Wing Color Predicts Future Mating Success in Male Monarch Butterflies

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ABSTRACT Predictors of male monarch butterfly mating success have eluded researchers for years. Although it has long been known that there is variation in male mating success in this species, the source of this variation remains unclear. We used digital image analysis techniques to measure fine-scale variation in three components of the orange color (hue, saturation and brightness of the orange) of the forewings of 174 males at a level of detail that has not been possible until recently, and we compared this information to the mating success of the males in captivity. A second experiment involved addition of orange coloration to the wings of 93 males before mating trials. Our results indicate that one component of the orange color (saturation) correlated with mating success in our first experiment. Furthermore, wing color manipulations had no effect, but the original saturation values of the males in the second experiment were directly related to mating success. Our results provide the first evidence that wing color in monarch butterflies relates to mating success, and they serve as an important starting point for future research.

KEY WORDS *Danaus plexippus*, mating success, wing color

A growing body of evidence demonstrates a relationship between morphological characters and mating success in butterflies (Knuttel and Fiedler 2001; Breuker and Brakefield 2002, 2004). In most cases, studies have examined species in which males choose females based on some morphological character such as wing size (Jimenez-Perez and Wang 2004) or wing color (Wiernasz 1995, Ellers and Boggs 2003). However, in monarch butterflies, *Danaus plexippus* (L.), it is unclear which is the choosier sex. Monarchs are unusual in that males use a "take-down" strategy, whereby they grab a female in flight (or at rest) and wrestle with her in an attempt to couple (Pliske 1975, Hill et al. 1976). Males may exercise mate choice by selecting which females to pursue. Females nearly always resist coupling during the wrestling phase. Only 30–40% of mating attempts end in copulation (Solensky and Oberhauser 2004), suggesting some degree of control by females. However, observations reveal no evidence of female mate choice for particular males (Solensky and Oberhauser 2004), although females vary in their degree of resistance based on their own mating history (Oberhauser 1989, Frey 1997).

It has long been known that there is variation in male mating success in monarch butterflies (Oberhauser 1989), although considerable research on the subject has revealed no consistent morphological correlates (Van Hook 1993, Frey 1997, Oberhauser and Frey 1997, Solensky and Oberhauser 2004). It is unknown whether particular male characters are selectively favored by differential female resistance or whether certain male characters confer advantages during copulations. It is known, however, that male mating success is not attributable to differential effort among males, as defined by the duration of struggles or number of mating attempts (Solensky and Oberhauser 2004), nor is male mating success associated with wing length in captivity (Solensky and Oberhauser 2004). Observations of wild monarchs at overwintering sites in Mexico and California indicate that mating males tend to be smaller than roosting males (Van Hook 1993, Oberhauser and Frey 1999), although this pattern seems to result from an effect of male size on the timing of mating during the overwintering period rather than an effect of male size on mating success per se. In these same studies, neither male size nor wing condition differed between successful and unsuccessful mating attempts (Van Hook 1993, Oberhauser and Frey 1999).

Despite the lack of evidence for consistent effects of male characteristics on mating success, Solensky and Oberhauser (2004) found that captive male monarchs that were successful at mating were also more likely to be successful on any given attempt than less successful males, leading them to suggest that some as yet unidentified characteristic makes some males either preferred by females or better able to force copulations. Furthermore, there is a significant genetic component to male mating frequency (Solensky and Oberhauser 2004). The particular gene or genes involved have yet to be identified, but Solensky and Oberhauser (2004) showed that male siblings were

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more similar in mating frequency than were unrelated males, which indicates significant heritability of this trait.

Recent advances in computer imaging and analysis software now allow for the measurement of butterfly wings in considerable detail (Windig 1991) and provide researchers with the ability to evaluate the importance of previously immeasurable wing characters on the life histories of butterflies. Researchers can scan or photograph butterfly wings and use computer software to measure characters on the wing images. These methods are especially useful for the measurement of wing color variation. For example, Davis et al. (2005) recently used these methods to determine that variation in rearing temperatures resulted in fine-scale variations in the wing patterning in monarch butterflies. Moreover, Davis et al. (2005) found that imaging software uncovered considerable variation in monarch wing colors that was nearly imperceptible to the human eye.

