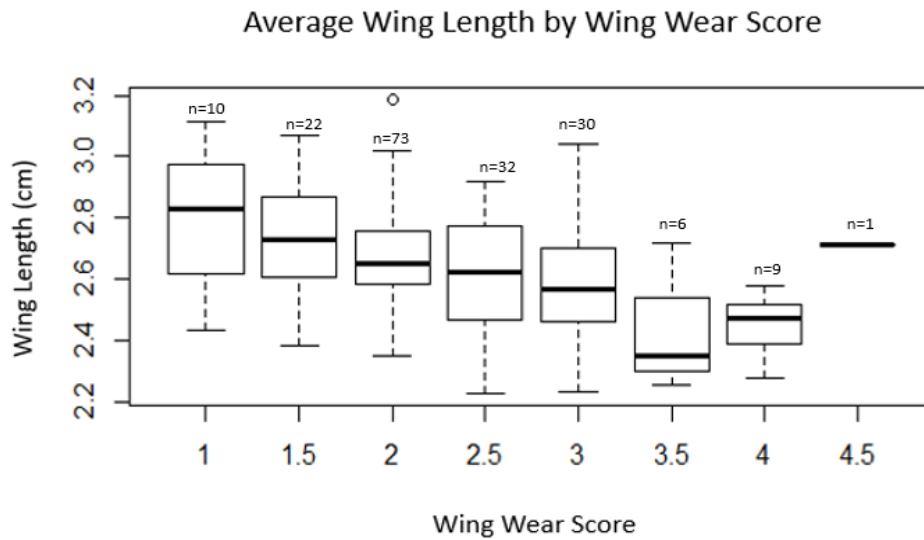


Figure 3.8: A box plot showing average wing length values as a function of wing wear score. The data was pooled from 2017 and 2018. Wing wear is negatively related to wing length.

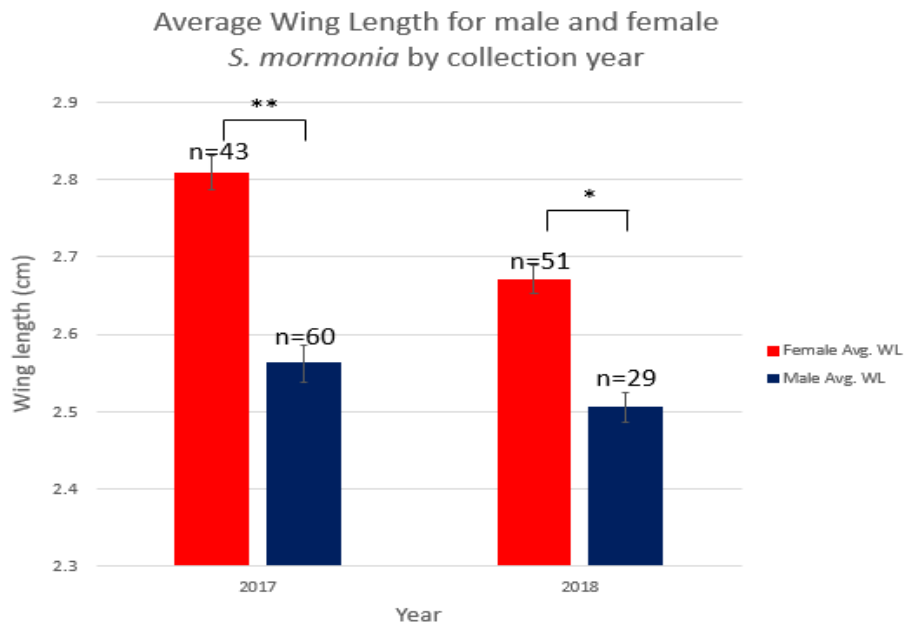


Figure 3.9: A bar graph showing the average wing length of male and female *S. mormonia* by year of collection. There seems to be a greater disparity between the average wing lengths of males and females in the 2017 data than there is in the 2018 data.

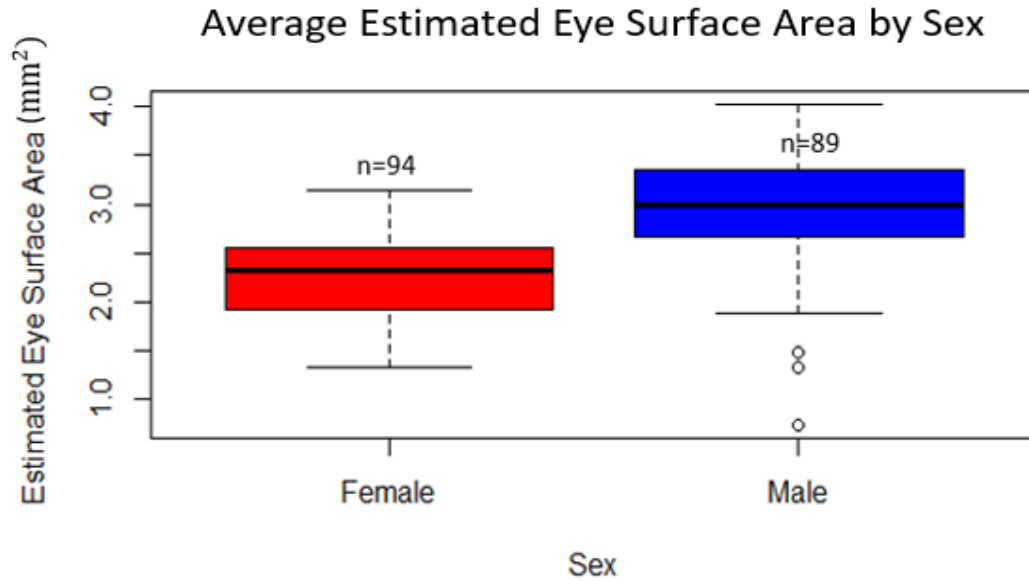


Figure 3.10: A box plot showing the average estimated eye surface area for male and female *S. mormonia*. This data was pooled from 2017 and 2018. The average estimated eye surface area of males ($2.95 \pm 0.6 \text{ mm}^2$) was significantly larger than that of females ($2.82 \pm 0.4 \text{ mm}^2$).

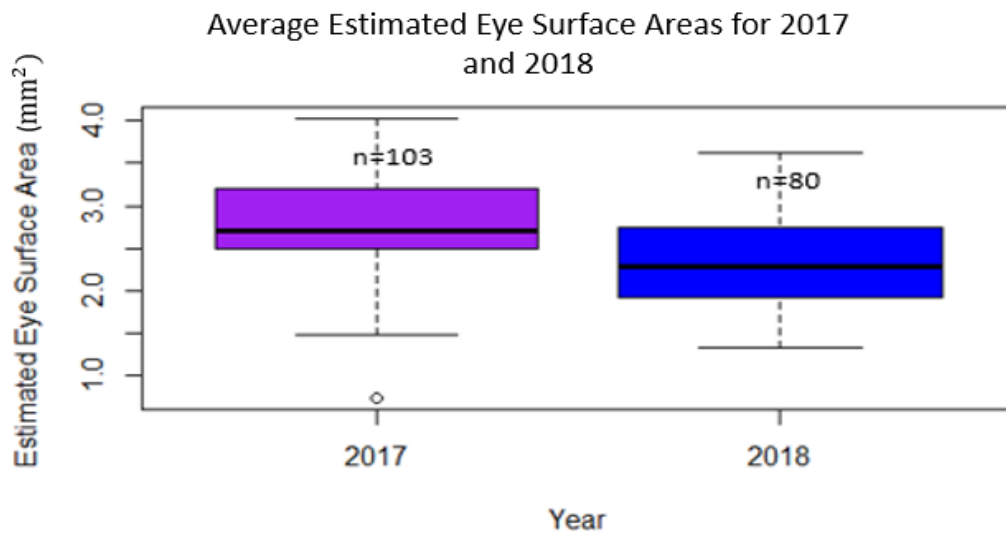


Figure 3.11: A box plot showing the average estimated eye surface areas of butterflies collected in 2017 and 2018. The average estimated eye surface area of butterflies collected in 2018 ($2.32 \pm 0.52 \text{ mm}^2$) was smaller than that of butterflies collected in 2017 ($2.82 \pm 0.59 \text{ mm}^2$).

3.1.5 Rhabdom length

A two-sample t-test revealed that male *S. mormonia* have longer average rhabdom lengths than female conspecifics, especially in the frontal and dorsal regions of the eye (Fig 3.12). A total of 8 cryosections from 5 male and 9 cryosections from 6 female eyes were used to measure rhabdom length. In our sample population, rhabdom length ranged from 379 ± 19 to 481 ± 15 μm in males, and from 316 ± 18 μm to 394 ± 30 μm in females. The overall average rhabdom length was 437 ± 1 μm in males and was significantly greater than the average rhabdom length in females, which was 353 ± 3 μm ($t_{15,0.05} = -3.67$, $p = 0.0023$).

