Hind Wing Eyespots of Brassolini Butterflies (Lepidoptera, Nymphalidae): Evolutionary Diversification and Functions in Anti-predator Defense and Mating Behavior

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Hind wing eyespots of Brassolini butterflies (Lepidoptera, Nymphalidae): evolutionary diversification and functions in anti-predator defense and mating behavior

A Thesis

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by

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# Table of Contents

List of Figures ........................................................................................................ iv  
List of Tables ........................................................................................................... v  
Abstract ................................................................................................................. vi  
Introduction ............................................................................................................. 1  
Methods ................................................................................................................. 4  
Results ....................................................................................................................... 8  
Discussion ............................................................................................................... 14  
Bibliography ............................................................................................................. 18  
Appendix: Supplemental Information ................................................................... 21  
Vita ............................................................................................................................ 32
List of Figures

Figure 1 .........................................................................................................................6
Figure 2 ..........................................................................................................................9
Figure 3 ..........................................................................................................................10
Figure 4 ..........................................................................................................................11
Figure 5 ..........................................................................................................................12
Figure 6 ..........................................................................................................................13
Supplementary Figure 1: .........................................................................................28-29
Supplementary Figure 2: .........................................................................................30
Supplementary Figure 3: .........................................................................................31
List of Tables

Table 1 .................................................................................................................................8
Supplementary Table S1 ..................................................................................................... 21-27
Abstract

Ventral hind wing eyespots are prominent pattern elements in Brassolini butterflies, likely functioning in predator-prey interactions and reproductive activities. *Caligo* and *Opsiphanes* differ in male mate-seeking behaviors and it has been suggested that *Caligo* females use the male cua1 eyespot as a mate-locating cue, but *Opsiphanes* females do not seem to do so. We predict *Caligo* males should have larger eyespots than congeneric females, but the sexes would not differ in eyespot size in *Opsiphanes*. Our analyses supported both these predictions. Displacing the eyespot to the center of the wing makes eyespots more conspicuous, we asked if eyespot position and size covaried across the Brassolini phylogeny. While we did observe a positive relationship, we found these two variables contained significant phylogenetic signal. Our study suggests that the cua1 eyespot performs multiple functions in Brassolini and might be evolving under natural and sexual selection.

Keywords: *Caligo*, *Opsiphanes*, mimicry
Introduction

The diversity of color patterns produced by wing scales is one of the most striking characteristics of Lepidoptera. Various butterfly and moth groups have evolved eyespots, wing pattern elements generally composed of concentric rings formed around a circle of solid color that contains a cluster of white scales (termed the focus). Eyespot morphology can range from a simple dot to a complex series of colorful rings, and can also vary in number across the wing (see examples in Nijhout 1991). Eyespots are found in all butterfly families (Schwanwitsch 1924) and are particularly common throughout the Nymphalidae, where they represent the best-studied component of the nymphalid groundplan (border ocelli or pattern element $h$ in Nijhout 1991, see also Otaki 2012). The variation in size, number, complexity, and position on the wings sparked interest on eyespot development, genetics and evolution (e.g., Nijhout 1980, Brakefield et al. 1996, Monteiro 2008). The framework for the research outlined here lies in the association between eyespot morphology and their function in butterfly natural history and behavior.

Eyespots of adult Lepidoptera are generally considered to serve as visual signals within the context of predator-prey interactions. The deflection hypothesis is an early explanation for eyespot function, and states that these patterns serve as a target to draw predator attacks away from critical portions of the body (Poulton 1890, see also Blest 1957). As such, eyespots must be clearly distinguishable from other color patterns on the wings. A recent experimental study provided support for this hypothesis by showing that reflective scales on the wing margins of *Lopinga achine* (Scopoli, 1763) (Nymphalidae, Satyrinae) efficiently deflected great tit (*Parus major*, Paridae) predatory attacks, but only in low light and high UV conditions (Olofsson et al. 2010). In another study, Pinheiro et al. (2014) examined the frequency of beak marks on field-collected *Junonia evarete* (Cramer, 1779) (Nymphalidae), and showed that predator attacks were more frequent on the eyespots than expected by chance alone. These studies are consistent with the finding that the hind wing tornus, which often contains an eyespot, is weaker than neighboring wing areas (DeVries 2002, DeVries 2003, Hill and Vaca 2004). In contrast, the intimidation hypothesis (Poulton, 1890, see also Blest 1957) suggests that eyespots might confuse or startle predators preventing an attack altogether. Within this context, eyespots might strongly resemble vertebrate eyes, or convey a conspicuous signal that is avoided because it is startling. The importance of distinguishing between vertebrate eye resemblance and a startling...
signal lies in the perception of the signal receiver (Stevens 2005, Quicke 2017). For example, great tits presented with images of *Caligo martia* (Godart, 1824) (Nymphalidae, Satyrinae) with intact and disfigured eyespots avoided those that were intact (De Bona et al. 2015). The classic startle display, in turn, involves a contrasting color signal that incites an avoidance reaction in naïve predators (e.g., Sargent 1978). Such a display might not show any resemblance to an eye, but the strong color contrast might be sufficient to deter a predator attack and be considered an aposematic signal (Stevens 2005).

