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The Relative Importance of Flower Color and Shape for the Foraging Monarch Butterfly (Lepidoptera: Nymphalidae)

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Abstract Flowers attract and guide pollinators via a wide array of sensory stimuli, including colors, odors, textures, and even sounds. Bees are known to respond to and learn multimodal and multicomponent floral cues, whereas, historically, studies of learning in butterflies have focused on a single visual stimulus component, most often color. In this study, we examine whether Monarch butterflies (*Danaus plexippus* L.) can learn to associate a compound visual stimulus, i.e., color and shape *together*, with a nectar reward. We also examine the relative importance of color and shape as cues for foraging butterflies. Our results indicate that within the visual modality, foraging Monarchs learn color more readily than shape. Monarchs, however, are capable of learning to associate shape with a sugar reward independent of color, and they may also be capable of learning the compound stimulus of color and shape in the context of foraging. We suggest that the hierarchical importance of cues is likely to vary depending on ecological context, and that although color may be most relevant for a nectar-foraging butterfly, shape may be a more useful cue for a butterfly searching for an oviposition substrate.

Keywords Monarch butterfly · *Danaus plexippus* L. · learning · multicomponent signal

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Introduction

Flowers have been described as 'sensory billboards' (Raguso 2004), as these often elaborate organs can attract and guide pollinators via a wide array of stimuli, including colors, odors, textures, and even sounds (Barth 1991; Kevan and Lane 1985; Weiss 1997; von Helversen et al. 2003; Goyret and Raguso 2006; Raguso 2008). Recent work has addressed the complexity and context-dependence of such floral signals, which commonly involve interactions amongst different types of information conveyed through different sensory modalities (e.g., Andersson and Dobson 2003; Goyret et al. 2009; Balkenius and Dacke 2010; Hebets and Papaj 2005; Raguso and Willis 2005; Leonard et al. 2011a, b; Leonard and Masek 2014). Historically, however, many studies of pollinators' responses to flowers have focused on individual floral traits within a given sensory modality, and have examined, for example, butterflies' preference for or ability to learn floral colors (Weiss 1995; Kinoshita et al. 1999; Weiss and Papaj 2003; Blackiston et al. 2011), bees' use of nectar-guide patterns on a petal (Giurfa et al. 1999; Leonard and Papaj 2011), or moths' attraction to floral odors (Willis and Arbas 1991; Balkenius et al. 2006). Even within a single sensory modality, floral signals can involve multiple components (Hebets and Papaj 2005; Raguso 2008; Leonard et al. 2011a). When looking at a flower, for example, a visually-oriented pollinator such as a bee or a butterfly will perceive not only the flower's color, but also its shape, surface properties, and any patterns that may be present, and may thus respond to an integrated suite of visual cues. The relative importance of such signal components may vary across taxa or behavioral context. With respect to visual cues in the context of nectar foraging, for example, butterflies generally rely on innate and learned color preferences to locate flowers (Weiss 1995; 1997; Kinoshita et al. 1999), whereas in the context of oviposition, females respond to leaf shape to locate appropriate host plants (Rausher 1978; Mackay and Jones 1989), and can learn to associate shape with the presence of an oviposition stimulant (Allard and Papaj 1996; Papaj 1986). It is not known whether butterflies attend to shape in the context of flower foraging, or how color and shape might interact to provide information to a foraging butterfly.

In this study, we ask whether monarch butterflies can learn to associate a compound visual stimulus, i.e., color and shape *together*, with a nectar reward. We also examine the relative importance of color and shape as cues to foraging butterflies. Specifically, we trained butterflies to four different color/shape combinations, and then examined their preferences for color irrespective of shape, shape irrespective of color, and both color and shape together. Monarch butterflies are well-suited to this line of inquiry, as they are long-lived and learn color cues rapidly in the context of nectar foraging (Blackiston et al. 2011; Rodrigues et al. 2010; Rodrigues and Weiss 2012).