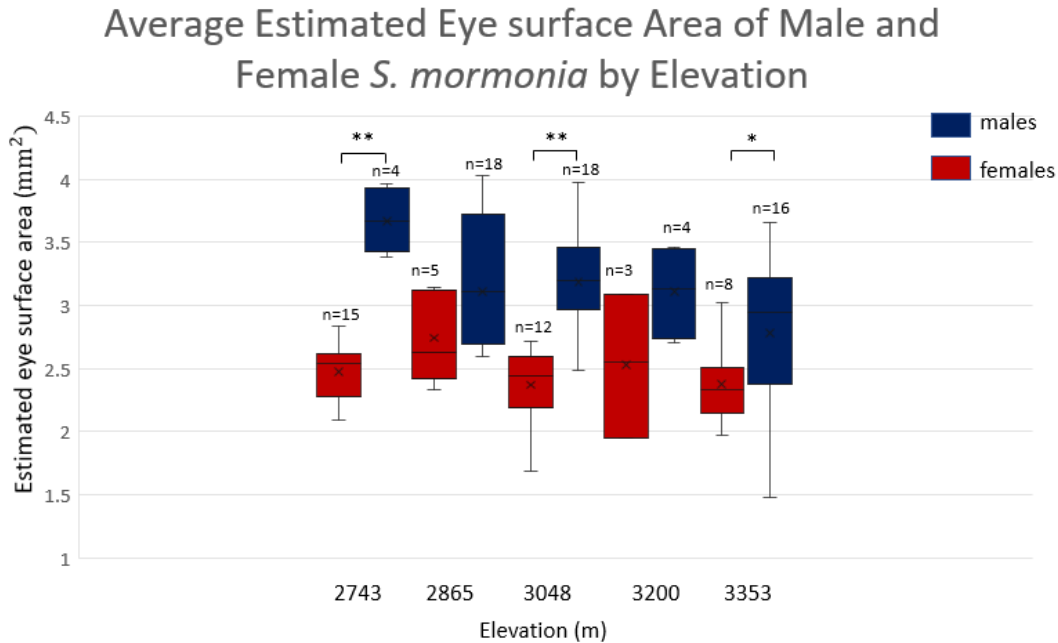


Figure 3.12: A box plot showing the average estimated eye surface area data from the 2017 field season plotted separately for males and females, across elevations. In a separate ANOVA test, there was an interaction effect by sex and elevation on the average estimated eye surface area values. The average estimated eye surface area of males tended to be smaller at higher elevations ($F_{4,101} = 3.109$, $p = 0.019$). The male-biased sexual dimorphism in estimated eye surface area was significant at the 2743- ($p = 4.99\text{E-}08$), 3048- ($p = 1.08\text{E-}06$), and the 3353- ($p = 0.042$) meter elevation sites.

3.1.6 Relationship between eye size and wing length

Using a Pearson regression analysis on RStudio, I found that only butterflies collected from the first field season showed a statistically significant, slight negative correlation between eye surface area and wing length (Fig 3.13). No such correlations existed between the two variables in butterflies collected during the second field season.

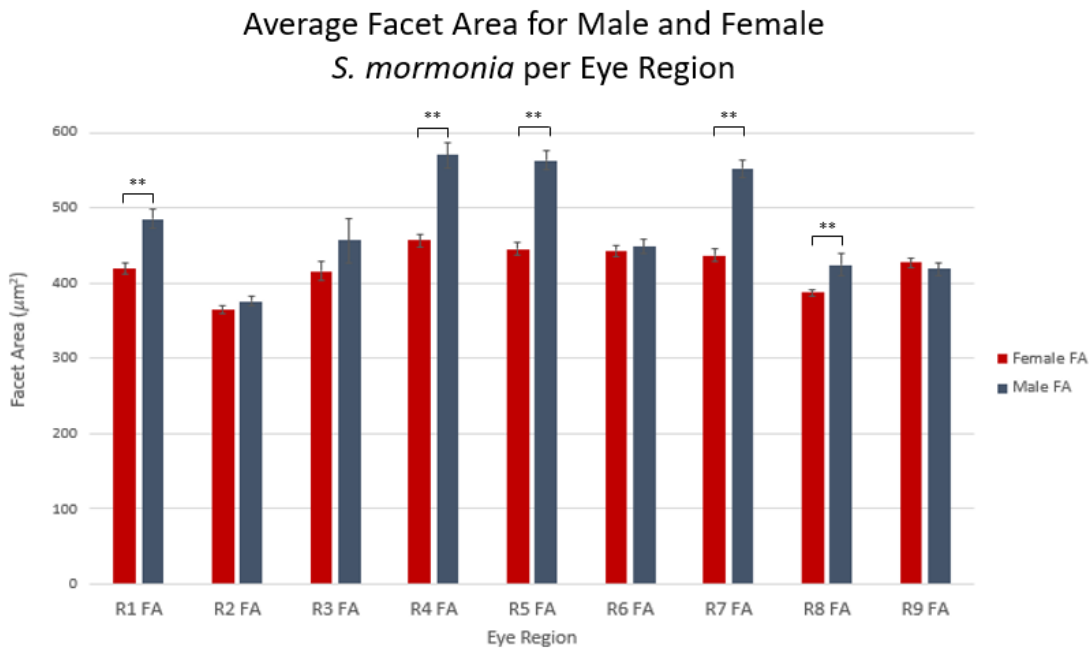


Figure 3.13: A bar graph showing the average facet areas for male and female *S. mormonia* for each region of the eye. I sampled 31 female eyes and 18 male eyes to obtain these results. The average facet areas within region 1 (M: $486 \pm 52.1 \mu\text{m}^2$; F: $419 \pm 42.9 \mu\text{m}^2$), 4 (M: $570 \pm 64.1 \mu\text{m}^2$; F: $456 \pm 41.8 \mu\text{m}^2$), 5 (M: $563 \pm 48.6 \mu\text{m}^2$; F: $445 \pm 43.7 \mu\text{m}^2$), 7 (M: $552 \pm 48.1 \mu\text{m}^2$; F: $437 \pm 42.9 \mu\text{m}^2$), and 8 (M: $425 \pm 60.8 \mu\text{m}^2$; F: $387 \pm 24.8 \mu\text{m}^2$) were found to be significantly larger in males compared to females.

3.2 WING COLOR MEASUREMENTS

3.2.1 Dorsal forewing chromaticity

A multi-factor ANOVA analysis with sex, collection year, and wing wear scores yielded the following results. Average dorsal forewing chromaticity values were greater in males compared to females (Fig 3.16; $F_{1,100}=7.613$, $p=0.007$) and in butterflies collected from

the 2018 field season compared to those collected in the 2017 field season (Fig 3.15; $F_{1,100}=28.392$, $p=0.000$). Average dorsal forewing chromaticity also decreased as wing wear increased (Fig 3.14; $F_{5,100}=6.008$, $p=0.000$).

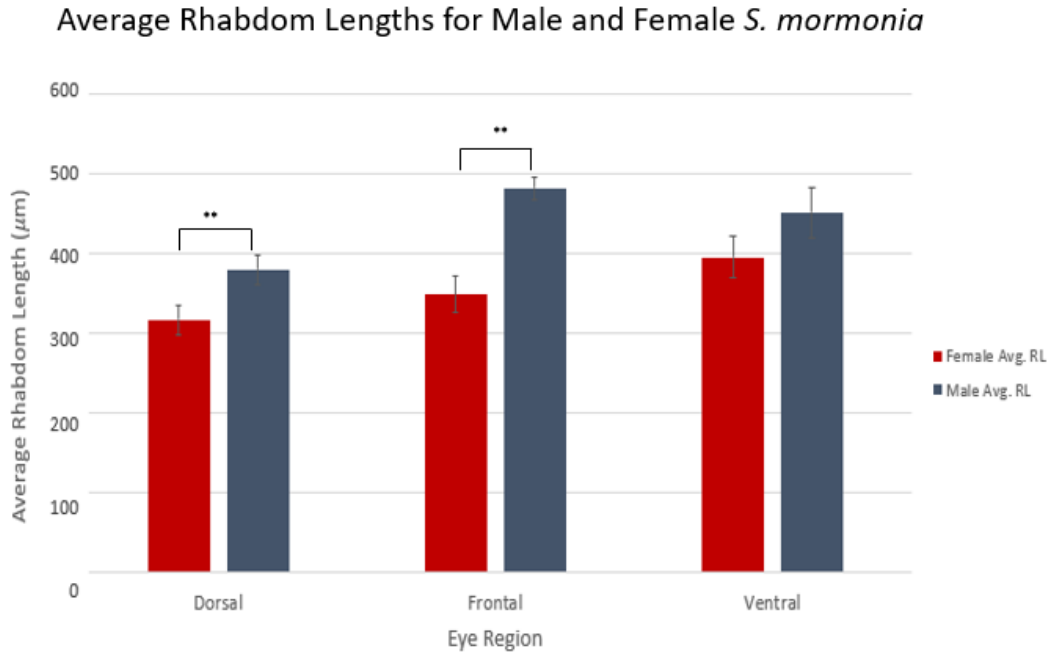


Figure 3.14: A bar graph showing the average rhabdom lengths of male and female *S. mormonia* for the dorsal, frontal and ventral regions of their eyes. I sampled 8 cryosections from 5 male eyes and 9 cryosections from 6 female eyes to obtain these results. The average rhabdom length of males was larger than that of females in every eye region tested, with statistical significance at the dorsal ($t_{15,0.05}=-2.4$, $p=0.0296$) and frontal regions of the eye ($t_{15,0.05}=-4.69$, $p=0.0003$).

