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Seasonal plasticity and correlations among butterfly wing color patterns: Insights into modularity

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Seasonal plasticity and correlations among butterfly wing color patterns: Insights into modularity

A Thesis

Presented to the Department of History

College of Liberal Arts and Sciences

and

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In Partial Fulfillment

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Rachel Urban

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Abstract

Dealing with the wide ranges of temperatures throughout the year can be a challenge for ectothermic animals like insects. Phenotypic plasticity, the development of different phenotypes depending on the environment, is one way that animals can combat this problem. However, traits are often integrated and share developmental or genetic mechanisms which can limit the adaptive response of an individual trait. Modules, groups of traits that vary together but are independent of other traits, often share developmental mechanisms and therefore can facilitate the evolution of these adaptive traits. The seasonally variable melanistic wing patterns elements of the *Colias* butterfly are an ideal model to study modularity because of their homologies and shared developmental requirement of the pigment, melanin. The seasonal variation of the ventral hindwing is adaptively advantageous for thermoregulation, but the variation of other melanistic traits has received far less attention. This goal of this project was to identify which melanistic traits vary seasonally and examine how they correlate with each other and with the ventral hindwing in *Colias* butterflies using statistical analyses. The results show that melanistic wing pattern elements are seasonally plastic and modular. Furthermore, these modules align with elements of the nymphalid groundplan. Because melanized basal trait elements show similar patterns of seasonal plasticity as the ventral hindwing, they may share its thermoregulatory function. Additionally, the potential underlying mechanisms of the seasonal plasticity and modularity of these melanistic wing pattern elements are discussed.

Introduction

Temperate regions, such as North America, experience a range of ambient temperatures throughout the year because of the seasons. In order to properly function, animals must be able to handle these changes in temperature. However, ectotherms, such as insects, rely on environmental conditions to maintain their body temperature and therefore changes in ambient temperature pose a greater challenge to them (Heinrich, 1995). Additionally, while one phenotype may be optimal in one environmental condition, another phenotype may be optimal in another environmental condition. Therefore, it is unlikely that a single phenotype is best suited for the wide range of environmental conditions an individual may experience. Phenotypic plasticity, or the development of different phenotypes depending on the environment (Murren, 2012), is one mechanism that allows organisms to cope with a wide range of environmental conditions (Snell-rood & Ehlman, 2021). Phenotypic plasticity is particularly useful when there are reliable cues for future environmental conditions, such as those preceding seasonal changes in temperature (Snell-rood & Ehlman, 2021). Therefore, many animals have evolved developmental processes that respond to current conditions to produce phenotypes better suited for future conditions (Snell-rood & Ehlman, 2021).

Because animals are formed by the combination of many traits, individual traits usually do not develop or evolve independently. Instead, they are often integrated, or form relationships, with other traits through common developmental or genetic mechanisms, such as through pleiotropy (the effect of one gene on multiple traits) or a shared reliance on a resource (Murren, 2012). This developmental integration may limit how well individual traits can adaptively respond to conditions during development, especially if selective pressures differ between integrated traits (Murren, 2012). However, integration can also result in modules, or groups of traits that are associated through variation, function, or other process and are relatively independent of other traits of that organism (Murren, 2012; Wagner et al., 2007). Because traits within a module share functions or developmental mechanisms, modularity can not only reduce constraints on adaptive development or evolution but even facilitate such processes (Murren, 2012; Wagner et al., 2007). Because traits within a module are strongly correlated with each other but not correlated with other traits (Wagner et al., 2007), phenotypic plasticity serves as an excellent framework for investigating modularity. This is because phenotypic plasticity increases total variation of traits in a population which in turn increases the ability to statistically identify correlations; this is the first step in identifying modules. Modularity is thought to be important for the continuing evolution of complex phenotypes because modularity allows traits within a module to develop, and evolve, relatively independently of other traits in other modules (Murren, 2012; Wagner et al., 2007). However, because modularity is not always absolute, variation in the degree of modularity can also serve as a constraint (Murren, 2012).

Butterfly wing color patterns are ideal model systems for studying modularity and plasticity because their structure consists of similar repeated pattern elements in each wing compartment, the area contained by the wing veins, that can develop independently of other, potentially adjacent, wing compartments or patterns (Nijhout, 1994). These repeated pattern elements form systems of homologies called the nymphalid groundplan (NGP) (Fig. 1), named after the butterfly family, Nymphalidae, in which it was first described, and can be identified in most major families of moths and butterflies (Nijhout, 1991b, 1994). The NGP consists of three symmetry systems or paired anterior-posterior bands of homologous wing pattern elements (Fig. 1) (Nijhout, 1991b, 1994). Although these symmetry systems are homologous, they are often highly differentiated and appear radically different from each other (Nijhout, 1991b). Within these symmetry systems, wing pattern elements often evolve independently from each other resulting in changes in the color, shape, size, or presence of the trait (Nijhout, 1991b). This idea is illustrated by modularity in the development of the familiar repeated "eyespots" (concentric rings of color on the wing) of differing size on the marginal hindwing of many species of butterflies (Fig. 1) (Monteiro et al., 2003). These eyespots are homologous structures, meaning they share common evolutionary origins, yet due to their independent development, almost every eyespot is part of a separate module (Monteiro et al., 2003). Comparing variation in individual wing pattern elements using statistical methods can reveal groups of strongly correlated wing pattern elements that comprise a module (Murren, 2012).

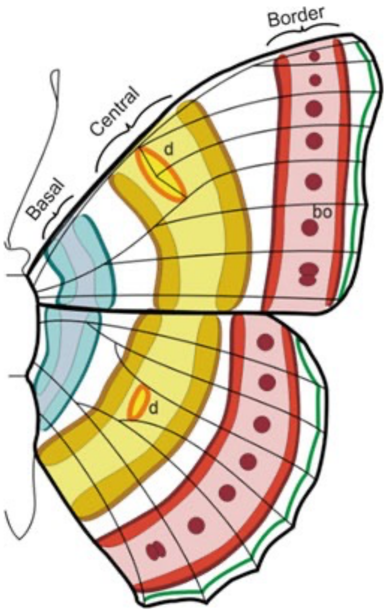

Figure 1. Nymphalid groundplan symmetry systems (basal, central, and border). Darker areas represent the bands of each symmetry system. The red ovals in the central symmetry system show the discal spot elements (d). Red spots in the border symmetry system are border ocelli (bo) which can develop into eyespots. Reproduced from Nijhout (2017).

