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Associative color learning and age in Heliconius butterflies

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Associative color learning and age in *Heliconius* butterflies

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ABSTRACT

Flower color is an important attractant and many pollinators show distinct color preferences. This study determines if color preference and its lability differ between experienced and naïve *Heliconius* butterflies. Butterflies were offered nectar in *Lantana camara* inflorescences, which naturally have flowers of two colors (yellow and red) on a single inflorescence. Butterflies visited yellow flowers more frequently, with 76% of visits to yellow flowers when both flower colors offered nectar. When nectar was offered only by red flowers, yellow preference decreased significantly over time. Newly eclosed butterflies offered inflorescences where only red flowers rewarded showed a 56% yellow visitation rate over two days, 22% less than for butterflies previously offered inflorescences where both flowers rewarded. These results suggest that yellow preference in *Heliconius* is both strong and innate, but can be weakened by experience. Further, it appears that naïve butterflies are more labile, allowing them to track changes in rewards more quickly. This reward-associated learning may help optimize foraging success by enabling individuals to adapt behaviorally to different environments.

RESUMEN

El color de las flores es un atractivo importante y varios polinizadores muestran distintas preferencias. Este estudio determina si la preferencia por color y su labilidad difieren entre mariposas con experiencia e ingenuas del género *Heliconius*. Se les ofreció néctar a las mariposas en inflorescencias de *Lantana camara*, que naturalmente tiene flores de dos colores (rojo y amarillo) en una misma inflorescencia. Las mariposas visitan flores amarillas con mayor frecuencia, con un 76% de visitas a flores amarillas cuando ambos colores fueron ofrecidos con néctar. Cuando el néctar se ofreció solo en las flores rojas, la preferencia por las flores amarillas decreció significativamente con el tiempo. Mariposas recién eclosionadas a las que se les ofreció néctar solamente en flores rojas mostraron un 56% de visitación en dos días, 22% menos que las mariposas a las que se les ofreció con recompensa en ambos colores. Estos resultados sugieren que la preferencia por el color amarillo en *Heliconius* es tanto fuerte como innata, pero puede ser disminuida por la experiencia. Además, parece ser que las mariposas ingenuas son más lábiles, permitiéndoles determinar los cambios en la recompensa más rápido. Este comportamiento asociado a la recompensa puede ayudar a optimizar el éxito de forrajeo permitiéndole a los individuos adaptarse a diferentes ambientes.

INTRODUCTION

Animal-mediated pollination occurs when plants reward animals to move pollen to conspecific stigmas (Faegri & Van der Pijl 1971, Proctor and Yeo 1973; both in Keister *et al.* 1984). To improve conspecific transfer of pollen, many plants have evolved specific morphologies, phonologies and rewards to attract a specific group of potential pollinators (Kiester *et al.* 1984, Aigner 2006). Further, animals have coevolved with plants to be more effective at extracting rewards, causing them to be restricted in the flowers they visit (Waser *et al.* 1996). Such specialization reduces pollen waste and stigmatic fouling as pollinators are more likely to move between flowers of the same species, and reduces time and energy expenditure by pollinators in their search for rewarding flowers (Keister *et al.* 1984). Thus, floral adaptations such as size, shape, color, and presentation of reward and pollen help ensure maximum pollination success and pollinator foraging efficiency (Kevan & Baker 1983).

Color is an important floral characteristic that can select for certain pollinators (Kevan & Baker 1983) and is an important component of pollinator syndromes: suites of traits that assure a certain subset of potential pollinators (Bawa 1990). Many butterflies, for example, prefer bright colors, such as yellows (Kevan & Baker 1983) while most hummingbirds preferred (Schatz 1990, Aigner 2006). Thus, pollinators can more quickly recognize flowers that allow them to extract appropriate rewards more easily (Kevan & Baker 1983, Weiss 1995). An overly narrow use of color cues may constrain the pollinator, however, as they may miss reward opportunities (Waser *et al.* 1996). In the case of floral color, it may be advantageous for pollinators to sample and learn which colors are currently most rewarding, rather than be constrained to innate color preferences that would overly limit flower choice.

