12-12-2016

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A dissertation submitted to the Graduate School at the University of Missouri-St. Louis in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Biology with an emphasis in Ecology, Evolution and Systematics

December 2016

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Acknowledgments

A Ph.D. does not begin in graduate school, it starts with the encouragement and training you receive before even setting foot into a University. I have always been fortunate to have kind, helpful and brilliant mentors throughout my entire life who have taken the time to support me. For the last decade, one of those mentors has been Dr. Godfrey R. Bourne (Godfrey). Godfrey, formally introduced me to behavioral ecology and taught me the skills to observe the natural world, for this I am eternally indebted as he had turned my fascination of animal behavior into a passion and my passion into a future career. The understanding and support Godfrey has shown me through all of the lows of my Ph.D., especially after the death of my father is one of the reasons I was able to return to St. Louis to complete my degree. Words cannot describe how grateful I am for the kindness and hospitality him and his wife (Dr. Carol Bourne) have shown me from the moment I arrived in St. Louis. As an academic advisor, Godfrey, has not only taught me observational skills, he has honed my critical thinking, experimental design and writing skills. Godfrey has worked tirelessly with me, putting in countless hours and providing never ending patience and guidance over the course of my Ph.D.

After Godfrey retired, Dr. Aimee S. Dunlap became my supervisor and it has been a pleasure to work with Aimee. In my second semester at UMSL, Aimee gave me the opportunity to work with her on one of her projects as a research assistant and this experience has helped me to develop confidence and numerous behavioral experimental design ideas, which I have used to develop my own research. Even before Aimee was my supervisor, she has always made time for me to discuss my ideas and has often acted as a sounding board. In addition to Aimee, there are others such as Dr. Yuefeng Wu, Dr.

Nathan Muchhala and Dr. Jessica Ware who have all been very generous with their time. Dr. Wu has made substantial contributions to the success of my final field season as he helped me with the design of my field experiments and patiently guided me through the analyses of my data. Dr. Wu has made me appreciate behavioral ecology through the eyes of a Mathematician! Without the guidance of Dr. Muchhala, none of my color work would be possible, he was a tremendous help along every step—from choosing the correct spectrophotometer to the creation of color spaces. Dr. Jessica Ware, helped me to link behavior to its genetic underpinnings, she personally trained me to do PCRs, gels and genetic analyses in her lab and encouraged me, through all my failures.

I am also very thankful to Oceana O'Dean and Hannah Stowe who have been excellent field assistants and Brian Waldrop without whose help I could not have completed my butterfly house experiments. I would like to express my gratitude to Joyce Wade, who always ensured I was well taken care of at CEIBA Biological Center and the kind and friendly staff of the Chesterfield Butterfly house, especially Laura Chisholm and Tad Yankoski for all their help with procuring the specimens and facilitating our work at the butterfly house. To Maryann Hempen and Kathy Burney- Miller, thank you for helping me sort through all my administrative issues. To the staff of the Guyana EPA, especially Diana Fernandes and Vidyanand Mohabir, for all of their assistance with my research permits. And, the University of Guyana for this staff development opportunity, in particular Diana Seecharran, Raihaana Ali, Calvin Bernard and Dr. Adil Ansari. And of course my funding agencies, Government of Guyana, UMSL, Idea Wild, Rufford, CIEBA and the Biology Graduate Student Association.

Finally, to my parents who never saw the limit to what I can achieve, my father who taught me kindness to the very end and to my mother who instilled unbreakable strength and to my all wonderful family and friends in Guyana and the USA who not only, shared their technical expertise, they have always supported me and kept me sane! Samoa Asigau, Ryan Russell, Isabel Loza, Isabel Rojas, Oyomoare Osazuwa-Peters, Fidisoa Rasambainarivo, Haydee Hernández, Mellisa Sanchez-Herrera, Ciara Mendoza, Catalina Guzman and so many others, thank you!

Contents

Dissertation Abstract

Colorful floral signaling and resulting insect foraging behaviors have only been extensively examined in hymenopteran pollinators, especially bees, in comparison to flies, beetles, and butterflies regardless of their ecological importance. Therefore, my study provides novel information by focusing on foraging behaviors of adult passionflower butterflies, *Heliconius melpomene* and *Dryas iulia*, to the color changing flowers of *Lantana camara*. My dissertation which is divided into four chapters, aims to explore various aspects of color mediate foraging in passionflower butterflies by combining observations in the wild with controlled field and laboratory experiments. In the first chapter I reviewed flower color development and pollinators' sensory mechanisms to detect color changes to first elucidate the evolution of communication tactics from the senders (plants), and the detection mechanisms used by receivers (pollinators). In the second chapter I examined the relationship between sexual and foraging color biases of butterflies. In my third chapter I determined how color change associated with reward differences affected pollinator-plant attraction; and for my final chapter I investigated foraging movement patterns as butterflies fed on *L. camara* plants in their natural habitat. Overall, I presented evidence that indicated the following: 1) *L. camara* evolved a generalized pollination visitation system based on honest signaling—of reward quantity and quality tied to color changing visual signals acting in consort to produce a billboard effect that was easily perceived and deciphered by both passionflower butterflies; 2) experienced butterflies fed at flowers and were attracted to inflorescences that were of similar color to their wings, however, newly emerged butterflies exhibited different but species specific behaviors; 3) foraging behaviors were

subject to change based on light environment, with yellow flower color eliciting feeding responses under blue light (open sky), and red elicited foraging under green light conditions (under forest canopy); 4) butterflies partitioned food resources spatially and temporally from each other, and from aggressive territorial hummingbirds; and 5) butterfly species changed the number of visits to plants, number of plants visited, and time spent foraging in order to successfully coexist with heterospecific competitors that shared the same space and food resource.

Chapter 1

Floral color signals and their Heliconiid butterfly receivers

Abstract

Signals vary in type and function. However, regardless of the signal, effective transmission and receiver detection is needed in order for communication to exist. This review focuses on visual color signals used by plants to attract pollinators. It specifically focuses on the relationship between floral color and Lepidopteran pollinator attraction. I focus on butterflies because, although, the effect of floral color signals on the behavior of pollinators has been studied extensively in bees, little work has been carried out on non-hymenopteran pollinators, despite their ecological importance. In addition, signal detection work has strongly focused on epigamic signals; therefore, this review adds to the body of knowledge on non-sexual signal communication. In this review I investigate what are visual signals as it relates to pollinators, why they develop and how the presence of these signals in the environment affect the behaviors of animals with which they communicate. I focus my review specifically on visual color signals used by Angiosperms flowers and I look at the pollinators' need to forage balanced by the plants' need for pollination. I also detail the visual systems used by pollinators, specifically Heliconiid butterflies, to detect these signals. I have found that signals in nature vary, however, the two of the main driving forces in the evolution of signal for all organisms is the need to find food and mates. In order to attract potential pollinators, Angiosperms have evolved many characteristics, that serve as signals and exploit these driving forces in order to attract animals. One of the primary signals used by Angiosperms include floral

color that attract a variety of visually-oriented pollinators, such as butterflies. Butterflies possess compound eyes with ultraviolet, blue and longwave length sensitive opsin genes and many duplications of these genes allowing them to have one of the widest visual ranges in the animal kingdom. As such, they use color signals in many different aspects of their lives, including mating and foraging. However, although their color preferences for these behaviors has been demonstrated independently, similarities/differences between their preferences have not yet been shown.

Keywords: signal, pollinator, floral color, visual system, butterfly

Introduction

It is recognized that various signals such as color, sound, vibration, scent among others, play a pivotal role in attracting animals to con- and heterospecifics (Shettleworth, 2009). Receiver's choice is based on an evaluation process whereby these signals are detected and subsequently discriminated (Heinrich 1975, Gumbert 2000, Andersson and Dobson 2003, Goulson *et al.* 2007, Raine and Chittka 2007, Ings *et al.* 2009). Although the idea of biological signals and its detection have existed since Darwin (1871) and his theories on sexual selection, the theory of signal detection was based on the founding work of Green and Swets (1966). Initially this idea of detecting a signal was used by radar researchers and in 1954 Peterson, Birdsall, Fox, Tanner, Green and Swets developed the theoretical framework for the signal detection theory (SDT) with Green and Swets (1966) going on to develop methods for psychophysics, many of which are used today (Abdi 2007). The central strategy of SDT is to manipulate the decision criterion through experiments in order to expose the sensitivity factors that remain unchanged (Macmillan 2002). Recent work on signal detection varies from fields such as biology, diagnostics, and psychology, among others. This review focuses on signal detection theory as it relates to color bias in butterflies, where they are more likely to respond to one color than another. Specifically, this review focuses on color bias of Lepidopteran pollinators and their response to plant signals. I focus on Lepidopteran as they represent an understudied group of taxa in the area of color detection despite their importance as pollinators, their known reliance on color in variety of behavioral contexts and their range of light perception is one of the broadest of all animals (Briscoe and Chittka 2001, Blackiston *et al.* 2011).

Color is one of the most important signals used in nature and the diversity in physical appearance of both plants and animals coupled with their receivers' ability to detect these traits is a testament to this, as it is in part explained by the multiplicity of signals used for communication between and within taxa (Endler 1992). Color and the use of visual displays that incorporate color are quite ubiquitous in many animal and plant taxa and these signals are used for a wide range of behavior such as; foraging, mate recognition and selection, recognition of members of their own species and various other forms of inter- and intra-specific communication, such as those between predator and prey and pollinator and plants, etc. (Osorio and Vorobyev 2008). Angiosperms in particular exhibit many colors and these are often used to communicate with their pollinators (Quattrocchio *et al.* 1999, Muchhala *et al*. 2014). These pollinators in turn have complex visual systems that allow for the discrimination of various wavelength of light (Sison-Mangus *et al*. 2006).

Although signal use spans such a wide range, the study of signals in organisms have been very narrow, mainly focusing on sexual selection (Schaefer *et al.* 2004, Pohl *et al.* 2011, Ryan and Cummings 2013). This review chapter aims to add to the body of knowledge on biological signals by focusing on floral color signals used by plants to attract their butterfly pollinators, it highlights *Heliconius* butterflies as they are known to use color in elaborated mimicry rings and as aposematic signals (Bybee *et al.* 2012). It looks at the evolution of visual signals and the use of these signals by these pollinators. This review also examines floral color and factors that drive its development and the mechanisms used by these Lepidopteran pollinators to detect this signal thereby adding to

the sparse non-hymenopteran, specifically non-bee, literature available in this area of study.

Visual Signals

Natural selection only favors signaling behavior if the signal strength is greater than background noise and thus can be detected clearly and effectively by receptors (Endler 1992). As such, signals, receptors and behavior are evolutionarily dependent traits and the evolution of one is likely to influence the evolution of another. For example, the visual signals of many fishes evolved in tandem with their visual systems (Briscoe *et al.* 2009). The factors driving the evolution of signals, receptors and behavior include: the *environment* in which the organism is found, *biophysics* such as communication between sender and receiver, ability to sensing the environment and foraging choices and, the *neurobiological systems* of the taxa, a few of which are seen in figure 1 (Endler 1992).

Plants use many types of visual signals involving both vegetative and reproductive parts (Hamilton and Brown 2001, Schaefer *et al.* 2004). Although I focus this review on flower color and insect attraction, it is recognized that this idea of use of floral color signals by plants is not in restricted to flowers, as fruits (Schaefer *et al.* 2004) and even leaves (Hamilton and Brown 2001) exploit insect color preferences. I focus on plant-pollinator signals as this provides unique insights into plant communication and the animals that interact with them and a direct way in which facets of signal theory can be directly tested such as honesty signals and sensory drive hypothesis.

Visual Signals — why did they evolve?

Two of the main factors driving the evolution of signals, receptors and signaling behavior stem from the basic need of all organisms to find food and mates (Ryan and Cummings 2013). Work by Allen (1879) links these two basic needs, as he proposed that color vision evolved as a food finding tool used to locate the edible parts of plants and this lead to secondary color preferences such as those for mate attraction and conspecific identification (Osorio and Vorobyev 2008, Bybee *et al.* 2012). Ryan and Cummings (2013) show that in addition to the cognitive processes of the receiver such as its preference for a particular trait of its potential mate there are many organisms in which intraspecific mating preferences can also be influenced by various perceptual biases such as foraging (Ryan and Cummings 2013). Owing to the need to feed, many males are able to use food biases to attract females (sensory bias). Examples of these include guppies that exploit the female penchant for orange food, water mites that vibrate their legs like prey and male swordtail characins that mimic prey (Rodd *et al.* 2002, Kokko *et al.* 2003, Smith *et al.* 2004, Bourne and Watson 2009, Ryan and Cummings 2013). Thus these senders evolved signals to exploit preexisting biases for food in receivers.

In butterflies, in addition to food, visual color signals are needed for mate selection and conspecific identification, especially in *Heliconius* due to the presences of elaborate Müllerian mimicry rings, where several distasteful species in a given area share a common warning signal used in predator deterrence, that show a convergence of pattern both between close and distantly related species (Mallet *et al.* 1998, Jiggins *et al.* 2004, Briscoe *et al.* 2009, Klein and de Araujo 2010). From research using colored models of the mimetic *Hypolimnas misippus* and melanic mimic forms of *Papilio glaucus,* it has

been shown that butterflies depend on wing color for mate recognition and selection (Jigging *et al.* 2004). Further, research by Arikawa *et al.* (2005) demonstrates that there is a co-evolutionary relationship between wing color and color vision as seen by the sexually dimorphic violet receptors of *Pieris rapae crucivora* which are used to discriminate between male and females. Briscoe and colleagues (2009) also show that *Heliconius* spp. possess positively selected UV opsins that allow detection of distinct yellow colors found on the wings of conspecifics. Additionally, *Heliconius* spp. are able to use these yellow wing markings to recognize and attract mates; e.g., in *H. pachinus, H. cydno, H. melpomene* and *H. erato* where females lacking these markings were less attractive to males (Jiggins *et al.* 2001, Briscoe *et al.* 2009). Therefore, in these species and among other *Heliconius* spp. mate preference is known to co-evolve with wing color as races are more attracted to their own color patterns (Jiggins *et al.* 2004, Briscoe *et al.* 2009)

In addition to conspecific communication, organisms also communicate with other completely unrelated taxa. One such relationship is clearly seen in plant-pollinator interactions. Flowers signal presence of rewards through the corolla or other floral parts (Schaefer *et al.* 2004) and these signals, including flower color, shape, and size, can play an important role in flower detection and choice (Waser and Price 1983).

Visual Signals — how do pollinators interact?

Owing to the decoupling of reward and signal in flowers, pollinators must visit flowers to ascertain rewards offered (Schaefer *et al.* 2004). While foraging, pollinators increase foraging efficiency by making two decisions based on distance: from long distances they decide 1) *which plants should be approached* and from shorter distances i.e., when they

are at the plant 2) *which flower/s should be visited*. Both of these decisions are based on visual attractiveness of plants and flowers, respectively (Oberrath and Böhning-Gaese 1999). In many cases pollinators usually visit one type of flower per foraging trip even if they routinely collect pollen from multiple sources (floral constancy) (Gullan and Cranston 2009). This behavior in turn benefits plants by reducing deposition of heterospecific pollen and increasing conspecific pollen (Schaefer *et al.* 2004) and benefits pollinators by reducing handling times (Andersson and Dobson 2003). In addition to being faithful to one species of plant, pollinators can also display faithfulness to a specific feeding area where they trapline i.e. they collect food at steady, repeatable sequences from the same plants within the site (Williams and Thomson 1998, Ohashi and Thomson 2008, Ohashi and Thomson 209 Lihoreau *et al.* 2010 and citations *therein*). This behavior has been reported in many taxa, included *Heliconius* butterflies (Gilbert 1980), and offers a deeper understanding of floral attraction and pollinators' ability to track rewards offered by flowers displaying honesty signals.

It is posited that plant constancy coupled with color preference behaviors of animal pollinators exert such strong selective pressures it is the major driving force behind the diversity in flower color (Meléndez-Ackerman *et al.* 1997). Flower color as a result of pollinator interaction can then be explained by two scenarios. First, pollinator behavior is constrained by its limited ability to perceive and distinguish different color. Hence, flower visiting animals show fixed color preferences and these preferences differ according to taxa. Therefore, different color signals are aimed at different pollinator groups (Fenster *et al.* 2004). An alternate scenario states that pollinators are relatively unconstrained by their ability to perceive color as many exhibit true color vision (Sison-

Mangus *et al.* . 2006) and flower color thus acts as an advertising mechanism to signal visitation that is induced by quality of reward offered (Meléndez-Ackerman *et al.* 1997). In the following sections, I will, in more detail, discuss flower color, its use in communication and how it is detected.

Floral Color

Color signals are an important attractant to pollinators, as flowers communicate with pollinators through overt advertising of large brightly-colored showy petal to subtle presentation of color combination that act as guides (Kevan 1972). It is recognized that although color does play an important part in pollination, its function in plants is not limited to pollinator communication (Rausher 2008, Campbell *et al.* 2012).

Floral Color — how is it produced?

Plants produce many different types of compounds, many of which are pigmented (Tanaka 2008). Humans recognize the color of a compound by perceiving reflected or transmitted light of wavelengths between 380 and 730 nm, while insects recognize light of shorter wavelengths (Tanaka 2008). Most flower colors are a result of chemical pigments present in the cells of the flower petals. Three major groups of pigments, *betalains*, *carotenoids*, and the *flavonoids*, are responsible for the attractive natural display of flower colors (Tanaka *et al.* 1998, Grotewold 2006).

Betalains are water soluble nitrogen containing compounds synthesized from tyrosine by the condensation of betalamic acid, with a derivative of dihydroxyphenylalanine (Grotewold 2006). This reaction results in the formation of the red to violet betacyanins. While yellow to orange betaxanthins are formed by the

condensation of betalamic acid with an amino acid or amino acid derivatives (Grotewold 2006). Unlike carotenoids, and the anthocyanins which are broadly distributed among a wide taxonomic range of flowering plants, betalains are restricted to the order of Caryophyllaceae (Grotewold 2006).

Plant carotenoids are 40-carbon isoprenoids with polyene chains that may contain up to 15 conjugated double bonds (Hirschberg 2001). They are split in to two major groups xanthophylls and carotenes (Kevan 1972). These are the red, orange and yellow lipid soluble pigments found embedded in the membranes of chloroplasts and chromoplasts and contribute to the bright colors of fruits and flowers, which attract animals (Bartley and Scolnik 1995, Hirschberg 2001).

Flavonoids are a large class of secondary plant metabolites of which anthocyanins are the most conspicuous and thus function to attract pollinators when in petals (Holton and Cornish 1995). Flavonoids have a wide range of colors from white, pale yellow to red, purple and blue (Tanaka *et al.* 1998). Anthocyanins are water-soluble pigments that possess a hydroxylated 2-phenylbenzopyrilium chromophore of which there are six types and increases in the number of hydroxyl groups result in increases in the visible absorption maximum (Tanaka *et al.* 1998, Yoshida *et al.* 2009). Anthocyanins which occur in the vacuoles of almost all vascular plants and are responsible for the majority of the orange, red, purple, and blue colors of flowers (Grotewold 2006, Tanaka 2008). Anthoxanthins, a less popular group of flavonoids, are responsible for white, cream to pale yellow coloration of plants that absorb ultraviolet light (Kevan 1972).

Plants also exhibit morphological characteristics that allow for enhancing the perceived color of the petal. Kay (1981, 1988 as cited in Glover and Martin 1998) show

that conical or papillate cells found on the adaxial epidermis of the petal increased the amount of light absorbed by the floral pigments (Glover and Martin 1998). Glover and Martin (1998) and Dryer *et al.* (2007) further provided experimental evidence from their study of *Antirrhinum majus* that flowers with conical cells received more pollinator attention than those with flat cells.

In addition to these structural color enhancers, contrasting floral color traits such as iridescent patches in some orchids, bulls-eye images caused by striations in certain region of the petal as in *Hibiscus trionum* or darken flower centers as in *Tulipa humilis*, or nectar guides in many groups which contrast the flower by absorbing light in the UV range, increases the attractiveness of a flower by increasing visibility from a distance and help pollinators orient themselves on the flower prior and post landing (Whitney *et al.* 2009).

Researchers observed that various floral phenotypes serve to signal or advertise the presence of nutrition rewards (Raguso and Willis 2005) with communication between flowering plants and their pollinators involving a combination of sensory signals which include color, morphology, odor, among others which in turn act in concert with each other to become "sensory billboards" (Willmer *et al.* . 2009; Raguso 2004).

Floral Color — why did it develop?

The importance of color as a signal in floral parts in attracting pollinators has led to the common interpretation that pollinators are the primary selective agents influencing flower color. Transitions to different colors represents adaptation to different suites of pollinators as selection of one functional group may cause divergence of color while another functional group may maintain that trait (Fenster *et al.* . 2004; Rausher 2008). In

addition, competition for pollinators can also account for color divergence as this promotes species level specialization by pollinators, thus decreasing the cost of intraspecific pollen deposition (Muchhala *et al.* 2014). Observations on the correlations between floral-trait combination and pollinator type by Darwin (1862 as cited by Fenster *et al.* 2004) and many others suggest that different pollinators promote selection for diverse floral forms that produce an array of "pollination syndromes," (Fenster *et al.* 2004, Ollerton *et al.* 2009). The primary evidence supporting this contention is the existence of groups of floral traits that occur together associated with attraction and utilization of a specific group of animals as pollinators (Fenster *et al.* 2004, Rausher 2008). Examples include: bird-pollinated flowers, which are typically red or orange and have elongated floral tubes, reduced floral limbs, exserted stigmas, and copious dilute nectar; butterfly pollinated flowers which are bright red or orange and have a landing platform and a narrow deep corolla tube, while bee-pollinated flowers, which are typically blue or purple and have short, wide tubes, wide limbs, inserted stigmas, and small amounts of concentrated nectar among many other specialized examples (Andersson and Dobson 2003; Fenster *et al.* 2004; Rausher 2008).

In addition to the pollinator-shift and the competition models as explanations for why flowers have colors, researchers also recognized the importance of flower pigmentation in other functions aside from visual signaling (Campbell *et al.* 2012). Enzymes used in the synthesis of anthocyanin pigments are also used to synthesize other flavonoid compounds which effect flower color and other ecological and physiological traits such as flower temperature. Thus, flower color evolution may be influenced by selection on these pleiotropic effects (Rausher 2008). For example, flower color mutants

not expressing anthocyanins can be less tolerant of stresses such as drought and heat (Campbell *et al.* 2012). Other selective pressures such as herbivory also select for flower color, as pigmentation in flowers often correlates with green pigmentation in vegetative tissues, caused by chlorophyll a and b (Kevan 1972), and affect the level of resistance to herbivores (Campbell *et al.* 2012). If selection is all together discounted another view on color divergence is based on the neutrality hypothesis which suggests that genetic drift is responsible for flower color transitions (Rausher 2008).

Floral Color — how is it used?

Color signals in plants are important to pollinators as they show color preferences due to reward associations (Campbell *et al.* 2012). Flower color can remain constant or it can change due to factors such as age, pollinators or the environment (Weiss 1991, Yoshida *et al.* 2009). However, regardless if flower color is stable or dynamic, it functions to communicate with its animal pollinators. Therefore, the evolution of floral color change is most likely the result of visually orientated pollinator color preference behavior (as was discussed above).

The physiological mechanisms responsible for the color change of the flower include the gain or loss of pigments such as anthocyanin, carotenoid and flavonol, the appearance of betalain, change in pH, or movement of floral part such as curling of petals (Robinson 1939, Weiss 1995, Tanaka *et al.* 1998, Yoshida *et al.* 2009). In fact, one of the first theories used to explain red and blue coloration was based on change in pH by Willstatter and Everest (1913) where plants would exhibit blue coloration under alkaline conditions and red when acidic (as cited in Yoshida *et al.* 2009). The rivaling theory at the time was by Shibata *et al.* (1919) who proposed the metal complex theory that

showed the yellow pigments of plants, flovone and the flavonal series when reduced with compounds such has sodium amalgamate obtained red anthocyanin solutions (Shibata *et al.* 1919).

Floral color change provides important information that benefit both plant communicators and animal receivers, as plants receive potential pollinators and animal usually gain food rewards. Color change usually occur in fully turgid flowers and differ in the locations which they affect, as dictated by pollinator type. For example, the entire flowers of bat or moth plants change color, while butterfly, bee and fly pollinated plants show changes to only specific floral parts (Weiss 1995). Regardless of location of color change, pre-change flowers generally signal the provision of rewards and the availability of receptive stigmas, while post change flowers that are often retained, to increase attractiveness of displays, are generally unrewarding and sexually inviable. (Gori 1989, Weiss 1995, Willmer *et al.* 2009). For example, as seen in Lungworth flowers (*Pulmonaria collina*) which change from red to blue with age (Oberrath and Bohning-Gaese 1999) or Sweet sage (*Lantana camara*) which employ honest signals where one day old yellow flowers offer the most rewards, while day two orange or day three scarlet flowers offer little or no rewards but are retained to serve as a large billboard for long distance attraction and larger landing platforms (Barrows, 1976, Weiss 1991, Nuttman *et al.* 2005). Therefore, floral color change is an adaptive trait that benefits both the plant and its insect pollinators by cuing the insects to visit the flowers at the optimal reproductive stage and with the greatest reward (Willmer *et al.* 2009).

Visual Systems

Color vision offers a remarkable point of entry to understand mechanisms underlying complex behaviors as many taxa are users and receivers of color signals. Among terrestrial animals, only vertebrates and arthropods possess the ability to discriminate wavelengths independent of color intensity, characteristic of ‘‘color vision’’ (Sison-Mangus *et al.* . 2006). Although the origin of color vision is still unknown one explanation is based on the fact that light reflected from objects lacks UV wavelengths but possess green/yellow middle energy wavelengths. Therefore, if an organism is able to detect UV and middle wavelengths then it can tell the difference between an open space with high UV from an UV low space that can serve as a habitat or has the presences of food and other organisms. This theory is further supported by the presence of UV and green sensitive pigments of primitive arthropods (Pichaud *et al.* 2002).

Visual Systems — what does it comprise?