3.2.2 Dorsal hindwing Chromaticity

A multifactor ANOVA analysis with year and wing wear as co-factors showed the following results. Average dorsal hindwing chromaticity was slightly higher in butterflies

collected in 2018 compared to those collected in 2017 (Fig 3.18; $F_{1,105}=5.644$, $p=0.0193$) and decreased as wing wear increased (Fig 3.17; $F_{1,105}=5.676$, $p=0.019$).

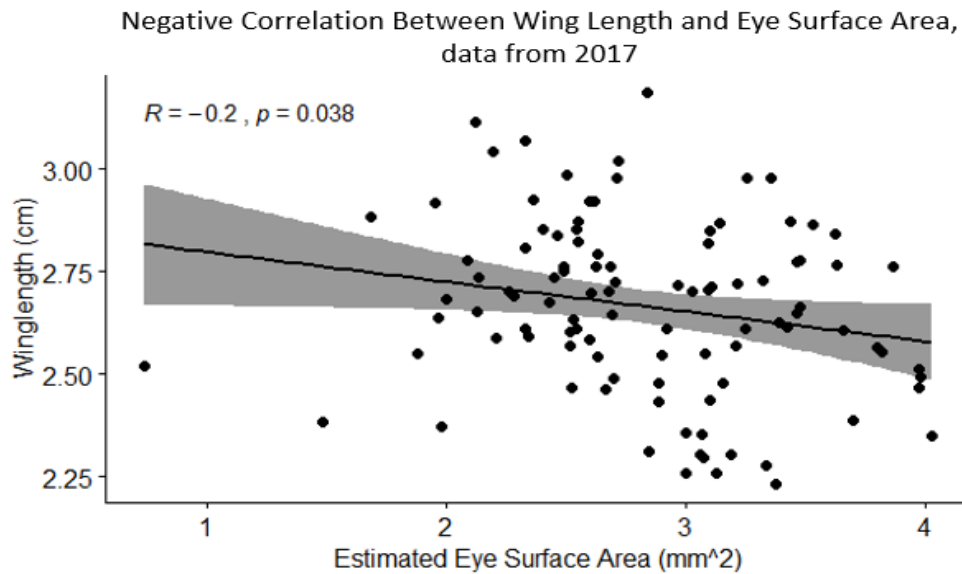


Figure 3.15: A scatterplot showing the negative relationship between wing length and eye surface area values from the dataset corresponding to the 2017 field season ($r=-0.2$).

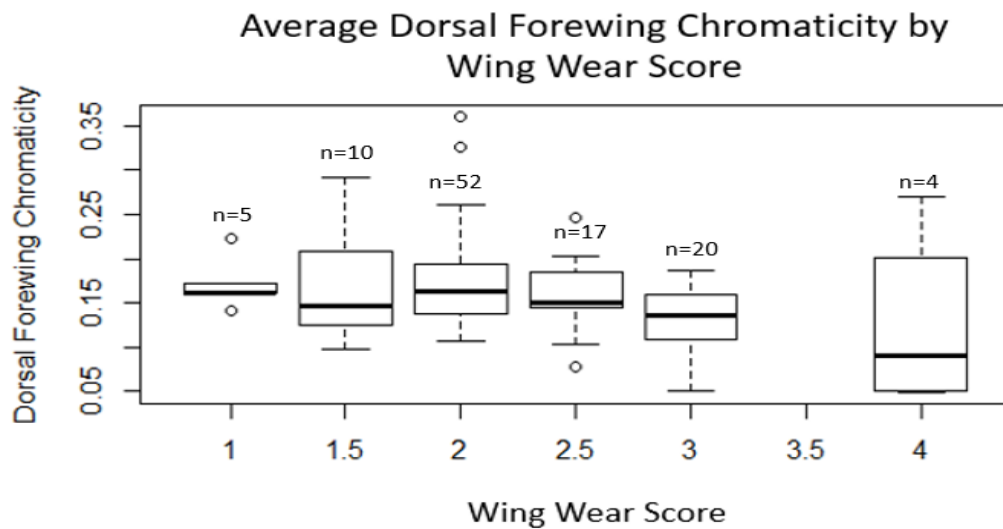


Figure 3.16: A box plot showing the average dorsal forewing chromaticity value as a function of wing wear score. Dorsal Forewing decreases as wing wear increases. The average dorsal forewing chromaticity values were particularly different between butterflies with wing scores of 1 ($p=0.002$ and 0.003), 1.5 ($p=0.001$), and 2 ($p=0.002$) when compared to those with scores of 3.

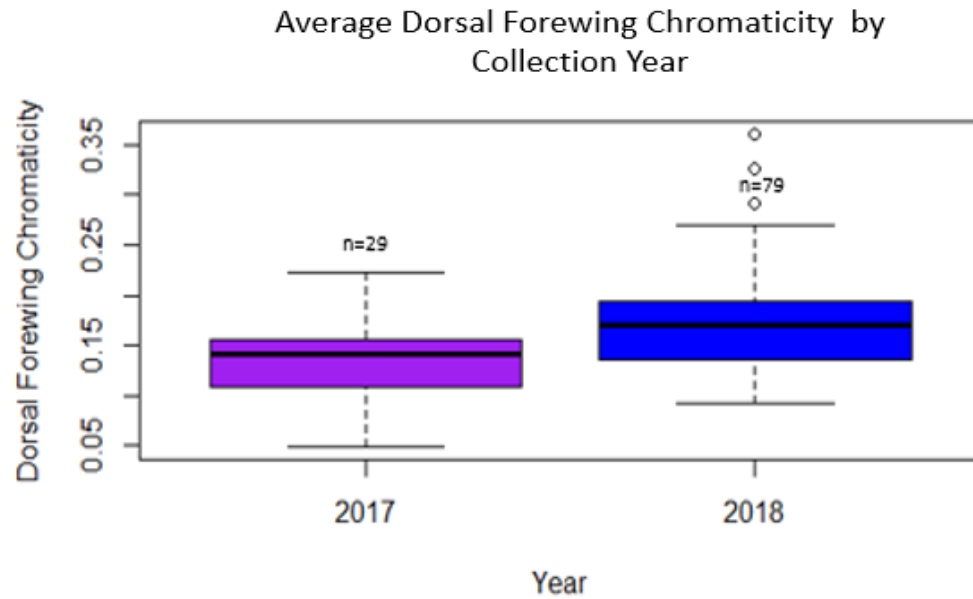


Figure 3.17: A box plot showing the average dorsal forewing chromaticity by year of collection. The average dorsal forewing chromaticity from the butterflies collected in 2018 (0.17 ± 0.05) is greater than those collected in 2017 (0.13 ± 0.05).