Some butterflies are able to learn which flowers are most rewarding by their floral color (Swihart & Swihart 1970, Swihart 1971, Weiss 1991). While butterflies may have innate color preferences, they sometimes switch preferences between flower colors depending on presentation of a consistent reward. For example, *H. charitonius* butterflies showed an increased preference for a given flower color if they were raised with rewarding flowers of only that color (Swihart & Swihart 1970, Swihart 1971). Further, another heliconiine, *Agraulis vanillae*, switched color preference from yellow to orange to avoid toxic nectars (Masters 1991).

Butterflies favor yellow flowers on the bicolor inflorescences of *Lantana camara* (Swihart & Swihart 1970, Weiss 1991). In *L. camara*, yellow flowers offer more nectar while red flowers are largely emptied of nectar (Weiss 1991). This study sought to determine whether preference for yellow flowers in *Heliconius* butterflies is learned or innate, and if it can be reversed through altered reward cues. It differs from previous studies (Masters 1991, Weiss 1991, Weiss 1997) in altering reward and investigating change in preference over time, and with the inclusion of newly eclosed butterflies to determine if there is a naïve preference. It was predicted that older butterflies may prefer yellow flowers because they reinforce preference with a better reward. Naïve butterflies, on the other hand, would have no reason to select for yellow flowers over red if both offered equal reward, unless there were an innate preference for yellow. All butterflies should eventually learn to favor red flowers if only red flowers contain nectar.

MATERIALS AND METHODS

Study organisms

Heliconius charitonius butterflies were raised in the Monteverde Butterfly Garden, Monteverde, Costa Rica. To feed adult butterflies, the garden fills both yellow and red flowers on *L. camara* inflorescences with 20% sucrose solution. Therefore, the *H. charitonius* used for this study were previously exposed to equal rewards in both yellow and red flowers.

L. camara natural infloresences naturally have a bulls-eye pattern, with older red flowers forming a ring around younger, yellow flowers (Weiss 1991). Mature inflorescences normally contain around 30 flowers. For this study, inflorescence color was controlled by 1. selecting inflorescences that were entirely or almost entirely one color, and 2. removing a small number of flowers of the opposite color to create monochromatic inflorescences. These were then offered to butterflies to assess color preferences independently of flower placement or inflorescence size.

Study site and conditions

The study site was the *Heliconius* room (Room 2) of the Monteverde Butterfly Garden in Monteverde, Costa Rica. A grid array of "feeding stations" consisted of 30 infloresceness in a 5 x 6 array, each approximately 15 cm apart (Figure 1A). Each station was a small vase of water containing a single *L. camara* inflorescence of only one color, either yellow or red, with 10-20 flowers (usually around 15) per inflorescence (Figure 1B). Yellow/red stations alternated throughout the grid to yield 15 of each color. Stations were mounted on posts 1-1.5 m tall. Nectar was inserted into the flowers with a syringe twice each morning, once at the beginning of the observation interval and once at the end. Most observation intervals fell between 9:00am and 11:00am.

Figure 1A&B. Grid array of *L. camara* flowers in the *Heliconius* room of the Monteverde butterfly garden. **A.** Full grid of 5 x 6 feeding stations each with one *L. camara* infloresence, either yellow or red, mounted on poles 1-1.5m tall. Colors alternated throughout the grid.. **B.** Yellow (left) and red (right) *L. camara* inflorescences in vases, before placement on poles.