Materials and Methods

Study Species

Monarch pupae were obtained from Sassyfrass Butterfly Ranch in Little Falls, MN in the summers of 2010 and 2011. The pupae eclosed in 30 cm by 30 cm by 30 cm mesh

cages that were kept indoors at 24–27 °C under 60 % relative humidity and a natural light/dark cycle. Butterflies were maintained under these same conditions. Males and females were housed in separate cages for the duration of the experiment. For identification purposes, butterflies were individually marked with a unique number at least 24 hours after eclosion. Each butterfly's unique number was written on its forewing using a fine-tip indelible marker.

Flower Models

Flower models were created by inserting 10 μl polypropylene pipette tips into the centers of paper squares or circles constructed of matte-finish, saturated purple (V-hue) or green (G-EX) Color-aid paper (Color-aid Corp., Hudson Falls, NY) (see Blackiston et al. 2011 for reflectance spectra of the papers). These simple closed (convex) shapes have been used in studies of shape discrimination by bees (Campan and Lehrer 2002). The pipette tips were firmly attached to the flower models with a glue gun. Models had approximately equal surface areas; squares were 3 × 3 cm, and circles had a radius of 1.75 cm. To create an array, the pipette tips of the paper models were inserted into a Styrofoam background covered in white printer paper, with models approximately 10 cm apart, center to center (Fig. 1). Arrangements of the models for each array are described in the methods for each test.

Innate Color Preference Tests

We selected the colors green and purple because our earlier studies had shown that monarchs did not have strong innate preferences for these colors; they did not seem to favor one over the other (Blackiston et al. 2011; Rodrigues et al. 2010). Thus, 2010 butterflies were not tested for innate color preference. However, after the experimental results from 2010 revealed a preference for purple over green, we subsequently tested all 2011 butterflies for innate color preference.

Three days after each butterfly eclosed, and just prior to its innate testing trial, we primed the butterfly to feed by gently unrolling its proboscis into a black star-shaped
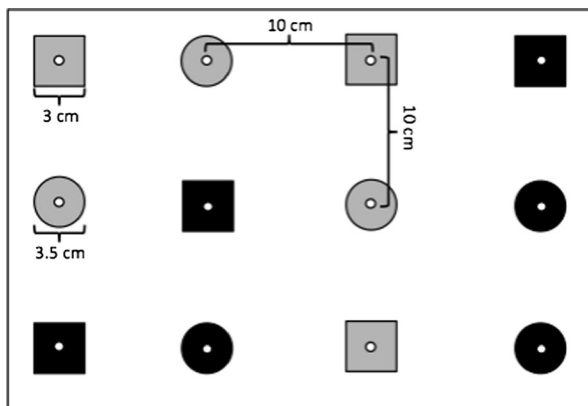
Fig. 1 Diagram of training and testing array. Black shapes represent purple models, and gray shapes represent green models

paper model that contained 20 % sucrose solution; we intentionally used a color and shape that would not be encountered in training or testing (see Rodrigues et al. 2010; Blackiston et al. 2011).

Testing took place in a 60 × 60 × 60 cm mesh cage containing 12 circular models, six purple and six green; none of the models contained sucrose. Butterflies were placed individually in the cage, and observations began immediately. Testing trials were conducted under natural light and lasted ten minutes.

A butterfly's innate preference was recorded as the first color that it landed on in the array. Only butterflies that flew to and landed on a model were counted as having made a choice; those that walked to or fell on a model were not counted. A butterfly that did not make a choice in its first innate preference test was tested again on the same array two to four hours later. Between each trial the arrays were rotated clockwise 90 degrees to avoid the possibility of positional learning.

Innate Shape Preference Tests

Butterflies from 2010 and 2011 were tested for innate shape preference. Testing was conducted as described above, but we used models of the same color that differed in shape. A butterfly's innate preference was recorded as the first shape that it landed on in the array. One set of tests utilized an array of six green squares and six green circles, the other an array of six purple squares and six purple circles. In 2011 each butterfly was tested for innate color and shape preferences during one day, with two to four hours between each trial. Following its last innate trial, each butterfly was fed for one minute on the black star model.

