Ambient Light Environment and the Evolution of Brightness, Chroma, and Perceived Chromaticity in the Warning Signals of Butterflies

by

Jonathan Marion Douglas

A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved April 2013 by the
Graduate Supervisory Committee

Ronald L. Rutowski, Chair
Kevin J. McGraw
Juergen Gadau

ARIZONA STATE UNIVERSITY

May 2013

ABSTRACT

1. Aposematic signals advertise prey distastefulness or metabolic unprofitability to potential predators and have evolved independently in many prey groups over the course of evolutionary history as a means of protection from predation. Most aposematic signals investigated to date exhibit highly chromatic patterning; however, relatives in these toxic groups with patterns of very low chroma have been largely overlooked.
2. We propose that bright displays with low chroma arose in toxic prey species because they were more effective at deterring predation than were their chromatic counterparts, especially when viewed in relatively low light environments such as forest understories.
3. We analyzed the reflectance and radiance of color patches on the wings of 90 tropical butterfly species that belong to groups with documented toxicity that vary in their habitat preferences to test this prediction: Warning signal chroma and perceived chromaticity are expected to be higher and brightness lower in species that fly in open environments when compared to those that fly in forested environments.
4. Analyses of the reflectance and radiance of warning color patches and predator visual modeling support this prediction. Moreover, phylogenetic tests, which correct for statistical non-independence due to phylogenetic relatedness of test

species, also support the hypothesis of an evolutionary correlation between perceived chromaticity of aposematic signals and the flight habits of the butterflies that exhibit these signals.

Key-words: achromatic, aposematism, bird tetrahedral color space, complex light environment, forest shade, mimicry, predator-prey interaction, toxic, unprofitable prey, vision.

Dedicated to my father, Matthew M. Douglas, for his unending inspiration and encouragement throughout this project and everything that came before it.

ACKNOWLEDGEMENTS

I would first and foremost like to thank my advisor, Ron Rutowski, who has provided me with a fantastic amount of patience and enthusiasm which has been invaluable in the completion of this study. Similarly, committee members Kevin McGraw and Juergen Gadau have been incredibly encouraging in my tenure as a graduate student and in the completion of this study. Joseph Macedonia has also provided me with innumerable hours of stimulating conversation and dedicated support that will not soon be forgotten. Justin Merry was a solid sounding-block for many early ideas and also a trailblazing member of the Rutowski laboratory, who instilled in me needed confidence. Darrell Kemp was another inspiring supporting figure during the inception of this project. I would further like to thank Dr. Jim Doyle for his vital encouragement at the onset of my graduate career. For all of their time and patience, I am sincerely grateful.

I also thank the graduate program office staff at the School of Life Sciences for their tireless support, especially that of Wendi Simonson. This work was supported by the Graduate College and the School of Life Sciences.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

Introduction

Aposematic signals function and features:

Animals with chemical or mechanical defenses against predation often advertise their defenses by displaying conspicuous signals—known as aposematic signals—to warn predators of their unprofitability as prey items (Poulton 1890). These signals are often visual, but acoustic and chemical warning signals also abound (Ratcliff & Nydam 2008; Weldon 2010). Aposematic signals are thought to evolve because they effectively communicate unprofitability to potential predators and thus decrease predation while increasing the fitness of the individual organism, population, or species (Roper & Redston 1987; Prudic, Skemp & Papaj 2006; Svádová *et al*. 2009). Though the mechanisms by which aposematic signals confer fitness upon organisms have frequently been investigated and debated, selection pressures that produce the many different types of aposematic signals are not well-understood (Merilaita & Ruxton 2006; Halpin, Skelhorn, & Rowe 2008; Chouteau & Angers 2012; Holen & Svennungsen 2012). Among potential selection pressures, the interaction of ambient light environment with visual aposematic signals has yet to be thoroughly investigated.

The most commonly studied aposematic patterns and coloration schemes are those that incorporate patches that reflect long-wavelengths in the portion of the electromagnetic spectrum visible to humans (i.e. red, yellow, or orange). To a much lesser extent, shorter

wavelengths such as green, blue, or ultraviolet on dark/black backgrounds have also been investigated (Rutowski, Nahm, and Macedonia 2010; Mäthger, et al., 2012). However, broadly reflecting color patches with very low chroma but high brightness (i.e. achromatic or "white") are often ignored as potential aposematic signals (though see Cloudsley-Thompson, 1976; Shine, 1980; Lyytinen *et al*, 1999; Hunter, 2009; Stankowich *et al*, 2011; and Nokelainen *et al*, 2012). This is perhaps because of biases within the human visual system as well as historical precedence in the scientific literature.

Aposematic displays and light environment

Signals are expected to evolve in concert with the visual systems of intended receivers and the light environments in which they are displayed and perceived (Endler 1992, Endler 1993, Douglas *et al*. 2007). Specifically, aposematic signals should reliably indicate the unprofitability of a prey species by maximizing conspicuousness, thereby increasing detectability while enhancing learning by predators (Prudic *et al*. 2006; Halpin *et al*. 2008). Aposematic signals also should be easily detected and recognized by potential predators in the light environment in which they are displayed, during the times at which they would commonly be encountered, and against the backgrounds that are typical for these places and times (Endler 1992). An easily detectable and memorable aposematic signal confers increased safety from predation upon an individual or a population of defended organisms by reducing future attacks on visually similar prey (Ham *et al*. 2006).

We can predict the features of visual aposematic signals that natural selection should produce given selection pressures on the signals exerted by predators in various environments. Butterfly predators are predominantly avian, visually oriented, and diurnal. Thus, we generally predict that aposematic signals in butterflies should consist of bright visual signals, which is largely supported in the literature (Merilaita & Ruxton, 2006; Stankowich Caro & Cox, 2011). More specific predictions can also be made with regard to the type of bright patch that would be most effective in warning visual predators of unpalatability in differentlight environments (Endler, 1992). In general, color patches should reflect those wavelengths that are most abundant in the environment because they will appear brightest to signal receivers, while those that do not match the ambient light spectrum will be less easily perceived (Endler 1992; Endler 1993; Endler & Théry 1996). We propose that bright signals with low chroma and low perceived chromaticity are an effective evolutionary strategy for aposematic organisms living in complex low light environments. Correlations between color patterns and light environment have previously been noted and investigated, but studies of correlations between light environment and low-chroma signals are rare (Fleishman 1992).

Highly chromatic signals are often utilized at close range by organisms for detailed information about their environment (Streinzer, Paulus & Spaethe 2009). Many angiosperm plants have increased pollen dissemination and seed dispersal through the evolution of highly chromatic blossoms and fruits (Burger, Dötterl & Ayasse 2010). Bright plumage, integument, or other tissues are used by many animals including birds,

mammals and insects to attract mates or indicate status (Papke, Kemp & Rutowski 2007; Kemp 2008; McGraw, Dale & Mackillop 2003; Macedonia 2001). In bright open environments where light energy from a broad range of wavelengths is available such as the sun, sky, fields, gardens, and above forest canopies, we predict that aposematic signals will or can be selectively reflective and thus highly chromatic and still be effective.

