BEHAVIOURAL AND ECOLOGICAL INTERACTIONS BETWEEN *HELICONIUS* BUTTERFLIES, THEIR PREDATORS AND HOST PLANTS

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“Não contavam com minha astúcia.”

Chapolin Colorado

DECLARATION

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface or specified in the text. It has not been previously submitted, in part or whole, to any other university or similar institution for any degree, diploma, or other qualification except as declared in the Preface and specified in the text. It does not exceed the prescribed word limit of 60,000 words for the Degree Committee in Biology.

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SUMMARY

Heliconius butterflies exhibit Müllerian mimicry, in which two or more unpalatable species share a mutual advantage from having a common conspicuous colour pattern. These tropical butterflies have impressive visual signals, which are under conflicting selection pressures, as they are used in choosing potential mates and defending against visual predators through aposematic coloration. As both selection pressures are likely to be strong, different elements of the signal might be adapted for different receivers. Here, I combine sensory ecology with behavioural ecology to explain *Heliconius* colours signals of different co-mimic pairs. I explore how mimicry in *Heliconius* is perceived both from the perspective of predators and conspecifics, using visual abilities of both butterflies and birds.

The different visual sensitivities of avian predators, *H. erato* females and males make them to perceive *Heliconius* coloration in different ways. My work suggests that having the ability to see in the ultra-violet light range enables higher discrimination between co-mimics both for birds and butterflies. *Heliconius* warning colours transmit a consistent signal across time of the day and habitat in a tropical forest for avian vision. In contrast through *Heliconius* vision there is evidence that patterns are more conspicuous in their own habitats. All these traits could facilitate communication between co-mimics and reduce the cost of confusion in courtship while still maintaining the advantages of Müllerian mimicry against predation.

I conducted a field experiment to show that attack rates on a novel distasteful butterfly reduced over time, suggesting that *Heliconius* wing colouration can enhance aversion among predators. Finally, I have shown that *Heliconius* butterflies use leaf shape as

a cue to approach their host plants, demonstrating the potential for *Heliconius* to drive negative frequency dependent selection on the leaf shape of their *Passiflora* host plants. Overall these results highlight ecological interactions between *Heliconius* butterflies, their predators and host plants.

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COLLABORATIONS

Four chapters of this thesis have been written for publication, and are intended as individual manuscripts. As such there is some repetition, particularly in the introduction of each chapter. In addition, much of the work presented here is inevitably the result of collaboration with others.

My fieldwork was performed in the Smithsonian Tropical Research Institute (STRI), Panama, with the support of W. Owen McMillan. Martin Stevens and Jolyon Troscianko developed the colour analysis and visual model methodology used in **Chapter 2**, **Chapter 3** and **Chapter 4**.

In **Chapter 2** and **Chapter 3**, I collected the data, conducted the analysis and wrote the manuscripts with help from Chris Jiggins and Martin Stevens, both of whom contributed to designing the concept of these two chapters. In addition, in **Chapter 2**, Patricio A. Salazar C. provided wing samples from Ecuador, Matthew Miller helped with the access to the STRI Cryological Collection, Natasha Bloch kindly provided the SWS1 primers, María Eugenia Losada conducted the mating experiment and Derya Akkaynak performed the red colour measurements.

In **Chapter 4**, I designed and performed the experiment, conducted statistical analyses and wrote the manuscript with help from Chris Jiggins and Martin Stevens. Rebecca M. Kilner and Hannah M. Rowland provided helpful comments on the experimental design and earlier version of the chapter.

Chapter 4 has already been published as:

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In **Chapter 5**, Chris Jiggins and I conceived and designed the experiments. María Eugenia Losada and I conducted the experiments. I analysed the data and wrote the manuscript with the help from Chris Jiggins. Elizabeth Evans and Oscar Paneso provided assistance in the insectaries.

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CONTENTS

CHAPTER 3. THE CONSPICUOUSNESS OF *HELICONIUS* BUTTERFLIES ACROSS TIME AND HABITAT

CHAPTER 1

THESIS INTRODUCTION

HELICONIUS BUTTERFLIES: APOSEMATISM AND ECOLOGY

Coloration is widespread in nature and is one of the first traits used in the study of evolution by natural and sexual selection. Darwin (1871) observed that conspicuous colours were brought into play in courtship, and that colours and patterns have been gradually modified by the preference of the females for the most beautiful males, since sexual selection accounts for many of the most beautiful displays in animal kingdom. Wallace (1867, 1877), in turn, discussed conspicuous colours as anti-predator adaptations, and described how natural selection leads to camouflage and warning signals. Subsequently, Poulton (1890) considered the fact that colour signals can play both roles, in natural and sexual selection, and that these can act together in the evolution of warning coloration. Animals often use coloration as a warning signal advertising distastefulness or harmful chemicals. Conspicuous colours are associated by predators with a bad experience and subsequently learn to avoid attacking them. Such signals are known as aposematic (Poulton, 1890; Ruxton *et al.*, 2004; Mappes *et al.*, 2005).

Bates (1862) and Müller (1879) described the two most classical forms of mimicry among insects. *Heliconius* butterflies exhibit Müllerian mimicry, in which two or more

unpalatable species share a mutual advantage from having a common conspicuous colour pattern (Müller, 1879; Brown, 1981). Specifically, this reduces the number of individuals that need to be killed per species before predators learn to identify them as distasteful (Benson, 1972; Langham, 2004). *Heliconius* are chemically defended by cyanogenic glycosides, which can be either synthesized *de novo* or sequestered from host plants, and thus represent a case of aposematism (Engler *et al.*, 2000; Engler-Chaouat & Gilbert, 2007; Cardoso & Gilbert, 2013). The genus *Heliconius* is widely distributed in Central and South America, and despite their great geographical variation in wing colour pattern, different species show almost identical colour patterns wherever they co-occur, with two or more sympatric species often mimicking each other (Brown, 1981; Mallet & Gilbert, 1995; Joron & Mallet, 1998) (Figure 1.1).

Mimicry pattern diversity within *Heliconius* is an excellent system in which to examine the idea of gradual evolution. Poulton (1913) suggested that mimicry could rise in small steps, beginning with similar appearances between the mimic and its model, followed by further mutations that would refine mimetic forms. Later, Fisher (1927) presented an alternative, in which mimicry evolution would be gradual and driven by occasional predator attack mistakes, where variation is equally frequent in either of the directions around the mean appearance. Deviations in any direction could result in loss of predator protection, although with another protected species co-occurring, variation in the direction towards that appearance might be beneficial. Early studies showed that phenotypic plasticity is not involved in shifts between *Heliconius* phenotypes, but major colour pattern elements are controlled by a relatively small number of Mendelian loci (Joron *et al.*, 2006; Kronforst *et al.*, 2006; Pardo-Diaz & Jiggins, 2014).

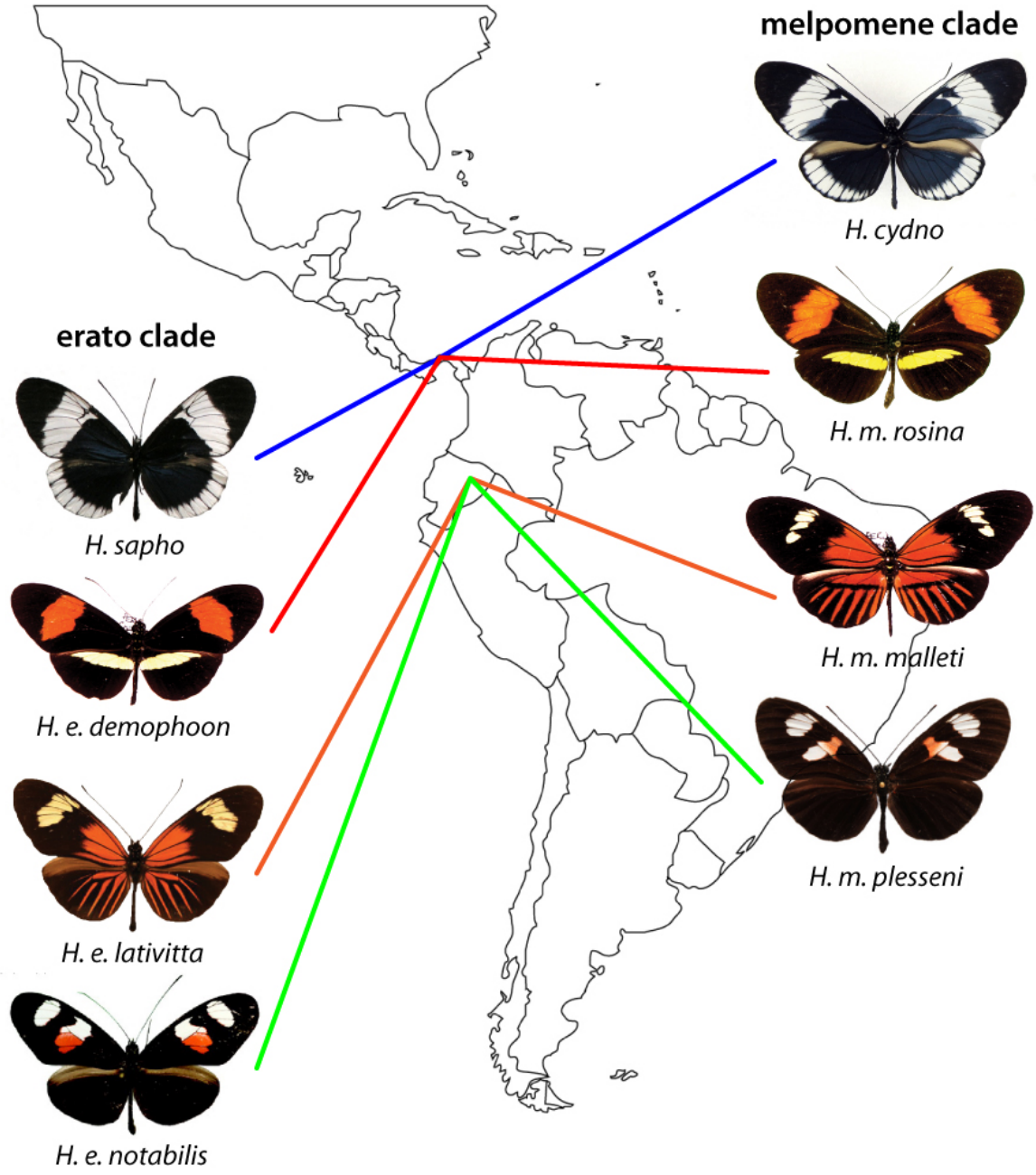


Figure 1.1. Different *Heliconius* species show almost identical colour patterns wherever they occur, with sympatric species mimicking each other. Locations of field collected samples used in this thesis. Mimetic pairs collected in Panama: *H. cydno* and *H. sapho*; *H. melpomene rosina* and *H. erato demophoon*. Collected in Ecuador: *H. melpomene malleti* and *H. erato lativitta*; *H. melpomene plesseni* and *H. erato notabilis*.

Colour patterns play a special role in speciation among *Heliconius* butterflies. Not only is colour pattern under strong frequency-dependent selection due to predation, implying that rare hybrids will be selected against, but also *Heliconius* butterflies find and choose potential mates based on colour signals which leads to reproductive isolation (Mallet & Barton, 1989; Mallet & Joron, 1999; Jiggins *et al.*, 2001; Finkbeiner *et al.*, 2014). Females *Heliconius* often mate soon after emergence when they cannot reject males, so that although females that are not mated quickly may have a role in choosing a mate, male preferences are more important to assortative mating (Estrada & Jiggins, 2008; Klein & de Araújo, 2010; Merrill *et al.*, 2011a). Two sympatric sister species *H. melpomene rosina* and *H. cydno* differ in microhabitat and host plant which reduce potential mating encounters, as well as strong mating preferences that has been enhanced in sympatry (Mallet et al., 1998; Jiggins et al., 2001; Merrill et al., 2013). Although hybrids between these two species are rare in nature, gene flow occurs at a low level (Martin et al., 2013).

Local adaptation of *Heliconius* mimicry rings, groups of unpalatable species that converge to the same warning colour, tends to involve adaptation to specific microhabitats (Mallet & Gilbert, 1995; Estrada & Jiggins, 2002; Elias *et al.*, 2008; Jiggins, 2008). *Heliconius* habitats are associated with their use of adult food plants, sexual behaviour, gregarious roosting and especially use of their larval host-plants, *Passiflora* vines (Mallet & Gilbert, 1995). In addition, different light environments should create microhabitats where butterfly signals might differ in their efficiency. Endler (1993) proposed that certain colour combinations would do better in some light habitats. For example, light found in the canopy should favour species with blue colours, and close to the forest floor and small gaps species should use red and oranges. Moreover, conspicuousness should vary between light

environment, as seen in the colour patterns of guppies which are more conspicuous at the times and places of courtship and relatively less conspicuous at times and places of predator risk (Endler, 1991). Similar effects are known for *Anolis* lizard dewlap colours used in sexual display, which match with their habitat use (Leal & Fleishman, 2002; Fleishman et al., 2009). In *Heliconius*, red band patterns were characterized as occurring in open and dry areas while white band with iridescent blue patterns occur more in the canopy (Brown & Benson, 1977; Turner & Mallet, 1996; Estrada & Jiggins, 2002).

The *Heliconius-Passiflora* interaction is already well established as an example of insect-plant co-evolution (Benson et al., 1975). *Passiflora* species possess a range of defensive traits against *Heliconius* butterflies, such as production of chemical compounds that deter herbivores (Smiley, 1985a; Engler et al., 2000), mechanical protection such as hooked trichomes that are able to pierce caterpillars (Gilbert, 1971) and high diversity in leaf shape which makes harder for females to detect host plants (Gilbert, 1982). In addition, extra-floral nectaries of some species are similar to *Heliconius* eggs to deter ovipositing females (Williams & Gilbert, 1981) while others produce nectar that attracts ants to the plant, which in turn, attack *Heliconius* larvae and eggs (Smiley, 1985b; Apple & Feener Jr., 2001).

Hence, *Heliconius* butterflies face a range of uncertainties associated with a varying environment, mate location, oviposition site, foraging, and enemy avoidance. It seems likely that sexual and natural selection has tuned the warning signals to transmit relevant information in the face of this ecological diversity. Strong, conspicuous colours such as *Heliconius* wing patterns, favour rapid discrimination and facilitate more rapid avoidance learning among predators (Guilford & Dawkins, 1991; Speed, 2000). In spite of the benefits

against predators, *Heliconius* co-mimics often demonstrate signal confusion during courtship due to their similar appearances (Jiggins et al., 2001; Estrada & Jiggins, 2008).

Multiple selective factors affect the evolution of butterfly wing coloration and both butterflies and predators need to identify and discriminate these signals according to their interests in mating or feeding. Human eyes easily recognize aposematism and mimicry, yet birds and insects have different sensory systems. In order to understand the trade-off between courtship and predation, in my thesis I consider the appearance of *Heliconius* warning signals from the perspective of both butterfly and bird vision.

HELICONIUS VISION

In recent years there have been substantial advances in our knowledge of the spectral sensitivities of visual receptors and their evolutionary history, together with increased interest in the evolutionary relationship between animal vision and communication signals. Butterflies show the most diverse wing colour patterns among insects, and with further studies came the realization of an enormous diversity in receptor number and spectral sensitivities (Briscoe & Chittka, 2001; Briscoe, 2008; Osorio & Vorobyev, 2008). Butterfly vision is based on three major classes of photoreceptors, with peak sensitivity in the ultraviolet (UV), blue (B) and long wavelength (LW). Differences in vision are due to gene duplications followed by mutations, which affect amino acids in the opsin (visual protein) producing a diversity of spectral sensitivities between species (Briscoe, 2008). For example, both butterflies *Pieris rapae* and *Lycaena rubidus* have gene duplications on the B opsin, however each duplication leads to different extended visual spectrum range and moreover, to sexual dimorphism in *Lycaena* eyes (Briscoe, 2008).

The reasons for this diversity of butterfly colour vision is not fully understood but likely includes their need to find food, host plants and select mates. In many insects, colour receptors are not uniformly distributed and visual pigment expression patterns across the eye can correlate with visual ecology. In *Lycaena* butterflies, although two different species have exactly the same four photopigments, species with blue wing colours possess blue receptors in the ventral eye, while species that reflect red and UV in the wings, lack blue receptors in the ventral eye (Bernard & Remington, 1991). Thus, the expression of blue visual pigment might be involved in perceiving sexual signals. Another example of adaptive tuning of photoreceptors is in *Photuris* fireflies, in which sexual communication involves bioluminescent signals. Each species of *Photuris* has spectral sensitivities of the LW-receptors matched with its own bioluminescent emission (Cronin *et al.*, 2000). Vision appears to be evolutionary tuned for maximum discrimination of conspecific signals.

Similarly, *Heliconius* butterflies rely on visual cues when searching for potential mates. Multiple mimetic colour patterns pose an additional challenge since individuals must recognize conspecifics from co-mimics to successfully reproduce. In the eyes of *Heliconius erato* five receptor sensitivities have been identified with spectral peaks at approximately 360 nm (UV1), 390 nm (UV2), 470 nm (Blue), 560 nm (Green LW) and 600 nm (Red LW) (Briscoe *et al.*, 2010; Yuan *et al.*, 2010; Bybee *et al.*, 2012; McCulloch *et al.*, 2016) (Figure 1.2). The compound eye of *H. erato* is sexually dimorphic and males express only UV2 while females express both UV opsins in separate photoreceptors (McCulloch *et al.*, 2016). Differences in photoreceptor ratios therefore might play a role in sexual selection and identification of mates. Other *Heliconius* species also possess the additional UV receptor, but to date nothing is known of its expression patterns. Research on reproductive behaviour

in *Heliconius* has focused on male attraction as male mate preferences appear to have co-evolved with changes in mimetic colour patterns (Jiggins *et al.*, 2004; Estrada & Jiggins, 2008; Merrill *et al.*, 2011b). However, the duplicate UV opsin might give females an advantage in recognizing conspecifics and avoiding co-mimic male harassment, which it is known to reduce individual fitness (Andersson *et al.*, 2000; Estrada *et al.*, 2011).

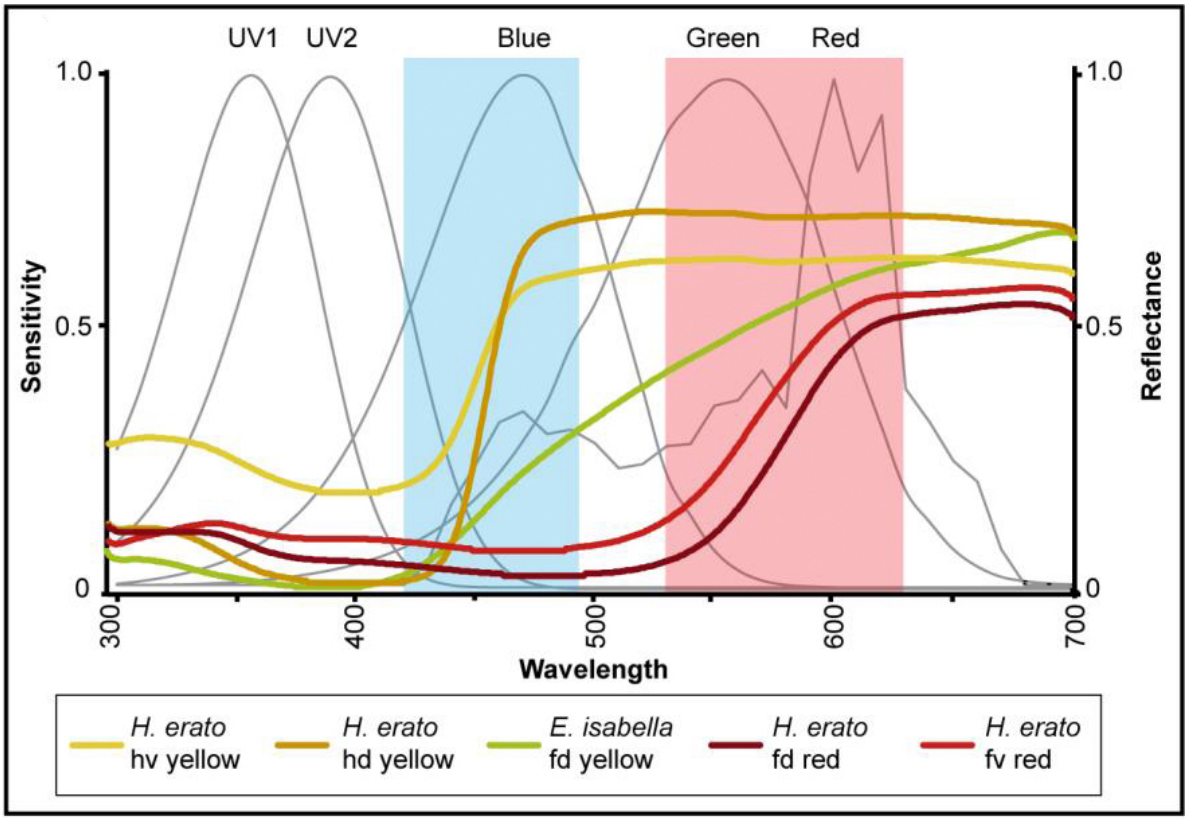


Figure 1.2. Spectral sensitivity curves for each of the five photoreceptor types of *Heliconius erato* vision overlaid with wing reflectance spectra from *H. erato* and *Eueides isabella*. Blue shaded area indicates the overlap of the yellow reflectance spectra with the sensitivity peak of the blue receptor, while the pink shaded area indicates the overlap of red wing reflectance spectra with the sensitivities of the green and red receptors. Figure from McCulloch *et al.* (2016). f, forewing; h, hindwing; d, dorsal; v, ventral.

This ability to see in the UV range might enable *Heliconius* to use hidden channels of communication in which species-specific signals are not detected by predators (Bybee *et al.*, 2012). Both the use of *3-hydroxykynurenine* as a UV yellow wing pigment and the additional UV opsin arose at the origin of the genus *Heliconius* (Briscoe *et al.*, 2010). In *H. erato* females, the two UV opsins might allow a greater degree of discrimination of UV-reflecting yellow wing patches when compared with *Eueides isabella* non-UV yellow (McCulloch *et al.*, 2016) (Figure 1.2). This has led to the suggestion that the visual system and wing colouration have co-evolved, and might offer a unique channel of communication. The role of colour in mate choice in butterflies has been frequently investigated, although only a few studies have evaluated preferences for variation in UV reflectance. For example, UV brightness is a strong component of male attractiveness in both *Colias* and *Eurema* butterflies (Silberglied & Taylor Jr., 1973; Rutowski *et al.*, 2007; Kemp, 2008) while *Polymmatus icarus* males prefer UV-absorbing females (Knüttel & Fiedler, 2001), as well as *Bicyclus*, in which small UV-reflective spots played a role in female choice (Robertson & Monteiro, 2005).

Moreover, *Heliconius* can be trained to associate a color stimulus with a food reward, demonstrating a high precision of discrimination and learning (Swihart & Swihart, 1970; Swihart, 1971; Blackiston *et al.*, 2011). The presence of red lateral filtering pigments shifts red photoreceptor cell sensitivity from 560nm to 600nm and allows *H. erato* to precisely distinguish colours in the red-green spectrum even though they only have a single LW-sensitive opsin (Zaccardi *et al.*, 2006; McCulloch *et al.*, 2016) (Figure 1.2). Therefore, *Heliconius* butterflies have a unique colour vision that might enable them to reliably detect and recognize food sources, host plants and mate partners. Previous research on the colour

patterns of two Müllerian mimic butterflies, *Heliconius* and *Melinaea*, has revealed that co-mimetic species have highly similar wing colour patterns. However, small differences between co-mimics can be perceived better by butterflies than birds (Llaurens *et al.*, 2014). A further study on Batesian mimics found differences between the sexes and wing surfaces, with females being better mimics and the dorsal side having better resemblance to co-mimic models (Su *et al.*, 2015).

However, the more common challenge faced by many *Heliconius* is not to distinguish between conspecifics and other genera of butterflies, but rather to distinguish between co-mimics within *Heliconius*. Using *Heliconius erato* vision, I explore the hidden channels of communication hypothesis in the **Chapter 2** and **Chapter 3**, and study colour differences and habitat conspicuousness between co-mimics. Furthermore, in **Chapter 5** I investigate vision in terms of perception of shape and the hypothesis that ovipositing females use leaf shape as a cue to find host plants.

BIRD VISION AND PREDATOR BEHAVIOUR

Mimicry provides an example of an adaptation improved by natural selection, but imperfect mimics appear to work as well as perfect mimics. For example the conspicuous red-black banded colour pattern of some harmless snakes is thought to have arisen from mimicry of venomous coral snakes (Rabosky *et al.*, 2016) and also many hoverflies species are harmless mimics of stinging Hymenopterans (Penney *et al.*, 2012). One explanation for this is that predators might generalize between prey appearances, responding to a novel stimulus from previous experience with another similar stimulus. This ability of the predator to generalize is important for the evolution of aposematism and mimicry, as shown in

behaviour experiments (Ham *et al.*, 2006; Svádová *et al.*, 2009) and theoretical predictions (Ruxton *et al.*, 2008). Additionally, another explanation is that mimicry might be in the 'eye of the beholder', such that poor mimics to human eyes remain good mimics to natural predators. Predators differ in vision or perception, which highlight the importance of exploring predator cognition in studies of colouration (Endler & Mappes, 2004; Mappes *et al.*, 2005).

Humans have trichromatic vision with three cone types that detect light in the wavelength range of 400-700 nm. In contrast, birds are tetrachromatic possessing four cones with a range of 300-700 nm and double cones for luminance (Hart *et al.*, 2000; Osorio & Vorobyev, 2005; Hart & Hunt, 2007) (Figure 1.3). All these cones are associated with a system of coloured droplets acting as filters that improve discrimination by sharpening the peaks of spectral absorbance (Vorobyev *et al.*, 1998; Vorobyev, 2003). These properties allow birds to perceive hues that humans are incapable of perceiving. Hence, understanding how birds perceive colours is crucial for interpreting and studying visual signals such as aposematism and mimicry in butterflies.

Bird predators see in the UV range, and diurnal birds, which likely have excellent colour vision, fall into two different classes of colour vision: a violet sensitive (VS) and an ultraviolet sensitive (UVS) group (Bennett & Cuthill, 1994; Ödeen & Håstad, 2013) (Figure 1.3). The differences between these groups are due to amino acid substitutions in specific sites of the UV/V cone opsin gene (SWS1) that confer changes in light absorption (Wilkie *et al.*, 2000; Carvalho *et al.*, 2007; Ödeen & Håstad, 2009). From 21 avian orders studied, the SWS1 gene has shifted between VS and UVS at least 14 times showing that avian colour vision is not strongly conserved between species (Ödeen & Håstad, 2013). As UV/V vision

is important for mate choice, foraging and predator avoidance, spectral tuning of the SWS1 gene offers an example of the molecular basis for ecological adaptation (Bennett & Cuthill, 1994; Church *et al.*, 1998; Siitari *et al.*, 1999; Håstad *et al.*, 2005; Werner *et al.*, 2012).