In central Indiana, one common group of butterflies, those in the genus *Colias*, commonly referred to as "sulfur butterflies," are particularly well-suited for this type of investigation because of their multiple dark discrete wing pattern elements consisting of scales pigmented with melanin (Fig. 2, 3). Additionally, there is variation in the degree of a more diffuse melanisation at the base of the ventral hind wing (VHW) (Fig. 2, 3) in *Colias* butterflies (Gerould, 1927; Roland, 1982; Watt, 1968, 1969). Melanisation of the VHW is phenotypically plastic, meaning that shorter days during butterfly development result in a higher degree of melanisation and longer days produce a lower degree of melanisation (Gerould, 1927; Hoffmann, 1973; Shapiro, 1976; Watt, 1968, 1969). Watt (1969) showed that the degree of melanisation of the VHW over the course of a year showed a "U-shaped curve," with the highest degree of melanisation occurring in the colder months (i.e. both spring and fall) and the lowest degree of melanisation occurring in the warmer summer months.

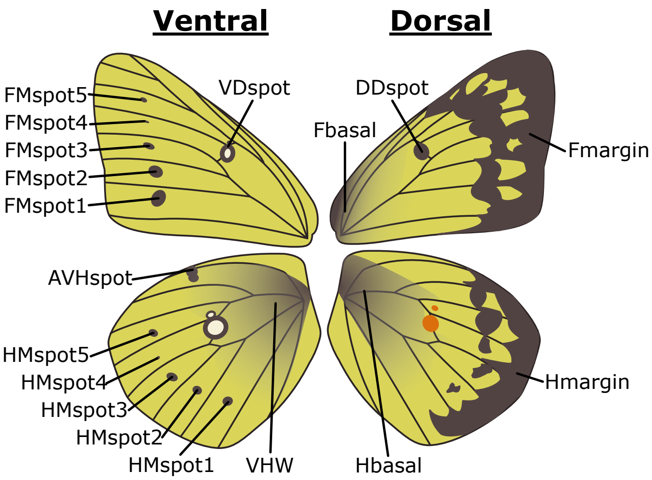

Figure 2. Measured wing pattern traits on the ventral (left) and dorsal (right) wing surfaces. The diagram depicts a female *Colias* butterfly, but the locations of these traits are the same for male *Colias* butterflies. Full names of abbreviated traits are included in Table 1.

Figure 3. Photographs of the ventral and dorsal surfaces of female and male *Colias* butterflies.

Furthermore, this variation in VHW melanisation was shown to be adaptive for effective thermoregulation (Roland, 1982; Watt, 1968, 1969). Butterflies require a certain body temperature (between about 35-37°C) in order to fly (Watt, 1968, 1969). If they are too cold, they will fold their wings over the body and bask with the VHW surface oriented perpendicular to the sun to increase solar absorption, and therefore their body temperature, to fly (Watt, 1968, 1969). Similarly, if they are too hot, they will find shade and orient their wings to be parallel to the sun to decrease their body temperature to fly (Watt, 1968, 1969). Flight is necessary for *Colias* butterflies for feeding and reproduction during their relatively short lifespan (Watt, 1968, 1969), so an adaptation that allows them to better thermoregulate and fly for longer periods of time is advantageous. Because the melanisation of the VHW is located near the body, these dark scales can absorb light to aid in increasing body temperature (Roland, 1982; Watt, 1968, 1969). This increased melanisation of the VHW is advantageous in colder environments because darker butterflies increase their body temperatures faster in the sun than lighter butterflies (Watt, 1968, 1969). This allows the butterflies with darker VHWs in cold temperatures to fly for longer durations of time, giving them an adaptive advantage over lighter butterflies (Roland, 1982). On the other hand, in warmer temperatures decreased melanisation of the VHW is advantageous in preventing overheating (Roland, 1982; Watt, 1968, 1969). Therefore, butterflies with lighter VHWs are able to stay active during the hottest parts of the day while darker butterflies seek shade to avoid overheating (Roland, 1982).

These thermoregulatory advantages clearly explain the seasonal variation of VHW melanisation observed in *Colias* butterflies. **However, variation in other melanized wing traits, such as the dorsal forewing margin (Fmargin) (Fig. 2, 3), have received far less**

attention. While it has been suggested that other melanized wing traits are plastic, none of this work has been rigorous or quantitative (Ellers & Boggs, 2004; Gerould, 1927; Hoffmann, 1973; Hovanitz, 1945). Therefore, the goal of this project is to identify the patterns of variation and covariation (i.e. correlation) of these other melanized traits and compare them to the known seasonal variation in the VHW. Depending on how these traits vary and covary compared to the VHW, we can gain insight into their potential modularity and/or adaptive significance.

I have developed three hypotheses that could explain covariation among wing pattern elements in *Colias* butterflies. First, all of the melanized wing traits are part of the same module with no substructure with respect to variation because they are all melanin-based. In other words, we might just think of butterflies as being heavily melanized or not. In this case, I would expect *all of the melanized wing elements to be strongly and positively correlated with each other*, resulting in relatively lighter or darker butterflies overall. This could indicate that these melanized wing patterns may serve a similar thermoregulatory function as the VHW or that they share a developmental pathway (Murren, 2012; Wagner et al., 2007).

Alternatively, because melanized wing traits compete for necessary resources (in particular, the amino acid tyrosine) for melanin production, melanin that is allocated to some traits may be unavailable to others (Stoehr, 2006). In other words, rather than butterflies being either heavily melanized or not, this second hypothesis, the "trade-off hypothesis," predicts that *some modules (defined by the traits that form them) will be heavily melanized at the same time that other modules are less melanized*. As a result, we might observe strong positive correlation within given modules but negative or absent correlations between modules.

Finally, it is possible that melanized wing traits in *Colias* butterflies largely conform to the nymphalid groundplan (NGP), as has been suggested by Schwanwitsch (1956). Therefore, wing pattern elements may be modular due to homologies in the symmetry systems and the divergence between them. In this case, I would expect groups of *melanized wing pattern elements that have derived from the same symmetry system to be strongly correlated with each other but much less or not correlated with traits in other symmetry systems*.

The central objective of this project is to test multiple alternative hypotheses that could identify and explain wing pattern modularity in *Colias* butterflies. These hypotheses are not necessarily fully mutually exclusive because module boundaries are not absolute (Murren, 2012), but it may still be possible to identify which hypothesis or hypotheses best explain wing pattern variation. To do so, I will identify which melanistic traits vary seasonally and examine how they covary (i.e. correlate) with each other. Therefore, this project would be the first step in exploring potential modularity in *Colias* wing patterns while also suggesting possible underlying mechanisms for that modularity.