Part 1: Experienced Butterflies, Nectar in All Flowers

For the first 13 days (April 11-24, 2011), all flowers contained nectar. Observations were taken on ten of those days (discounting April 15, 18, 23, and 24) using *H. charitonius* that were previously released in the garden and had been feeding at least several days, and up to several months, on *L. camara* with nectar added to all flowers of bicolored inflorescences. Each observation period lasted 30 minutes, and most days contained two with less than ten minutes between periods. Exceptions were 4/16 and 4/11 with one observation period, and 4/17 and 4/22 with three. During each period, the total number of visits by *H. charitonius* butterflies to each color inflorescence was recorded. A visit was defined as a butterfly landing on an inflorescence, pausing long enough to cease fluttering its wings, and attempting (regardless of success) to probe the flowers.

Part 2: Experienced Butterflies, Nectar Only in Red Flowers

For the next seven days (April 25-May 1), nectar was inserted into red flowers only. Observations were taken on six of those days (discounting April 29). For this "red treatment" period, three 30-min observation intervals were taken each day, usually consecutive or with less than ten minutes between intervals. Again, total number of visits by *H. charitonius* to each flower color was recorded.

On April 26 and 27, in addition to visit number, visit duration was recorded for all visits to both flower colors for one 30-min interval each day. Visits were timed from the time a butterfly ceased fluttering its wings after landing to the moment it flew away.

Part 3: Naïve Butterflies, Nectar Only in Red Flowers

On April 30, 13 newly eclosed *H. charitonius* were marked on the abdomen and released, and their visits were recorded separately for two days: 4/30-5/1.

RESULTS

Total number of visits in each 30-minute interval ranged from 8 - 68 with an average of 33 visits. Because individual observation periods differed markedly in total number of visits, rather than comparing number of flower visits, the percent visits to yellow was calculated for each period. The data reported are the mean percents yellow, where n = the number of observation periods for that particular treatment and the mean is the average of the percent visits to yellow flowers for all pertinent observation periods, regardless of total number of visits per observation period.

Part 1: Experienced Butterflies, Nectar in All Flowers

When all flowers contained nectar, experienced butterflies displayed a preference for yellow flowers (paired t-test, DF = 20, N = 21, t = -6.51, $p < 0.0001$) with a mean percent of visits to yellow flowers per observation period ± 1SD of 76.4% ± 15.9% (Figure 2). One outlying observation period had 25% yellow visitation rate, and that interval comprised of only eight visits total, the lowest count observed. Because of small sample size for this observation period, the unusually low relative visits to yellow flowers may be spurious. If this observation period is excluded, the mean percent of visits to yellow flowers per observation period is 79.0% ± 10.9%. Since the trends hold even including the outlier, it will remain included for further discussion.

Part 2: Experienced Butterflies, Nectar Only in Red Flowers

When only red flowers contained nectar, an overall preference for yellow still existed (paired t-test, DF = 17, N = 18, t-ratio = -2.42, p = 0.02), but with a lower mean percent yellow visitation rate per observation period of 60.2% ± 19.3% (Figure 2). Yellow preference when all flowers contained nectar was 16% higher than when just red flowers contained nectar, and this difference was significant (unpaired t-test, DF = 1, F = 8.09, p = 0.007; Figure 2).

