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# HONEST SIGNALLING AND THE BILLBOARD EFFECT: HOW HELICONIID POLLINATORS RESPOND TO THE TRICHROMATIC COLOUR CHANGING *LANTANA CAMARA* L. (VERBENACEAE)

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*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 flowers will be visited more frequently by pollinators than post change orange, or red flowers; 2) pre-change yellow flowers will produce higher quality and greater quantities of sucrose rewards than post-change orange, or red 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). About 90% of the more than 240,000 species of flowering plants are pollinated by more than 200,000 animal species (Graham et al. 2003; Holland 2011). These plants employ three broad strategies for achieving pollination: (1) deception, where "gullible" animals are tricked by mimicry of "false" rewards into providing pollen transfer among flowers (Wickler 1968; Ackerman 1986; Nilsson 1992; Graham et al. 2003); (2) imprisonment, where flowers attract insects most of which are already covered with conspecific pollen, and 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).

Plants signal honestly to a wide range of organisms using many types of signals involving both vegetative and reproductive parts (Hamilton & Brown 2001; Schaefer et al. 2004). Many plants employ sensory signals which include colour, morphology, odour, among others, which in turn acting in concert with each other to become "sensory billboards" (Weiss & Lamont 1997; Raguso 2004; Willmer et al. 2009) . These sensory signals can function "honestly" in their communication with pollinators by reliably signalling the presence and/or quality of nectar, pollen, oil, or fragrance rewards (Nilsson 1992; Proctor et al. 1996; Schaefer et al. 2004; Raguso & Willis 2005; 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). As such, plant colour signals, especially floral colour, exemplify the evolution of

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floral traits driven by ecological interactions between plants and pollinators (Weiss 1997; Oberrath & Böhning-Gaese 1999; Ida & Kudo 2003; Ida & Kudo 2010; Willmer et al. 2009; Suzuki & Ohashi 2014).

Flower colour can remain constant during the entire anthesis stage or it can experience colour change due to multiple factors including; the environment, age or receptivity status (Weiss 1991; Yoshida et al. 2009). Changes in colour which occur in fully turgid flowers 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). 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 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 & Böhning-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 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 red flowers? (2) Do newly opened yellow flowers attract more *L. camara* pollinators than older orange and red flowers? And (3) how does inflorescence size and ratio of rewarding to unrewarding flowers influence butterfly pollinator visitation? 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: (P1) first stage yellow flowers will attract more pollinators because they contain higher concentrations and volumes of sucrose than later orange and red stages; (P2) 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 (P3) inflorescences with a combination of rewarding yellow and orange flowers and unrewarding red 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 (320m2) 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 red), 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 ~ 85 mm); 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 of butterflies to flower colour changes as seen by humans. However, we do acknowledge that butterflies typically have long-wavelength opsins, screening pigments that modify spectral sensitivities, 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*) and furthermore some have four or five photoreceptor types (Zaccardi et al. 2006, Stavenga & Arikawa 2006, Briscoe 2008). Consequently, our study species are not only capable of distinguishing changes in long wave length red markings, they are likely to have a visual system significantly different from that of human observers, in terms of both short- and long-wavelength sensitivity, and colour discrimination abilities. 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 (red) — was taken on the following morning. To estimate colour and sucrose measurements of the nine sub-divisions of these three major colour stages a total of 25 flowers were selected for nine days (July 2014). The colour stages were as follows: Stg. 1 — orange yellow centre with spectrum orange edges, Stg. 2 — orange yellow centre with chrome orange edges, Stg. 3 — orange yellow with flame scarlet edges, Stg. 4— spectrum orange with flame scarlet edges, Stg. 5 — chrome orange with flame scarlet edges, Stg. 6— chrome orange, Stg. 7— flame scarlet, Stg. 8— flame scarlet with scarlet edges and Stg. 9 — scarlet (colour swatches in Smith 1975). 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, from both the three day and nine stage experiments, were fresh, 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 m2 quadrat was sampled for 2 min 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 $cm^2$). 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 per $cm^2$) 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 fruitflies (*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 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 mins) of all *L. camara* visitors during sunny periods, from 09:00-14:59 h, to vases with mixed inflorescences (all three flower colours), yellow, orange and red only inflorescences matched by floral numbers. Vials of flowers were presented on wooden dowels, 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 of *H. melpomene* and *D. iulia* were observed for 30 d (June–July 2014) and 10 d (December–January 2015), with the total number of visits 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: — (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 (un-manipulated mixed i.e. comprised of all three colour morphs in natural combinations), (ii) manipulated