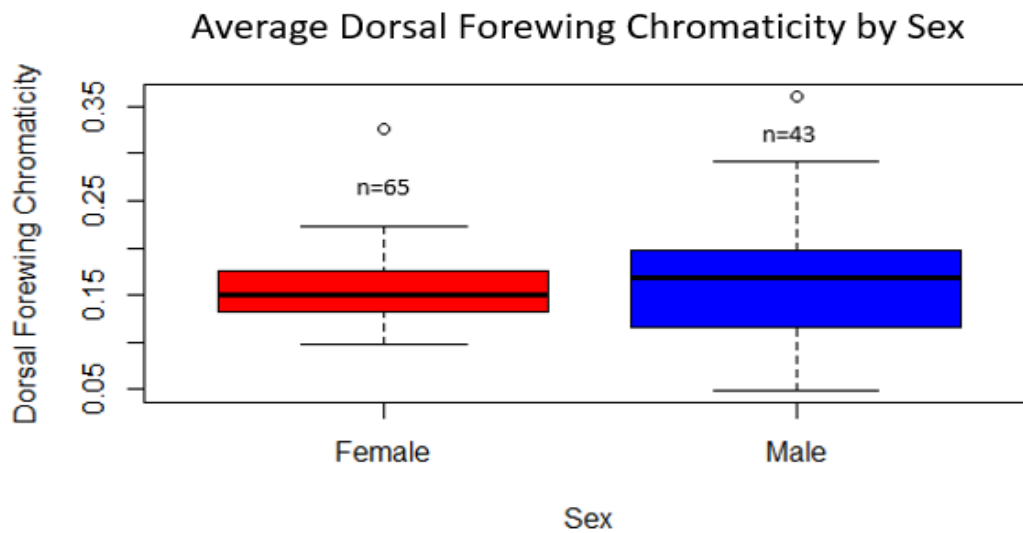


Figure 3.18: A box plot showing the average dorsal forewing chromaticity for females (0.15 ± 0.05) and males (0.18 ± 0.06). The average dorsal forewing chromaticity for males was greater than that of females.

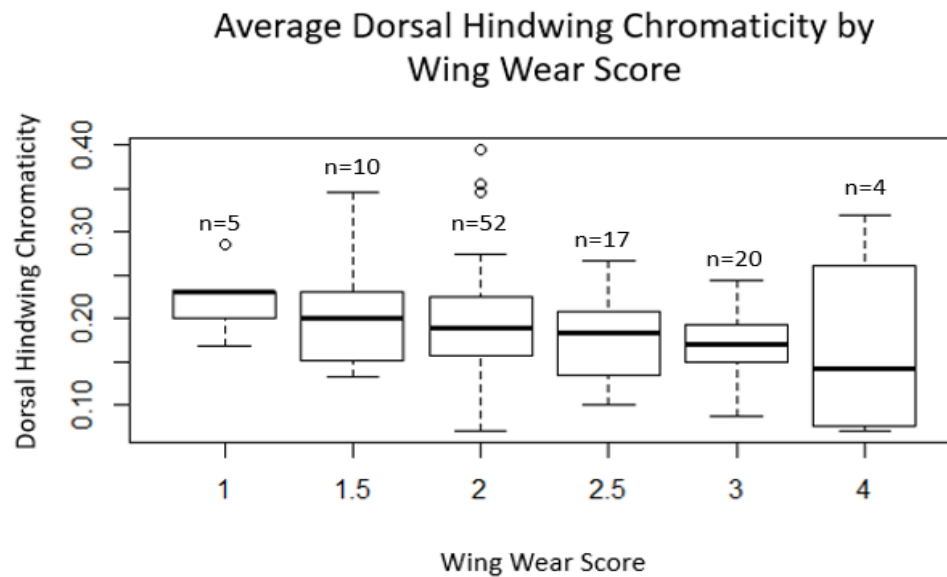


Figure 3.19: A boxplot showing average dorsal hindwing chromaticity values plotted against wing wear scores. Dorsal hindwing chromaticity has a negative relationship to wing wear score.

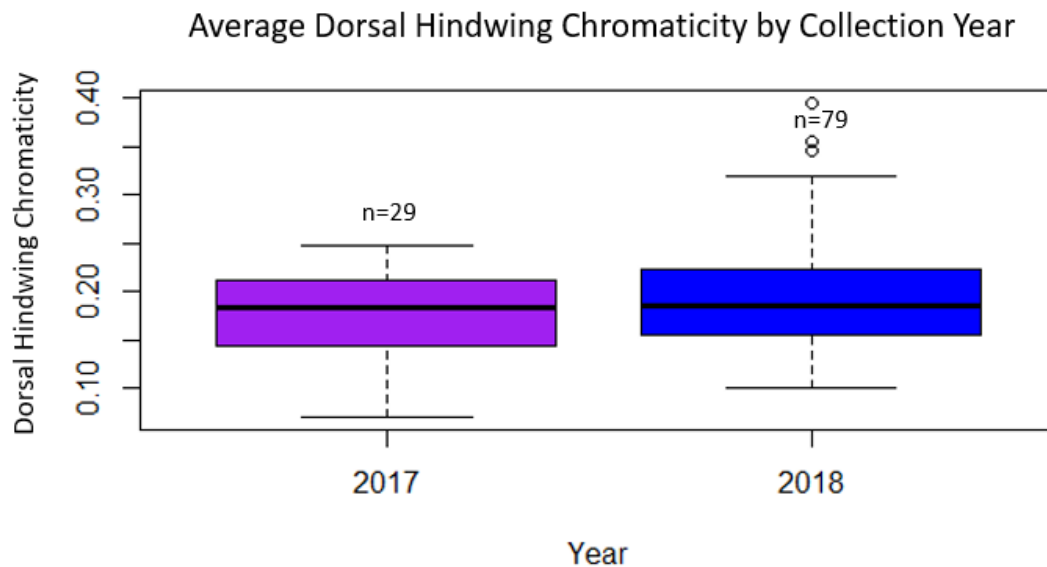


Figure 3.20: A box plot of the average dorsal hindwing chromaticity values of butterflies collected in 2017 and 2018. The average dorsal hindwing chromaticity was greater for the butterflies collected in 2018 (0.19 ± 0.06) than for butterflies collected in 2017 (0.17 ± 0.05).

3.3 COMPUTATIONAL MODEL OF VISUAL SENSITIVITY

3.3.1 Results from the electroretinography trials

The data points composing the MoR curve represent the absolute value of the difference between the highest and lowest voltage responses of eyes from *S. mormonia* for each wavelength (from 400 to 700 nm). The shapes of the response curves of male and female *S. mormonia* were similar, so I averaged the data from both sexes to create an MoR curve, which estimates spectral sensitivity (Fig. 3.19). A long-wavelength visual pigment with a peak wavelength of absorbance of 550 nm was a poor fit for the long-wavelength tail of the MoR curve, despite the long-wavelength visual pigment of *Speyeria leto* having a wavelength of peak of absorbance of 550 nm (Yuan et al. 2010). When I changed the peak wavelength of absorbance to 570 nm instead, the long-wavelength sensitive pigment absorbance curve was a better fit for the MoR curve (Fig 3.20) Therefore, I used both the 550 nm and 570 nm wavelengths as absorbance maxima corresponding to the long-wavelength sensitive visual pigment curve in the computational model. I also compared how these differences in lambda max values of the long-wavelength sensitive visual pigment affected the resulting ΔS^t output values. Since I only exposed the butterflies to wavelengths of 400-700 nm, it was not possible to determine whether the short-wavelength absorbance curve fit the MoR curve produced from the ERG data. Given that I was only interested in determining the discriminability of stimuli from dorsal wing regions, which are orange in color, sensitivity to long wavelengths was my focus, so this was not an issue.