The compound eyes insects are made up of 8-9 photoreceptor cells surrounded by support and visual pigment cells that are organized in optical units called ommatidia (Pichaud *et al.* 2002). Ommatidia are classified as either open, fused or tiered based on the structure of their rhabdoms which in turn affects the spectral sensitivities of the photoreceptor cells (Briscoe and Chittka 2001). If open, such as in flies, receptor cells 1-6 each have their own rhabdomere that receives its own image (broadening spectral sensitivity), if fused rhabdomeres which have different photopigments act as lateral filters for each other thus narrowing spectral sensitivity, and if tiered distal photoreceptor cells filter light from the proximal cells, narrowing the spectral sensitivity of the animals such as butterflies and dragonflies (Briscoe and Chittka 2001, Pichaud *et al.*2002). In addition to visual

pigments, screening/filtering pigment found surrounding the rhabdom vary in spectral absorption and distribution, and affect the spectral sensitivity of the eye, although the interaction between these pigments are not clearly understood (Briscoe and Chittka 2001). For example, in *Papilio* butterflies their UV screening pigments superimpose onto their UV or green sensitive opsins causing an increase in spectral sensitivity allowing these butterflies to be able to detect six different colors; UV, violet, two kinds of green and red (Pichaud *et al.*2002).

Regardless of all the factors that affect the color sensitivity of the eye, for color vision of any kind to exist opsin genes (Fig. 2 modified from Frentiu *et al.* 2007), which encode visual pigments sensitive to different wavelengths of light, are obligatory (Briscoe 1998, Frentiu *et al.* 2007, Koyanagi *et al.* 2008). Visual pigments are made of two components; a light-sensitive retinal base chromophore (e.g. 11-cis-3- hydroxyretinal) (Smith and Goldsmith, 1990) attached by a Schiff- base linkage to an opsin protein (Briscoe and Chittka 2001). An opsin belongs to the family of G-protein-couple receptor and they contain transmembrane domains which form a binding pocket within which the chromophore is located (Briscoe and Chittka 2001). The spectral tuning of the visual pigment wavelength of peak absorbance, λ max, is achieved through the interaction of the chromophore with critical amino acid residues within the opsin. Changes in the polarity of amino acids in the chromophore-binding pocket of opsins affect the distribution of electrons in the π-electron system of the chromophore, producing a diversity of λ max values (Honig *et al.*1976). However, although the amino acid sequence and the chromophore both affect the maximum absorption λ max the fact that most organisms make a single chromophore, the diversity of the visual pigment absorption spectra

primarily depends on the amino acid of the visual pigments (Briscoe and Chittka 2001). Selection for amino acid substitutions at these key sites has led to the spectrally diverse array of visual pigments present in different classes of photoreceptor cells (Briscoe 2000). It is believed that photo pigment sensitivities represent adaptations to an animal's light environment, therefore these amino acid sites may be under positive selection from selective pressures such as finding food, shelter, oviposition sites (butterflies), mates and conspecifics (Frentiu *et al.* 2007).

Visual Systems — how did they evolve?

Phylogenetic analyses confirm that opsin genes duplicated many times before the radiations of the metazoans giving rise to several protein subfamilies (Frentiu *et al.* 2007). In Arthropods four phylogenetic groups of opsins have been identified (Briscoe and Chittka 2001) with most butterflies possessing three, as in most insects. Peak sensitivities of these opsins include: the ultraviolet (UV, 300-400 nm), blue (B, 400-500 nm) and long wavelength (L, 500-600nm) part of the light spectrum (Briscoe 2008, Bybee *et al.* 2012). Exceptions, in the insect kingdom, include the loss of the blue sensitive receptors in Dictyoptera and Hymenoptera, the gain of an additional short wavelength in Odonata and Diptera and the presences of a red-sensitive receptor in some Lepidoptera, Odonata and Hymenoptera (Briscoe 2000).

In bees, moths and most butterflies each ommatidium has six or seven receptors expressing long wavelength opsins, and two receptors expressing two blue and short wavelength opsins or just one of each (Zaccardi *et al.* 2006). The spectrum visible to butterflies (ultraviolet through the red) is one of the broadest in the animal kingdom (Bybee *et al.* 2012), therefore making them ideal study specimens in color vision studies.

Most butterflies possess the three major spectral classes encoded by ancient duplications, which produced distinct UVRh, BRh and LWRh opsin genes (Bybee *et al.* 2012). Although all butterflies share this similarity, butterfly eyes are extremely diverse in terms of their spectral organization (Sison-Mangus *et al.* 2006; Briscoe *et al.* 2010), as some have kept this ancestral arrangement while many other butterflies have many more (Osorio and Vorobyev 2008). For example, swallowtail butterflies *Papilio* spp. have at least three L opsins expressed in the compound eye owing to repeated gene duplication events (Kitamoto *et al.* 2000) whereas in the family Pieridae, B opsins are duplicated (Awata and Wakakuwa 2009). Overall it has been found that representative species of each butterfly family have different number of opsins due to lineage specific duplication events of the three basic classes of opsins (Yuan *et al.* 2010). Butterflies also show diversity in terms of the spectral sensitivities of their photopigments and their intraocular filters (Osorio and Vorobyev 2008).

Butterflies of the genus *Heliconius* (Nymphalidae) are considered examples of an adaptive radiation due to the spectacular diversity of mimetic wing color patterns that evolved in species and races throughout Mexico and Central and South America (Yuan *et al.* 2013). They also have unique visual systems because, besides the pressures of finding food they must also be able to recognize mates from the multitudinous arrays of mimics (Yuan *et al.* 2010; Zaccardi *et al.* 2006). As such they exhibit a remarkable radiation of photoreceptor sensitivities (Osorio and Vorobyev 2008). These Nymphalid butterflies have eyes that typically contain three spectrally distinct rhodopsins, including, as was stated before, one ultraviolet, one blue, and one long-wavelength. For example, *Dryas iulia*, a close *Heliconius* relative, has eyes that contain three rhodopsins with λ

max=385, 470, and 555 nm, whereas *Heliconius erato* has eyes that contain four rhodopsins with λ max = 355, 398, 470, and 555 nm. *Heliconius erato* eyes also contain four opsin-encoding mRNAs UVRh1 (UV Rhodopsin 1), UVRh2 (UV Rhodopsin 2), BRh (Blue Rhodopsin), and LWRh (Long wavelength Rhodopsin) in contrast to the usual three found in *D. iulia* (UV, B, L) (Briscoe *et al.* 2010, Yuan *et al.* 2010). This diversity of the eye design reflects the diversity of its evolution and of the lifestyles of the different species (Awata *et al.* 2010). For example, the gene duplication events such as that of the UVRh into UVRh1 and UVRh2 opsin genes have occurred at the same time that UV–yellow pigments of the wings appeared (Briscoe *et al.* 2010) suggesting that the duplicate UV opsin genes has evolved for species recognition and by extension mate selection, in Heliconiid group (Briscoe *et al.* 2010, Yuan *et al.* 2010).

Conclusion

Generally, photoreceptor sensitivities are adapted for universal vison and do not focus on specific communication signals (Osorio and Vorobyev 2008). However, this is definitely not the case for butterflies that possess a wide diversity of photoreceptors, owing to its multitudinous uses, such as recognition of green leaves for oviposition, yellow, blue, among other color flowers for feeding (Weiss 1997, Blackiston *et al.* 2011, Nuzhnova and Vasilevskaya 2013), yellow for mate recognition (Briscoe *et al.*2010, Yuan *et al.* 2010) etc.

Bodies of work showing clear cut evidence for the co-evolutionary relationship between butterfly receptors and mating signals have been substantial (Naisbit *et al.* 2001, Jiggins *et al*. 2004, Arikawa *et al.* 2005, Sison-Mangus *et al.* 2006, Bybee et al 2012). It is also shown that butterflies exhibit innate color preferences associated with feeding

(Hsu 2001) and the color of flowers play an important role in attracting pollinators (Quattrocchio *et al.* 1999). Additionally, Angiosperms employ a variety of strategies to encourage pollinators to approach, of these; color and changing color appears to be particularly important for flower recognition (Weiss 1997; Willmer *et al.* 2009). In particular, the flowers of Angiosperms exhibit tremendous diversity in color that ranges across the UV and visible spectrum (Muchhala *et al.* 2014). These flowers also differ from pale to nearly black in intensity with closely related sister species or populations of the same species differing in the intensity, hue, or patterning of the corolla (Rausher 2008, Muchhala *et al.* 2014) caused by numerous evolutionary transitions attributed to pollinator-mediated selection (Rausher 2008, Muchhala *et al.* 2014).

Thus, although, separate bodies of work focused on communication signals as it relates to conspecific identification and mate selection and plant-pollinator communication this review highlights the need to on focus relationships and correlations between these signals, especially in light of Ryan and Cumming's (2013) recent review linking the color biases for food and sex in other taxa. More so, this review shows that it is essential to bring more attention to plant-pollinator communication as this facilitates an increase in knowledge in the area of signal theory that has, historically, been biased towards epigamic signals. Further, future work in plant-pollinator communication should offer insights into non-hymenopteran pollinator behavior and how visual signals affect these behaviors and should not be limited to stable floral signals but include understudied areas such as floral color change.

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Figures

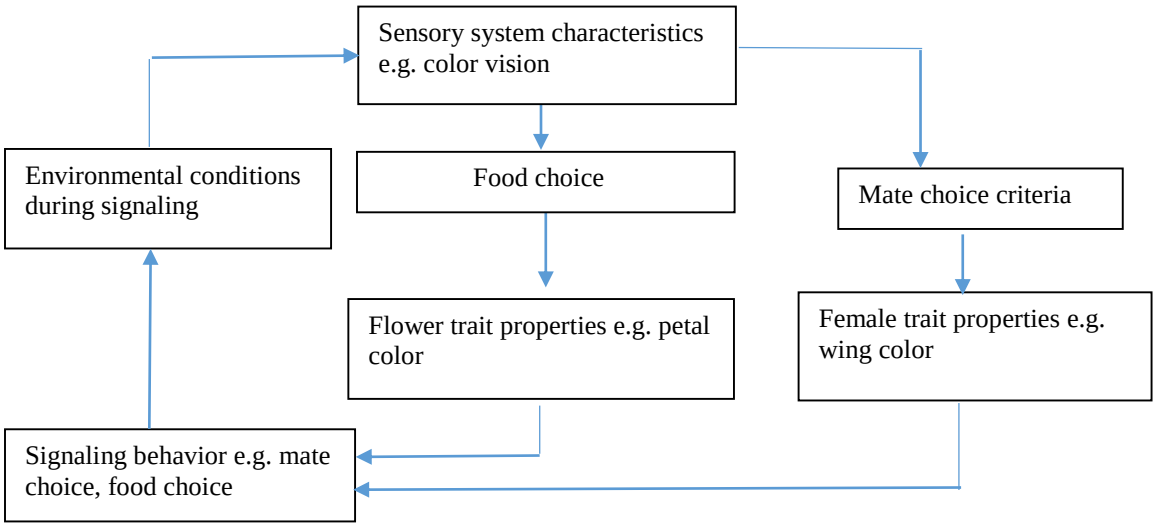
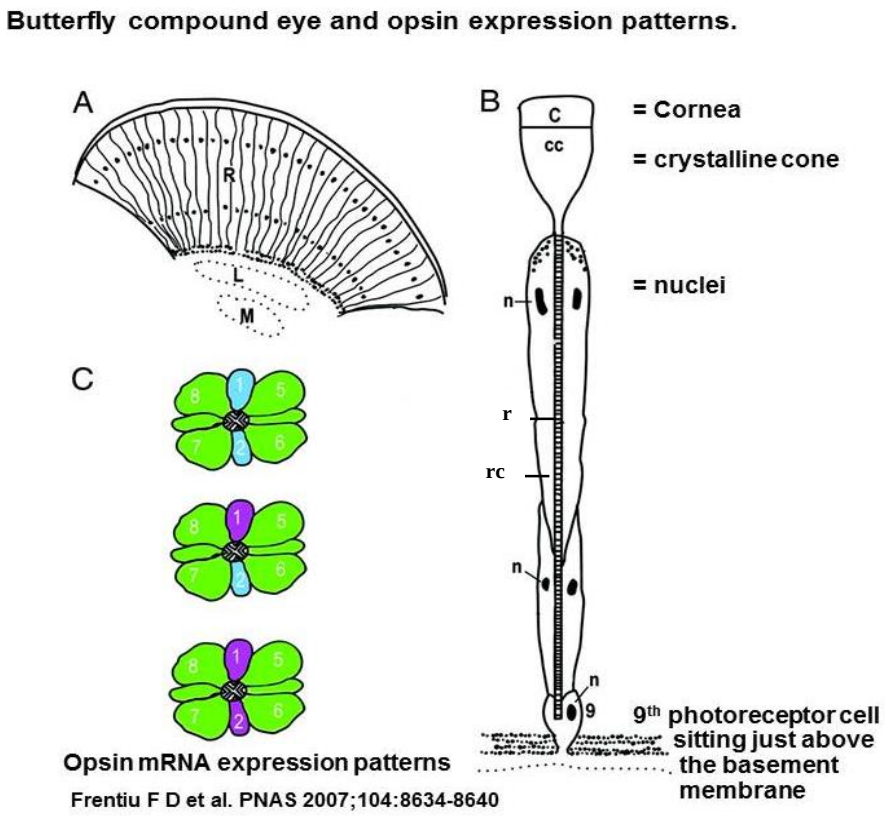


Fig.1 Process of sensory drive (sensu Endler 1992) as seen in innate food choices and sexual selection. Arrows indicate evolutionary influences.



***A*. Diagram of a longitudinal section through the compound eye showing the ommatidial units. Black dots indicate location of photoreceptor nuclei. R, retina; L, lamina; and M, medulla.**
***B*. Schematic of an ommatidium. C, cornea; CC, crystalline cone; n, nuclei; 9, the ninth photoreceptor cell that sits just above the basement membrane, r, rhabdom, rc, retinula cell, pigment cells not represented located as outer layer of cc and rc.**
***C*. Opsin mRNA expression patterns. The cross-sections of three ommatidia are shown. The cross-hatched area in the middle of each depicts the fused microvillous membranes of the rhabdomeres that contain the visual pigment proteins. Numbers refer to the photoreceptor cells (R1–R8), and the colors refer to the opsin expression patterns: violet, UV opsin mRNA; blue, blue opsin; green, long-wavelength opsin.**
Modified from Frentiu *et al* .2007.

Fig. 2 Butterfly compound eye and opsin expression patterns (Frentiu *et al.* 2007).

Chapter 2

Passionflower butterflies, *Heliconius melpomene* and *Dryas iulia* prefer flowers that match their wing colors

Manuscript draft to be submitted to *Journal of Behavioral Ecology and Sociobiology* with Godfrey R. Bourne and Jessica L. Ware.

Lay Summary

Color in nature serves many functions, and many animals exhibit pre-existing color biases for food which in turn sub-serve other functions such as finding mates. We show that experienced passionflower butterflies fed on flowers that were similar in color to their wings. Similarly, naïve flambeau (*Dryas iulia*) demonstrated the same color preferences for both food and mates. However, although naïve postman (*Heliconius melpomene*) butterflies showed preferences for red wings they did not show foraging preferences for flower color.

Abstract

Colors are one of the most ubiquitous and important cues exploited as signals by animals in nature. We investigated the relationship between colored signals used for foraging and mate selection by two passionflower butterflies, *Heliconius melpomene* and *Dryas iulia*, by testing the hypothesis that sensory bias for easy food detection was exploited during mate acquisition. We did this by presenting naïve butterflies with model yellow, orange and red *Lantana camara* flowers and same color model mates of each species. We also observed the feeding preferences of experienced butterflies at *L. camara* flowers and we ascertained from literature that these butterflies are attracted to mates with same conspecific wing color. We found support for the sensory bias hypothesis in naïve *D. iulia* that chose the same colored model flowers and model wings. However, *H. melpomene* butterflies showed no preference for flower choices, but chose red wing models as potential mates, as was noted in experienced butterflies. However, we also found that these feeding preferences were subject to change under simulated sky or forest environments. When we observed experienced butterflies in nature, we found support for the sensory bias hypothesis for both of our study species as the spectral reflectance measurements of conspecific wing color more closely matched their foraging plants in comparison to non-foraging plants present in the study site with flower color being as conspicuous to potential pollinators as wing color was to potential conspecifics against the visual back ground.

Key Words: color preference, sensory bias, *Lantana camara*, foraging, mate choice, floral preference, *Heliconius melpomene*, *Dryas iulia*

Introduction

Elucidating the origin and evolution of mating preferences and cues that influence signal evolution is an important goal of evolutionary ecology because the origin of these preferences and the cues that trigger them are usually unknown (Ryan and Cummings 2013). As early as 1879, Allen surmised that color vision was primarily adapted to finding edible parts of plants, and this lead to secondary color preferences such as those due to sexual selection—however he had no evidence for his conclusion (Osorio and Vorobyev 2008; Bybee et al. 2012a). We now know that either preference or cue can evolve first and then be favored by sexual selection, or both preference and cue can coevolve simultaneously in the sensory or receiver bias model (Arak and Enquist 1995; Ryan 1997; Ryan 1998; Payne and Pagel 2001; Andersson and Simmons 2006; Ryan and Cummings 2013). This model, which posits that preference for a trait did not evolve through sexual selection but rather in a non-mating context and is then exploited by one sex to increase their probability of mating (Ryan 1998; Endler and Basolo 1998) has been receiving growing empirical support (Grether et al. 2003; Kokko and Brooks 2003; Smith et al. 2004). For example, investigations in sexual selection have demonstrated that females have preferences for traits that are not yet exhibited by conspecific males (Basolo 1990a;Ryan 1997; Ryan 1998). These females show preferences for conspecific males with manipulated phenotypes, preferring males with an added trait such as a colored sword or complex call components, over the typical male phenotype (Basolo 1990; Shaw 1995; Ryan 1998; Ryan and Cummings 2013). Other sources of support for the receiver bias model come from empirical studies of foraging behavior (Endler 1992; Rodd et al. 2002; Smith et al. 2004; Fuller et al. 2005; Bourne and Watson 2009). The sexual

preference of female guppies (*Poecilia reticulata*), stickle backs (*Gasterosteus aculeatus*), and pentamorphic livebearing fish (*P. parae*) for males with orange spots, red throats, and yellow and red swaths respectively are explained by the idea that orange, yellow, and red coloration resemble the colors of their food sources (Rodd et al. 2002; Kokko and Brooks 2003; Smith et al. 2004; Bourne and Watson 2009).

The expression of many ornamental traits depends on carotenoids that animals cannot synthesize *de novo* (Brush 1990) but can only be obtained through ingestion (Olson and Owens 1998). Moreover, carotenoids are antioxidants and immunostimulants (Britton 1995), with a tradeoff between carotenoid allocation for maintaining health and enhancing ornamentation (Negro et al. 2002). Thus, when females exhibit preferences for males or males for females with the most intense carotenoid coloration they are choosing mates with strong immune systems (Blount et al. 2003) and foraging abilities (Rodd et al. 2002), and these colorful traits are in fact honest signals (Garcia and Ramirez 2005; McGraw 2005; Maan et al. 2006). Observations of guppies foraging on fruits rich in carotenoids in Trinidad led Rodd et al. (2002) to propose, test, and corroborate the hypothesis of a non-sexual origin of the female mate preference by using colored discs to test female and male guppy preferences for colors by recording approaches to and nibbling at these discs. They also showed that the visual system of guppies is tuned to preferentially detect orange food items, thereby providing further evidence for the predictions by Basolo (1990, 1995) and Endler and Basolo (1998) that demonstrate male orange coloration evolved because of a pre-existing female receiver bias. As far as we can determine, all tests of the sensory bias hypothesis to date focus on vertebrates (Ryan and Cummings 2013), and this prompted us to test this hypothesis on passionflower

butterflies, the flambeau, *Dryas iulia*, a member of the high-flying orange-patterned Müllerian mimicry complex, and postman, *Heliconius melpomene*, a member of low-flying red and black patterned mimicry ring (Papageorgis 1975; Mallet and Gilbert 1995). In Guyana, adults of both species feed on pollen and nectar (The Heliconius Genome Consortium 2012) from flowers that were red, yellow, orange, yellow-green and pink (G. R. Bourne and G. Maharaj pers. observ.).

In order to test this hypothesis, we needed to provide proof of Basolo and Endler's (Basolo 1990, 1995; Endler and Basolo 1998) four predictions to demonstrate that a male trait evolved because of female receiver bias. These are as follows: 1) preference for the trait is ancestral, however, 2) the trait itself must be absent or in a primitive form in ancestors, that is, the trait is derived; 3) there is a bias in the psychosensory system that matches the direction of preferences, that is, it predicts the direction of the preferences; and 4) male choice relies on heritable variation in the trait. So if the trait is present, there is a preference for it and the trait is used in mate choice. Previous work suggests that primitive arthropods developed color vision to first distinguish between open spaces that reflected high amounts of UV light and green/yellow food rich environments (Pichaud et al. 1999). This simple system then evolved into the three major classes of photoreceptors, with peak sensitivity (λ max) in the ultraviolet (UV, 300–400nm), blue (B, 400–500) and long wavelength (LW, 500–600nm) UV (ultra-violet) part of the light spectrum, seen in many insects today (Briscoe 2008). In butterflies, recent duplications of these ancestral opsin genes and changes in the kind and distribution of lateral filtering pigments has led to the evolution of novel λ max values (Briscoe 2001; Briscoe and Chittka 2001; Briscoe 2008), thereby increasing color

discrimination (Kelber and Pfaff 1999; Kinoshita et al. 1999; Bybee et al. 2012b). Many of these changes correlate with the evolution of wing color pigments, thus butterflies that developed additional wing colors simultaneously exhibited changes in the ancestral opsin expression and their filtering pigments (Briscoe et al. 2010a) and, as such, demonstrate respective changes in their mating preferences (Cook et al. 1994; Jiggins et al. 2001; Ellers and Boggs 2003; Jiggins et al. 2004). Thus lending support for predictions 1) the trait must be absent in ancestors; and 2) trait preference is ancestral (Endler and Basolo1998). Since we failed at extracting opsin genes from our specimens following protocols provided by Briscoe et al. (2010), we had no way of directly evaluating tuning in the visual systems of *H. melpomene* and *D. iulia.* Therefore, we were unable to directly and definitively test prediction three, bias in the psychosensory system matches the direction of preferences (Endler and Basolo1998). However, we assume that these butterflies' visual systems are tuned preferentially for detecting carotenoid-colored flowers (flowers with colors in the long-wavelength range) as reported for other Heliconiinae, as they both possess LW opsins and *Heliconius melpomene* possesses screening pigments that selectively absorb short-wavelength light and, thus, fine-tune the sensitivity spectrum of long-wavelength receptors (Stavenga 2002ab; Zaccardi et al. 2006). Therefore, for our study as a test for the sensory bias hypothesis we provide evidence for the final prediction using Passionflower butterflies, i.e. 4) male choice relies on heritable variation in the trait (Endler and Basolo 1998). So if the trait is present, there is a preference for it and the trait is used in mate choice.

Passionflower butterflies (Nymphalidae: Heliconiinae) exhibit sexually selected color dimorphism with males exhibiting greater color saturation than females (G. R.

Bourne unpubl. data). Male *H. melpomene* only approach and court females with species typical larger red spots on black wings (Fig. 2a), and females prefer males with these distinctive red-and-black badges as mates (G. R. Bourne and G. Maharaj pers. observ.), but the origin of this preference is unknown. We hypothesize that mate preference for females with larger or more chromatic red spots might be a pleiotropic effect of selection in a foraging context as is seen in several fish taxa i.e. mate preference developed as a result of a unrelated non-mating preference (Rodd et al. 2002; Ryan and Cummings 2013). Our aim was to devise experiments to determine whether *H. melpomene* with its bold red wing patches, and the mostly orange winged *D. iulia* had a pre-existing sensory-bias for carotenoid coloration. Specifically, we tested four predictions about the foraging and mating preferences of *H. melpomene* and *D. iulia*. First, newly eclosed (naïve) male and female butterflies should approach and uncurl proboscises to flowers with colors that match their own wing coloration; i.e. naïve *H. melpomene* and *D. iulia* will choose red and orange model flowers respectively. Second, spectral reflectance measurements of conspecific wing color patterns should match flower color preferences of experienced butterfly foraging than colors of non-visited flowers. Third, both butterfly species should more often approach butterfly models displaying conspecific wing color patterns. And finally, flower color will be as detectable to potential pollinators, as wing color is to potential conspecifics in similar light environments i.e. wing color and flower color will exhibit equal contrast against the visual back ground.

Materials and Methods

Study area

We conducted our study at CEIBA Biological Center (N 06° 29/.945//, W 058° 13/.106//), Madewini, Guyana (Fig. 1). This white sand forested area is comprised of low seasonal forest dominated by the fast-growing *Eperua falcate* (Caesalpiniaceae), and tall primary growth flooded forests dominated by *Mora excelsa* (Fabaceae) (Bourne and Bourne 2010). A mixed farm was established in this habitat and consisted of an 80×40m ($320m^2$) plot with numerous *L. camara* stands interspersed among *Citrus spp*. (lime, orange and citrus hybrids) and *Ananas comosus* (pineapples). In order to tract butterfly behavior we followed line transects that started in the northern forested margin and south of the biological station's complex. This transect extended towards the path to the mixed farm south of the biological station, it then continued west bound to the flooded forest, then north towards the spring, finally culminating in an easterly direction to the camp complex.

Study Species

Passionflower butterflies, or Heliconiids, are associated with a suite of derived life-history and ecological traits, including pollen feeding, extended lifespans, traplining foraging behaviors, gregarious roosting, and complex mating behaviors (*Heliconius* Genome Consortium, 2012). We have chosen to work with this group because they are tractable to study in the laboratory and the wild, and have been the focus of a large body of work in evolutionary biology, genetics, and animal behavior (Hsu *et al.* 2001). Additionally, Heliconiids vary considerably in the way they use visual signals to find flowers, mates, and communicate (Hsu *et al.* 2001).