mixed (25:25:50 - this is an inflorescence comprised of 25% yellow, 25% orange and 50% red flowers), (iii) yellow and orange (50:50, made up of 50% yellow and 50% orange flowers), and (iv) All red (100% red flowers) (Gori 1989).

(B) different size different colour:

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

### *Statistical analyses*

A Kruskal–Wallis 1-Way Analysis of Variance (ANOVA) model with pairwise comparisons (Tukey Test) 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 with pairwise comparisons (Holm-Sidak method) was used to compare sucrose concentrations of the three major flower stages and for the nine sub-colour stages. We employed a generalized linear model (Poisson distribution) to test for significant differences among treatments, while a binomial logistic regression was employed to predict the probability of either short or long distance attraction based on single versus multiple visits (coded as a dichotomous variable) 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*

We observed a significant decrease in both sucrose volume ($H^2 = 49.06$, $P < 0.001$, $N = 20$, statistically significant differences between all pairwise comparisons) and sucrose concentration ($F_{2, 57} = 619.84$, $P < 0.001$, $N = 20$, statistically significant differences for pairwise comparisons, Tab. 1), as flowers changed from yellow to orange to red.

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, with earlier stages characterized by lower volumes and higher concentration and later stages having higher volumes and lower concentrations, with the exception of the final stage that offered no reward. Overall measurements of sucrose concentration and volume by colour stage showed substantial variability (Tab. 2). 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 (Tab. 2). 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 statistically significantly different from 6, 7, 8, and 9, and Stgs. 6, 7, and 8 were different from 9 (Tab. 2).

### *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. 1). Controls, butterflies, carpenter bees and hummingbirds had significantly better fruit set percentages than Trigonid bees, wasps and ants (Fig. 1). The numbers of diurnal pollinator butterfly taxa observed on *L. camara* are presented in Tab. 3. 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 Tab. 3), however only *H. melpomene* and *D. iulia* were used in experiments because of their high abundancies.

TABLE 1: Mean and SD values of sucrose (nectar) volumes and concentrations for the three gross colour stages indicated declining production with time.

| | Yellow | Orange | Red |
|---|---|---|---|
| Volume | 0.91 (± 0.144) | 0.67 (± 0.128) | 0.03 (± 0.028) |
| Concentration | 27.00 (± 2.046) | 21.06 (± 1.769) | 8.11 (± 1.312) |

TABLE 2. Comparison of means and SD values of sucrose volumes and concentration for fine temporal colour stages (Stg.1= Stage 1 etc.) showing an increase in volume after stage 3 and no reward offered in stage 9 and a decrease in concentration in later stages (5-9). Stage 1 correspond to yellow flower colour, Stage 4 to orange and Stage 7 to red as identified in Tab. 1.

| | Stg. 1 | Stg. 2 | Stg. 3 | Stg. 4 | Stg. 5 | Stg. 6 | Stg. 7 | Stg. 8 | Stg. 9 |
|---|---|---|---|---|---|---|---|---|---|
| Volume | 0.62 (±0.23) | 0.77 (±0.41) | 0.58 (±0.20) | 1.18 (±0.88) | 1.09 (±0.96) | 0.91 (±0.41) | 0.85 (±0.32) | 1.36 (±0.75) | 0.00 (±0.00) |
| Concentration | 21.41 (±1.25) | 20.52 (±2.57) | 20.40 (±2.42) | 20.74 (±2.39) | 19.03 (±1.96) | 16.02 (±5.40) | 15.99 (±2.93) | 16.22 (±4.81) | 0.00 (±0.00) |