In addition to functioning as defense against predation, two lines of evidence support the idea that eyespots might also play a role in male-female interactions. First, mate choice experiments on *Bicyclus anynana* (Butler, 1879) (Nymphalidae) showed that females select mates with larger dorsal eyespots (Breuker & Brakefield 2002), and larger eyespot focus size with higher UV reflectance (Robertson & Monteiro 2005). These findings suggest that male *B. anynana* dorsal color pattern is under sexual selection, while natural selection drives the ventral pattern (Oliver et al. 2009). Secondly, a survey of 450 species in 399 nymphalid genera found that in species showing sexual dimorphism, males had a larger number of ventral hind wing eyespots than females (Tokita et al 2013). If ventral eyespots were to function mainly as a defense against predation, this begs the question why the number of eyespots is reduced in females.

The butterfly tribe Brassolini is a Neotropical group that includes over 100 species in 16 genera (Penz 2007, Matos-Maraví et al. submitted). There is tremendous variation in wing size between genera (e.g., *Caligo* Hübner, 1819 wingspan is ca. six times that of *Bia* Hübner, 1819), and some members of this group (e.g., *Opsiphanes* Doubleday, 1849) have large, robust thoraces (Penz & Williams 2020). These fruit-feeding butterflies are mostly found in the shady understory, but some species inhabit the forest canopy (DeVries et al. 2011, Fordyce and DeVries 2016). Adult Brassolini are predominantly crepuscular, with courtship and oviposition occurring at dawn and dusk (Fruhstorfer 1910, DeVries 1987), although diurnal habits evolved independently in some taxa (*Caligo martia*, Fruhstorfer 1910; *Dasyophthalma* Westwood, 1851, Casagrande & Mielke 2000; three species of *Opoptera* Aurivillius, 1882, Penz & Heine 2016). Compiling available information, Penz & Williams (2020) noted that little is known about Brassolini male reproductive behavior. *Caligo* males generally perch in leks (Freitas et al. 1997, Srygley and Penz 1999), and as the large hind wing eyespots are conspicuous in perching males,
they might offer a cue to females approaching the lek (Penz 2017). In contrast, *Opsiphanes* males use rapid flight to patrol forest edges or to perform aerial displays (Anton Fassl in Fruhstorfer 1910, Srygley 1994), and their hind wing eyespots seem less likely to play a role in mate finding. A comparative study of 75 species in all Brassolini genera investigated color pattern variation (Penz & Mohammadi 2013), and two findings are particularly relevant here. First, most Brassolini species have two conspicuous ventral hind wing eyespots (in cells sc+r and cua1). Secondly, “*Catoblepia*” orgetorix (Cramer, 1775), now placed in *Selenophanes* Staudinger, 1887 (Matos-Maraví et al. submitted), evolved a mimetic resemblance to *Caligo atreus* (Kollar, 1850) via simple modifications of dorsal color bands associated with two groundplan pattern elements. In most of its range, mimicry in *S. orgetorix* is limited to females, except in northern Colombia where both sexes are mimetic. Penz & Mohammadi (2013) also discussed wing color sexual dimorphism, which is uncommon in Brassolini.

The ventral hind wing eyespot in cell cua1 is nearly universal within Brassolini (Penz & Mohammadi 2013), and constitutes a conspicuous signal in many members of this tribe. To document the range of variation of this eyespot, we measured its size and position for 389 specimens representing 28 species and 14 genera. We asked two questions about the potential relationships of eyespot morphology to the natural history and mating behavior of these butterflies. First, if *Caligo* females use the male eyespot as a cue to locate or select a mate as previously suggested (Penz 2007), we might predict that males with larger eyespots would be more visible to females, which could then lead to sexual dimorphism in eyespot size. In contrast, eyespot visibility might not be as important for patrolling males such as in species of *Opsiphanes*, in which case sexual dimorphism would not be expected. We then asked: (1) Is there sexual dimorphism of eyespot size in *Caligo* and *Opsiphanes*? Secondly, inasmuch as both size and position might potentially enhance the visual signal of eyespots, we investigated a possible evolutionary association between these two characteristics. We asked: (2) Does eyespot size covary with its position on the wing in Brassolini? Finally, given the wide-ranging interest in butterfly eyespot genetics and development (e.g., Monteiro 2015), we also compared size variation in two eyespot components (the outermost ring and the dark inner core) for selected species. Our analyses allowed us to revisit unusual cases of mimicry and sexual dimorphism in the Brassolini.
Methods

Specimens

Measurements
To measure wing and eyespot size, individual butterflies were photographed next to a metric scale. We used ImageJ (version 1.48v, https://imagej.nih.gov/ij/, last accessed 03 March 2020) to measure the sizes of the outer ring and inner core of the eyespot in cell cua1 (Fig. 1a), the relative position of the eyespot within the cell, and the adjusted hind wing area (Fig. 1b). The left hind wing was used for all measurements. This wing was damaged in a few individuals, and for those cases all measurements were performed on the right hind wing. To minimize error in
our measures of the outer ring, inner core, and eyespot position, each specimen was measured three times and the mean was used in the analyses.

The outer ring constitutes the outermost ring of dark color and, unless stated otherwise, eyespot size refers to the measurement of the outer ring. Measurements of the outer ring were taken at its exterior boundary (Fig. 1a). The inner core is defined as the black circle that forms the center of the eyespot, which was measured by following the exterior boundary of the black oval. In some species, the boundaries of the outer ring and inner core were blurred due to a gradual transition of colors, and in these instances measurements were performed around an area of solid color (Fig. 1a).

Some taxa differed from the general Brassolini eyespot morphology. The eyespot of *Narope cyllastros* is comprised of only the inner core, which corresponds to the total eyespot size in our analyses. Species in the genera *Caligopsis* Seydel, 1924 and *Eryphanis* Boisduval, 1870 have contiguous eyespots in cells m3 and cua1. In these taxa the measurement of the outer ring was taken along wing vein CuA1 and, depending on the species, followed its regular perimeter within cell cua1 and sometimes cua2.