Methodology

Specimen acquisition and preparation

The lab's principal investigator, past lab members, and I caught two species of *Colias*, *C. eurytheme* and *C. philodice*, (and their hybrids) throughout central Indiana between 2017 and 2022. We froze then pinned and dried the butterflies using standard entomological techniques.

Photography

I photographed the dorsal and ventral surfaces of the pinned butterflies using a Canon EOS Digital Rebel T3i camera mounted with a Canon EF 100 mm macro lens and Canon MR-14EX macro ring flash at 1/4 power. Additionally, I mounted the camera perpendicularly to a copy stand with the flash 38.4 cm from the wing surface. To ensure standardized exposure, I photographed the butterflies using the same settings (shutter speed = 1/200 second, f-stop = F14, ISO = 100). I placed the butterflies on a piece of blue cardstock with a vertical cutout for the pin and legs allowing for the wing surface to lay flat on the cardstock. Anterior to the butterfly, I placed an Image Science Associates M color standard, which could be used in the future as a standard to investigate variation non-melanin wing pattern elements. Posterior to the butterfly, I placed a 1 cm scale bar and ID number. I sorted the butterflies into boxes, more-or-less, by ID and photographed them by box, with box order randomized.

Image analysis

Current lab members and I analyzed all photographs using the ObjectJ plugin for ImageJ. I created measurement groups of about 80 butterflies by randomly sampling, without replacement, 5 males and 5 females from each month, or if fewer than 5 individuals were available, I placed all remaining individuals into the group. Due to the uneven distribution of butterflies by month and sex, sometimes all butterflies of a certain sex and month had already been sampled as more groups were created. Therefore, to keep measurement groups around 80 individuals, I increased the number of males and females randomly sampled from each month with individuals remaining. We measured a total of 526 butterflies, of which 280 were male (M) and 244 were female (F). For each month, we measured between 10 and 39 butterflies of each sex, but most months contained 39 butterflies of each sex.

We measured a total of five dorsal and thirteen ventral wing pattern traits and their names, abbreviations, and locations are shown in Table 1 and Figure 2. To quantify wing pattern melanism, we measured the area of "black" above the designated threshold within the traced area. We converted the images to grayscale, with 0 = white, 255 – black, and then quantified the area above a pre-set threshold. The thresholds that we used for each wing pattern are listed in Table 1. I chose the thresholds so that black (melanized) scales were clearly separated from the background yellow or white scales. Because the melanized scales and background colors vary slightly among wing regions, I chose different thresholds for different traits. However, at the beginning of the study after visually inspecting several butterflies, we used the chosen threshold for all butterflies. For all spot wing pattern elements (FMspot1-5, HMspot1-5, AVHspot, DDspot, and VDspot), we included

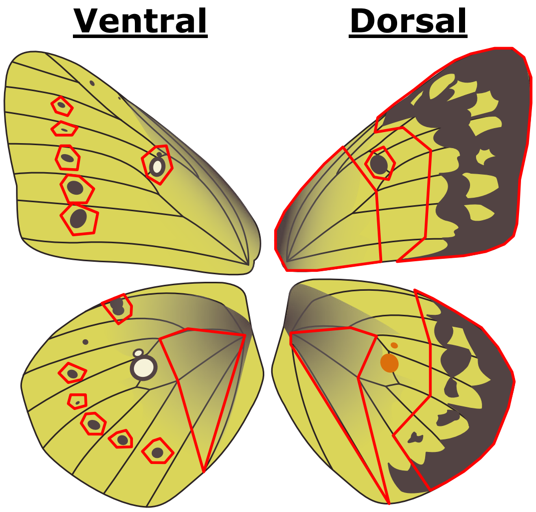

Figure 4. Measured areas for each wing pattern trait on the ventral (left) and dorsal (right) wing surfaces. The specific landmarks used for non-spot wing pattern elements are shown.

all melanin scales associated with the spot in the measured area (Fig. 4). We completely traced the margin elements (Fmargin and Hmargin) with certain cutoffs associated with wing veins (Fig. 4). For all other wing pattern elements, we defined the areas by specific intersections of wing veins, or a wing vein and the wing margin as shown in Figure 4. We recorded forewing length (FWL), not pictured, as a measure of size and defined it as the distance between the point where the wing joins the body and the furthest point on the wing margin.

To estimate measurement repeatability, three lab members and I measured the dorsal and ventral wing patterns of one set of 80 butterflies using the same designated wing vein landmarks.

Table 1. Measured wing pattern trait name, abbreviation, and threshold value.

Wing Surface	Trait Name	Abbreviation	Threshold
Dorsal	Forewing margin	Fmargin	100
	Hindwing margin	Hmargin	
	Basal forewing	Fbasal	
	Basal hindwing	Hbasal	
	Dorsal discal spot	DDspot	
Ventral	Ventral discal spot	VDspot	108
	Forewing margin spot 1	FMspot1	
	Forewing margin spot 2	FMspot2	
	Forewing margin spot 3	FMspot3	
	Forewing margin spot 4	FMspot4	
	Forewing margin spot 5	FMspot5	
	Anterior ventral hindwing spot	AVHspot	107
	Hindwing margin spot 1	HMspot1	117
	Hindwing margin spot 2	HMspot2	
	Hindwing margin spot 3	HMspot3	
	Hindwing margin spot 4	HMspot4	
	Hindwing margin spot 5	HMspot5	
	Ventral hindwing basal	VHW	120

Statistical analyses

I analyzed the sexes separately due to sexual dimorphism of wing pattern traits (males n = 280, females n = 244). In addition, I made no distinction between the two species (i.e. both species are lumped together in the analyses) because they have very similar wing patterns and regularly hybridize. I used R (version 4.2.0; R Core Team, 2022) in the RStudio IDE (version 2022.7.1.554; RStudio Team, 2022) to conduct all data analyses. For general data wrangling and manipulation, I used the tidyverse packages (Wickham et al., 2019). Additionally, I formatted dates using lubridate (Grolemund & Wickham, 2011).

To examine the seasonal plasticity of wing pattern elements, I conducted an ANCOVA using a linear model predicting melanism (area of black above the threshold) by month of capture while factoring out wing size (trait ~ FWL + month).