TABLE 3. Frequency of Lepidopteran pollinators observed foraging on *L. camara* over a 15-day period. Top four foragers include *Heliconius melpomene*, *H. sara*, *Dryas iulia* and *Papilio thoas*.

| Butterfly Species | Count/Total no. visits (15d) |
|---|---|
| *Agraulis vanillae* | 1 |
| *Aphrissa boisduvalii* | 9 |
| *Aphrissa statira* | 37 |
| *Astraptes fulferator* | 1 |
| *Battus polydamas* | 4 |
| *Dryas iulia* | 115 |
| *Emesis aurimna* | 3 |
| *Emesis fatimella* | 8 |
| *Heliconius bruneiye* | 27 |
| *Heliconius erato* | 1 |
| *Heliconius hecale* | 22 |
| *Heliconius melpomene* | 357 |
| *Heliconius numata* | 14 |
| *Heliconius sara* | 212 |
| *Lemonias emylius* | 2 |
| *Mechanitis lysimnia* | 1 |
| *Melinaea lilis* | 3 |
| *Nymphidium ascolia* | 1 |
| *Nymphidium oleamun* | 5 |
| *Papilio thoas* | 95 |
| *Philatria dido* | 3 |
| *Phoebis argante* | 11 |
| *Phoebis sennae* | 7 |
| *Phoebis statira* | 1 |
| *Stalachitis phlegia* | 9 |
| *Synargis tytia* | 2 |
| *Thecla sp.* | 7 |

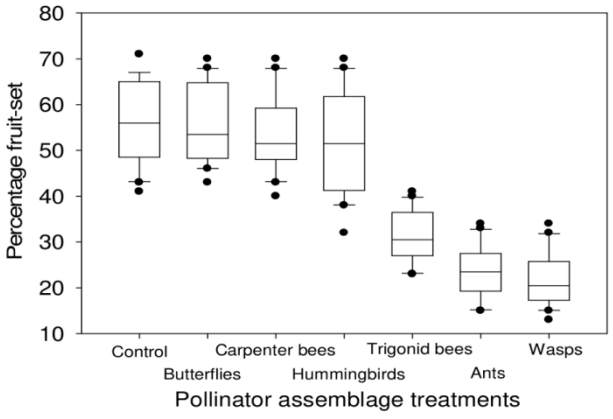


FIGURE 1. Pollinator taxa and effectiveness of visits on the percentage of fruit set in *Lantana camara*. The Control variable consists of the effects of all pollinating taxa visits on fruit set. Butterflies, Carpenter bees and Hummingbirds were found to be more effective pollinators than Trigonid bees, ants and wasps.

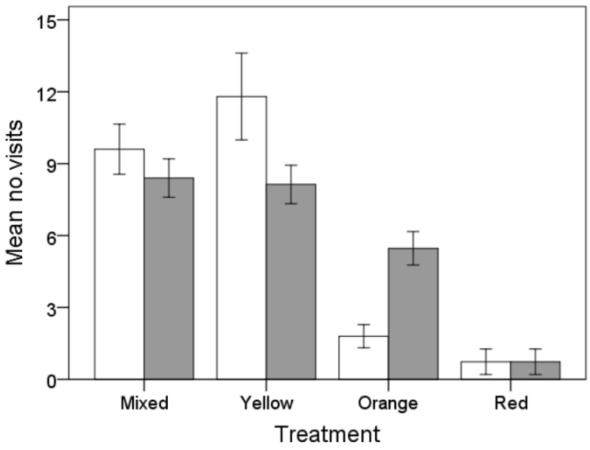


FIGURE 2. Mean (± 2SE) pollinator visitation of inflorescences presented in nature (white bars) and in vials (grey bars). Highest visitation observed at plants with mixed and all yellow inflorescences but lowest at orange and red inflorescences.