Training

The day following the innate testing trials, we began training the butterflies to associate a compound stimulus of color and shape with a food reward. Butterflies were randomly assigned to a training group, with the constraint that each group contain approximately equal numbers of males and females. To prevent courtship behavior and mating, butterflies were trained and housed in single-sex groups. We repeated the entire experiment three times, once each in July 2010, August 2010, and August 2011. In each case, the training and testing arrays included purple squares, purple circles, green squares, and green circles, but the subset of models that offered a reward differed within and across experiments. Within each experiment, two groups of butterflies were trained; each training group received its reward from a different combination of color and shape stimuli, providing an internal control for comparison. In both experiments conducted in 2010, one training group of butterflies was rewarded on purple squares (PS), and another on green circles (GC). In 2011 we reversed the stimulus pairing and rewarded one training group on purple circles (PC) and another on green squares (GS). Data from the two experiments conducted during 2010 were combined and analyzed together.

To begin training, butterflies were fed for five seconds on their assigned training stimulus model, then placed into a mesh cage containing an array consisting of 12 models – three purple squares, three purple circles, three green squares, and three green circles – with a maximum of six butterflies per cage. Only the training stimulus models

in the array were filled with 20 % sucrose solution; the other models were left empty to reinforce that the training model was the only rewarding shape/color combination. For example, in 2011, for butterflies in the PC training group, purple circles contained nectar, while purple squares, green circles, and green squares were empty; for the GS training group, green squares offered nectar, while the other 3 types of models did not. During the two-hour-long session, the butterflies essentially trained themselves, as they were free to visit any of the flower models and develop associations between the models and the presence or absence of food reward. Butterflies were exposed to the rewarding arrays 2 h a day, for 5 consecutive days.

For each day of training, the location of models in the array was determined haphazardly. Models of the training stimulus were refilled with sucrose solution as needed, so that they were always rewarding. After the two-hour session, butterflies were fed for one minute on their assigned training model to ensure that each received a minimum of 65 s of reward on the training stimulus (5 s before +60 s after the training period), in addition to the time spent on the rewarding models during training.

Testing

Butterflies were tested after five consecutive days of training. Before testing, each butterfly was fed sucrose solution on its trained stimulus for five seconds in order to encourage feeding behavior, a standard practice in butterfly training (see Rodrigues et al. 2010; Blackiston et al. 2011). Each butterfly was then released individually into a cage containing an array of empty models identical to the training models (three purple squares, three purple circles, three green squares, and three green circles); none of the models had ever contained nectar. Over a ten minute period, we recorded the color and shape of the first model visited, the number of visits to each model, and the number and duration of probes to each model. A visit was defined as a landing from flight that resulted in a probe; landing on a model without probing was not counted as a visit. Timing of a probe began when the butterfly's proboscis first touched the model and ended when the butterfly re-rolled its proboscis. If a butterfly stopped probing a model, but remained on the model for a period before it began probing the model again, the second round of probing was not counted as a new visit and only the time spent probing was recorded.

Statistical Analyses

In our analyses we included only butterflies that probed models for a cumulative total of at least 20 s during a ten-minute trial, in order to avoid biasing the data with butterflies that were not motivated to forage. Statistics were performed using GraphPad Prism version 5.00 for Mac (GraphPad Prism 2010). For all statistical tests other than the innate preference tests, in which we used number of first visits to a shape or color, we analyzed the percentage of time spent probing models because that measure seemed to best represent a butterfly's determination to find nectar in the model. However, analyses of both number and percentage of visits to models showed essentially the same patterns as we saw in our analyses of percentage of time spent probing models.

Innate Preference Tests Innate color and shape choices were analyzed using a chi-square test, based on an initial null expectation of equal visitation to green and purple in the color test, and to squares and circles in the shape test.