I created the plots illustrating seasonal plasticity of wing pattern elements using date as a continuous variable. I corrected trait melanism for wing size by dividing the trait value by FWL. Then, I scaled the size-corrected trait values to have a mean of 0 and standard deviation of 1 where a higher scaled trait value indicates increased melanisation (i.e. a darker or larger trait). I created the smoothed lines using LOESS regression. To create all plots, I used the R packages, ggplot2 (Wickham, 2016) and cowplot (Wilke, 2020), and to arrange the plots, I used the packages, ggpubr (Kassambara, 2020) and gridExtra (Auguie, 2017). Additionally, I changed the fonts in the figure using the packages, showtext (Qiu, 2022a) and sysfonts (Qiu, 2022b).

I assessed the modularity of wing pattern elements using conditional independence and graphical modeling as described by Magwene (2001). I calculated the partial correlations between all pairs of wing pattern elements controlling for wing size (FWL) using the R package, ppcor (Kim, 2015). Edge Exclusion Deviance (EED) is a theoretical measure of whether a particular edge (connection between traits) can be excluded from the model including all possible edges (Magwene, 2001). I calculated EED using: $-N*\ln(1-p_{ij}^2)$, where N is the sample size and p_{ij} is the partial correlation coefficient between traits *i* and *j*. Then I tested the calculated EED for each trait pair against the χ^2-distribution (df = 1). EED values less than 3.84 ($p>0.05$) were rejected and I did not include them in the graphical model (Magwene, 2001). Then, I calculated the edge strength, or how predictable one trait is given the other, for each trait pair using: $-0.5*\ln(1- p_{ij}^2)$, where p_{ij} is the partial correlation coefficient between traits *i* and *j* (Magwene, 2001). I used the EED values and edge strengths of trait pairs to create an edge list, a list of edges to include in the graphical model. To construct the graphical model, I used the R package, qgraph (Epskamp et al., 2012), illustrating the integration between wing pattern elements.

I measured repeatability by estimating the intraclass correlation coefficient (ICC) for each wing pattern element using the R package, ICC (Wolak et al., 2012).

Results

Repeatability

Wing pattern measurements were highly repeatable with all having repeatability values (intraclass correlation coefficients) above 90%, except for HMspot4, which had a repeatability value of about 0.86 (Table 2). Because HMspot4 is usually very small or absent, sometimes the melanized scales on the ventral hindwing that are not a part of HMspot4 will be above the set threshold for HMspot4. When the trait is measured, those "background" scales are included in the area of "black" for HMspot4. Therefore, the slightly lower repeatability value for HMspot4 is due to variations in the traced area of HMspot4 and the amount of "background" scales included. Although repeatability for HMspot4 is lower than those of the other traits, the repeatability value is still high.

Table 2. Repeatability values (intraclass correlation coefficients) for each measured wing pattern trait.

Trait	Repeatability Value
Fmargin	0.999
Hmargin	0.997
Fbasal	0.990
Hbasal	0.988
DDspot	0.979
VDspot	0.985
FMspot1	0.994
FMspot2	0.992
FMspot3	0.989
FMspot4	0.967
FMspot5	0.976
AVHspot	0.945
HMspot1	0.928
HMspot2	0.962
HMspot3	0.990
HMspot4	0.860
HMspot5	0.927
VHW	0.988
FWL	0.975

Seasonal Plasticity

Wing pattern elements of both male and female *Colias* butterflies exhibit seasonal plasticity. Most traits, with the exception of the male Fmargin, HMspot2, and HMspot5, varied significantly across the months (Table 3). (Note, too, that while those three male traits were not statistically significant against a significance threshold of 0.05, their associated p-values are not particularly large.) Because of the nonlinear relationships between trait melanism and time, it was simpler and more straightforward to analyze melanism by month (a categorical variable) rather than date (a continuous variable). However, as a means for visualizing and understanding the general shapes of plasticity, wing pattern trait melanism was plotted against date. For these reasons, I chose to present seasonal plasticity in this way, however the two analyses control for wing size in a slightly different way than the treatment of wing size in the graphs. In most cases, this does not change the way we interpret seasonal plasticity. However, in a few cases, traits that are not significantly plastic in the analysis are plastic in the graph.

In both sexes, the VHW exhibits a distinct, “U-shaped curve” in melanisation throughout the year and is most melanized in the colder months and least melanized in the warmer months (Fig. 5a, d). Although less dramatic, Hbasal melanisation also follows a “U-shaped curve” throughout the year (Fig. 5a, d). All spots located on the ventral forewing (FMspots and VDspot) show similar patterns and degrees of seasonal plasticity (Fig. 5b, e). In general, these traits have little variability in melanisation across the season except for an increase between August and October. Additionally, all spots located on the ventral

Table 3. Results of the ANCOVA predicting each wing pattern traits by month after factoring out wing size (FWL).

	Female			**Male**		
Trait	**Df**	**F value**	**p-value**	**Df**	**F value**	**p-value**
Fmargin	7, 233	12.482	1.455e-13 ***	7, 265	1.7797	0.09149 .
Hmargin	7, 232	11.116	4.101e-12 ***	7, 268	2.5972	0.01313 *
Fbasal	7, 235	7.8247	1.618e-08 ***	7, 271	4.6156	6.763e-05 ***
Hbasal	7, 234	19.047	< 2.2e-16 ***	7, 271	18.590	< 2.2e-16 ***
DDspot	7, 235	3.9218	0.0004569***	7, 271	3.1606	0.003144 **
VDspot	7, 235	7.0948	1.081e-07 ***	7, 270	3.2171	0.002719 ***
FMspot1	7, 235	9.6104	1.675e-10 ***	7, 268	5.1701	1.540e-05 ***
FMspot2	7, 235	7.6269	2.704e-08 ***	7, 266	3.4578	0.001463 **
FMspot3	7, 235	8.5875	2.265e-09 ***	7, 269	3.2604	0.002434 **
FMspot4	7, 235	2.6745	0.01109 *	7, 268	3.8240	0.0005583 ***
FMspot5	7, 235	3.4049	0.001746 **	7, 269	3.0675	0.004001 **
AVHspot	7, 235	13.717	7.343e-15 ***	7, 267	2.9308	0.005681 **
HMspot1	7, 232	9.4060	2.913e-10 ***	7, 269	6.8160	1.849e-07 ***
HMspot2	7, 232	5.6931	4.363e-06 ***	7, 269	1.8598	0.076342 .
HMspot3	7, 234	4.3639	0.0001438 ***	7, 269	2.0932	0.0445199 *
HMspot4	7, 232	5.1577	1.789e-05 ***	7, 269	3.1452	0.003276 **
HMspot5	7, 234	4.8313	4.202e-05 ***	7, 269	1.8141	0.08462 .
VHW	7, 234	31.651	< 2.2e-16 ***	7, 270	36.034	< 2.2e-16 ***