### *Flower colour preference & billboard effect*

Experiment 1 — Colour preference

Our Poisson regression showed that there was no difference in visitation by set-up (vials vs nature), however there was a statistically significant difference in which treatments were visited by pollinators. Pollinator interest (mean pollinator visitation rates – number per 2 min) in the arrays of *L. camara* bouquets presented in vials away from the plants and in nature had highest and statistically similar visitation rates at the yellow and mixed inflorescences (Exp (B) = 0.903, 95% CI 0.766-1.065, $P = 0.224$). Pollinator visitation significantly declined when presented with orange inflorescences (Exp (B) = 0.365, 95% CI 0.293-0.454, $P < 0.001$) and even more so with red inflorescences (Exp (B) = 0.074, 95% CI 0.048-0.113, $P < 0.001$) (Fig. 2).

Experiment 2A — Billboard effects

A Poisson regression was run to predict the number of visits to a *L. camara* plant based on the butterfly species and the type of treatment carried out to the inflorescence of that *L. camara* plant. Our results show that there are statistically significant differences by species and treatment in visitation. *D. iulia* (Exp (B) = 0.300– CI 95% 0.255-0.353, $P < 0.001$) visited fewer plants than *H. melpomene*. Butterflies were most likely to visit large mixed inflorescences (Exp (B) = 1.298– CI 95% 1.095-1.539, $P = 0.003$) and large red inflorescences (Exp (B) = 1.204 – CI 95% 1.013-1.432, $P < 0.035$) in comparison to small yellow inflorescences. With *H. melpomene* visiting large mixed and large red more than small yellow, while *D. iulia* preferred large mixed and small yellow to large red (Fig. 3). 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

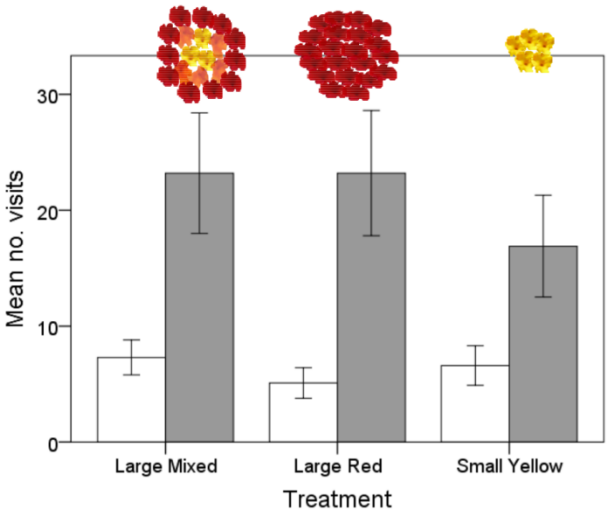


FIGURE 3. Mean (± SE) pollinator visitation of inflorescences by study species. Highest visitation by *D. iulia* (white bars) observed at Large Mixed plants while *H. melpomene* (grey bars) visited both Large Mixed and Large Red inflorescences more than Small Yellow.

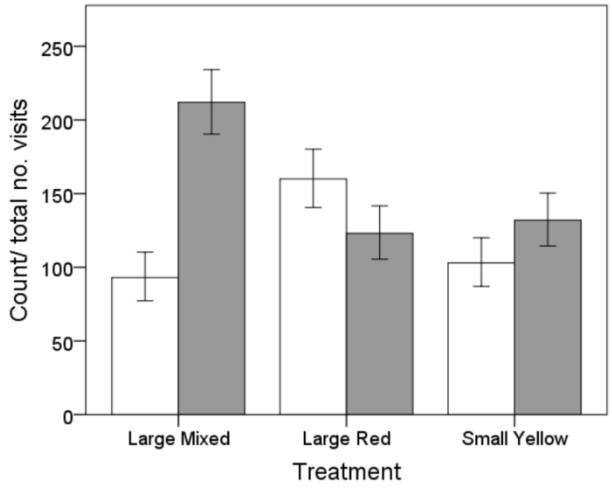


FIGURE 4. Total number (95% CI) of single (white bars)/multiple (grey bars) visits to inflorescences by study species. Highest number of multiple visits by butterflies occurred at Large Mixed inflorescence whereas, highest number of single visits occurred at Large Red inflorescences.