In forested environments, ambient light is low in intensity and spectral composition changes in space and time (Endler, 1992). Organisms that travel through a light environment such as the forest understory are generally unable to change their visual signals to match the spectrum of changing ambient light. For this reason, relatively achromatic features and signals in mobile organisms such as butterflies may be more reliable as aposematic signals than chromatic features in dim light environments. In contrast to chromatic signals, bright features of organisms and objects with low chroma in the environment are generally understood to be used by birds in detection of motion, small objects, details, and edge detection (Kram, Mantey & Corbo 2010, Osorio, Miklósi & Gonda 1999), which may be of particular ecological importance to birds in the forest understory. Bright signals with low chroma also differ from chromatic signals in that they are highly reflective across a broad range of wavelengths and are therefore brighter in general than chromatic signals. This also means they may appear more visually contrasting with the background against which they are viewed.

Butterflies as a study system for aposematism

We chose butterflies to address these issues because they generally exhibit bright and contrasting patterns on their wings—which are disproportionately large when compared with their body size. Butterfly color patterns are known to serve in both intra- and interspecific signaling (Chai, 1986; Papke Kemp & Rutowski, 2007; Rutowski Nahm & Macedonia, 2010). Many of the patterns may serve as warning signals, making them an ideal group for studying aposematic coloration.

Butterflies are perhaps the best-studied group of organisms that frequently display aposematic coloration in conjunction with chemical or mechanical defenses. Indeed, butterflies comprised many of the study species used to establish the founding principles of aposematism (Poulton 1890). However, perusing a museum collection of toxic or distasteful groups of butterflies leaves one questioning what exactly defines an aposematic pattern. Many species in these groups have color patterns that do not exemplify those that are commonly considered to be aposematic in that they exhibit low-chroma white and black pattern elements rather than highly chromatic patches on black backgrounds (pers. obs.). We hypothesize that many of these toxic species evolved bright signals with very low chroma in response to varying light environments, such as forest understories.

Prey species in this study were selected based upon their general geographic origins—neotropics, afrotropics, or australiasian tropics—and their known or inferred toxicity or distastefulness to predators. Tropical origins allows reasonable inference to be made regarding the similarities between light habitats that butterflies and predators will encounter (Endler 1993). Toxicity inferences are made based on close evolutionary conservation of toxic larval host plants used by the groups studied (e.g. Passifloraceae, Apocynaceae, and Aesclepiaceae, among others) and the understanding that many species in each group are known to harbor toxic secondary metabolites of these host plants (Owen 1970, Hay-Roe & Nation 2007, Mebs et al., 2012). All species belong to one of three Lepidopteran clades: Heliconiini, Acraeini, or Danainae. Papilionidae was used as the out-group for phylogenetic analyses.

Predator perception and aposematic signals

Birds are common visual predators of Lepidoptera and so we used the avian visual system to model perception of aposematic signals. Absorbance spectra of visual pigments that reside in light sensitive cones are known for many avian species. We use the visual system of the blue tit and peafowl as our model because most parameters pertinent to modeling visual perception are known for these species. These species also encompass both basic types of bird visual system, the violet-sensitive and the ultraviolet-sensitive. Using absorbance spectra of avian visual pigments, a model of avian visual systems has been devised by Endler and Mielke (2005) using a 3-dimensional tetrahedral color space.

The tetrahedral color space model allows one to infer how color patches and contrasts are seen by birds, rather than by the direct comparison of reflectance spectra. In this manner, one can quantify contrast, brightness, hue, and perceived chromaticity in ways that are ecologically relevant to birds.

If ambient light environment works in conjunction with predatory visual systems in shaping the evolution of aposematic signals, we should be able to test for correlations between light environment and the type of visual display presented by prey species. To this end, we compared reflectance measurements and descriptive measures of reflectance spectra to test for differences between butterfly groups. We also used visual modeling to describe how aposematic butterflies appear to bird predators using the appropriate light environment under which they would be viewed. After plotting these data onto a constructed phylogeny, we tested for correlations between perceived chromaticity and flight habits in open or closed habitat.

Materials and methods

Natural history data

We chose for this study 90 species of butterflies that were either known or inferred to be aposematically colored using criteria laid out in the Introduction. Natural history information regarding habitat preferences of adult butterflies for all species was obtained

from the literature (DeVries 1987, Larsen 1996, Ackery & Vane-Wright 1984). Key words such as "full-sun, open, and city gardens" were taken to indicate species that frequented open areas whereas words such as, "forest understory, deep forest, under forest canopy" were indicative of those species that spent most of their adult flight time within forest. Butterflies that frequented open habitat were classified as "open-dwelling" species, while those described as flying primarily in forests were deemed "forest-dwelling" species.

Reflectance spectra

Reflectance spectra of salient or major pattern elements were collected from wings of preserved butterfly specimens housed in the Los Angeles Natural History Museum. The largest contributing color patch that was not the background color (generally black) was determined by simple visual examination. Ascertaining the largest patches on butterflies is akin to measuring the largest patches on birds and methods resembling this have been used in the literature (Stoddard & Prum, 2008). Spectra were recorded from the dorsal wing surfaces as these are day-flying species and the dorsal pattern elements are likely the most visible to birds and most brightly illuminated during flight. Reflectance spectra were measured in a darkened room using a PX-2 light source. Pattern elements were illuminated with a ~5mm circular beam of light focused through a collimating lens oriented normal to the wing surface. A collector probe fitted with a collimating lens and with its optic path oriented at 45° relative to normal, gathered reflectance spectra through

an Ocean Optics spectrophotometer. Reflectance spectra were gathered using OOIbase3.2 (Ocean Optics) and were made relative to an MgO white standard.

Comparison and analyses of reflectance spectra

Reflectance spectra of the largest wing pattern element (determined by visual examination) that was not the background or ground color (usually black) were compared between open-dwelling and forest-dwelling species based upon their overall brightness, chroma, and ultraviolet reflectance according to methodologies summarized in Hill & McGraw (Montgomerie, 2006). Brightness was calculated as the integral of the spectral curve from wavelengths of 300 to 700 nanometers; the range of wavelengths thought to be visible to birds. Chroma, a measure of spectral purity or saturation, was calculated as the difference between the maximum and minimum reflectance percentages within a given reflectance spectrum. Finally, ultraviolet reflectance was calculated by integrating the area under the curve of a reflectance spectrum within the ultraviolet portion of the spectrum (300 to 400 nanometers). Ultraviolet reflectance was considered separately because bird predators are sensitive to this band of wavelengths. A higher reflectance in the UV concommitant with a similarly bright reflectance in the remaining wavelengths suggests a flatter overall reflectance patch that will likely be perceived as less chromatic. Angle-dependent colors were uncommon and were not measured in this study.