Significance labels: *** < 0.001, ** < 0.01, * < 0.05, . < 0.1

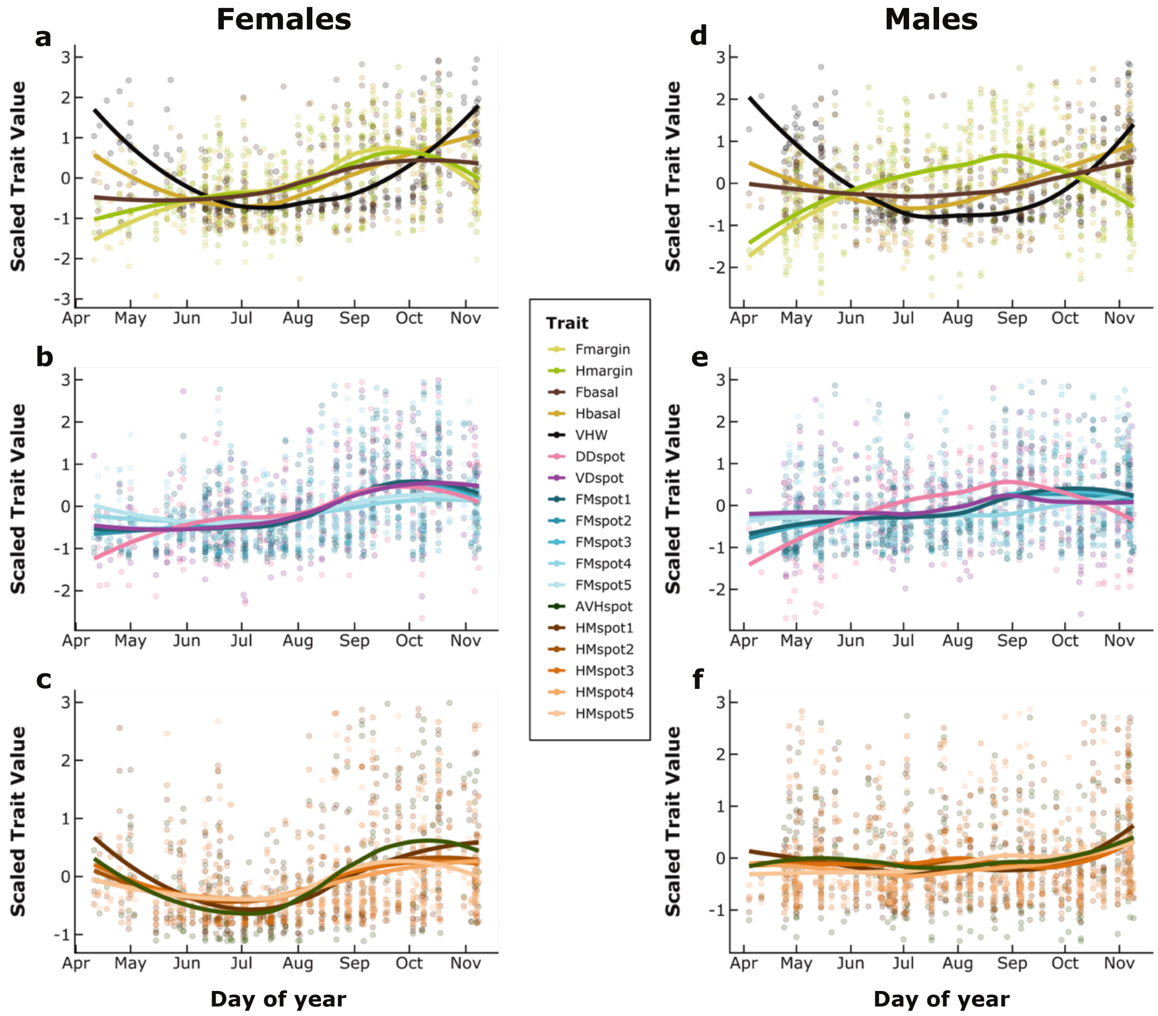

Figure 5. Melanisation of wing pattern elements in females (a-c) and males (d-f) by day of the year. The area of black scales of each trait was divided by FWL then scaled to have a mean of 0 and standard deviation of 1 with a higher scaled trait value indicating increased melanisation and a darker trait. Smoothed lines were created using LOESS regression.

hindwing (HMspots and AVHspot) show similar patterns and degrees of seasonal plasticity, although these differ between the sexes (Fig. 5 c, f). In females, melanisation of these spots decreases until about mid-July, then increases until it plateaus in the fall (Fig. 5c). In males, melanisation of these spots is less variable seasonally and only slightly increases throughout the fall (Fig. 5f). Interestingly, the pattern of seasonal plasticity in the DDspot more closely resembles that of the margins (Fmargin and Hmargin) than of the other spots (Fig. 5a-b, d-e). The margins and DDspot exhibited slightly different patterns of plasticity in males and females (Fig. 5a, d). Fbasal patterns of plasticity also differed between males and females, however, there was generally less variation in melanisation of this trait compared to the other traits. In the females, the pattern of plasticity is similar to that of the ventral forewing spots while in the males, the pattern of plasticity resembles a very shallow “U-shaped curve” (Fig. 5a, d).

Modularity

In both male and female *Colias* butterflies, all partial correlations between traits were positive (Fig. 6). In general, female wing pattern elements are more strongly correlated to each other than male wing pattern elements. Proposed modules were determined through visual assessment of the strength of edges and clustering of traits. The wing pattern elements included in each proposed module were the same in both males and females (Fig. 6, 7). All ventral hindwing spots (HMspots1-5 and AVHspot) and FMspot5 formed a module which will be referred to as the “hindwing margin spots” module (Fig. 6, 7, shown in blue). In both males and females, the partial correlation between HMspot2 and HMspot3 is the strongest in the module (Fig. 6). FMspots1-4 form another module, the “forewing margin spots” module, which is highly correlated to the traits in the hindwing margin spots module (Fig. 6, 7, shown in red). Additionally, FMspot5 is correlated with traits in both the hindwing margin spots module and forewing margin spots module (Fig. 6).