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, i.e. butterflies were more likely to visit one inflorescence (long distance attraction) or multiple inflorescence (short distance attraction) on a plant based on type of inflorescence treatment only. Butterflies visiting Large Mixed (Exp (B) = 1.783, – CI 95% 1.250-2.544, $P = 0.001$) and small yellow inflorescences were more likely to visit multiple inflorescences on a plant, whereas butterflies visiting Large Red inflorescences were less likely to visit multiple flowers (Exp (B) = 0.603, – CI 95% 0.425-0.857, $P = 0.005$), i.e. they were more likely to visit a single inflorescence only (Fig. 4).

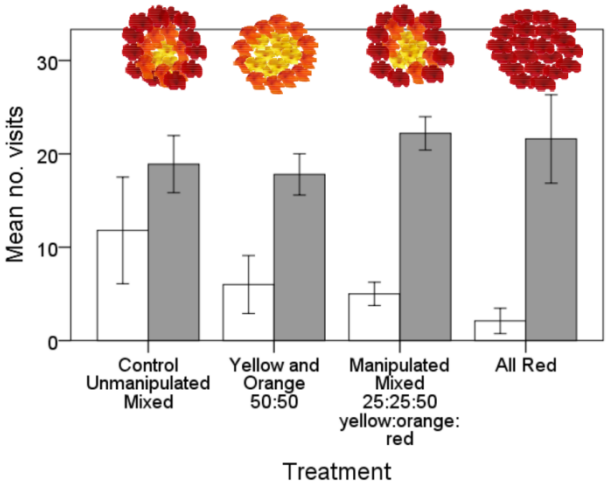


FIGURE 5. Mean (± SE) pollinator visitation of inflorescences by study species. Highest visitation by *D. iulia* (white bars) was observed at Control plants while *H. melpomene* (grey bars) visited Manipulated Mixed inflorescences and All Red inflorescences the most.

Experiment 2B — Billboard effects

Our Poisson regression predicted the number of visits to a *L. camara* plant based on the butterfly species and the type of treatment on that *L. camara* plant. Our results show that there is a significant difference in visitation by species and treatment. Similar to before, *D. iulia* butterflies (Exp (B) = 0.309 – CI 95%0.268-0.357, $P < 0.001$) visited fewer plants in comparison to *H. melpomene*. Overall, butterflies were most likely to visit control (un-manipulated mixed) inflorescences (Exp (B) = 1.295 – CI 95% 1.093-1.535, P = 0.003), followed by manipulated mixed (25:25:50-yellow: orange: red) (Exp (B) = 1.148 – CI 95% 0.965-1.366, $P = 0.121$), yellow and orange (50:50) (Exp (B) = 1.004 – CI 95% 0.839-1.202, $P = 0.963$), and least likely to visit all red inflorescences. However, visits to mixed manipulated, yellow and orange and all red inflorescences were not statistically significantly different from each other. We found that *H. melpomene* visited all red and manipulated mixed more in comparison to the other treatments while *D. iulia* preferred control and yellow and orange (Fig. 5). 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 (un-manipulated mixed), manipulated mixed (25:25:50 - yellow:orange:red), yellow and orange (50:50), 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, $\chi^2_4 = 60.954$, $P < 0.001$. The Wald criterion demonstrated that both predictor variables were statistically significant. We found that *H. melpomene* (Exp (B) = 1.430, 95% CI 1.057-1.935, $P = 0.02$) was more likely to visit multiple inflorescences than *D. iulia*. Overall, butterflies were more likely to visit multiple inflorescences on the following plants, i.e. yellow and orange