Visual adaptation in the UV range makes birds more able to discriminate cues in food that reflect strong UV relative to the background, such as fruits, seeds and insects. In experiments focusing on the importance of UV light in predation, bird predators could find their prey quicker when UV cues were present (Church *et al.*, 1998) while others found no evidence that the presence of UV would enhance learnt avoidance of aposematic signals (Lyytinen *et al.*, 2001). In addition, field experiments reveal increased predation on moths with UV reflectance compared with moths lacking UV (Lyytinen *et al.*, 2004). However, UV signals should be considered as part of general colour vision, rather than being particularly special as compared to other wavelengths (Stevens & Cuthill, 2007).

In colour vision, it is not only the stimulation of visual receptors that matters, but also the relative stimulation of two or more receptor types and their neuronal interactions, such as opponent colour channels. The perception of colour contrast is maximized by these mechanisms (Kelber *et al.*, 2003; Lovell *et al.*, 2005; Renoult *et al.*, 2015). Colour conspicuousness is a very important aspect of the ecology and evolution of aposematism. The conspicuousness of a colour is defined as the distance between the studied trait and the background, and the use of opponent channels to address contrast has been used to predict the behaviour of perceivers, as seen in opisthobranchs where conspicuousness and toxicity are strong correlated (Cortesi & Cheney, 2010).

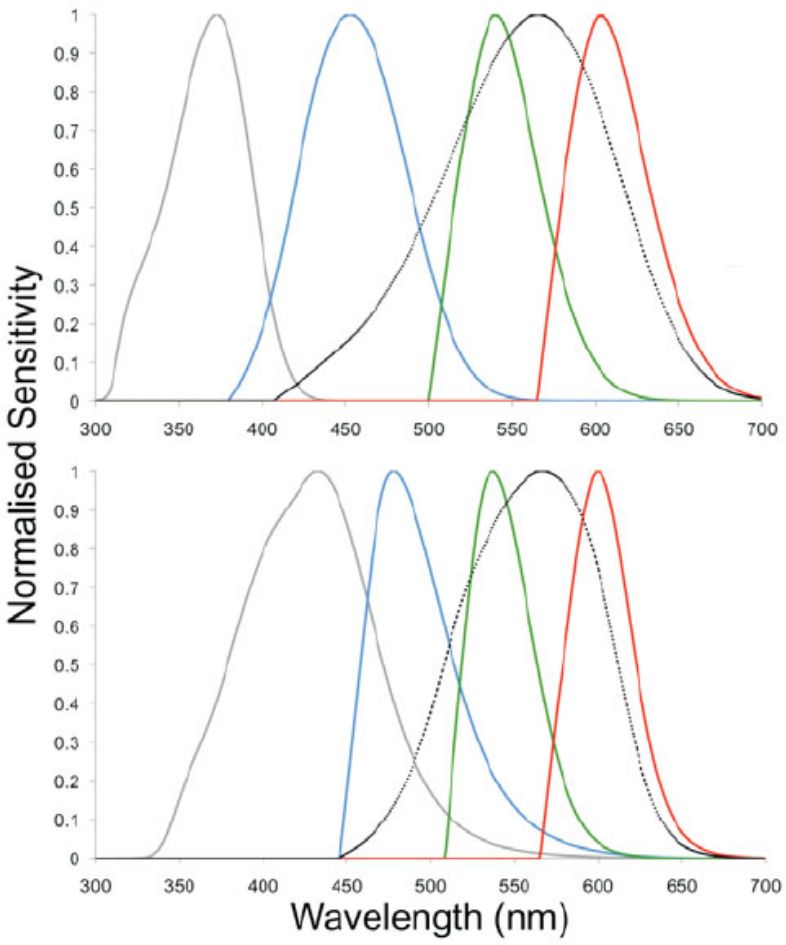


Figure 1.3. Birds can be divided in two different classes of colour vision. Spectral sensitivity curves for each of the four cone types for blue tit (*Cyanistes caeruleus* – UVS; top) and peafowl (*Pavo cristatus* – VS; bottom). Long-wave (red), medium-wave (green), short-wave (blue), UV/V (grey) and double cones (dotted black). Figure from Stevens (2011) and data from Hart *et al.* (2000) and Hart (2002).

Furthermore, the receptor noise-based model of visual discrimination can be used to calculate the distance between colours, assuming that colours are encoded by opponent mechanisms and limited by noise, which are determined by the relative proportion of each photoreceptor; however, environment luminosity is ignored in this model (Vorobyev & Osorio, 1998; Vorobyev *et al.*, 2001). Colour richness and distinctiveness can be estimated from a matrix of pairwise colour distances expressed in JND units (just noticeable

difference) by counting the number of values above the discrimination threshold, which is calculated from behavioural experiments and are only known for a few experimental organisms (Vorobyev *et al.*, 2001; Siddiqi *et al.*, 2004; Olsson *et al.*, 2015).

Birds have the most complex vision system of any vertebrate and knowledge of how they perceive prey can improve our understanding of the evolution of warning colouration and mimicry (Hart, 2001; Hart & Hunt, 2007). Birds are widely considered to be the primary selective agent for the aposematic coloration of butterflies. After unpleasant experiences with an unpalatable prey, bird predators learn to avoid similar morphs (Ham *et al.*, 2006; Lindström *et al.*, 2006). This learning ability leads to selection favouring the most abundant colour patterns in a local area in which predator attacks are reduced through aversion learning of locally common aposematic patterns (Mallet & Joron, 1999).

It is already known that avian predators are capable of learning and can select against unfamiliar coloration of experimental *Heliconius* in the field (Pinheiro, 2003; Langham, 2004). Several studies have investigated predator behaviour in cages using wild-caught rufous-tailed jacamars (*Galbula ruficauda*), which are specialist predators of fast-flying insects and exhibit specific butterfly handling strategies. Jacamars readily discriminate *Heliconius* from other butterfly species and reject them by sight and by taste (Chai, 1986; Langham, 2004). Field experiments using other butterfly predators, kingbirds and flycatchers, also showed taste-rejection of *Heliconius* butterflies (Pinheiro, 2003, 2011). Previous field studies have also demonstrated mimicry selection by releasing live butterflies and monitoring recapture rates (Benson, 1972; Mallet & Barton, 1989).

However, it is very difficult to observe predation in the wild, which means that there is limited knowledge of the actual communities of predators that attack butterflies and of

direct estimates of predation rates. The dynamics of mimicry evolution depend on unknown factors such as predator identity, avoidance rate learning, when and where predation take place, and predator resistance to toxicity. In **Chapter 2** and **Chapter 3** I investigate how predators perceive *Heliconius* butterflies mimicry and conspicuous coloration using bird vision models, and in **Chapter 4** I quantify the extent of avoidance of aposematic signals in wild regarding distastefulness and colour.

CHAPTER 2

HELICONIUS BUTTERFLY MIMETIC COLOURS: BETTER WITH UV VISION

ABSTRACT

Heliconius colour signals are under two conflicting selection pressures: choosing potential mates and defending against visual predators through warning coloration. As both selection pressures are likely to be strong, different elements of the signal might be adapted for different functions, and in particular colour differences between mimetic species that could be used in mate discrimination might be favoured. Here, I investigated how mimicry in four co-mimics pairs of *Heliconius* is perceived both from the perspective of butterflies and birds. First, the visual sensitivities of eight insectivorous avian predators were assessed through genetic analysis of their visual pigments. I then used digital image colour analysis, combined with bird and butterfly visual system models, to investigate how predators and conspecifics perceive mimetic patterns. I found that avian predators and conspecifics perceive coloration in *Heliconius* butterflies differently. Ultra-violet (UV) bird vision systems are able to discriminate between the yellow and white colours of co-mimics better than Violet bird vision. *Heliconius* vision showed differences between males and females in the ability to discriminate co-mimics. My work suggests that the presence of an extra UV opsin in females and lateral filtering pigments have an effect on the perception of some colours, such as the yellow band in *H. erato lativitta / H. melpomene malleti* co-mimic

and the red ventral in *H. erato demophoon / H. melpomene rosina* co-mimic. In contrast, variation in the red patches is largely a result of aging. A behavioural experiment showed that UV cues are used in mating behaviour; removal of such cues was associated with males showing an increasing tendency to approach co-mimics instead of their conspecifics. These traits could facilitate communication between co-mimics and reduce the cost of confusion in courtship while still maintaining the advantages of mimicry against predation.

INTRODUCTION

Natural selection has tuned sensory systems to detect specific and biologically relevant signals (Stevens, 2013; Cronin *et al.*, 2014). Many signals reflect a balance between the strength of sexual selection and the pressure of predation. Arguably the most widely studied sensory modality is vision, and colour signals provide clear examples of signals that are influenced by both mate choice and predation, for example in guppies (Endler, 1980). Where predation is a relatively stronger selective force than sexual selection, colouration will be more conspicuous for aposematic species or more cryptic for camouflaged species. If predation is relatively weaker, colour patterns will be closer to the optimum for mate choice (Endler, 1978, 1992).

Similarly, multiple selective factors affect the evolution of butterfly wing coloration, notably among defended mimics that are communicating both with their own species and predators. *Heliconius* butterflies exhibit Müllerian mimicry (Müller, 1879), in which two or more defended species share a mutual selective benefit from shared colour patterns (Brown, 1981; Mallet, 1999). These mimetic butterflies exhibit conspicuous colour as a warning that they are toxic and should be avoided and benefit one another by protecting themselves from

visual predators (Benson, 1972). Predators in turn learn to avoid these unpalatable butterflies (Chai, 1986; Pinheiro, 2003; Langham, 2004), which are chemically defended by cyanogenic glycosides (Engler-Chaouat & Gilbert, 2007). The coloration and patterns exhibited by different *Heliconius* species vary geographically, but within a given geographical area, two or more sympatric species commonly mimic each other (Brown, 1981; Mallet & Gilbert, 1995; Jiggins, 2008).

Heliconius butterflies find and choose potential mates based on colour signals (Jiggins *et al.*, 2001; Sweeney *et al.*, 2003; Kronforst *et al.*, 2006). It has been shown that closely related mimics often demonstrate signal confusion during courtship due to their similar appearances (Jiggins *et al.*, 2001; Estrada & Jiggins, 2008). *Heliconius erato* males use wing colour pattern in mate recognition, and are more likely to approach and court with models of their own coloration (Estrada & Jiggins, 2008). *Heliconius* butterflies are therefore a useful system for investigating the two conflicting selection pressures of predation and mate preference.

Heliconius butterflies might exploit hidden channels of communication in which signals are not detected by predators. Since the discovery of an additional UV opsin in *Heliconius* vision, it has been suggested that UV-based signals could facilitate species-specific recognition while not compromising Müllerian mimicry. In *H. erato*, the two UV opsins confer sensitivity from ~355 nm (UVRh1) to ~398 nm (UVRh2), and it has been suggested that this might allow a greater degree of discrimination of UV-reflecting yellow wing patches (Briscoe *et al.*, 2010; Bybee *et al.*, 2012). The compound eye of *H. erato* is sexually dimorphic and males express only UVRh2 while females express both UV opsins in separate photoreceptors (McCulloch *et al.*, 2016). Differences in photoreceptor ratios

therefore might play a role in sexual selection and identification of mates. Moreover, the use of *3-hydroxykynurenine* as a yellow wing pigment and the additional UV opsin arose at the origin of the genus *Heliconius*, giving species yellow wing patches with UV reflectance. Yellow colours within *Heliconius* are generally more distinct than yellow colours within non-*Heliconius* species (Briscoe *et al.*, 2010). This has led to the suggestion that the visual system and the wing colouration have co-evolved, and might offer a unique pathway of communication.

Furthermore, recent work has provided new information on red filtering pigments and their distribution in the eyes. The presence of red lateral filtering pigments shifts red cell sensitivity from 560nm to 600nm and allows *H. erato* to distinguish colours in the red-green spectrum even though they only have a single LW-sensitive opsin (Zaccardi *et al.*, 2006; McCulloch *et al.*, 2016). *Heliconius* mating preference is highly linked to colour and in *H. melpomene*, the gene responsible for red colour pattern is genetically linked to the preference for the same pattern (Jiggins *et al.*, 2001; Naisbit *et al.*, 2001; Merrill *et al.*, 2011b). Most studies of colouration in *Heliconius* butterflies focus on the genetic basis for variation in colour (Reed *et al.*, 2008, 2011; Pardo-Diaz *et al.*, 2012). However, there is also phenotypic variation, most notably due to age, with a change in colour from bright red to dark red, orange or brown as individuals age due to oxidation of the red dihydroxanthommatin pigment (Crane, 1954; Ehrlich & Gilbert, 1973; Gilbert *et al.*, 1988). This is likely due to a combination of sun exposure and pigment oxidation through time.

Previous research on the colour patterns of two polymorphic Müllerian mimic butterflies, *Heliconius numata* and *Melinaea*, has revealed that co-mimetic species have highly similar wing colour patterns (Llaurens *et al.*, 2014). The contrasts were computed

between yellow/orange against the black of the wing, and small differences in contrast between co-mimics can be perceived better by butterflies than birds (Llaurens *et al.*, 2014). Another co-mimic pair analysed was *Heliconius sara* and *Mimoides pausanias*, and although they have wing colour differences, they look more similar under avian violet vision (Thurman & Seymoure, 2016). A further study on Batesian mimics found differences between the sexes and wing surfaces, with females being better mimics and the dorsal side having better resemblance to mimic models (Su *et al.*, 2015). However, the more common challenge faced by many *Heliconius* is not to distinguish between other genera of butterflies, but rather to distinguish between sympatric co-mimics within *Heliconius*. The use of UV signals in signalling between different species has not yet specifically been addressed. The precise Müllerian mimicry among Neotropical *Heliconius* shows that wing pattern evolution within the genus is under strong selection for mimicry, making wing patterns almost identical where they co-occur. Here I analyse the colours of these co-mimics from the perspective of both bird and butterfly vision.

I tested whether *Heliconius* are able to distinguish between their co-mimics using UV cues in mate choice. Speciation of *Heliconius* butterflies has been facilitated by colour-based assortative mating (Jiggins *et al.*, 2001), and co-mimics that do not differ in colour pattern can be confused and court the wrong species (Estrada & Jiggins, 2008). The role of colour in mate choice in butterflies has been frequently investigated, although few studies have evaluated preferences for variation in UV reflectance. For example, UV brightness is a strong component of male attractiveness in both *Colias* and *Eurema* butterflies (Silberglied & Taylor Jr., 1973; Rutowski *et al.*, 2007; Kemp, 2008) while *Polymmatus icarus* males prefer UV-absorbing females (Knüttel & Fiedler, 2001).

In order to understand the trade-off between courtship and predation, we need to consider the appearance of butterflies from both a bird and butterfly visual perspective. Bird predators see in the UV range, and diurnal birds, which likely have excellent colour vision, fall into two different classes of colour vision: a violet sensitive (VS) and an ultraviolet sensitive (UVS) group (Bennett & Cuthill, 1994; Ödeen & Håstad, 2013). Recent research has highlighted the use of ultraviolet colour vision of birds for mate choice and foraging (Church *et al.*, 1998; Maddocks *et al.*, 2001; Kelber & Osorio, 2010). It is already known that avian predators are capable of learning and can select against unfamiliar *Heliconius* coloration in the field (Pinheiro, 2003; Langham, 2004). Jacamars (Galbulidae) and flycatchers (Tyrannidae) are suggested as important predators and agents of selection in butterfly mimicry system (Chai, 1986; Pinheiro, 1996).

Nonetheless, there are no data on the visual ability of these tropical species. Furthermore, avian visual systems are likely to be more complex, with less phylogenetic conservation than has previously been thought (Ödeen & Håstad, 2003, 2013). Hence, it is important to understand the visual system of the natural predators of tropical butterflies. Although all diurnal birds are though to be sensitive to UV light to some degree, small differences between VS and UVS systems can produce a large variation in the perception of colours in this part of the spectrum (Ödeen *et al.*, 2012). Here I aim to also better understand the visual systems of tropical birds that are potential *Heliconius* predators.

In this study, the aim was to examine the colouration of *Heliconius* co-mimic pairs and investigate visual signalling relevant to mimicry both from the perspective of butterflies and birds. Here I aimed to: (1) investigate the visual pigments of potential avian predators determined from amino acid sequences; (2) analyze differences in colour between four co-

mimic pairs to estimate the capacity of *Heliconius* butterflies and birds to effectively perceive the differences within and between mimetic species, using digital photography; (3) investigate the influence of butterfly age on red colour; (4) use behavioural tests to explore whether UV reflectance might be important for recognition of conspecifics. These data are used to test the hypothesis of cryptic channels of communication between butterflies, which would reduce the cost of confusion in courtship while still maintain the advantages of Müllerian mimicry against predation.

MATERIAL AND METHODS

Avian predator vision

Eight species of birds were selected to ascertain the visual system of potential *Heliconius* predators: white-whiskered puffbird (*Malacoptila panamensis*), blue-crowned motmot (*Momotus momota*), rufous-tailed jacamar (*Galbula ruficauda*), black-tailed trogon (*Trogon melanurus*), slaty antshrike (*Thamnophilus atrinucha*), great kiskadee (*Pitangus sulphuratus*), ochre-bellied flycatcher (*Mionectes oleaginous*) and Panama flycatcher (*Myiarchus panamensis*). Although not all of these species are known to feed on butterflies, all occur near my study site in Panama, are mainly insectivorous, and most show the 'sit-and-wait' foraging behaviour of capturing insects during flight.

The samples used were archived in the Smithsonian Tropical Research Institute Cryological Collection in Panama (Table S2.1 for biorepository ID). Total DNA was extracted from muscle tissue with the DNeasy Blood and Tissue Kit (QIAGEN) using standard procedures. The difference between two types of bird visual system is the sensitivity of their short-wavelength sensitive type 1 pigment (SWS1), which is shifted from

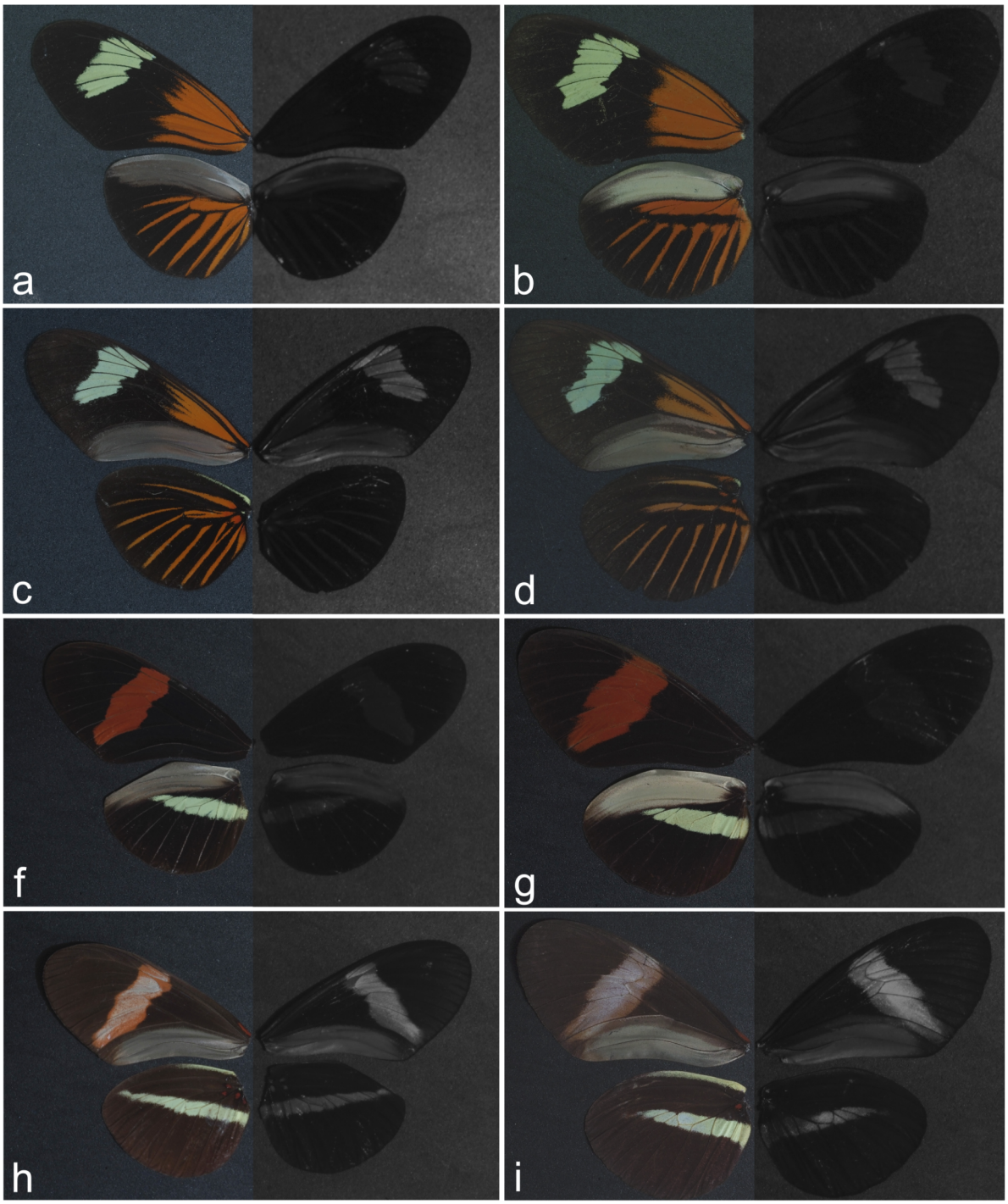
ultraviolet to violet by amino acid replacements at the sites 84-94 (Ödeen & Håstad, 2003). The primers used amplified a gene fragment coding the specific sites located in the SWS1 opsin (Ödeen & Håstad, 2003; Bloch, 2015).

A PCR was conducted on a G-Storm cycler (Somerton, UK). Each 20µl reaction volume contained 2 µl total DNA extracts, 1x BIOTaq DNA-polymerase (Bioline), 2 µl 10x NH_4 reaction buffer, 1 µl of each primer, 0.2 mM of each dNTP, 0.8 µl 50mM $MgCl_2$ and 0.6 µl DMSO. Reaction conditions were 120 s at 94ºC, 4 x (20 s at 94ºC, 20 s at 62ºC, 10 s at 72ºC), 6 x (20 s at 94ºC, 20 s at 60ºC, 11 s at 72ºC), 30 x (20 s at 94ºC, 20 s at 57ºC, 12 s at 72ºC) and 10 min at 72ºC. In case of amplification of multiple products, the product was purified from a 1.5% agarose gel using MinElute Gel Extraction Kit (QIAGEN). PCR products were cleaned using the ExoSAP-IT system (USB, Cleveland, Ohio) on 30 min at 37ºC and 15 min at 80ºC. PCR products were verified using Sanger sequencing. DNA sequences were translated into amino acids to identify the sites 86, 90 and 93, where the mutations of spectral tuning of each species are located (Wilkie *et al.*, 2000; Ödeen *et al.*, 2009).

Study species and image collection in dark room

Four pairs of *Heliconius* mimics that live in sympatry were selected for this study. The specimens were selected from the available collection of *Heliconius* butterflies wings in the Butterfly Genetics Group, Cambridge, UK. The co-mimic pairs were *H. erato lativitta* and *H. melpomene malleti* (*Hel/Hmm*; n = 14), *H. erato notabilis* and *H. melpomene plesseni* (*Hen/Hmp*, n = 10) collected in Ecuador, *H. erato demophoon* and *H. melpomene rosina* (*Hed/Hmr*, n = 10), *H. sapho* and *H. cydno* (*Hs/Hc*, n = 10) collected in Panama (Figure 2.1).

Colouration was investigated using digital photography, following the methodology described recently using the image analysis toolbox (Stevens *et al.*, 2007; Troscianko & Stevens, 2015). Dorsal and ventral wings of each specimen were photographed with a Fujifilm IS Pro UV-sensitive digital camera with a quartz CoastalOpt UV lens (Coastal Optical Systems), fitted with a UV/IR blocking filter (Baader UV/IR Cut filter; transmitting between 400nm and 700 nm) and a UV pass filter (Baader U filter; transmitting between 300 and 400 nm). The spectral sensitivity of the camera sensors was derived prior to photography (Stevens *et al.*, 2007; Troscianko & Stevens, 2015). Two photographs were taken in sequence, one in human-visible spectrum and other in UV spectrum with the respective filters (Figure 2.1). The photography setup used for the experiments consisted of a sheet of black ethylene-vinyl acetate (EVA) used as a low-UV reflective background, including a 40% grey standard (Spectralon® Labsphere) used for calibration. All the photographs were taken in constant light conditions, in a dark room with an UV/white light (300nm to 700nm), a tripod in a 90º in relation to the butterfly's wing surface and at the same distance. Photographs were at 90º for two reasons, first, wing pattern would not be distorted in the photos, and second, the angle that signal is observed varies in the environment and so I chose a standardised angle here.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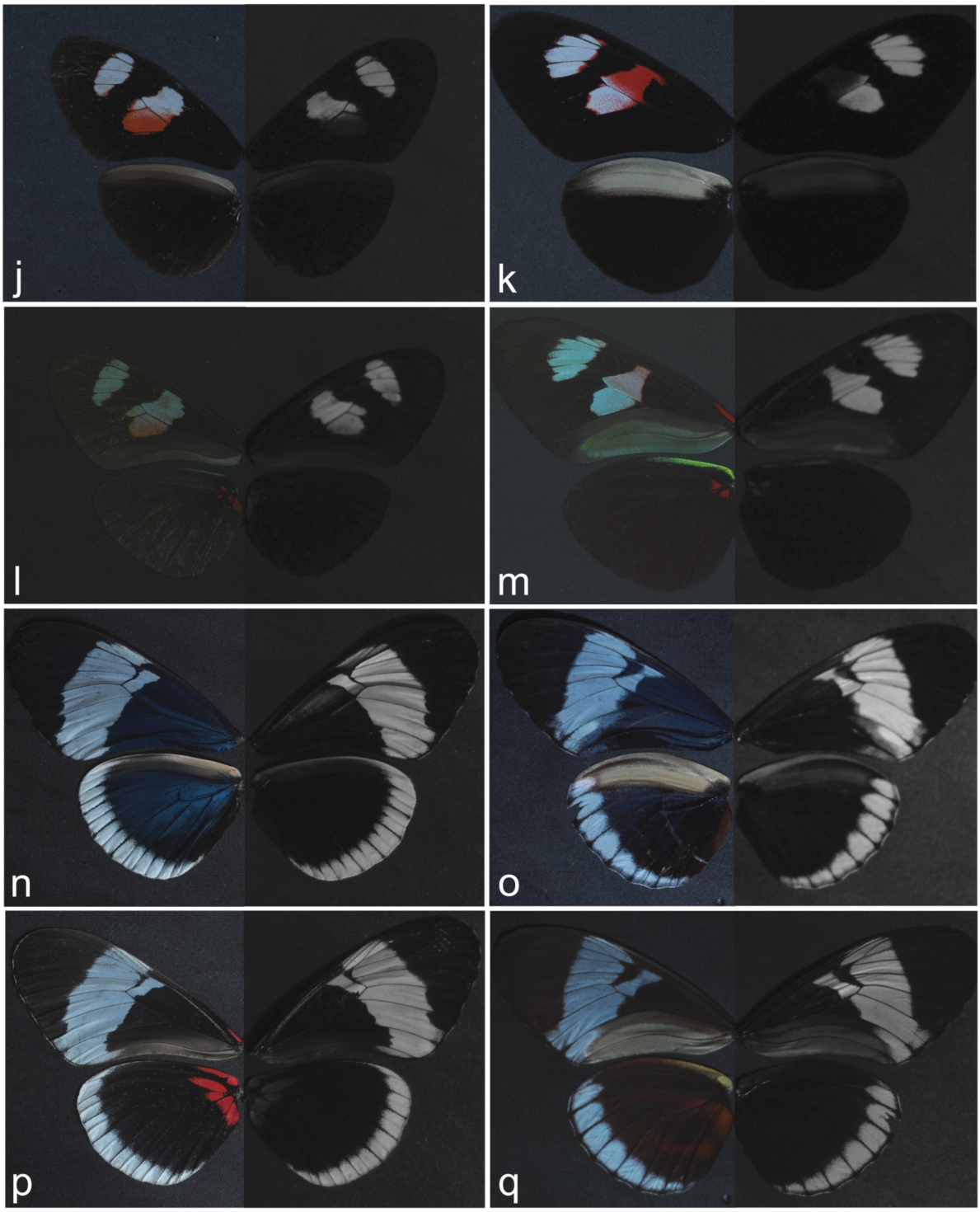
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Figure 2.1. Higher UV reflectance is perceived on the ventral side of the yellow and red bands. Photographed species; left, RGB photo; right, UV photo: *Heliconius erato lativitta*, (a) dorsal, (c) ventral; *H. melpomene malleti*, (b) dorsal, (d) ventral; *H. e. demophoon*, (f) dorsal, (h) ventral; *H. m. rosina*, (g) dorsal, (i) ventral; *H. e. notabilis*, (j) dorsal, (l) ventral; *H. m. plesseni*, (k) dorsal, (m) ventral; *H. sapho*, (n) dorsal, (p) ventral; *H. cydno*, (o) dorsal, (q) ventral. All photographs were taken at the same distance.