The margins (Fmargin and Hmargin) form a module, referred to as the margins module, but number and strength of correlations between traits inside and outside of the

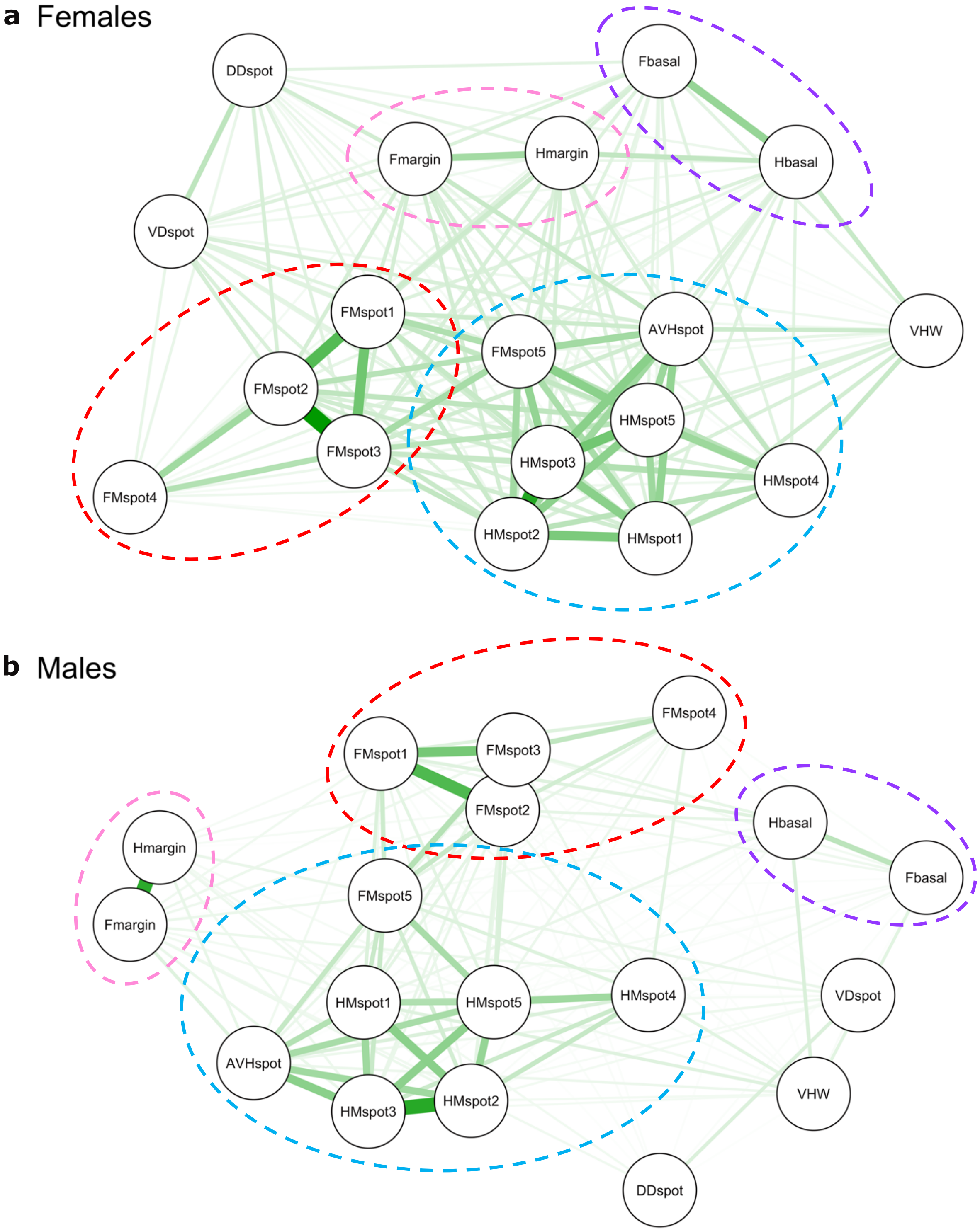

Figure 6. Partial correlation networks of all wing pattern elements for females (a) and males (b). All partial correlations were positive. Edge strength is indicated by edge thickness and length with a darker, shorter edge representing a stronger partial correlation between traits. Proposed modules are represented by colored dashed ellipses (blue: hindwing margin spots, red: forewing margin spots, pink: margins, purple: basal).

module differ between males and females (Fig. 6). In females, the margins are moderately correlated with each other and with most other traits (Fig. 6a). On the other hand, in males, the margins are strongly correlated with each other but very weakly correlated with traits in the forewing margin spots and hindwing margin spots modules (Fig. 6b). Similarly, the basal traits (Fbasal and Hbasal) form a module, the "basal" module, where the numbers and strength of correlations differ by sex (Fig. 6, 7, shown in pink). In females, the basal traits are strongly correlated with each other and moderately correlated with most other traits (Fig. 6a). In males, the basal traits are moderately correlated with each other and weakly correlated with some spots in the forewing margin spots module and hindwing margin spots module and with the VHW (Fig. 6b).

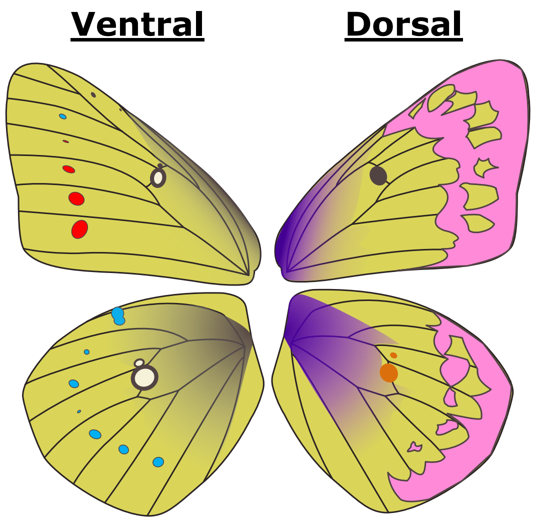

Figure 7. Diagram of proposed modules. A female *Colias* butterfly is depicted, but the proposed modules are the same for both males and females. Wing pattern elements in the same module share the same color. Melanized wing pattern elements that are not colored are not considered modular. Module colors are the same as those in Figure 6.

Neither the discal spots (DDspot and VDspot) nor the VHW were modular in either sex (Fig. 6). However, in females, the moderate and weak correlations between these traits and the other traits indicate that they interact with the modules, despite not being modular themselves (Fig. 6a). This interaction is limited in males as shown by the few weak correlations between the non-modular traits and all the other traits (Fig. 6b).