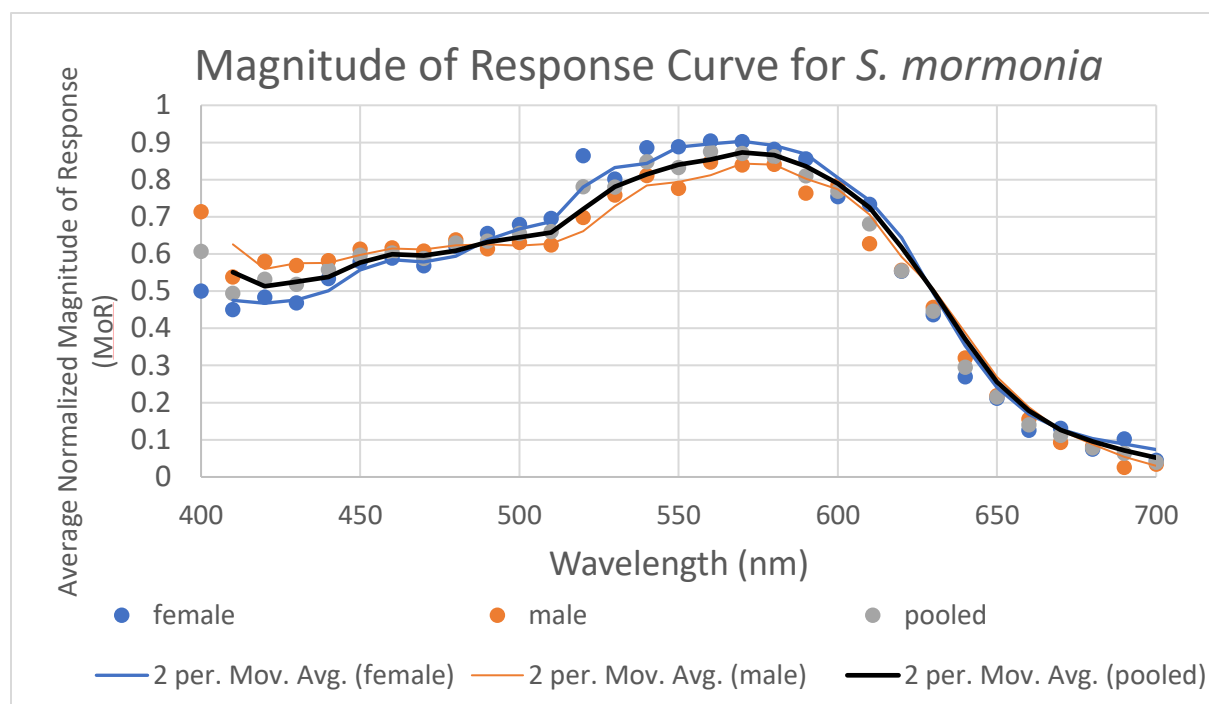


Figure 3.19: A scatterplot showing magnitude of response (MoR) as a function of wavelength. Data was obtained from DC electroretinography recordings of 3 male and 3 female adult *S. mormonia*. Responses from male (green) and female (red) butterflies were similar. The pooled data from this MoR curve was used to approximate the wavelengths of peak spectral sensitivity of the visual pigments of *S. mormonia*.

3.3.2 Results from the Computational Model for Color Discriminability

The results of the computational model indicate that *S. mormonia* can distinguish between the hues of orange displayed by the wings of conspecifics. When average reflectance data corresponding to the dorsal hindwings and dorsal forewings from butterflies collected in the 2017 and 2018 field seasons were applied to the model, the ΔS^t output values of these models were all greater than 1. Using different wavelengths of maximum sensitivity (i.e. 550 nm versus 570 nm) for the long-wavelength sensitive visual pigment absorbance data influenced ΔS^t values but did not change the outcome of the model. In the models with 2017 and 2018 reflectance data inputs, setting the webber fraction to 0.01 and the wavelength of maximum sensitivity to 570 nm

generally yielded larger ΔS^t values (Table 3.1 and 3.2).

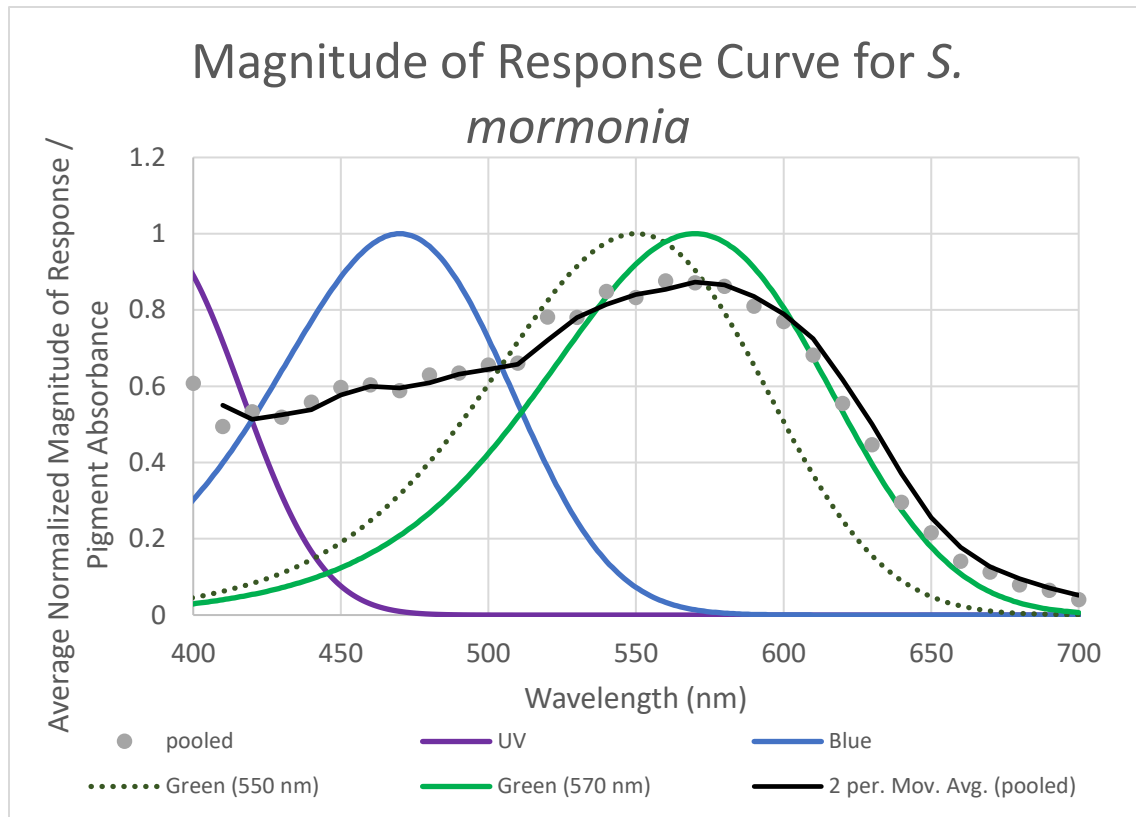


Figure 3.20: A pigment absorbance template (Stavenga et al.1993) with maximum sensitivity peaks at 385 (purple), 470 (blue), and 570 nm was used to obtain the pigment absorbance curves corresponding to the short-, middle-, and long-wavelength sensitivity visual pigments. These are plotted alongside the MoR curve. When the absorbance of the long-wavelength sensitive pigment has its maximum sensitive wavelength set to 550 nm (black dotted curve), which was determined to be the peak wavelength of sensitivity for one *Speyeria leto* female, the long-wavelength sensitive curve was to the left of the MoR data curve. However, changing the long-wavelength sensitivity peak to 570 nm (solid green) instead shifted the absorbance curve closer to the MoR curve. Therefore, 570 nm was also plugged into the computational model as a maximum absorbance peak.

The ΔS^t output values indicate that males have more perceivable variations in wing reflectance than females and that these variations in male wing reflectance are discriminable by the *S. mormonia* visual system (Figure 3.23-3.24). Models which compared inter-elevational differences in the average reflectance data from male dorsal forewings and dorsal hindwings consistently yielded higher ΔS^t values than the models

which compared inter-elevational differences in the average reflectance data collected from female wings. This trend occurred when reflectance data from both collection seasons were plugged into the model. This means that the variation in male dorsal forewing chromaticity and dorsal hindwing chromaticity across elevations are functionally relevant, because they are perceivable by *S. mormonia*.

Finally, setting the wavelength of maximum absorbance at 570 nm in the models generally yielded larger ΔS^t output values than when 550 nm was used as the sensitivity peak of the long-wavelength visual pigment. This may indicate that *S. mormonia* has greater spectral sensitivity at higher wavelengths, which may allow *S. mormonia* to distinguish between hues of orange that related butterflies, like *Dryas iulia* or *Speyeria leto*, may not be able to distinguish between. However, follow up studies are needed to corroborate this observation.

Table 3.1: Color discriminability values (ΔS^t) corresponding to comparisons between the average male and female dorsal hindwing reflectance spectra from 2017 and 2018

	wavelengths of max. sensitivity	
Collection Year	λ max=550nm	λ max=570nm
2017	$\Delta S=6.87$	$\Delta S=7.66$
2018	$\Delta S=6.25$	$\Delta S=6.7$

Table 3.2: Color discriminability values (ΔS^t) corresponding to comparisons between the average male and female dorsal forewing reflectance spectra from 2017 and 2018

	wavelengths of max. sensitivity	
Collection Year	λ max=550nm	λ max=570nm
2017	$\Delta S=5.72$	$\Delta S=6.36$
2018	$\Delta S=27.21$	$\Delta S=25.29$