Heliconius melpomene and *Dryas iulia* are common at CEIBA and belong to two different mimicry color rings as defined by their wing colors: red for *H. melpomene* and

orange for *D. iulia* (Mallet and Gilbert 1995), caused by yellow, orange and red scales containing 3-OH kynurenine and ommochrome pigments (Stavenga et al. 2014). *Heliconius melpomene* (Fig. 2a) wing color consists of red and black color patterns. The race used in this study can be identified by three features, viz., the color of the forewing band, the absence of the red patch on the proximal portion of the forewing, and the absence of red hindwing rays (Sheppard et al. 1985; Papa et al. 2008; Wallbank et al. 2016). This species is often encountered as solitary individuals along forest edges and old second growth (Barcant 1970; DeVries 1987). *Dryas iulia* (Fig. 2b) have elongate forewings characterized by bright orange dorsal surfaces with black margins (DeVries 1987). This butterfly is frequently found in secondary growth forest, gardens, and roadways feeding on pollen and nectar of flowers (Barcant 1970; G. Maharaj and G.R. Bourne unpublished data).

Study System

We focused our study on floral visitation as flowers are relatively constant in time and space and convey unambiguous messages to their receivers thus presenting a suitable system with which to study the effects of these signals on animal behavior (Schaefer et al. 2004). In addition flower visitation and foraging has been extensively studied in hymenopteran pollinators in comparison to butterflies regardless of their ecological importance (Weiss 1991; Weiss 1997). We record all foraging at non-foraging plants of our study species at CEIBA, however we focus our experiments on *Lantana camara* (Verbenaceae).

Lantana camara is a weedy Neotropical herb that has spread to various parts of the world as an invasive (Fig. 2c). It is usually found growing on human disturbed sites

(Sharma et al. 2005). This shrub has many inflorescences with 20–25 flowers per inflorescence placed in whorls (Fig. 2c; G.R. Bourne unpublished data). There are many horticulture varieties of *Lantana* that have small 5-lobed flowers in a variety of colors which include white, yellow, orange, red and purple that are often mixed in the same cluster (Ghisalberti 2000; Sharma et al. 2005). Common floral visitors include ants, carpenter bees, honey bees, black and brown stingless bees (usually as nectar robbers), and wasps, but especially butterflies belonging to diverse families. This plant has been the focus of many studies on color vision and color preference (Weiss 1991, 1997) and its tri-phasic color system provides unique insight to floral color signals and pollinator perception. Additionally, the major color phases of *L. camara* match the wing color of our study species i.e., red and orange, therefore this model system provides a unique opportunity to investigate whether each of my study species will be more attracted to colors matching their wing color.

Data collection

Feeding and wing preferences of naïve butterflies

We tested individuals for preference in terms of flower color (feeding) and wing color pattern (mating) using newly emerged imagoes of *H. melpomene* and *D. iulia* at the Chesterfield Butterfly House, Chesterfield Missouri, from (March 2015-September 2016). These butterflies were reared and shipped as pupa from the Bosque Nuevo Butterfly Farm, Santa Cecilia, Guanacaste, Costa Rica. Prior to testing, butterflies were kept in black cages and food was withheld for 12-24 h following eclosion (Nuzhnova and Vasilevskaya 2013). Each butterfly was tested in a 1 m^3 black mesh cage (feeding arena) that was only illuminated by two 15 W Philips Natural Light bulbs - color rendering

index = 92, Color temperature 5000K, full spectrum light, at a distance of 20 cm and no natural daylight was admitted into the testing cage. For our feeding experiments two types of additional colored filters were used to simulate blue and green lighting environments. All models were produced from pictures taken with a Nikon D90 DSLR camera (June 2015) printed on 100% white reflectance paper using PG-240/CL-240 ink on a Cannon MX432 printer.

Innate feeding

Before initiating the innate flower color preference trials, we gently unrolled each individual's proboscis with a dissecting needle and guided it into a black paper model flower containing 50% sucrose solution in order to expose the butterfly to the model and to stimulate interest in foraging. Initial models were black, as this was the only color butterflies were exposed to subsequent to eclosion and this prevented any color bias. Each butterfly was allowed to feed for 5s after which we placed them into the feeding arena.

Model flowers 3 cm in diameter consisting of four rays projecting from a circular center were made from matte paper to reduce glare of the three main colors viz. red, orange and yellow, of the natural wild-type *L. camara.* These models were created from pictures of flowers that were taken from the day one yellow flowers, day two orange flowers and day three red flowers. All pictures were taken at 08:00 h each day to ensure consistency as flower color changes temporally. Flowers were printed, cut to shape and attached to leaf green cardboard. Flowers were placed 10 cm apart in a completely random array and presented in the feeding arena. The light generated by the Philips Natural Light bulbs were diffused by a single sheet of UV-transmitting white diffusion

screen (no. 216, Rosco, Munich) to provide even, homogenous illumination (Blackiston et al. 2011). Blue illumination was generated by Mist blue Rosco 061(transmittance – 13%) filter sheets placed on top of the arena cover to simulate a blue sky-lit foraging environment. Green illumination, simulating conditions under forest canopy, was provided by placing a Rosco 139 Primary Green (transmittance – 15%) filter sheet on top of the flight arena (Lotto and Chittka 2005).

We conducted two experiments, the first to determine innate flower color preferences and the second to elucidate preferences for spatial orientations of color whorls on inflorescences. The first experiment utilized three single model flowers (red, yellow and orange) under the two lighting conditions discussed above. We tested a total of 104 *D. iulia* and 61 *H. melpomene* butterflies of both sexes. Butterflies were randomly chosen to be tested using blue ($N_{D.\ iulia} = 45$, $N_{H.\ melpomene} = 32$) or green filters ($N_{D.\ iulia} = 59$, $N_{H.\ melpomene} = 29$). In the second experiment we made *L. camara* inflorescences composed of model flowers in three colors whorls (red, yellow, orange). We altered the order of these colored whorls in six different orientations (Fig. 3). Orientation tests were carried out using 95 *D. iulia* and 52 *H. melpomene* butterflies of both sexes under blue illumination only.

In both of these experiments one butterfly was released into the array and its behavior recorded for 15 mins. None of the models contained sugar solution rewards. We recorded the color and location of the first model probed and then the butterfly was removed. Butterfly settlings without probing were not tallied. We used the location data to ensure that butterflies visited different individual flowers choices, rather than a particular area/location in the array.

Wing preferences

Butterfly models were made from pictures of *Heliconius melpomene* and *Dryas iulia* taken in the field. The coloration of their respective wings were manipulated to reflect yellow, orange, and red color markings, e.g., the red patches found on the fore wings of the *H. melpomene* were altered to look orange and yellow (matching day-2 and day-1 *L. camara* flowers, as taken from pictures). *H. melpomene* were presented with models that had red (control), and orange and yellow wing patches, whereas the wings of the *D. iulia* were altered to look yellow and red. After models were printed they were mounted on a black plastic stand (Jiggins et al. 2001; Ellers and Boggs 2003). All three models were presented together in the $1m^3$ mesh cage with one butterfly at a time. Butterflies were observed for 15 mins and the first approach was recorded. An approach was tallied if we observed the butterfly flying towards the models or settling on the model. We tested 76 *D. iulia* (29 females and 47 males) and 58 *H. melpomene* (33 females and 25 males).

Flower and wing preferences of experienced butterflies

We used an Ocean Optics STS-VIS-50-400-SMA microspectrometer with a HL-2000-HP light source to measure flowers of all foraging and non-foraging plants and butterfly wing color reflectance. The system permits reflectance relative to a white reflectance standard to be measured over a working wavelength range of 337-821 nm. We followed butterflies along fixed transects in open and closed canopy habitats at CEIBA, as described in the method, and selected flowers of foraging and non-foraging plants. Foraging plants were classified as those whose flowers were probed by imagoes of our study species, while non-foraging plants were those that were available to butterflies but whose flowers were

not visited. These flowers were taken back to the field station where we took spectral measurements on the upper surface of the petals using a probe holder and fix the fiber-optic probe at a 45° angle relative to the tissue surface. We randomly selected and measured five flowers from each foraging and non-foraging plant for each butterfly species (Muchhala et al. 2014). In order to take wing color measurements the wings were mounted on a black plastic backing and the fiber optic probe with the stand was placed on different colored dorsal sections of the wings (Luke et al. 2009). Reflectance spectra were taken from ten members of each species in order to account for variation in reflectance across conspecifics.

Data analyses

Innate flower and wing color preferences

For this experiment, the first model probed/approached by each individual were tallied across all butterflies and compared with an expected even distribution using a chi-square goodness-of-fit test (Blackiston et al. 2011).We do recognize that only males search for females, whether it is actively by patrolling in search of resting females or passively by perching and waiting for females (Scott 1975; Rutowski 1991). Therefore, we first used a chi-squared test to compare male and female innate mate preferences, before combining these data (male and female choices) and comparing them to the feeding color choices of flower models under blue illumination.

Flower and wing color measurements

We used the model specified by Gomez and Théry (2007) to better understand the differences in contrast between the butterfly wing color and food source relative to

natural light conditions and its background as perceived by the butterflies themselves. This model was chosen because it describes the discriminability of two colors against a chromatic background by their 'distance', in perceptual space, where perceptual space is defined by quantum catches of receptors of the animals looking at the colors analyzed. We used the quantum catches calculated from Gomez and Théry's model (as detailed in supplementary information) to plot in a color space flower, flower models and wing color for each species. We divided all reflectance spectra into three categories, feeding plants (background—mean of leaves of feeding and non-feeding plants), non-feeding plants (background—mean of leaves of feeding and non-feeding plants), flower model (back ground—leaf green cardboard on which models were presented) and wing color (background—mean of border around color), and we calculated the color differences from their respective backgrounds in units of just noticeable differences (JNDs). We calculated chromatic contrast (hue). That is, the distance between any two points representing a pair of colors and achromatic contrast (brightness) for a hypothetical white target (reflectance = 1 across all wavelengths) (analysis conducted as in Gomez and Théry 2007) and compared these values using a One-way ANOVA with accompanying post-hoc analyses for flowers and wings and a t-test for flower models under the two light environments.

Results

Feeding and wing preferences of naïve butterflies

Chi-square analyses of food preference under blue and green illumination show that there was a significant relationship between color choice and filter for *D. iulia* ($\chi^2 = 13.696$, df = 2, p = 0.001) but not for *H. melpomene* ($\chi^2 = 2.666$, df = 2, p = 0.264). With orange

being favored under the blue filter for *D. iulia* and yellow for *H. melpomene*, while red was favored under the green filter by both butterflies (Fig 4a, b). There were no differences for color choices between sexes for either *D. iulia*, ($\chi^2 = 3.477$, df = 2, p = 0.176) or *H. melpomene* ($\chi^2 = 2.578$, df = 2, p = 0.276).

Our analyses of our model flowers under the different filters revealed that there was no statistically significant difference between the chromatic ($t_{D.\ iulia} = 0.994$, df = 2, p = 0.425, $t_{H.\ melpomene} = 0.705$, df = 2, p = 0.554) and achromatic measurements ($t_{D.\ iulia} = 0.261$, df = 2, p = 0.819, $t_{H.\ melpomene} = 0.263$ df = 2, p = 0.817) of any flower model for either butterfly. However, we did note for *D. iulia* red flower models under the green light filter were 6.411 times brighter than under the blue filter while yellow and orange models were less bright i.e. their achromatic contrast against the background was less under green filters. Additionally, orange flower models were 15.512 times brighter and yellow models 1.624 times brighter, while the blue filters. For *H. melpomene* red flowers were 9.341 times blighter under blue light filters while orange and yellow were more bright under the green filter. Orange was 8.751 times brighter under green light filters, while yellow was 1.606 times brighter.

Our analyses of wing color and food color preferences for *D. iulia* ($\chi^2 = 6.207$, df = 2, p = 0.045), and *H. melpomene* ($\chi^2 = 7.319$, df = 2, p = 0.026) were significantly different, both butterflies preferred orange and yellow to red for food choices, and *D. iulia* preferred orange for mating choices, and *H. melpomene* red (Fig 5a, b). When we examined the orientation for feeding choice *D. iulia* butterflies preferred the OYR (orange, red, yellow), ORY and RYO orientations over YOR, YRO and RYO whereas *H. melpomene* preferred the ORY and YRO over the others. Although, our butterflies

displayed an observable preference for certain orientations, these preferences were not statistically significant for either species ($\chi^2 = 6.672$, df = 5, p = 0.246) (Fig. 6).

Flower and wing preferences by experienced butterflies

Color spaces for wing color and feeding plants (Table 1) were clustered, but both were separated from non-feeding plants, although there was some overlap for both species (Fig. 8). When we compared the just noticeable differences (JNDs) for *D. iulia*, feeding and non-feeding plants had larger achromatic contrasts than wings, but similar chromatic contrasts (Fig. 8). Our one-way ANOVA showed that there were no statistically significant differences in chromatic contrasts between wings, flowers and non-feeding plants (F = 2.167, df = 2, p = 0.119). However, there were statistically significant differences between the achromatic measurements of these groups (F = 3.949, df = 2, p = 0.022), specifically wings and non-feeding plants (Tukey HSD). For *H. melpomene* achromatic contrasts for wing, non-feeding and feeding plants were similar, however there were noted differences in chromatic contrasts between feed and non-feeding plants (Fig. 8). Our one-way ANOVA revealed that there were no statistically significant differences between the achromatic measurements of wings, food and non-feeding plants (F = 1.677, df = 2, p = 0.191). However, we found between group statistically significant differences in the chromatic values (F = 9.003, df = 2, p = 0.000) of feed and non-feeding plants but not wings and feeding plants (Tukey HSD).

Discussion

To date, proof of the sensory bias hypothesis had been shown mainly in fishes. Our study, similar to Rodd and colleagues (2002) show that our study species, Heliconiid butterflies,

show a similar color preference for food and mates, thereby specifically providing evidence for Basolo and Endler's final prediction. In our study we also show that color choice is also species specific and subject to change with experience and various lighting environments.

Feeding and wing color preferences of naïve butterflies

Flower visiting animals have innate sensory biases evolved to detect flowers by traits such as color, pattern, odor, size of these traits color is often used to locate, recognize and discriminate among flowers (Menzel and Shmida 1993; Lunau and Maier 1995; Gumbert 2000). Thus, due to these innate sensory biases, we see patterns, known as pollination syndromes, with blue and yellow bee pollinated flowers, red hummingbird flowers, and orange or red butterfly flowers although for butterflies, preference for flower color varies depending on family membership (Menzel and Shmida 1993; Weiss 1997; Andersson and Dobson 2003; Fenster et al. 2004; Chittka and Raine 2006). Such pollination syndromes arose by bilateral coevolution between flower color signals and the pollinators' ability to detect and exploit these signals to identify specific food plants (Menzel and Shmida 1993; Gumbert 2000).

Evidence of innate color preferences for food plant types by bees was presented as early 1881 by Müller (Gumbert 2000), and additional evidence presented in the early to mid-1900s by Knoll, for hawk-moths, and Ilse (1928) and Eltringham (1933) and butterflies (Ilse and Vaidya 1956; Lunau and Maier 1995; Hsul et al. 2001). Butterfly color preferences, in particular, were the subject of many studies that followed later because the taxon demonstrates innate preferences for flower color across many species, and this can vary depending on family or even species affiliation (Weiss 1997; Goyret et

al. 2008). For example, in cognitive studies, purple is preferred by several Papilionids and Pierids, and yellow by several Pierids and Nymphalids (Weiss 1997; Blackiston et al. 2011; Nuzhnova and Vasilevskaya 2013). However, preferences varies among species, e.g., newly emerged *Papilio aegues* (Papilionid) preferred human perceived blue, while *Pieris brassicae* (Pierid) preferred human perceived red (Lunau and Maier 1995).

In our study, feeding preferences changed with the quality of illumination (open sky vs canopy) for *D. iulia* but not *H. melpomene*. Both *D. iulia* and *H. melpomene* butterflies demonstrated a preference for yellow and orange flowers under blue filters that mimic open sky, and red flowers under forest green filters. However, for *H. melpomene* observed choices were not statistically significantly different. As such, only *D. iulia* support the prediction that newly eclosed (naïve) male and female butterflies should approach and uncurl proboscises to flowers with colors that match their own wing coloration, but both butterflies supported our prediction, that they should more often approach models displaying conspecific wing color patterns.

These results are similar to findings by Weiss (1997) and Andersson et al. (2003) where under controlled conditions, yellow elicited strong butterfly feeding responses, and under field conditions, butterflies favored the yellow and orange flowers of *L. camara* (our model plant) that grow in open unshaded habitats (Darwin 1877). Endler (1992) also found similar results and elegantly explained in his sensory drive model how heterogeneity in light environments, e.g., cloudy, clear skies, woodland shade, forest shade, can affect the overall conspicuousness of a color by affecting both the brightness and color contrast of adjacent patches and thus color choices. For example, he stated that "…..a color pattern of gray, blue, yellow-green, and red patches show high color and

brightness contrast in white light, but under the yellow-green light of forest-shade the yellow-green is very bright, whereas the blue and red patches are darker and duller. In woodland shade the blue is brightest (reflecting the greatest proportion of ambient light), whereas the other colors are duller" (Endler 1992). Therefore, he concludes that this varied light can affect the appearance of an organism sending a color signal and thus in turn affect the behavior of their receiver, as exemplified by guppies (Endler 1992), cichlid fishes, *Pundamilia pundamilia* and *P. nyererei* (Seehausen et al. 2008). For *D. iulia* yellow and orange appeared brighter under blue light while red was brighter under green lighting as such, depending on the lighting conditions certain colors were darker and duller and as a result were not chosen. However, for *H. melpomene* although red was brighter under blue light while orange and yellow were brighter it is possible that *H. melpomene* did not show a statistically significant difference in color choice when food was presented under various lighting conditions because similar to its co-mimic, *H. erato,* opthalmoscope studies reveal two classes of ommatidia resulting from lateral filtering pigments that can affect the long wavelength of light to which receptors are sensitive that are present in *Helconius* spp. unlike other Nymphalidae such as *Vanessa atalanta, V. cardi, Siproeta steneles, Inachis io and Polygonia c-album,* and possibly *D. iulia* (Stavenga 2002ab; Briscoe and Bernard 2005; Zaccardi et al. 2006). Additional studies should be conducted to confirm this. Moreover, *Heliconius* spp., unlike *D. iulia,* have coevolved new mechanisms for producing and detecting yellow wing pigments; a double duplication of their UV opsins, which likely favored the evolution of distinct yellow colors on the wing compared to non-*Heliconius* spp. which can now distinguish among several shades of yellow with increased sensitivity (Briscoe et al. 2010). Due to the

increased sensitivity in color range detection driven by these lateral filtering pigments and the presence of a duplicated UV opsin it is now possible that *H. melpomene* can differentiate among types of yellow, orange and red pigments under open sky and canopy filtered lighting conditions. In addition, it is interesting to note that although naïve *H. melpomene* did not discriminate among the colors presented when feeding, we observed that experienced butterflies under blue sky open field conditions visit *L. camara* inflorescences with greater numbers of red flowers although, they foraged more on plants with yellow and orange flowers (Maharaj et al. 2016 manuscript in review). Therefore, it is possible that although *H. melpomene* may be attracted to red flowers as red appears brighter under blue light, yellow elicits feeding behaviors because for *L. camara*, yellow flowers produce greater quantities and quality of nectar than red flowers (Maharaj et al. 2016 manuscript in review), which was the behavior tested in our experiments. This is strong evidence supporting the prediction that if the trait is present, in this case carotenoid flower coloration, there is a preference for it and the trait is used in mate choice, i.e., selection of similar carotenoid colored wings (Endler and Basolo 1998).

Flower and wing color preferences of experienced butterflies

Color vision enables animals to reliably detect and recognize food types and mates (Zaccardi et al. 2006; Ryan and Cummings 2013), and Lepidopterans are no exception (Swihart and Swihart 1970; Jiggins et al. 2001). In particular, the ability to discriminate colors in the red spectrum is vital as it can increase the number of flower species that can be perceived, facilitating the finding of better hosts for larvae and aiding mate detection of butterflies with orange-red coloration of their wings (Zaccardi et al. 2006). As such, it is not surprising that our results show the feeding plants (human detected yellow, orange

and red) of both of our butterfly species clustered with wing color (orange and red), but separated from the non-feeding plants (mostly human detected white and blue). Thus supporting our second prediction—spectral reflectance measurements of conspecific wing color patterns should match more closely experienced butterfly foraging flower color preferences than colors of non-visited flowers. Again, this is strong evidence corroborating the final prediction of Endler and Basolo, as there is a preference for orange/red food and this color trait is used in mate choice.

Our results, also demonstrated that the flowers of the feeding plants of our butterflies are as noticeable as the wings of their conspecifics thereby facilitating the increase in communication efficacy between conspecifics and pollinators in order to maximize detection by potential receivers. This supported our final prediction—flower color will be as detectable (equal contrast against the visual back ground) to potential pollinators, as wing color is to potential conspecific mates in similar light environments. Specifically, there were no statistical differences between the chromatic and achromatic contrasts of feeding plants and wings for either butterfly, i.e., butterflies were able to detect the hue and brightness of the flowers and conspecific wing color equally well against their backgrounds. In Heliconiinae, wing coloration serves as both defense (aposematic signals) and interspecific communication (epigamic signals) (Jiggins et al. 2004; Estrada and Jiggins 2008; Bybee et al. 2012a). Therefore wings evolved to be visible to Lepidopteran conspecifics that possess opsins in the ultraviolet (λ_{max} 349-399 nm), blue (λ_{max} 460-470 nm) and long wavelength (λ_{max} 550-560 nm) (Briscoe 2008; Yuan et al. 2010), and to avian predators such as tyrant flycatchers and tanagers (G. Maharaj and G. R. Bourne pers. obser.), which possess opsins similar to the blue tit (λ_{max}

372, 451, 537, and 605nm) (Shultz 2011; Bybee et al. 2012b). The flowers that are fed on by our study species are also shared by other pollinators from a broad range of taxa which possess opsins with differing and similar λ max values than *H. melpomene* and *D. iulia*. For example, other Lepidopterans such as Papilionids with five different photoreceptors (360 nm, 390 nm, 440 nm, 540 nm and 600 nm) (Kelber and Pfaff 1999; Kelber et al. 2003), hummingbirds (370 nm, 455 nm, 515 nm, 575 nm) (Muchhala et al. 2014), and hymenopterans such as *Xylocopa* spp. (360 nm, 428 nm, 544 nm) (Peitsch et al. 1992). Thus, our butterflies and the plants in their environment, whether feeding or non-feeding, evolved to send colorful signals with strong contrasts to many different taxa with similar and varying opsin sensitivities. As such, in our study, there were no statistical differences in chromatic contrasts between wings, and feeding and non-feeding plants for *D. iulia*, nor were there differences in achromatic contrasts for *H. melpomene*.

In summary, our findings implied that the mate acquisition of *D. iulia* and *H. melpomene* butterflies probably originated as a result of a sensory-bias for orange- red- and yellow-colored objects, such as rare flowers which might be sources of high quality pollen and nectar as foods (The *Heliconius* Genome Consortium 2012). However, the relationship between response to carotenoid models and mating preference suggests experiments should be conducted to elucidate causal relationships such as exposing multiple generations of butterflies to multicolored foods and noting if changes will also occur mate choice in models. Also, further study is needed to clarify to what extent geographical variation in male preference for orange, red, and yellow flowers is a result of natural selection influencing foraging behavior, and to what extent mate acquisition may have been co-opted by sexual selection mechanisms (Ryan and Cummings 2013).

We presented evidence that innate attraction to carotenoid colored model flowers and wings were similar for *D. iulia* and *H. melpomene* butterflies. Overall, our results suggest a strong association between a potential trigger of a mate choice preference and a sexually selected trait, thereby corroborating the receiver-bias hypothesis for carotenoid coloration (Ryan and Cummings 2013). Our study suggested both an association between a potential trigger of a mate preference and a sexually selected trait, thereby corroborating the sensory-bias hypothesis for the evolution of male mating choice in *D. iulia* and *H. melpomene* butterflies.

Funding

This work was supported by a Small Grant from the Rufford Foundation, UK, support from the J. L. Ware Lab, three CEIBA Biological Center Grants awarded to G. Maharaj, NSF-1453157 to J. L. Ware, and three anonymous foundation grants to G. R. Bourne. This paper was constructed from a chapter in G. Maharaj's unpublished dissertation presented to the University of Missouri-St. Louis.

We are grateful to O. O'Dean for field assistance and support during field work and to Brian Waldrop for assistance in all controlled color experiments carried out at the Chesterfield Butterfly House. A. Dunlap, N. Muchhala and Y. Wu provided guidance and comments during the planning and execution of this research. We thank the Guyana EPA for permits that facilitated data collection in Guyana, and the Chesterfield Butterfly House, Chesterfield, Missouri, for the provision of all butterfly specimens and allowing us to conduct our innate preference experiments at their facilities. We especially thank Laura Chisholm and Tad Yankoski for their assistance during the execution of these experiments.

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Figure Legends

Fig.1 Satellite view of study site, CEIBA Biological Center, Madewini, Guyana, 18 September 2002, 6°29′ 57.75″N, 58°13′ 07.23″ (Google© 2009 Europa Technologies).

Fig.2 Study species, a) Dorsal views of the postman, *Heliconius melpomene*, (b) the flambeau, *Dryas iulia* and (c) multiple inflorescences of the study plant, wild type of the sweet sage, *Lantana camara*, showing central position of unopened flower buds, inner whorl of yellow, newly opened flowers, followed by a whorl of orange 2-day old flowers, flanked by inflorescences of pollinated, non-nectar producing red flowers that are 3-days or older. These flowers also occur in the outer most whorls of inflorescences, however this is not captured in this photograph.

Fig.3 Model of inflorescence used to test spatial preferences of color whorls. These inflorescences were assembled by placing single model flower in concentric rings/whorls of six color orientations, viz., 1) center yellow, second ring orange, third ring red (YOR), yellow/red/orange (YRO), orange/yellow/red (OYR), orange/red/yellow (ORY), red/orange/yellow (ROY) and red/yellow/orange (RYO).

Fig. 4 Comparison of observed and expected color choices of food under blue and green lighting environments based on chi-squared contingency table values. Butterflies show preference for different colors while foraging in different lighting environments, i.e. open sky (blue filter) vs forest canopy (green filter); a) *D. iulia* preferred orange under the blue filter while red was favored under the green filter, and yellow favored equally under both filters b) *H. melpomene* preferred yellow under the blue filter but red under the green filter.