Irradiance spectra and radiance calculations

Irradiance spectra typical of forest shade and open habitats were collected by J. Macedonia from Grand Caymen Island during the middle of the day? (Macedonia 2001). A hemispherical cosine-corrected collector was used to measure downwelling ambient light on sunny days (when the sky was at least 1/3 blue) by orienting the lens of the collector upward in open and forest habitats when the sun was not obscured by clouds. These irradiance spectra are considered representative of open and forest habitats due to the general similarities between the geometry of forests and the effects forest geometry has on ambient light environment (Endler 1993). We justify using these irradiance spectra because ambient light habitats have been shown to vary little across multiple types of forests and open areas (Endler 1993) and the middle of the day is when butterflies are most likely to be on the wing.

The radiances of colored or bright (non-black) patches under both forest shade and open enviroments were calculated by multiplying the reflectance spectrum of each patch by the spectrum of illumination under which it would be perceived. We also calculated radiances of patches with illumination under which they would not typically be perceived. This allowed comparisons of overall reflected light within groups between light environments and between groups and light environment.

Modeling of bird vision tetrahedral color space

Birds' visual system responses to butterfly reflectance patches were modeled using Endler & Mielke's tetrahedral color space model (2005). To describe how a bird would visualize that particular color patch this model combines information about the reflectance of the color patch of interest as gathered in the laboratory, irradiance under which the patch would commonly be viewed, and the transmission and absorbance properties of the bird visual system (ocular media, oil droplets, light-sensitive cone cells). Ocular transmission properties and absorbance spectra of blue tit and pea fowl cones and double-cones were obtained from microspectrophotometric studies performed by Nathan Hart (Hart 2002).

Bird visual system response is characterized by three coordinates (x,y,z) that reside in a tetrahedral color space. The vertices of this tetrahedron indicate absolute excitation of only one of four light-sensitive cones (UV, S, M, L) in the retinae of birds while the center of the tetrahedron indicates an equal excitation of all 4 cones, and thus, an achromatic stimulus with a “flat” reflectance spectrum (i.e. black, white, or gray). The relative excitation of each of these cone classes by a given color patch places it somewhere in a unique position within this tetrahedron. The exact method by which color patches are placed within the tetrahedron are discussed in detail in Endler & Mielke, 2005; but will be covered only briefly here.

Reflectance spectra of the primary and ground color patches of butterfly wings, and irradiance spectra under which these patches would commonly be viewed, were input into a visual modeling program called Avicol (Gomez 2011), which uses absorbance spectra of bird cones to generate output as raw excitation of bird cones. The relative excitation of each cone was calculated by dividing each raw excitation value by the total excitation of all four cone classes. Relative excitation values are then used to determine x,y, and z coordinates within the tetrahedral color space using the following formulae:

$$x = ((1-2s-m-u)/2)\ \sqrt{3}/2$$

$$y = (-1+3m+u)/2\sqrt{2}$$

$$z = u-1/4$$

Double-cone inputs were also calculated using raw output data from Avicol. Because double-cones are thought to be a separate visual channel primarily responsible for perception of intensity-contrast and motion-detection, we compared the inputs for the two main color patches on each species using in order to calculate an intensity contrast value. We report only the blue tit perception model data in this report, as they give very similar results and the visual system of the blue-tit may more closely resemble the common predators of butterflies.

Interpreting visual modeling coordinates

By definition color patches that excite all cones to a very low degree, i.e to reside at the center of the tetrahedron one or near (x,y,z) = (0,0,0), are assumed not only to be achromatic, but perceived as "black." In practice, a color patch that produced an excitation of less than 0.10 for all four raw cone inputs was considered to occupy the center of the tetrahedron. This 0.10 criterion was set to allow for the noise of spectra that have very low reflectance over all wavelengths.

Using the tetrahedral coordinates for each patch we calculated the theoretical chromatic contrast between the two largest color patches for each species as observed by birds. The perceived chromatic contrast between these patches is estimated by the Euclidean distance between the patches within the color space. Additionally, we estimated perceived chromaticity of a color patch as its distance from the center of the tetrahedron. Because ground color patches tended to have very low reflectance and were either black or extremely dark brown, their perceived chromaticity measurements were not considered in our comparative analyses of perceived butterfly chromaticity. Instead, only the colorful and/or bright patches were considered.

Phylogeny and trait mapping

Phylogenies were compiled from the most recently available phylogenies in the scientific literature and from the Tree of Life Project (www.tolweb.org) and entered into both Mesquite and MacClade (Maddison and Maddison, 2005; Maddison and Maddison, 2011) phylogenetic analysis tools.

Natural history traits and perceived chromaticity were binary characters and mapped as such upon the constructed phylogeny. Open-dwelling species were given a value of "zero" for the trait of flight habitat, while forest-dwelling species were given a value of 1. Likewise, those species that exhibited perceived chromaticity values of less than 0.5 were considered to be less chromatic or "low-chroma" and were given a value of 1 for the chromatic trait, whereas species that showed perceived chromaticity values of greater than 0.5 were considered to be given a value of "zero." The cut-off of 0.5 was used because, while technically a perceived chromaticity of approximately 1 is possible, this would require monochromatic light, which would not occur in nature. Chromaticity values of near 1, however, are conceivable, and thus we chose half of that measure, 0.5, as a cut-off value for distinguishing between patches with high and low chromaticity.

Statistical analyses

t-tests were performed to compare averages of brightness, chroma, hue and ultraviolet brightness between groups of forest-dwelling species and field-dwelling species. *T*-tests were also used when comparing the brightness contrast values obtained from double-cone inputs. These tests were carried out using the Open Office Calc program (www.openoffice.org).

We used Fisher's Exact Test to test for correlations between discrete variables so each species was scored with respect to light environment and perceived chromaticity as described above. To determine whether evolution of high or low chromaticity in aposematic groups was correlated with ambient light environment, non-parametric statistical analyses that correct for phylogenetic non-independence are needed. We used two such analytical methods to test this hypothesis Pagel's test of correlated discrete character evolution (Pagel, 1994; Maddison and Maddison, 2011) and the concentrated changes test of MacClade (Maddison and Maddison, 2005). We utilized these two phylogenetic tests because they focus on slightly different aspects of the phylogenetic tree and corroboration of the separate results strengthens hypotheses tested.

Pagel's test for correlated evolution of two binary traits uses simulations of distributions of traits and hypothetical ancestral traits on a phylogeny to generate complex matrices of

probability distributions at node on the phylogenetic tree. These are used to arrive at two logarithmic likelihood values, the difference of which produces a test statistic that corresponds to a chi-squared distribution.

MacClade's concentrated changes test uses different methodologies to test for correlated evolution of binary traits. It is similar to Pagel's test for correlated evolution in that it compares simulations of distributions of characters across a phylogeny to generate a likelihood probability that two traits co-evolved at a frequency that is significantly greater than random. It also corrects for the non-parametric and non-independent nature of phylogenetic data.

Results

Brightness, chroma, hue and ultraviolet of the reflectance spectra

As predicted the non-black color patches of forest-dwelling species were significantly brighter ($p = 0.0005$; Table 1) and less chromatic ($p < 10^{-7}$)than than those of open-dwelling species. The average chroma of open-dwelling species was 2.92, while that of forest-dwelling species was 1.61. These results indicate that field species of aposematic butterflies tend to exhibit more saturated color patches generated by pigments.