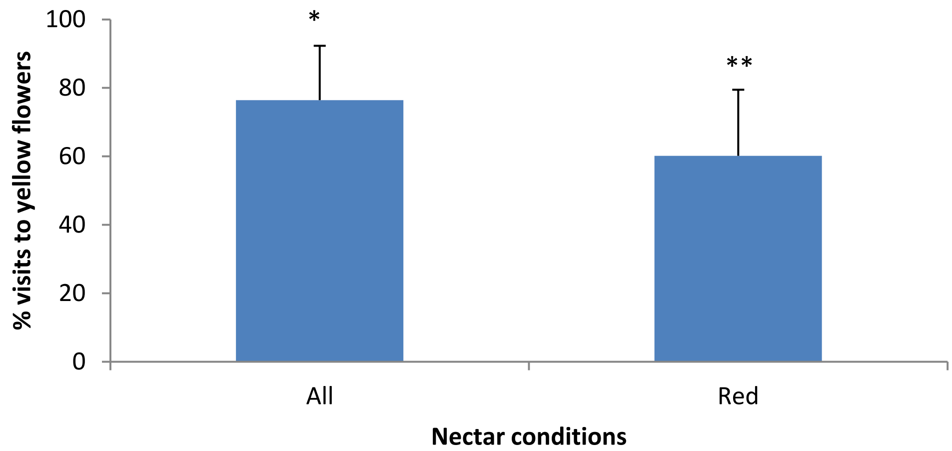

Figure 2. Mean proportion of visitation to yellow *L. camara* inflorescences, out of an array of equal numbers yellow and red, by *H. charitonius* across all 30-minute observation intervals. Nectar was present in all flowers for 13 days (n = 21) followed by red flowers only for seven days (n = 18). Yellow was preferred for both treatments, but yellow preference was higher with nectar in all flowers. Asterisks over bars represent significant differences. Error bars represent 1 SD.

Change in yellow preference over time

Yellow preference over the first 11 days with nectar in all flowers showed no significant change over time (Regression test, $R^2 = 0.168$, N = 9, p = 0.177), whereas over the following seven days with nectar in red flowers only, yellow preference decreased over the week (Regression test, $R^2 = 0.853$, N =6, p = 0.0005; Figure 3).

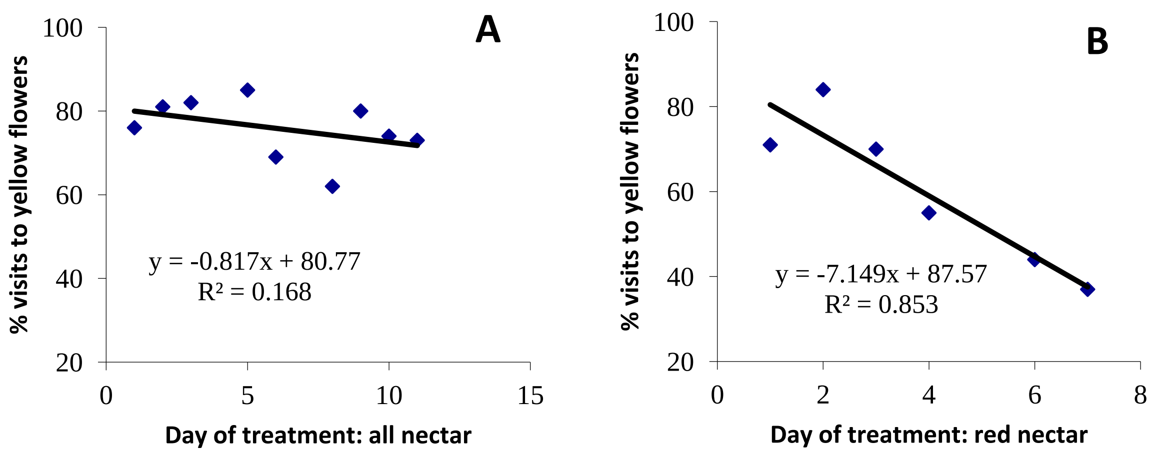

Figure 3. Yellow preference of experienced *H. charitonius* butterflies, measured by percent of visits which occurred to yellow *L. camara* flowers, remained constant over time while all flowers had nectar for 13 days, with nine days of data collection (**A**) and decreased while only red flowers had nectar for seven days, with six days of data collection (**B**). Equations and R^2 values refer to the trendline for yellow visitation rate over time.

The average yellow preference during the red treatment (Figure 2) is not an accurate representation of butterfly behavior since visitation preference changed significantly over time (Figure 3B). Yellow visitation was 78.2% in the first two days of the red treatment and dropped to 40.6% over the last two days.