Fig. 5 Comparison of observed and expected color choices of food and mates based on chi-squared contingency table values. Butterflies show preferences for similar colors while foraging in blue light conditions but species specific color when choosing mates. a) *D. iulia* preferred orange and yellow to red for food choices and orange for mate choices, while b) *H. melpomene*, similarly preferred orange and yellow for food choices but chose red winged mates.

Fig. 6 Newly emerged a) *Dryas iulia* and b) *Heliconius melpomene* preferred test arrays with orange in the center. Orange, yellow, red (OYR) arrays were favored by *D. iulia*, while ORY arrays were visited more often by *H. melpomene*. However, there were no statistical differences among the color choices made by either butterfly species.

Fig. 7 Triangular and tetrahedral color spaces for (a) *D. iulia*, and (b) *H. melpomene,* produced by plotting relative quantum catches of opsins, show clumping of the floral spectral reflectance readings for feeding plants and wing color measurements. There was separation of non-feeding plants from feeding plants, although there was some overlap between the color reflectance of feeding and non-feeding plants.

Fig. 8 JNDs (chromatic and achromatic differences), (a) *D. iulia,* and (b) *H. melpomene*, based on reflectance readings from flowers of feeding and non-feeding plants and leaf backgrounds, and contrasts between colored and black portions of conspecific butterfly wing color patterns show no difference between butterfly foraging plants and wing colors.

Figures

Figure 1

a)

b)

c)

Figure 2 a, b and c

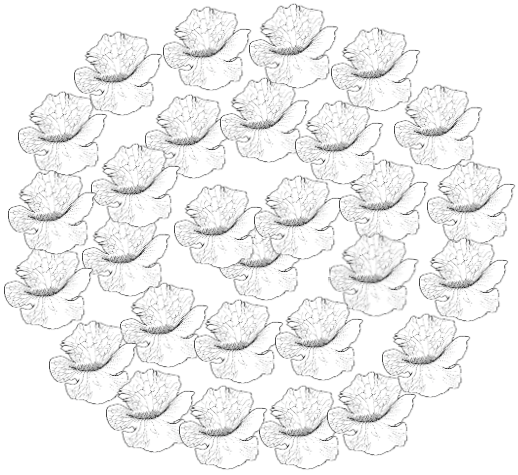
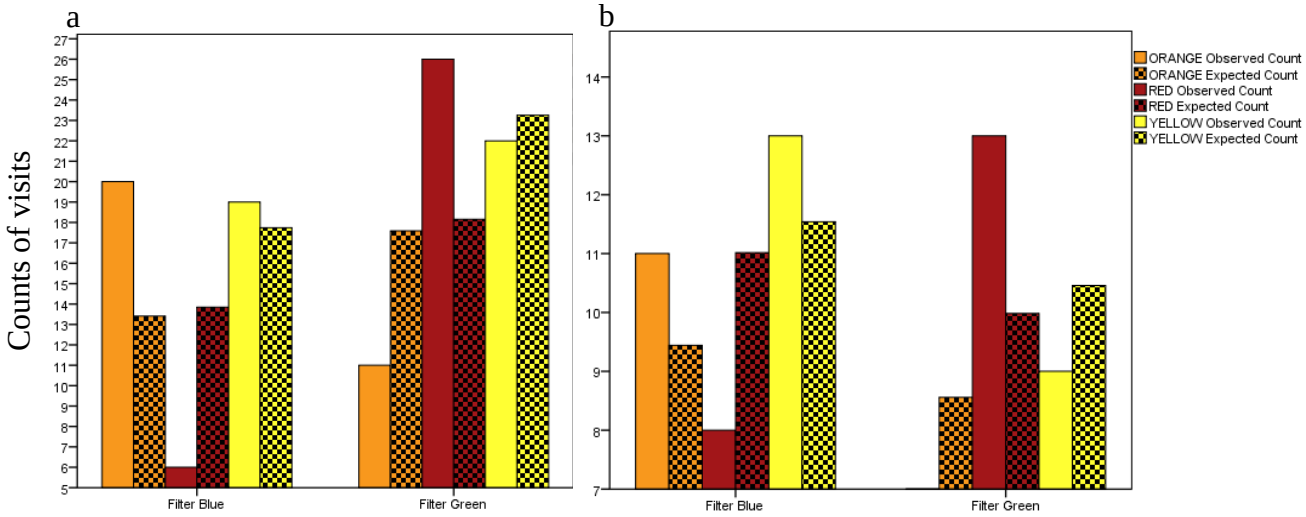


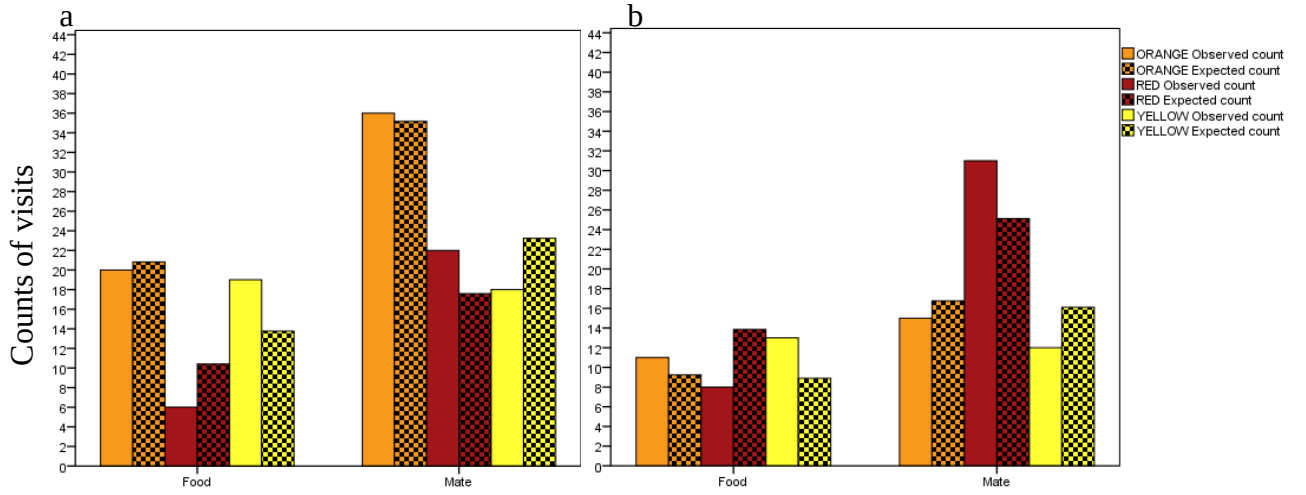
Figure 3



D. iulia color choices under different filters

H. melpomene color choices under different filters

Figure 4 a, b



D. iulia color choices during different activities

H. melpomene color choices during different activities

Figure 5 a, b

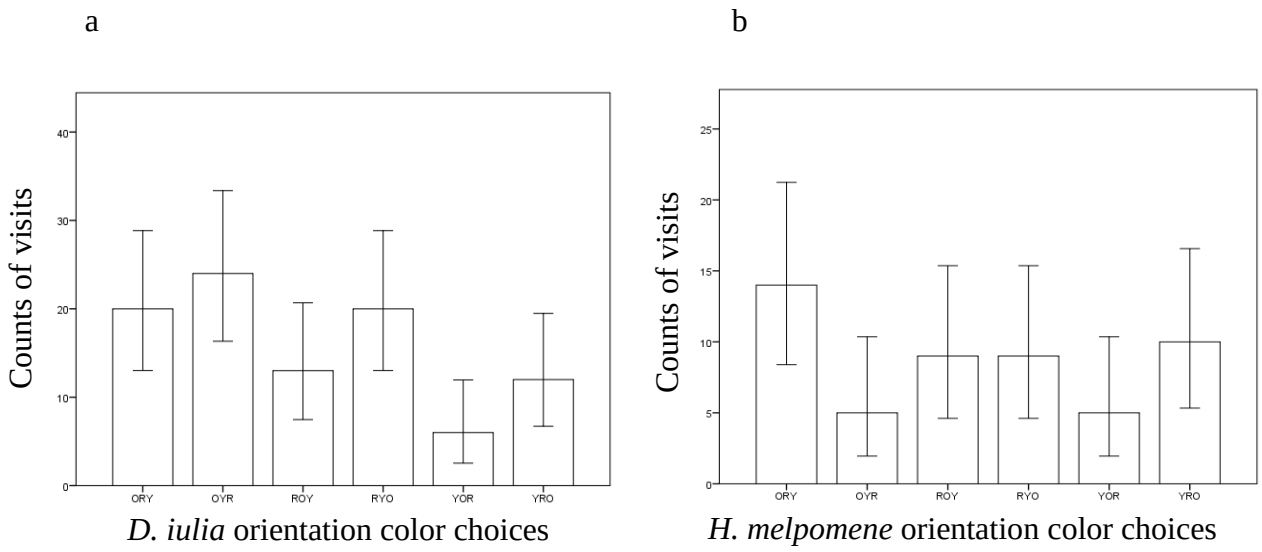


Figure 6 a, b

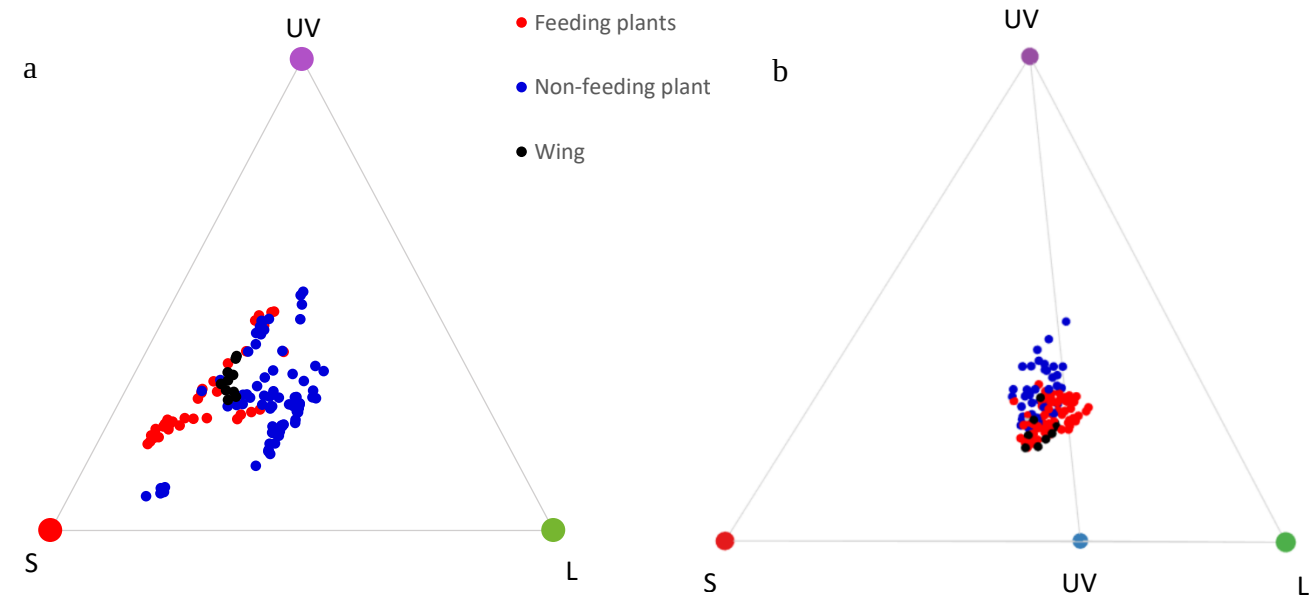
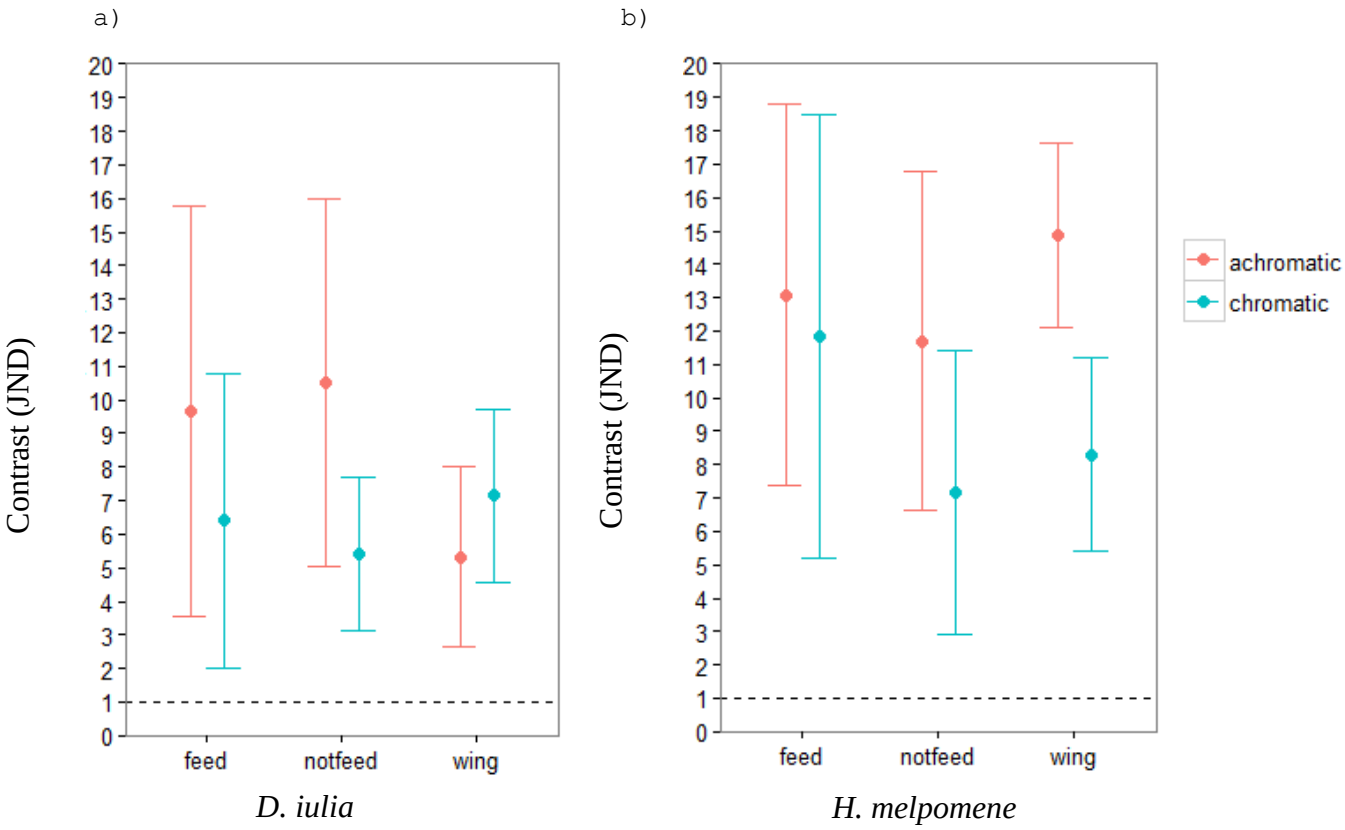


Figure 7 a, b


a)
b)
Contrast (JND)
Contrast (JND)
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19
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8
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4
3
2
1
0
feed
notfeed
wing
achromatic
chromatic
D. iulia
H. melpomene

Figure 8 a, b

Table 1: Adult feeding plant preferences of butterflies with associated petal color as observed by researchers identified using Smithe (1975)

Family	Plant species	Color of petals (as seen by humans)	Butterfly species
Bromeliaceae	*Aechmea nudicaulis*	Spinel pink bracts with parrot green flowers	*H. melpomene*
Anacardiaceae	*Anacardium occidentale*	Ruby center with lime green tips	*H. melpomene*
Bromeliaceae	*Billbergia pyramidalis*	Spinel pink bracts with spinel red flowers with spectrum violet tips	*H. melpomene*
Asteraceae	*Erechtites sp.*	Spinel red tips and trogon yellow petals	*H. melpomene*
Verbenaceae	*Lantana camara*	Spectrum orange with flame scarlet edges	*H. melpomene*
Verbenaceae	*Lantana camara*	Orange yellow with flame scarlet edges	*H. melpomene*
Verbenaceae	*Lantana camara*	Chrome orange	*D. iulia* *H. melpomene*
Verbenaceae	*Lantana camara*	Flame scarlet	*D. iulia* *H. melpomene*
Verbenaceae	*Lantana camara*	Orange yellow with chrome orange edges	*D. iulia* *H. melpomene*
Verbenaceae	*Lantana camara*	Orange yellow center with spectrum orange edges	*D. iulia* *H. melpomene*
Cucurbitaceae	*Psiguria spp. 1*	Spectrum orange	*D. iulia* *H. melpomene*
Cucurbitaceae	*Psiguria spp. 2*	Chrome orange with orange yellow center	*D. iulia* *H. melpomene*
Anacardiaceae	*Tapirira guianensis*	Straw yellow center with olive yellow petals	*H. melpomene*

Asteraceae	*Wulffia baccata*	Spectrum yellow petals and spectrum orange center	*D. iulia* *H. melpomene*

Supplementary information

Quantal catch calculations

We used measured irradiance measurements, with previous measurements of flower color and wing color to compute quantal catch (Q -amount of light captured for each photoreceptors (i)) for wing and flower color as $Q_i = \int_{337}^{700} R\ (\lambda)\ I\ (\lambda)\ T\ (\lambda)\ Si\ (\lambda)\ d\lambda$, where λ = the wavelength in nanometers, *R* is the reflectance of the stimulus (butterfly wing color or flower petals), *I* is spectral irradiance of the illuminant (the light environment), *T* is transmittance in air (taken perfect transmittance (T = 1) as readings were taken in areas without, fog or dust at close distances), and S_i refers to the spectral sensitivity of the respective cone *i* (Gomez and Théry 2004; Gomez and Théry 2007; Muchhala et al. 2014) (as taken from literature, *D. iulia*—(UVRh λmax 385 nm, BRh λmax 470 nm, LWRh λmax 556 nm as cited in Yuan 2010), *H. melpomene*—(UVRh1 λmax 355 nm, UVRh2 λmax 398 nm, BRhλ max 465 nm, LWRhλ max 550 nm as cited in Zaccardi et al. 2006, Briscoe et al. 2010, Bybee et al. 2012). We then corrected quantal catch to take into account receptor saturation and model color constancy (*sensu* Gomez and Théry 2007): $q_i = Q_i/(Q_i + Q_i^B)$ where Q_i^B is the response of cone to background.

Chapter 3

Honest signalling and the billboard effect: how Heliconiid pollinators respond to the trichromatic colour changing *Lantana camara* L. (Verbenaceae)

Currently, resubmitted to be reviewed in *Journal of Pollination Ecology* with Godfrey R. Bourne.

Abstract

Plants communicate with their pollinators through an astonishing range of signals that serve as either honest or deceptive cues which draw in and inform potential visitors of possible rewards. In wild type sweet sage, *Lantana camara*, floral colour signals were associated with nectar volume and sucrose concentration, and many pollinator taxa quickly learned to associate these varying colour signals with rewards. We tested the hypothesis that if sweet sage is employing a generalist pollinator strategy based on a trichromatic changing floral presentation system of honest rewards for pollinators, then the following predictions will be realized: 1) pre-change yellow coloured flowers will be visited more frequently by pollinators than post change orange, or scarlet flowers; 2) pre-change yellow flowers will produce higher quality and greater quantities of sucrose rewards than post-change orange, or scarlet flowers; 3) inflorescences with higher ratios of rewarding flowers to unrewarding flowers are more attractive at short distances; and 4) inflorescences with a combination of pre-change rewarding and post-change rewarding and unrewarding flowers will act as a multi-coloured advertising billboard and as such be most attractive at long distances. We found corroboration for all of the aforementioned predictions. Thus, sweet sage evolved a generalized pollination visitation system based

on honest signalling—of reward quantity and quality tied to colour changing visual signals acting in consort to produce a billboard that was easily perceived and deciphered. These resulted in high visitation rates by many different taxa of pollinators, thus contributing to higher individual plant fitness.
Keywords: colour change, Guyana, honesty signals, billboard effect, *Lantana camara*, pollinator

Introduction

The evolution of the great array of floral traits seen in Angiosperms rely on the diversity of animal pollinators to visit regularly and inadvertently transfer pollen efficiently from anthers of one flower to the stigmas of conspecifics (Graham et al. 2003; Kaesar et al. 2006). Approximately, 90% of the more than 240,000 species of flowering plants are pollinated by over 200,000 animal species (Graham et al. 2003; Holland 2011). These plants employ three broad strategies for achieving pollination: (1) deception, where animals are tricked by mimicry of real rewards into providing pollen transfer among flowers (Wickler 1968; Ackerman 1986; Nilsson 1992; Graham et al. 2003); (2) imprisonment, where flowers, that often offer rewards, attract insects most of which are already covered with conspecific pollen, and they are then delayed for several hours until pollen is released (Lack & Diaz 1991; Proctor et al. 1996; Gibernau et al. 2004; Bolin et al. 2009); and (3) honesty, in which the plant produces something of value to the animal (Nilsson 1992; Graham et al. 2003). Here the plant usually invests in food rewards—nutritious nectar fortified by sugars and amino acids, modified food pollen devoid of sperm; or provides safe and food−rich oviposition sites for insects to lay eggs, or produce fragrances that enhance males' mating success through female choice (Simpson & Neff 1981; Seymour & Matthews 2006; Wright & Schiestl 2009; Goodrich 2012). In honest signalling, these rewards are positively correlated with the presence and intensity of display signals (Kaesar et al. 2006; von Arx 2012).

Many plants employ sensory signals which include colour, morphology, odour, among others, which in concert become "sensory billboards" (Raguso 2004; Willmer et al. 2009) . These sensory signals can function "honestly" in their communication with

pollinators if they reliably signal the presence and/or quality of nectar, pollen, oil, or fragrance rewards (Nilsson 1992; Proctor et al. 1996; von Arx 2013). Colour signals are of particular importance to pollinators as they are able to perceive and distinguish colours and many show innate and learned colour preferences due to reward associations (Campbell et al. 2012). Flower colour can remain constant during the entire anthesis stage or it can experience colour change due to multiple factors (Weiss 1991; Yoshida et al. 2009). In some plant taxa, floral signals can also change with the environment, age or receptivity status (Weiss 1991; Yoshida et al. 2009)—with younger pre-change flowers signalling receptive stigmas and the provisioning of rewards for animal visitors. While older post change flowers are generally unrewarding and sexually inviable (Gori 1989; Weiss 1995; Willmer et al. 2009). Floral colour change (pollination-induced or an age-dependent pattern) has most likely evolved in response to selection by visually orientated pollinators, and reflects a widespread functional convergence within flowering plants (Weiss 1991). Von Linne 1793 (cited in Oberrath & Böhning-Gaese 1999) noted that floral colour change is a common phenomenon among flowering plants with diverse life histories and growth forms from over 78 families and 250 genera of angiosperms, distributed worldwide, visited by approximately 15 families of insects and four families of birds (Weiss 1991; Weiss 1995; Weiss & Lamont 1997, Oberrath & Böhning-Gaese 1999).

Despite the wide prevalence of flower colour change (Ida & Kudo 2010) and the well−developed hypotheses offered to explain the adaptive nature of this trait, this phenomenon has been experimentally examined in only a few species (Weiss 1995; Oberrath & Bohning−Gaese 1999). In addition, many of these studies focus on non-

lepidopterans (see Ida & Kudo 2003; Ida & Kudo 2010; Pereira et al. 2011, Suzuki et al. 2014) or multiple groups of pollinators (Weiss & Lamont 1997; Oberrath & Böhning-Gaese 1999). Our study is unique because we compare the feeding behaviours of two major lepidopterans in a natural setting. Thus offering a unique perspective of how colour change of one plant differentially affects two pollinators that share a similar feeding niche (G. Maharaj unpubl. data). Our goal was to examine the relationships among floral colour change, and nectar volume and sucrose concentration in wild type sweet sage, *L. camara* on pollinator visitation rates at CEIBA Biological Center, Madewini, Guyana. Specifically, we asked the following questions of the sweet sage pollinator system: (1) Do younger yellow flowers produce greater quantities and higher sucrose concentration nectar than older orange and scarlet flowers? (2) Do newly opened yellow flowers attract more *L. camara* pollinators than older orange and scarlet flowers? And (3) how does inflorescence size and ratio of rewarding to unrewarding flowers influence butterfly pollinator visitation rates? Thus, we tested the hypothesis that if *L. camara* is employing a generalist pollinator strategy based on a trichromatic colour changing floral presentation system of honest rewards for pollinators, then the following predictions will be realized: (P_1) first stage yellow flowers will attract more pollinators because they contain higher concentrations and volumes of sucrose than later orange and scarlet stages; (P_2) inflorescences with greater proportions of rewarding to unrewarding flowers will be more attractive over short distances as this will result in multiple visits to an individual plant due to butterflies' tendencies to visit particular colours that are associated with greater sucrose rewards; and (P_3) inflorescences with a combination of rewarding yellow and orange flowers and unrewarding scarlet will be most attractive to butterflies over

long distances as these large multi-coloured inflorescences will provide large landing platforms (Barrows 1976) and serve as an advertising billboard drawing in potential pollinators from greater distances (Barrows 1976; Weiss 1991; Raguso 2004; Nuttman et al. 2005; Willmer et al. 2009).

Materials and Methods

Study site

Experiments on the pollination biology of sweet sage, *L. camara* were conducted at CEIBA Biological Center (CEIBA; 06°29′57″N, 58°13′06″W), on the Soesdyke-Linden Highway, Madewini, Guyana, South America. Observations were conducted in a sustainable demonstration farm site (320m^2) filled with numerous *L. camara* stands. The study plot was bordered by a seasonally flooded white podsolized sand area comprised of low seasonal forest dominated by the fast-growing *Eperua falcate* (Caesalpiniaceae), and tall primary growth flooded forests dominated by *Mora excelsa* (Fabaceae) (Hughes 1947; Bourne & Bourne 2010).