Color Learning Across Shape The butterflies' very first color choices after training were compared to their very first innate color choices, using a chi-square test. In addition, the percentage of time spent probing purple vs green models of either shape after training were compared to one another, using a two-tailed, paired Wilcoxon test.

Shape Learning Across Color The butterflies' very first shape choices after training were compared to their very first innate shape choices, using a chi-square test. In addition, the percentage of time spent probing square vs circle models of either color after training were compared to one another, using a two-tailed, paired Wilcoxon test.

Compound stimulus learning (trained shape within trained color) To test whether butterflies learned the compound stimulus of trained shape *and* trained color, we compared the proportion of time butterflies spent probing each shape within their trained color, using a two-tailed, paired Wilcoxon test. For example, we determined the proportion of time butterflies trained to purple squares spent probing purple squares and purple circles. We did not look at the proportion of time spent on each color within the trained shape because the butterflies demonstrated a clear ability to learn color, so that this measure would not have been informative. For each training group, Friedman's tests were used to compare the percentage of time spent probing each model, and pairwise comparisons of each combination of stimuli were conducted using Dunn's all pairs with joint ranks.

Effect of butterfly sex on learning To test whether males and females differed in learning ability, we compared the percentage of time each sex spent probing a) their trained color, b) their trained shape, and c) each shape within their trained color, all using a Wilcoxon test.

Results

Innate Color Preference

Out of 48 butterflies tested, 20 visited a model during innate color testing. Choices differed significantly from random and revealed a strong innate preference for purple (Fig. 2; chi-square goodness of fit, $\chi^2 = 8.45$; df = 1; $P = 0.0037$).

Innate Shape Preference

Out of 50 butterflies tested in 2010 and 2011, 34 visited a model during innate shape testing. Choices did not differ significantly from random (Fig. 3; chi-square goodness of fit, $\chi^2 = 0.118$; df = 1; $P = 0.7316$). It should be noted that when the data from each
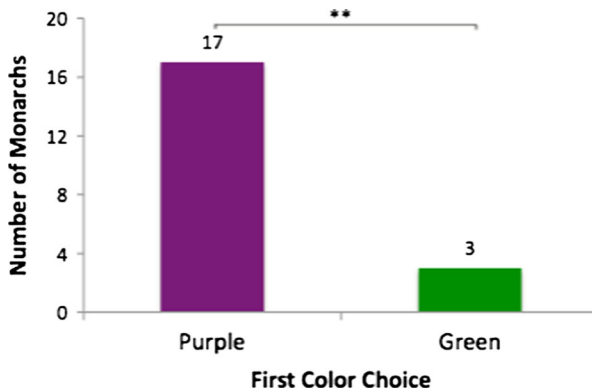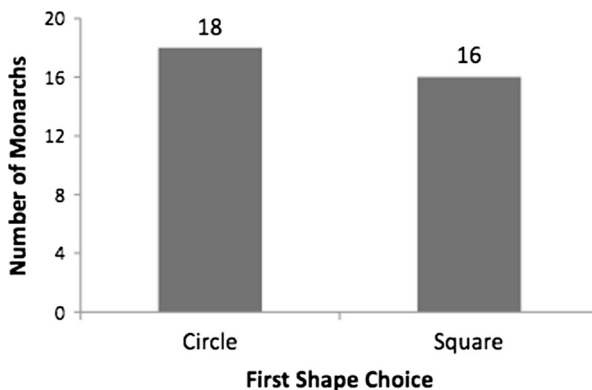
Fig. 2 Number of first visits monarchs made to each color during innate color preference trials. Monarchs showed a significant innate color preference for purple. **$P < 0.01$

year are analyzed independently, the choices did not differ significantly from random, suggesting that prior exposure to models in the innate color preference test in 2011 did not influence the results of the innate shape test (chi-square goodness of fit, 2010: $\chi^2 = 1.06$; df = 1; $P = 0.3032$; 2011: $\chi^2 = 1.90$; df = 1; $P = 0.1681$).