The lower calculated chroma of the forest-dwelling species means they have quantitatively flatter reflectance curves (Fig. 1).

Brightness in ultraviolet wavelengths (300-400 nm) was also significantly different (p-value = 0.0014) between butterfly species that live in open habitat and those that dwell in the forest with the latter group exhibiting far higher reflectance in the ultraviolet wavelengths. When considered with chroma, brightness and hue, the UV reflectance further indicates that the reflectance patterns of forest-dwelling species are consistently less chromatic than their field-dwelling counterparts.

Chroma and brightness of radiance

We compared the radiance for the four combinations of butterfly habitat preference and ambient illumination. The chroma of forest-dwelling butterflies was significantly less under forest illumination while differences between chroma approached significance under open illumination. The brightness of forest-dwelling butterflies was significantly higher than that of open-dwelling butterflies under both open and forest ambient light conditions. However, neither brightness or chroma changed significantly within open-dwelling or forest-dwelling butterflies when radiance of patches was calculated using illumination under which they would not typically be viewed (Table 2).

Comparing bird-perceived chromaticity and intensity contrast

Chromaticity calculations refer to the distance of a color patch on a butterfly wing from the achromatic center of the bird vision tetrahedron. Chromaticity measurements of darkly colored pattern elements were disregarded in these tests because they generally present cone inputs well below the cut-off of 0.1 and thus provide little information regarding the perception of the signal. They are, however, considered in perceived contrast measurements. Higher chromaticity measurements indicate a patch that is perceived as more colorful when viewed within the ambient light environment in which it is generally displayed, while lower measurements signify a less colorfully perceived patch.

Perceived color contrast between the primary pathes and the dark ground color was significantly lower on average in forest-dwelling butterflies than those that live in open environments ($p = 0.000123$; Table 2). Perceived chromaticity was also much lower in forest-dwelling butterflies ($p = 5.02E-13$). Estimates of perceived intensity contrast using double-cone inputs were significantly different between open- and forest-dwelling species ($p = 0.001$). These calculations incorporated the environmental irradiance under which the color patches would be perceived. Perceived intensity contrast was higher in forest-dwelling species than in open-dwelling species, indicating brighter and less chromatic patterning in forest-dwelling species.

Phylogenetic tests

Phylogenies generated in Mesquite and MacClade were used to test for correlated evolution of low-chroma patterns within low-light/forested environments. The distribution of chromaticity and natural history across the phylogeny is represented in Table 4. The phylogenies contain identical information regarding the relations of study species, but each program provides unique ways of testing for correlated evolution of traits (see Methods). Therefore we only show one phylogeny for reference. Fig. 2 shows the phylogenetic relations of all test species with habitat and chromaticity coded as branch color. Pagel's test for correlation between perceived chromaticity and flight habits produced a p-value of less than 0.001. When we analyzed the constructed tree using MacClade's concentrated changes test we produced a test statistic with a p-value of 0.022.

Discussion

Reflectance spectra show that that the warning signals that toxic butterflies have evolved differ fundamentally between species that live the in forest and those that live in open enviroments. As expected the major non-black patches on the wings of unpalatable forest understory butterflies generally have reflectance spectra that are flatter, brighter in the ultraviolet, and brighter across all visual wavelengths (Table 1 & Fig. 1) and will produce warning colorations that are more effective in this environment. Also, forest-dwelling butterflies

had a average hue value shifted toward shorter wavelengths, further indicating in objective terms that they are not red, orange, or yellow. In contrast and also as predicted, distasteful butterflies that inhabit open environments tend to have patches that display chromatic reflectance spectra with strong inflection points and generally reflect wavelengths longer than 550 nanometers (i.e. yellow-red).

Radiance measurements of non-black patches corroborated findings from reflectance parameter measurements. Under forest illumination, the radiance from patches in forest-dwelling species were significantly be brighter over all visible wavelengths than those from open environment species. Interestingly, this was also true when patches from both groups were illuminated with open irradiance. Chroma of forest butterflies was lower than open-dwelling species when radiance was calculated with open or forest irradiance, but was significantly different only in the forest. This, taken with brightness comparisons of radiances further suggests that chroma is less important in forest illumination, where brightness is essential for effective visual warning signals. What is more, within groups of butterflies the chroma of neither group changed significantly between open and forest irradiance. This finding may have further implications regarding the effects of light-environment on chroma and the importance of chroma of signals which have evolved under illumination that differs in intensity and wavelength availability (see Endler, 1993).

Warning signals seen through the eye of avian predator

In addition to objective spectral differences between open and forest-dwelling toxic butterflies, we have demonstrated that bird predators perceive the aposematic patterns of butterflies in their respective environments in very different ways. Species that live in open environments tend to have a higher perceived chromaticity according to bird visual modeling and thus are likely to be perceived as highly chromatic under the irradiance typical of open environments. In contrast, those butterflies that fly in closed light environments tend to exhibit very low measures of perceived chromaticity and therefore are likely to be perceived as less chromatic or nearly achromatic. But high brightness contrast?

The reflectance spectra and tetrahedral color space measurements of open-dwelling butterfly species follow Endler's hypothesis that color patches that most closely match the available ambient light prevail as effective signals. However, this study suggests that, while less chromatic signals do not necessarily match the wavelengths that are most abundant in all of the ambient light spectra found within the forests, their spectra may still be efficient signals indicating toxicity because they reflect most visible wavelengths (~330-700nm). Thus, they should act as general reflectors, reflecting whatever wavelengths of light are available. This may be an adaptive strategy for producing bright memorable signals that indicate distastefulness or unprofitability.

The analysis incorporating double-cone receptors also provides evidence that the two groups of butterflies are different with regard to their pattern elements. Double-cones are the most prevalent light sensitive cell in avian retinae, next to rods, and may be responsible for achromatic, brightness, motion, and edge detection, however, they are not useful in hue discrimination or color vision (Vorobyev & Osorio, 1998; Osorio & Vorobyev, 2005). When internal contrast between pattern elements is calculated, those butterflies that primarily fly in forest light environments have a significantly higher internal contrast when we consider the quantum catch of double-cones. This may be another evolutionary avenue by which less chromatic but highly contrasting aposematic butterflies alert birds to their distastefulness. Bright low chroma signals in forest environments may also be detectable from a longer distance than that allowed by color contrast conferred by the other four light-sensitive cones.

Correlated evolution of warning coloration and habitat preferences

Correlated evolution of bright and low chroma warning signals with flight habits within forest light environments was supported by all parametric and non-parametric tests performed in this study. Though the concentrated changes test performed was slightly above generally accepted statistical significance at the 0.05 level (0.056), we suggest that in conjunction with Pagel's test indicating evolutionary correlation and our other parametric tests (with p-values lower than 0.05) that this study provides strong evidence for a correlation between forest light environments and bright warning signals with low chroma.