Image processing and analyses

The images were processed using a toolbox in the imaging software ImageJ (Rasband, 1997), in which each photograph was linearized and normalised with regards to a grey standard (Stevens *et al.*, 2007; Troscianko & Stevens, 2015). Image data was mapped to the visual sensitivity of the relevant visual system using an image calibration and analysis toolbox, based on mathematically mapping from camera sensitivity to animal sensitivity (Stevens *et al.*, 2007; Pike, 2011; Troscianko & Stevens, 2015). Predicted photon catch values were obtained for each colour, using the entire patch, applying spectral sensibility of each cone type of the blue tit (*Cyanistes caeruleus*) for the UV-sensitive vision (Hart *et al.*, 2000), peafowl (*Pavo cristatus*) for the Violet-sensitive vision (Hart, 2002), and *Heliconius erato* (Briscoe *et al.*, 2010; McCulloch *et al.*, 2016).

The colour patches chosen were orange and yellow for *Hel*/*Hmm*, red and yellow for *Hed*/*Hmr*, red and white for *Hen*/*Hmp* and white for *Hs*/*Hc* (red ventral, due to its considerable differences in colour and shape, and iridescent blue pattern, due to its polarized light reflection not captured by the camera, were not used, Figure 2.1). Black areas of the wings were not analysed because values for these regions were consistently very low and uninformative for *Heliconius* races. In order to determine how well *Heliconius* co-mimics colours are matched, chromatic and achromatic contrasts were quantified according to the receptor noise model (Vorobyev & Osorio, 1998). Achromatic contrast was calculated using

bird double cones sensitivity. To account for receptor noise, I used a Weber fraction value of 0.05 for the most frequent cone type, as has been used in other models of bird and butterfly colour vision (Vorobyev & Osorio, 1998; Briscoe *et al.*, 2010). Relative proportions of cone types were used to calculate chromatic contrast for the blue tit: LW = 1, MW = 0.99, SW = 0.71, UV = 0.37 (Hart *et al.*, 2000), for peafowl: LW = 0.95, MW = 1, SW = 0.86, V = 0.45 (Hart, 2002), and for *H. erato*: females, LW = 1, B = 0.17, UV2 = 0.076, UV1 = 0.086 and males, LW = 1, B = 0.2, UV2 = 0.13 (McCulloch *et al.*, 2016). In *Heliconius*, it is not clear how the presence of filtering pigments might influence colour perception, I therefore used both possible wavelength sensitivities of the LW photoreceptors separately; Red-LW (λ_{max} = 600nm) and Green-LW (λ_{max} = 555nm) (Zaccardi *et al.*, 2006; McCulloch *et al.*, 2016).

The degree of discriminability between two colours is expressed in 'just-noticeable-differences' (JND), based on a model of colour distance which predicts that colour contrasts result from a set of opponent colour channels (Vorobyev & Osorio, 1998). Normally, JND of less than 3.00 should be difficult to discriminate in natural light conditions, and larger values allow increasingly easy discrimination (Siddiqi *et al.*, 2004; Olsson *et al.*, 2015). JND values were calculated for all pairwise within co-mimics and within conspecifics, separated by colour and side of the wing, for *Heliconius*, UVS and VS vision models. For *Heliconius* vision, JND within conspecifics were calculated using *erato* clade species, as *H. melpomene* has a different retina (Adriana Briscoe, personal communication).

Change in red colour through aging

My preliminary analysis showed considerable within-species variation in red colours for all studied vision systems. To investigate this further, since there is a strong male preference for

red patterns (Merrill *et al.*, 2011b), I used the co-mimic pair *Hed*/*Hmr* which contain the red forewing band. The samples were assorted using subjective age/colour categories based on previous work in *H. ethilla* (Ehrlich & Gilbert, 1973), whereby categories were based on how colours appear to human vision: pink (fresh wings), red (intermediate) and faded/dark red (worn). Also, a different set of 55 *H. m. rosina* butterflies raised in insectaries (from Owen McMillan's collection in Gamboa, Panama) of known age (in days) were used to quantify changes in the dorsal forewing red band and were also assorted in these categories.

For this different *H. m. rosina* set, another methodology was used. Images were captured using an Olympus OM-D EM-1 digital camera with an Olympus Zuiko Digital ED 60 mm f/2.8 macro lens (Olympus, Inc.). Forewing specimens were photographed against a Kodak R-27 Gray card, with Munsell 18% reflectance (Eastman Kodak, Inc). Camera RAW images were converted to Adobe DNG format using the Adobe DNG Converter (Adobe, Inc.), and white balanced and colour corrected (Akkaynak *et al.*, 2014) using an Xrite Color Checker (Xrite, Inc). For illumination, a Bolt VM-110 LED macro ring light was used (Gradus Group, LLC). Image manipulations were done using custom scripts written in MATLAB (Mathworks, Inc) in wide gamut Kodak ProPhoto RGB colour space. Following colour calibration, RGB images were projected to L*a*b* colour space (Wyszecki & Stiles, 1982) and in this colour space, higher the a* value, the "redder" an image appears. I used this methodology instead of the previous one in order to estimate human vision perception of age of the wing, and be able to assign age based on the redness of the forewing red band.

UV mating experiment

To investigate whether the UV reflectance of natural butterflies affects mate preference, a mate choice test was carried out under natural daylight conditions. Adult males of *H. erato demophoon* were collected around Soberanía National Park, Panama, and kept in insectaries facilities in Gamboa, Panama. Although males express only one UV opsin compared to females, male mate preferences were used in this study following previous work on courtship in these species.

Butterfly wing models were made with wings dissected from *H. erato demophoon* and *H. melpomene rosina* female bodies and glued to adhesive black tape. The adhesive tape kept the wings together in an open wing position but also allowed movement of the model in a simulated flight, following methodology of earlier studies (Jiggins *et al.*, 2004; Estrada & Jiggins, 2008). To block the UV, a sunscreen (Soltan© Invisible SPF30) cover was spread over the coloured region of one pair, covering the red and yellow bands on both sides (UV-). By covering the wing colour bands using transparent sunscreen, UV reflectance was removed without changing the colour (Heiling *et al.*, 2005), confirmed with a UV photograph using the methodology described above (Figure S2.1). Sunscreen was also spread on the black part of the wings of the other pair, not covering any colour, in order to control for smell (UV+).

I used 41 males to test their response to models with UV blocked of the two different species inside a cage (2 x 1 x 2 m). Prior to experimental use, males were acclimated to the cage environment for at least 24 hours. Each male were tested twice and always offered the choice of two females: *H. erato* UV+ versus *H. melpomene* UV+, or *H. erato* UV- versus *H. melpomene* UV-. The models were placed 1 m apart, fixed on the ends of zip-ties attached to

a PVC pipe suspended between two metal bars. The PVC pipe was manipulated so that the models simulate butterfly flight (Jiggins *et al.*, 2001; Finkbeiner *et al.*, 2014).

Each pair of models was presented for 30 min to a single male, starting at the first sign of activity by the male. When a male flew towards the model to within a distance of 15 cm, the behaviour was recorded as 'approach', and when a male came flying close to the model in a hovering or circling behaviour, the behaviour was recorded as 'courtship' (Jiggins *et al.*, 2001, 2004). Two replicates 30 min observation periods were carried out for each comparison and replicates were combined for analysis.

Statistical analyses

First, I used the average of the pairwise JND values of each individual, between its co-mimics and between its conspecifics, which were grouped by co-mimic species, colour, side of the wing and visual system. Then, to test whether differences between co-mimics were significantly higher than between conspecifics, I compared JNDs between co-mimics against JNDs between conspecifics using analysis of variance (one-way ANOVA). Normality tests showed that JND data were not normally distributed; therefore the data was transformed to normality using square-root transformation for statistical analyses. Raw JND data was plotted to illustrate the results.

To determine whether the high red variability in the samples was due to differences in age, I performed a MANOVA using the photon catches values of the three visual systems against age/colour categories on the comimic pair *Hed*/*Hmr*. Then, to assure whether red categories were actually representing age, a linear regression was performed correlating age (days after the emergence) with red measurement (a*) using the *H. m. rosina* data set.

To evaluate mate choice experiments, a weighted binomial GLM was used to compare *H. erato* male proportion of successes of 'approach' and 'courtship attempts' towards its co-mimic *H. melpomene* female and to evaluate interaction between treatments, where the weight was the number of total successes and fails of each individual. All statistical calculations were processed in the software R 3.2.1 (R Core Team, 2015).

RESULTS

Predator vision sensitivity

I amplified the SWS1 fragment sequence from all eight species (Table 2.1). I could confirm some of the sequences with previous studies that used same species, genus or family (Ödeen & Håstad, 2003, 2013). I discovered that half of possible butterfly predators chosen for this study have mutations that designate a UVS opsin, and the other half have a VS opsin (Table 2.1).

Table 2.1. Type of vision in examined bird species. SWS1 amino acid sequences for the eight potential avian predators, showing sites from 84 to 94. In bold, sites 86, 90 and 93 are shown as sites where mutations are responsible for spectral tuning according to Wilkie *et al.* (2000).

				aa seq 84-94											
Order	Family	Species	Common name			**86**				**90**			**93**		Type
Trogoniformes	Trogonidae	*Trogon melanurus*	Black-tailed Trogon	F	I	**F**	C	V	F	**S**	V	F	**T**	V	UVS
Coraciiformes	Momotidae	*Momotus momota*	Blue-crowed Motmot	F	I	**F**	C	S	F	**S**	V	F	**T**	V	UVS
Piciformes	Bucconidae	*Malacoptila panamensis*	White-whiskered Puffbird	F	I	**S**	C	I	F	**S**	V	F	**T**	V	VS
Piciformes	Galbulidae	*Galbula ruficauda*	Rufous-tailed Jacamar	L	M	**C**	C	I	F	**S**	V	F	**T**	V	VS
Passeriformes	Thamnophilidae	*Thamnophilus atrinucha*	Slaty Antshrike	F	M	**C**	C	I	F	**C**	I	F	**T**	V	UVS
Passeriformes	Tyrannidae	*Pitangus sulphuratus*	Great Kiskadee	F	M	**C**	C	I	F	**S**	V	F	**T**	V	VS
Passeriformes	Tyrannidae	*Mionectes oleagineus*	Ochre-bellied Flycatcher	F	M	**C**	C	I	F	**S**	V	F	**T**	V	VS
Passeriformes	Tyrannidae	*Myiarchus panamensis*	Panama Flycatcher	F	M	**C**	C	I	F	**S**	V	F	**T**	V	VS

Colour mimicry contrasts through avian vision

For both the *Hel/Hmm* and *Hen/Hmp* mimicry rings there were many JND values that were greater than the threshold of perception, especially for the UVS bird visual system (Figures 2.2a and 2.2c). Butterflies in these mimicry pairs were more similar to the VS visual system, where pairwise JNDs for white and yellow colours were close to the perception threshold. Nonetheless, in none of these comparisons there was any evidence for significantly greater JNDs in comparisons between co-mimics as compared to within conspecifics (Table S2.2). This indicates that, despite considerable individual level variation, there was no informative information between the co-mimics that could be used by predators to distinguish co-mimic pairs.

In contrast, for both the *Hed/Hmr* and *Hs/Hc* mimicry rings, there was evidence for significant differences between co-mimics that might be perceptible to predators (Table S2.2). This was the case for red, yellow and white patterns, especially with UVS visual systems (Figures 2.2b and 2.2d). Under the VS visual system, there were significant differences (Table S2.2) but in some cases these were not far above the perceptibility threshold and may not therefore have much relevance in the wild (Figure 2.2).

Achromatic contrast did not show significant difference between conspecifics and co-mimics in both vision systems, with the exception of the ventral red patch in *Hen/Hmp* (Table S2.3). All JNDs were above the threshold for great discrimination (Figure 2.3).

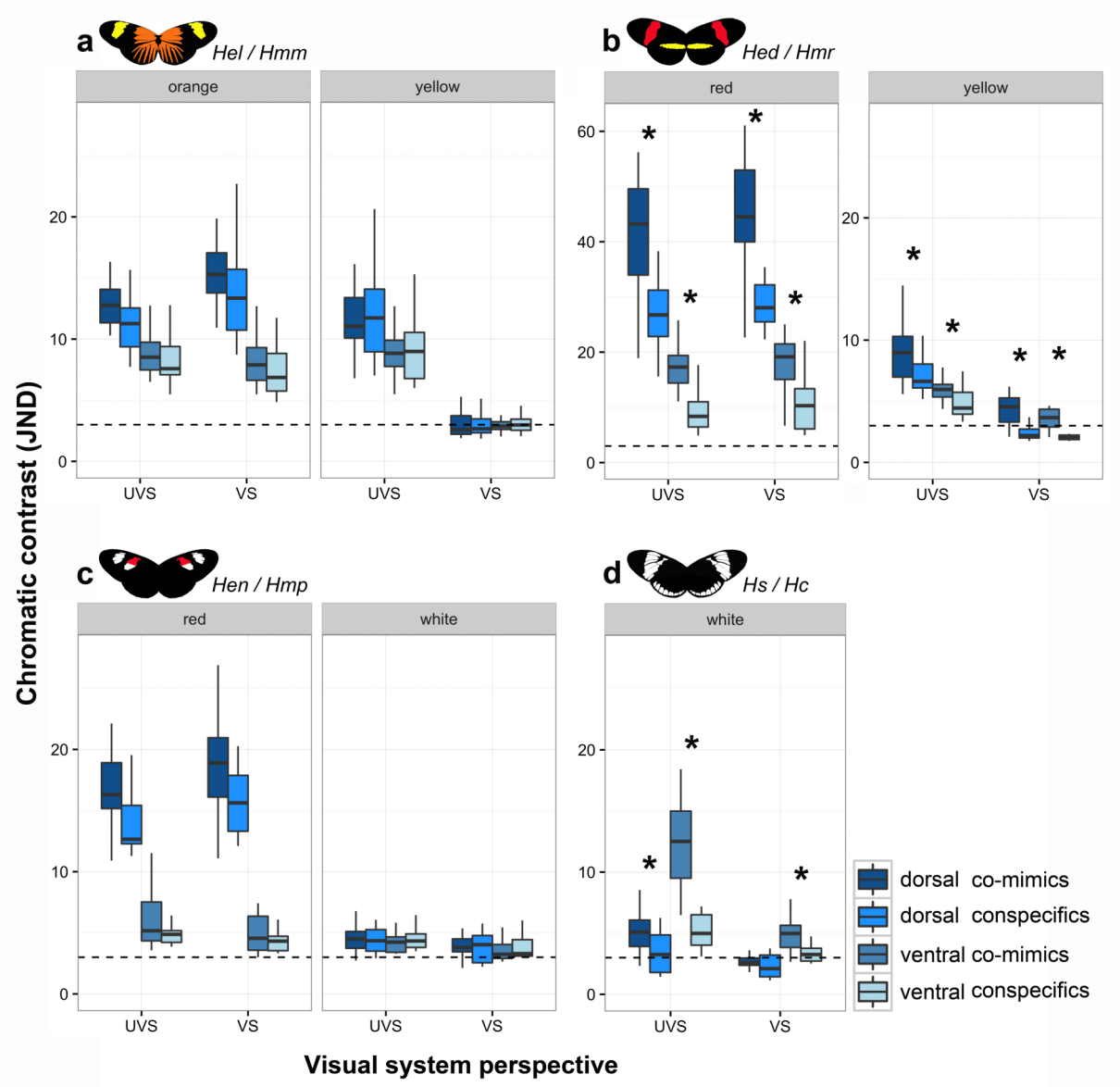

Figure 2.2. Chromatic comparison of colour patches between conspecific and co-mimic specimens. Butterfly pictures illustrate co-mimics' colours and patterns. Box plots show UVS and VS visual system JNDs between co-mimics and between conspecifics in each colour and wing side: (a) *H. erato lativitta* and *H. melpomene malleti* (*Hel/Hmm*); (b) *H. e. demophoon* and *H. m. rosina* (*Hed/Hmr*); (c) *H. e. notabilis* and *H. m. plesseni* (*Hen/Hmp*); (d) *H. sapho* and *H. cydno* (*Hs/Hc*). Values > 3 JND denote an increasing ability to discriminate colours, whereas values ≤ 3 JND are generally difficult to distinguish (dash line = 3). Box plots show median, upper and lower quartile, maximum and minimum. Asterisks (*) show co-mimics' JNDs that are statistically higher than conspecifics' JNDs ($P < 0.05$, Table S2.2).

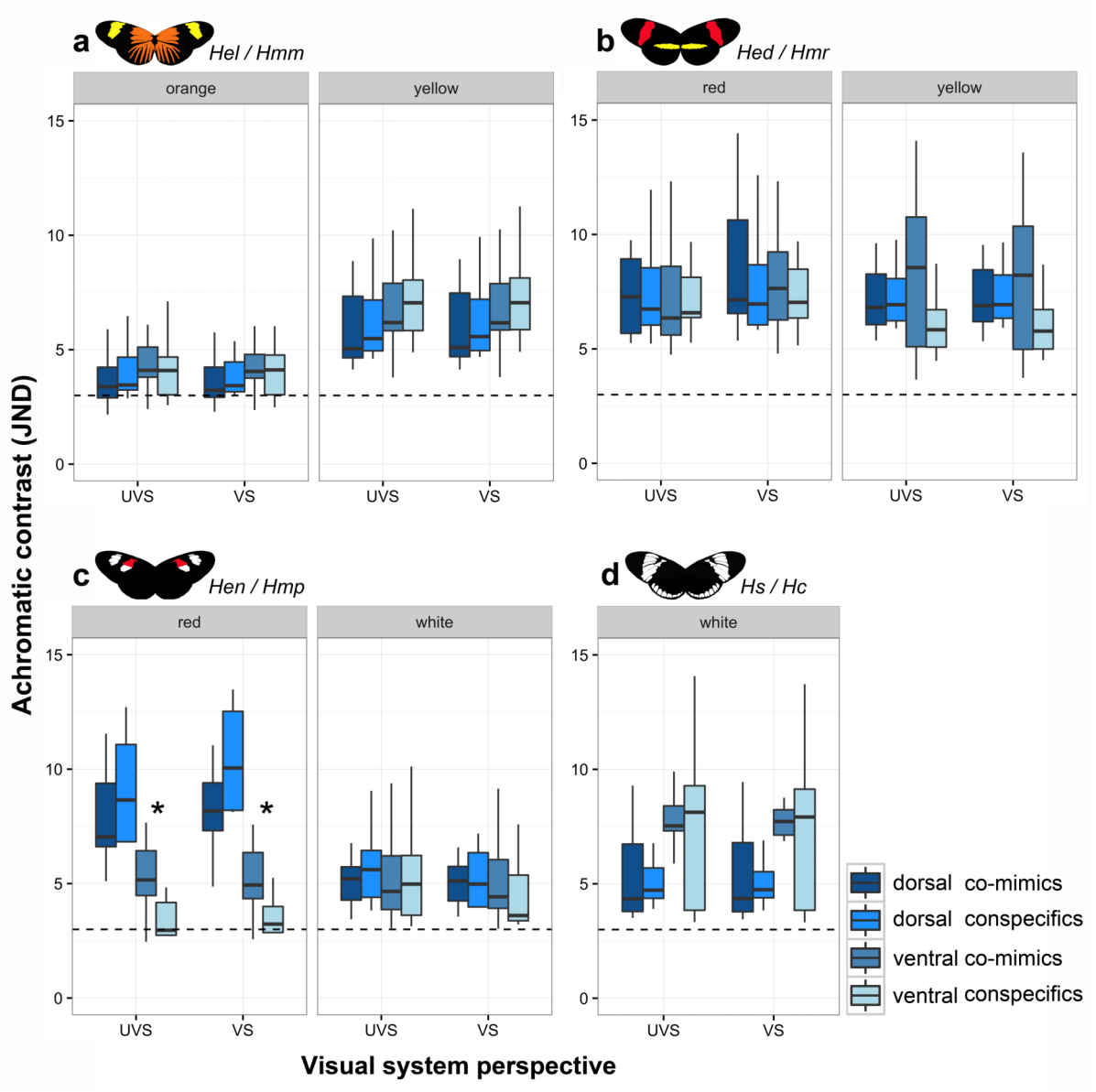

Figure 2.3. Achromatic comparison of colour patches between conspecific and co-mimic specimens. Butterfly pictures illustrate co-mimics' colours and patterns. Box plots show UVS and VS visual system JNDs between co-mimics and between conspecifics in each colour and wing side: (a) *H. erato lativitta* and *H. melpomene malleti* (*Hel/Hmm*); (b) *H. e. demophoon* and *H. m. rosina* (*Hed/Hmr*); (c) *H. e. notabilis* and *H. m. plesseni* (*Hen/Hmp*); (d) *H. sapho* and *H. cydno* (*Hs/Hc*). Values > 3 JND denote an increasing ability to discriminate colours, whereas values ≤ 3 JND are generally difficult to distinguish (dash line = 3). Box plots show median, upper and lower quartile, maximum and minimum. Asterisks (*) show co-mimics' JNDs that are statistically higher than conspecifics' JNDs ($P < 0.05$, Table S2.3).

Colour mimicry contrasts through Heliconius vision

Once again, similar to the bird vision models, both red and orange colours showed high JND values in the comparisons but these were mostly not significantly different between conspecifics and co-mimics. Only the red ventral pattern of the *Hed/Hmr* mimicry ring showed significant differences that might indicate a consistent difference between co-mimics (Table S2.4). It is worth noting that the Red-LW sensitivity increase co-mimics difference perception for red ventral and yellow dorsal for males in *Hed/Hmr* (Figure 2.4b).

In contrast, yellow and white colours commonly showed greater differences between co-mimics than conspecifics, most especially to the female visual system (Table S2.4). In particular the yellow band of the *Hel/Hmm* mimicry ring showed strong and significant differences in the female but not the male visual system (Figure 2.4a, Table S2.4). This perhaps indicates that females could distinguish the co-mimics based on this colour patch.

White colours in the *Hs/Hc* mimicry ring, similar to the pattern seen for the bird visual system, were significantly different between co-mimics and conspecifics for all comparisons (Table S2.4). However, the JND values were only above the perception threshold for the ventral side of the wing (Figure 2.4d), again suggesting ventral patterns may be adapted for signalling to potential mates.