Color Learning Across Shape

After five days of training, butterflies rewarded on purple squares (Fig. 4a) or purple circles (Fig. 4c) spent significantly more time probing purple models of either shape than they did probing green models (PS: paired Wilcoxon, two-tailed, $n = 14$, $P = 0.00015$; PC: paired Wilcoxon, two tailed; $n = 16$, $P = 0.0001$). However, the learned preference for purple, as measured by the color of the first flower chosen after training, did not differ significantly from the butterflies' strong innate preference for that color (chi-square goodness of fit, $\chi^2 = 0.0785$, df = 1, $P > 0.05$).

Similarly, after five days of training, butterflies rewarded on green circles (Fig. 4b) or green squares (Fig. 4d) spent significantly more time probing green models of either

Fig. 3 Number of first visits monarchs made to each shape during innate shape preference trials. Monarchs did not show a significant innate shape preference. $P > 0.05$
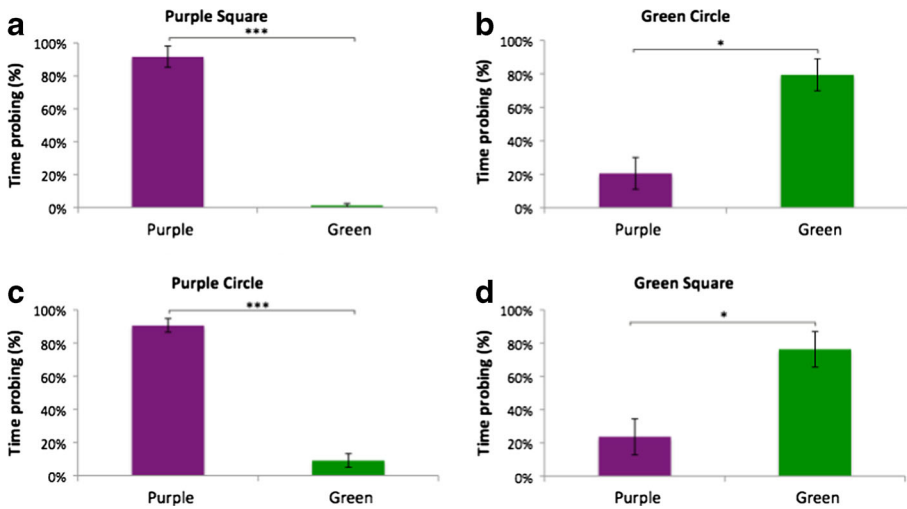
Fig. 4 Amount of time monarchs spent probing each color across shape after five days of training. All butterfly training groups spent significantly more time probing their trained color compared to their non-trained color. Title above each graph (e.g., Purple Square, Green Circle) indicates the color and shape of the rewarding models for that training group. Values are means ± SE. ***$P < 0.001$; *$P < 0.05$

shape than they did probing purple models (GC: paired Wilcoxon, two-tailed, $P = 0.0096$, $n = 13$; GS: paired Wilcoxon, two tailed, $P = 0.0208$, $n = 9$). The learned preference for green differed significantly from the weak innate preference for that color (chi-square goodness of fit, $\chi^2 = 27.819$, df $= 1$, $P < 0.0001$).

The significant differences in probing times reflect a common trend of the majority of butterflies: 92 % of butterflies spent more than 50 % of time probing their trained color, and 83 % probed their trained color more than 80 % of the time.

Shape Learning Across Color

After five days of training, butterflies rewarded on purple squares did not show a significant preference for squares over circles of either color (Fig. 5a, paired Wilcoxon, one-tailed; $P = 0.1447$, $n = 13$), but the butterflies rewarded on green squares did show a significant preference for squares over circles of either color (Fig. 5d, paired Wilcoxon, two-tailed $P = 0.0244$, $n = 9$).