The suggestion of correlation between bright but low-chroma warning signals and forest light environments is perhaps more convincing given the disjunct and disparate geographic origins of the butterfly groups examined in this study. These include groups from three tropical areas: Central and South America, Africa, and Asia. When we consider that the groups studied evolved in disparate regions of the world, but likely under similar ambient light habitats, the evolutionary convergence on largely achromatic pattern elements further bolsters the hypothesis that there is an evolutionary correlation between these patterns and the light environments in which they evolved.

Implications for low-chroma patterning in other animals

Many achromatic pattern elements exhibit a relatively flat reflectance spectrum in the range of wavelengths to which human are sensitive and so appear white to humans. However, many of these spectra show sharp drops in reflectance in the ultraviolet wavelengths and so would not appear white but chromatic to the many animals that have visual sensitivities that extend into the ultraviolet, such as insects, fish, reptiles, birds, and some mammals (Maier, E. J. & Bowmaker, J. K. 1993; Sillman, A. J., Govardovskii, V. I. & Loew, E. R. 1997; Palacios, A. G., Srivastava, R. & Goldsmith, T. H. 1998; Leal & Fleishman 2002; Peichl *et al*. 2005; Skorupski & Chittka 2010). For example, if we consider pierid butterflies (many of which appear white to humans) we must consider that they actually possess pterin pigments that absorb ultraviolet light (with few exceptions). Therefore, these patterns would exhibit considerably different reflectance spectra than

those many species examined in this study. In fact, these patterns likely would appear highly chromatic to both butterflies and birds. This supposition is supported by the fact that there is little evidence to suggest that pierid butterflies are aposematic (Lyytinen *et al*. 1999). Laboratory measurements—spectrophotometric, chromatographic, or otherwise— are critical to assess whether what is being described appears as a truly achromatic stimulus to the species in question.

However, a small number of bright markings with low chroma in a number of other groups of organisms have been implicated in aposematism, supporting the hypothesis that relatively achromatic signals can function in aposematic displays, especially when produced and viewed in low light. The Bandy-Bandy (*Vermicella annulata*), a primarily nocturnal snake, exhibits bright pattern elements with low chroma that are thought to be aposematic (Shine 1980). White or low-chroma markings in aggressive and defensive mammalian carnivores such as skunks have been found to be correlated with nocturnal nocturnal (Stankowich, Caro & Cox 2011). Some deep-water nudibranchs, a group of poisonous molluscs, have been noted to be curiously white-ish as well (Valdés 2001). Thus, bright pattern elements with very low chroma and chromaticity should be considered as potentially important informative portions of patterns of animal integument.

Future directions

Forest butterflies fly not only within a heterogeneous light environment, but also against an ever-changing background. Using bird visual modeling to compare butterfly color patterns with the background (or an averaged background) of the forest understory would be a promising avenue of research to further test the hypothesis that bright warning signals with low chroma are reliable and more likely to have evolved in forested environments.

Furthermore, while visual modeling has advanced greatly within the last decade, it is also always important to test hypotheses in as many ecologically relevant contexts as possible. To further test the potentiality of the adaptive nature of low-chroma signals, it is important to investigate how live birds react to bright visual stimuli with low-chroma that many toxic butterflies exhibit under field conditions. Furthermore, it is possible that conspecifics of each of the study species have shaped the evolution of their patterning for species recognition or mating signals. Investigations into how butterlfy visual systems interpret these signals may provide insight into other pressures that shape butterfly patterns.

Mechanisms by which broadband low-chroma coloration are generated, and how they differ in intensity and reflectance characteristics, may also be a fruitful area of future research. Broadband scattering requires cuticle (or integument in the case of vertebrates)

containing microstructures that randomly and efficiently scatter light of all visible wavelengths (Giraldo, Yoshioka, & Stavenga, 2008; Stavenga, Giraldo & Leertouwer, 2010). It is likely that bright low-chroma scales in butterflies arose from scales that already contained pigment or some type of selective reflector. The evolutionary mechanisms that would cause these changes and select for them may be a fertile foray into a unique type of aposematic signaling.

References

Ackery, P. R. & Vane-Wright, R. I. (1984) *Milkweed butterflies, their cladistics and biology, being an account of the natural history of the Danainae, a subfamily of the Lepidoptera, Nymphalidae*. Cornell University Press, Ithaca, New York.

Burger, H., Dötterl, S. & Ayasse, M. (2010) Host-plant finding and recognition by visual and olfactory floral cues in an oligolectic bee. *Functional Ecology*, **24**, 1234–1240.

Chai, P. (1986) Wing Coloration of Free-Flying Neotropical Butterflies as a Signal Learned by a Specialized Avian Predator. *Biotropica*, **20**, 20-30.

Chouteau, M. & Angers, B. (2012) Wright's shifting balance theory and the diversification of aposematic signals. *PloS One*, **7**, 1-8.

DeVries, P. J. (1987) *The Butterflies of Costa Rica and their Natural History*. Princeton University Press, Princeton, New Jersey.

Douglas, J. M., Cronin, T. W., Chiou T-H. & Dominy, N. J. (2007) Light habitats and the role of polarized iridescence in the sensory ecology of neotropical nymphalid butterflies. (Lepidoptera: Nymphalidae) *The Journal of Experimental Biology,* **210**, 788-799.

Endler, J. A. (1992) Signals, signal conditions, and the direction of evolution. *The American Naturalist*, **139**, S125-S153.

Endler, J. A. (1993) The color of light in forests and its implications. *Ecological Monographs*, **63**, 1-27.

Endler, J. A. & Théry, M. (1996) Interacting effects of lek placement, display behavior, ambient light, and color patterns in three neotropical forest-dwelling birds. *The American Naturalist*, **148**, 421-452.

Endler, J. A. & Mielke P. W. Jr (2005) Comparing entire colour patterns as birds see them. *Biological Journal of the Linnean Society*, **86**, 405–431.

Fleishman, L. J. (1992) The Influence of the sensory system and the environment on motion patterns in the visual displays of anoline lizards and other vertebrates. *The American Naturalist*, **139**, S36-S61.

Giraldo, M. A., Yoshioka, S. & Stavenga, D. G. (2008) Far field scattering pattern of differently structured butterfly scales. *Journal of Comparative Physiology A*, **194**, 201-207.

Gomez, D. (2006) AVICOL. A program to analyse spectrometric data. Version 6. http://sites.google.com/site/avicolprogram/.
Guilford, T. (1988) The evolution of conspicuous coloration. *The American Naturalist*, **131**, S7-S21.

Halpin, C. G., Skelhorn, J. & Rowe C. (2008) Being conspicuous and defended: selective benefits for the individual. *Behavioral Ecology*, **19**,1012–1017.

Ham, A. D., Ihalainen E., Lindström L. & Mappes, J. (2006) Does colour matter? The importance of colour in avoidance learning, memorability and generalisation. *Behav Ecol Sociobiol*, **60**, 482–491.