Study species

Sweet sage, *Lantana camara* is a perennial shrub of the Vervain or Teak family (Verbenaceae) (Munir 1996) native to tropical regions of Central and South America (Graham 1963; Myint 1994). It is a readily available, easily tractable, common plant of CEIBA found in open habitats, especially on human disturbed sites (Sharma & Singh 2005) that provides food to a variety of pollinators. This plant has been the focus of many studies on colour vision and colour preference (Weiss 1991; Weiss 1997). This hairy herb with very aromatic leaves sometimes assumes either climbing or woody shrub growth

forms. Wild type *L. camara* usually attains heights between 1 and 2 m and has square stems armed with short coarse spines (Ghisalberti 2000). *L. camara* plants used in this study were large shrubs that were approximately 1 m in height as these smaller plants were easier to work with i.e. manipulate. Leaves are simple and opposite, emanating at right angles from each node to leaves of the nearest neighbouring node. Leaf surfaces are wrinkled and scabrous or rough textured, while leaf edges are regularly serrate. In addition, leaf shapes vary from broadly lanceolate to cordate with distinctive pointed drip tips; leaves vary in measurement from 75.0−102.4 mm long by 25.3−56.7 mm wide, and with petiole lengths of 21.2−32.8 mm (G.R. Bourne unpubl. data). When leaves or stems are damaged, a distinctive odour is released. There are many horticulture varieties of *Lantana* that have small 5-lobed flowers in a variety of colours which include white, yellow, orange, pink, red and purple that are often mixed in the same cluster (Ghisalberti 2000; Sharma & Singh 2005). Inflorescences of our studied variety (wild-type) present trichromatic succession flowers (i.e. yellow to orange to scarlet), held in close heads of umbel form, ranging from 31.3−42.6 mm in diameter, and with 9−30 flowers with four stamens. Thus, the inflorescences of *L. camara* allow for manipulation experiments testing the effect of colour of rewarding and unrewarding flowers on short and long distance attractiveness. Regular floral visitors include ants, carpenter bees, honey bees, black and brown stingless bees (usually as nectar robbers), wasps and hummingbirds (Weiss 1991), but especially butterflies belonging to diverse families such that many Guyanese classify sweet sage as a butterfly bush (G.R. Bourne and G. Maharaj pers. obs.). Fruits are smooth, round, two−celled berries (Graham 1963) with diameters of 4.2−6.6 mm presented in ball−like clusters 21.4−31.7 mm in diameter. When immature

they are a shiny lime green in colour changing to indigo blue when ripe (Sharma & Singh 2005), and whose seeds are dispersed by many bird taxa including barbets, flycatchers, and tanagers.

We focused our experiments on two common butterfly species (Nymphalidae, Heliconiinae) at CEIBA, *Heliconius melpomene* and *Dryas iulia*. The first species is characterised by black wings with a red blurred patch on forewing (fwl ~ 41mm) and a yellow line on underside of hind wing curves towards the posterior. This species is often encountered as solitary individuals along forest edges and old second growth groves (DeVries 1987), and is frequently observed feeding on *Lantana camara* (Verbenaceae) (G. Maharaj & G.R. Bourne pers. obs.). Whereas, *D. iulia* is characterised by bright orange wings with black margins and with elongate forewings (fwl ~ 85mm); males are typically brighter than females (DeVries 1987). This species is usually found in second growth forest imbibing nectar from many flower species, it is also a noted gregarious feeder of *L. camara* (G. Maharaj & G.R. Bourne unpubl. data). We chose to work with species of Heliconiinae because they are tractable to study in the laboratory and the wild, and have been the focus of a large body of work in evolutionary biology, genetics, and animal behaviour (Hsu *et al.* 2001). Heliconiids also vary considerably in the way they use visual signals to find flowers (food sources), mates, and communicate (Hsu *et al.* 2001).

General sampling protocols

Flower colour and diurnal sucrose measurements

In order to determine flower colour and respective rewards offered we used destructive sampling to measure daily diurnal spectral reflectance change, nectar volume and sucrose

concentrations. For each flower, we used type colour swatches in Smithe (1975) to measure and name flower colour (as perceived by humans). Flowers were placed directly onto swatch and colour was determined by researcher and research assistant. If both investigators were unable to agree on colour nomenclature, a third researcher was consulted. Although human colour nomenclature and just noticeable differences were used in this study, we do recognise the need to refer to colour differences in terms of insect perceptions as both study species and most insect classes possess three classes of opsin genes, ultraviolet (UVRh λmax ~350nm), blue (BRh λmax ~ 440nm) and long-wavelength (LWRh λmax ~ 530nm) (Briscoe & Chittka 2001; Sison-Mangus et al. 2006; Briscoe 2008; Yuan et al. 2010). In this study we first aimed to establish whether there is an actual difference in behaviours to flower colour changes as seen by humans, taking into consideration that due to the presence of long-wavelength opsins and long-wavelength opsins and the possible presence of lateral filtering pigments that filter short wavelength light, thus shifting the sensitivity of the visual pigments to the longer wavelengths, such as red filtering pigments seen in *Heliconius erato*, our study species are capable of distinguishing changes in long wave length red markings (Zaccardi et al. 2006, Briscoe 2008). In a different study we investigated these floral colour changes in our respective butterflies' colour spaces (Maharaj et al. manuscript in prep.).

For this current study, we used 1µL Drummond Microcap® tubes and a digital calliper to estimate nectar volumes, and a SPER Scientific Sugar−Brix Refractometer to measure nectar concentration (Waser & Price 1981). In order to determine colour and sucrose measurements of the three major colour stages, a total of 20 flowers were used for each cohort of each colour type. These flowers were haphazardly selected from

several inflorescences of ten marked plants at 09:00 h for three days during May 2010. Day 1(yellow) — was taken as the first day after buds bloomed, day 2 (orange) — was taken the morning after that and day 3 (scarlet) — was taken on the following morning. To estimate colour and sucrose measurements of the sub-divisions of these three major colour stages a total of 25 flowers were selected for nine days (July 2014). These flowers were picked during four 3-hour time blocks (TBs); (TBI 06:00−9:00 h, TBII 09:00−12:00 h, TBIII 12:00−15:00 h and TBIV 15:00−18:00 h). All sampled flowers were fresh and turgid and picked from previously bagged inflorescences. These inflorescences were placed in light-admitting bags as buds initially and remained bagged for the duration of the study to prevent nectar consumption by pollinators. Our sampled colour change flowers did not include colour changes from bud to flower or wilted flowers.

Pollinator species & fruit set

In order to determine which visitor taxa were effective pollinators we conducted visitation watches and fruit set experiments. We counted the number of diurnal animals visiting wildtype *L. camara* inflorescences of nine selected plants that differed in floral density, number of inflorescences per 0.5 m^2, (high [20+], medium [6-19], and low [2-5]), and colour for 60 d during May–July 2010 at CEIBA. Each 0.5 m^2 quadrat was sampled for 2 mins only during sunny periods in time block II (TB II, 09:00–11:59 h), the peak pollinator activity period at CEIBA. Every visiting animal taxon was photographed to aid in identification using various guides (Barcant 1970; Borror & White 1970; Milne & Milne 1980; Pyle 1981; DeVries 1987; Opler 1992; Restall et al. 2007a, b; Marshall 2008; Maharaj et al. 2010), and foraging behaviours recorded. If a floral visitor had pollen on any part of its body, it was considered a pollinator of *L. camara*. A checklist

was made of all pollinators, a special focus was made on butterflies due to their proclivity for visiting this plant and the two major pollinators (as characterized by frequency of visits and abundance), *Dryas iulia* (Fabricius, 1775) (Nymphalidae) and *Heliconius melpomene* (Linnaeus, 1758) (Nymphalidae) (DeVries 1987) were our focal animals for our inflorescence manipulation experiments due to ease of observations.

Fruit-set experiments were carried out by inclusion (focal taxon)/exclusion (other taxa) in 1 m^3 mesh cages (fine gauge mosquito netting 3000 holes per cm). Prior to initiation of fruit-set studies, 240 immature inflorescences on 10 *L. camara* bushes were bagged using see-through, home-made pollinator bags during May 2010. Pollinator bags, 133×99 mm were constructed from perforated (by safety-pins, 26±8 holes$^{1cm^2}$) white printing paper on one side and clear plastic from ZipLock® freezer bags on the other, stitched together by 17 mini-staples. As inflorescences matured some were unbagged and the mesh cages set up in the evening (18:00 h) after diurnal pollinator activity ceased. Each pollinator tested (included all captured as we did not control for pollinator sex) was introduced by hand and held for a 72 h period. Inflorescences were then rebagged to prevent cross species visitation, after which the mesh cages were removed. Hummingbird diets were supplemented by 25% sucrose solution and adult fruit flies (*Drosophila* spp.).

Flower colour preference and billboard effect

In order to demonstrate whether or not pollinators exhibited a pattern of colour preferences, and that clustering of floral displays had a billboard effect, we conducted two field experiments in which we manipulated *L. camara* inflorescence densities by removing variable numbers of individual coloured flowers to create multiple treatments. We then observed visitation rates to these treatments. The first experiment, called, colour

preference, was a generalized study that examined colour choices of all animal visitors to *L. camara* flowers. The second experiment, entitled, billboard effect, followed two focal butterfly species, *H. melpomene* and *D. iulia*. These were major visitors to *L. camara* where they navigated different concentrations of colour combinations in their choices of inflorescences that may explain why non-rewarding red flowers persist in displays.

Experiment 1 — Colour preference

These field studies were conducted over 60 days during May–July 2010. We first measured pollinator visitation rates (counts/2 min) of all *L. camara* visitors during sunny periods, from 09:00-14:59 h, to vases with control inflorescences (all three flower colours), yellow, orange and scarlet only inflorescences matched by floral numbers. Vials of flowers were presented on wooden dowels, completely randomly arrayed across the study site. The number of flowers in each treatment was standardized at nine and sample size was established at 15. This experiment was repeated by removing flowers from inflorescences on randomly selected plant stands to determine whether patterns of general pollinator visitation patterns were similar for flowers detached from plants (vase presentation) and those still attached to plants (natural presentation).

Experiment 2 — Billboard effect

For Experiment 2, a 0.5 m^2 quadrat was placed on an individual *L. camara* plant to delineate the area in which inflorescences were manipulated to reflect treatments described below. After the quadrat was removed, the entire plant with the exception of the 0.5 m^2 manipulated portion was covered with fine gauge mosquito netting, 3000 holes per cm, to prevent access of pollinators to un-manipulated inflorescences. Visitation rates of *H. melpomene* and *D. iulia* were observed for 30 d (June–July 2014) and 10 d

(December–January 2015), with visitation rates estimated over a 2-hour observational period. A butterfly was characterized as a visitor if it perched on the inflorescence. Visits were further categorized as either, long distance—number of approaches to a single plant stand or short distance—number of successive visits to multiple inflorescences on a single *L. camara* plant (Oberrath & Böhning-Gaese 1999). All experimental manipulations were done at 08:00 h just after yellow flowers had first opened but before focal butterfly species had begun to forage (G. Maharaj unpubl. data). All visitation observations were initiated 2-hours after experimental set-up. Each treatment was replicated five times on different plants randomly chosen form 25 marked plants with each replication carried out on different days to account for variability in butterfly behaviours and weather conditions.

Treatments were as follows (Fig. 1): — a) same size (app. 19-24 flowers) different colour: Each day we randomly selected a total of eight plants (two per treatment). Inflorescences on these plants were manipulated in the following ways — (i) control (not manipulated), (ii) 25:25:50 ratios of yellow: orange: red flowers, (iii) 50:50 ratios of yellow and orange flowers only, and (iv) All red flowers only (Gori 1989).

b) different size different colour:

We modified a total of six inflorescences per day (two plants per treatment) to offer the following three pairs of choices — (i) large red inflorescences (20 red flowers) versus large mixed inflorescences (three yellow, five orange and 12 red), (ii) large red versus small yellow (five yellow flowers), and (iii) large mixed versus small yellow (Weiss 1991).

Statistical analyses

A Kruskal−Wallis 1-Way Analysis of Variance (ANOVA) model was employed to assess differences among volumes for each of the major floral colour stages (yellow, orange and red) in wild-type sweet sage. A 1-Way ANOVA was used to compare sucrose concentrations of the three major flower stages and for the nine sub-colour stages. We employed a χ^2 goodness of fit model with a null hypothesis of equal attractiveness in test for significant differences among treatments (Weiss 1991), while a logistic regression was employed to compute short and long distance attraction based on single versus multiple visits to each treatment plant. All statistical analyses were carried out using IBM SPSS Statistics Version 23 (IBM Corp. 2015) and R Version 3.2.2 (R Development Core Team 2015).

Results

Flower colour and sucrose measurements

A Kruskal-Wallis 1-Way ANOVA revealed significant differences among sucrose volumes for all colour stages ($H_2 = 49.06$, $P < 0.001$, $N = 20$, Fig. 2) with significant differences between all pairwise comparisons (Tukey Test). Similarly, a 1-Way ANOVA model also showed significant differences among sucrose concentrations ($F_{2, 57} = 619.84$, $P < 0.001$, $N = 20$, Fig. 3) with significant differences for pairwise comparisons (Holm-Sidak method).

A more detailed look at the wildtype *L. camara* flower colour change system revealed that it can be subdivided into nine stages characterized by variations of the three main colours, yellow, orange, and red. These were as follows: Stg. I — orange yellow centre with spectrum orange edges, Stg. II — orange yellow centre with chrome orange

edges, Stg. III — orange yellow with flame scarlet edges, Stg. IV— spectrum orange with flame scarlet edges, Stg. V — chrome orange with flame scarlet edges, Stg. VI— chrome orange, Stg. VII— flame scarlet, Stg. VIII— flame scarlet with scarlet edges and Stg. XI — scarlet (colour swatches in Smith 1975). Measurements of sucrose concentration and volume by colour stage showed substantial variability (Figs. 4 & 5). However, there were significant differences among stages for both nectar sucrose concentrations and volumes. The 1-Way ANOVA for volume ($F_{8,216}$ = 12.906, $P < 0.05$, $N = 25$) and post-hoc analyses (Tukey Test) revealed that Stg. 1 flowers were statistically different from stages 4, 8 and 9, Stg. 2 was different from 8 and 9, Stg. 3 differed significantly from 4 and 5, while Stg. 4 differed from Stg. 9 (Fig. 4). For our concentration measurements analyses ($F_{8,216}$ = 117.32, $P < 0.05$, $N = 25$) we found that Stgs. 1, 2, 3, 4 and 5 were different from 6, 7, 8, and 9, and Stgs. 6, 7, and 8 were different from 9 (Fig. 5).

Pollinator taxa & fruit set

When percentage fruit set is considered, butterflies were the most effective taxon of diurnal pollinators, followed by carpenter bees (Apidae; *Xylocopa* spp.), and hummingbirds (Trochilidae; Fig. 6). Controls, butterflies, carpenter bees and humming birds had significantly better fruit set percentages than Trigonid bees, wasps and ants (Fig. 6). The numbers of diurnal pollinator butterfly taxa observed on *L. camara* are presented in Fig. 7. Therefore, we focused our study on butterflies due to their proclivity to visit *L. camara*, and their efficacy as pollinators. Of the butterflies, the most frequent visitors were *Heliconius melpomene* followed by *Heliconius sara, Dryas iulia* and

Heraclides thoas (syn *Papilio thoas*) (as seen in Fig. 7), however only *H. melpomene* and *D. iulia* were used in experiments because of their high abundancies.

Flower colour preference and billboard effect

Experiment 1 — Colour preference

Pollinator interest (mean pollinator visitation rates – number per 2 min) in the arrays of *L. camara* bouquets presented away from the plants had highest visitation rates at the all yellow only and control inflorescences ($F_{3, 56} = 98.34$, $P < 0.001$, $N = 15$). These results were similar to what was found in nature ($H_3 = 47.60$, $P < 0.001$, $N = 15$), where we observed butterflies showing preferences for inflorescences with all colour morphs (control) and inflorescences with only yellow morphs.

Experiment 2a — Billboard effects

A χ^2 test indicated a significant relationship between species and the frequency of visits to treatments (Tab. 1), $\chi^2_2 = 7.520$, $P = 0.02$, $N = 823$ *Heliconius melpomene* visited Large Mixed and Large Red flower more than Small Yellow whereas *D. iulia* visited Small Yellow and Large Mixed more than Large Red. A logistic regression analysis predicted the likelihood that our focal butterflies ($N_{H.\ melpomene} = 633$, $N_{D.\ iulia} = 190$) visited either single or multiple inflorescences on a single plant. For our model we used species and the three treatments (Small Yellow, Large Mixed and Large Red) as predictors. We did this to elucidate how species and treatment affects long and short distance attraction. A test of the full model against a constant only model was statistically significant indicating that the predictors as a set reliably distinguished between single versus multiple flower visitation, $\chi^2_3 = 41.23$, $P < 0.001$. The Wald criterion demonstrated

that of the two predictor variables only treatment was statistically significant. Butterflies visiting Large Mixed were 1.783 (P = 0.001) times more likely to visit multiple inflorescences on a plant, whereas butterflies visiting Large Red inflorescences (Exp (B) = 0.603, P = 0.005) were less likely to visit multiple inflorescences in comparison to plants with only small yellow inflorescences (see Tab. 3).

Experiment 2b — Billboard effects

A significant relationship between species and the frequency of visits to treatments was elucidated by a χ^2 test (Tab. 2), χ_2^2 = 70.434, P < 0.001, N = 1054. *Heliconius melpomene* visited 25:25:50 and all red more in comparison to the other treatments, while *D. iulia* preferred Control and 25:25:50 treatments. A logistic regression analysis was employed to predict the likelihood that our focal butterflies ($N_{H. melpomene}$ = 805, $N_{D. iulia}$ = 249) visited either single or multiple inflorescences on a single plant—in this model we used species and the four treatments (Control, 25:25:50-yellow: orange: red, 50:50-red and orange, and All Red) as predictors. We did this to determine how species and treatment affects long and short distance attraction. A test of the full model against a constant only model was statistically significant indicating that the predictors as a set reliably distinguished between single versus multiple flower visitation, χ_4^2 = 60.954, P < 0.001. The Wald criterion demonstrated that both predictor variables were statistically significant (Tab. 4). We found that *H. melpomene* (Exp (B) = 1.430, 95% CI 1.057-1.935) was more likely to visit multiple inflorescences than *D. iulia* and overall, butterflies were more likely to visit multiple inflorescences on the following plants, i.e. 50:50 (yellow: orange) (Exp (B) = 3.563, 95% CI 2.433-5.219), control (un-manipulated mixed) (Exp (B) = 3.562, 95% CI 2.464-5.148), and 25:25:50 (yellow:

orange: red) (Exp (B) = 2.618, 95% CI 1.822-3.761), in comparison to all red (see Table 4).

Discussion

Plants signal to a wide range of organisms using many types of visual signals involving both vegetative and reproductive parts (Hamilton & Brown 2001; Schaefer et al. 2004). In particular, many floral features, including but not exclusive to colour, odours, shapes, act as advertisements for potential pollinators (Weiss & Lamont 1997; Raguso 2004; Willmer et al. 2009). Researchers have also observed that various floral phenotypes serve to signal or advertise the presence of nutrition rewards (Schaefer et al. 2004; Raguso & Willis 2005). Angiosperms, such as sweet sage (*L. camara*), Lungwort flowers (*Pulmonaria collina*) and *Weigela middendorffiana* and *W. coraeensis* employ a variety of strategies to encourage pollinators to approach, of these; colour and changing colour appears to be particularly important for flower recognition and it exemplifies the evolution of floral traits driven by ecological interactions between plants and pollinators (Weiss 1997; Oberrath & Bohning-Gaese 1999; Ida & Kudo 2003; Ida & Kudo 2010; Willmer et al. 2009; Suzuki & Ohashi 2014). Changes in colour which occur in fully turgid flowers and differ from fading or darkening associated with floral senescence (Weiss 1995). These changes differ in the locations which they affect and may take place in any of the four floral whorls. It may affect the entire whorl, several whorls or parts of whorls in combination, or it may be completely localized to specific areas (Weiss 1995). The location of colour changes in Angiosperms are dependent on pollinator type, for example, plants pollinated by bats or moths generally have colour changes in the entire

flower while those that are butterfly, bee and fly pollinated usually have localized changes to specific floral parts, whereas bird pollinated flowers can encompass both types of changes (Weiss 1995). However, regardless of area affected it provides important information for pollinators that benefit both plants (communicator) and animals (receiver)—with pre-change flowers signalling the provision of rewards and the availability of receptive stigmas (Weiss 1991; Kudo et al. 2007). While post change flowers, are often retained though unrewarding and sexually inviable as plants benefit from larger floral displays that attract pollinators over long distances and indicating, at close range, pre-change flowers that are still viable (Gori 1989; Weiss 1991; Weiss 1995; Willmer et al. 2009, Ida & Kudo 2010).

Flower colour and sucrose measurements

When we examined flowers for three consecutive days our results, (pre-change) flower higher sucrose concentration and volume in comparison to day 2, orange, and day 3, scarlet, (post-change) flowers, mirrored that of Fritz Müller who reported to Charles Darwin (1877) that *Lantana camara* flowers in Brazil are viable for three days, changing from yellow on day−one, to orange on day− two, and scarlet on day−three with these floral colour signals correlating with nectar volume and sucrose concentration in many varieties (Darwin 1877). Thus *L. camara* flowers signal honestly to their pollinator as each colour stage reliably conveys information about an associated reward. However, when we examined nine colour stages (Figs 4 and 5) we noticed that as time progressed i.e., as the flowers aged, there was a no significant change in sucrose volume, although we noted earlier stages 1-3 having lower volumes than the later 5 stages with the exception of stage 9 that did not offer sucrose. This was also evident for concentration

with stages 1-5 having higher mean concentration than the later 4 stages including the final scarlet stage when no reward was offered. Additionally, although we were able to distinguish a colour change using the colour swatches in the first three stages they offered statistically indistinguishable rewards. However, when we only examined three colour stages (Figs. 2 & 3) we noted a decrease in both sucrose volume and concentration. The lower nectar volumes noted for initial stages of these the nine stages could be caused by environmental differences in temperature, relative humidity and soil moisture (Wolff 2006) between the two field seasons as we were unable to control for these factors at our study site. In addition, although Carrión-Tacuri et al. (2012), showed that the nectar volumes of bagged *L. camara* flowers did not change significantly throughout the day, they presented evidence that volumes oscillated between 0.9 and 1.1 µl. These variations were probably reflected in the measurements of the 9-stage *L. camara* readings but not in the 3-stage because these readings were only taken once per day.

Pollinator species & fruit set

Colour change in *L. camara* occurs for several reasons, these include attraction of pollinators such as hummingbirds, bees, wasps, ants, but especially butterflies (G.R. Bourne and G. Maharaj unpubl. data). The pollination syndrome hypothesis posits that different pollinators prefer different floral cues, with butterflies and bees preferring colours ranging from ultraviolet to yellow or red coloured flowers, and birds, orange, deep-pink and red flowers (Proctor et al. 1996; Weiss 1997; Johnson & Steiner 2000; Graham et al. 2003). Therefore, the presence of different colours on individual inflorescences served to attract the high taxon diversity of pollinators observed (Ostler & Harper 1978; Kampny 1995; Campbell & Hanula 2007; Suzuki & Oashi 2014). We do

acknowledge that in order to test the direct effect of colour on diversity we would have to manipulate inflorescences to reflect individual colour morphs and observe changes in visit diversity. However, from our study we did observe that inflorescences with all colour morphs were visited more often by all pollinators, although butterflies, carpenter bees and hummingbirds were the main visitors and most effective pollinators. The fact that this plant attracts these three ubiquitous taxonomic groups, may account for its spread globally.

Flower colour preference and billboard effect

Our findings suggested that *L. camara* signals honestly as their colour cues correlated with nectar rewards, with early more receptive yellow stages offering better rewards (higher concentration of sucrose, although volume was variable). While sexually inviable stages such as final stage scarlet flowers offered no reward (Oberrath & Bohning-Gaese 1999; Keasar et al. 2006). Therefore, this floral colour change is an adaptive trait that benefits both the plant and its insect pollinators by cuing the insects to visit the flowers at the optimal reproductive stage and thus minimizing the probability of illegitimate visits to non-reproductive flowers by changing colour and reward value, as we have seen with yellow, orange and scarlet flowers in our experiments (Willmer et al. 2009). Our evidence clearly supported prediction one (P_1) that first stage yellow flowers attract more pollinators as they contain greater quality of rewards (greatest concentration of sucrose) than later orange stages (lower quality reward) and scarlet stages (no reward). Thus the pollinators of *L. camara* displayed a greater preference for these pre-change yellow flowers than orange or scarlet flowers. We do acknowledge that our results may represent a combination of innate and learned preferences since many pollinators are able to

associate colour with reward (Menzel 1967, 1985; Waser & Price 1985; Weiss 1991; Waser et al. 1996; Weiss 1997; Campbell et al. 2012). In order to determine whether our pollinators have innate colour biases for these colours we would have to test naïve pollinator or carry out experiments in which post-change flowers offer greater rewards than pre-change flowers (Lanau & Maier 1995; Weiss 1997).

When we tested for the billboard effect we noted that although pollinators were more attracted to yellow flowers there were other factors that also affected visitations rates. While foraging, pollinators increase foraging efficiency by making two decisions based on distance—at long distances pollinators decide: 1) which plants should be approached, and at shorter distances, i.e., when they are on the plant, and 2) which flower(s) should be visited. Both of these decisions are based on visual attractiveness of plants and flowers, respectively (Oberrath & Böhning-Gaese 1999). Work by Gori (1989), Weiss (1995) and Willmer et al. (2009) also demonstrate that plants benefit from larger floral displays that attract pollinators over long distances. Plants offering both rewarding pre-change flowers and provision less post-change flowers served as a superior attractant to pollinators at greater distances—a strategy that results in increased pollinator visitation (Barrows 1976; Weiss 1991; Nuttman et al. 2005). These results corroborated our findings and supported our second and third predictions. We observed inflorescences with greater proportions of yellow and orange flowers i.e., small yellow, 25:25:50 (yellow: orange: red), 50:50 (yellow: orange), control and large mixed were more attractive over short distances (P_2) as this resulted in multiple visits to individual flowers on each because butterflies learned to associate colour with reward, thus pre-change yellow flowers were favoured at close range (Gori 1989; Weiss 1995; Willmer et al.