Of the butterflies rewarded on circles (Fig. 5a) the purple circle butterflies showed a significant preference for circles over squares of either color (Fig. 5c paired Wilcoxon, two-tailed; $P = 0.0019$, $n = 16$), but the green circle butterflies did not (Fig. 5b, paired Wilcoxon, two-tailed $P = 0.1683$, $n = 13$). Overall, 68 % of butterflies spent more than 50 % of time probing their trained shape.

Compound Stimulus Learning (Trained Shape Within Trained Color)

In order to determine whether the butterflies learned a compound stimulus, we examined the proportion of time butterflies spent probing each shape within their trained color. For example, we determined the proportion of time butterflies
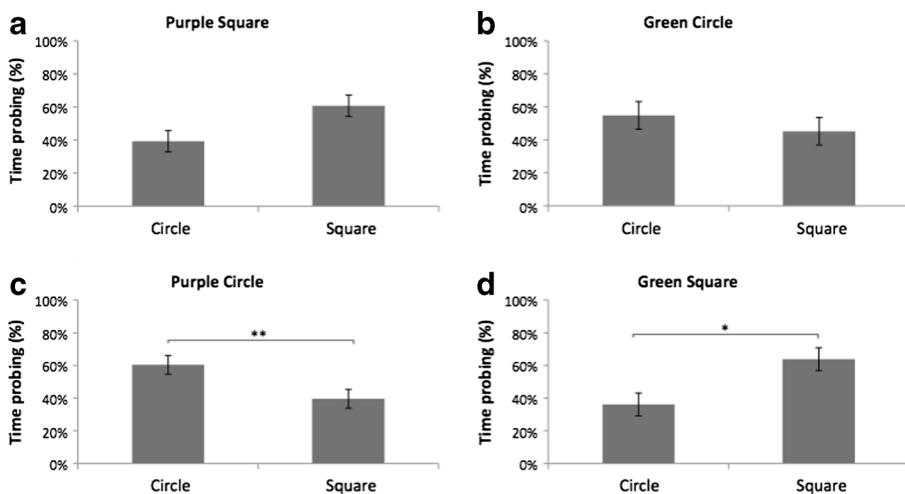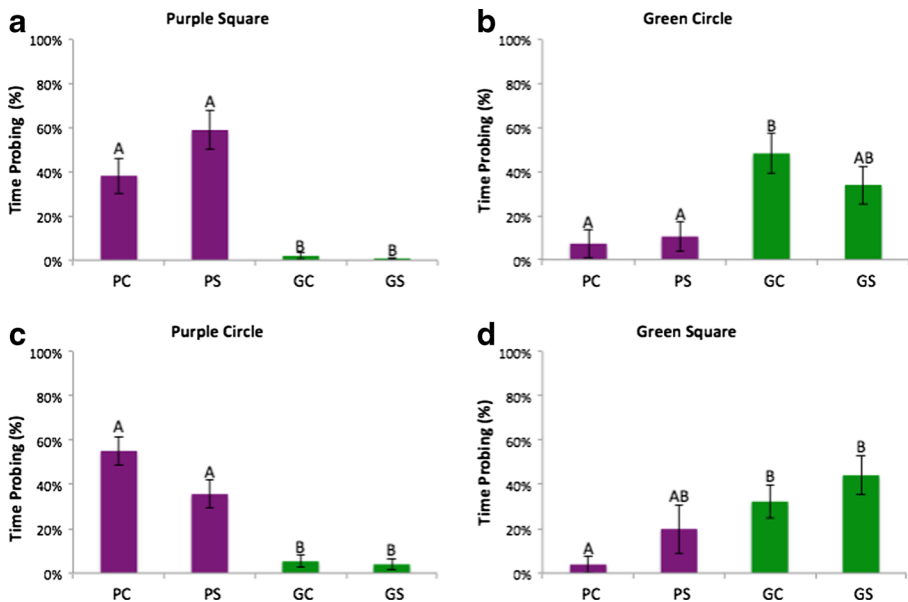
Fig. 5 Amount of time monarchs spent probing each shape across color after five days of training. Butterflies trained to purple circles and green squares spent significantly more time probing their trained shape compared to the non-trained shape, while butterflies trained to purple squares and green circles did not. Title above each graph (e.g., Purple Square, Green Circle) indicates the color and shape of the rewarding models for that training group. Values are means ± SE**$P < 0.01$; *$P < 0.05$