Hart, N. S. (2002) Vision in the peafowl (Aves: *Pavo cristatus*). *The Journal of Experimental Biology*, **205**, 3925–3935.

Hay-Roe, M. M. & Nation, J. (2007) Spectrum of Cyanide Toxicity and Allocation in *Heliconius erato* and *Passiflora* Host Plants. *Journal of Chemical Ecology*, **33**, 319-329.

Holen, Ø. H. & Svennungsen, T. O. (2012) Aposematism and the handicap principle. *The American Naturalist*, **180**, 629-641.

Kemp, D. J. (2008) Female mating biases for bright ultraviolet iridescence in the butterfly *Eurema hecabe* (Pieridae). *Behavioral Ecology*, **19**, 1-8.

Kram, Y.A., Mantey S. & Corbo J. C. (2010) Avian Cone Photoreceptors Tile the Retina as Five Independent, Self-Organizing Mosaics. *PLoS ONE*, **5**, e8992.

Larsen, T. B. (1996) *The Butterflies of Kenya and their Natural History*. Oxford University Press, New York.

Leal, M. & Fleishman, L. J. (2002) Evidence for habitat partitioning based on adaptation to environmental light in a pair of sympatric lizard species. *Proceedings of the Royal Society London B*, **269**, 351-359.

Lyytinen, A., Alatalo, R. V., Lindstrom L. & Mappes, J. (1999) Are European white butterflies aposematic? *Evolutionary Ecology*, **13**, 709-719.

Macedonia, J. M. (2001) Habitat light, colour variation, and ultraviolet reflectance in the Grand Cayman anole, *Anolis conspersus*. *Biological Journal of the Linnean Society*, **73**, 299-320.

Maddison, D. R. & Maddison, W. P. (2005) MacClade 4: Analysis of phylogeny and character evolution. Version 4.08a. http://macclade.org.

Maddison, W. P. & Maddison, D.R. (2011) Mesquite: a modular system for evolutionary analysis. Version 2.75 http://mesquiteproject.org.

Maier, E. J. & Bowmaker, J. K. (1993) Colour vision in the passeriform bird, *Leiothrix lutea*: correlation of visual pigment absorbance and oil droplet transmission with spectral sensitivity. *Journal of Comparative Physiology A*, **172**, 295-301.

Mäthger, L. M., Bell, G. R. R., Kuzirian, A. M., Allen, J. J. & Hanlon, R. T. (2012) How does the blue-ringed octopus (*Hapalochlaena lunulata*) flash its blue rings? *Journal of Experimental Biology,* **215**, 3752-3757.

McGraw, K. J., Dale, J. & Mackillop, E. A. (2003) Social environment during molt and the expression of melanin-based plumage pigmentation in male house sparrows (*Passer domesticus*). *Behavioral Ecology & Sociobiology*, **53**, 116-122.

Merilaita, S. & Ruxton, G. D. (2006) Aposematic signals and the relationship between conspicuousness and distinctiveness. *Journal of Theoretical Biology*, **245**, 268-277.

Mebs, D., Wagner, M. G., Toennes, S. W., Wunder, C. & Boppré (2012) Selective sequestration of cardenolide isomers by two species of *Danaus* butterflies (Lepidoptera: Nymphalidae: Danainae) *Chemoecology*, **22**, 269-272.

Montgomerie, R. (2006) Analyzing colors. *Bird Coloration Mechanisms and Measurments* (eds G. E. Hill & K. J. McGraw), pp. 90-147. Harvard University Press, Cambridge.

Osorio, D., Miklósi, A. & Gonda, Z. (1998). The visual ecology and perception of coloration patterns by domestic chicks. *Evolutionary Ecology,* **13**, 673-689.

Osorio, D. & Vorobyev, M. (2005) Photoreceptor spectral sensitivities in terrestrial animals: adaptations for luminance and colour vision. *Proceedings of the Royal Society B*, **272**, 1745-1752.

Owen, D. F. (1970) Mimetic polymorphism and the palatability spectrum. Oikos, **21**, 333-336.

Papke, R. S., Kemp, D. J. & Rutowski, R. L. (2007) Multimodal signalling: structural ultraviolet reflectance predicts male mating success better than pheromones in the butterfly Colias eurytheme L. (Pieridae). *Animal Behaviour*, **73**, 47-54.

Peichl, L., Chavez, A. E., Ocampo, A., Mena, W., Bozinovic, F. & Palacios, A. G. (2005) Eye and vision in the subterranean rodent cururo (*Spalacopus cyanus*, Octodontidae). *The Journal of Comparative Neurology*, **486**, 197-208.

Palacios, A. G., Srivastava, R. & Goldsmith, T. H. (1998) Spectral and polarization sensitivity of photocurrents of amphibian rods in the visible and ultraviolet. *Visual Neuroscience*, **15**, 319-331.

Poulton, E. B. (1890) *The Colours of Animals their Meaning and Use, Especially Considered with the Case of Insects*. D. Appleton and Company, New York.

Prudic, K. L., Skemp, A. K. & Papaj, D. J. (2006) Aposematic coloration, luminance contrast, and the benefits of conspicuousness. *Behavioral Ecology*, **18**, 41-46.

Ratcliffe, J. M. and Nydam, M. L. (2008) Multimodal warning signals for a multiple predator world. *Nature*, **455**, 96-100.

Roper, T. J. & Redston, S. (1987) Conspicuousness of distasteful prey affects the strength and durability of one-trial avoidance learning. *Animal Behaviour*, **35**, 739-747.

Rutowski, R. L., Nahm, A. C. & Macedonia, J. M. (2010) Iridescent hindwing patches in the Pipevine Swallowtail: differences in dorsal and ventral surfaces relate to signal function and context. *Functional Ecology*, **24**, 757-775.

Shine, R. (1980) Reproduction, Feeding and Growth in the Australian Burrowing Snake *Vermicella annulata*. *Journal of Herpetology,* **14**, 71-77.

Sillman, A. J., Govardovskii, V. I. & Loew, E. R. (1997) The photoreceptors and visual pigments of the garter snake (*Thamnophis sirtalis*): a microspectrophotometric, scanning electron microscopic and immunocytochemical study. *The Journal of Comparative Physiology A*, **181**, 89-101

Skorupski, P. & Chittka, L. (2010) Photoreceptor Spectral Sensitivity in the Bumblebee, *Bomus impatiens* (Hymenoptera: Apidae). *PloS One*, **5**, 1-5.

Stankowich, T., Caro, T. & Cox, M. (2011) Bold Coloration and the Evolution of Aposematism in Terrestrial Carnivores. *Evolution*, **65**, 3090-3099.

Stavenga, D. G., Giraldo, M. A. & Leertouwer, H. L. (2010) Butterfly wing colors: glass scales of *Graphium sarpedon* cause polarized iridescence and enhance blue/green pigment coloration of the wing membrane. *The Journal of Experimental Biology*, **213**, 1731-1739.

Stoddard, M. C. & Prum, R. O. (2008) Evolution of avian plumage color in a tetrahedral color space: A phylogenetic analysis of New World buntings. *The American Naturalist*, **171**, 755-776.