To compare eyespot size among taxa we needed to account for variation in wing size. Thus, measurements of hind wing area were used to calculate the relative size of eyespot outer ring and inner core (e.g., outer ring area / hind wing area). Brassolines in general, and *Caligo* in particular, have an extended hind wing anal region that curves around the abdomen. The anal hind wing area of pinned specimens is not uniformly curved, which would affect whole wing area measurements and the estimated eyespot size, given that the area of eyespot is much smaller than the area of the wing. To minimize error and standardize our protocol, we measured a portion of the wing that was flat in all images (shaded in gray in Fig. 1b), and excluded the fringe.

To estimate the position of the eyespot within cell cua1, its center was marked with a red dot using Photoshop CS5.1, and two measurements were taken using this landmark: linear distance from the base of vein CuA1 to the center of the eyespot (M1 in Fig. 1b), and from the center of the eyespot to the point where CuA1 meets the wing margin (M2 in Fig. 1b). The
position of the eyespot was then calculated as $M_2/(M_1+M_2)$, which represents the relative distance between the center of the eyespot and the wing margin. As eyespot position varied less than its size (results not shown), we used a random number generator in Microsoft Excel to select a subset of six males and six females of each species of *Caligo* and *Opsiphanes* for position measurements. All specimens of other genera were measured. As some species within Satyrinae show seasonal polyphenism in eyespot size (Brakefield 1987), we performed t-tests to ask if locality or seasonality had an influence on eyespot size or position, but found no effects (results not shown).

**Analyses**

All statistical comparisons were performed using R version 3.6.3 (R Core Team), and ancestral character state reconstructions were performed in Mesquite (version 3.6 build 917). To test for sexual dimorphism in eyespot size in *Caligo* and *Opsiphanes*, a Mann-Whitney U test was run with species pooled by genera. To test for covariance between eyespot position and size, a phylogenetic generalized least squared regression was performed for 28 species (sexes pooled). We pruned the most recent Brassolini phylogeny (Matos-Maraví et al. submitted) to exclude species that were not in our data set. Branch lengths used in the phylogenetic generalized least
squared analysis were obtained from Matos-Maraví et al. (submitted). Finally, to document variation in eyespot morphology we plotted the size ranges of the outer ring and inner core for selected taxa.
Results

In perching *Caligo* males, the hind wing eyespot is a potential cue to approaching females, hence our prediction of sexual dimorphism in eyespot size. When species were pooled, we found that male *Caligo* had significantly larger eyespots than females (Table 1, p=0.03; example in Fig. 2a, b), which supports our prediction. In contrast, we found no sexual dimorphism in *Caligo* with respect to inner core size (Table 1, p=0.36).

Table 1. Mean size and ranges for the eyespot outer ring and inner core of *Caligo* and *Opsiphanes.*

<table>
<thead>
<tr>
<th></th>
<th>Outer ring</th>
<th></th>
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<th>Inner core</th>
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<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
<td></td>
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<tr>
<td><strong>Species pooled</strong></td>
<td></td>
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<tr>
<td><em>Caligo</em> (94M, 69F)</td>
<td>0.124 ±0.023</td>
<td>0.115 ±0.019</td>
<td>0.037 ±0.011</td>
<td>0.035 ±0.009</td>
<td></td>
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<tr>
<td><em>Opsiphanes</em> (58M, 28F)</td>
<td>0.048 ±0.009</td>
<td>0.047 ±0.008</td>
<td>0.017 ±0.003</td>
<td>0.016 ±0.002</td>
<td></td>
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</tr>
<tr>
<td><em>Caligo</em></td>
<td></td>
<td></td>
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<tr>
<td><em>C. atreus</em> (10M, 11F)</td>
<td>0.149 ±0.027</td>
<td>0.131 ±0.014</td>
<td>0.055 ±0.009</td>
<td>0.049 ±0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. eurilochus</em> (25M, 19F)</td>
<td>0.127 ±0.019</td>
<td>0.117 ±0.019</td>
<td>0.036 ±0.010</td>
<td>0.033 ±0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. idomeneus</em> (23M, 9F)</td>
<td>0.120 ±0.016</td>
<td>0.104 ±0.011</td>
<td>0.028 ±0.004</td>
<td>0.025 ±0.005</td>
<td></td>
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<tr>
<td><em>C. illioneus</em> (22M, 19F)</td>
<td>0.103 ±0.018</td>
<td>0.099 ±0.015</td>
<td>0.036 ±0.002</td>
<td>0.033 ±0.007</td>
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<tr>
<td><em>C. martia</em> (4M, 5F)</td>
<td>0.128 ±0.017</td>
<td>0.129 ±0.009</td>
<td>0.041 ±0.007</td>
<td>0.037 ±0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. telamonius</em> (10M, 6F)</td>
<td>0.137 ±0.017</td>
<td>0.132 ±0.006</td>
<td>0.044 ±0.009</td>
<td>0.041 ±0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Opsiphanes</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>O. cassina</em> (43M, 19F)</td>
<td>0.048 ±0.008</td>
<td>0.048 ±0.008</td>
<td>0.017 ±0.0031</td>
<td>0.017 ±0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>O. invirae</em> (15M, 9F)</td>
<td>0.049 ±0.0121</td>
<td>0.044 ±0.009</td>
<td>0.018 ±0.0029</td>
<td>0.016 ±0.002</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sample sizes for male (M) and female (F) specimens are in parentheses. SD is the standard deviation.
In both sexes, the range of variation in size was broader for the outer ring than the inner core (Supp. Fig. 2). As male *Opsiphanes* patrol to search for females, it seemed less likely that their eyespots play a role during mate-searching activities. When species were pooled, we found no evidence of sexual dimorphism in total eyespot size in *Opsiphanes* (Table 1, p=0.61; example in Fig. 2c, d), nor for the size of the inner core (Table 1, p=0.19). The outer ring showed broader variation in size than the inner core in both sexes (Supp. Fig. 2).