2009). Inflorescences with unrewarding red flowers were found to be most attractive to pollinators over long distances, as inflorescences on these plants were only visited once, however overall, most visits to plants were made to large mixed and control due to the billboard effect that results from larger multi-coloured displays (P_3) (Barrows 1976; Gori 1989; Weiss 1991; Weiss1995; Nuttman et al. 2005; Willmer et al. 2009). The retention of provision less scarlet flowers function to increase the inflorescence size, and advertisement attractiveness so making a bigger landing platform for large butterflies (Barrows 1976), thereby making these inflorescences more attractive than just small yellow all rewarding inflorescences of our focal plant system. Although we found that retention of scarlet flowers benefitted our study plants Ida and Kudo (2003) demonstrated that this is not case for all colour change plants i.e. *Weigela middendorffiana*. It was also noted that although the size of the landing platform and its effect on proclivity to land was not measured, it was noted that *D. iulia*, a medium sized butterfly, as is *H. melpomene*, preferred both large mixed and small yellow to large red, whereas *H. melpomene* preferred large red to large mixed. This suggests, although not conclusively that butterflies will feed on inflorescences of both sizes.

Overall we found differential preferences by our two focal species, with *D. iulia* visiting inflorescences many yellow flowers, viz. small yellow, 50:50 (yellow: orange) or control, more frequently while *H. melpomene* tended to frequent inflorescences with many red flowers; large red, large mixed, 25:25:50 (yellow: orange: red) and all red treatments. We also noted that when presented with small yellow, large mixed and large red inflorescences butterflies were more likely to visit the flowers of large red inflorescences only once. Similarly, when presented with control plants, 25:25:50 (yellow: orange: red),

50:50 (yellow: orange) and all red plants butterflies visited single flowers on all red inflorescences. Therefore, although red flowers draw in pollinators from a long distance, only plants with rewarding flowers facilitate short distance feeding behaviours.

In summary our results suggested that *L. camara* incorporates two main strategies to visually attract pollinators at long and short distances. First, they signal honestly as the rewards offered reliably correlated with colour stage. Secondly, by offering multiple coloured inflorescences with centrally located scarlet flower buds surrounded by pre-change yellow flowers and older post-change orange and older scarlet flowers, plants behave like billboards communicating their attractiveness to pollinators at greater distances; a strategy that resulted in visitations by a diversity of pollinators at both long and short distances (Weiss 1991; Nuttman et al. 2005), the overall effect being that individual *L. camara* plants have increased fitness. Our study also highlighted species specific visitation preferences based on flower colour morphs presented, although both study species exhibit generalized learned preferences when it came to feeding, i.e., choosing flowers with greatest rewards. These visitation preferences may be due to inherent colour preferences of each butterfly species and linked to their abilities and genetic mechanisms to decipher colour (Hsu et al. 2001; Briscoe 2008). This study further identified areas of future work as we try to tease apart the specific visual signals that are used by each butterfly species and its impacts on pollination efficacy.

Acknowledgements

We are grateful to O. O'Dean, H. Stowe, H. Chaney, and S. France for field assistance and support during field work and to A. Dunlap, N. Muchhala and Y. Wu for

their guidance and comments during the planning and execution of this research. This work was supported by a Small Grant from the Rufford Foundation, UK, and three CEIBA Biological Center Grants awarded to G. Maharaj, and three anonymous foundation grants to G.R. Bourne. This paper was constructed from a chapter in G. Maharaj's unpublished dissertation presented to the University of Missouri-St. Louis.

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Figures

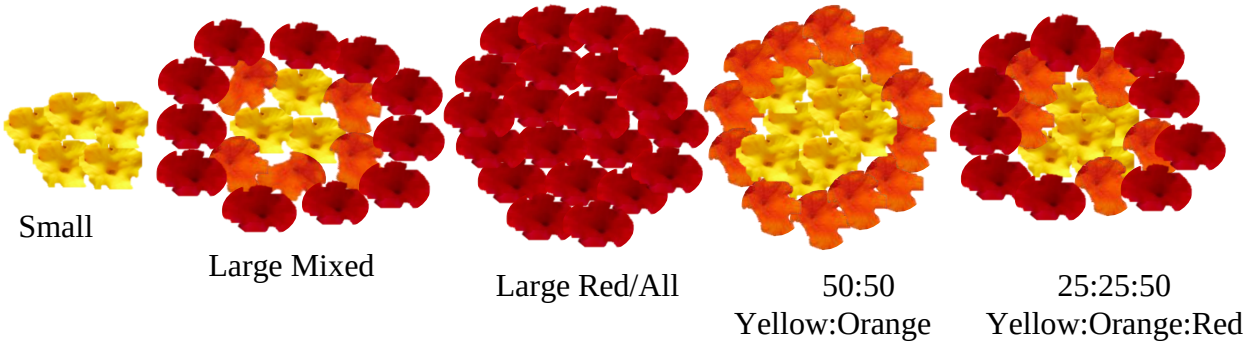

Figure 1. Manipulated treatments of flower colour preference & billboard effect experiments

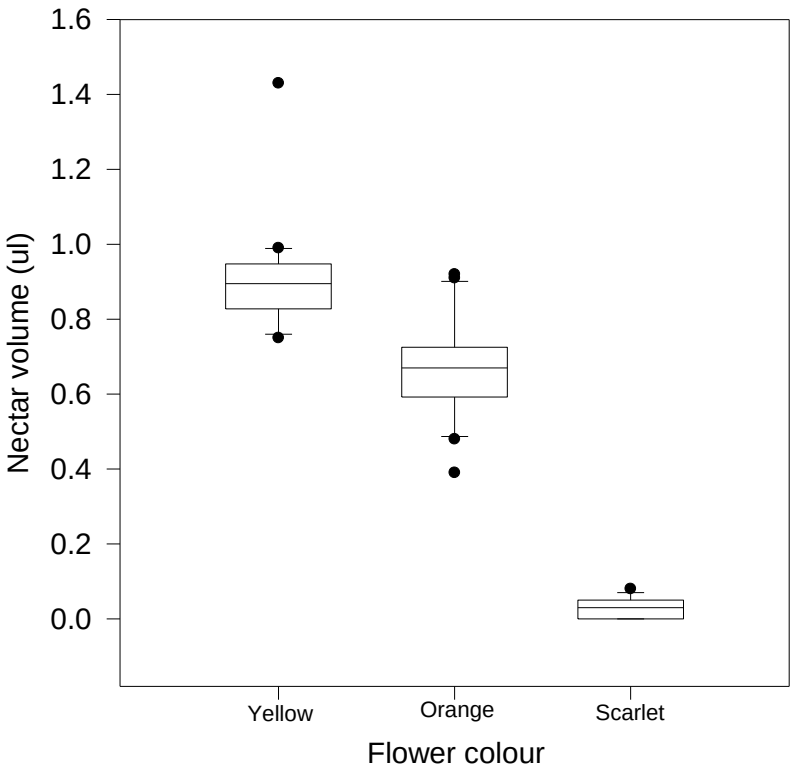

Figure 2. Sucrose (nectar) volumes for the three gross colour stages indicated declining production with time.

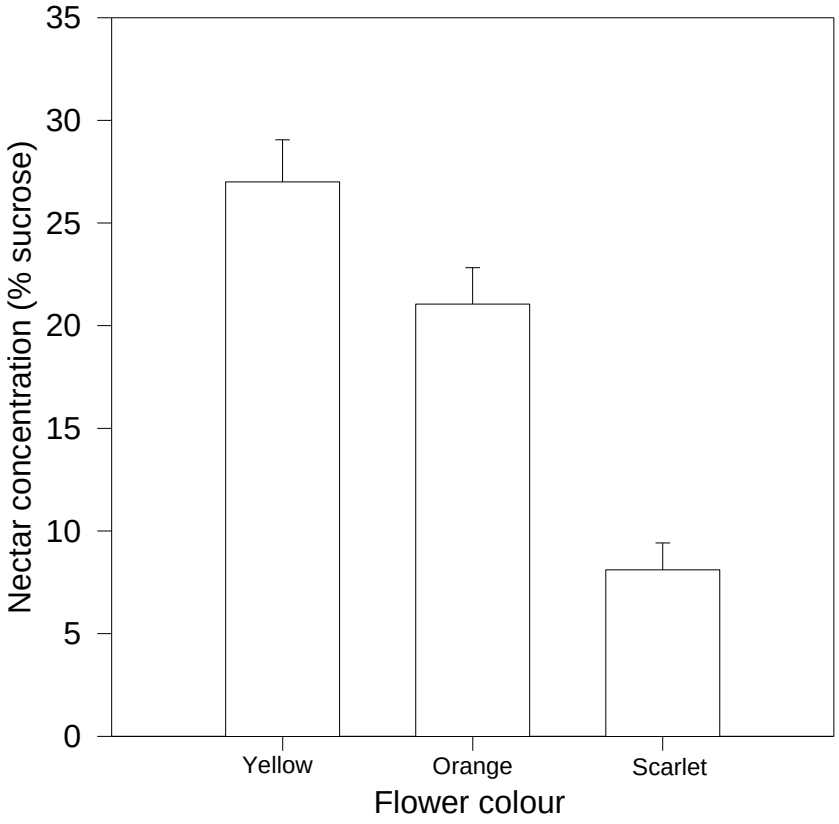

Figure 3. Comparisons of percentages of sucrose concentrations for the three gross colour stages, these comparisons also indicated reduced quality with time.

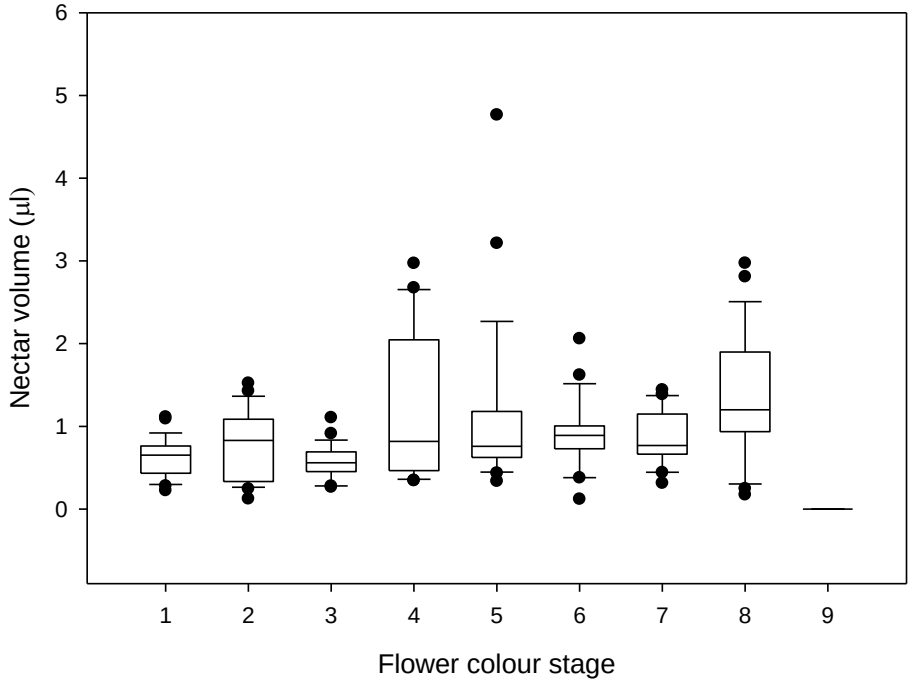

Figure 4. Comparison of sucrose volumes for fine temporal colour stages (1= Stage 1 etc.) showing an increase in volume after stage 3 and no reward offered in stage 9.

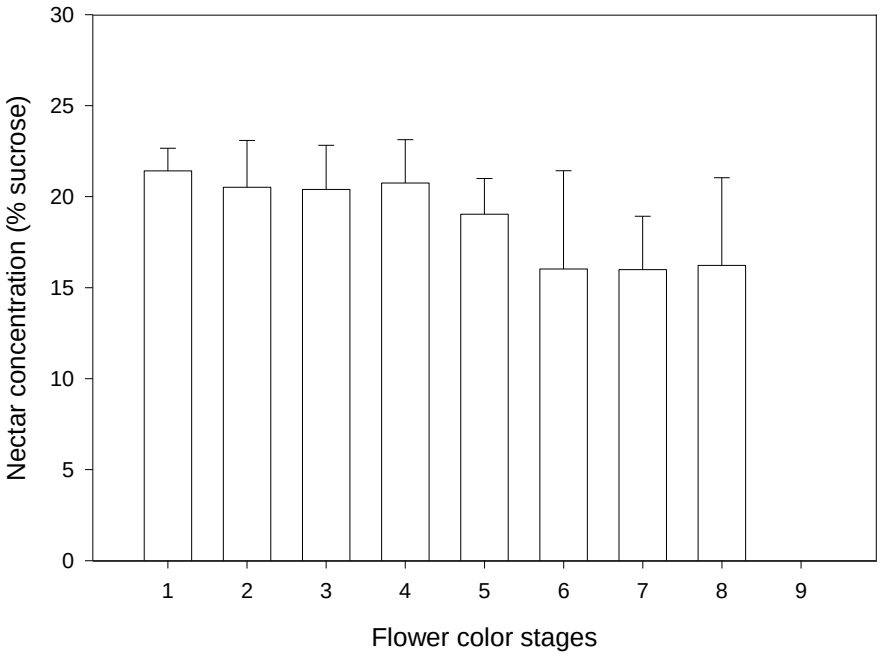

Figure 5. Comparisons of sucrose concentrations by colour stage showing a decrease in concentration in later stages (5-9).

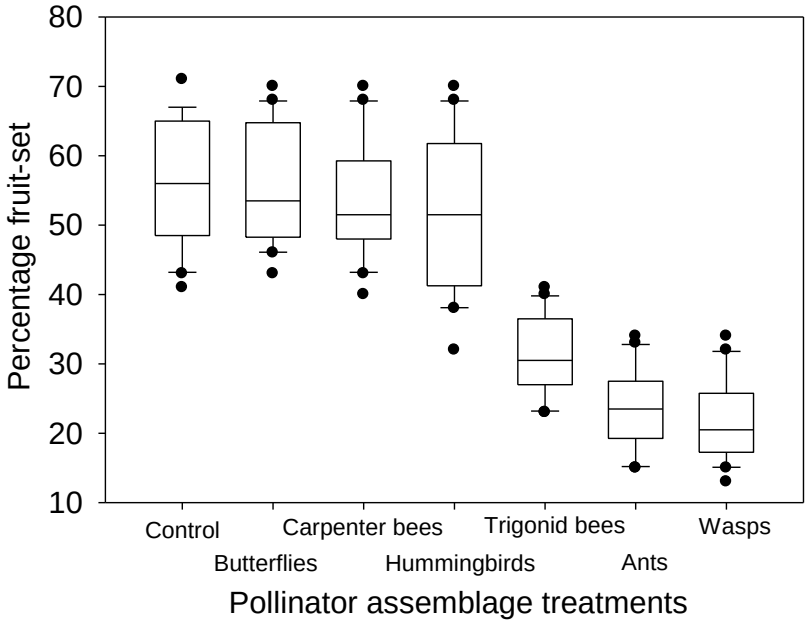

Figure 6. Pollinator taxa and effectiveness of visits on the percentage of fruit set in *Lantana camara.* The Control variable consists of the effects of all pollinating taxa visits on fruit set. Note that butterflies were as effective as the combined control taxa.

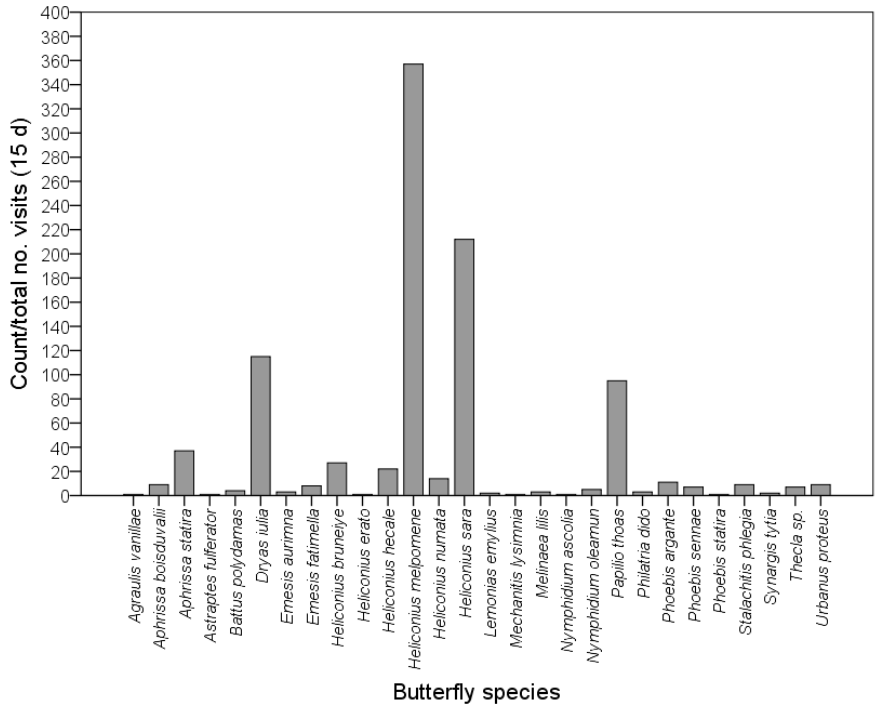

Figure 7. Frequency of Lepidopteran pollinators observed foraging on *L. camara*.

Table 1. Frequency of visits by *H. melpomene* and *D. iulia* to treatments of same size different colours.

	Variables	Frequency
Treatment	25:25:50	272
	Control	307
	50:50	238
	All Red	237

Table 2. Frequency of visits by *H. melpomene* and *D. iulia* to treatments of different sizes different colours.

	Variables	Frequency
Treatment	Large Mixed	305
	Large Red	283
	Small Yellow	235
Species	D. iulia	190
	H. melpomene	633

Table 3. The Wald criterion identified treatment (Small Yellow, Large Mixed and Large Red) as a significant predictors of the likelihood that our focal butterflies visited either single or multiple inflorescences on a single plant.

Variables		B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I. for EXP(B) Lower	Upper
Step 1[a]	SP(1)	−0.060	0.173	0.121	1	0.728	0.942	0.672	1.321
	Treat			39.235	2	0.000			
	Treat(1)	0.578	0.181	10.197	1	0.001	1.783	1.250	2.544
	Treat(2)	−0.505	0.179	7.984	1	0.005	0.603	0.425	0.857
	Constant	0.291	0.181	2.591	1	0.108	1.338		

[a] Variable(s) entered on step 1: SP = Species, Treat = Treatments (viz. Small Yellow, Large Red and Large Mixed).

Table 4. The Wald criterion identified treatment (Control, 25:25:50 (yellow: orange: red), 50:50 (red and orange) and All Red) and species (*D. iulia* and *H. melpomene*) as a significant predictors of the likelihood that our focal butterflies visited either single or multiple inflorescences on a single plant.

Variables		B	S.E.	Wald	Df	Sig.	Exp(B)	95% C.I. for EXP(B)	
								Lower	Upper
Step 1[a]	Sp(1)	0.358	0.154	5.386	1	0.020	1.430	1.057	1.935
	Treat			57.113	3	<0.001			
	Treat(1)	0.962	0.185	27.072	1	<0.001	2.618	1.822	3.761
	Treat(2)	1.270	0.188	45.662	1	<0.001	3.562	2.464	5.148
	Treat(3)	1.271	0.195	42.609	1	<0.001	3.563	2.433	5.219
	Constant	−1.002	0.198	25.673	1	<0.001	0.367		

[a]Variable(s) entered on step 1: Sp = Species, Treat = Treatment (viz. 50:50 (yellow and orange), All Red only, 25:25:50 (yellow: orange: red) and Control).

Chapter 4

Butterfly foraging patterns disrupted by the presence of heterospecific butterflies and hummingbirds

Chapter draft to be submitted to *Journal of Behavioral Ecology* with Yeufeng Wu and Godfrey R. Bourne.

Lay Summary

When organisms share a limiting resource at the same time and in the same habitat it is best to either avoid each other or feed differently. Butterfly types divided feeding areas spatially from each other, and from aggressive territorial hummingbirds. When food types were shared butterflies fed at different times and on different plants. Additionally, the different butterfly species changed the number of visits to plants, number of plants visited, and time spent foraging thereby achieving successful coexistence.

Abstract

We determined whether freely foraging passionflower butterflies, postman (*Heliconius melpomene*) and flambeau (*Dryas iulia*) established regular foraging routes that matched the geometry of arrays created by covering and uncovering naturally blooming sweet sage shrubs (*Lantana camara*). They did not, but we traced movement patterns that minimized interplant flight distances influenced by the presence of heterospecific butterflies and very aggressive hummingbirds (Trochilidae). Both butterfly species exhibited territoriality that excluded each other. When hummingbirds defended *L. camara* flower patches both butterfly species divided flower resources spatially and temporally. Butterflies exhibited both similar and dissimilar foraging behaviors to solve problems associated with changing nectar resources. *Heliconius melpomene* was more sensitive to nectar availability than *D. iulia*, and responded by exhibiting two foraging tactics. One was unique, increasing the number of visits and foraging times, and the other, also exhibited by *D. iulia*, increasing the number of visits and decreasing foraging times. In addition, both species varied their feeding repertoire, incorporating new plants when current feeding plants were covered but continued to visit these new foraging locations even when access to previously covered plants were available again. Our results suggested that foraging patterns differed by species but were modified by the presence of heterospecific animals competing for the same flower resources with fluctuating rewards. Yet, movement patterns by the butterflies always minimized interplant flight distances.

Keywords: *Dryas iulia*, *Heliconius melpomene*, Guyana, *Lantana camara*, foraging patterns, passionflower butterfly, resource partition, heterospecific competition

Introduction

Individual organismal movement is a critical element of most evolutionary and ecological processes that are now attracting focused research attention (Holyoak et al. 2008; Nathan 2008). But few studies investigate movement relationships of invertebrates among plants at the individual level (Holyoak et al. 2008). Spatial-use strategies by foragers are key factors in their fitness, as they must move to locate and acquire their food (Ohashi et al. 2007). When using pollinators as model organisms, for example, researchers usually assume that they are choosing each flower from which they imbibe nectar naïvely, that is, foragers tend to 'meander' until an appropriate flower is encountered. However, Zimmerman (1979) indicated that foragers have prior knowledge of the locations and values of rewards(Zimmerman 1979; Zimmerman 1981). Pioneering studies of pollinator movement tended to model foraging patterns as outcomes of simple movement rules between successively visited flowers or plants as choices of ''movement distance'' and ''turning angle'' (Zimmerman 1979; Waddington 1980; Cresswell 2000; Ohashi et al. 2007). This approach, however, may not be sufficient to describe spatial use by pollinators given competitive interactions. Bees, for example, sometimes establish small foraging areas to which they return faithfully over many days (Thomson 1996; Williams and Thomson 1998; Ohashi et al. 2007). Similarly, leking species of hermit hummingbirds (*Phaethornis* spp.) repeatedly visit isolated and undefended flowers offering large rewards (Gill 1988). These pollinators are not only remembering the locations of resource sites, they also trapline, employing a foraging strategy in which the individual animals travel among food resources in a stable repeatable sequence in order

to gain optimal profit which reflects knowledge of reward (Heinrich 1976, Gill 1988, Thomson 1996).

Traplining is an efficient method of collecting food at steady intervals from renewable isolated resource locations, as is used by many species (Heinrich 1976; Gill 1988). Due to local knowledge, trapliners that learn locations of most rewarding resources can more efficiently exploit these resources. In the case of flowers, many animals are able to track nectar-refilling schedules and can outcompete 'naïve' conspecifics for resources (Williams and Thomson 1998; Ohashi and Thomson 2005; Ohashi et al. 2008; Ohashi and Thomson 2009; Lihoreau et al. 2010 and citations therein). Traplining behaviors are documented for many taxa such as bees, birds, and mammals (Thomson, Slatirin, Thomson, et al. 1997; Lihoreau et al. 2010). Yet very little work has statistically tracked feeding patterns of butterflies (Gill 1988). Gilbert (1980) indicated that *Heliconius* butterflies trapline (Gilbert 1980, *Heliconius* Genome Consortium 2012). However, little is known about their traplining behavior, i.e., whether they established regular foraging routes that confirm to the geometry of distributional patterns of plants, and how butterflies adjust in response to perturbations such as the loss of plants, and the presence of territorial competitors in their feeding circuits (Ohashi and Thomson 2005; Ohashi and Thomson 2009). Our aim was to document whether movements of two heterospecific passionflower butterflies conformed to the geometries of arrays created by covering and uncovering naturally blooming sweet sage *Lantana camara*.

We initially intended to document traplining behaviors in our study species because preliminary observations provided evidence of this behavior. However, during

our latest study season we noted that our butterflies visited far fewer plants, and did not establish regular foraging routes that matched the geometries of the treatment arrays, as such we were unable to statistically test for traplining behaviors as described in Thomson et al.(1997). Instead, we investigated short-distance foraging and movement patterns used by the two sympatric passionflower butterflies, *H. melpomene* and *D. iulia* at a long term feeding patch where they faithfully feed on *Lantana camara.* Specifically, we focused on changes to the feeding patch by two perturbations: 1) changes in nectar availability; and 2) presence of heterospecific competitors, both exploitative competitors (butterflies) that consume nectar thereby making it unavailable to other butterflies and interference competitors (hummingbirds) that aggressively exclude butterflies from nectar sources. Although early research focused on factors affecting nectar feeders at established feeding areas (Gill 1988; Heinrich 1976; Thomson 1996; Ohashi et al. 2007), the presence of competitors (Ackerman et al. 1982; Thomson et al. 1987; Ohashi et al. 2007) and changes in nectar availability (Goulson et al. 2007; Lihoreau et al. 2010), they focused on hymenopteran groups. Therefore, our study was unique as we focused on topics such as resource partitioning, foraging behavior, and competition in the understudied Lepidoptera as we investigated short-distance foraging, movement patterns and competitor interactions.