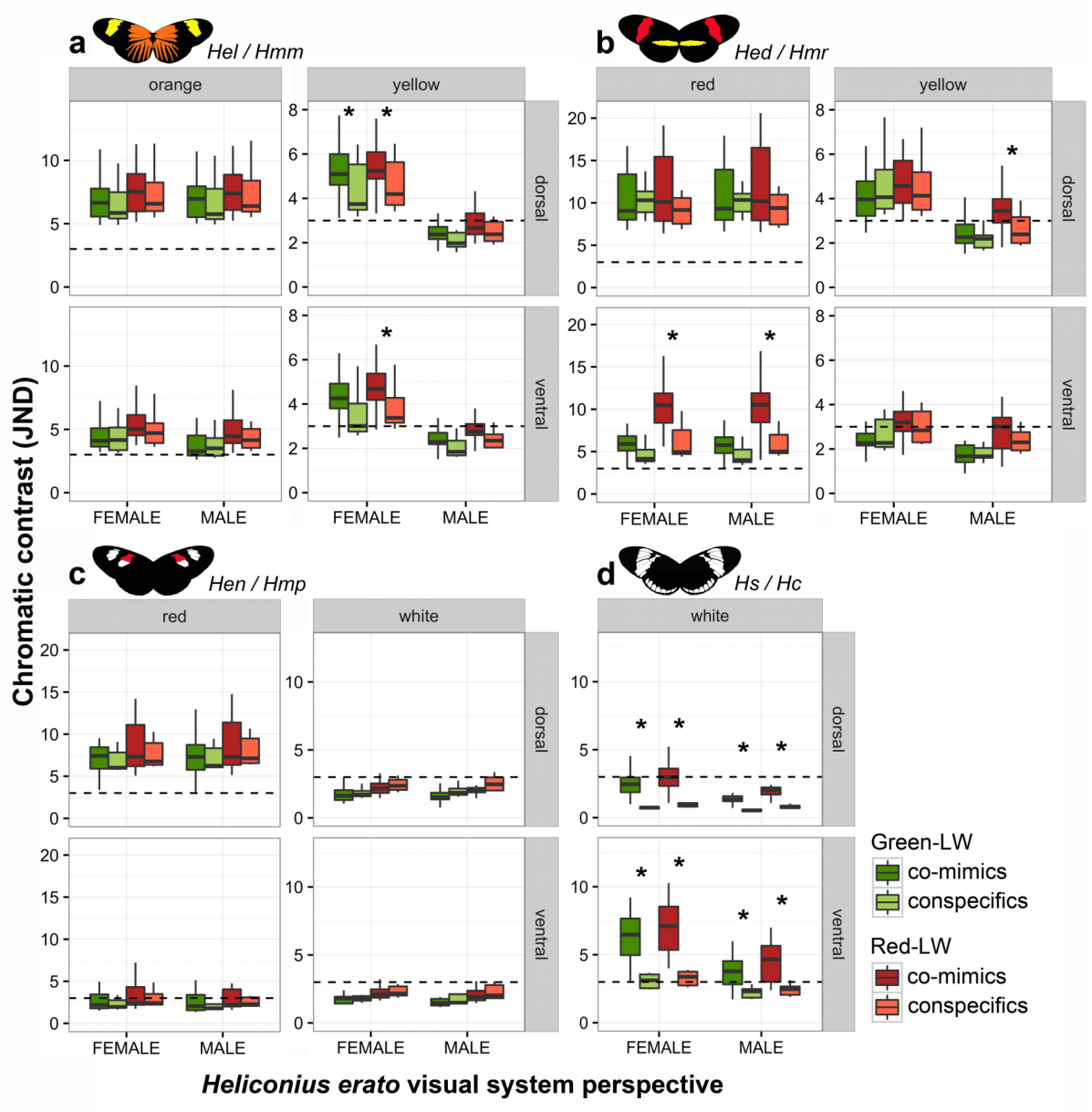

Figure 2.4. Chromatic comparison of colour patches between conspecific and co-mimic specimens. Butterfly pictures illustrate co-mimics' colours and patterns. Box plots show *Heliconius erato* female and male visual system JNDs, using Green-LW and Red-LW sensitivities, between co-mimics and between conspecifics in each colour and wing side: (a) *H. erato lativitta* and *H. melpomene malleti* (*Hel/Hmm*); (b) *H. e. demophoon* and *H. m. rosina* (*Hed/Hmr*); (c) *H. e. notabilis* and *H. m. plesseni* (*Hen/Hmp*); (d) *H. sapho* and *H. cydno* (*Hs/Hc*). Values > 3 JND denote an increasing ability to discriminate colours, whereas values ≤ 3 JND are generally difficult to distinguish (dash line = 3). Box plots show median, upper and lower quartile, maximum and minimum. Asterisks (*) show co-mimics' JNDs that are statistically higher than conspecifics' JNDs ($P < 0.05$, Table S2.4).

Effect of age on colouration

The largest JND values were consistently found between individuals in their red wing patches. Anecdotally it is known that there is considerable plasticity in the colour of red patches, especially as butterflies age. In order to test this, I compared the colour of both yellow and red patches from *Hed/Hmr* co-mimic pair. Using photon catch data, age was influencing the results for red differences in all visual systems (*Heliconius*: $F_{15,20} = 5.556$, $P = 0.005$; UVS: $F_{15,20} = 6.544$, $P = 0.002$; VS: $F_{15,20} = 8.004$, $P = 0.001$), but not for the yellow band (*Heliconius*: $F_{15,20}=2.748$, p=0.067; UVS: $F_{15,20}=1.912$, p=0.161; VS: $F_{15,20}=1.618$, p=0.221), demonstrating that age-related fading was a characteristic of the red pigment only.

Also, at the *H. m. rosina* data set, redness (*a value) and age were correlated ($t_{53,55} = -7.461$, $P < 0.001$, Figure 2.5), showing that the forewing dorsal red band changes colour with age (Figure 2.5). Although human visual categories do not fit especially well with this correlation, categories "faded red" included specimens that were all older than 25 days, and "pink" had specimens that were less than 10 days old (Figure 2.5).

UV light as a cue in species recognition

I next evaluated the role of UV in species recognition using mate choice experiments between co-mimics. The responses of males were recorded when presented with female wings from both species simultaneously with either UV signals present (UV+) or blocked (UV-). Overall, 709 approaches and 62 courtships were recorded. The *H. erato* males approach *H. melpomene* females more frequently than their conspecifics in the UV- treatment showing that the absence of UV in wing colouration led to maladaptive male

choice (z = 4.967, $P < 0.001$, Figure 2.6). There was no difference in courtship behaviour between species (z = 1.024, $P = 0.306$). Furthermore, there was a significant interaction between species and treatments for approach behaviour (z = -2.719, $P = 0.006$) but not for courtship attempts (z = -0.327, $P = 0.743$), indicating that disruption of UV influences mating signals. Although males might be expected to approach females of their own species more than co-mimics, I found that approach to the two species was close to random in the +UV treatment (Figure 2.6).

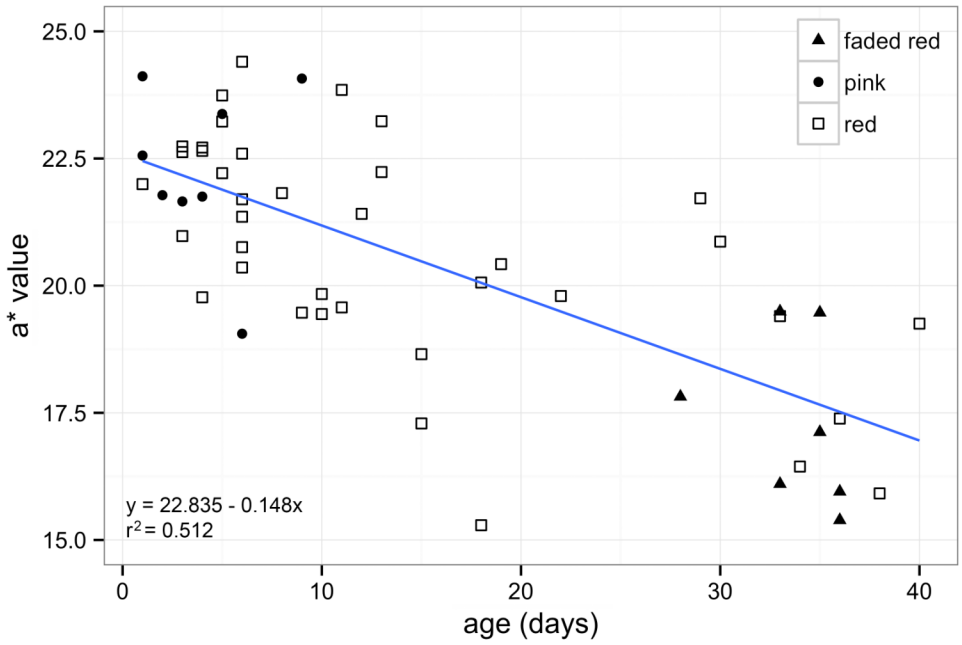

Figure 2.5. Forewing red band changes colour with age. Association between redness (a* value) and age in days after emergence in *Heliconius melpomene rosina* forewing dorsal red band. Human visual categories: pink (filled circles, n = 9), red (open squares, n = 39) and faded red (filled triangles, n = 7). Redness and age were correlated (solid line, y = 22.835 – 0.148x, $r^2 = 0.512$).

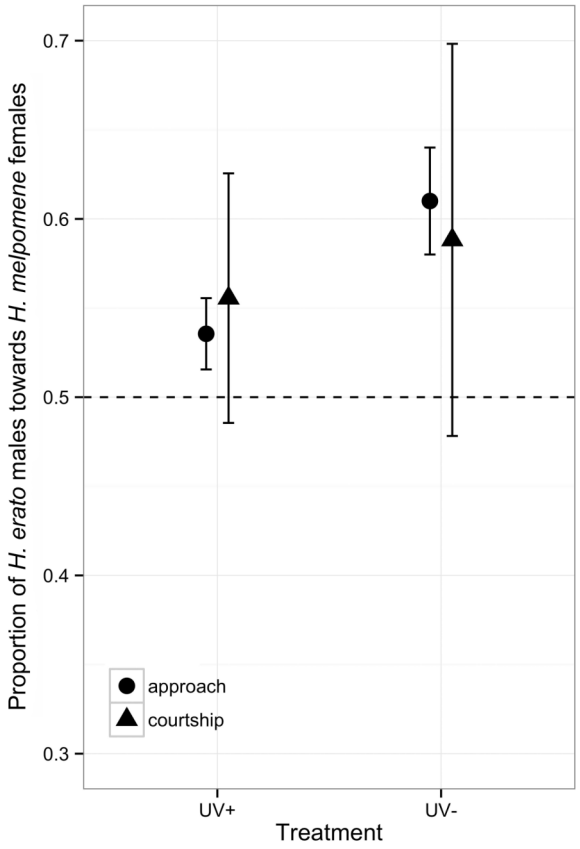

Figure 2.6. Males approach more frequently their co-mimic in the absence of UV. Proportion of *Heliconius erato* males to perform approach (circles) and courtship (triangles) behaviour towards their co-mimic female *H. melpomene* over their own species female in two treatments, UV+ and UV- (mean ± SE).

DISCUSSION

I have shown that both birds and butterflies can potentially see differences between some of the colours of mimetic *Heliconius*. This is perhaps not so surprising as birds and butterflies share considerable similarities in the adaptations of their visual systems; both groups use their colour vision to find food, such as flowers and fruits, and have evolved in similar light environments (Frentiu & Briscoe, 2008). Moreover, the wings of butterflies clearly evolve to send signals via the sensory systems of bird predators through of

aposematic patterns (Endler & Mappes, 2004). Nonetheless, despite the overall similarities it has been proposed that the gene duplication of the UV sensitive opsin in *Heliconius* might confer visual abilities that would permit differentiation of co-mimics specifically by butterflies (Briscoe *et al.*, 2010).

In order to consider the abilities of birds to distinguish co-mimics, it is essential to understand the visual system of relevant predators. However, remarkably little is known about the visual systems of tropical insectivorous birds that are likely to be *Heliconius* predators. From 21 avian orders studied, results of the SWS1 gene showed that avian colour vision shifted between VS and UVS at least 14 times showing that avian colour vision is not conserved between species (Ödeen & Håstad, 2013). My findings match previous SWS1 sequences of some groups, such as *Momotus momota* (Ödeen & Håstad, 2013), genus *Trogon* and *Myiarchus* and families Bucconidae and Tyrannidae (Ödeen & Håstad, 2003). The only exception is *Thamnophilus atrinucha*, the slaty antshrike, in which mutations confer UVS vision, different from other Thamnophilidae species which have the VS vision (Seddon *et al.*, 2010). I have shown that among eight species of birds found in the Canal Zone area of Panama, both UVS and VS visual systems are represented. Although I assume that these species are potential butterfly predators, the only species tested experimentally with *Heliconius* were Jacamars and Flycatchers (Chai, 1986; Pinheiro, 1996). The UVS/VS distinction in visual systems is likely to be only part of the variation in visual abilities among birds; my results nonetheless demonstrate that we need to take into account both visual systems in considering *Heliconius* mimicry.

In the light of this information on predator visual systems, there is some support for the hypothesis of a 'cryptic channel' of communication available to the butterflies.

Previously it has been shown that in *Heliconius numata* and its *Melinaea* comimics, colour contrasts for yellow patches were predicted to have lower error rates when seen by butterflies and UVS than by VS bird vision (Llaurens *et al.*, 2014). Similarly, the dorsal yellow colours on the *Hel/Hmm* pair are not distinguishable to either bird visual system, but my visual modelling suggests that female *H. erato* would be able to distinguish these patterns. The fact that the female visual system specifically is able to distinguish these patterns is also consistent with a role in sexual behaviour. This therefore supports the earlier suggestion that yellow colours might act as species-specific cues in *Heliconius* (Bybee *et al.*, 2012). However, this pattern is far from general, as the yellow band of the *Hed/Hmr* mimicry pair is perhaps more readily distinguishable by birds, in particular those with a UVS visual system, than it is for butterflies.

Similarly, in the case of red and white colours there is little evidence for a 'private channel' of communication. In both avian visual systems, *Hed/Hmr* red and yellow bands and *Hs/Hc* white co-mimics chromatic contrasts were significantly higher than those between conspecifics. This suggests that there is at least the potential for predators to perceive the differences between these species. However, given the precision of mimicry in *Heliconius* in other aspects such as wing pattern and flight, it seems likely that these colour differences are sufficiently similar that predators generalise between the co-mimics. Indeed, although differences between co-mimics were shown to be greater than those between conspecifics, in some cases the latter also showed quite high JND values, such that some degree of predator generalization is likely.

This work benefited from recent advances in our understanding of *Heliconius* vision, and in particular the discovery of sexual dimorphism in the visual system of *H. erato*

(McCulloch *et al.*, 2016). This dimorphism is likely to play a role in conspecific recognition. For example, in the *Hel/Hmm* mimicry pair, the yellow dorsal and ventral, JND values for comparisons between co-mimics were significantly higher than for conspecifics in the female vision model, but not in that for males. This perhaps suggests that the presence of an extra UV opsin in females might allow them to better distinguish conspecific mates (Bybee *et al.*, 2012; McCulloch *et al.*, 2016). Furthermore, the sensitivity shifted to red in the LW photoreceptor by the presence of filtering pigments clearly makes some colours to be more distinguishable than with the Green-LW sensitivity, for example *Hed/Hmr* red ventral. It has been suggested that differences in certain parts of the eye may be adaptations for specific visual tasks (Briscoe & Chittka, 2001), therefore I can propose that LW photoreceptors that contain red filtering pigments might be used for mate choice. Sexual dimorphism in photoreceptors and the presence of red filtering pigments in *H. erato* eyes help discrimination between co-mimics, possibly avoiding confusion between close mimetic colour patterns.

The differences between dorsal and ventral wings might suggest signal partitioning as has been demonstrated in other butterflies (Rutowski *et al.*, 2010). For example in *Bicyclus*, dorsal wing characters are involved in sexual signalling while the ventral wing with eyespots have a role in predator avoidance (Robertson & Monteiro, 2005; Oliver *et al.*, 2009; De Bona *et al.*, 2015). Also, blue *Morpho* butterflies show intense iridescent blue coloration on the dorsal side that is involved in males flight patrolling, whereas on the ventral side cryptic colour and big eyespots may have been selected against visual predators (DeVries *et al.*, 2010). In *Heliconius*, it seems likely that dorsal colours might have evolved through selection for aposematism as anti predator protection, while ventral surfaces are

selected for sexual signalling. It is notable that during courtship behaviour males show off their ventral side while hovering over the female, which may make it easier for females to recognize conspecific males (Klein & de Araújo, 2010).

I also provide behavioural support for a role of UV signals in sexual selection. My analysis shows in particular that ventral red regions of the *Hed/Hmr* mimicry pair are readily distinguishable by both males and females. I therefore tested the ability of *H. erato* males to distinguish conspecifics from co-mimics both with and without the availability of UV signals. The results show that removing UV reflectance has a strong influence on mate choice. It is rather surprising that *H. erato* males seems to prefer wings of *H. melpomene*, perhaps due to an absence of other pheromonal and behavioural cues in my experiments. However this work contributes to previous studies showing that UV light influences mating behaviour of other butterflies, such as Pieridae butterflies, which can visually discriminate between sexes using UV cues (Silberglied & Taylor Jr., 1973; Kemp, 2008), as well as *Bicyclus*, in which small UV-reflective spots played a role in female choice (Robertson & Monteiro, 2005). Such experiments are somewhat unnatural, as removing only the UV part of a visual signal likely results in a colour that looks odd or unnatural to conspecifics (Stevens & Cuthill, 2007).

One of the most variable colours in my analyses was red, which is partly related to age. The samples included individuals of all age categories, reflecting the natural age structure of wild populations resulting in high chromatic contrast results between the individuals for red colour (Ehrlich & Gilbert, 1973). Previous studies have taken advantage of this phenomenon to measure age structure in *Heliconius* using wing condition such as wear, dull colours and scale loss (Ehrlich & Gilbert, 1973; Walters *et al.*, 2012). Here I

quantified the fading of red pigment, showing a strong correlation between age and colour. In the future, calibrated photographs could be used to assign age based on the redness without relying on subjective human vision. Age fading has also been shown in *Colias eurytheme*, in which LW was the most accurate predictor of male age. Females of this species choose their partners based on age, since new males produce more nutritious spermatophores such that colour might be a useful cue for mate choice (Kemp, 2006). It is less clear whether there would be a similar benefit to such age discrimination in *Heliconius*. Females mate only once or a few times in their lifetime, depending on the species, and the first mating occurs soon after eclosion (Walters *et al.*, 2012). In contrast males can mate throughout their life and there is no evidence that spermatophore quality is influenced by male age.

In summary, it is clear that avian predators and conspecifics perceive coloration in *Heliconius* butterflies differently. In general, UVS birds can perceive differences between co-mimics and conspecifics better than VS birds, perhaps suggesting that *Heliconius* mimicry is primarily directed at VS predators. Furthermore there is evidence that sexually dimorphic vision in *H. erato* might confer an advantage to females in perceiving differences betweens co-mimics. Moreover, *Heliconius* could use UV signals for mate choice, indicating that conflicting forces of natural and sexual selection affect visual signals, both reducing cost of confusion in courtship and maintaining the advantages of Müllerian mimicry against predation. Apart from aposematic colouration, *Heliconius* butterflies have other adaptations that might also help to reduce risk of predation, such as levels of toxicity, anti-predator behaviour and chemical cues. Future work should consider looking at predation more closely and find how specific predators interact with these adaptations.

SUPPLEMENTARY INFORMATION

Table S2.1. Biorepository ID for bird samples used archived in the Smithsonian Tropical Research Institute Cryological Collection in Panama.

Species	Common name	Biorepository ID
Trogon melanurus	Black-tailed Trogon	BBT19878
Momotus momota	Blue-crowed Motmot	BBT18191
Malacoptila panamensis	White-whiskered Puffbird	BBT10765
Galbula ruficauda	Rufous-tailed Jacamar	KDKC0583
Thamnophilus atrinucha	Slaty Antshrike	BBT11166
Pitangus sulphuratus	Great Kiskadee	BBT17670
Mionectes oleagineus	Ochre-bellied Flycatcher	BBT10964
Myiarchus panamensis	Panama Flycatcher	BBT17572

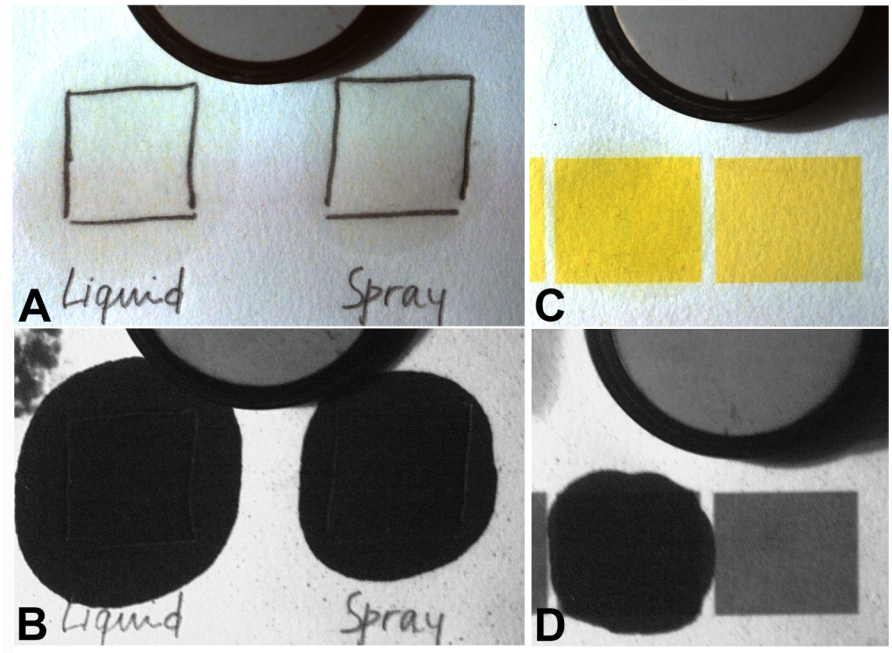

Figure S2.1. Sunscreen completely block UV light reflectance. UV block test photographs: left, testing liquid and spray sunscreens (**A**, RGB; **B**, UV); right, testing colour changes with the liquid sunscreen, which was applied only on the left yellow square, blocking the UV but not changing colour (**C**, RGB; **D**, UV).

Table S2.2. One-way ANOVA *F* and *P* values, and degrees of freedom (df) for chromatic JND comparisons between co-mimics and conspecifics for Figure 2.2. Results are shown for UVS and VS avian vision system in each co-mimic pair, colour patch and side of the wing. *Hel/Hmm*: *H. erato lativitta* and *H. melpomene malleti*. *Hed/Hmr*: *H. e. demophoon* and *H. m. rosina*. *Hen/Hmp*: *H. e. notabilis* and *H. m. plesseni*. *Hs/Hc*: *H. sapho* and *H. cydno*. Number in bold indicate $P < 0.05$.

Co-mimics	Colour	Wing side	Avian vision system					
			UVS			VS		
			F	P	df	F	P	df
Hel / Hmm								
	Yellow	dorsal	0.13	0.720	1	0.03	0.872	1
		ventral	0.09	0.771	1	0.06	0.802	1
	Orange	dorsal	3.57	0.064	1	1.90	0.174	1
		ventral	2.19	0.145	1	2.69	0.107	1
Hed / Hmr								
	Yellow	dorsal	6.44	**0.015**	1	32.43	**1.49E-06**	1
		ventral	7.61	**0.009**	1	29.77	**3.17E-06**	1
	Red	dorsal	24.29	**1.66E-05**	1	22.55	**2.90E-05**	1
		ventral	45.64	**5.27E-08**	1	18.74	**0.0001**	1
Hen / Hmp								
	White	dorsal	0.05	0.829	1	0.21	0.650	1
		ventral	0.72	0.403	1	0.55	0.464	1
	Red	dorsal	4.42	0.052	1	1.57	0.218	1
		ventral	3.07	0.088	1	1.83	0.185	1
Hs / Hc								
	White	dorsal	8.00	**0.007**	1	2.99	0.092	1
		ventral	73.86	**1.93E-10**	1	17.97	**0.0001**	1

Table S2.3. One-way ANOVA *F* and *P* values, and degrees of freedom (df) for achromatic JND comparisons between co-mimics and conspecifics for Figure 2.3. Results are shown for UVS and VS avian vision system in each co-mimic pair, colour patch and side of the wing. *Hel/Hmm*: *H. erato lativitta* and *H. melpomene malleti*. *Hed/Hmr*: *H. e. demophoon* and *H. m. rosina*. *Hen/Hmp*: *H. e. notabilis* and *H. m. plesseni*. *Hs/Hc*: *H. sapho* and *H. cydno*. Number in bold indicate $P < 0.05$.

Co-mimics	Colour	Wing side	Avian vision system					
			UVS			VS		
			F	P	df	F	P	df
Hel / Hmm								
	Yellow	dorsal	1.37	0.247	1	1.26	0.266	1
		ventral	1.07	0.305	1	1.08	0.302	1
	Orange	dorsal	2.17	0.147	1	1.64	0.206	1
		ventral	0.40	0.532	1	0.40	0.529	1
Hed / Hmr								
	Yellow	dorsal	0.01	0.939	1	0.01	0.929	1
		ventral	4.92	0.052	1	4.37	0.053	1
	Red	dorsal	1.12	0.298	1	0.08	0.781	1
		ventral	0.07	0.792	1	0.72	0.401	1
Hen / Hmp								
	White	dorsal	0.95	0.337	1	0.12	0.728	1
		ventral	0.10	0.756	1	0.68	0.415	1
	Red	dorsal	2.32	0.136	1	6.22	0.067	1
		ventral	9.25	**0.004**	1	8.08	**0.007**	1
Hs / Hc								
	White	dorsal	0.17	0.680	1	0.19	0.667	1
		ventral	0.65	0.425	1	0.79	0.379	1

Table S2.4. One-way ANOVA *F* and *P* values, and degrees of freedom (df) for chromatic JND comparisons between co-mimics and conspecifics for Figure 2.4. Results are shown for *Heliconius erato* female and male vision system in each co-mimic pair, colour patch, side of the wing and LW sensitivity. *Hel/Hmm*: *H. erato lativitta* and *H. melpomene malleti*. *Hed/Hmr*: *H. e. demophoon* and *H. m. rosina*. *Hen/Hmp*: *H. e. notabilis* and *H. m. plesseni*. *Hs/Hc*: *H. sapho* and *H. cydno*. Number in bold indicate $P \leq 0.05$.

Co-mimics	Colour	Wing side	LW	Vision system					
				Females			Males		
				F	P	df	F	P	df
Hel / Hmm									
	Yellow	dorsal	Green	5.42	**0.025**	1	3.15	0.084	1
			Red	4.64	**0.037**	1	1.28	0.264	1
		ventral	Green	3.92	0.055	1	2.36	0.133	1
			Red	4.38	**0.043**	1	2.84	0.100	1
	Orange	dorsal	Green	0.69	0.412	1	0.83	0.368	1
			Red	0.55	0.463	1	0.67	0.417	1
		ventral	Green	0.001	0.974	1	0.02	0.898	1
			Red	0.96	0.333	1	0.49	0.490	1
Hed / Hmr									
	Yellow	dorsal	Green	1.03	0.318	1	0.66	0.422	1
			Red	0.26	0.615	1	6.22	**0.019**	1
		ventral	Green	0.87	0.359	1	0.22	0.640	1
			Red	0.59	0.450	1	1.05	0.315	1
	Red	dorsal	Green	0.01	0.92	1	0.01	0.913	1
			Red	1.77	0.194	1	1.78	0.193	1
		ventral	Green	3.53	0.071	1	3.99	0.056	1
			Red	23.14	**4.65E-05**	1	17.93	**0.0002**	1
Hen / Hmp									
	White	dorsal	Green	0.46	0.503	1	3.62	0.068	1
			Red	0.92	0.345	1	3.15	0.087	1
		ventral	Green	0.40	0.534	1	1.03	0.320	1
			Red	0.38	0.541	1	1.01	0.323	1
	Red	dorsal	Green	0.002	0.966	1	0.06	0.816	1
			Red	0.03	0.859	1	0.00	0.968	1
		ventral	Green	1.02	0.321	1	0.64	0.429	1
			Red	0.51	0.481	1	0.30	0.587	1
Hs / Hc									
	White	dorsal	Green	39.87	**7.89E-07**	1	29.40	**8.76E-06**	1
			Red	32.01	**4.61E-06**	1	20.55	**9.93E-05**	1
		ventral	Green	28.76	**1.03E-05**	1	11.68	**0.002**	1
			Red	36.90	**1.50E-06**	1	20.28	**0.0001**	1

CHAPTER 3

THE CONSPICUOUSNESS OF *HELICONIUS* BUTTERFLIES ACROSS TIME AND HABITAT

ABSTRACT

Forests are a mosaic of light spectra, and colour signal efficiency and perception might change in different light environments. Local adaptation in *Heliconius* butterflies is linked to microhabitat use and the colourful wing colour patterns may also be adapted for signalling in different light environments. These butterflies exhibit conspicuous colours as a warning to predators that they are toxic and should be avoided, but also find and choose potential mates based on colour signals. The two conflicting selection pressures of predation and mate preference are therefore acting together. In this study I analyzed the contrast of two *Heliconius* mimicry rings in their natural habitats under varying degrees of forest fragmentation and light conditions. I used digital image analyses and mapped the images to bird and butterfly vision colour space in order to examine whether warning colours have greater contrast against green foliage and if they transmit a consistent signal across time of the day and habitat in a tropical forest. I tested conspicuousness of *Heliconius* colours using opponent colour channels against a natural green background. For avian vision, colours are generally very stable through time and habitat. While for butterfly vision, there is some evidence that species are more contrasting in their own habitats, where conspicuousness is higher for red and yellow bands in the border and for white in the forest. Light environment affects

Heliconius butterflies' warning signal transmission to a higher degree through their own vision, but to a lesser degree through avian predator vision. My work provides insight into the use of colour signals in sexual and natural selection in the light of ecological adaptation.