Large eyespots would appear to be more obvious than small ones, but their position on the wing might also affect the visual signal they convey. Ancestral state reconstructions of both eyespot position and size (Fig. 3) revealed three patterns. First, there was a trend for larger eyespots to be more centrally located on the wing (Fig. 4), which is evident in the clade including *Caligo*, *Caligopsis* and *Eryphanis*. Nonetheless, the phylogenetic generalized least squared analysis indicated the association between these two characters was not significant (Fig. 4), and that there is strong phylogenetic signal for both characters instead (λ=0.962, not significantly different from 1; p=0.52). Second, ancestral state reconstructions indicate that eyespot size decreased independently in some clades (*Narope* Doubleday, 1849; *Dynastor* Doubleday, 1849, *Brassolis* Seydel, 1824) and increased independently in others (*Dasyophthalma*, *Caligo*), which was also the case for eyespot position. Lastly, *Mielkella singularis* and *Selenophanes orgetorix* showed considerable divergence from closely related taxa (Fig. 3, see node and branch values). *Mielkella singularis* has a larger, more centrally located eyespot than other members of its clade (Supp. Fig. 1). The eyespot size of mimetic *Selenophanes orgetorix* matches the ancestral state for *Caligo* and approximates the value for *C. atreus*, but differs from *Selenophanes cassiope* (Fig. 3). The two *Selenophanes*
species also diverged in eyespot position, which is more centrally located in *S. orgetorix* than in *S. cassiope*.

In most Brassolini, the eyespot core is usually confined to the boundaries of cell cua1 (some *Caligo* species are exceptions), but the outer ring often expands beyond this compartment (see Supp. Fig. 1 for examples of all genera included in the analyses). Thus we hypothesized that the outer ring size should vary to a greater extent than the inner core, and this was supported by a

![Figure 3](image-url)
comparison of selected species (Fig. 5, see also Supp. Fig. 2). The plot in Fig. 5 also allowed us to assess evolutionary divergence among closely related taxa. Although they are members of the same clade (Fig. 3), and their outer rings are comparable in size, the inner core of *Caligopsis seleucida* is much smaller than those possessed by *Caligo*. In both *Opsiphanes* species the outer ring is not much larger than the inner core. *Mielkella singularis* is closely related to *Opsiphanes* and has a similar size inner core, but the outer ring is markedly larger. The inner core is similar in size in both *Selenophanes* species, but the mimetic *S. orgetorix* has a much larger outer ring than that of the non-mimetic *S. cassiope*.

In summary, we found evidence of sexual dimorphism in eyespot size for *Caligo* but not *Opsiphanes*. Although the association of eyespot position and size within Brassolini contains strong phylogenetic signal, *M. singularis* and *S. orgetorix* nonetheless differed from their close relatives. Finally, for the species in our analyses, we found the range of variation of the outer ring surpassed that of the inner core.

![Figure 4. Phylogenetic generalized least squares regression of hind wing eyespot position and relative size (28 species, sexes pooled).](image)

*adjusted R² = 0.1056, p = 0.054*
Figure 5. Eyespot inner core and outer ring relative sizes for selected Brassolini species (sexes pooled). Vertical bars indicate the mean size. Note the slight overlap in the range of values for *Opsiphanes invirae*, *Selenophanes cassiope* and *Caligo illioneus*. 
Figure 6. Dorsal (left) and ventral views of selected Brassolini. a, *Caligo atreus* male (Panama). b, *Selenophanes orgetorix* male (Panama, Colón), and c female (Ecuador, Tinalandia). d, *Selenophanes cassiope* male (Peru, Tingo Maria). e, *Caligo uranus* (Mexico, Chiapas). f, *Mielkella singularis* male (Mexico, Comitán [de Domínguez]) and g, female (Mexico, Comitán [de Domínguez]). h, *Opsiphanes invirae* male (Brazil, Rondônia, Fazenda Rancho Grande). Arrows indicate color pattern modifications involved in mimicry (*S. orgetorix*, compare to *C. atreus*) or sexual dimorphism (*M. singularis*, compare to *C. uranus*).
Discussion

Hind wing eyespots are prominent color pattern elements in Brassolini butterflies, and our comparative study explored variation in the cua1 eyespot between sexes and among genera. We used our findings to propose possible eyespots functions in these butterflies in light of natural history and behavioral observations, and discuss how ventral hind wing eyespots appear to involve sexual dimorphism and mimicry in two species.