We began our research by observing interplant movement and foraging by passionflower butterflies, postman (*H. melpomene*) and flambeau (*D. iulia*) under natural conditions (no resource restriction with competitor). Subsequently we investigated interplant movement, foraging patterns and competitor interaction (butterflies and hummingbirds) when the numbers of flowers available were reduced and then made

available again. For these experiments, we posited the following hypotheses: (H_1) if resources in an established feeding patch are reduced butterflies will adjust their existing movement patterns to accommodate for this change. The predictions generated are that butterflies will: (P_1) include more plants in their feeding circuit; (P_2) increase number of floral visits; and (P_3) increase time spent in order to acquire sufficient nectar to meet their caloric needs. (H_2) when previously unavailable plants become available again butterflies will include them in their feeding circuits. Thus, we predict that: (P_1) butterflies will return to previously established routes when plants in these locations were available again. Finally: (H_3) that the presence of competitors, butterflies (exploitative competitors) and hummingbirds (interference competitors), in a feeding patch will affect the feeding patterns of butterflies. We predicted that: (P_1) the two butterfly species will partition their resources spatially in order to avoid confrontation with the larger hummingbirds, which aggressively displace them, butterflies should avoid patches defended by territorial hummingbirds and (P_2) when hummingbird defended plants are unavailable hummingbirds will establish new territories within the feeding habitat, thereby displacing butterflies from their established feeding plants.

Method

Plant

Lantana camara L. (Verbenaceae) is a readily available, easily tractable common shrub found in open habitats in the CEIBA area that provides food to a variety of pollinators including our study species, which are among the top three foragers as characterized by frequency of visits (G. Maharaj manuscript in preparation). This shrub has multiple inflorescences with 20–25 flowers per inflorescence placed in whorls (G.R. Bourne

unpublished data). There are many horticulture varieties of *Lantana* that have small 5-lobed flowers in a variety of colors which include white, yellow, orange, red and purple that are often mixed in the same cluster. Wild-type *L. camara* used in this study presented potential visitors with day-1 yellow flowers, day-2 orange flowers and day-3 flame scarlet flowers that then become scarlet until abscission.

Pollinators

We focus on *Heliconius melpomene* (black wings with red a blurred patch on forewing and a yellow line on underside of hind wing curves towards the posterior) and *Dryas iulia* (bright orange wings with black margins, forewings elongate with dorsal fore and hind wing surface brighter than ventral side). Both butterflies are members of the Nymphalidae family and are common pollinators/foragers of *Lantana camara* present at our study site (G. Maharaj and G.R. Bourne unpublished data). We have chosen to work with these butterflies because they are easily tractable in the wild, and have been the focus of a large body of work in evolutionary biology and animal behavior (Hsu et al. 2001).

Site

We conducted our field studies at CEIBA Biological Center, N 06° 29/.945//, W 058° 13/.106//), on the Soesdyke−Linden Highway, Madewini, Guyana. Observations were carried out in a sustainable demonstration farm site (320m^2) comprised of numerous *L. camara* plants. Surrounding this site is a white sand area is comprised of low seasonal forest dominated by the fast-growing *Eperua falcate* (Caesalpiniaceae), and tall primary

growth flooded forests dominated by *Mora excelsa* (Fabaceae) (Bourne and Bourne 2010).

Procedures

We spent two days (23-24 June 2015) hand netting and marking butterflies with small round unique color combination tags (Ehrlich and Gilbert 1973, Gill 1988). To reduce butterfly stress, we released them within 60 s of capture to a foraging plant. With the help of University of Missouri St. Louis and University of Guyana field assistants we observed all marked butterflies but noted that only a few marked *H. melpomene* butterflies and no marked *D. iulia* butterflies survived through every day of each experimental treatment listed below. Therefore, we continued to mark new butterflies as needed for the duration of the study to reduce identification errors and ensure we were tracking the same individual for their entire feeding bout.

We also marked by color flagging and mapped positions of all flowering *L. camara* plants in study area using a Garmin eTrex 10 Worldwide Handheld GPS Navigator (N = 25). We also calculated the size of the average crown spread of each plant using the cross-method by measuring the longest spread (drip-tip to drip-tip) and the longest cross-spread perpendicular to the first cross-section through the central mass of the crown (Blozan 2006). This produced the variable, average crown spread = (longest spread + longest cross-spread)/2. The crown density of each plant was also determined by using Crown density-foliage transparency cards to estimate the percentage of light that was being blocked by the crown mass. These estimates were based on measurements

made from two directions at right angles to each other and reconciled to determine the amount of branches, foliage and flowers on each plant (USDA Forest Service 2010).

Foraging patterns of butterflies under natural conditions – Control

We monitored inter-plant movement and foraging times by individually marked butterflies and we observed plants that were used by butterflies only, those shared with hummingbirds and those used by hummingbirds only (Fig. 1.1). These observations were made for 3-hours, 08:00-11:00 h, and 14:00-17:00 h for 6 days, 25-29 June 2015. We also recorded movement sequences at plants visited by butterflies, number of plants visited, time spent foraging at each plant, and duration of entire foraging bouts. We used these measurements to indicate changes in forging patterns as an expression of the distribution of number of visits and foraging time per visit.

Reduction and subsequent return of food resources – Treatment 1

After identifying the plants fed on by butterflies and hummingbirds, we chose "shared" plants (fed on > five times by both butterflies and hummingbirds) and covered 50% of these plants, (Treatment 1a, Fig.1.2) from 30 June –4 July 2015. These were later completely covered (100%; Treatment 1b, Fig. 1.3) form 5–9 July 2015 with fine gauge mosquito mesh (3000 holes per cm) to prevent butterfly access and observed plants visited and time spent foraging. All "shared" plants were then made available again (Treatment 1c, Fig. 1.4) from 10–14 July 2015, and inter-plant foraging movement and time spent foraging were observed for another five days.

Interference competitor presence on feeding behaviors – Treatment 2

In addition to observing butterfly/hummingbird interactions in natural un-manipulated and manipulated treatment 1 settings, to investigate the effect of competitor presence on feeding behaviors we also covered hummingbird plants (>10 hummingbird feedings) by the three territorial species of the Trochilidae family, *Chlorostilbon mellisugus*, *Amazilia fimbriata* and *A. leucogaster*. We subsequently observed and recorded any reciprocal changes in hummingbird and butterfly feeding plants, and time allocated by each species to foraging (Fig. 1.5) from 15–21 July 2015.

Analyses

Due to the low number of survivors of marked butterflies in all treatments we did not analyze foraging times and number of visits to individual plants by individual butterflies. Instead, we grouped individuals into three broad categories viz. *H. melpomene*, *D. iulia* and territorial hummingbirds (*Chlorostilbon mellisugus*, *Amazilia fimbriata* and *A. leucogaster*). We employed IBM SPSS Version 23 (IBM Corporation 2015), R version 3.2.5 (2016), and Microsoft Excel (2016) programs to analyze data sets, and to generate graphs and tables. To determine whether there were relationships among specific plants and animal species we used a Fisher's Exact Test. A two-way factorial ANOVA with post-hoc analyses Tukey's HSD was used to compare the average time spent between treatment by species, and a Pearson Chi-square was used to investigate how well the observed distribution of the total number of visits by each species per treatment fitted its expected distribution. In order to test for overlap in forging time we used the formula **(Start time butterfly/hummingbird 1 <= End time butterfly/hummingbird 2) and (End time butterfly/hummingbird 1 >= Start time butterfly/humming bird 2).**

Finally, we created a model that described the visiting and feeding pattern for the butterflies and hummingbirds in our study site:

Model

$$\mathbf{Y_{i,j,k} \sim Poisson\ (\lambda_{i,j,k}),} \quad \mathbf{(1)}$$

Where i = 1, 2, 3, …, 25 – 25 different plants, j = 1, 2 - two species, k = 1, 2, …, 5 – five treatments,

then

$$\mathbf{Log\ \lambda_{i,j,k} = \beta 0_{j,h} + \beta 1_{j,h} * resource_i} \quad \mathbf{(2)}$$

where h denotes the "zone" and h itself is a function of plants and treatments:

$$h=h(i,k) \quad \mathbf{(3)}$$

Priors on the parameters:

B0,β1~normal (0, 10000000) which means we give almost 0 prior information on these parameters

h~ unif (1,2,3), we tried 1234 or 1,2 for different number of zones, but 3 is the best.

The model assumes almost no prior information on feeding behaviors such as preferences for a specific plant. The expected times of foraging bouts were determined by the resource (linearly), and the intercept and the slope differed not only between species but also among different plants that belong to different "zones". With h changes for the same

L. camara plant under different treatments, which reflects the change of the feeding pattern due to the presence of hummingbirds and available resources.

Results

Foraging patterns of butterflies under natural conditions – Control

Under unmanipulated conditions (control) we observed that plants 1-5 and 22-25 are shared by both butterflies and the hummingbirds (Fig. 2). Those shared by a particular species of butterfly and hummingbird included (hummingbirds + *H. melpomene* = 6 and 14-17, hummingbirds + *D. iulia* = 7 and 19), while there were others that were visited exclusively by hummingbirds (9, 10, 11 and 21) or butterflies (8 butterflies only, 12 and 13 *H. melpomene* only). Number of visits to these plants and time spent on each plant changed depending on the treatment. However, although *H. melpomene* and *D. iulia* shared the same some resources we observed in control conditions that *H. melpomene* occupied the upper right portion of the study site and focusing on plants 1, 3, 4, 5, 12 and 13. Here, we observed 69% of their feeding bouts. Whereas, *D. iulia* feed mostly (64% feedings) in the lower left focusing on plants 19, 22, 23, 24 and 25 ($p<0.01$, Fisher's Exact Test). Hummingbirds defended territories in the center of the farm on plants 9, 10, 11 and 21. This behavior therefore facilitated very little overlap with each other and hummingbirds as shown in Fig. 3.

Reduction and subsequent return of food resources – Treatment 1

When the "shared" plants (1, 5, 22 and 24) were covered by 50%, time spent on these plants varied from the control with *H. melpomene* spending more time on these plants, and included new plants 9 and 10 in their feeding territories. *Dryas iulia* spent less time

on plants 22 and 24 and did not feed at all on plant 1 or 5. Like *H. melpomene*, *D. iulia* increased time spent on surrounding plants such as 3, 4, 6 and included a hummingbird defended plants 10, 14,15, and 17. Hummingbirds spent more time on plants such as plants number 11, 12, 13, 14, 15, 16, 17, and 18.

When all "shared" plants (1, 5, 22 and 24) were covered completely (100%) hummingbirds, *H. melpomene* and *D. iulia* displayed a "hold over" behavior where they revisited many of these plants after they were covered (Total number of visits observed = 34) although no reward was provided. We also observed that *H. melpomene's* feeding trend was similar to when plants were covered by 50% with plants 3, 4, 12, 13, 15, 18 and 23-25 being popular. *Dryas iulia*, interestingly, returned to many of the plants that they feed on in the control but later abandoned when 50% of these plants were covered. Hummingbirds fed more frequently on the plants that they had moved to when shared plants were covered by 50%. When all shared plants were reopened *H. melpomene*, *D. iulia* and hummingbirds incorporated both old and new plants into their feeding repertoires. Thus, there was a great amount of overlap, Fig. 3, however, there was still a significant relationship with species and various plants ($p<0.01$, Fisher's Exact Test). We observed that *H. melpomene* fed on 1, 3, 4, 5 12 and 13 (52%) and also started to incorporate plants 2, 10 and 22-25. *Dryas iulia* feed on 19, 22-25 (40%) but also depended on many other plants such as 1,2,3,5,7,8,10,11,15,16, and 18 (both *H. melpomene* and hummingbird frequented plants).

Table I shows that *D. iulia* increased the number of visits and reduced time spent when shared plants were completely covered. When all plants were again available *D. iulia* increased their visits and reduced time spent foraging. *Heliconius melpomene*,

however, increased visits and time spent when only 50% of plants were covered but increased visits and reduced time when 100% were covered. Additionally, when plants became available again we observed that *H. melpomene* returned to the approximate number of visits and time spent as in the control treatment.

Interference competitor presence on feeding behaviors – Treatment 2

When hummingbird plants were covered, and shared plants were left open, *H. melpomene* increased feeding on shared plants, 1 and 25, *D. iulia* continued feeding on new and old plants as they did in the open treatment, as the overlap continued, Fig. 3, and there was a marked decrease in time spent by hummingbirds on the study area.

From our two-way factorial ANOVA (Table II) based on mean time spent presented in Table I, the mean time/s spent foraging in each did change, however those changes were not statically significant. The p-value for species is however significant. Tukey's HSD, Table III, shows there was no significant difference in the time spent foraging by hummingbirds and the *D. iulia* butterflies. However, there was a difference in both groups compared to *H. melpomene* butterflies, which on average spent more time foraging on plants in comparison to the very short feeding bouts noted for *D. iulia* and hummingbirds. This is in keeping with the slower flight observed for *H. melpomene* in comparison to faster erratic flight pattern of *D. iulia*, and the more focused speedy darting flight of hummingbirds. Our χ^2 test on total number of visits by each species per treatment, however, revealed a significant relationship ($\chi^2 = 79.623$, df = 8, $p < 0.01$) N = 1724). Therefore, although the time spent foraging does not change statically with varying treatments the number of visits to plants is statically different.

However, when individual plants were examined (Fig. 4) the number of visits by butterflies did not change regardless of the treatment. We noted that plants on which butterflies feed were located on the periphery of the farm whereas plants unaffected by treatments were located in a central zone of the study site. In this zone there was an abundance of hummingbird feeding plants. As we examined this concept of various zones seen in Figures 5a and 5b we noticed that *H. melpomene* exhibited feeding in three separate zones, i.e., Interaction Zone (Hummingbirds + Butterflies Share), No Interaction Zone (Butterfly Safe, Hummingbirds absent) and Hummingbird Defended Zone (Butterflies unsafe, Hummingbirds territorial). However, when we looked at *D. iulia* there was a cross over from the interaction zone and the no interaction zone, caused by the high number of interactions between *D. iulia* and hummingbirds. In addition, we found that individual plants were not fixed in particular zones but changed zone assignment in response to treatments, (see Fig. 6—Class 1 – Interaction Zone, Class 2 – No interaction Zone, Class 3 – HBD Zone).

Discussion

When foraging, movement patterns of nectarivores are dependent on resource availability i.e. the plant itself through spacing, floral density and nectar production (Levin and Kerster 1969; Scott 1975; Cresswell 2000, Fermon et al. 2003), and on exploitative and interference competitors (Milinski 1982; Belovsky 1997), as all of these factors places limits on nectar intake per visit. Therefore, it is necessary for foragers to adopt spatial use strategies i.e. foraging movement patterns and behaviors, that facilitate maximum nectar intake from available resources while simultaneously reducing competition. We found that our study species, *D. iulia* and *H. melpomene*, varied their foraging patterns by

adjusting the location of their feeding plants, feeding times, the number of visits to plants, number of plants visited, and time spent foraging, in order to satisfy their caloric needs and to avoid being outcompeted by heterospecifics.

Foraging patterns of butterflies under natural conditions – Control

Our results show, that initially, when resources were available but competitors limited nectar intake, *D. iulia* and *H. melpomene* butterflies use a variety of resource partitioning feeding patterns in order to promote a long term coexistence with each other and other nectar feeders such as hummingbirds (Graham and Jones 1996). This is not surprising as it is advantageous for sympatric species that share food resources to avoid each other whenever possible. This in turn promotes the use of different resources or the use of resources differently, i.e. resource partitioning, which facilitates reduced competition and increased food intake (Pianka 1981; Walter 1991; Graham and Jones 1996).

Resource partitioning methods used, included spatial partitioning of feeding plants by location, where butterflies mainly fed on the periphery of the farm, with *H. melpomene* concentrating its feeding in the upper right portion of the farm while *D. iulia* focused in the lower left, while humming birds fed in the center (Fig. 4). Thus, we found very little overlap for feeding times (Fig. 3). Our results, although novel for Lepidoptera, are also known for other taxa such as fishes (Ross 1986; Sala and Ballesteros 1997), reef-building corals (Porter 1976), and even other insects such as ants (Albrecht and Gotelli 2001), and bees (Graham and Jones 1996) which also spatially partition resources in order to successfully coexist with competitors.

Although we observed overall spatial partitioning among both butterflies and hummingbirds we noted that the extent of this behavior was species specific, with *D. iulia* having more interactions with hummingbirds in comparison to *H. melpomene*, i.e. *D. iulia* foraging movement patterns intersected more with hummingbirds than did *H. melpomene* (Figs. 5a, and 5b). This may be attributed to *D. iulia's* faster flight, statistically similar to the feeding bouts times of Hummingbirds, which permitted it to feed on some hummingbird defended plants and escape attacks unscathed. This contrasted with *H. melpomene*, which is a slower flyer and slower feeder, thus, making it more susceptible to hummingbird attacks. These species specific behaviors are in keeping with the findings of Toft (1985) where she concluded that resource partitioning varies in organisms as factors that contribute to partitioning operate independently on individual species.

Whenever, the butterfly species shared plants with each other, and/or hummingbirds, they utilized two additional partitioning strategies to reduce encounters, i.e.,: i) they fed at mutually exclusive times, as seen in bats which use temporal resource partitioning when feeding at water holes (Kunz 1973; Adams and Thibault 2006). Or, ii) when feeding at the same time they feed on spatially different parts of the plants. Similar to *Anolis* lizards that occupy various spatial arrangements on plants (Schoener 1974).

These results support our hypothesis that the presence of competitors, butterflies (exploitative) and hummingbirds (interference/exploitive), in a feeding patch will affect the feeding patterns of butterflies. Specifically, our findings prove our prediction that the two butterfly species will partition their resources spatially to avoid patches defended by larger aggressive hummingbirds in order to reduce confrontation and displacement.

However, our results also show that our butterfly species partition resources spatially—by feeding on different parts of the plants and temporally—by feeding at different times (Pianka 1967; Case and Gilpin 1974; Pianka 1981). Therefore, by employing a combination of these strategies each butterfly species is able to reduce exploitative and interference competition.

Reduction and subsequent return of food resources – Treatment 1

As the resource availability changed, we found that butterflies used different foraging patterns when adjusting to these changes. This is not surprising as resource availability directly affects behavior of consumers, which try to balance the benefit and cost of feeding on specific items (Justino et al. 2011). Therefore, use of select foraging behaviors play a key role in nectarivore fitness as it reduces time and energy spent acquiring their food (Ohashi and Thomson 2005; Ohashi et al. 2007).

Dryas iulia adopted an "increase number of visits and reduce time spent" foraging pattern but only when the resources were very limited, i.e. 100% of shared plants were covered. When plants were again available *D. iulia* retained this pattern (Table I), and included both old and new plants into their feeding route (Fig 1). In comparison, the more sensitive *H. melpomene* used multiple patterns depending on the resource availability. At first, they used an "increase visits increase time spent foraging" strategy when only 50% of shared plants were covered. Then they adopted the "increase visits and reduce time spent" foraging strategy similar to that of *D. iulia* when 100% of the shared plants were covered. However, they subsequently returned to their initial forging movement patterns i.e. similar number of plants visited and time spent as in the control treatment when

plants became available again (Table I). *Heliconius melpomene* behaved similar to *D. iulia* in that they incorporated both old and new plants into their feeding patches, thus, although there was a noted difference in time spent foraging in each treatment it was not statistically significant as was the number of visits to plants due to the inclusion of new plants which resulted in more overlaps in butterfly foraging bouts as seen in comparison to the control treatment (Fig 3). These differing foraging movement patterns could be owing to a partial break down in the spatial habitat partition patterns used initially, as more "designated" feeding areas now were shared, because butterflies included new plants as their feeding patches as productive flowers became limiting resources. *Heliconius melpomene* may have also switched between patterns to reduce competition and to avoid being out-competed by *D. iulia,* as dictated by the competitive exclusion principle (Zaret and Rand 1971), as flight speed constraints prevented it from sharing plants with hummingbirds as seen in *D. iulia* (Fig. 5a and 5b).

In addition to the adjusting number of visits and time spent per visit we also found that each butterfly species exhibited visit consistency to specific plants. In the beginning of this study as butterflies spatially partitioned plants based on location they showed visit consistency to specific plants and this changed to some extent with resource availability (Fig. 6). However, although they started to include more plants into their feeding routes as resources decreased, many butterflies of both species exhibited a "hold over" behavior where they revisited plants for a few days before moving on to new plants and they returned to some of these plants after they were made available again. Early researchers believed that visit consistency only existed in hymenopterans (Bennett 1883; Christy 1883), specifically bee species because of their eusociality and learning abilities (Lewis

1989). However, Gilbert (1980) and Lewis (1989), showed that butterflies such as Heliconiids and *Pieris rapae*, support our findings of visit consistency, where they not only establish constant feeding areas but faithfully visit specific plants within these areas, bypassing potentially rewarding plants, in an attempt to reduce search and handling times and outcompete naïve foragers (Lewis, 1989; Laverty 1994; Laverty 1994b; Raine and Chittka 2007).

We also noted that both butterfly species also visited many inflorescences on the same plant and plants in close proximity to each other (Fig. 1). In addition, butterflies increased visits to plants close to their primary food source when resources were reduced (Fig 1). This behavior is also observed in many floral foragers that move only short distances between plant visits by mainly visiting flowers on the same plant or neighboring plants in an attempt to minimize time travelling and reduce energy costs incurred (Waser 1982; Cresswell 2000).

Overall all our findings supported our predictions of our first hypothesis, if resources in an established feeding patch are reduced butterflies will adjust their existing movement patterns to accommodate for this change thus they will; (P_1) include more plants in their feeding circuit; (P_2) increase number of floral visits; and (P_3) increase time spent in order to acquire sufficient nectar to meet their caloric needs when resource availability changed. However, these behaviors varied by species depending on resources available. In addition, although butterflies demonstrated plant consistency behaviors overall, our second hypothesis, when previously unavailable plants become available again butterflies will include them in their feeding circuits, and its prediction of butterflies returning to previously established feeding routes, was not supported, as

butterflies adopted new feeding routes by including new plants into their feeding repertoire, many of which were located in close vicinity to abandoned plants.

Interference competitor presence on feeding behaviors – Treatment 2

Many animals use territoriality in order to exploit limiting resources such as food, breeding sites and mates. However, territoriality is only economical when the benefits of exclusive use of a resource outweigh the costs of its defense (Kodric-Brown and Brown 1978). For territorial hummingbirds, which actively defended clumped flower resources in the center of the farm for their own use, we found a significant difference in number of visits and time spent of each plant due to treatment. This differed for the butterflies that exhibited only significant difference in number of plants visits due to treatments because they included previously unexploited plants into their feeding repertoires to compensate for a decrease in flower resource quantities. The effect of treatment on hummingbirds was especially apparent when we considered the drastic decrease (>50%) in number in visits when the hummingbird plants covered in comparison to the other treatments and control. This may be explained by the small size and high metabolic demands of hummingbirds that caused them to respond quickly to changes in resource availability at a given site because they cannot sustain a negative energy budget for a long period of time, and must secure high quality nectar at low costs (Wolf and Hainsworth 1971; Justino et al. 2011). Therefore, when hummingbirds can no longer economically defend territories due to increasing numbers of potential interlopers driving up costs per intruder, they then abandon uneconomical territories and seek nectar resources away from the study area (Wolf and Hainsworth 1971; Justino et al. 2011). Although we have found evidence to support our hypothesis that the presence of competitors, butterflies

(exploitative) and hummingbirds (interference/exploitive), in a feeding patch will affect the feeding patterns of butterflies our findings when hummingbird plants are covered are not congruent with our prediction that when hummingbird defended plants are unavailable they will displace butterflies as in our study they abandoned the feeding habitat.

We conclude that our study species adjusted their feeding times, movements, and even plant visit consistency when resource availability was experimentally changed. This is strong evidence in support of hypothesis one. However, we note that behaviors were species specific. When previously unavailable resources were made available again both butterfly species included only some of these resources into their feeding circuit—they tended to adopt a new feeding pattern where they incorporated newly discovered plants and older plants—therefore hypothesis two was not corroborated. This is especially so because the butterflies did not return to their previously established feeding patterns. Finally, in response to competitors, we presented evidence that butterflies partitioned floral resources spatially. Thus, lending support for hypothesis three. However, they also partitioned resources temporally to reduce the use of the same plants by sympatric butterflies, and aggressive encounters with hummingbirds dictated plant visit consistency.

In the near future, we intend to investigate the effects of exploitative and interference competition on nectar availability, by removal and/or introductions (Schoener 1983) of both butterflies and hummingbirds. We also plan to conduct controlled foraging experiments, such as those described by Thomson et al. (1997), Ohashi et al. (2007) and Lihoreau et al. (2010) to better describe optimal flight paths and movement among patches, and holdover patterns of individuals of each butterfly species.

Acknowledgements

We are grateful to O. O' Dean and B. Waldrop for all of their help and support during fieldwork and to A. Dunlap and N. Muchhala for guidance and comments during the planning and execution of this research. G. Maharaj received three grants, a Rufford Small Grant (the Rufford Foundation, UK), an Idea Wild grant, Fort Collins, Colorado, USA, and a CEIBA Biological Center grant. G.R. Bourne was supported by an Anonymous Foundation grant.

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Figure legends

Figure 1.1: Feeding patterns for *Heliconius melpomene*, *Dryas iulia*, and hummingbirds in control (un-manipulated) environment. Each plant was uniquely numbered, and the color of the dots around the number denotes the total number of flowers of this plant. There were more flowers on the purple side and fewer on the red side. Each dot represents a single visit to a plant. The circle corresponded to *D. iulia* butterfly visits, the triangle to *H. melpomene* butterfly, and the cross to hummingbird visits. The sizes of these symbols corresponded to the time spent for each visit.

Figure 1.2: Feeding patterns for *H. melpomene*, *D. iulia*, and hummingbirds when 50% of the resource from "shared" plants were covered, and made unavailable as foraging sites. Each plant was assigned a unique number. See Figure 1.1 legend for details and explanations of symbols.

Figure 1.3: Feeding patterns for *H. melpomene*, *D. iulia* and hummingbirds when 100% of the resource from the "shared" plants were covered. Detailed explanations for symbols are available in Figure 1.1 legend.

Figure 1.4: Feeding patterns for *H. melpomene, D. iulia* and hummingbirds when covers on the "shared" plants were removed. Details are provided in Figure 1.1 legend.

Figure 1.5: Feeding patterns for *H. melpomene, D. iulia* and hummingbirds when uniquely numbered "hummingbird bushes" were covered. See Figure 1.1 legend for details.

Figure 2a: Individual plants visited and time spent on each plant by focal butterflies and hummingbirds varied each treatment as both species abandoned old feeding plants and incorporated new plants in their feeding repertoires.