Visit duration

On the first three days experienced butterflies were exposed to the red treatment, which all had higher yellow visitation rates than the mean for that treatment (71%, 84%, and 70%, respectively; Figure 3), butterfly behavior was notably different. During two 30-minute intervals on the second and third days of red treatment, experienced butterfly visits to red flowers were significantly longer than yellow visits (Wilcoxon sign rank test, $p < 0.0001$; Figure 4). They repeatedly visited many yellow flowers in a row for a very short period of time (mean 12.3 seconds, Wilcoxon Score Mean 31.3, n = 57), but remained for a long time on red flowers (mean 93.4 seconds, Wilcoxon Score Mean 58.3, n = 17), although with less frequency (Figure 3).

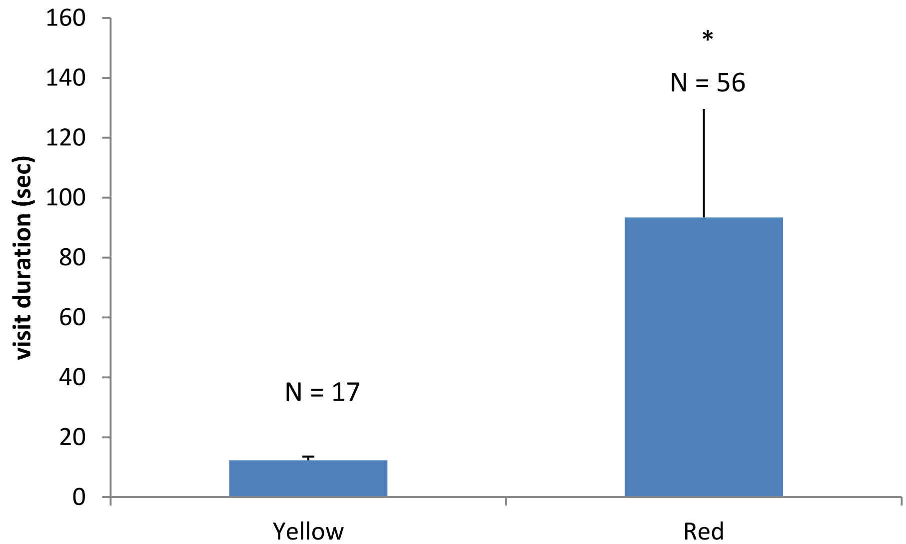

Figure 4. Mean visit duration by *H. charitonius* to yellow and red *L. camara* flowers during two 30-minute intervals, one each on 4/26 and 4/27, the second and third days of experienced butterfly exposure to red-only reward. Asterisk indicates that visits to red flowers were significantly longer. Error bars represent 1SE. Numbers above bars indicate sample size.

Part 3: Naïve butterflies, Nectar Only in Red Flowers

The naïve butterflies released on April 30 were exposed to conditions of nectar in red flowers only for two days, for a total of six observation intervals. The total number of naïve visits during that time was 66, and 37 of those visits to yellow flowers. The overall yellow visitation rate of 56.0% was not a significant preference for yellow (Chi square test, $\chi^2 = 0.48$, $p > 0.05$), even though it was within 4% of the mean yellow visitation rate of experienced butterflies during the red treatment, which was significant (figure 5). However, over only the first two days of the red treatment for experienced butterflies, yellow visitation rate was 78.2%, 22% higher than the naïve butterflies' yellow visitation rate over two days, and significantly different from both the naïve yellow preference (Chi square test, $\chi^2 = 40.0$, $p < 0.05$) and total experienced yellow preference during red treatment (Chi square test, $\chi^2 = 22.2$, $p < 0.05$; Figure 5).