Discussion

Seasonal plasticity and trait function

As expected, the degree of melanisation of the VHW exhibited a "U-shaped curve" throughout the year with the highest degree of melanisation occurring in the colder months (i.e. both spring and fall) and the lowest degree of melanisation occurring in the warmer summer months. This matches previous descriptions of the seasonal plasticity of the VHW (Gerould, 1927; Shapiro, 1976; Watt, 1968, 1969). Additionally, the other melanized basal wing pattern elements (Fbasal and Hbasal) exhibited relatively "U-shaped curves" of melanisation throughout the year (Fig. 5). The potential seasonal plasticity of these basal surfaces have been previously noted, but no quantitative work had yet been conducted (Ellers & Boggs, 2004; Hoffmann, 1973; Shapiro, 1976). Therefore, this study is the first to provide quantitative data supporting the seasonal plasticity of these basal traits. The similar pattern of seasonal plasticity exhibited by the VHW and other basal wing pattern elements indicates that these basal wing pattern elements may also serve a thermoregulatory function. Lateral basking is a thermoregulatory posture exhibited by *Colias* butterflies in which their wings are folded over their body with their VHW surface oriented perpendicular to the sun (Watt, 1968, 1969). Because of the butterfly's orientation during lateral basking, only the VHW surface is capable of solar absorption when basking. It has been hypothesized that the dorsal basal wing pattern elements may aid in thermoregulation during flight because these dorsal wing surfaces are exposed when the butterfly is flying (Ellers & Boggs, 2004; Tsuji et al., 1986). The results of the current study would support this hypothesis as dorsal basal wing pattern elements were generally shown to be most melanized in the colder months and least melanized in the warmer months (Fig. 5a, d).

Because some traits were most heavily melanized in the spring and fall (e.g. the VHW and Hbasal), a few were more heavily melanized in the summer (e.g. the margins and DDspot), and still others showed limited plasticity (e.g. FMspots), it would not be correct to describe plasticity as generally resulting in either highly melanized or lightly melanized butterflies (Fig. 5). Instead, we have to consider plasticity of traits, or at least trait groups or modules. Notably, the patterns of seasonal plasticity for many of these traits (i.e. the spots and margins) seem unrelated to thermoregulation because these traits are not most heavily melanized in the cooler months of spring and fall, relative to those of summer (Fig. 5). This raises the question: Why are these other melanin traits seasonally plastic? Melanistic wing pattern elements located at the wing margins in *Pieris* butterflies are seasonally plastic and were hypothesized to function in “reflectance basking” (Kingsolver, 1985b, 1985a). Reflectance basking is a posture in which the wings are held open at an angle between 10° and 60° with the dorsal surfaces oriented toward the sun (Kingsolver, 1985a). This orientation allows the dorsal white wing surfaces to reflect solar radiation to the body to increase body temperature (Kingsolver, 1985a). Additionally, Kingsolver (1985b) suggests that because increased melanisation of the margin wing pattern elements decreases the surface area of white on the wings, it may decrease the amount of solar radiation that is reflected and absorbed by the butterfly. It is unlikely that this is the function of melanized marginal wing pattern elements in *Colias* butterflies for two reasons. The first is because their yellow or orange wing color would be much less effective in reflecting solar radiation (Kingsolver, 1985b). The second reason is that *Colias* butterflies have only been observed to utilize lateral basking (Kingsolver, 1985b; Watt, 1968, 1969).

The most likely explanation for the seasonal plasticity of these other melanistic wing pattern elements is trade-offs. Although no wing pattern elements are negatively correlated (Fig. 6), the results do suggest that some modules, particularly the wing margin and discal spot modules, which tend to show heavier melanisation in the summer, develop mostly independently from some of those that are most melanized in the cooler months. In other words, it may be that heavy allocations of melanin to traits such as the VHW, basal wing regions, and some spots in the cooler months reduces melanin available for, and therefore the size of, the wing margins. Furthermore, there may also be trade-offs between wing melanisation and other (unmeasured) traits resulting from differential resource allocation between molecular pathways. For example, melanin is used in the immune response to encapsulate parasites (Stoehr, 2006; True, 2003). Additionally, tyrosine, a precursor in melanin synthesis, is also a precursor for sclerotin, which hardens the cuticle, and dopamine, a neurotransmitter (Stoehr, 2006; True, 2003). Therefore, trade-offs are most likely not limited to the melanisation of wing pattern elements and may occur in other pathways.

Furthermore, the lack of negative correlations between these melanin traits may be due to differences in individual acquisition of resources (Metcalf, 2016; van Noordwijk & de Jong, 1986). If variation in resource acquisition is much greater than the variation of resource allocation, then trade-offs between competing traits may be "invisible" (Metcalf, 2016; van Noordwijk & de Jong, 1986). In insects, tyrosine is obtained through diet and phenylalanine catabolism (Stoehr, 2006). Both tyrosine and phenylalanine are acquired from the insect's diet (Stoehr, 2006) and must be acquired before pupation to be used for wing pattern melanisation. Therefore, the variation in the acquisition of these amino acids through diet in *Colias* butterflies may obscure trade-offs in the melanisation of wing pattern elements.

Modularity and the nymphalid groundplan

The results of the correlation networks provide support for the modularity of wing pattern elements in *Colias* butterflies. While all melanistic wing pattern elements were correlated, the strengths of these correlations differed among traits, with some being strongly correlated and others just weakly so. This indicates that the wing pattern element modularity is not as simple as all melanistic traits forming a single module with no substructure regarding variation. However, it seems clear that these modules best align with the hypothesis stating that modules are based on the nymphalid groundplan (NGP).

All wing margin spots in Pierid butterflies, the family including *Colias*, belong to the same symmetry system, the border symmetry system (Fig. 1) (Nijhout, 1991c, 1994). The hindwing margin spots and forewing margin spots modules described in this study, therefore, are based on this border symmetry system (Fig. 1). Although two separate modules are proposed due to their location on different wing surfaces (either the ventral forewing or ventral hindwing), the forewing margin spots module and hindwing margin spots module interact through correlations between wing pattern elements (Fig. 6). This most likely results from their common inclusion in the border symmetry system in which the forewing and hindwing pattern elements are homologous. Although traits within a module must be relatively independent from other traits, modules must interact with each other for the proper function of the whole organism (Murren, 2012). It might be reasonable to think of these two spots modules, as defined in Figure 6 by their ellipses, as constituting an unshown higher level and less "rigid" spots module. Additionally, the forewing margin spots are much more correlated to each other than with the hindwing margin spots and vice versa. Interestingly, FMspot5 is included in the hindwing margin spots module rather than the forewing margins spots module due to its stronger correlations with hindwing margin spots (Fig. 6). This may be because the high interactivity of the forewing margin spots module and hindwing margin spots module creates a "fuzzy" border between them, allowing FMspot5 to be more closely correlated to hindwing margin spots.