trained to purple squares spent probing purple squares or purple circles. Notably, butterflies in all four different training groups spent more time probing their respective trained compound stimulus than they did the other three choices (Fig. 6a-d). In only one treatment, however, were differences in time spent probing trained shape within trained color statistically significant: butterflies trained to purple circles spent significantly more time probing purple circles than they did purple squares ($P = 0.0362$, $n = 16$). There was not a significant difference between the amount of time purple square butterflies spent probing purple squares and purple circles ($P = 0.2893$, $n = 13$). Green circle butterflies did not probe green circles significantly more than green squares ($P = 0.3440$, $n = 13$); nor did green square butterflies probe green squares significantly more than green circles ($P = 0.4609$, $n = 9$). We then used a nonparametric Friedman test followed by a Dunn's post hoc test to compare the amount of time butterflies spent probing each model stimulus for each training group. For all training groups, the Friedman test was significant (PS: $F_{3,12} = 23.03$, $P < 0.0001$; PC: $F_{3,15} = 33.20$, $P < 0.0001$; GC: $F_{3,12} = 17.16$, $P = 0.0007$; GS: $F_{3,8} = 12.61$, $P = 0.0055$), indicating that butterflies in each training group spent significantly more time probing some models relative to others. For each group, different multiple comparisons were significant (Fig. 6a-d).

Effect of Butterfly sex on Learning

We saw no differences between male and female butterflies in percentage of time spent probing trained color (Wilcoxon, $\chi^2 = 0.3272$, df $= 1$, $P = 0.56$), trained shape (Wilcoxon, $\chi^2 = 0.0498$, df $= 1$, $P = 0.83$), or trained shape within trained color (Wilcoxon, $\chi^2 = 0.4128$, df $= 1$, $P = 0.52$).

Fig. 6 Amount of time monarchs spent probing each model color/shape combination after five days of training. Title above each graph (e.g., Purple Square, Green Circle) indicates the color and shape of the rewarding models for that training group. Values are means ± SE. Different capital letters indicated significant differences at the $P < 0.05$ level

Discussion

Our results demonstrate that in the context of nectar foraging, Monarchs readily learn to associate color, and to a lesser extent, shape, with a food reward. Our data also suggest that monarchs may be capable of learning the compound stimulus of color and shape together.

Monarchs quickly learned to associate a color with a sugar reward. During testing, all groups of butterflies showed a significant preference for their trained color, irrespective of model shape. Initial flower choices made by butterflies trained to purple (across both shapes) did not differ significantly from their strong innate preference for purple, although training is likely to have reinforced this innate preference, as indicated by the very high proportion of time spent by purple-trained butterflies on that color. Butterflies trained to green shapes, on the other hand, demonstrated a learned preference for green that significantly differed from their innate color preference. For those butterflies trained to green, visitation to the innately preferred color (purple) diminished significantly, as would be expected after exposure to an alternate rewarding color (Goyret et al. 2009; Kelber 1996; Weiss 1997; Blackiston et al. 2011). These results confirm that the monarchs were able to learn a color cue independent of shape in a foraging context, as has been demonstrated by several previous studies (Blackiston et al. 2011; Rodrigues et al. 2010; Rodrigues and Weiss 2012).

Although monarchs were able to associate floral shape with a sugar reward, this cue was not learned nearly as well as color. Whereas 92 % of the color-trained butterflies spent more than 50 % of time probing their trained color, 68 % of the shape-trained butterflies spent more than 50 % of time probing their trained shape. After training, all

groups of butterflies showed a preference for their trained shape, and butterflies trained to purple circles and green squares (the 2011 experiment) showed a significant preference for their trained shape independent of color. These results suggest that, like *Apis* and *Megachile* bees (Campan and Lehrer 2002), butterflies are able to associate a shape with a food reward in the foraging context, which to our knowledge has not previously been demonstrated.