Eyespots of Brassolini show remarkable variation in size and morphology (Supp. Fig. 1). Our ancestral state reconstructions (Fig. 3) suggest the common ancestor of Brassolini had a relatively small cua1 eyespot located near the wing margin, and that morphological diversification proceeded in different directions during the taxonomic diversification of this group (including a complete loss of hind wing eyespots in Penetes, not studied here). The cua1 eyespot is composed of a simple white or black marking in the genus Narope, but Caligo species possess the largest eyespots in the Nymphalidae (Ho et al. 2016), which exceeded 400 mm² in some specimens studied here. Among the Brassolini, Caligo eyespots are also the most centrally located on the wing. Caligopsis and Eryphanis (sister taxa to Caligo) have a contiguous eyespot in cell m3 that increases the overall effect of the cua1 eyespot. Several genera exhibit small to medium-sized eyespots containing all the same morphological features of the larger eyespot (i.e., inner core, outer ring), which vary in size between and within genera. We found that the outer ring showed greater variation in size than the inner core (Fig. 5), which is usually confined to the boundaries of the cua1 cell. The absence of a particular evolutionary trend for cua1 eyespot size among the Brassolini corresponds to that described for dorsal hind wing eyespots in 22 species of Junonia Hübner, 1819 (Nymphalidae). As their eyespots in color, size, number and complexity vary randomly across the phylogeny, Kodandaramaiah (2009) suggested that diversification in eyespot morphology was possibly due to different selective forces operating in the environments where each Junonia species evolved. We can provide a comparable example within Brassolini. Opooptera includes four crepuscular and three diurnal species, and adult time of activity influenced the evolution of wing morphology attributes that relate to flight (Penz and Heine 2016). While we cannot establish causation, diurnal Opooptera species have more vividly
colored and larger hind wing eyespots than crepuscular ones (compare crepuscular *O. aorsa* to diurnal *O. sulcius* in Fig. 3 and Supp. Fig. 1).

Studies on eyespots tend to categorize them as either having a deflection or an intimidation function (e.g., Stevens et al. 2009, Oloffson et al. 2010). Typically, deflection eyespots tend to be relatively small and occur as a series near the wing margin, while larger, individual eyespots located towards the center of the wing more likely function in intimidation (Kodandaramiah 2011). Based on this rationale, we can identify examples of deflection and intimidation eyespots in Brassolini butterflies. Taxa with putative deflection eyespots include *Catoblepia berecynthia* and *C. xanthisles*, *Opsiphanes invirae* and *O. cassina* (Fig. 2, Supp. Fig. 1). In comparison to other Brassolini, the eyespots of these species are small (single or multiple), located near the wing margin, and composed of a distinct inner core and outer ring, with the ripple pattern completely surrounding the eyespot. In contrast, the large eyespot of *Caligo* species that rest during the day on tree trunks might serve as an intimidation signal given their size, contrast and position on the wing (Fig. 2, Supp. Fig. 1). The absence of a ripple pattern around the cua1 eyespot enhances its visual effect, and this pattern is found in *Caligopsis*, *Eryphanis* and some species of *Caligo* (Penz and Mohammadi 2013, Supp. Fig. 1). Nonetheless, the patterns of wing tears produced during predation attempts (see Quesnel and Stradling 2012, Supp. Fig. 3) suggests that *Caligo* butterflies are exposed to, and attacked by multiple bird and lizard predators, as would any other Brassolini species. In fact, given the diversity of vertebrate insectivores in the Neotropics, it is likely that the cua1 eyespot could intimidate some potential predators and act as a target by others. As a deflection function cannot be positively ruled out for *Caligo*, this serves as a cautionary note for generalizing eyespot function based on experiments with European birds; they do not approximate the complexity of tropical communities. Finally, if predator attacks on butterflies occur more often around hind wing veins CuA1-CuA2 (i.e., DeVries 2002, 2003, Hill and Vaca 2004, Pinheiro et al. 2014), then displacement of the cua1 eyespot towards the center of the wing would help prevent damage to this visual signal in *Caligo*, something particularly important if male eyespots function in mating activities.

Wing colors play an important role in mate selection in various butterfly groups (Silberglied 1984, Fordyce et al. 2002, Kronforst et al. 2006, Davis et al. 2007, Kemp and Rutowski 2011). In sexually dimorphic species, divergence in color may result from either female preference for brightly colored males or by the evolution of dull, protective coloration in the female sex.
(Darwin vs. Wallace mechanisms; Oliver and Monteiro 2011). Nonetheless, butterflies also use seemingly monomorphic color patterns to select mates, found on dorsal or ventral wing surfaces. Laboratory choice experiments showed that female *Bicyclus anynana* prefer males with larger, complete dorsal eyespots with highly UV reflective foci (Breuker and Brakefield 2002, Robertson and Monteiro 2005, Oliver et al. 2009). In contrast, male *Lycaeides idas* (Linnaeus, 1761) (Lycaenidae) use female ventral hind wing orange spots and aurorae to recognize potential mates before courtship is initiated (Fordyce et al. 2002), and these eyespot-like color elements are present in both sexes. Such examples highlight the importance of visual stimuli at the onset of male-female interactions. Although never tested empirically, *Caligo* females appear to use the large ventral hind wing cua1 eyespot as a visual cue to locate lekking males (Penz 2007; see also Srygley and Penz 1999). Indeed our analyses here show that male *Caligo* generally have larger eyespots than females (Table 1, Fig. 2, Supp. Fig. 2), suggesting a function during sexual interactions. This hypothesis is reinforced by the observations that males aggregate in single or multispecies leks (Freitas et al. 1997, Srygley and Penz 1999), which presumably provide an opportunity for females to choose high quality males (but see Wickman and Jansson 1997). We also note that *Caligo illioneus* males readily respond to approaching females and conspecific males, implying good visual acuity during their crepuscular leks (CMP per. obs.). Sexual signals are expected to occur in one sex only (see Kemp and Rutowski 2011 for a review), but in male and female *B. anynana* courtship-role reversal between wet season (males court) and dry season (females court) was interpreted as a mechanism for the evolution and maintenance of sexual ornaments (Prudic et al. 2011). In *Caligo*, the maintenance of large cua1 eyespots in both sexes could be explained by its dual function as a signal in predator-prey, and male-female interactions. As such, both natural and sexual selection presumably operate simultaneously towards the evolution of this eyespot. Studies investigating male and female behavioral responses to conspecific eyespots will be required to assess our interpretations proposed here.