Streinzer, M., Paulus, H. F. & Spaethe, J. (2009) Floral colour signal increases short-range detectability of a sexually deceptive orchid to its bee pollinator. *The Journal of Experimental Biology,* **212**, 1365-1370.

Svádová, K., Exnerová, A., Stys, P., Landová, E., Valenta, J., Fuciková, A. & Socha, R. (2009) Role of different colours of aposematic insects in learning, memory and generalization of naïve bird predators. *Animal Behaviour*, **77**, 327-336.

Valdés, Á. (2001) Depth-related adaptations speciation processes and evolution of color in the genus *Phyllidiopsis* (Mollusca: Nudibranchia). *Marine Biology*, **139**, 485-496.

Vorobyev, M. & Osorio, D. (1998) Receptor noise as a determinant of colour thresholds. *Proceedings of the Royal Society London B*, **265**, 351-358.

Vorobyev, M., Osorio, D., Bennett, A. T. D., Marshall, N. J. & Cuthill, I. C. (1998) Tetrachromacy, oil droplets and bird plumage colours. *The Journal of Comparative Physiology A*, **183**, 621-633.

Weldon, P. J. (2010) Nuisance arthropods, nonhost odors, and vertebrate chemical aposematism. *Naturwissenschaften*, **97**, 443-448.

Table 1: Comparison of average brightness (overall), chroma, and UV brightness between open-dwelling and forest-dwelling species of aposematic butterflies. Results of t-test comparisons are shown.

	Open	Forest	p-value
Brightness	5293	8754	0.0005
Chroma	2.92	1.61	3.80E-08
UV Brightness	378	1130	0.001

Table 2: Comparison of chroma and brightness of radiances between open and forest-dwelling butterflies under open and forest illumination .

		Open-dwelling	Forest-dwelling	p-value
Brightness	Open irradiance	215.5	302.22	**0.0059**
	Forest irradiance	181.52	278.43	**0.0011**
	p-value	0.14	0.27	
Chroma	Open irradiance	1.93	1.63	0.106
	Forest irradiance	1.98	1.58	**0.036**
	p-value	0.77	0.68	

Table 3: Comparison of avian-perception model parameters between open-dwelling and forest-dwelling species of aposematic butterflies.

	Open	Forest	T-Test
Perceived Contrast	0.37	0.23	0.000123
Chromaticity	0.49	0.24	5.02E-13
Double-cone absorbance	2.27	3.63	0.001

Table 4: Using the criteria in the Methods, butterflies were scored as open-dwelling or forest-dwelling and as more or less chromatically perceived by birds (n = 90). The p-value for the Fisher's exact two-tailed statistic is less than 0.0001, indicating a correlation between forested environments and aposematic butterflies with patches that exhibit low chroma.

	More Chromatic	Less Chromatic
Open-dwelling	12	1
Forest-dwelling	9	68

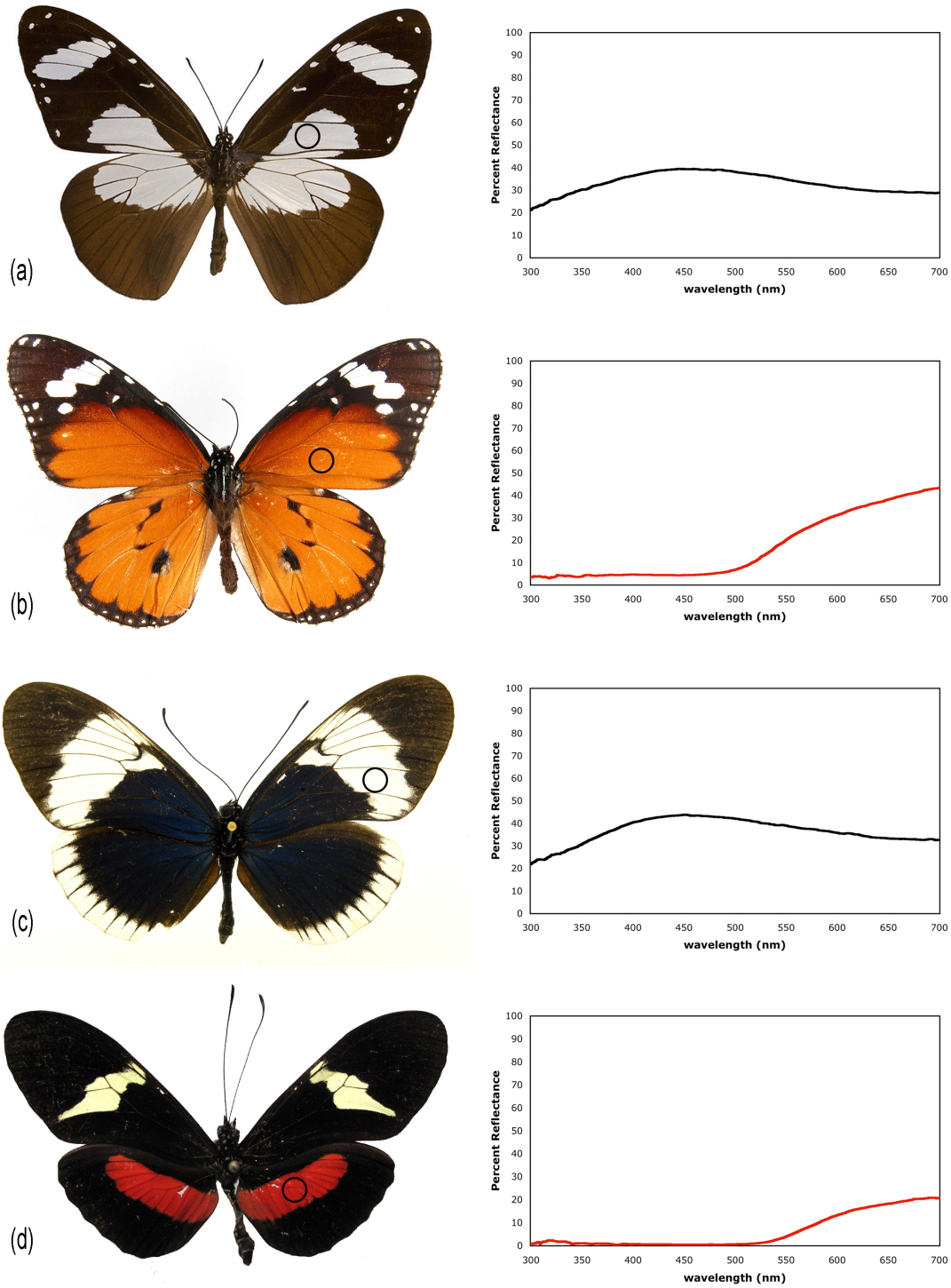
(a)
(b)
(c)
(d)
Percent Reflectance
wavelength (nm)
0
10
20
30
40
50
60
70
80
90
100
300
350
400
450
500
550
600
650
700

Fig. 1. Images of four select study species and the reflectance of their largest non-black patch. Measurement areas are indicated by black circles on one of the wings. Figs 1a (*Amauris niavius*) and 1b (*Danaus chrysippus*) belong to the subfamily danainae; while figs 1c (*Heliconius cydno*) and 1d (*Heliconius clysonymus*) belong to the heliconinae. The reflectance spectra of figs 1a and c are examples of forest-dwelling butterflies and illustrate the relatively flat appearance of the reflectance spectrum indicating that most visible wavelengths are reflected to a similar degree. The reflectance spectra of figs 1b and d, however are those of open-dwelling butterflies and show a selectively reflecting patch indicated by the low reflectance of wavelengths below 550nm.