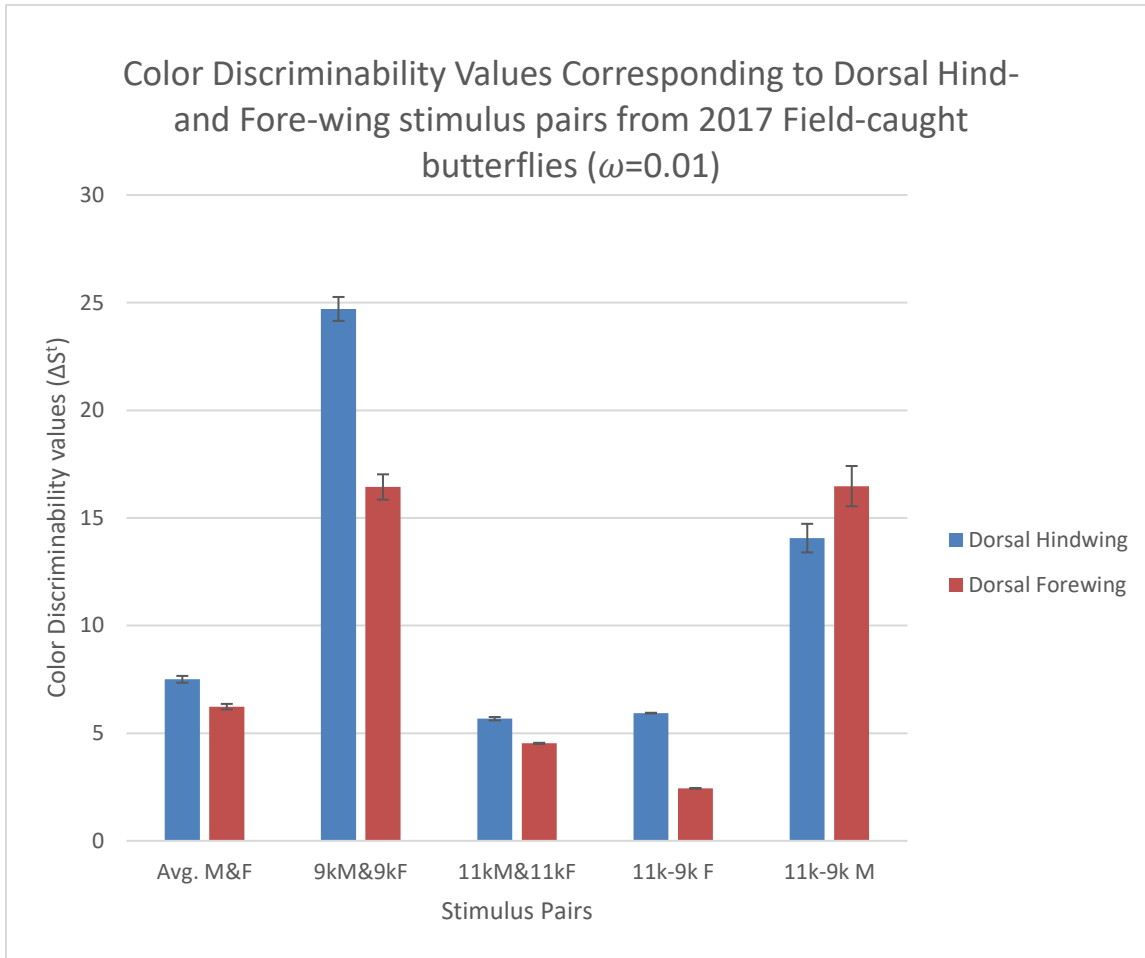


Figure 3.23: A bar graph showing the ΔS^t output values of computational models comparing the average reflectances from males versus females from different elevations in the 2017 field season. The difference between the average reflectances of dorsal forewings and dorsal hindwings of males versus females were both discriminable. Two-sample t-tests demonstrated that the ΔS^t value corresponding to the models comparing inter-elevational differences between male dorsal hindwing and dorsal forewing average reflectances were significantly larger than the ΔS^t output values of models comparing corresponding inter-elevational stimuli in females (dorsal hindwing: $t_{4,0.05}=2.78$ and $p=0.0003$; dorsal forewing: $t_{4,0.05}=2.78$ and $p=0.0001$). Also, the inter-sexual differences of dorsal hindwing and dorsal forewing average reflectances are greater on butterfly wings collected from low elevation sites than in those collected at high elevation sites (dorsal hindwing: $t_{4,0.05}=2.78$ and $p=4.55E-06$; dorsal forewing: $t_{4,0.05}=2.78$ and $p=0.0003$).

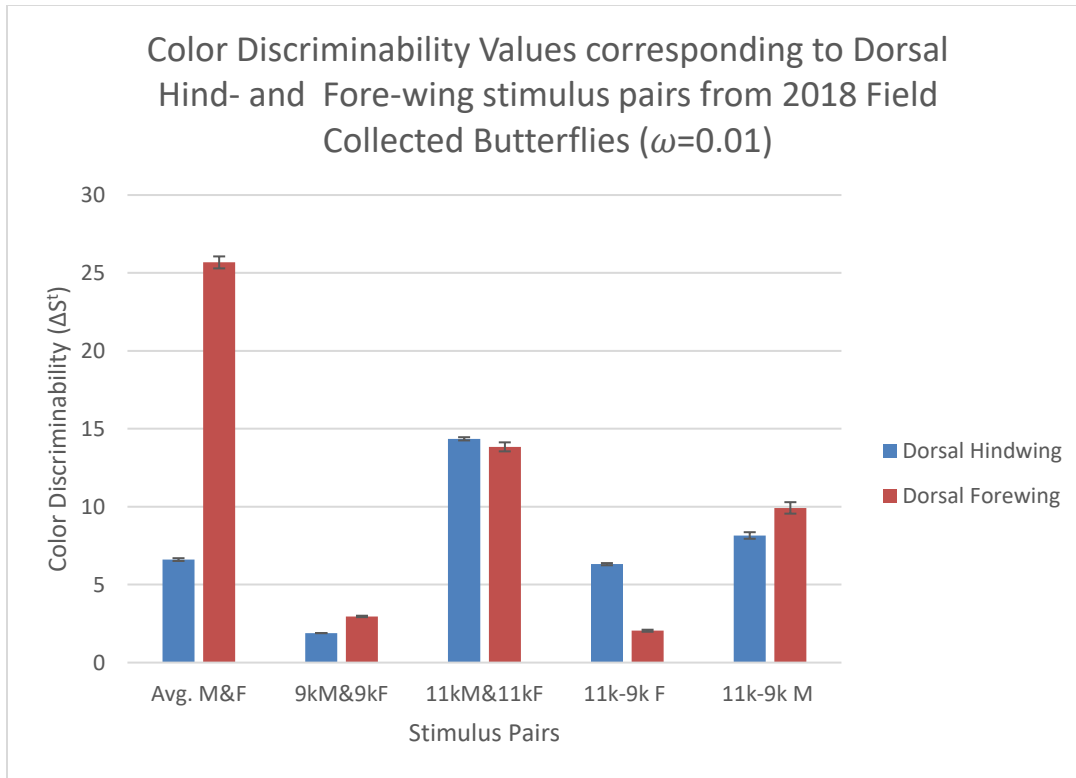


Figure 3.24: A bar graph showing the ΔS^t values corresponding to the comparison of dorsal hindwings and forewing reflectance data from male versus females of different elevations from the 2018 field season. Two-sample t-tests revealed that the ΔS^t values corresponding to the models comparing inter-elevational differences between male dorsal hindwing and dorsal forewing average reflectances were significantly larger than the ΔS^t output values of models comparing corresponding inter-elevational stimuli in females (dorsal hindwing: $t_{5,0.05}=2.57$ and $p=0.000422$; dorsal forewing: $t_{4,0.05}=2.78$ and $p=2.99E-05$). Also, the intersexual differences of dorsal hindwing and dorsal forewing average reflectances are greater on butterfly wings collected from high elevation sites than in those collected at low elevation sites (dorsal hindwing: $t_{4,0.05}=2.78$ and $p=2.88E-08$; dorsal forewing: $t_{4,0.05}=2.78$ and $p=3.15E-06$, respectively).

CHAPTER 4

DISCUSSION

4.1 Sex-specific energy investment strategies between male and female *S. mormonia* in response to resource-dependent shifts across elevations

Overall, the data corresponding to morphometric parameters (i.e. wing length and eye size) for these study populations of *S. mormonia* suggest that this species may have sex-specific energy investment strategies in response to the local energy resources which are available to them. Females consistently had longer wings than males (Fig 3.5). This is a typical sexual dimorphism observed in *S. mormonia* (Boggs 1987). Longer wings in females have been linked to a greater success in egg-laying ability, because this allows females to maintain flight performance as they carry heavy egg-loads during oviposition (Turlure et al. 2016). Males had larger eyes than females (Fig 3.9); this was consistently true at all elevations. This was partly because males had larger facets within regions 1, 4, 5, 7 and 8 of their eyes (Fig 3.11) and longer rhabdoms compared to females (Fig 3.12). Visual sensitivity increases as rhabdoms get longer and facets get wider (Land and Nilsson 2012). Therefore, the males likely have greater visual acuity and sensitivity than females, especially in the frontal, ventral, and dorso-medial regions of the eye, presumably to optimize their ability to find mates. This finding is consistent with observations made of other patrolling species of butterflies such as *Boloria aquilonaris* or *Coenonympha tullia* (Turlure et al. 2016; Rutowski 2000). Males which patrol as their

mate-seeking strategy may benefit from having acute zones located along the frontal and ventral regions of the eye, as females usually appear in front of or beneath them (Rutowski 2000). This is different than species with perching males, like *Pararge aegeria* or *Coenonympha pamphilus*, wherein males benefit from having frontal and dorsal acute zones because females fly in front of or on top of them while they sit and wait (Rutowski 2000).