Although the forewing and hindwing margins form a module in both males and females, the wing margin patterns between males and females differ dramatically (Fig. 3). This pattern of sexual dimorphism in which the male margin consists of a single uninterrupted band of melanin while the female margin consists of an interrupted band of melanin is common in the subfamily Coliadinae, which include *Colias* butterflies (Shapiro, 1984). From this, it has been hypothesized that the female margin is the ancestral character consisting of an "interzone" of ground color between two melanin bands (Hovanitz, 1945; Shapiro, 1984). It is possible that the proximal (inner) melanin band in the margins is derived from a band in the border symmetry system (Fig. 1), which is significantly reduced in the males. This could explain the stronger correlations between the margin spots and margins in females, compared to those in the males (Fig. 6) and may provide some insight into why the male Fmargin was not found to be significantly seasonally plastic while the female Fmargin was (Table 3). Therefore, it may be that the width of the margins, in both sexes, depends on the degree of melanisation of both bands.

In the NGP, discal spots are a distinct wing pattern element around which the central symmetry system is centered (Fig. 1) (Nijhout, 1991b, 2017). Because these discal spots are the only melanized wing pattern element in the central symmetry system in *Colias* butterflies (Nijhout, 1991c), it makes sense that they do not form a module with any other melanized wing pattern elements. The reason why the ventral and dorsal discal spots do not form a module may be because they are located on separate wing surfaces and have therefore diverged in pattern. The independent evolution of dorsal and ventral wing surface traits in butterflies is widespread (Nijhout, 1991a).

Basal melanin is not included in the NGP and is instead considered as "background" to the NGP (Nijhout, 1991b). Therefore, the relative independence of the basal module and VHW from wing pattern elements considered part of the NGP in *Colias* butterflies makes sense. The basal module is not strongly correlated with the VHW, which may be because of their locations on different wing surfaces (dorsal and ventral) or differences in the mechanism of thermoregulation (i.e. basking vs flight; see previous section).

The NGP is suggested to be composed of many developmentally independent modules with each corresponding to an element of the NGP (Suzuki, 2017). In the moth, *Thyas juno*, each module involved in the formation of the wing's camouflage pattern corresponds to a symmetry system in the NGP and provides support for a developmentally modular NGP (Suzuki, 2017). However, these developmental modules can be coupled or uncoupled to form functional modules that diverge from the NGP (Suzuki, 2017). The results of this study further support the modularity of the NGP because the modules found in the *Colias* melanized wing pattern elements closely align with the trait groupings proposed by the NGP. Additionally, the sexual dimorphism found in the forewing margin may be a result of the coupling of two NGP elements to form the margins module.

Modularity and development

The developmental mechanisms involved in the formation of melanin traits in Pierid butterflies is relatively unknown; however, the expression of a couple of genes known to play a role in eyespot development in nymphalid butterflies has been investigated in species of *Colias* and *Pieris* (Stoehr et al., 2013; Wee et al., 2022). The transcription factor, Spalt (Sal), was found to be expressed in developing pupal wing cells in the regions that ultimately develop the black scales in the margins, margin spots, and discal spots of developing *Colias*, but not in the black scales of basal traits (Stoehr et al., 2013). Similarly, Sal expression was found in the regions corresponding to spots and wingtip patches of developing *Pieris rapae* and *P. oleracea* but not in the basal traits (Stoehr et al., 2013). In both *Colias* and *Pieris*, Sal is only expressed in the melanized wing pattern elements that are included in the NGP which suggests that melanized NGP wing pattern elements are controlled by different developmental mechanisms than the melanized "background" wing pattern elements, such as the basal traits.

Additionally, the transcription factor, Distal-less (Dll), was found to be expressed in the wing tips and margins, but not the spots of developing *Pieris candida* (Wee et al., 2022). Because Sal expression is similar between species of *Colias* and *Pieris*, Dll may also be expressed in *Colias* margins. It was also found that both *sal* and *dll* genes are necessary for the development of the melanized wing tip and margin while only *sal* is necessary for the development of melanized spots in *Pieris* (Wee et al., 2022). If this is also true in *Colias*, the margins module and the forewing margin spots and hindwing margin spots modules differ in their developmental requirement of Dll expression for melanin trait formation. Ultimately, this would illustrate one of the developmental differences between the modules separating symmetry systems in Pierids. While the developmental regulators of the formation of melanistic wing pattern traits in *Colias* are poorly understood, our results provide some insight into which of these traits may share developmental pathways and the reasons why they may be grouped in the first place.

Conclusions

The VHW in *Colias* butterflies is seasonally plastic and exhibits the highest degree of melanisation in the colder months (i.e. both spring and fall) and the lowest degree of melanisation in the warmer summer months (Gerould, 1927; Shapiro, 1976; Watt, 1968, 1969). Furthermore, this seasonal variation in the VHW has been shown to be adaptive for thermoregulation (Roland, 1982; Watt, 1968, 1969). My study found that the dorsal basal trait elements also show this seasonal pattern of melanisation and therefore may also play a role in thermoregulation. The other melanistic wing pattern elements are also seasonally plastic, however, the adaptive significance of their plasticity is unknown. The most likely explanation for the seasonal plasticity of these wing pattern elements is based on trade-offs in pathways involved in both melanin synthesis and other processes.

Melanized wing pattern elements in *Colias* butterflies align with the symmetry systems of the nymphalid groundplan. The forewing margin spots, hindwing margin spots, margins, and basal traits each form their own module. Although the forewing margin spots and hindwing margin spots modules interact and are both included in the border symmetry system (Nijhout, 1991c, 1994), their location on different wing surfaces may explain their inclusion in separate modules. Additionally, the wing pattern elements within the margins module differs by sex in the strength of correlations with other wing pattern elements and the significance of seasonal melanisation patterns. This supports the hypothesis that the inner melanin band in the female margin is formed from a band in the border symmetry system. The modularity exhibited in the melanized wing pattern elements of *Colias* butterflies also provides support for the developmental modularity of the nymphalid groundplan. Furthermore, differential expression of transcription factors, Spalt (Sal) and Distal-less (Dll), in melanized wing pattern elements may be one developmental difference between the modules of wing pattern elements in *Colias* butterflies. Ultimately, this study found evidence that melanized wing pattern elements in *Colias* butterflies are both seasonally plastic and modular.

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