In order to determine whether the butterflies learned a compound stimulus of color and shape, we compared the proportion of time butterflies spent probing each shape within their trained color. Only the butterfly training group rewarded on purple circles (the group with the highest N) demonstrated a significant preference for their trained compound stimulus. However, for all four training groups, the butterflies spent the greatest proportion of time probing their trained color/shape combination; thus our results follow the pattern that would be expected if the monarchs did indeed learn to associate a compound stimulus with a reward. Further studies will need to be conducted to confirm this result.

Some studies of butterflies have revealed differences between the sexes in innate color preferences or learning abilities, while others have not. Kandori et al. 2009 found that for two of four species of butterflies examined, the females learned to associate floral color with a reward more rapidly than did the males. With respect to monarchs, Rodrigues et al. (2010) found no difference between the sexes in learned risk-averse foraging behavior; nor did Blackiston et al. find sex-based differences in innate color preferences or in ability to learn orange (Blackiston et al. 2011). In this study we saw no differences between male and female butterflies in percentage of time spent probing trained color, trained shape, or trained shape within trained color.

Our results indicate a hierarchy within the visual modality with respect to monarchs' foraging behavior: color is the more important cue in a butterfly's recognition of flowers, as it is learned more readily than shape in all training groups. For all compound stimuli (as seen in Fig. 6), each group spent the most time on their trained color/shape combination, and the second largest amount of time on their respective trained color/non-trained shape combination. If, however, shape were the more important cue, we would expect that the butterflies would spend the second highest proportion of their time on their trained shape/non-trained color models. It is possible that for monarchs learning a trained color, floral shape serves as a secondary, facilitative cue that helps to improve foraging accuracy (Hebets and Papaj 2005; Leonard et al. 2011a).

The behavioral context in which a butterfly operates may affect the hierarchical importance of perceived visual cues. In the context of nectar foraging, for example, color is a more important visual cue than shape, and the eyes of visually-oriented insect pollinators are well-tuned to perceive floral colors (Barth 1991; Chittka 1996; Briscoe and Chittka 2001; Arikawa 2003). Thus floral color, which has presumably been selected to contrast with a background of vegetation, is likely to be easier to discriminate than floral shape at a distance. In the context of oviposition, however, shape may be a more important visual cue than color. Because leaves are commonly green (of one shade or another), color alone may not provide a robust cue for female butterflies when searching for a particular host plant, and leaf shape may be a more reliable indicator for species recognition. Female butterflies are able to form a search image for leaf shape (Rausher 1978; Mackay and Jones 1989), and can learn to associate shape models with an oviposition stimulant (Papaj 1986; Allard and Papaj 1996).

Because our experiment was conducted in the context of nectar foraging, it is not surprising that we found color to be a more salient cue than shape. However, it is also possible that the apparent importance of color over shape was further enhanced by the degree of difference between the two pairs of stimuli. The particular colors of the green and purple papers used in our study are well separated in a color triangle that represents the monarch's color vision space (Blackiston et al. 2011), suggesting that the butterflies could readily perceive the difference between them. However, the circle and square are both convex or closed shapes, and are likely to be less easily discriminable than a closed and an open shape, such as a cross and a square.

Floral complexity promotes pollinator learning and memory, improving the ability of pollinators to respond to floral signals and facilitating pollination of conspecific flowers (Raguso 2004; Leonard et al. 2011a). Several studies have demonstrated that multimodal cues, e.g., those involving both color and scent, improve foraging speed and/or accuracy for bumblebees (Kulahci et al. 2008; Leonard et al. 2011b) and hawkmoths (Balkenius and Dacke 2010). Our results demonstrate that multicomponent cues within a single sensory modality can also enhance a pollinator's recognition and learning of flowers.

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