Figure 3: Overlap interactions between each butterfly and hummingbirds increased as resources decreased.

Figure 4: Number of visits by butterflies per treatment remained unchanged. Note that butterflies fed on the periphery of the farm while hummingbirds fed in center.

Figure 5a and 5b: *Heliconius melpomene* and *D. iulia* butterflies respectively, exhibited feeding in three zones, i.e., Blue line-Interaction Zone, Red line-No Interaction Zone, Green line-Hummingbird Defended Zone.

Figure 6: Resource availability affected hummingbird and in turn butterfly plant choices. The three classes represent feeding zones: Class 1, Interaction Zone; Class 2, Non-interaction Zone; and Class 3, Hummingbird Zone.

Table I: Total number of visits (count) with mean time spent (s) for each treatment for all plants.

Treatment	Species					
	D. iulia		*H. melpomene*		Hummingbirds	
	Count	Mean	Count	Mean	Count	Mean
Control	28	40.89	119	58.85	101	42.33
50cover	23	34.65	183	61.60	171	38.19
100cover	69	36.89	192	51.38	177	29.87
Open	52	28.75	124	60.27	159	28.90
Hbplants	58	33.26	196	48.99	70	27.37

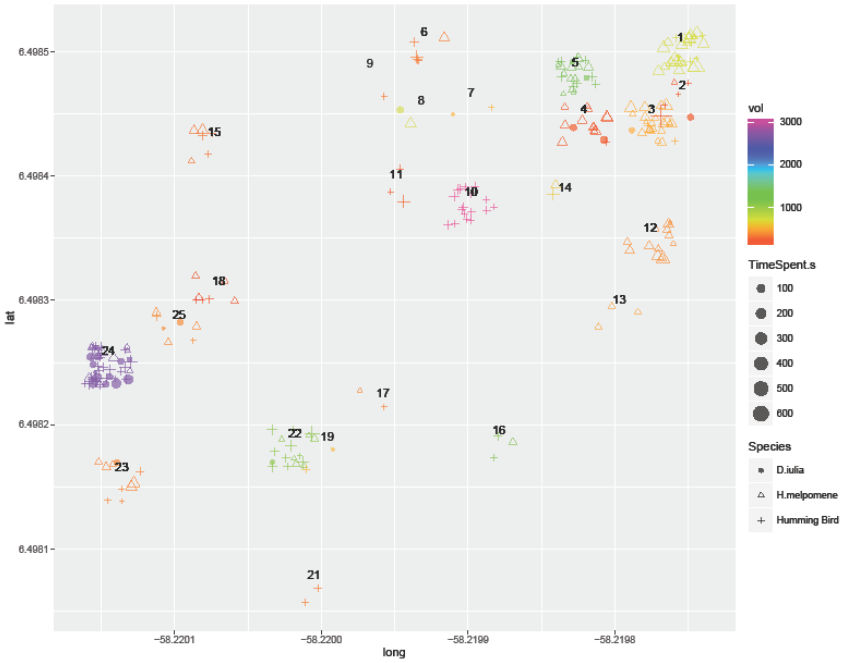
Table II: Time spent on individual plants varies by species by not treatment.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	247406.729[a]	14	17671.909	5.445	.000
Intercept	1856560.881	1	1856560.881	571.999	.000
Treatment	15289.536	4	3822.384	1.178	.319
Species	192279.757	2	96139.878	29.620	.000
Treatment * Species	12457.849	8	1557.231	.480	.871
Error	5397670.663	1663	3245.743		
Total	8902227.000	1678			
Corrected Total	5645077.393	1677			

Table III: *D. iulia* and hummingbirds differ from *H. melpomene* in the amount of time spent per plant.

(I) Species	(J) Species	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
D. iulia	H. melpomene	-21.34*	4.311	.000	-31.46	-11.23
	Hummingbirds	.88	4.404	.978	-9.45	11.21
H. melpomene	D. iulia	21.34*	4.311	.000	11.23	31.46
	Hummingbirds	22.22*	3.000	.000	15.18	29.26
Hummingbirds	D. iulia	-.88	4.404	.978	-11.21	9.45
	H. melpomene	-22.22*	3.000	.000	-29.26	-15.18

Figures



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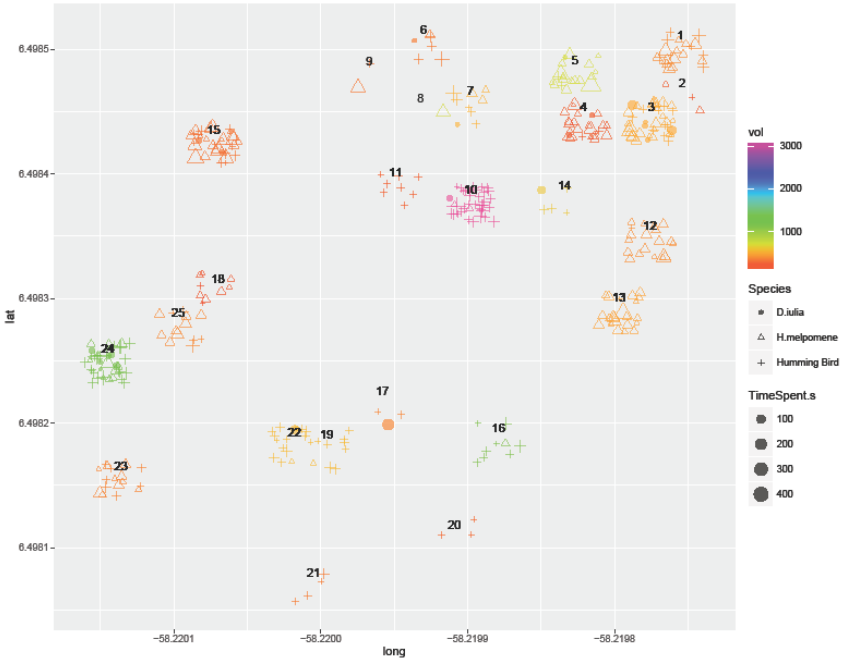

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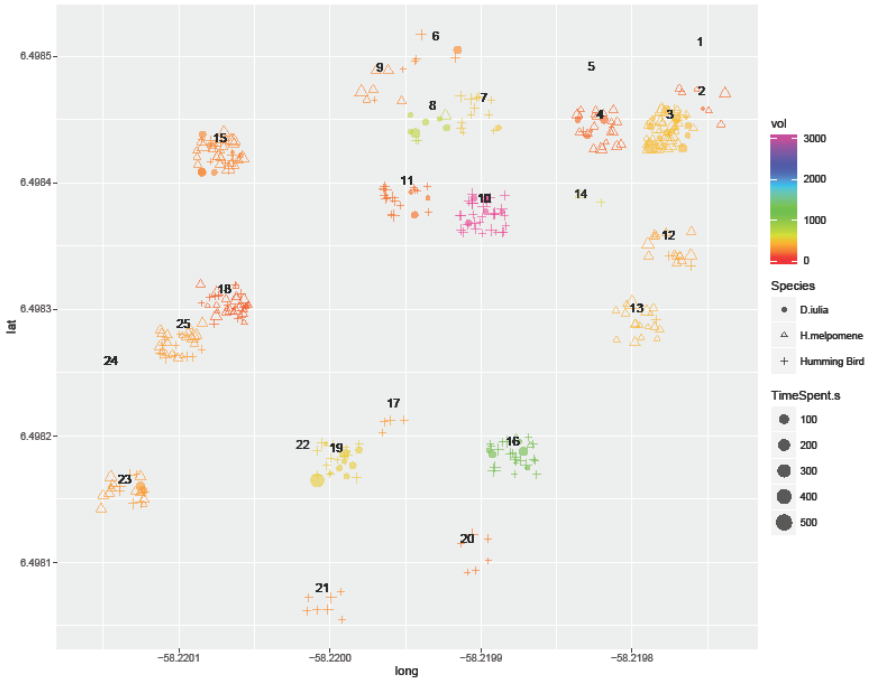
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vol
3000
2000
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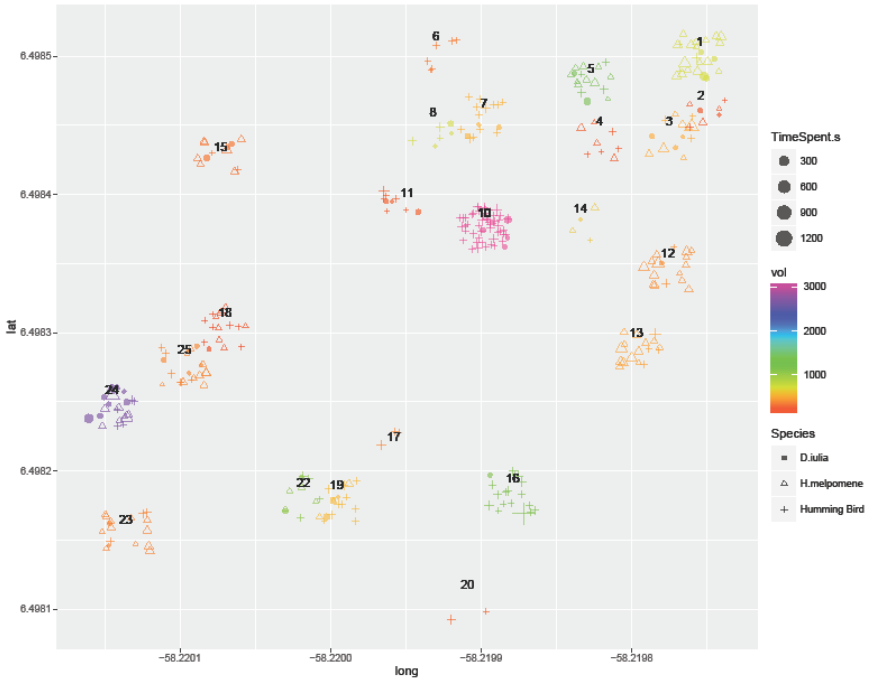
Species
D.iulia
H.melpomene
Humming Bird
TimeSpent.s
100
200
300
400
500

Fig. 1.3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TimeSpent.s
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vol
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2000
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Species
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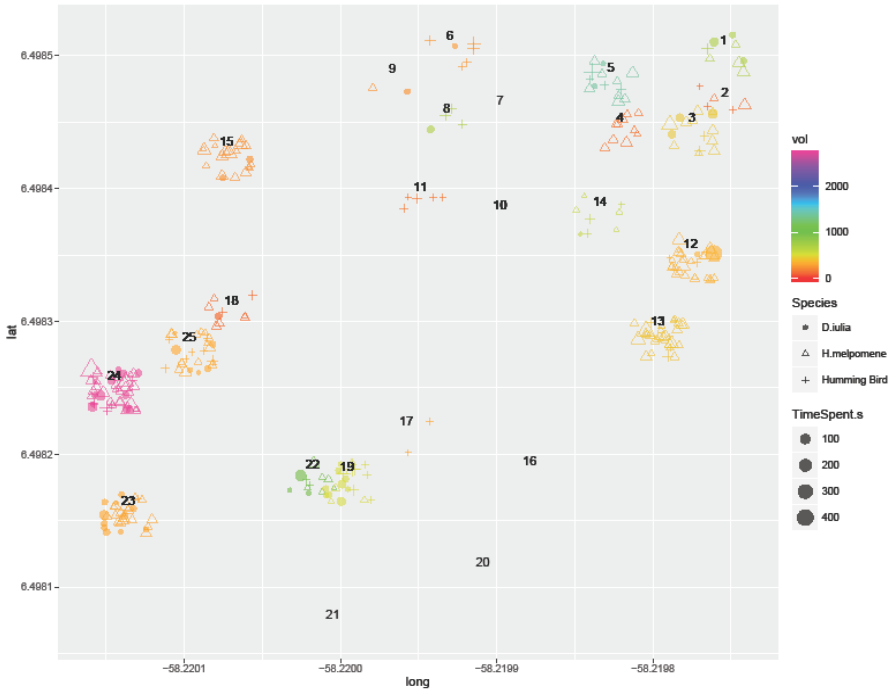
H.melpomene
Humming Bird

Fig. 1.4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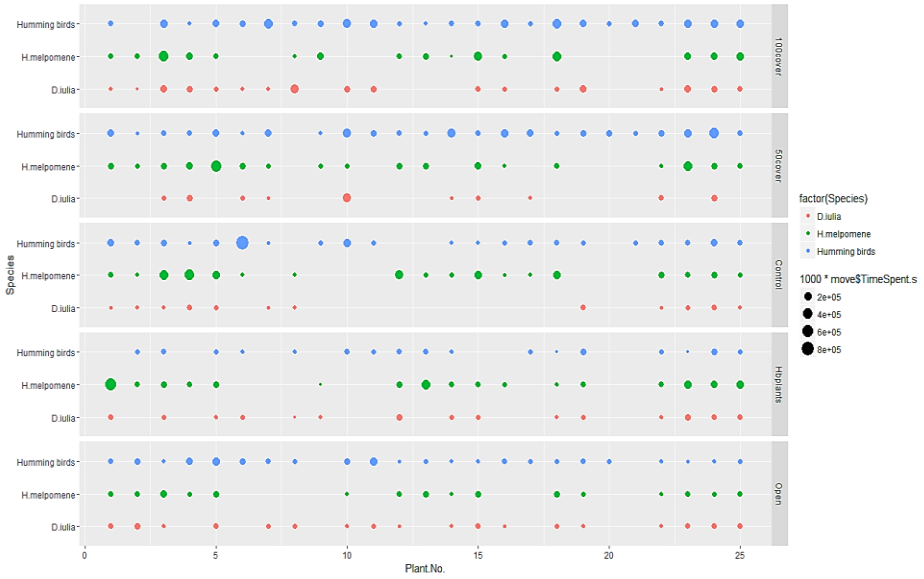
H.melpomene
Humming Bird
TimeSpent.s
100
200
300
400

Fig. 1.5


Humming birds
H.melpomene
D.iulia
100cover
50cover
Control
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Species
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H.melpomene
Humming birds
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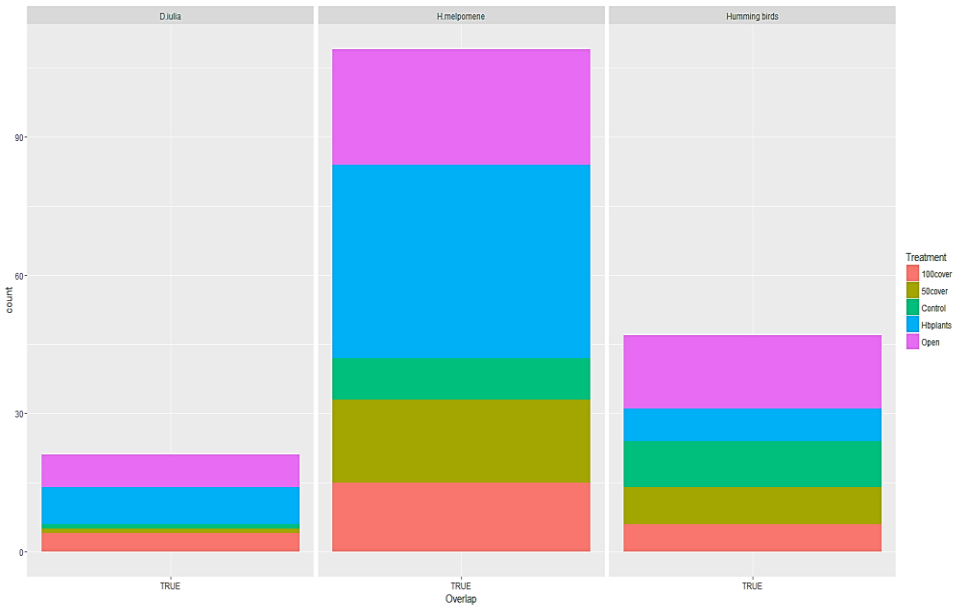
Fig. 2


D.iulia
H.melpomene
Humming birds
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30
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TRUE
Overlap
Treatment
100cover
50cover
Control
Htplants
Open

Fig. 3

Fig. 4

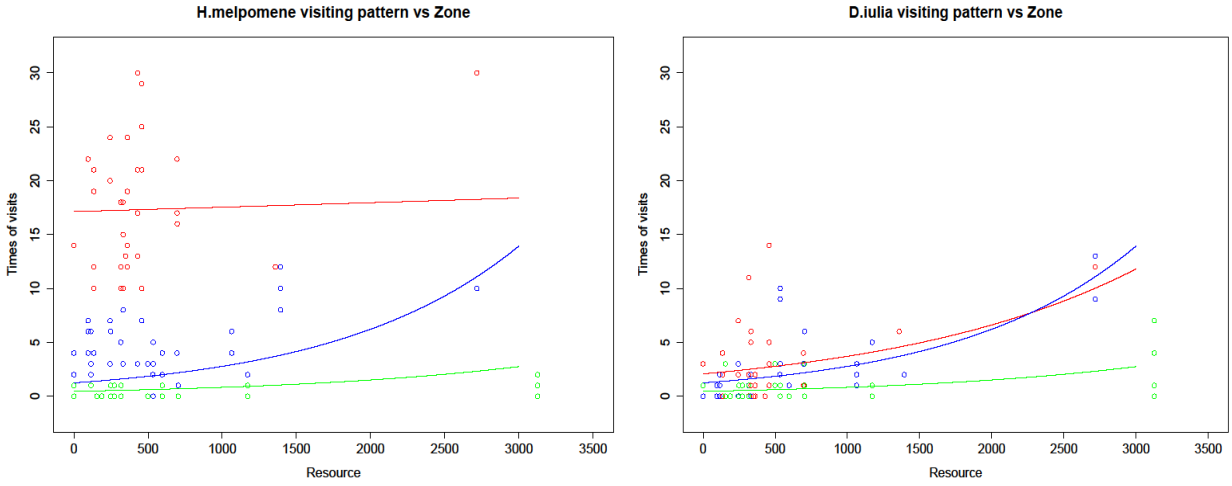
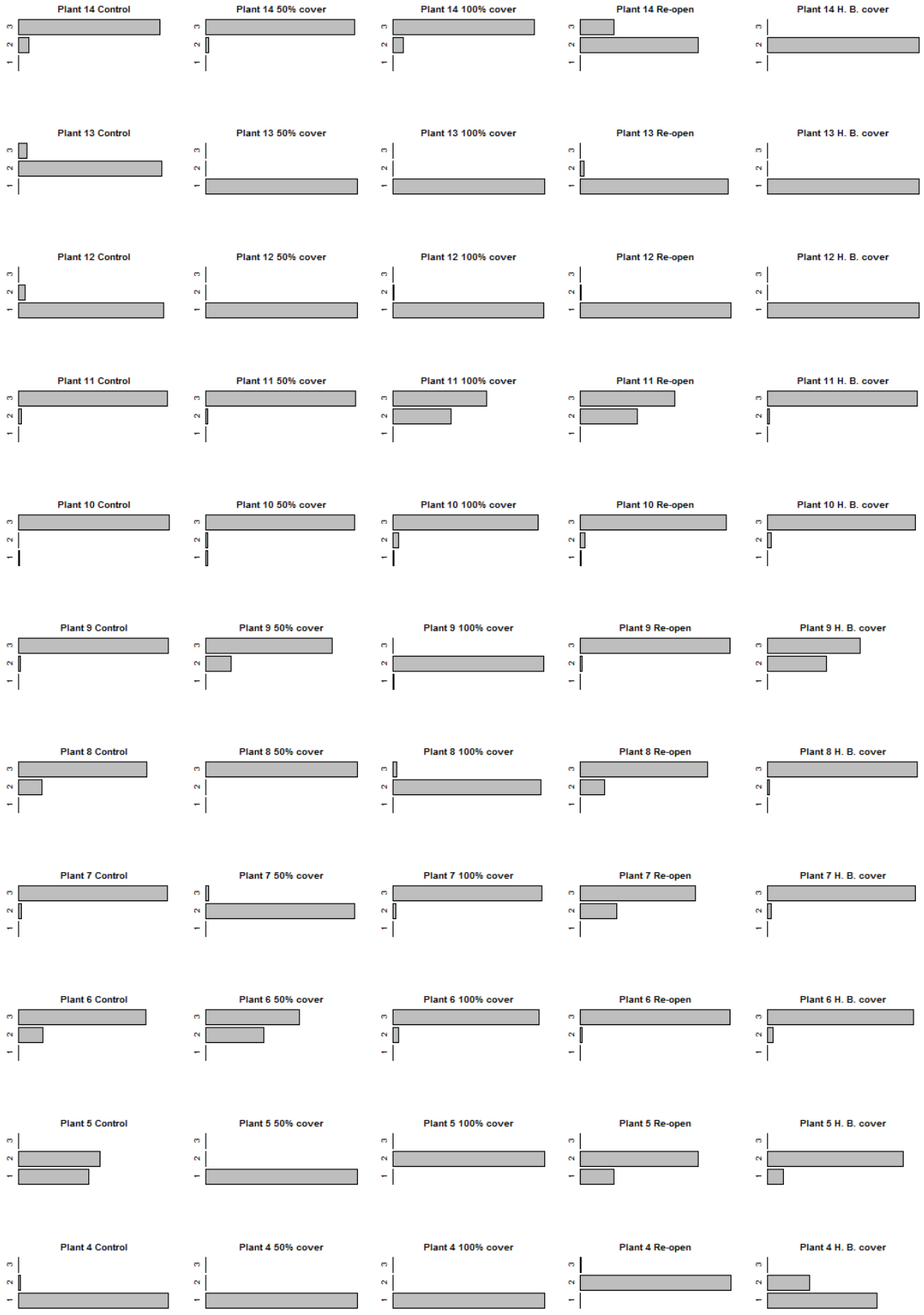


Fig. 5a and 5b


Plant 25 Control
Plant 25 50% cover
Plant 25 100% cover
Plant 25 Re-open
Plant 25 H. B. cover
Plant 24 Control
Plant 24 50% cover
Plant 24 100% cover
Plant 24 Re-open
Plant 24 H. B. cover
Plant 23 Control
Plant 23 50% cover
Plant 23 100% cover
Plant 23 Re-open
Plant 23 H. B. cover
Plant 22 Control
Plant 22 50% cover
Plant 22 100% cover
Plant 22 Re-open
Plant 22 H. B. cover
Plant 21 Control
Plant 21 50% cover
Plant 21 100% cover
Plant 21 Re-open
Plant 21 H. B. cover
Plant 20 Control
Plant 20 50% cover
Plant 20 100% cover
Plant 20 Re-open
Plant 20 H. B. cover
Plant 19 Control
Plant 19 50% cover
Plant 19 100% cover
Plant 19 Re-open
Plant 19 H. B. cover
Plant 18 Control
Plant 18 50% cover
Plant 18 100% cover
Plant 18 Re-open
Plant 18 H. B. cover
Plant 17 Control
Plant 17 50% cover
Plant 17 100% cover
Plant 17 Re-open
Plant 17 H. B. cover
Plant 16 Control
Plant 16 50% cover
Plant 16 100% cover
Plant 16 Re-open
Plant 16 H. B. cover
Plant 15 Control
Plant 15 50% cover
Plant 15 100% cover
Plant 15 Re-open
Plant 15 H. B. cover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Plant 14 50% cover
Plant 14 100% cover
Plant 14 Re-open
Plant 14 H. B. cover
Plant 13 Control
Plant 13 50% cover
Plant 13 100% cover
Plant 13 Re-open
Plant 13 H. B. cover
Plant 12 Control
Plant 12 50% cover
Plant 12 100% cover
Plant 12 Re-open
Plant 12 H. B. cover
Plant 11 Control
Plant 11 50% cover
Plant 11 100% cover
Plant 11 Re-open
Plant 11 H. B. cover
Plant 10 Control
Plant 10 50% cover
Plant 10 100% cover
Plant 10 Re-open
Plant 10 H. B. cover
Plant 9 Control
Plant 9 50% cover
Plant 9 100% cover
Plant 9 Re-open
Plant 9 H. B. cover
Plant 8 Control
Plant 8 50% cover
Plant 8 100% cover
Plant 8 Re-open
Plant 8 H. B. cover
Plant 7 Control
Plant 7 50% cover
Plant 7 100% cover
Plant 7 Re-open
Plant 7 H. B. cover
Plant 6 Control
Plant 6 50% cover
Plant 6 100% cover
Plant 6 Re-open
Plant 6 H. B. cover
Plant 5 Control
Plant 5 50% cover
Plant 5 100% cover
Plant 5 Re-open
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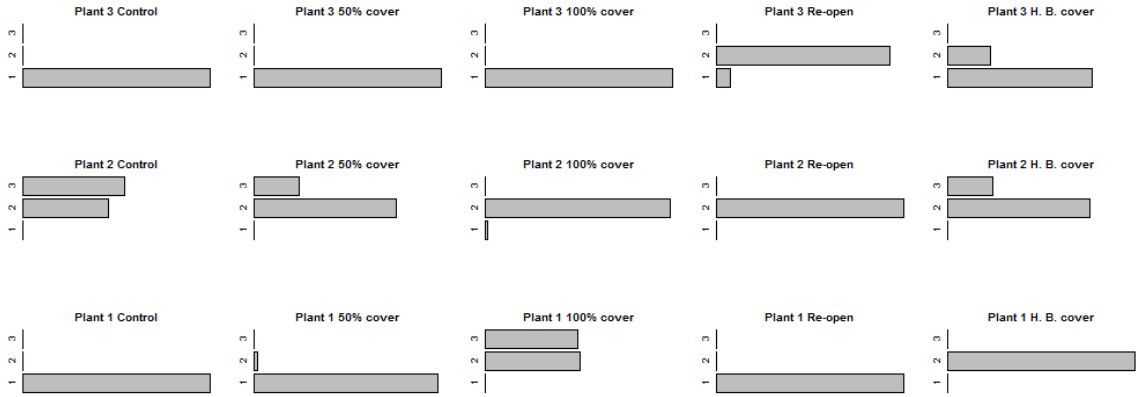
Plant 4 100% cover
Plant 4 Re-open
Plant 4 H. B. cover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Plant 2 50% cover
Plant 2 100% cover
Plant 2 Re-open
Plant 2 H. B. cover
Plant 1 Control
Plant 1 50% cover
Plant 1 100% cover
Plant 1 Re-open
Plant 1 H. B. cover

Fig. 6