The combination of mimicry and strong sexual dimorphism within the Brassolini is uncommon, and apparently occurs in only two species. Female *Selenophanes orgetorix* converges onto both the dorsal and ventral patterns of *Caligo atreus* (Fig. 6a, c). Dorsally, *S. orgetorix* has a thin, iridescent forewing cross band and a broad, yellow hind wing marginal band (see arrows in Fig. 6c), and these relatively small changes from the typical *Selenophanes-Catoblepia* phenotype produce a strong visual effect (Penz and Mohammadi 2013). The ventral
The forewing has a large eyespot in cell m1 plus a corresponding broad band (pattern element h), and the hind wing has a pale wide marginal band and a large cua1 eyespot (Fig. 6c). Our analyses showed that this eyespot is larger and more centrally located in S. orgetorix than in S. cassiope (Figs 3, 5, 6d), and this observation also holds for other Selenophanes species not studied here (S. josephus (Godman and Salvin, 1881), S. supremus Stichel, 1901). This suggests the evolution of mimetic convergence due to position displacement and expansion of the outer ring only, since the inner core size is similar among S. orgetorix, S. cassiope and other members of the Opsiphanes clade (Fig. 5). Although males are not mimetic in most of this species range, they too have a large cua1 eyespot (Fig. 6b), which could be due to genetic convergence between the sexes. Female Mielkella singularis differs in dorsal color pattern from congeneric males by having white dorsal forewing spots, a pale yellow forewing band, and a broad orange hind wing band (see arrows in Fig. 6g). In a similar way to S. orgetorix females, the large ventral hind wing eyespot of M. singularis results from an increased size of the outer ring only (Figs 3, 5, 6g), which is in contrast to the eyespot configuration of closely related taxa (Fig. 6h). Although we cannot explain the evolution of sexual dimorphism in this species, we hypothesize that this might be a case of imperfect female-limited mimicry with Caligo uranus Herrich-Schäffer, 1850 as the model (Fig. 6e). Finally, we note that parallel color pattern modifications evolved independently in S. orgetorix and M. singularis leading to convergence onto Caligo species (see phylogeny in Fig. 3).

This study examined the diversity in size, position and configuration of a ventral hind wing eyespot that is nearly universal in the Brassolini. Based on behavioral observations, we discussed potential functions of eyespots in some taxa. We propose that ventral hind wing eyespots might function simultaneously as defense and sexual signal in Caligo butterflies, which might also be the case in other Brassolini taxa. We showed that variation in size of the eyespot outer ring is greater than that of its inner core, which corresponds to expectations of pattern development models (see Nijhout 2017). In fact, the increased size of the outer ring alone contributes to mimetic resemblance in two Brassolini species, Selenophanes orgetorix and Mielkella singularis, whose color patterns converge on Caligo. Our research provides a framework for future studies that seek to investigate eyespot morphology and function in Brassolini butterflies, but we emphasize that this can only be accomplished by broadening our understanding of the natural history and behavior of these iconic butterflies.
Bibliography


Appendix: Supplementary Information

Supplementary Table S1: Locality data of examined specimens from the Florida Museum of Natural History (University of Florida, US), Milwaukee Public Museum (US), American Museum of Natural History (US), Smithsonian Institution (US), Phil DeVries Collection (US), Museu de Zoologia da Universidade de São Paulo (Brazil), and Museu de Zoologia da Pontifícia Universidade Católica do Rio Grande do Sul (Brazil). Species are listed in the order they appear in the phylogeny (Fig. 2).

Narope cyllastros
M, Brazil, Paraná, Rio das Cobras, Feb 1942; M, no locality, no date; F, Brazil, Santa Catarina, Nova Teutônia, 14 Feb 1961; F, Paraguay, no date.

Opoptera sulcius
M, Brazil, Santa Catarina, Taió, Feb 1959; M, Brazil, Rio Grande do Sul, Pinhal, Feb 1950; F, Brazil, (south), Nov 1973; F, Brazil, Santa Catarina, Feb 1964; F, Brazil, Santa Catarina, São Bento do Sul, 10 Mar 1984; F, Brazil, Santa Catarina, Taió, 2 1986.

Opoptera aorsa
3M, Brazil, Espírito Santo, no date; M, Brazil, Paraná, no date; F, Brazil, Espírito Santo, no date; F, Brazil, Paraná, no date.

Dynastor darius
M, Mexico, Oaxaca, Chimalapa, Jul 1956; M, Colombia, no date; M, Brazil, Santa Catarina, Joinville, Feb 1984; M, Peru, Huanuco, Tingo Maria, 15-22, Aug 1981; M, Costa Rica, no date; 4M, no locality, no date; F, Panama, Panama, Las Cumbres, 12, Dec 1960; F, Colombia, Antioquia, no date; F, Brazil, Santa Catarina, Joinville, Feb 1984; F, Peru, Leoncio Prado, Tingo Maria, 30, Jul 1980; F, Ecuador, Chimborazo, Riobamba, no date; F, Mexico, Oaxaca, Tuxtepec, Jun 1954; F, Puerto Rico, La Ceiba, Sep
(Supplementary Table S1 continued)

1971; 2F, Mexico, Chiapas, 20-26, Nov 1973; F, Mexico, Oaxaca, Palomares, 8 Sep 1961; F, no locality, Aug 1952; F, no locality, no date.