INTRODUCTION

The success of a signal is related to its effectiveness in a specific environment and how strongly it influences the behaviour of the receiver (Endler, 1978). Forests are a mosaic of light colours, and the same colour pattern can have an altered appearance in different light environments (Endler, 1993). If an individual shows high reflectance of a specific wavelength, but the environment lacks light in that part of the spectrum, the region of high reflection will be unimportant as a signal (Stevens *et al.*, 2007). Ambient light spectra vary not only over different environments but also from dawn to dusk, hence species that signal only at certain times and places are expected to evolve characteristics and predictable combinations of colours for particular environments (Endler, 1993). Therefore, ambient light characteristics should be included together with the receiver visual system to understand the microhabitat choice and behaviour of animals.

Signals depend on the habitat where animals live in, since light conditions can alter colour perception by filtering wavelengths and altering visual backgrounds (Endler, 1993; Lovell *et al.*, 2005). Sensory drive explains the process of adaptation of signalling and sensory systems to the local environment (Endler, 1992; Endler & Basolo, 1998). Environment tuned spectral sensitivity is better known in aquatic habitats, such as in guppies (Endler, 1980) and cichlid fish (Seehausen *et al.*, 2008), as compared to terrestrial light environments. On land, colour depends on the reflection of the surroundings and has greater

variability over time (Boughman, 2002). Habitat signal transmission can favour diversification of mating signals through local adaptation, leading to reproductive isolation. One example are *Anolis* lizards male dewlaps found in different microhabitats (Fleishman *et al.*, 1997). Male dewlap colours are more conspicuous in their own habitat than in other habitats, mainly because of the contrast against the background in the ultraviolet (UV) range (Leal & Fleishman, 2002). Perception of colours in different light conditions can also influence attacks by predators, for example among butterflies in an environment with high UV light, birds aimed at the butterfly wings, more specifically the marginal white eyespots that have UV reflectance, instead of the head (Olofsson *et al.*, 2010).

Local adaptation in *Heliconius* butterflies commonly involves adaptation to specific microhabitat use (Estrada & Jiggins, 2002; Elias *et al.*, 2008; Jiggins, 2008). Mimicry rings are groups of unpalatable species that share the same warning colour, and these tend to be found in different microhabitats such as forest or open areas. The *Heliconius* habitats are associated with the use of larval host-plants, adult food plants, sexual behaviour and gregarious roosting (Mallet & Gilbert, 1995). Species that lay eggs on *Passiflora* species that occur in second growth tend to be seen in open areas, while species that lay eggs on canopy *Passiflora* vines are seen flying more commonly in the forest. The choice of microhabitat also might be connected with light differences between those environments, such as the choice of using very shady areas in communal roosting (Mallet & Gilbert, 1995; Finkbeiner, 2014). In addition, different light environments should create microhabitats where butterfly signals would be more efficient. Although mimicry rings differ in their microhabitat, the light environment has not been measured to verify whether colour patterns could be specifically adapted to particular light environments.

The colourful wing colours of *Heliconius* butterflies may also be subject to evolution caused by sensory drive due to their potentially conflicting roles in predation and mate preference. Many species exhibit Müllerian mimicry (Müller, 1879), in which two or more species share the same conspicuous colour as a warning to predators that they are toxic and should be avoided (Benson, 1972). Also, these butterflies find and choose potential mates based on colour signals, which can lead to reproductive isolation (Jiggins *et al.*, 2001; Sweeney *et al.*, 2003; Kronforst *et al.*, 2006). Conversely, closely related mimics often demonstrate signal confusion during courtship due to their similar appearance (Jiggins *et al.*, 2001; Estrada & Jiggins, 2008). Furthermore, communication between conspecifics might be based on UV signals, since *H. erato* females express the duplicate UV opsin gene, which allows a greater degree of discrimination of the UV-yellow wing patches (Briscoe *et al.*, 2010; Bybee *et al.*, 2012; McCulloch *et al.*, 2016).

This microhabitat structuring allows mimicry rings to remain distinct. This may be because there are sets of predators in different habitats, each of which perceive a different mimicry ring as the most abundant pattern (Joron & Mallet, 1998). Although little is known of the specific predators that attack *Heliconius*, it seems likely that their aposematic signals are directed at several predators with different visual abilities and spectral sensitivities. Ambient light together with predator sensitivity can interfere with the interpretation of the information perceived from colour signals. Warning coloration should, therefore, be easy to detect and memorize even in heterogeneous environments and light conditions (Guilford & Dawkins, 1991; Endler, 1992). Warning signals are often dominated by red, yellow and orange, frequently contrasting with black, which are the main colours in *Heliconius*. The reason why these long-wavelength colours are widely represented in aposematic coloration

is that they are highly conspicuous against natural backgrounds, are more stable across light conditions, allowing long distance discrimination and detectability, are distinctive from profitable species and influence memorability (Guilford & Dawkins, 1991; Stevens & Ruxton, 2012; Arenas *et al.*, 2014).

Perception of colour depends on several neurophysiological mechanisms, such as the presence of opponent colour channels. This chromatic mechanism involves comparisons of receptors outputs, in which opposite neural pathways are either activated or inhibited depending on the stimuli reaching the eye (Kelber *et al.*, 2003; Renoult *et al.*, 2015). This mechanism is useful especially regarding colour stability against spatial and temporal variation in illumination (Lovell *et al.*, 2005; Renoult *et al.*, 2015). For example birds, the major predator of aposematic butterflies, have tetrachromatic vision and seemingly have at least three opponent channels, as found in domestic chicks (Vorobyev *et al.*, 1998; Osorio *et al.*, 1999b). Opponent channels have also been described for insects (Chittka *et al.*, 1992; Chittka, 1996) and butterflies (Kelber, 1999), and have been hypothesized for *Heliconius* butterflies (Swihart, 1971, 1972; Bybee *et al.*, 2012) although more behavioural analyses are needed to confirm which opponent channels are actually used. In female *Papilio aegeus* butterflies, host plant choice involves chromatic interactions of at least three photoreceptors, with high green receptor quantum catches against low red and blue receptor quantum catches (Kelber, 1999). Butterflies in the genus *Papilio* have duplicate LW opsin genes to see in the red and green range (Kelber, 1999; Briscoe, 2008), while *Heliconius* has only one LW opsin to see red and green, and differences in sensitivity are associated with the presence of red filtering pigments in the ommatidia (Zaccardi *et al.*, 2006; McCulloch *et al.*, 2016). Thus, I

expect avian predators and butterflies to rely on these high-contrast systems to process information under a changing light environment.

The aim of this study was to analyse *Heliconius* warning colouration under different light conditions in their natural habitats. In particular, to test conspicuousness as the distance between the colour patch of the wing and a natural green background, encoded by opponent colour channels. Using digital image analyses, I photographed butterfly wings and mapped the images to UVS and VS avian predator vision and to *Heliconius erato* vision. My predictions are that (1) signal contrast and conspicuousness for avian predators should have constancy, that should be stable throughout the day and in different light environments (Stevens & Ruxton, 2012; Arenas *et al.*, 2014). Warning signals might be honest indicators of prey unprofitability to predators, if signals fluctuate through the day and between light environments I would predict that this could delay learning by predators and be costly to the prey. Similarly for internal contrasts (i.e. contrast between black and the coloured bands), therefore conspicuousness would not rely totally on background contrast but also on internal patterns which account for close-distance conspicuousness (Endler, 1978; Aronsson & Gamberale-Stille, 2009). (2) From a *Heliconius* butterfly perspective, I predict that signal contrast and conspicuousness should show habitat-specific maximum background contrast and higher colour differences in their own habitats (Table 3.1), which would facilitate detection and species identification. However, since females mate readily during or soon after eclosion, there is not likely to be strong selection for signal constancy for mating purposes (McMillan *et al.*, 1997). I therefore predict that selection for signal constancy will be much stronger in the avian visual system as compared to the butterfly visual system.

Table 3.1. Colour patches for each co-mimic pair studied and typical microhabitats and light conditions where these co-mimics occur. Microhabitats descriptions are based on Estrada and Jiggins (2002) and light conditions based on Endler (1993) and personal observations.

Co-mimics	Colour	Microhabitat	Light conditions
H. erato demophoon & *H. melpomene rosina*	red & yellow	forest border open area	partial shade no shade
H. sapho & *H. cydno*	white	closed forest canopy	full shade partial shade

MATERIAL AND METHODS

Study site and species

Fieldwork was performed during the dry season, along Pipeline Road, a tropical lowland rainforest in the Panama Canal Zone (Parque Nacional Soberanía, 9º7'33"N, 79º42'90"W). Pipeline Road makes a transect through the forest, creating a heterogeneous habitat with open sunny areas and close canopy exceeding 10 m in height. All specimens were collected in the area. Two pairs of co-mimics that live in sympatry were selected, *H. erato demophoon* (n = 8) and *H. melpomene rosina* (n = 8), *H. sapho* (n = 5) and *H. cydno* (n = 5), belonging to two different mimicry rings, red and yellow, and white, respectively (Table 3.1).

Digital photography

The general approach and methodology for this work was based on previous work with colour stability using opponent signals (Lovell *et al.*, 2005; Arenas *et al.*, 2014). The spectral reflectance of mimetic pairs was investigated using digital photography. This provides a way to control for natural variation in luminance intensity (shadowing) that is not captured by spectrometry, and also allows non-invasive colour measurements easily applied in the field

(Stevens *et al.*, 2007; Troscianko & Stevens, 2015). Therefore, through this method I could obtain colour measures under the sensitivity of all receiver photoreceptors (300-750 nm) in the actual viewing conditions of conspecifics and avian predators.

Fresh wings of each specimen were photographed with a Fujifilm IS Pro UV-sensitive digital camera with a quartz UV lens (Coastal Optical Systems), fitted with a UV/IR blocking filter (Baader UV/IR Cut filter; transmitting between 400 nm and 700 nm) and a UV pass filter (Baader U filter; transmitting between 300 and 400 nm). Two photographs were taken in sequence, one in the human-visible spectrum and the other in the UV spectrum with the respective filters. The camera was fitted to a tripod and pointed towards the ground (90º) at a height of approximately 80 cm. The photography setup used for the experiments consisted of a sheet of black ethylene-vinyl acetate (EVA) used as a low-UV reflective background. Each photo setup included two individuals, one of each species of the co-mimic pair, a 40% grey standard (Spectralon® Labsphere) used for calibration and a leaf freshly collected to make background measures. The species used was *Guazuma ulmifolia* (Sterculiaceae), a small abundant tree across all Pipeline Road, which facilitated the collection of fresh leaves.

Photos were taken under three different arboreal canopy conditions, forest border, closed forest, and open area, where those butterflies are usually seen (Table 3.1). All photos were taken under sunny to part-cloudy days, with three replicates in each habitat making sure that the amount of light was similar. In order to standardize the replicates, light measures were taken with a digital light meter (Digital Lux meter, Tondaj LX-1010B), which measures the total amount of LW (555 nm) per square meter (Lux) (Figure 3.1). Also photographs of the canopy were taken in order to measure vegetative cover, which was

81.5% (SE ± 0.2) for closed forest, 59.8 % (SE ± 3.4) for forest border, and 0% for open area. The aim was to analyze how colour signals are perceived throughout the morning when butterflies are most active. Therefore, photos were taken at dawn (7 am), morning (9 am) and noon (12 pm) during a short period of 15min as light conditions change rapidly. Sunrise during dry season was around 6h40. Direct sunlight was used for open area photographs, which makes impossible to have this light at dawn and also photos were overexposed because of high sunlight incidence in the tropics and technical limitations of the camera. Nevertheless I choose to show data for 12 pm (Figure 3.1) in my analysis to represent a highly used environment by butterflies at this period of day.

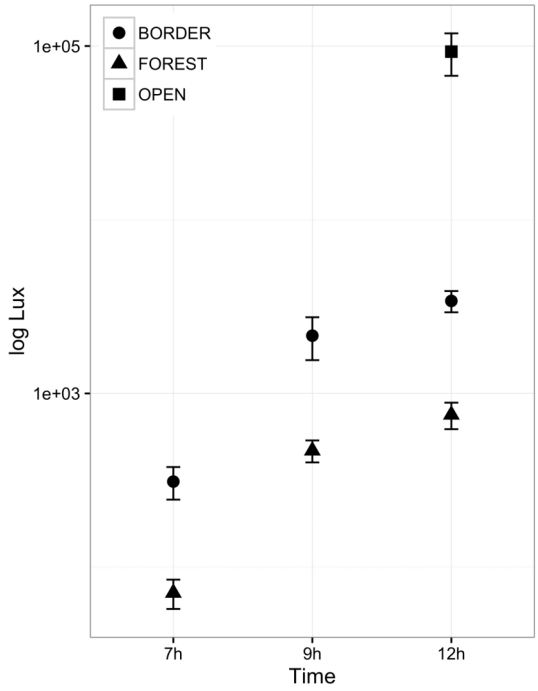

Figure 3.1. Amount of light differs across light environment and time of day. Showing the average log Lux between the three replicates of each habitat (± SE). Lux represents the amount of long-wave (555 nm) per square meter.

Image analysis and visual modelling

All images were processed and analyzed into the imaging software ImageJ (Rasband, 1997-2012). RAW human-visible and UV images were linearized and aligned following the methodology of Troscianko and Stevens (2015) and Arenas et al. (2014). Normally, photos would be normalized to the grey standard, which removes effects of light conditions (Arenas *et al.*, 2014). Since my main interest was to measure how coloration changes in different light environments, the images were not normalized to each grey standard. Instead, an average grey standard was obtained from all photographs. Photon catch values were obtained for each colour using the entire patch from linearized photos, and subsequently these values were multiplied by each photo exposure time and normalized with the average grey standard. With this methodology I was able to calculate how particular environment and time varies from the natural average light, and also assume colour constancy, a neural mechanism that compensates for changes in illumination (Stevens *et al.*, 2007; Arenas *et al.*, 2014). I used the average photon catch results from the three habitats replicates. Predicted photon catch values were obtained using spectral sensitivity for each cone type of the blue tit (*Cyanistes caeruleus*) for the UV-sensitive vision (UVS) (Hart *et al.*, 2000), peafowl (*Pavo cristatus*) for the violet-sensitive vision (VS) (Hart, 2002) and *Heliconius erato* (Briscoe *et al.*, 2010; McCulloch *et al.*, 2016).

Background of many terrestrial habitats is dominated by greenish vegetation; therefore a green leaf was chosen to make contrast calculations. Differences between light environment and time of the day were calculated using the contrast of warning colours against an average green leaf. Channel activation in avian vision was calculated using the Red-Green (RG), Blue-Yellow (BY) and Blue-UV opponent channel (Osorio *et al.*, 1999b;

Lovell *et al.*, 2005; Stevens *et al.*, 2009). For the achromatic signal, I used avian double cones (DBL). Using a ratio-based approach suggested by Lovell et al. (2005), I calculated the opponent channel responses as follows:

$$RG = LW - MW / LW + MW$$

$$BY = SW - (LW + MW) / SW + (LW + MW)$$

$$Blue\text{-}UV = SW - UV / SW + UV$$

$$Achromatic = DBL$$

Opponent channels for *Heliconius* were based on existing bird opponent channels and on what has been proposed in earlier studies (Swihart, 1971, 1972; Bybee *et al.*, 2012). The *H. erato* compound eye has red filtering pigments that shift LW photoreceptor sensitivity from green to red and as the physiological mechanisms underlying these two LW photoreceptors are not known, sensitivities in Green (560 nm) and Red (600 nm) were used (McCulloch *et al.*, 2016). To investigate differences between co-mimic species, I calculated opponent channel activation based on the prediction that *Heliconius* mating system might use UV2-UV1 and RG contrasts for mate choice (Bybee *et al.*, 2012; McCulloch *et al.*, 2016). Channel activation in *Heliconius* vision was calculated using the opponent channels as follows:

$$RG = Red - Green / Red + Green$$

$$BY = Blue - (Red + Green) / Blue + (Red + Green)$$

$$Blue\text{-}UV2 = Blue - UV2 / Blue + UV2$$

$$UV2\text{-}UV1 = UV2 - UV1 / UV2 + UV1$$

To examine whether warning colours have greater contrast against green background I calculated the Weber Contrast (Whittle, 1994), which takes into account the image value of the objects of interest as a fraction of background appearance using the formula:

$$C = (object - background) / background$$

Where *background* corresponds to the green leaf opponent channel values, and *object* corresponds to warning colour opponent channel values. This measure is suited to comparisons between small objects against larger backgrounds, such as butterflies against the green forest. For internal contrast, I used achromatic values of the warning colours against the black of each individual wing as background (Arenas *et al.*, 2014). I plotted the mean absolute contrast of each colour signal as a function of time and light environment for the three vision models.

Statistical analyses

All statistical calculations were processed in the software R 3.2.1 (R Core Team, 2015). My approach was to model colour contrasts over the course of a day and under different habitats in term of both predator and butterfly vision. Normality tests showed that contrast data were not normally distributed, therefore data were transformed to normality using square-root transformation and the transformed data were used in all statistical analyses. Raw data was plotted to illustrate the results. To test my predictions, I performed General linear mixed models using Satterthwaite approximations with random effects (packages *lme4* and *lmerTest*) and Tukey's post-hoc (package *multicomp*). I fitted the models accordingly to the predictions outlined above. Analyses were carried out using contrast values as the dependent variable, and fixed and random factors varied depending on the question. Factors were

individuals, colour (red, yellow, white), habitat (border, forest, area), time (7am, 9am, 12pm), and bird vision (UVS, VS). For *Heliconius* vision, I added side of the wing (dorsal, ventral) because this trait might be more important for butterflies than for their avian predators. To test contrast stability through time and habitat, I used the coefficient of variation (CV) of each colour in each opponent channel for each visual system. The CV is an effective measurement to determine how relatively stable a measurement is around a mean value, following methodology from Arenas et al. (2014).

RESULTS

Signal contrast and conspicuousness for avian predators

Red was generally the most contrasting colour against a green background in the RG opponent channel as compared to yellow ($z = -11.10$, $P < 0.001$, Table S3.1) and white ($z = -18.0$, $P < 0.001$, Table S3.1). In contrast, white had higher contrasts against a green background in the BY channel, as compared to red ($z = 22.88$, $P < 0.001$, Table S3.1) and yellow ($z = -31.47$, $P < 0.001$, Table S3.1) (Figure 3.2). Colours in open areas showed a higher contrast, such in the RG channel for red band ($t = 7.54$, $P < 0.001$, Table S3.2) with no difference between border and forest ($z = -0.31$, $P = 0.94$, Table S3.2) (Figure 3.2). In the Blue-UV opponent channel, UVS and VS birds could perceive red and yellow with less stability (Table 3.2), and yellow showed higher contrast early in the morning than at noon (7 am: $z = 12.15$, $P < 0.001$; 9 am: $z = 14.24$, $P < 0.001$, Table S3.3).

Internal achromatic contrast was higher for yellow, compared to red ($z = 41.89$, $P < 0.001$, Table S3.1) and white ($z = 12.42$, $P < 0.001$, Table S3.1). Moreover, yellow has more

contrast in the border, which is the preferred habitat of yellow band butterflies, than in the forest ($z = -3.42$, $P = 0.001$, Table S3.4) (Figure 3.2).

Signal contrast and conspicuousness for Heliconius conspecifics

In some cases, contrasts followed my prediction that species would be more contrasting in their own habitats (Figure 3.3). The yellow colour was more contrasting in the border in the UV2-UV1 channel, especially early hours such as 7 am ($t = -23.1$, $P < 0.001$, Table S3.5) and 9 am ($t = -13.3$, $P < 0.001$, Table S3.5). White was more contrasting in the forest than in the border at 7 am in the UV2-UV1 channel ($t = 2.32$, $P = 0.014$, Table S3.5) and also at 7 am in the Blue-UV2 channel ($t = 6.12$, $P < 0.001$, Table S3.5). Also in the Blue-UV2 channel, while white colour contrast decreased during the day in the forest, it increased at 12 pm in the border ($z = -4.11$, $P < 0.001$, Table S3.5) (Figure 3.3). The red colour showed large differences in the RG channel between dorsal and ventral side, with dorsal side with the higher contrast ($t = -40.04$, $P < 0.001$, Table S3.6). Same results were found for red in the Blue-Yellow and Blue-UV2 opponent channel (Table S3.6) (Figure 3.3).

The contrast of signals against the background revealed greater differences across habitat and time when seen through the *Heliconius* vision model as predicted (Table 3.2). *Heliconius* vision had higher values of coefficient of variation, which revealed greater fluctuations across habitat and time in most of the opponent channels. The only exception was in the Blue-UV opponent channel for red and yellow colour, which showed more instability for avian vision than for *Heliconius* vision (Table 3.2).

Species differences in opponent channel activity for Heliconius vision

In order to investigate differences between co-mimics, I calculated opponent channel activity across habitat and time of day. In the UV2-UV1 opponent channel activity, yellow was higher at 7 am and decreased with time in both habitats and species (dorsal, t = 7.44, $P < 0.001$; ventral, t = 7.57, $P < 0.001$, Table S3.7), and species contrast differences were only significant in the forest for yellow dorsal (t = 2.22, $P = 0.031$, Table S3.8) (Figure 3.4). In RG, species contrasts were significantly different only for red colour in both sides of the wing (dorsal, t = 3.6, $P = 0.003$; ventral, t = -4.6, $P < 0.001$, Table S3.9) and especially marked for the ventral colour, higher in *Heliconius erato* (Figure 3.4). There are therefore potentially visible differences between co-mimic species across all light environments, for red and yellow colours, although it remains unknown whether these are biologically relevant.

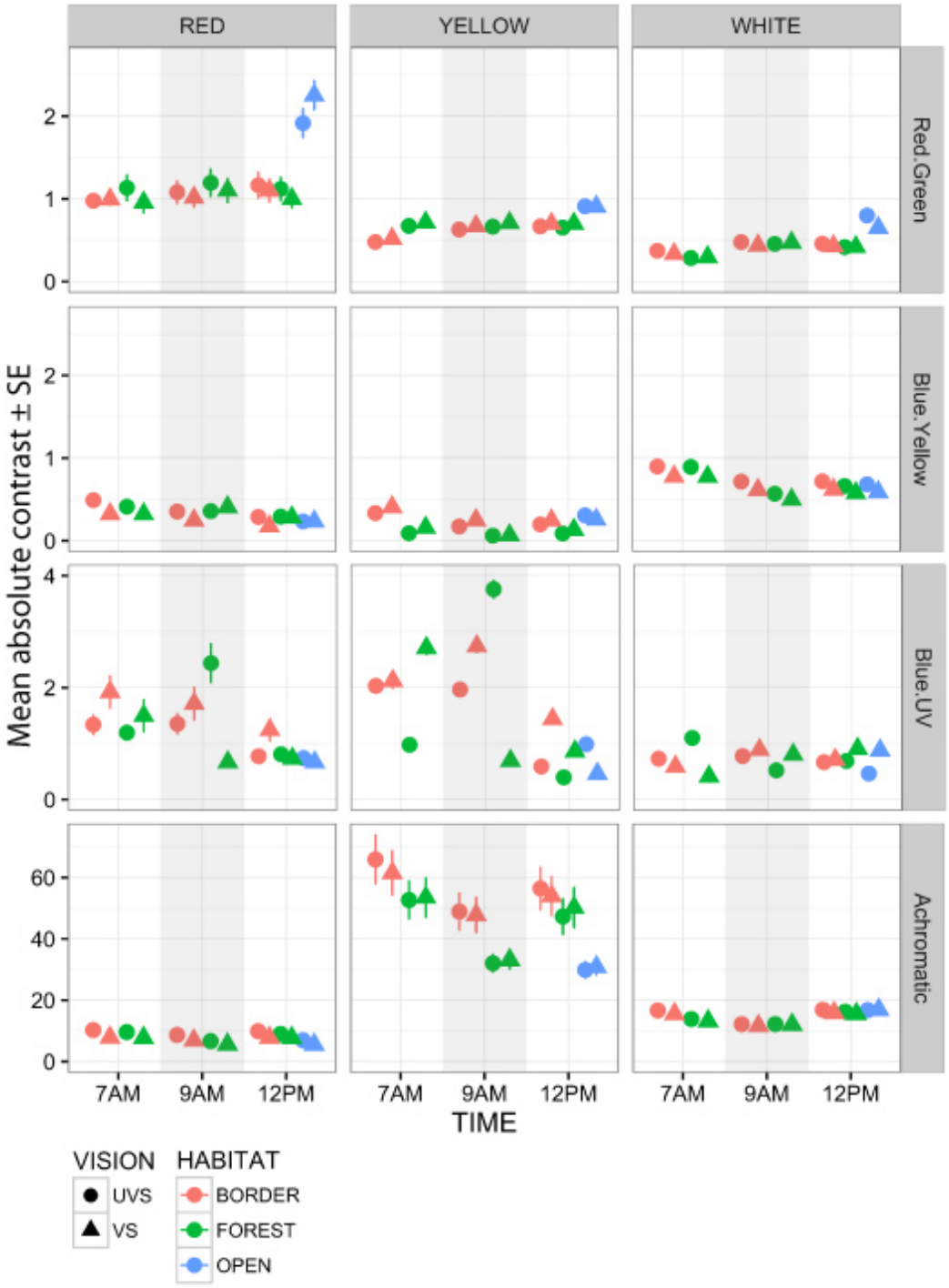

Figure 3.2. *Heliconius* colours' conspicuousness for avian predators. Mean absolute contrast of colour signals (±SE, standard error) in the bird vision systems analyzed (circles, UVS; triangles, VS) through habitats (red, border; green, forest; blue, open) and time (7am, 9am, 12pm). Vertical panels show the three colour signals (red, yellow and white), horizontal panels show opponent channels against green leaf (top, Red-Green; middle, Blue-Yellow and Blue-UV) and against the black of the wing (bottom, Achromatic). Note: Channels have different y-axis values. Error bars smaller than data points are not shown.