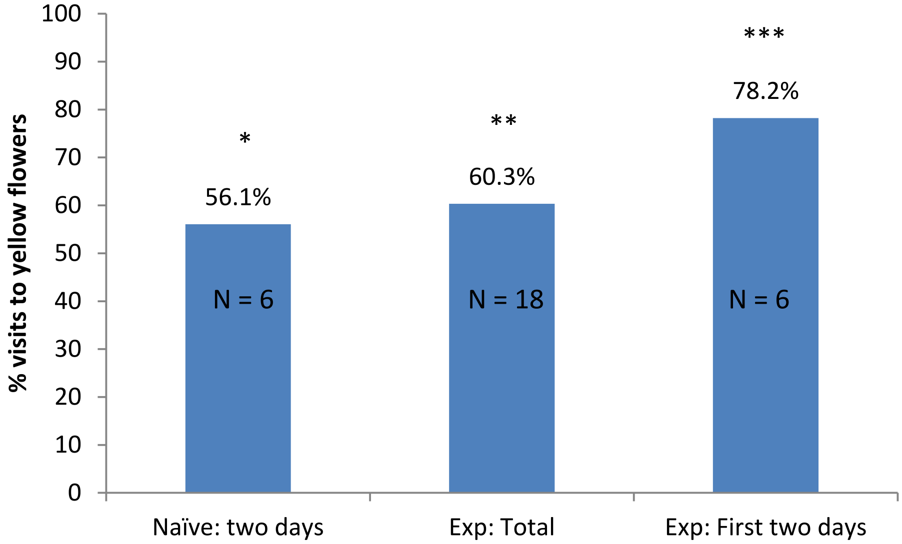

Figure 5. Mean proportion of visitation by *H. charitonius* to yellow *L. camara* inflorescences, when only red flowers contained nectar. Yellow preference was not significant for naïve butterflies over two days (left bar) and was significant for experienced (Exp.) butterflies across all days of red treatment (middle) and the first two days of experienced butterflies in red treatment (right). Asterisks represent significant differences. Percent above bars is the mean, and number inside bars is the number of 30-min observation periods included in each mean.

DISCUSSION

Color preference of experienced butterflies

Since *H. charitonius* preferred yellow flowers even when all flower colors were equally rewarding, the preference for yellow is probably innate, an adaptation which optimizes foraging success by favoring flowers which most efficiently reward butterflies. Preference for yellow gradually changed to a preference for red in experienced butterflies when only red flowers rewarded. Although the six data points for this second treatment yielded a significant preference for yellow, I believe that given more time under the red treatment the visitation preference would have stabilized below 50% as seen in the final two days. I attribute this to butterflies learning to associate the color red with exclusive reward. The learning process was quantified by the significantly shorter visits to yellow flowers than red during the initial days of the second treatment, when mean visitation rate still significantly favored yellow. Butterflies were observed moving fairly quickly from one yellow inflorescence to another in search of nectar, and leaving each inflorescence after probing the flowers and finding no reward. When they finally tried a red inflorescence, upon discovering the nectar reward they would sit on the inflorescence for a long time to feed, and then often leave the array without visiting any more red flowers, presumably satisfied.

An innate color preference can be overridden by reward and associative learning. The ability to learn and shift foraging behavior based on experience also optimizes foraging success, by enabling individuals to more efficiently extract a reward across a variety of environments. This balance between innate and learned preferences demonstrates how coevolution can provide enough specialization to create mutually beneficial interactions while allowing generalist flexibility which buffers the instability of high specialization (Waser *et al.* 1996).

Color preference of naïve butterflies

Naïve butterflies showed no significant yellow preference even though only red flowers were rewarding. This could be explained by a strong innate preference for yellow, mediated by the immediate effects of the opposite reward distribution, or because two days was not long enough for them to learn to favor red, and so before learning they visited the two colors arbitrarily. An innate yellow preference seems most likely because the experienced butterflies also demonstrated a yellow preference even though they were in an environment where reward did not differ between colors.

Since naïve butterflies were only exposed to red-only reward for two days, it makes biological sense to compare their yellow preference with that of experienced butterflies for only the first two days of the experienced red-reward trial. The experienced yellow preference was significantly higher than naïve for those two days, and the naïve butterflies showed no statistical color preference, so I conclude that naïve butterflies were able to respond more readily to an alternate reward. If butterflies can learn more quickly when they are young, they demonstrate some phenotypic plasticity which enables them to adjust their behavior to the environment into which they were born.

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