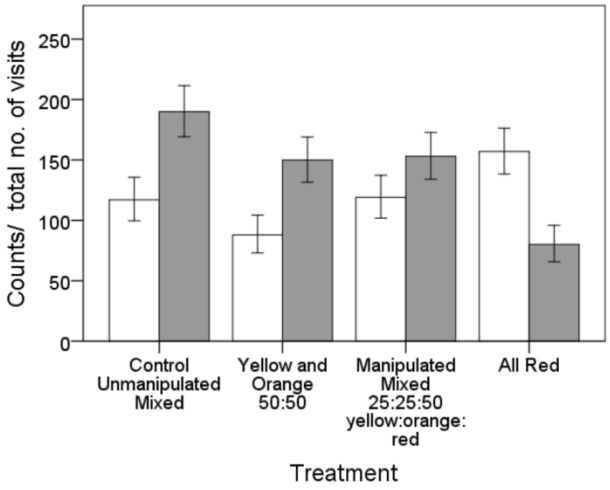


FIGURE 6. Total number (95% CI) of single (white bars) /multiple (grey bars) visits to inflorescences by study species. Highest number of multiple visits by butterflies occurred at Control inflorescence whereas, highest number of single visits occurred at All Red inflorescences.

(50:50) (Exp (B) = 3.563, 95% CI 2.433-5.219, $P < 0.001$), control (un-manipulated mixed) (Exp (B) = 3.562, 95% CI 2.464-5.148, $P < 0.001$), and manipulated mixed (25:25:50 yellow: orange: red) (Exp (B) = 2.618, 95% CI 1.822-3.761, $P < 0.001$), in comparison to all red inflorescences (Fig. 6).

## DISCUSSION

### *Flower colour and sucrose measurements*

When we examined flowers for three consecutive days our results, pre-change yellow flowers having higher sucrose concentration and volume in comparison to day 2, orange, and day 3, red, 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 red 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 (Tab. 2) we noticed as time progressed i.e., as the flowers aged, there was not a 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 (Tab. 1) 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. Carrión-Tacuri et al. (2012), showed that the nectar volumes of bagged *L. camara* flowers did not change significantly throughout the day but nectar 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 seem to serve the purpose of attracting 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 *L. camara* attracts these three ubiquitous pollinator groups, birds as seed dispersers and humans who commercialized many colourful forms may account for its spread globally (Ghisalberti 2000).

### *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 & Böhning-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 red flowers in our experiments (Willmer et al. 2009). Our evidence clearly supported prediction one (P1) that first stage yellow flowers attract more pollinators as they contain greater quality of rewards (greatest concentration of sucrose) than later orange and red stages (lower quality reward). Thus the pollinators of *L. camara* displayed a greater preference for these pre-change yellow flowers than orange or red 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, manipulated mixed (25:25:50 - yellow:orange:red), yellow and orange (50:50), control (un-manipulated mixed) and large mixed were more attractive over short distances (P2) 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. While overall, most visits to plants were made to large mixed and control (un-manipulated mixed) due to the billboard effect that results from larger multi-coloured displays (P3) (Barrows 1976; Gori 1989; Weiss 1991; Weiss1995; Nuttman et al. 2005; Willmer et al. 2009). The retention of provision less scarlet flowers or red flowers that produce little reward 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 red 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, yellow and orange (50:50) or control (un-manipulated mixed), more frequently while *H. melpomene* tended to frequent inflorescences with many red flowers; large red, large mixed, manipulated 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 (un-manipulated mixed), manipulated mixed (25:25:50 - yellow:orange:red), yellow and orange (50:50) and all red, butterflies visited single flowers on all red inflorescences. Therefore, although unrewarding red flowers draw in pollinators from a long distance, only plants with rewarding flowers facilitate short distance feeding behaviours, while plants with both such as, large mixed and control (un-manipulated mixed), attracted the most visits overall.

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 red 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.

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