These sexually dimorphic traits in *S. mormonia* may be linked to sex-specific constraints of nitrogen consumption during larval development (Carol Boggs, personal communication). Therefore, the fact that males develop larger eyes compared to females while females develop larger wings may suggest that males are optimizing mate-seeking ability strategies while females optimize egg-laying rates and fecundity (Turlure et al. 2016). Both investment strategies, though different, may be predicted to increase the reproductive fitness of members in this species. Further, these sexually dimorphic traits may impact traits relevant to visual ecology by causing modifications to the quality of the visual signal (i.e. wing length) and to the visual system (i.e. eye size).

I expected to see larger butterflies at higher elevations because the temperature size rule and Bergmann's law predict that organisms should have larger body sizes (i.e. wing lengths) at colder temperatures, which are usually associated with higher elevations (Angilletta Jr. et al. 2003). However, *S. mormonia* from these sample populations showed cross-elevational wing length patterns consistent with the converse Bergmann's law instead (Mousseau 1997): butterflies had shorter wings at higher elevations instead of lower elevations. More importantly, there was an effect of sex in the average wing length values across elevations. The female-biased sexual dimorphism observed in the pooled

average wing length values was generally greater at lower elevations. The degrees of sexual size dimorphism observed in the estimated eye surface area values from the dataset collected in 2017 (Fig 3.12) and in the average wing length values from the pooled data set (Fig 3.9) were generally more extreme at lower elevations compared to higher elevations. These results resemble those of a study which investigated how morphometric parameters changed in species of grasshoppers along an elevational gradient (Laiolo et al. 2013). The sexual size dimorphism displayed by the grasshoppers was less extreme at higher elevations. This has been linked to shifts in climatic features from one elevation to the next, particularly precipitation and temperature. It seems that male and female grasshoppers reacted differently to variations in their environment. Since precipitation and temperature, which directly impact the abundance of host plants, both tend to decrease at higher elevations, female grasshoppers were largest (relative to males) at low elevations in comparison to high elevations. This is because female grasshoppers showed greater plasticity than males in response to increases in host plant abundance. Overall, these disparities in sexual size dimorphisms across elevations have also been shown to apply to many other species of insects as well and seem to be a by-product of sex-dependent responses to changes in local environmental conditions (Tedder and Tammaru 2005; Nylin and Svard 1991).

My results suggest that male *S. mormonia* invest in making larger eyes that may be associated with better vision, whereas females invest in making larger bodies (i.e. longer wings). In this case I predict that the environmental factor to which *S. mormonia* are responding may be larval host plant abundance. *S. mormonia* are relatively nutrient-limited in the adult stage, as they primarily feed on carbohydrate-rich nectar, and derive

most of their nitrogenous amino-acid compounds from the leaf-based diets consumed in their larval state. Females may have longer wings than males, in part, because they spend relatively more time consuming host plant than males do in the larval stage as a result of protandry (Boggs 1987). This is reinforced by the fact that female *S. mormonia* have significantly greater body masses in pupation and eclosion than males do (Boggs 1987; Karlsson 1994), which is directly related to the degree of larval host plant intake (Carol Boggs, personal communication). Moreover, the way in which *S. mormonia* redistribute their body mass in adulthood occurs in a sex-specific manner which reinforces the suggested presence of sex-specific energy investment strategies in this species (Karlsson 1994). At eclosion, females have greater abdomen to total body mass ratios while males have greater thorax to total body mass ratios. Since they cannot feed on pollen to supplement their larval diet, both male and females lose body mass over time, but the rate at which males lose body mass is much slower than females, especially at the thorax region. Females also redirect the body mass at the thorax to their abdomens over time to optimize egg-laying success despite their nitrogen-limited adult diet (Karlsson 1994). This is likely the case because males need energy in their flight muscles, which are in the thorax, to patrol for mates, while females need more fat reserves in their abdomens to optimize egg-laying success.

Therefore, this sex-specific energy investment strategy of *S. mormonia* seems to be characterized by the following: females spend a longer time in the larval stage feeding than males do as a result of protandry, which causes them to eclose with larger body sizes (i.e. wing lengths) in comparison to males (Boggs 1987). The later emergence in females may also cause them to have more consistently chromatic wing colors as a result of their

significantly lower average wing wear scores in comparison to males, since wing wear negatively influences wing color (Dell’Aglia et al. 2017). Greater wing lengths in females may allow them to have greater success in egg-laying pursuits to optimize fecundity. Due to the earlier relative eclosion periods of males within this protandrous species, they cannot forage on host plants for as long a time as females in the larval stage, which makes them significantly more nitrogen limited and smaller in size as adults (Carol Boggs, personal communication). However, with the limited amount of host plant they can consume, males still consistently manage to produce larger eyes than their female counterparts, which increases their likelihood of detecting potential mates.

Furthermore, if male *S. mormonia* spend more of their time patrolling for females instead of feeding on nectar like other fritillaries, including *Boloria aquilonaris* (Turlure et al. 2016), I predict that they may not only have to face nutrient deficiency from their restricted larval diet, but also from their adult diet. Adult nectar uptake has been linked to greater reproductive ability and fecundity in butterflies (Karlsson 1994), so it would make sense if female *S. mormonia* feed on nectar more frequently than males do, especially given their life history traits. There is no data regarding the amount of time male *S. mormonia* allocate towards feeding on nectar in comparison to females, but they do in general imbibe nectar at faster rates (Boggs 1988). If there is a relative decrease in the amount of nectar feeding males do in comparison to females, this could add to their energy deficiency problem which began at the larval stage.

The Ghiselin-Reiss small male hypothesis may explain why something like this may occur, however. The hypothesis claims that in a species where there is scramble competition (competition of a resource, which is not monopolizable by any given

individual within a community), when there is a dearth of energy resources, it is beneficial for males to be smaller (Blanckenhorn et al. 1995). Small males will need to forage for less food than larger males would in order to have enough energy for mating. This means they will put more energy into mating because they are small enough that they don't need large amounts of energy from flower nectar to fuel their mating pursuits. Small males will therefore be more successful at mating than larger males. This is particularly true in species where conspecific females are significantly larger than males. In populations like these, food intake is correlated with female fecundity and male-mating success (Blanckenhorn et al. 1995). If this is the case for *S. mormonia*, then males of this species could possibly be putting what little energy resources they have been able to accumulate as larvae towards making large eyes to better detect potential mates, and a general decrease in nectar intake in adulthood is non-detrimental because they are relatively small and they can dedicate more time towards patrolling for mates instead of nectaring.

4.2 Evidence for context-dependent variations in the degree of protandry occurring at different elevations and the possible effects this may have on the visual signal of *S. mormonia*

Protandry is the tendency for certain species of organisms (esp. arthropods) to have males eclose and arrive at sites of reproduction earlier than females. This process allows males to optimize mating opportunities and helps decrease the probability of pre-reproductive death in females (Morbey 2013). The peak eclosion period of male *S. mormonia* occurs 2-3 weeks before that of females (Carol Boggs, personal communication). However, temperature can have sex-specific effects on developmental rates which can yield different degrees of protandry in different environments (Fischer and Fiedler 2001). In one particular study, lab reared lycaenid butterflies (also

protandrous) were exposed to a range of temperatures during development, from the egg stage through the adult stage. The butterflies showed sex-specific reactions in response to exposures of elevated temperatures (Fischer and Fiedler 2001). At higher rearing temperatures, males had significantly faster development rates in the pupal stage, which led to earlier than normal male emergence periods. This led to a decrease in pupal and adult weight in males of high temperature treatments compared to their female counterparts because they were unable to eat as much host plant during the larval stage. The reaction of lycaenid females in response to the high temperature treatment was different; they chose to optimize body size instead and did not significantly increase their pupal development rates. On top of this, another study on the phenology of British butterflies (including *Argynnis phaphia* and *Argynnis aglais*, both close relatives of *S. mormonia*) in response to climate change has proven that in a wide range of species, there has been an advancement in butterfly eclosion periods ranging between 1-10 days in response to the increases in spring temperatures onset by climate change (Roy and Sparks 2000). This just reinforces the idea that varying temperature can shift phenological schedules of butterflies.