*Dynastor macrosiris*

M, Peru, Pichis, no date; M, Costa Rica, no date; M, French Guiana, Bas-Maroni, no date; F, [Mexico], Santecomapan, Aug 1952; F, Puerto Rico, La Ceiba, Sep 1971; F, Mexico, Oaxaca, Tuxtepec, Jun 1954; 2F, Mexico, Chiapas, 20-26 Nov 1973; F, Mexico, Oaxaca, Palomares, 8 Sep 1961.

*Dasyophthalma rusina*

M, Brazil, Santa Catarina, São Bento do Sul, 10 Mar 1984; M, Brazil, Boitzenburgo, 1932; M, Brazil, Santa Catarina, 4 Jan 1968; F, Brazil, Alto da Serra, Morretes, 16, Mar 1990; F, Brazil, Alto da Serra, Morretes, 16, Mar 1990.

*Dasyophthalma creusa*

M, Brazil, Rio de Janeiro, 6 Dec 1944; M, Brazil, Santa Catarina, no date; M, Brazil, Santa Catarina, Taió, 1959; 5F, Brazil, Santa Catarina, 3 Feb 1962, 7 Feb 1966, 25 Feb 1962, 5 Mar 1966, and no date.

*Mielkella singularis*

M, Mexico, Chiapas, Comitán, Jun 1962; M, Mexico, Chiapas, no date; 4F, Mexico, Chiapas, Comitán, Mar 1960, May 1937, 12 May 1974, Jun 1962; F, Mexico, Chiapas, Ocosingo, Jul 1947; F, Mexico, Chiapas, no date.

*Orobrassilos ornamentalis*

M, Brazil, Paraná, Umuarama, 10 Feb 1937; F, Brazil, Paraná, Umuarama, 10 Feb 1937.

*Blepolenis batea*

2M, Brazil, São Paulo, Salesópolis, Jan 1952, 1968; M Brazil, Rio Grande do Sul, Porto Alegre, no date; M, no locality, no date; F, no locality, 2 May 1939; F, Brazil, São Paulo, Salesópolis, 2 March 1968; F, no locality, Feb 1929.
(Supplementary Table S1 continued)

**Opsiphanes invirae**


**Opsiphanes cassina**


**Selenophanes cassiope**
(Supplementary Table S1 continued)

M, Peru, Leoncio Prado, Tingo Maria; Jun 1980; M, Peru, no date; M, Peru, Loreto, Iquitos, no date; F, Peru, Loreto, Iquitos, 23 Nov 1988; F, Peru, Mariscal Cáceres, Juanjú, 22 Dec 1950.

*Selenophanes orgetorix*

*Catoblepia amphirhoe*
M, Brazil, Espírito Santo, Concepção da Barra, 27 Nov 1969; 2M, Brazil, São Paulo, Mendes, no date, no date; M, Brazil, Santa Catarina, São Bento do Sul, 10 Mar 1984; M, Brazil, Rio Grande do Sul, Pelotas, 20 Jan 1968; M, British Guiana, Bartica, (locality label considered incorrect), no date; F, Brazil, Espírito Santo, Linhares, Apr 1972; F, Brazil, Rio de Janeiro, no date.

*Catoblepia berecynthia*

*Catoblepia xanthicles*
M, Peru, Madre de Dios, Tambopata, 19 Oct 1988; M, French Guiana, Nouveau Chantier, no date; F, French Guiana, Nouveau Chantier, no date; F, Peru, Loreto, Iquitos, no date; F, Ecuador, Morona-Santiago, Macas, 21 Mar 1979.

*Catoblepia xanthus*
(Supplementary Table S1 continued)

*Brassolis astyra*

M, Brazil, Rio de Janeiro, Gávea, 1 Feb 1960; M, Brazil, Santa Catarina, no date;
M, Brazil, Santa Catarina, Corupa, no date; M, Brazil, Para, Óbidos, Amazon River, 16 Aug 1952; M, Brazil, Nov 1973; F, Brazil, Sep 1932; F, Brazil, São Paulo, Itaici, no date; F, Brazil, Rio de Janeiro, Gávea, 1 Feb 1960.

*Caligopsis seleucida*


*Eryphanis aesacus*

M, Mexico, San Luis [Potosí], Tamazunchale, 23 Jul 1937; M, Mexico, Catemaco, Nov 1965; F, Mexico, no date.