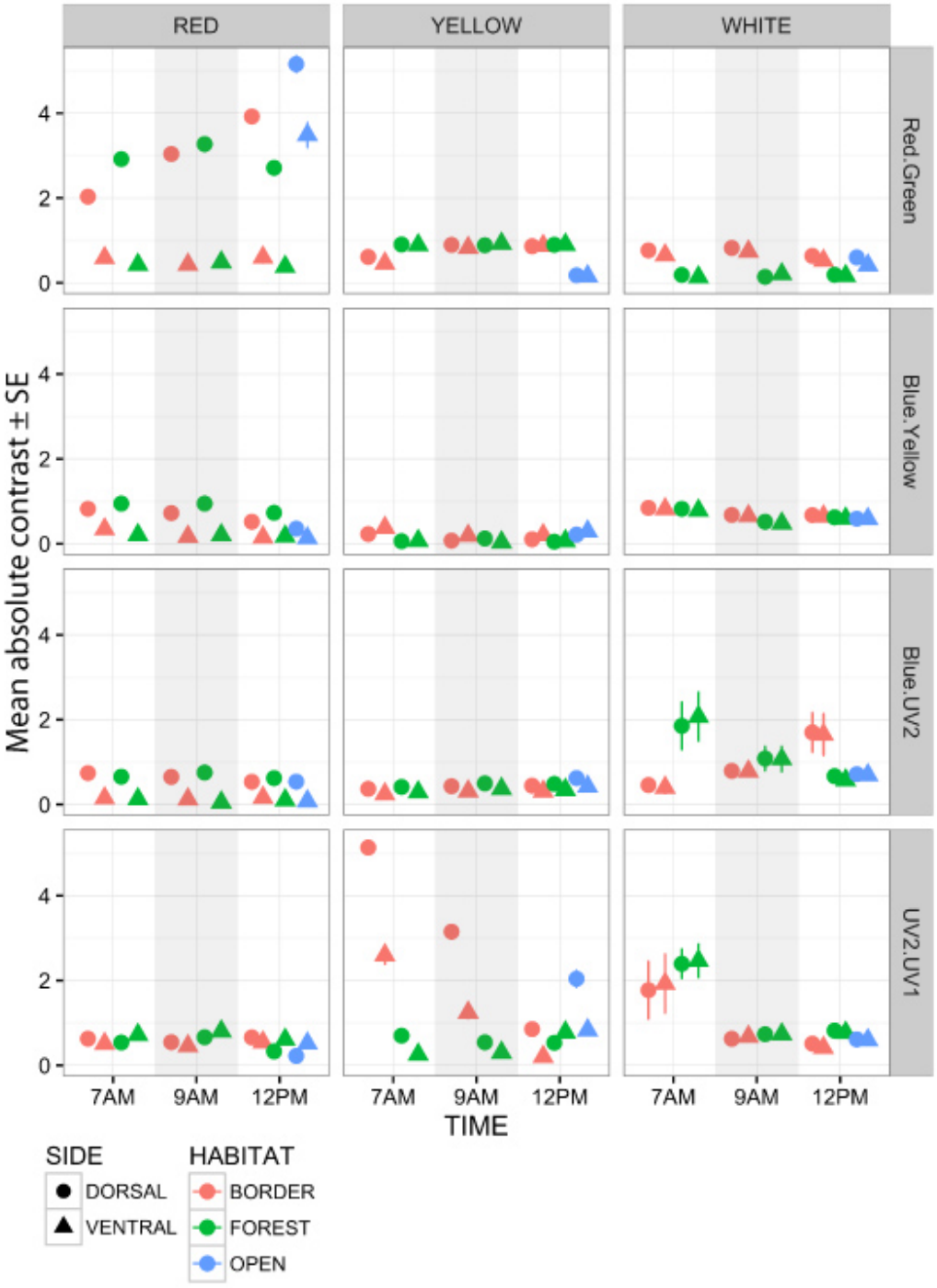

Figure 3.3. *Heliconius* colour's conspicuousness for conspecifics. Mean absolute contrast of colour signals against green leaf in *Heliconius* vision through habitats (red, border; green, forest; blue, open), time (7 am, 9 am, 12 pm) and side of the wing (circles, dorsal; triangles, ventral). Vertical panels show colour signals (red, yellow and white), horizontal panels show opponent channels against green leaf (top, Red-Green; middle, Blue-Yellow and Blue-UV2; bottom, UV2-UV1). Error bars: ± 1 standard error (SE), error bars smaller than data points are not shown.

Table 3.2. *Heliconius* vision has higher coefficient of variation in most of the colours compared with bird vision across time and habitats. Coefficient of variation for each visual system, colour and opponent channel, higher values are in bold.

Opponent channel	Colour	Visual system		
		Heliconius	UVS	VS
Red-Green				
	Red	**1.053**	0.704	0.695
	Yellow	**0.384**	0.211	0.178
	White	**0.722**	0.375	0.314
Blue-Yellow				
	Red	**0.699**	0.576	0.606
	Yellow	**0.735**	0.667	0.528
	White	**0.177**	0.134	0.169
Blue-UV				
	Red	0.783	0.897	**1.024**
	Yellow	0.265	**0.788**	0.637
	White	**1.035**	0.549	0.341
UV2-UV1				
	Red	0.525	-	-
	Yellow	1.047	-	-
	White	1.061	-	-
Achromatic				
	Red	-	0.367	0.436
	Yellow	-	0.717	0.699
	White	-	0.348	0.349

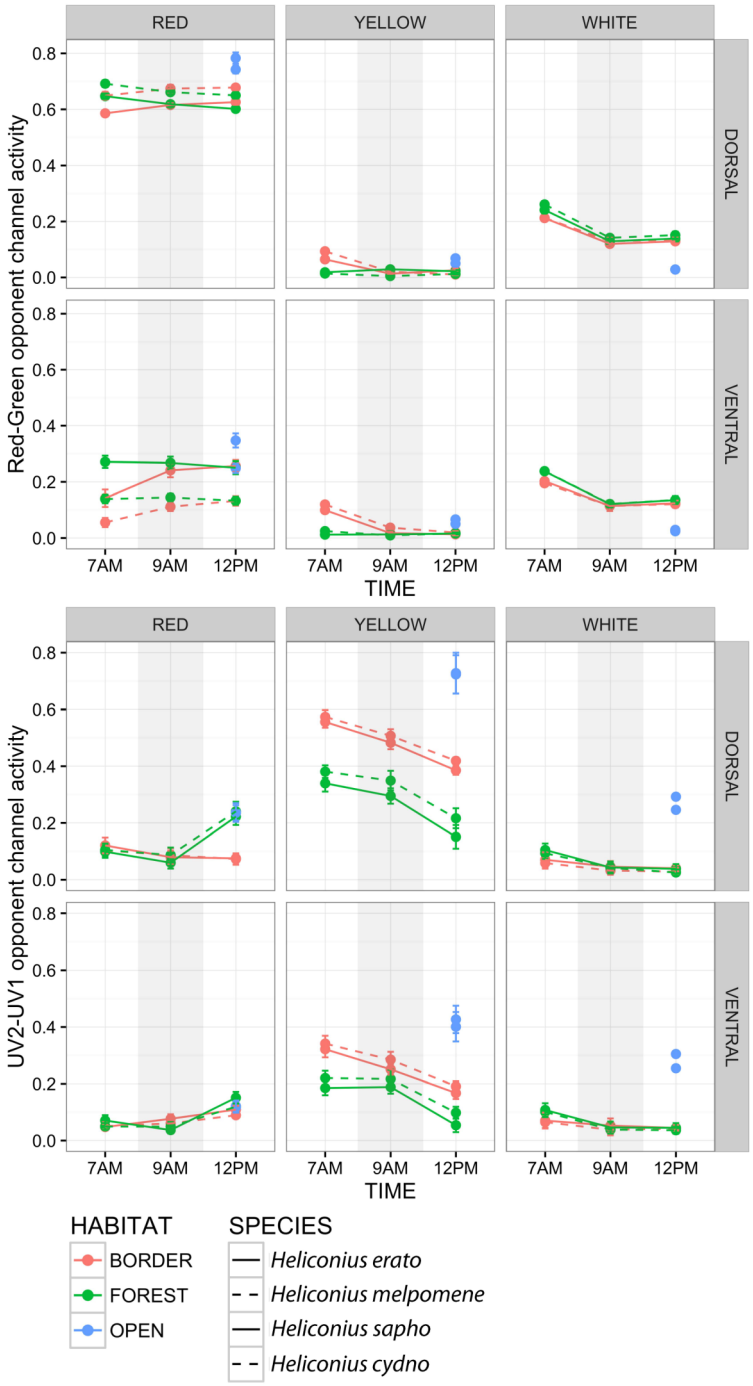

Figure 3.4. Colour signal differences between *Heliconius* species. Mean absolute Red-Green (top) and UV2-UV1 (bottom) opponent channel activity in *Heliconius* vision through habitats (red, border; green, forest; blue, open), time (7am, 9am, 12pm) and species (red and yellow for *H. erato* and *H. melpomene*; white for *H. sapho* and *H. cydno*). Vertical panels show the three colour signals (red, yellow and white), horizontal panels show side of the wing (dorsal and ventral). Error bars: ± 1 standard error. Error bars smaller than data points are not shown.

DISCUSSION

The bright and contrasting *Heliconius* wing patterns appear well adapted for signalling distastefulness to predators. However, their colour constancy and appearance in different light environments remains poorly studied. I have shown that colours are indeed very stable for avian predator vision, but somewhat less so for *Heliconius* vision. This is consistent with the idea that wing patterns are primarily selected for their role in signalling distastefulness to predators.

Signal stability and conspicuousness to avian predators

Generally, warning signals involve combinations of long-wavelength colours such as red, orange, and yellow which are highly conspicuous against natural backgrounds and stable under different natural conditions (Lovell *et al.*, 2005; Stevens & Ruxton, 2012). In my results, *Heliconius* red colouration has higher detectability against average green background in the RG output and these results are consistent regardless of habitat and time of the day. Previous work has investigated colour stability through opponent colour channels and also showed that red coloration is more contrasting and stable against green backgrounds over the course of a day and across light conditions to the bird visual system (Lovell *et al.*, 2005; Arenas *et al.*, 2014). Moreover, the *Heliconius* yellow colouration also is highly conspicuous against its internal black pattern in the achromatic output. Achromatic information is one of the main cues used for motion detection (Hämäläinen *et al.*, 2015). Therefore, my results suggest that red and yellow signals work together and are likely effective in stimulating avian opponent channels in order to be conspicuous in all light environments.

There is evidence that red and yellow colouration serve as reliable warning signal to avian predators (Ham *et al.*, 2006; Svádová *et al.*, 2009; Arenas *et al.*, 2015), but that this is less true of white colouration. One explanation is that white is more variable across time and habitat, so provides a less reliable signal under varied light conditions (Stevens & Ruxton, 2012; Arenas *et al.*, 2014). Moreover, field and aviary experiments with polymorphic yellow and white wood tiger moths, *Parasemia plantaginis*, showed that yellow males are avoided more than white males by predators, but white males have higher mating success (Nokelainen *et al.*, 2012). My results showed that white contrasts against green background were lower and rather variable for avian vision. The co-mimics *H. sapho* and *H. cydno* also contain iridescence blue that was not measured with this methodology. However, the lack of high contrast in white colouration might be balanced with the fact that polarized light might act as a signal, especially in forest habitats (Sweeney *et al.*, 2003; Douglas *et al.*, 2007; Pegram *et al.*, 2015).

Highly conspicuous warning signals are expected to evolve to be stable in their appearance throughout the day and between light environments, in order to remain honest indicators of prey unpalatability (Blount *et al.*, 2009; Cortesi & Cheney, 2010; Stevens & Ruxton, 2012; Arenas *et al.*, 2015). If warning signals fluctuate through time and space this could alter bird foraging experiences and reduce the effectiveness of the aposematic signal. The final decision on whether or not to attack a prey results from a combination of information reaching the predator brain, and for greater efficiency, aposematic coloration needs to be easy to remember (Endler, 1988). My results support this prediction, as colours were generally stable through time and light environments in all opponent systems with only a few exceptions. Notably these occurred where contrasts were higher in open areas and in

the early morning. This might also be favourable as the prey would be more conspicuous when they are most vulnerable to predation, since birds are more active and forage early in the morning (Buskirk *et al.*, 1972; Poulin *et al.*, 2001; Steiger *et al.*, 2009). In agreement with this, *Heliconius* predation and roost disturbance has been observed in the early morning (Mallet, 1986; Finkbeiner, 2014).

Habitat and time influence conspicuousness in Heliconius conspecifics

Butterflies belonging to the two mimetic rings studied here tend to be segregated between habitats, corresponding to areas where the photographs were taken, although there is considerable overlap (Estrada & Jiggins, 2002). My results showed that the colours were more unstable when seen through *Heliconius* vision as compared to avian vision and some colours tend to be more contrasting in their respective habitats.

My results provide some evidence that co-mimic rings are more conspicuous in their own habitat as seen through *Heliconius* vision, reinforcing the idea that ecological adaptation leads to spatial segregation to where detection would be facilitated. Some colours had higher contrast against green backgrounds in their respective habitat, such as yellow in the border and white in the forest. Nonetheless, red showed the opposite trend and was generally more contrasting in the forest. Differences across light environments could affect mating preferences by altering search costs for a specific colour pattern, and perhaps changing the fitness of different colour patterns. Adaptation in different microhabitats within the forest might have an influence on how closely related species commonly differ in pattern, while convergence in pattern occurs between more distantly related species (Joron & Mallet, 1998). Ecological adaptation is attributed to habitat preference and leads to assortative

mating (Jiggins, 2008). The two sister species studied here, *H. melpomene rosina* and *H. cydno*, are known to rarely hybridise in the wild, hence microhabitat segregation reduces potential mating encounters between these two species and reduces gene flow (Mallet *et al.*, 1998; Merrill *et al.*, 2013). Subtle environmental conditions could affect recognition in mating behaviour as seen in the jumping spider, *Habronattus pyrrithrix*, which red males were more successful in approaching females in the sunlight (Taylor & McGraw, 2013).

The activation of opponent channels was often higher in the early hours of the morning, at the time when the butterflies are more active and leave their roost or perches to forage (Mallet, 1986; Finkbeiner *et al.*, 2012). This was especially the case for *Heliconius* white and yellow wing colours in the UV channel, which might act in intraspecific communication (Briscoe *et al.*, 2010; Bybee *et al.*, 2012). There is some evidence that distinct UV colour signals are being transmitted between co-mimics (**Chapter 2**), which may reduce costs of mating confusion (Estrada & Jiggins, 2008). Similarly, in two species of newt, belly colour is distinct in the UV range and females often made mistakes choosing the wrong males in the absence of UV light (Secondi & Théry, 2014).

In this context, the duplicate genes encoding two distinct visual pigments with sensitivity peaks in the UV range in *H. erato* females offer the potential for enhanced spectral discrimination in light environments and time of the day where UV is more prominent. A UV2-UV1 opponent channel was proposed by Bybee et al. (2012), who showed that this receptor combination would have lower error rates for discrimination between *Heliconius* and *Dryas* yellows. There is no direct evidence for such a mechanism yet, but the fact that males and females show differences in the expression of the two UV proteins suggests that UV2-UV1 contrasts could be an important opponent channel for a

female specific behaviour, perhaps mate recognition or host plant finding (McCulloch *et al.*, 2016). There is similarly no direct evidence for a Red-Green opponent channel, although the presence of red filters in their eyes means that this is a possibility (McCulloch *et al.*, 2016). Differences between species in Red-Green channel activity for red colouration might have a role in mate recognition since *Heliconius* tend to be attracted to red (Merrill *et al.*, 2011b).

The sensory drive hypothesis describes evolutionary relationships among visual systems, conditions of the light environment and mating preferences (Endler & Basolo, 1998). *Heliconius* mating preference is highly linked to colour and in *H. melpomene*, the gene responsible for red colour pattern is genetically linked to the preference for the same pattern (Jiggins *et al.*, 2001; Naisbit *et al.*, 2001; Merrill *et al.*, 2011b). Visual sensitivity data used here is only from *H. erato petiverana* whereas there is no information yet for other species, which might differ in their visual systems and perhaps match with colour preference or habitat (Frentiu *et al.*, 2007; Briscoe *et al.*, 2010). In addition, mating behaviour might benefit from some habitats in maximizing conspicuousness, such as in tropical dwelling birds and wire-tailed manakins which visual contrast is increased during display by habitat choice (Endler & Théry, 1996; Heindl & Winkler, 2003). Habitats used by *Heliconius* vary in transmission properties and the ideal habitat differs among populations because of divergence in colour signals, as proposed by sensory drive (Endler, 1992). Nevertheless, our results suggest that selection for conspicuousness in the preferred habitat could explain in part the divergence in colour pattern in these species.

Conclusion

In conclusion, the transmission of *Heliconius* warning signals varies due to light environment to a much greater degree through their own visual system, but to a smaller degree through avian predator vision. Selection for signal detectability under different habitat conditions is a mechanism that is proposed to lead to evolution of signal diversity, as seen in species of *Anolis* lizards that occupy habitats that match their visual system and signal design (Leal & Fleishman, 2002), in species of warblers which different cone opsin gene expression correlate with sexual selection and habitat use (Bloch, 2015) and also colour patterns of guppies are more conspicuous to guppies at the times and places of courtship and relatively less conspicuous at times and places of predator risk (Endler, 1991). *Heliconius* butterfly warning colours are highly contrasting against the forest background and stable through time and habitat in terms of predator avoidance but also conspicuous to attract the attention of conspecifics. However, more extensive studies considering spectral sensitivities of different *Heliconius* species and their responses to environmental changes in their signal visibility are needed to confirm the conspicuousness to mates. Opponent channel colour contrasts can predict behaviour of perceivers, however, additional behavioural experiments on how light environment influences prey detectability, such with poison frogs (Rojas *et al.*, 2014), are necessary to verify our results.

SUPPLEMENTARY INFORMATION

Table S3.1. Colour contrast differences for avian vision per opponent channel. General linear mixed models results using Satterthwaite approximations with random effects and Tukey's post-hoc (y ~ Colour + (1 | Individuals) + (1 | Vision:Habitat:Time)).

Opponent channel	Colour	N	Linear mixed model			Tukey's post-hoc		
			df	t value	p-value	Contrasts	z value	p-value
Red-Green								
	Red (intercept)	1176	16.3	34.59	< 2E-16	White - Red	-18.00	< 2E-16
	White		1160	-18.00	< 2E-16	Yellow - Red	-11.10	< 2E-16
	Yellow		1160	-11.10	< 2E-16	Yellow - White	8.26	3.33E-16
Blue-Yellow								
	Red (intercept)	1176	17.4	29.01	4.44E-16	White - Red	22.88	<2E-16
	White		49.3	22.88	< 2E-16	Yellow - Red	-11.98	<2E-16
	Yellow		1140	-11.98	< 2E-16	Yellow - White	-31.47	<2E-16
Blue-UV								
	Red (intercept)	1176	14.5	16.84	6.44E-11	White - Red	-6.11	2.85E-09
	White		1160	-6.11	1.34E-09	Yellow - Red	6.81	< 1E-09
	Yellow		1160	6.80	1.57E-11	Yellow - White	12.08	< 1E-09
Achromatic								
	Red (intercept)	1176	36.6	16.27	< 2E-16	White - Red	4.69	5.42E-06
	White		28.4	4.69	6.25E-05	Yellow - Red	41.89	< 1E-07
	Yellow		1136	41.88	< 2E-16	Yellow - White	12.42	< 1E-07

Table S3.2. Habitat contrast differences for avian vision per colour for the Red-Green opponent channel. General linear mixed models results using Satterthwaite approximations with random effects and Tukey's post-hoc (y ~ Habitat + (1 | Individuals) + (1 | Vision:Time)).

Colour	Habitat	N	Linear mixed model			Tukey's post-hoc		
			df	t value	p-value	Contrasts	z value	p-value
Red								
	Border (intercept)	448	445	33.05	<2E-16	Forest - Border	-0.31	0.947
	Forest		445	-0.31	0.755	Open - Border	7.55	<1E-05
	Open		445	7.54	2.54E-13	Open - Forest	7.77	<1E-05
Yellow								
	Border (intercept)	448	9.2	53.37	7.90E-13	Forest - Border	10.77	<2E-16
	Forest		425	10.77	<2E-16	Open - Border	21.46	<2E-16
	Open		429.2	21.46	<2E-16	Open - Forest	14.63	<2E-16
White								
	Border (intercept)	280	9.05	24.88	1.21E-09	Forest - Border	-3.14	0.005
	Forest		262.9	-3.14	0.002	Open - Border	16.84	< 1E-04
	Open		265.3	16.84	<2E-16	Open - Forest	18.83	< 1E-04

Table S3.3. Time contrast differences for avian vision per colour for the Blue-Yellow opponent channel. General linear mixed models results using Satterthwaite approximations with random effects and Tukey's post-hoc (y ~ Time + (1 | Individuals) + (1 | Vision:Habitat)).

Colour	Time	N	Linear mixed model			Tukey's post-hoc		
			df	t value	p-value	Contrasts	z value	p-value
Red								
	12PM (intercept)	448	9.4	17.31	2.03E-08	7AM - 12PM	4.44	2.37E-05
	7AM		367.3	4.44	1.18E-05	9AM - 12PM	4.17	8.14E-05
	9AM		367.3	4.17	3.73E-05	9AM - 7AM	-0.25	0.964
Yellow								
	12PM (intercept)	448	5.7	14.04	1.15E-05	7AM - 12PM	12.15	<1E-04
	7AM		436.6	12.15	2.00E-16	9AM - 12PM	14.24	<1E-04
	9AM		436.6	14.24	2.00E-16	9AM - 7AM	2.07	0.0969
White								
	12PM (intercept)	280	5.1	25.46	1.41E-06	7AM - 12PM	-1.59	0.250
	7AM		250.1	-1.58	0.113	9AM - 12PM	-0.08	0.996
	9AM		250.1	-0.08	0.936	9AM - 7AM	1.46	0.306

Table S3.4. Habitat contrast differences for avian vision for the achromatic opponent channel per colour. General linear mixed models results using Satterthwaite approximations with random effects and Tukey's post-hoc (y ~ Habitat + (1 | Individuals) + (1 | Vision:Time)).

Colour	Habitat	N	Linear mixed model			Tukey's post-hoc		
			df	t value	p-value	Contrasts	z value	p-value
Red								
	Border (intercept)	448	12.5	22.28	2.00E-11	Forest - Border	-3.50	0.001
	Forest		425	-3.51	0.001	Open - Border	-7.52	< 1E-04
	Open		429.8	-7.52	3.19E-13	Open - Forest	-5.30	< 1E-04
Yellow								
	Border (intercept)	448	14.4	24.45	3.89E-13	Forest - Border	-3.42	0.002
	Forest		424.8	-3.42	0.001	Open - Border	-5.56	< 1E-04
	Open		376.1	-5.56	5.07E-08	Open - Forest	-3.34	0.002
White								
	Border (intercept)	280	13.9	21.02	5.89E-12	Forest - Border	-2.41	0.041
	Forest		263	-2.41	0.017	Open - Border	-0.02	1.000
	Open		267.7	-0.02	0.981	Open - Forest	1.51	0.280

Table S3.5. Habitat contrast differences for *Heliconius* vision for yellow and white colours, per opponent channel and time. General linear mixed models results using Satterthwaite approximations with random effects and Tukey's post-hoc (y ~ Habitat + (1 | Individuals) + (1 | Side)).

Colour	Channel	Time	Habitat	N	Linear mixed model			Tukey's post-hoc		
					df	t value	p-value	Contrasts	z value	p-value
Yellow	UV2-UV1	7AM								
			Border (intercept)	64	1	7.41	0.081			
			Forest		46	-23.1	<2e-16			
		9AM								
			Border (intercept)	64	1	6.56	0.090			
			Forest		61	-13.31	<2e-16			
		12PM								
			Border (intercept)	96	1	4.28	0.124	Forest - Border	2.20	0.071
			Forest		92	2.20	0.03	Open - Border	7.49	0.001
			Open		92	7.49	4.02E-11	Open - Forest	5.29	0.001
White	UV2-UV1	7AM								
			Border (intercept)	40	11.4	6.57	3.37E-05			
			Forest		29	2.62	0.014			
	Blue-UV2	7AM								
			Border (intercept)	40	13.3	4.52	0.001			
			Forest		29	6.12	1.14E-06			
		12AM								
			Border (intercept)	60	21.6	13.1	9.32E-12	Forest - Border	-4.11	1.24E-04
			Forest		48	-4.11	1.54E-04	Open - Border	-3.43	0.002
			Open		48	-3.43	0.001	Open - Forest	0.67	0.776

Table S3.6. Side of the wing contrast differences for *Heliconius* vision for the red colour, per opponent channel. General linear mixed models results using Satterthwaite approximations with random effects and Tukey's post-hoc (y ~ Side + (1 | Individuals) + (1 | Habitat:Time)).

Opponent Channel	Side of the wing	N	Linear mixed model		
			df	t value	p-value
Red-Green					
	Dorsal (intercept)	224	6.2	9.78	5.59E-05
	Ventral		201	-40.04	< 2E-16
Blue-Yellow					
	Dorsal (intercept)	224	7.3	22.24	5.63E-08
	Ventral		201	-25.5	< 2E-16
Blue-UV2					
	Dorsal (intercept)	224	16.6	36.51	< 2E-16
	Ventral		201	-34.61	< 2E-16
UV2-UV1					
	Dorsal (intercept)	224	8	17.89	8.76E-08
	Ventral		216	2.84	0.0048

Table S3.7. Time of the day contrast differences for *Heliconius* vision for the yellow colour on the UV2-UV1 opponent channel activity per side of the wing. General linear mixed models results using Satterthwaite approximations with random effects and Tukey's post-hoc (y ~ Time + (1 | Individuals) + (1 | Habitat)).

Side	Time	N	Linear mixed model			Tukey's post-hoc		
			df	t value	p-value	Contrasts	z value	p-value
Dorsal								
	12PM (intercept)	112	2	5.34	0.033	7AM - 12PM	7.44	<1E-04
	7AM		107	7.44	2.58E-11	9AM - 12PM	5.36	<1E-04
	9AM		107	5.36	4.70E-07	9AM - 7AM	-2.07	0.095
Ventral								
	12PM (intercept)	112	2	4.07	0.055	7AM - 12PM	7.57	<1E-04
	7AM		92.4	7.57	2.68E-11	9AM - 12PM	6.26	<1E-04
	9AM		92.4	6.26	1.18E-08	9AM - 7AM	-1.31	0.39

Table S3.8. Species contrast differences for *Heliconius* vision for the yellow colour at the forest on the UV2-UV1 opponent channel activity per side of the wing. General linear mixed models results using Satterthwaite approximations with random effects and Tukey's post-hoc (y ~ Species + (1 | Individuals) + (1 | Time)).