The average wing wear scores of male and female *S. mormonia* across collection sites suggest that there are different degrees of protandry occurring across elevations, possibly as a result of sex-specific phenological shifts in male eclosion periods. There was no significant difference between the male and female average wing wear scores at the 3352-meter elevation, which was the highest collection site. At the three lowest elevations, however, there were significantly large disparities between male and female wing wear scores. Wing wear is often used as a proxy for age in butterflies. With this in

mind and given that temperature tends to decrease at higher elevations, these results could suggest that males are emerging much earlier than females at lower elevation sites due to the higher temperatures occurring there, similarly to the males in the lycaenid study (Fischer and Fiedler 2001).

Visual cues from conspecifics are important for proper detection of potential mates by male *S. mormonia* (Boggs personal observation) and its close relatives (Hidaka 2010; Stride 1957). In fact, *Argynnis paphia* males, which belong to a European sister species to *S. mormonia*, are known to prefer females with more orange and larger wings with fast wing beat frequencies, even when these stimuli are supernormal (Magnus 1960). This affinity for the orange wing color of mates is reinforced by the tendency for male *S. mormonia* to land on orange-colored *Agoseris aurantiaca* flowers in the field with the apparent initial intent to mate (Carol Boggs, personal observation). This suggests that orange wings may act as behavioral release cues that induce males to pursue a target for mating. Orange wings are associated with higher chromaticity values because they have more saturated orange colors, likely because they are richer in wing pigment. This affinity for orange wing colors on females may come about because it is a cue or indicator of mate-quality, which is the case with other butterfly species, where the hue of wings may be a byproduct of the larval diet consumed by females (Johnson et al. 2014).

A promising follow up study would be to investigate whether there are sex-specific regions on butterfly wings where males lose wing scales or show greater wing wear in comparison to females, due to the differences between their life history traits. Males have higher average dorsal hindwing chromaticity values than females, while females are more chromatic at the dorsal forewing, which may indicate that males lose

more scales in the dorsal forewing, perhaps due to the strain imposed by flight on the wing surface.

If my assumptions concerning the temperature-dependent shift in phenology of *S. mormonia* are correct, these findings could hold implications regarding the effects that climate change has on the phenology of these butterflies. Global warming causes significant increases in average global temperatures. If my observations are correct and *S. mormonia* are indeed responding to temperature increases by emerging earlier than normal, this could have serious ecological consequences. This could cause desynchronization between *S. mormonia* adult flight periods and the flowering periods of their nectaring plants. This may lead to a potential decrease in the size of local populations, due to an increase in larval mortality, which may ultimately make the study population more susceptible to stressful environmental conditions, due to the subsequent depletion in within population genetic variation that may follow. Alternatively, in response to drought, the species might migrate to another area which bears an adequate abundance of food resources. This would cause them to overlap with other butterfly species, which may lead to interspecific competition (Kearny et al. 2010; Both et al. 2008; Sims 2017). Moreover, I predict that the sex-specific responses to temperature increases, particularly in the emergence dates of male *S. mormonia*, may cause there to be a longer time lapse between male and female flight periods, which may negatively affect their ability to mate.

4.3 How *S. mormonia* perceive the variations in wing chromaticity across elevations brought about as a result of wing wear

The average chromaticity of dorsal wing regions tended to decrease as the wing wear score increased. This makes sense as wing wear can affect the integrity of the pigments, and thereby affect the way that light interacts with the surface of the wing, which can dampen wing color or chromaticity (Dell’Aglia et al. 2017; Stavenga et al. 2014). Given this significant negative relationship between dorsal forewing chromaticity and wing wear scores, males presumably had more variable dorsal forewing chromaticity than females across elevations as a result of the variable wing wear scores assigned to males compared to females, due to the different degrees of protandry occurring across elevations.

In the model, I found that for both dorsal forewing and dorsal hindwing specific stimulus comparisons, *S. mormonia* could perceive the difference in wing color of males from low (i.e. 2743 meters) versus high (i.e. 3353 meters) elevations significantly more than they could perceive the difference between corresponding stimulus pairs of wings from females collected from low versus high elevation sites. This means that the variation in chromaticity of male wings, as a result of wing wear, is discriminable by *S. mormonia* and are therefore functionally relevant. Given that wing wear is often used as a proxy for age in butterflies, this means that *S. mormonia* can discriminate older or more worn males (i.e. collected at low elevations) from younger or less worn males (i.e. collected at high elevations) based on their wing reflectance or chromaticity. These findings may indicate that wing color is important for *S. mormonia* males to spot potential mates, and for females to distinguish between potential mates. Though vision plays a role in target detection, female butterflies in general do not solely rely on color for mate detection.

Smell is also important to help female butterflies choose mates because the quality of the pheromones emitted by male counterparts from their androconia at the time of courtship is often indicative of the quality of the male's spermatophores (Nieberding et al. 2012; Capinera 2008). Therefore, males do not necessarily need to have a certain hue of orange on their wings to be attractive to females, which may partially explain the large variation in male-specific wing chromaticity across the elevational gradient.

I also found that the greatest discriminable inter-sexual variation in wing reflectance values from 2018 field-caught butterflies is observed in the butterfly population collected from the 3353-meter elevation site. However, in the data from the 2017 collection season, the greatest discriminable inter-sexual variation in wing reflectance occurred between the wings of butterflies collected at the 2743-meter elevation site, which is also one of the sites at which the sexual dimorphism in wing wear was greatest. Since inter-sexual disparities in reflectance data from 2018 are presumably unlinked to the effect of wing wear, because the disparities in wing wear scores between males and females were not significant at high elevations, the chromaticity of butterfly wings collected at the high elevation sites during 2018 may indicate that there were differences between the sexes in the degree of plasticity in wing color in response to some environmental condition at this elevation site, including host plant abundance.

Finally, results from electroretinography suggest that the absorbance peak of the long-wavelength sensitive visual pigment of *S. mormonia* may be at a longer wavelength than expected. In the process of doing the computational models, I noticed that the long-wavelength sensitive pigment absorbance curve could fit closer to the magnitude of response curve when it had a sensitivity peak set to 570 nm than when the peak was

instead set to 550 nm. Using a spectral sensitivity peak wavelength of 570 nm instead of 550 nm for the long-wavelength sensitive pigment absorbance data also yielded higher ΔS^t output values from most computational models. These results were different than I expected, and in order to know for certain that the long-wavelength sensitivity peak for *S. mormonia* occurs at 570 nm, I would have to perform follow up electroretinography trials, using different light filters and epi-microspectrophotometry (emsp) to get a more accurate idea of the spectral sensitivity across different wavelengths for this species. If the follow up experiments reveal that the peak wavelength of sensitivity for *S. mormonia* occurs at 570 nm, then this may mean that *S. mormonia* can discriminate between stimuli which reflect long wavelengths of light better than close relatives can. I would then follow up with experiments involving genetic sequencing techniques or emsp to determine whether a gene duplication event or a filtering pigment is causing them to have a higher sensitivity to longer wavelengths of light.

Most nymphalids lack filtering pigments or tiered retinas, which are typical in pierids and papilionids and contribute to their extraordinary spectral sensitivities (Stavenga and Arikawa 2006). However, this possibility shouldn't be ruled out because red filtering pigments have been found in *Danaus plexippus* and *Heliconius erato*. Both species are relatives of *S. mormonia*, as *Heliconius* and *Speyeria* are in closely related tribes and *Danaus plexippus* and *Heliconius* butterflies are both part of the family Nymphalidae (Blackiston et al. 2011; Zaccardi et al. 2006). There is also evidence of various other butterflies, including lycaenids and colias, which have tuned their spectral sensitivities to the wing reflectances of their conspecifics (Sisson Mangus 2009; Sisson Mangus 2006; Arikawa 2005). If the sensitivity peak of *S. mormonia* does occur at 570

nm, this may be the case because of how important the color orange is as a mating-detection cue for this species (Carol Boggs, personal communication).

Given the observed trends in the wing chromaticity and wing wear scores of *S. mormonia*, I would be interested in following up with an experiment which investigates whether there is a link between male wing chromaticity and the way they perform in courtship events using behavioral trials. *S. mormonia* males, as a patrolling species, are non-aggressive. Though they need to have good flight endurance to obtain females (Wickman 1992), the quality of their pheromones at the time of courtship is an important trait for mating success in various butterfly species (Dunford 2007). It would be interesting to investigate whether there is a correlation between male dorsal forewing chromaticity and the quality of their nuptial flights or of the pheromones they produce, and whether females would be receptive to mating with them as a result of these factors.

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