*Eryphanis automedon*


*Caligo martia*


*Caligo idomeneus*

M, Brazil, Pará, Santarém, no date; F, Brazil, Pará, Itaituba, Aug; 3M, Brazil, Manaus, Rio Negro, Dec 1929, Dec 1929, Dec 1929; M, Peru, San Martín, Moyobamba, June 1948; M, Peru,
(Supplementary Table S1 continued)


*Caligo atreus*


*Caligo illioneus*

(Supplementary Table S1 continued)


*Caligo eurilochus*

M, no locality, Sep 1961; M, Mexico, Oaxaca, Tuxtepec, Oct 1968; M, Mexico, Chiapas, Bachajón, 2 Sep 1970; M, Mexico, Oaxaca, Río Sarabia, Sep 1958; M, Mexico, Chiapas, Arroyo Miranda, Río Jacatun, 30 May 1987; M, Costa Rica, Puntarenas, San Vito de Java, 3 Apr 1989; M, Costa Rica, Cartago, Turrialba, 5 Jun 1972; M, Trinidad and Tobago, Maraval, July 1891; M, Trinidad and Tobago, 1898; M, Ecuador, Quevedo, no date; 2M, Ecuador, Pichincha, S. Dom. Tinalandia, 5 Jun 1972, 28 Aug 1973; M, Ecuador, Cuenca, 10 Dec 1966; M, Ecuador, Morona-Santiago, Macas, R. Pumayacu, 21 Mar 1979; 5M, Peru, Madre de Dios, Los Amigos Biological Station, 11 Jan 2005, 14 Jan 2005, 12 Apr 2005, 15 Jul 2005, 10 Nov 2005; M, Brazil, Pará, Itaituba, Aug; M, Brazil, Pará, no date; M, Brazil, Pará, Obidos, Oct 1921; M, no locality, no date; F, no locality, Sep 1961; F, Mexico, Ocosingo, July 1947; F, Mexico, Ocosingo, July 1947; F, no locality, Aug 1952; F, no locality, Sep 1961; F, Costa Rica, Sandoval, 15 Mar 1926; F, Costa Rica, Turrialba, Catie, 16 12 1977; F, no locality, on date; F, Trinidad, Tabaquite, 18 Jan 1921; F, Trinidad, no date; F, Brazil, Pará, no date; M, Colombia, Cali, 4 Aug 1967; M, Colombia, Boyacá, Muzo, Jun 1917; F, Colombia, Cali, 4 Aug 1967; F, Colombia, Cali, 4 Aug 1967; F, Ecuador, Cuenca, 10 Nov 1966; F, Ecuador, Pichincha, S. Dom. Tinalandia, 25 Jul 1972; F, Ecuador, Cuenca, 8 May 1969; F, Ecuador, Cuenca, 10 Dec 1966; F, no locality, no date; F, Peru, Madre de Dios, Los Amigos Biological Station, 11 Nov 2005.
Supplementary Figure 1: Hind wings of male specimens in ventral view, sizes adjusted to facilitate comparison. Scale bars: 1 cm. 1, Narope cyllastros (no data); 2, Opoptera sulcius (Brazil, Taió), 3, Opoptera aorsa (Brazil, Espírito Santo); 4, Dynastor darius (Colombia); 5, Dynastor macrosiris (French Guiana, Bas-Maroni); 6, Miellka singularis (Mexico, Chiapas); 7, Dasyophthalma rusina (Brazil, Santa Catarina); 8, Dasyophthalma creusa (Brazil, Taió); 9, Brassolis astyra (Brazil, Gávea); 10, Orobrassolis ornamentalis (Brazil, Umuarama); 11, Blepelenis batea (Brazil, Salesópolis); 12, Opsiphanes invirae (Brazil, Ariquemes); 13, Opsiphanes cassina (Mexico, Oaxaca, Chiltoco); 14, Selenophanes cassiope (Peru, Tingo Maria); 15, Selenophanes orgetorix (Panama, Cerro Campana); 16, Catoblepia amphirhoe (Brazil, Pelotas); 17, Catoblepia berecynthia (Ecuador, Garza Cocha); 18, Catoblepia xanthicles (Peru, Puerto Maldodado); 19, Catoblepia xanthus (Ecuador, Garza Cocha); 20, Caligopsis seleucida (Peru, Los Amigos Biological Station); 21, Eryphanis automedon (Peru, Los Amigos Biological Station); 22, Eryphanis aesacus (Mexico, Catemaco); 23, Caligo martia (Brazil, São Francisco de Paula); 24, Caligo idomeneus (Brazil, Santarém); 25, Caligo atreus (Colombia, Muzo); 26, Caligo illioneus (Costa Rica, Chilamate); 27, Caligo eurilochus (Costa Rica, Puntarenas); 28, Caligo telamonius (Colombia, Bogotá).
Supplementary Figure 2: Relative sizes of the eyespot inner core (solid circles) and outer ring (open circles) for male (black) and female (gray) *Opsiphanes* and *Caligo* species. Vertical bars indicate the mean size. Note that the outer ring shows a broader variation in size than the inner core for both sexes and all species.
Supplementary Figure 3: a, Male *Caligo martia* (Brazil, Terra de Areia) showing beak marks from predator attempts that seemed to be using the eyespot as a target. Note that the displacement of the cual eyespot towards the center of the wing helps prevent damage to the eyespot even if the predator uses it as a target for attack. Preserving the integrity of this eyespot would be particularly important if it is used in male-female interactions. b, Male *Opsiphanes invirae* (Brazil, São Pedro da Serra) showing a beak mark towards the costal hind wing eyespot. As this is an asymmetrical damage, it was probably incurred during escape flight. c, Male *Opoptera sulcius* (Brazil, Barração, Parque Estadual Espigão Alto) with two asymmetrical beak marks that damaged the cual eyespot, likely acquired during escape flight. d, Female *Blepolenis batea* (Brazil, Terra de Areia) showing two sets of symmetrical beak marks, one of which is near the cual eyespot.
VITA

The author received his Bachelor of Science degree at Iowa State University in 2018. He joined the University of New Orleans in 2018 to pursue a Master of Biology degree and became a member of the Dr. Carla Penz and Dr. Phil DeVries research group.