Side	Species	N	Linear mixed model		
			df	t value	p-value
Dorsal					
	H. erato (intercept)	48	2.2	8.53	0.010
	H. melpomene		44	2.22	0.031
Ventral					
	H. erato (intercept)	48	2.2	5.26	0.027
	H. melpomene		14	1.84	0.087

Table S3.9. Species contrast differences for *Heliconius* vision for the red colour on the Red-Green opponent channel activity per side of the wing. General linear mixed models results using Satterthwaite approximations with random effects and Tukey's post-hoc (y ~ Species + (1 | Individuals) + (1 | Habitat:Time)).

Side	Species	N	Linear mixed model		
			df	t value	p-value
Dorsal					
	H. erato (intercept)	112	9.6	63.22	6.57E-14
	H. melpomene		14	3.60	0.003
Ventral					
	H. erato (intercept)	112	12.4	14.204	4.84E-09
	H. melpomene		14	-4.66	0.0004

CHAPTER 4

AVOIDANCE OF AN APOSEMATICALLY COLOURED BUTTERFLY BY WILD BIRDS IN A TROPICAL FOREST

ABSTRACT

Birds are considered to be the primary selective agents for warning coloration in butterflies, and select for aposematic mimicry by learning to avoid brightly coloured prey after unpleasant experiences. It has long been thought that bright coloration plays an important role in promoting the avoidance of distasteful prey by birds. I tested the hypothesis that warning coloration facilitates memorability and promotes predator avoidance by means of a field experiment using distasteful model butterflies. Artificial butterflies with a *Heliconius* colour pattern unknown to local birds were generated using bird vision models, either coloured or achromatic, and hung in tree branches in a tropical forest. Two sequential trials were conducted at each site in order to test avoidance by naïve and experienced predators. There was a significant reduction in predation in the second trial. In addition, coloured models were attacked less than achromatic models. Specifically, coloured butterflies were attacked significantly less in the second trial, but there was no significant decrease in predation on achromatic models. My results imply an important role for colour in enhancing aversion of aposematic butterflies. I have also demonstrated that previous experience of distasteful prey can lead to enhanced avoidance in subsequent trials, supporting mimicry theory.

INTRODUCTION

The aposematic signals of unpalatable prey are a defence against visually hunting predators. In particular, conspicuous coloration is strongly favoured in defended prey as it can increase detection efficiency and lead to rapid decision-making (Endler, 1988). Colours such as red, yellow and orange are normally highly contrasting with the background and are commonly used to advertise unpalatability (Stevens & Ruxton, 2012; Arenas *et al.*, 2014). Therefore, these brightly coloured signals support rapid discrimination from cryptic prey and have long been considered to facilitate avoidance learning when compared to less visible coloration (Guilford & Dawkins, 1991; Speed, 2000).

Birds are widely considered to be the primary selective agent for the aposematic coloration of butterflies. After unpleasant experiences with an unpalatable prey, bird predators learn to avoid similar morphs (Ham *et al.*, 2006; Lindström *et al.*, 2006). This learning ability leads to selection favouring the most abundant colour patterns in a local area and generates aposematism and Müllerian mimicry in which predator attacks are reduced through aversion learning of locally common aposematic patterns (Müller, 1879; Mallet & Joron, 1999).

Learning and forgetting are essential for the maintenance of Müllerian mimicry (Speed & Turner, 1999). Memory is linked to recognition, and if predators forget about experiences with prey, then recognition of an aposematic signal is not possible (Speed, 2000). Warning signals should therefore be selected to be memorable, in order to provoke low rates of forgetting and enhance predator aversion (Servedio, 2000; Speed, 2000). Among mimetic butterflies, long-term memorability of learned avoidance of the model is vital for protection of the co-mimic. There is a large body of evidence supporting the role of colour in

avoidance learning and memory, but this primarily comes from captivity experiments (Sillén-Tullberg, 1981; Osorio *et al.*, 1999b; Ham *et al.*, 2006; Sandre *et al.*, 2010).

Experiments in the wild with natural predators can better estimate the overall response of a local population and can complement cage studies. Responses from captive birds might be influenced by their appetite (Sandre *et al.*, 2010), food deprivation and artificial environments with constrained viewing, whereas natural environments are heterogeneous and offer a wider variety of alternative food, which might alter decision-making strategies. For example, a study with natural bird populations using artificial models of the wood tiger moth (*Parasemia plantaginis*) suggested that spatial heterogeneity in a predator community creates a mosaic of selection facilitating polymorphism (Nokelainen *et al.*, 2013). Also, another study with wild birds showed that achromatic (non-coloured) *Heliconius* models were attacked significantly more than coloured models of a local pattern, demonstrating the importance of aposematic signals in avoiding predation (Finkbeiner *et al.*, 2014). Furthermore, an experiment with model poison frogs (*Dendrobates tinctorius*) showed varying attack rates of wild tropical predators in different light conditions (Rojas *et al.*, 2014). Still, few studies to date have explored attack rates on different coloured models using wild birds and under natural conditions.

Neotropical *Heliconius* butterflies are one of the best-studied mimicry systems (Mallet & Joron, 1999), in which unpalatable sympatric species form mimicry rings. Many *Heliconius* species are highly variable in coloration and patterns (Mallet & Gilbert, 1995). Several studies have investigated predator behaviour towards *Heliconius* butterflies in cages using wild-caught rufous-tailed jacamars (*Galbula ruficauda*), which are specialist predators of fast-flying insects and exhibit specific butterfly handling strategies. Jacamars readily

reject *Heliconius* by sight or by taste, and discriminate them from other butterfly species (Chai, 1986; Langham, 2004). Field experiments using other butterfly predators, kingbirds and flycatchers, also showed taste-rejection of *Heliconius* butterflies (Pinheiro, 2003, 2011). Previous field studies have demonstrated mimicry selection by releasing live butterflies (Benson, 1972; Mallet & Barton, 1989) and monitoring recapture rates.

Therefore, to better understand the dynamics of *Heliconius* mimicry, more information from the predators' perspective in the wild is required. Here I investigate the role of coloration in attack rates, testing the ability of bird predators to avoid an unpalatable *Heliconius* warning signal in a tropical forest. The assumption is that wild birds would have a bias against aposematic colouration, which would facilitate the memory of novel butterfly colour pattern. I performed a field test of the hypothesis that aposematism facilitates avoidance of novel distasteful prey using artificial distasteful butterflies with a colour pattern unknown to local bird predators.

MATERIAL AND METHODS

Production of artificial butterflies

Artificial butterflies were produced based on wings of *Heliconius erato lativitta* which is found only in the Amazon basin, not in Panama (Brown, 1979; Hines *et al.*, 2011). I calibrated the appearance of the artificial wings to account for bird colour and luminance vision. Photographs of real wings and of a printer colour palette were taken with a Fuji calibrated UV SLR camera with an ultraviolet (UV) transmitting quartz lens (Jenoptic) with a UV pass filter (transmitting between 300 and 400 nm; Baader U filter) and a UV/IR-Cut pass filter (blocking UV below 400nm and IR above 700 nm; Baader UV/IR Cut Filter),

representing the UV and human visible spectrum respectively. Following this, predicted photon catch values of four single cones (used in colour vision) and double cones (likely used in achromatic vision) were calculated, based on the sensitivity of a UV vision bird receptors, Blue tit (*Cyanistes caeruleus*) (Hart *et al.*, 2000; Endler & Mielke, 2005), following the methodology created by (Troscianko & Stevens, 2015). Our criteria for selecting appropriate colours were that the "just-noticeable-differences" (JND) values (Vorobyev & Osorio, 1998) of the printer colours against real butterfly colours (Finkbeiner *et al.*, 2012; Merrill *et al.*, 2012) should be as close as possible to the threshold of discrimination of 3 JND (Siddiqi *et al.*, 2004) (Table S4.1). For achromatic models only achromatic contrast was used. Colours were closely reproduced as demonstrated in avian colour space vision (Figure S4.1). Afterwards, two types of artificial butterflies were designed, coloured and achromatic (Figure 4.1). These were printed on Whatman filter paper, which produces reflectance spectra close in brightness to actual wings (Finkbeiner *et al.*, 2012), using a HP Colour Laser Jet 4700dn printer. A 3-hydroxy-DL-kynurenine (3-OHK) pigment was applied to the yellow bands of the forewing to provide accurate UV reflectance (Finkbeiner *et al.*, 2012).

The artificial wings were attached with a nylon line to an edible pastry body (flour, lard, water and black food dye). To provide an unpleasant taste, quinine monohydrochloride dihydrate (4% solution) was sprayed on the body and wings of both model types. This concentration is aversive and has a similar effect to sampling a toxic prey (Rowe & Skelhorn, 2005). Finally, Krylon matte finishing spray was applied lightly to coat the artificial butterflies with a waterproofing element 24 h before placing the models out, without altering colour or smell.

Figure 4.1. Artificial butterfly models used in the experiment: chromatic (left) and achromatic (right).

Field experiment

The trials were conducted along three forest trails in Parque Nacional Soberanía, Panama. Models were hung by nylon line (~10 cm long) on tree branches (~1.70m high) in order to swing freely similar to a live butterfly. I aimed to maximise attack rates by butterfly predators that catch insects during flight and detect movement. Models were hung every 10 m in pairs, one coloured and one achromatic on opposite sides of the trail, with the assignment randomised.

In order to test memorability, the experiment had two trials. In the first trial 152 models of each type were placed for four days, followed by a second identical trial started five days after the first trial finished. In the second trial, the same procedure was repeated at the same location with new 152 new models of each type. The models were checked for attack marks after 48h and 96h. An artificial model was considered attacked if the body or wings included clearly visible beak marks, or part or all of the body was missing. If a model had more than one beak mark on it, this was counted as a single attack. Evidence of attack by animals other than birds, notably insects such as ants, was generally readily distinguished and was not counted as an attack (Salazar *et al.*, 2014).

Statistical analyses

I used the binomial response of attack (presence or absence) of two treatments (chromatic and achromatic) in two trials (1 and 2) across three localities. In order to test homogeneity of variance between localities a Bartlett test and Fligner-Killeen test were used. I used generalized linear mixed models (GLMM) with a binomial distribution, to test for the effect of trial, treatment and locality (as a random factor), as well as their interaction terms, on predation. Tests used the R packages *stats* and *lme4* in R statistical software (Bates *et al.*, 2015; R Core Team, 2015).

RESULTS

In total, 608 artificial butterflies were placed in the wild (152 chromatic and 152 achromatic on trial 1 and 152 chromatic and 152 achromatic on trial 2). The use of a nylon line allowed us to fully recover the models, 117 (19%) of which were attacked. Tests of homogeneity revealed no evidence that the three localities differed in predation events (Bartlett test: $K^2 = 0.85$, d.f. = 2, $p = 0.651$, Fligner-Killeen test: $\chi^2 = 2.71$, d.f. = 2, $p = 0.257$). The "locality" term did not explain much variation in our model ($s^2 = 0.033$, s.d. = 0.18). There were clear differences in the number of predation events on the models between the two trials (Figure 4.2). I observed no difference in predation of the achromatic butterfly between the two trials (37 on trial 1 and 31 on trial 2). A greater proportion of attacks occurred during the first trial (69 on trial 1 and 48 on trial 2, trial: $z_{604, 608} = -2.35$, $p = 0.018$). Also, aposematic colour models were attacked less overall (colour: $z_{604, 608} = -2.15$, $p = 0.031$). This was mainly due to a reduction in attacks in the second trial (32 on trial 1 and 17 on trial 2), but also compared with the achromatic pattern of the second trial (31 achromatic

and 17 chromatic). However, although the GLMM showed a significant effect of both trial and colour alone, the interaction between trial and colour was not significant (trial*colour: $z_{604, 608} = -1.06$, $p = 0.28$).

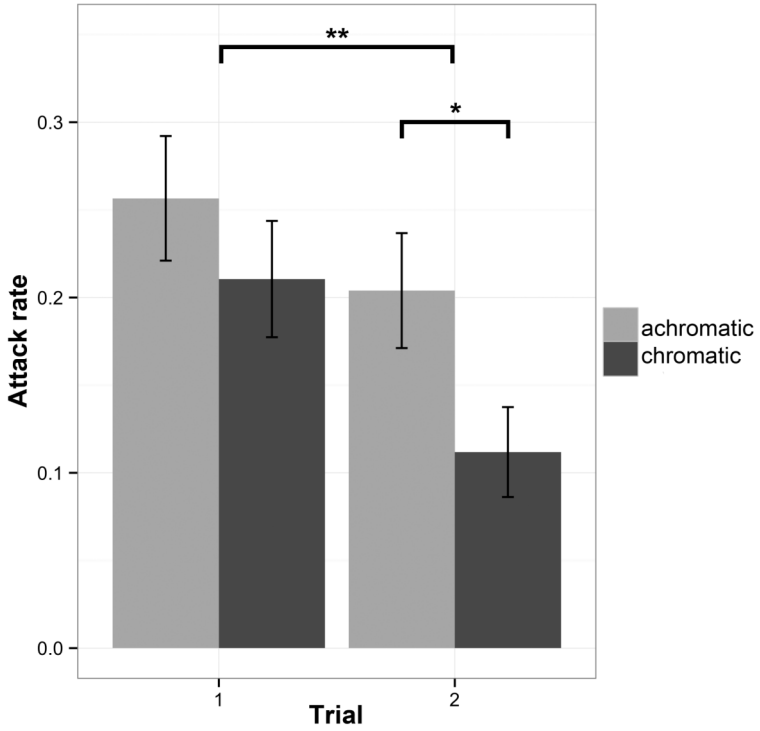

Figure 4.2. Attack rates on chromatic and achromatic models in sequential trials (± SE) during the first and second trial. Asterisks represent statistically significant *p*-values from GLMM comparisons, where * between colours, $p = 0.031$ and ** between trials, $p = 0.018$.

DISCUSSION

I evaluated the influence of aposematic colouration on attack rate by bird predators in a tropical forest. I observed a reduction in attack rates on coloured models as compared to achromatic models, demonstrating a role for colour in enhancing the avoidance of a novel distasteful prey. Many previous experiments have demonstrated the protective value of *Heliconius* warning colour patterns alone (Benson, 1972; Chai, 1986; Mallet & Barton,

1989; Kapan, 2001; Langham, 2004; Merrill *et al.*, 2012; Finkbeiner *et al.*, 2014), including one study which compared chromatic and achromatic prey (Finkbeiner *et al.*, 2014). My results therefore support previous work showing that bright colours enhance the avoidance of aposematic prey, and contributes to an explanation of why aposematic insects in general and *Heliconius* in particular, often evolve bright colouration.

There was a significantly reduced attack rate in the second trial, suggesting that the bad experience of the distasteful model in the first trial may have induced later aversion. Prey palatability is known to influence predator learning and memory of warning colours (Lindström *et al.*, 2006; Skelhorn & Rowe, 2006; Svádová *et al.*, 2009). Having both warning colouration and distastefulness can change predator decision-making and increase avoidance (Servedio, 2000). However, the short time period between trials means that I cannot distinguish between true 'memory' and a short term aversion reaction to explain these results. It would be interesting to repeat similar experiments over different periods of time to test for long-term memory. Predation field studies in tropical forests are challenging and it was not possible to identify predators to demonstrate that the same individual that had a bad experience later avoided the same prey type, so there may be other ecological explanations for my results. Nonetheless, whatever the cause, my experiment supports the prediction of mimicry theory that attack rates on aposematic prey should decline with predator experience.

The avoidance of aposematic patterns is often considered not only as a learned trait but also as an innate response to conspicuous colours, whereby predators are unwilling to eat prey with a novel appearance (Marples *et al.*, 1998; Lee *et al.*, 2010). In addition, a study comparing predation rates on aposematic and cryptic prey, also in field conditions, showed that aposematic prey were completely consumed less often than cryptic prey but partially

consumed more often suggesting "go-slow" predation, in which predators are more cautious with aposematic prey (Carroll & Sherratt, 2013). However, there was no strong support for this in my data, with the two novel patterns equally attacked in the first trial of the experiment. Similar results were found for another *Heliconius* predation experiment in which the "nonlocal" phenotype had higher attack rates (Finkbeiner *et al.*, 2014). Different predators are likely to have different aversion responses to colour, and so the heterogeneity of predators in the wild might explain this result (Endler, 1988; Servedio, 2000; Speed *et al.*, 2000; Endler & Mappes, 2004).

The least attacked prey were the coloured models in the second trial. This suggests that chromatic prey would have triggered a stronger aversion response than the achromatic prey, implying a role for colour in reducing attack rates. However, a test for the interaction between trial and pattern was not significant, so I cannot definitively conclude that colour influenced the reduced response in the second trial, although this seems likely. A power analysis suggested that I would need to approximately quadruple the size of my experiment in order to detect a significant interaction between colour and trial. The results are nonetheless consistent with the idea that colour enhances learning of aversion (Speed, 2000).

Predator psychology models assume that the rate of predation is dependent on learning and forgetting rates, and the absence of reinforcing experiences might lead to forgetfulness (Turner & Speed, 1996; Speed & Turner, 1999; Servedio, 2000; Speed, 2000). For instance Jacamars have been shown to forget novel colour morphs after an interval of two years (Langham, 2004), which might have been due to a lack of reinforcing encounters with the artificial prey. My artificial butterflies were in the sight of predators for 4 days during the trials, which may have led them to be seen several times and which could have

stimulated memory. Occasional sampling in nature also might reinforce memory provided that butterflies can be rejected by sight or by taste, which is a common behaviour among butterfly predators (Chai, 1986; Pinheiro, 2003). Further experiments would be needed to determine whether distasteful models or repeated exposure could trigger long term memory and faster learning rates.

In this experiment, there were no detectable effects of pattern itself as a warning signal, since the distasteful achromatic pattern was equally attacked in both trials. Previous experiments with chicks indicate that colour differences are more memorable than luminance contrast, whereas pattern attracts attention (Osorio *et al.*, 1999a). Nonetheless, previous studies have shown avoidance learning using different patterns (Rowe *et al.*, 2004; Aronsson & Gamberale-Stille, 2008; Rowland *et al.*, 2010) and benefits of pattern mimicry may emerge at later stage in the learning process (Rowe *et al.*, 2004). Given the precise mimicry seen in *Heliconius*, both pattern and colour seem to be vitally important for predator avoidance (Finkbeiner *et al.*, 2014).

The attack frequency of this study was significantly higher than in previous work using artificial *Heliconius* patterns (Merrill *et al.*, 2012; Finkbeiner *et al.*, 2014; Salazar *et al.*, 2014). This may be partly due to the fact that the models represented a novel morph that birds had not experienced before. However, our methodology using suspended butterflies that could move in the wind might also have attracted more predators. This method may therefore be useful for future experiments studying selection on butterfly models.

This experiment indicates that attack rates on novel aposematic butterflies are reduced over time, consistent with experiments on caged birds showing learning of warning colours. Furthermore, I have also shown a role for colour in enhancing aversion towards

aposematic prey. This experiment has shown avoidance of an aposematic butterfly in a tropical forest and contributes to a better understanding of the dynamics of *Heliconius* aposematic mimicry in the wild.

SUPPLEMENTARY INFORMATION

Table S4.1. Chromatic and achromatic contrast (JND) between real wing and printed wing perceived by Blue tit (*Cyanistes caeruleus*) vision. Values > 3 JND denote an increasing ability of discrimination, whereas values ≤ 3 JND denote colours generally indistinguishable from each other. Notice that yellow colour could be closely reproduced in the models and the orange was close but not possible to reproduce accurately.

	JND	
	Chromatic	Achromatic
Orange	7.3	8.7
Yellow	4.9	5.7
Grey Orange	-	7.8
Grey Yellow	-	2.8

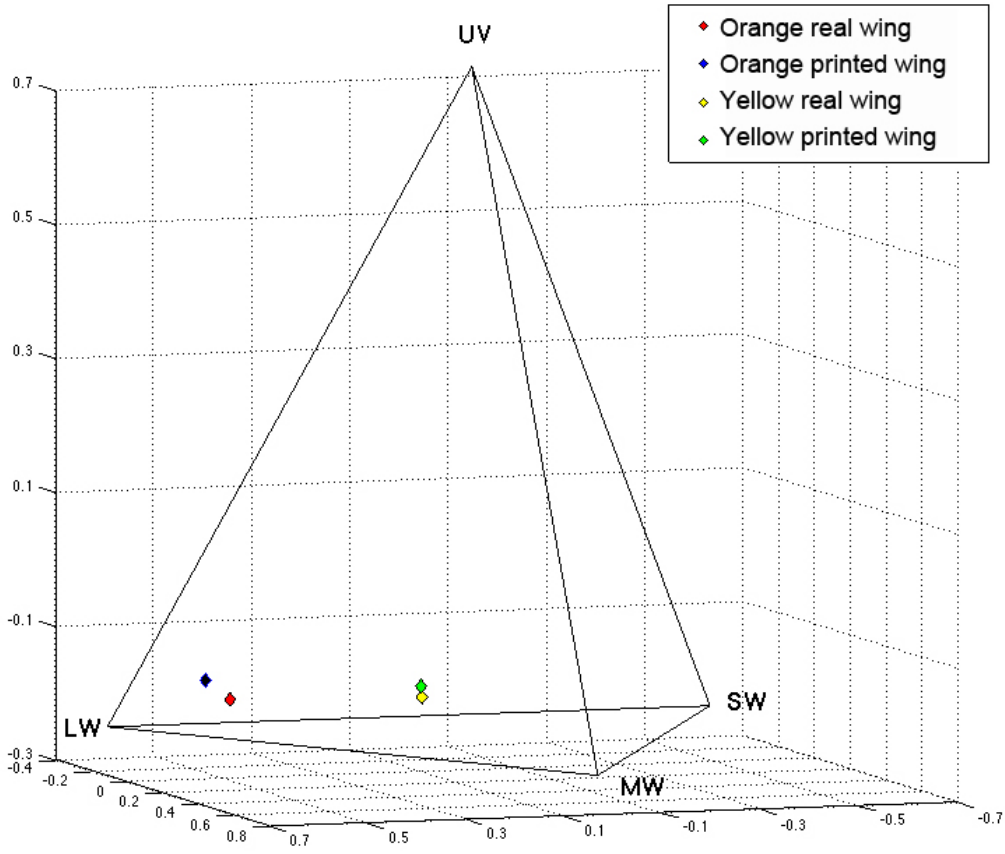

Figure S4.1. Distribution of colours perceived by Bluetit (*Cyanistes caeruleus*) vision in a tetrahedral colour space. Each point is determined by the relative stimulation of the four cone colour channels and each axis represent a channel: ultraviolet (UV), short (SW), medium (MW) and long (LW) wavelength sensitive cones. Notice that yellow colour could be reproduced in the models and the orange was close but not possible to reproduce accurately.

CHAPTER 5

BUTTERFLY LEARNING AND THE DIVERSIFICATION OF PLANT LEAF SHAPE

ABSTRACT

Visual cues are important for insects to find flowers and host plants. It has been proposed that the diversity of leaf shape in *Passiflora* vines could be a result of negative frequency dependent selection driven by visual searching behavior among their butterfly herbivores. Here I tested the hypothesis that *Heliconius* butterflies use leaf shape as a cue to initiate approach towards a host plant. I first tested for the ability to recognize shapes using a food reward conditioning experiment. Butterflies showed an innate preference for flowers with three and five petals. However, they could be trained to increase the frequency of visits to a non-preferred flower with two petals, indicating an ability to learn to associate shape with a reward. Next I investigated shape learning specifically in the context of oviposition by conditioning females to lay eggs on two shoots associated with different artificial leaf shapes: their own host plant, *Passiflora biflora*, and a lanceolate non-*biflora* leaf shape. The conditioning treatment had a significant effect on the approach of butterflies to the two leaf shapes, consistent with a role for shape learning in oviposition behavior. This study is the first to show that *Heliconius* butterflies use shape as a cue for feeding and oviposition, and can learn shape preference for both flowers and leaves. This demonstrates the potential for

Heliconius to drive negative frequency dependent selection on the leaf shape of their *Passiflora* host plants.

INTRODUCTION

Co-evolution between plants and herbivores is a major cause of both plant and insect diversity and adaptation (Ehrlich & Raven, 1964). The role of host shifts and key innovations as a driving force in herbivore diversification has been widely studied. Similarly, in recent years there has been considerable interest in the role of herbivores in promoting plant diversification, specifically through the Janzen-Connell effect (Janzen, 1970; Connell, 1971). This hypothesis states that herbivores could exert negative frequency dependent selection by adapting to exploit the commonest host plants in their local environment. This could in turn favor rare plant species and promote local plant species diversity.

The Janzen-Connell hypothesis has generally been discussed in the context of specialist herbivores preventing the local establishment of common plant species. However, an alternative mechanism is that more generalist herbivores might learn a 'search image' for locally common plant species. This could similarly generate negative frequency dependence, but on a much shorter timescale (Sinervo & Calsbeek, 2006). In visually searching predators, this could be driven by learning of distinctive cues for finding host plants, such as leaf shape. Variation in the size and shape of leaves is often considered to be mainly a result of the physiological and biomechanical demands imposed by different habitats (Brown *et al.*, 1991). The role of herbivores in influencing the evolution of leaf size and shape has mainly considered in the context of physical barriers to herbivory (Brown *et al.*, 1991). The role of leaf shape as an adaptation against visual herbivores has been less well studied, but one

example is leaf mimicry in the *Boquila trifoliolata* vine, which mimics the leaves of its supporting trees to avoid visual herbivores (Gianoli & Carrasco-Urra, 2014).

In order to test the idea that herbivores might use visual cues such as leaf shape in finding their host plants, we need to demonstrate that the relevant herbivores can indeed use shape cues. Shape perception in insects has primarily been studied from the perspective of foraging bees (Anderson, 1977; Zhang *et al.*, 1995), which show a preference for radial patterns when searching for nectar (Lehrer *et al.*, 1995). Monarch butterflies, *Danaus plexippus*, are capable of learning shape only in association with color, showing that both stimuli must appear together in the context of foraging for nectar (Cepero *et al.*, 2015). Furthermore, leaf shape detection and learning has been demonstrated in oviposition preference in *Battus philenor* (Rausher, 1978; Rausher & Papaj, 1983; Papaj, 1986; Weiss & Papaj, 2003) and in *Eurema*, which landed more often on leaves that resemble their host (Mackay & Jones, 1989).