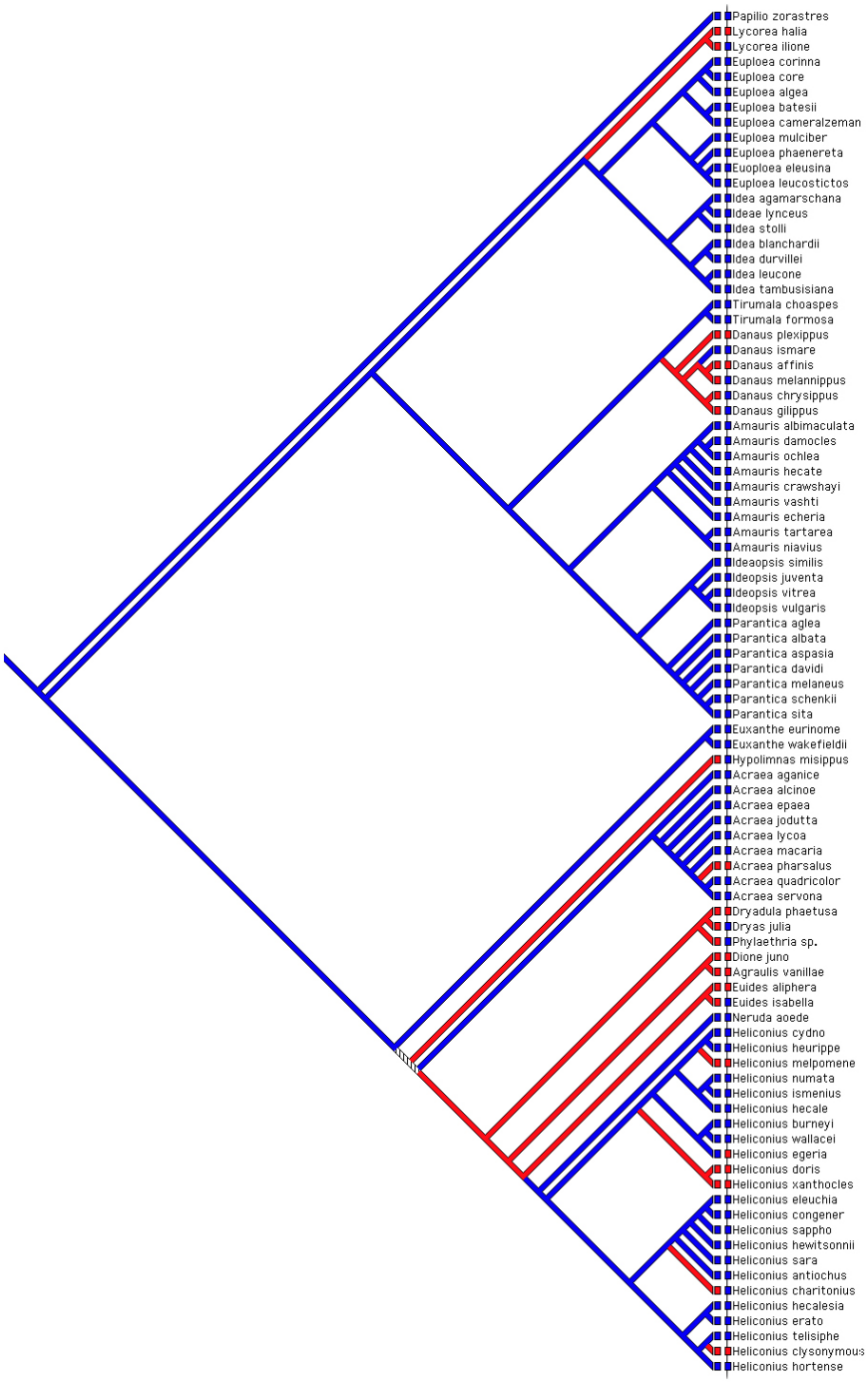
Papilio zorastres
Lycorea halia
Lycorea ilione
Euploea corinna
Euploea core
Euploea algea
Euploea batesii
Euploea cameralzeman
Euploea mulciber
Euploea phaenereta
Euoploea eleusina
Euploea leucostictos
Idea agamarschana
Ideae lynceus
Idea stolli
Idea blanchardii
Idea durvillei
Idea leucone
Idea tambusisiana
Tirumala choaspes
Tirumala formosa
Danaus plexippus
Danaus ismare
Danaus affinis
Danaus melannippus
Danaus chrysippus
Danaus gilippus
Amauris albimaculata
Amauris damocles
Amauris ochlea
Amauris hecate
Amauris crawshayi
Amauris vashti
Amauris echeria
Amauris tartarea
Amauris niavius
Ideaopsis similis
Ideopsis juventa
Ideopsis vitrea
Ideopsis vulgaris
Parantica aglea
Parantica albata
Parantica aspasia
Parantica davidi
Parantica melaneus
Parantica schenkii
Parantica sita
Euxanthe eurinome
Euxanthe wakefieldii
Hypolimnas misippus
Acraea aganice
Acraea alcinoe
Acraea epaea
Acraea jodutta
Acraea lycoa
Acraea macaria
Acraea pharsalus
Acraea quadricolor
Acraea servona
Dryadula phaetusa
Dryas julia
Phylaethria sp.
Dione juno
Agraulis vanillae
Euides aliphera
Euides isabella
Neruda aoede
Heliconius cydno
Heliconius heurippe
Heliconius melpomene
Heliconius numata
Heliconius ismenius
Heliconius hecale
Heliconius burneyi
Heliconius wallacei
Heliconius egeria
Heliconius doris
Heliconius xanthocles
Heliconius eleuchia
Heliconius congener
Heliconius sappho
Heliconius hewitsonnii
Heliconius sara
Heliconius antiochus
Heliconius charitonius
Heliconius hecalesia
Heliconius erato
Heliconius telisiphe
Heliconius clysonymous
Heliconius hortense

Fig. 2. Current consensus of phylogenetic relations of all species in this study as summarized by The Tree of Life web project (http://www.tolweb.org/). Both trees are identical, but the tree on the left has the trait for habitat mapped upon it, while the tree on the right has mapped upon it the trait of chromaticity. Blue branches on the left tree indicate forest-dwelling habits and blue branches on the right tree indicate low chromaticity. To the right of the species names are two colored squares that indicate first habitat, then chromaticity traits.