Perhaps the most promising system in which visually searching herbivores interact with a diverse community of leaf shapes is among *Heliconius* butterflies and their *Passiflora* host plants (Gilbert, 1982). Leaf morphology in the family Passifloraceae, both between and within species, is among the most variable observed in any plant group (Figure 5.1a). In any locality, *Passiflora* species exhibit a wide variety of leaf shapes even if they are closely related and inhabit similar physical conditions (Benson *et al.*, 1975; Gilbert, 1975, 1982). Some species also show a huge range of intra-specific variation in shapes, especially between young and old leaves (Gilbert, 1982). For example, *Passiflora suberosa* shows a high degree of leaf plasticity when raised in different light intensities (Barp *et al.*, 2006). In addition, some *Passiflora* are very similar in form and texture to other non-host plants,

which might be a form of mimicry. Gilbert (1975) speculated that visual searching behavior by *Heliconius* butterflies acts as a diversifying evolutionary force on *Passiflora* vines. *Heliconius* larvae feed almost exclusively on the family Passifloraceae, and can cause severe foliage damage (Gilbert, 1982). This close insect-plant interaction has led to the evolution of various defense mechanisms in *Passiflora* plants in response to selective forces imposed by *Heliconius* caterpillars.

The *Heliconius*-*Passiflora* interaction is already well established as an example of insect-host co-evolution. *Passiflora* species possess a range of defensive traits, such as production of chemical compounds that provide feeding barriers (Smiley, 1985a; Engler *et al.*, 2000) and mechanical protection such as hooked trichomes that are able to pierce larvae, resulting in death for the majority of *Heliconius* caterpillars on *P. adenopoda* (Gilbert, 1971). In turn *Heliconius charithonia* has evolved to overcome these trichomes and is the only species that can feed on this host. In addition, extra-floral nectaries on some *Passiflora* species are similar to *Heliconius* eggs (Gilbert, 1982). In *Passiflora cyanea* projections on the stipules resemble, in shape and color, eggs of *Heliconius ethilla* (Williams & Gilbert, 1981). Females avoid ovipositing in the presence of a conspecific egg on the host, as young larvae are often cannibalistic (Nardin & Araújo, 2011), and are therefore deterred by these egg mimics. Egg-mimicry by extra-floral nectaries provides strong evidence that ovipositing females use visual cues in the selection of suitable *Passiflora* vines. Another function of extra-floral nectaries against herbivores is the production of nectar that attracts ants to the plant, to collect this valuable food resource (Apple & Feener Jr., 2001; Izaguirre *et al.*, 2013). Ants in turn attack *Heliconius* larvae and eggs (Smiley, 1985b, 1986).

Based on field observations, females of *Heliconius* butterflies use visual cues while searching for host plants (Brown, 1981). Females may inspect objects that resemble a *Passiflora* structure, such as similarly shaped leaves or vines that look like tendrils (Benson *et al.*, 1975; Gilbert, 1982). A female searching for a specific *Passiflora* plant typically flutters slowly just above the vegetation, periodically approaching and landing on leaves (Figure 5.1b). Upon landing, she drums her forelegs and presumably stimulates tarsal chemoreceptors, allowing the female to "taste" the plant with her gustatory receptors (Briscoe *et al.*, 2013). It has been hypothesized that *Passiflora* leaf shape variation might make it harder for *Heliconius* females to detect host plants.

However shape detection has not yet been demonstrated in *Heliconius* butterflies. They can be trained to associate a color stimulus with a food reward, demonstrating a high precision of discrimination and learning (Swihart & Swihart, 1970; Swihart, 1971; Blackiston *et al.*, 2011). Here I extend these experiments to show that *Heliconius erato* can be trained to associate a shape cue with a food reward, demonstrating the perceptual ability to detect and distinguish shapes. Next, I tested shape perception for leaf morphology using ovipositing females trained on artificial leaves. *Heliconius erato* naturally feeds on three species with diverse leaf shapes in our study area, and our results show that learnt shape preference is therefore a plausible selective force on *Passiflora* leaf morphology in this community.

Figure 5.1. *Passiflora* species that occur in Gamboa or near by Soberanía National Park, Panama, highly differ in leaf morphology. **(A)** From left to right: top, *P. ambigua*, *P. biflora*, *P. edulis*; bottom, *P. coriacea*, *P. menispermifolia*, *P. auriculata*. **(B)** *Heliconius erato demophoon* female laying egg on a *P. biflora* shoot.

MATERIAL AND METHODS

Butterfly rearing

Experiments were performed between March 2014 and August 2015 in insectary facilities located in Gamboa, Panama. Wild adults of *Heliconius erato* Linnaeus, 1758 were caught in the surrounding areas and kept in insectary cages for egg collection. Caterpillars were reared on *Passiflora biflora* Lam. leaves. Adults were fed with sugar solution and pollen from *Psiguria* sp. flowers and were around maximum 2 weeks old at the beginning of the training period.

Flower shape experiment

This experiment was designed to test whether *Heliconius* butterflies can perceive shapes using a learning experiment with a food reward. As shapes are defined in terms of the luminance contrast at their boundaries against the background (Zhang *et al.*, 1995), five

flower shapes were chosen varying the number of petals (zero, two, three, four and five, Figure 5.2), generating marked differences in the shape edges and perimeter. Artificial flowers were constructed of red foam sheets (ethylene-vinyl acetate) with a 1 ml Eppendorf tube for sugar water solution attached in the centre. The color red was chosen to facilitate association of model flowers with food, because most of the flowers used by *Heliconius* have this coloration (Estrada & Jiggins, 2002). Prior to the experiment adult butterflies were fed with a sugar solution presented in feeders made of red card to increase the association of color and food. Butterflies were subjected to over-night food deprivation to ensure they would be willing to feed. Groups of five to six butterflies were separated in a different cage for the experiment, both females and males.

The first part of this experiment was designed to demonstrate spontaneous feeding preferences and to determine innate choice of flower shapes. A set of five shapes was presented, none of which contained a food reward. The relative position of flowers was randomized. The first choice of flower, and the number of feeding attempts in which the butterfly landed on the artificial flower and probed with its proboscis were recorded for 30min. Over the following 8 days, butterflies were presented with the least preferred shape from the first trial (the two-petal shape) with sugar solution, while the other shapes contained only water. The shape choice trial was then repeated by again presenting the set of five shapes without a food reward, using the same method described above. I aimed to determine whether feeding experience could modify initial feeding preferences through learning. All experiments were performed in the early morning when butterflies were active and willing to feed and the experimental flowers provided the first food source of the day. After the

experiments, butterflies were allowed to feed on *Psiguria* sp. flowers for pollen. Butterflies were tested only once and were not re-used for the subsequent experiment.

Leaf shape experiment

Following the results of the first experiment, I then wanted to determine whether shape perception also functions in the choice of plants for oviposition. Two artificial leaf models were constructed from green foam sheet (ethylene-vinyl acetate), one *P. biflora* leaf shape, which is depressed obovate with two lateral lobes, and one non-*biflora* lanceolate leaf shape (Figure 5.3). The shapes were generated using real leaves (approx. width x height and area: *biflora* = 10 x 8 cm and 25 cm^2; non-*biflora* = 6 x 12 cm and 21 cm^2). Four artificial leaves were attached to a metal frame onto which a young *P. biflora* shoot without leaves was also attached (~70 cm high). The shoot was placed in a bottle of water, located on the floor and at the center of the cage. *P. biflora* shoots were used, which is the most common host plant for *H. erato* in Gamboa. It was anticipated that preference for the leaf shape of this species might be the innate response for this species (Smiley, 1978). The stimulus combination of the green leaf with the real plant shoot odor and taste was shown to be sufficient to stimulate oviposition by *Heliconius* butterflies.

Adult females were kept in 2 m^3 insectaries cages without *Passiflora* plants. All females were mated prior to the experiments. Females were randomly separated into two different training cages: the *biflora* shape training with only *P. biflora* artificial leaves, and the non-*biflora* shape training with only lanceolate artificial leaves. Females were free to lay eggs on the young shoots, which were replaced daily. Eggs were counted and collected every day to confirm that females were actively laying eggs on the shoots with artificial leaves.

The females were kept in the training cage for a minimum of 8 days, and then moved to a cage without plants for 2 days. Next, a choice experiment was performed, presenting a single focal female with a choice between two leaf shapes, *biflora* and non-*biflora* artificial leaves in the same set up as the training period, and placed 1 m apart. The female was observed for 30 min, and the first leaf choice, the number of approaches (flying around the stimulus to within a 15 cm distance), number of landings and eggs laid on the shoot associated with each leaf shape were recorded. Each individual butterfly was tested twice using this choice experiment, totaling 1 hour of observation. Results from the two trials were combined for analysis.

Statistical analysis

All statistical analysis was carried out in R (R Core Team, 2015) with *multcomp* package (Hothorn *et al.*, 2008). In the flower shape analysis, I used a general linear model (GLM) in each trial for first choice (binomial) and number of feeding attempts (individuals as random factor), followed by *post hoc* tests for the significance of pairwise comparisons when relevant. I calculated the effect of flower area and perimeter on the number of feeding attempts and first choice. I also calculated the interaction between trial (innate and learnt) and flower shape (zero, two, three, four and five) for first choice and number of feeding attempts. There were no differences in behavior between females and males, so both sexes were considered together in the analyses. In the leaf shape analysis, I used a binomial GLM with prior weights, in which the proportion of successes was the response factor weighted by the total number of approaches, landing and eggs, to test for an interaction between leaf choice (*biflora* or non-*biflora*) and training regime (on *biflora* and non-*biflora*). I used a

Pearson's Chi-squared test (with simulated p-value) for first leaf approach data for given proportions.

RESULTS

Flower shape learning

I recorded a total of 112 feeding attempts during the innate behavior trial and 126 feeding attempts during the learned behavior trial of 53 butterflies. The results indicate that there was a distinctive preference for certain flower shapes. The butterflies showed a preference for the more flower like patterns, with the two-petal flower chosen significantly less than three and five petals as first choice (2 petals: $z_{260} = -2.957$, $P = 0.003$; *post hoc*: 2-3 petals, $P = 0.023$; 2-5 petals, $P = 0.022$) (Figure 5.2a). In contrast, the number of feeding attempts during the innate trial did not differ significantly (2 petals: $t_{237} = -1.285$, $P = 0.2$) (Figure 5.2b). Neither area nor perimeter influenced number of feeding attempts (area: $t_{239} = 0.195$, $P = 0.845$; perimeter: $t_{239} = 1.189$, $P = 0.236$), but perimeter influenced first choice (area: $t_{262} = 1.033$, $P = 0.302$; perimeter: $t_{262} = 3.353$, $P = 0.0009$).

I then trained the butterflies on their less preferred two petal shape. After the training period, the frequency of visits to the two petal artificial flower increased from 14% to 36% of all visits (46/126). The first feeding attempt preference shifted significantly to the two petal model flower ($z_{260} = 2.334$, $P = 0.019$) (Figure 5.2a), and number of feeding attempts also differed significantly ($t_{217} = 4.218$, $P < 0.001$) (Figure 5.2b). There was a significant interaction between petals and trials for first choice (2 petals*learnt: $z_{520} = 3.75$, $P = 0.0001$), and for number of feeding attempts (2 petals*learnt: $z_{485} = 3.446$, $P = 0.0006$). In addition, the learnt response in terms of number of feeding attempts was influenced by

flower perimeter, but not by area (area: $t_{219} = -1.198$, $P = 0.232$; perimeter: $t_{219} = -3.185$, $P = 0.0016$). Similar results are seen for the first choice data (area: $t_{262} = -0.482$, $P = 0.63$; perimeter: $t_{262} = -2.286$, $P = 0.023$).

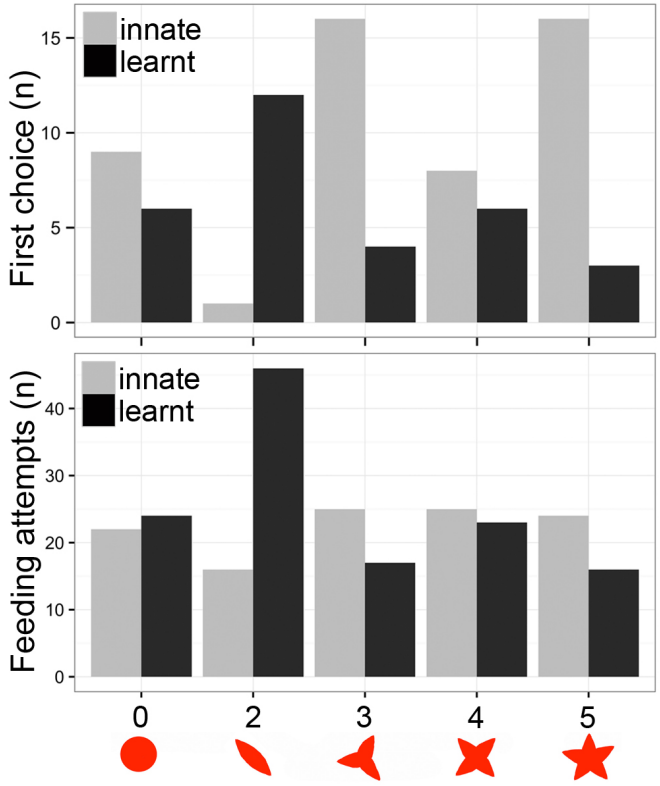

Figure 5.2. *Heliconius* butterflies learnt to associate flower shape with a food reward. Top: number of individuals that selected each shape as first choice. Bottom: number of feeding attempts to the five shapes during the assays. The five shapes correspond to no-petals, two, three, four and five petal artificial flowers. Trials: Innate response, grey bars; Learnt response, black bars.

Leaf shape choice

I trained 12 *H. erato* butterflies on *biflora* artificial leaves and 14 on non-*biflora* artificial leaves. There was a significant effect of both leaf shape and trial on approach probability. In addition, there was also a significant interaction between training regime and approach probability, demonstrating evidence for learning. Butterflies experienced with non-*biflora*

leaf models were subsequently more likely to approach the non-*biflora* leaf shape than butterflies experienced with *biflora* leaf models (training*leaf choice: z_{48}=2.592, *P*=0.0095) (Figure 5.3a). I also found significant differences for first leaf approach (χ^2 = 4.147, *p* = 0.041) (Figure 5.3c). However, the preference for landing did not differ between the two training groups (training*leaf choice: z_{48} = -0.116, *P* = 0.908) (Figure 5.3b).

Females trained on non-*biflora* leaves laid 46% on non-*biflora*, while females trained on *biflora* leaves laid 47% of the eggs on non-*biflora*. There was therefore no significant difference in eggs laid on the shoots between the two training regimes (z_{49} = -0.132, *P* = 0.895).

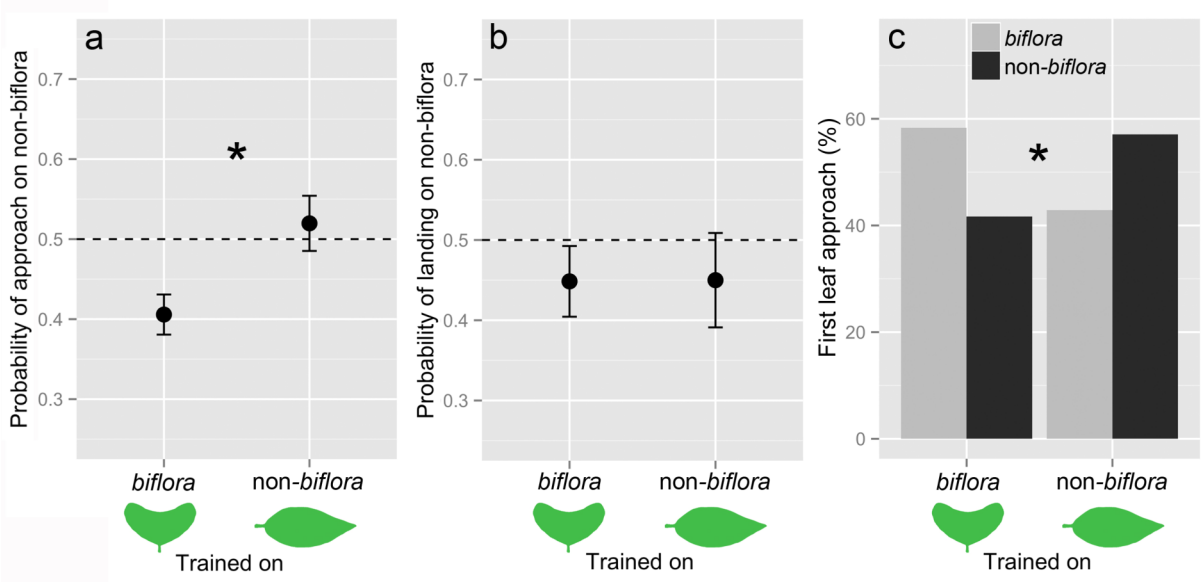

Figure 5.3. Female butterflies were more likely to approach the leaf shape on which they had been trained. Probabilities of (a) approach and (b) landing on the leaf shapes (mean ± SE). (c) Proportion of first leaf approach: *Biflora*, grey bars; Non-*biflora*, black bars; and * $P < 0.05$.

DISCUSSION

Here I have shown for the first time that *Heliconius* butterflies can use shape cues to search for both flowers and leaves during feeding and oviposition. One explanation for the observed spontaneous preference for three and five petal flower shapes is an innate preference for radial symmetry, which corresponds to the actinomorphic flowers used most commonly by *Heliconius* (Corrêa *et al.*, 2001). After training, I conditioned individuals to shift their shape feeding preference to an artificial flower with two petals, the least preferred shape initially. Previous studies have shown that conditioned *Heliconius* butterflies can shift their preference to yellow and green flowers, against their innate preference for orange and red (Swihart & Swihart, 1970). Here I show a similar effect for shape cues. Color is perhaps a more reliable visual cue for finding flowers since it is not affected by the angle of approach, as observed in Monarch butterflies (Cepero *et al.*, 2015). However, shape is a complementary cue and may be important to distinguish objects that are similar in color.

The pipevine swallowtail butterfly, *Battus philenor*, has long been known to use leaf shape in oviposition, in experiments in which the butterflies were trained on both real plants (Papaj, 1986) and artificial leaf models (Rausher, 1978; Allard & Papaj, 1996; Weiss & Papaj, 2003). Here I have provided the first evidence that *Heliconius* also use shape for leaf detection. Our results therefore support field observations of *Heliconius* female butterflies visually discriminating different leaves while searching for host plants. The butterflies exposed to lanceolate leaves approach the lanceolate shape more than those exposed to *P. biflora* shape. It is worth noting that my artificial plants did not provide a negative stimulus against laying eggs during the training period, in contrast to the way that the flower shape experiment could provide a negative stimulus in terms of the absence of sugar water. The

difference in response of the butterflies to the two leaf shape treatments is therefore the result of a learnt association between leaf shape cue and availability of an oviposition stimulus.

The lack of any significant difference between treatments in egg laying perhaps indicates that shape is not the only clue used to oviposit on host plants. Other cues such as leaf color, plant architecture and plant odor and taste are also likely to be extremely important (Rausher, 1978; Allard & Papaj, 1996). Specifically, in the confined insectary space female butterflies do not have trouble in eventually finding the shoots, irrespective of their associated leaf shape. Thus, once they have found both shoots the optimal strategy for a female is to distribute her available eggs evenly between the two shoots. Nonetheless, in the wild where long distance detection of host plants is likely to be more challenging than in a small insectary cage, it seems likely that leaf shape could play an important role in the location of host plants used by female butterflies.

I can therefore speculate about the potential for this learning behavior to influence the evolution of leaf shape in *Passiflora*. It has been previously suggested that diversification of leaf morphology might be a response to herbivore pressure (Gilbert, 1975; Rausher, 1978). Three elements of *Passiflora* leaf morphology may have evolved in response to *Heliconius* visual perception: mimicry, divergence in leaf shape between species and different adult and juvenile foliage (Gilbert, 1982). Negative frequency dependent selection could favor leaf polymorphism, as an unusual or rare leaf morphology would be more likely to escape the attentions of ovipositing butterflies using shape cues. My results support this and I suggest that this might be an example of "enemy free space" competition (Jeffries &

Lawton, 1984; Brown *et al.*, 1991) between *Passiflora* plants for survival against *Heliconius* caterpillars.

Heliconius females can show strong host plant preferences that may not be perfectly aligned with larval food preference and survival (Copp & Davenport, 1978; Smiley, 1978; Kerpel & Moreira, 2005; Silva *et al.*, 2014). The ability to learn to associate new leaf shapes with oviposition sites may allow females to tailor their search image to the local *Passiflora* community. Specifically, in the case of *H. erato*, there are three important host species in the Gamboa area which have dramatically different leaf shapes, *P. biflora*, *P. auriculata* and *P. coriaceae* (Merrill *et al.*, 2013). Furthermore, there is considerable variation in all three species both between individuals and between young and old leaves. It seems plausible that visual searching behavior by *H. erato* could play a role both in promoting the coexistence of these three species, and as a selective pressure favoring the evolution and maintenance of within species leaf shape diversity.

The Janzen-Connell hypothesis proposes that interactions between parasites and their host could be a driving force in maintaining plant species diversity (Wright, 2002) and even egg coloration polymorphism (Yang *et al.*, 2010). Here I have demonstrated the potential for behavioral plasticity in animal responses to play a role in maintaining plant species diversity. If generalist herbivores commonly learn a 'search image' for locally common plant species this could be an important source of negative frequency dependent selection favoring rare plant species. In the highly diverse and complex tropical rainforest environment, such an effect might play a role in maintaining species diversity and in particular in sustaining populations of rare species.

CHAPTER 6

THESIS CONCLUSION

In this thesis, I have combined sensory ecology with behavioural ecology to explain ecological interactions between *Heliconius* butterflies, their predators and host plants. I mainly focus on coloration and vision, and through that I provide insights into how differences in colouration may interact with behavioural traits and how this could influence the evolution of predation and reproductive isolation in *Heliconius* butterflies. Colour has long been the main topic for many behavioural and evolutionary studies, especially because as humans we are very 'visual animals' ourselves. However, other animals perceive visual signals in a very distinct manner as compared to human perception. The study of coloration has undergone great advances in recent years, primarily because of new technologies and methodologies (Stevens *et al.*, 2007).

Vision models have been applied to answer several questions in ecology and evolution. Opponent channels have been used to investigate signals of red and yellow fruit colours over the course of a day (Lovell *et al.*, 2005) and warning coloration of ladybird beetles against natural backgrounds (Arenas *et al.*, 2014). Also, the visual discrimination model by Vorobyev and Osorio (1998) has been widely applied in visual ecology to predict behaviour, such as to quantify host-parasite egg colouration (Spottiswoode & Stevens, 2012), micro-habitat choice by camouflaged lizards (Marshall & Stevens, 2014; Marshall *et al.*, 2016) and colour signals in the poison frog *Dendrobates pumilio* (Siddiqi *et al.*, 2004).

In the same way, I applied visual models using both bird and butterfly vision on *Heliconius* co-mimics, establishing a link between perception and behaviour, which is crucial to understand evolutionary ecology of communication systems.

Using new tools for colour analysis, I could address old questions about mimicry in *Heliconius*. In **Chapter 2** and **Chapter 3** I show differences in visual sensitivities of avian predators, *H. erato* females and males lead them to perceive *Heliconius* coloration in different ways. My results suggest that having the ability to see in the ultra-violet light range enables higher discrimination between co-mimics both for birds and butterflies. Although different mimetic rings of *Heliconius* butterflies occur in different light environments, their warning colours transmit a consistent signal across time of the day and habitat in a tropical forest for avian vision. In contrast through *Heliconius* vision there is evidence that patterns are more conspicuous in their own habitats. The increased conspicuousness of *Heliconius* colours to conspecifics compared to avian predators potentially enhances mate detection. Predator signalling and inter-specific communication are two confliction demands of natural and sexual selection that are acting together on *Heliconius* ecology, and are maintained by a balance between both.

All these traits facilitate communication between co-mimics and could reduce the cost of confusion in courtship while still maintaining the advantages of Müllerian mimicry against predation. Although my results make predictions about the behaviour of *Heliconius* predators and conspecifics, these need to be confirmed using behavioural experiments in which colour differences are manipulated. Also, future work should explore how vision differences within *Heliconius* correlate with the use of microhabitats, which might improve sexual signalling and potentially lead to reproductive isolation.

Studying the dynamics of *Heliconius* aposematic mimicry in the wild was always a challenge due to the difficulty of measuring predation in a tropical forest. In **Chapter 4**, I demonstrate that attack rates are reduced over time when predators face a distasteful coloured butterfly, showing the role of colour in enhancing aversion towards aposematic prey. Artificial models have successfully been used in several other non butterfly studies as a tool to test anti-predation responses from birds, such as in amphibians (Rojas *et al.*, 2014), lizards (Marshall *et al.*, 2015) and snakes (Niskanen & Mappes, 2005; Dell'Aglio *et al.*, 2012). Here, the methodology I used of suspending butterflies in branches may have attracted more predators than previous studies using *Heliconius* paper models (Merrill *et al.*, 2012; Finkbeiner *et al.*, 2014; Salazar *et al.*, 2014). This method may therefore be useful for future experiments studying selection on butterfly models.

In **Chapter 5**, I reveal the potential for behavioural plasticity in *Heliconius* responses to play a role in maintaining *Passiflora* species diversity. It has been previously suggested that diversification of *Passiflora* leaf morphology might be a response to herbivore pressure (Gilbert, 1975, 1982), but this has never been tested until now. The visual searching behaviour of *Heliconius* might play a role in promoting coexistence of *Passiflora* species and with these results I present new evidence relevant to *Heliconius*-*Passiflora* co-evolution. Studies of co-evolution show how specialized relationships between species can lead to reciprocal evolutionary changes. Interactions between parasites and their hosts could be a driving force in maintaining species diversity, such as egg colouration polymorphism seen in avian brood parasites and their hosts (Yang *et al.*, 2010; Spottiswoode & Stevens, 2011).

Overall my results highlight ecological interactions between *Heliconius* butterflies and their surroundings. It supports the understanding of the maintenance of diversity in

mimicry, such as new pattern emergence and establishment. Nonetheless, a number of questions remain to be answered. Future research directions in this field include understanding the ecology of mimicry, finding the main predators, and how they learn and when predation occurs in wild. Field and insectary behaviour experiments are necessary to provide more robust evidences for the vision models used here. Colour differences are only one modality used by mimetic butterflies during mate choice, which is also influenced by other mating cues like pheromones and behaviour. Also, it would be interesting to understand the genetic basis for more complex traits, such as behavioural traits that contribute to reproductive and ecological isolation.

Further studies should consider signal honesty and toxicity of *Heliconius* butterflies in terms of predation and physiological costs. Also, **Chapter 5** is just the beginning of solving the question of *Passiflora* leaf shape diversity. Research on plant volatiles and *Heliconius* chemosensory systems might lead to the identification of additional factors that drive host plant choice. The understanding of *Heliconius* ecological interactions have been increasing in the last 150 years and more progress is to come (Merrill *et al.*, 2015).

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