In this article, we investigated the relationship between fine-scale variations in wing color and male mating success in monarch butterflies by using image analysis techniques to measure wing morphology in much finer detail than has been attempted previously. In an initial study, we examined wing size and orange coloration of 174 males before initiating an experiment whereby male mating frequency was measured under controlled conditions. Based on patterns revealed in this study, we initiated a second experiment to investigate the cause of a correlation between wing coloration and male mating success. We manipulated the orange color of 93 males before mating trials in an attempt to establish whether initial or manipulated coloration more strongly predicts mating frequency. If females choose males based on wing coloration, then wing color manipulations should affect male mating success. However, if wing coloration correlates with some inherent characteristic of males that affects their ability to capture or couple with females (independent of female choice), then color manipulations should not alter the correlation between initial coloration and male mating frequency.

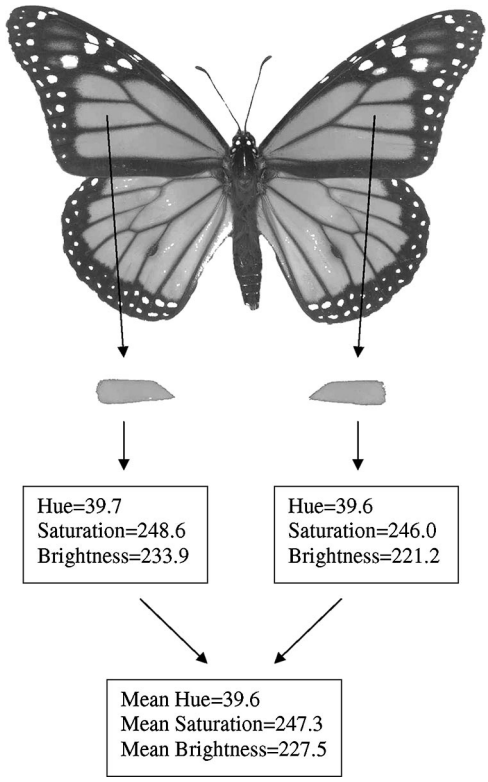

Fig. 1. Scanned image of a typical male monarch butterfly used in this study (shown in gray scale). In this image, all gray areas of the wings are orange in real life. Orange colors were calculated by digitally selecting the middle cells in the left and right forewing and calculating the mean color variables for the entire cell via image analysis. The left-right average (bottom box) of these variables was used in the analyses.

Materials and Methods

Scanning Males. We scanned males using a Hewlett–Packard ScanJet 6200C (experiment 1) or 3970 (experiment 2) flatbed scanner at a resolution of 300 dpi and with the same light and exposure settings throughout. Butterflies were chilled on ice for 10 min before scanning to minimize movement. Each was scanned in standard pinning position (Fig. 1). Once all scans were completed, we imported the images into Adobe Photoshop (Adobe Systems, Mountain View, CA) with the Fovea Pro plugin installed (Reindeer Graphics, Inc., Asheville, NC).

Measuring Forewing Size and Color Variables. We digitally cropped the right and left forewings from the image of each male and saved them separately for forewing measurements. To obtain measures of size, we ran the Fovea Pro measurement routine on each forewing image, which measures the total area of the forewing. We used the average of the left-right forewing areas of each butterfly as our measure of wing size. To measure the orange color of the monarch wings, we sampled the middle cell of the right and left forewing on all individuals (Fig. 1). We used this location because it is not near the wing margin where it could be tattered, and it is fully enclosed by black pigmentation, which provides a convenient boundary for tracing. Thus, on each forewing image, we digitally traced and cut out the middle cell (Fig. 1) and saved it in a separate file. When tracing the cell, we avoided areas beyond the boundary of the orange area, so that all cells selections contained orange pigmentation only. We then used the Fovea Pro color measurement routine on all cell images, which computes the average hue, saturation, and brightness values for all pixels within the cell (middle cells in this study had an average of 886 pixels each). The average hue value we obtained for each male can be thought of as what most people mean by "color" (i.e., the distinction between red, orange, yellow, green, and so on) and is measured in degrees (i.e., up to 360). The saturation value is

Table 1. Summary of orange color of males in all treatments in experiment 2

Treatment	Color variable	Premanipulation	SD	Postmanipulation	SD
Orange added (n = 32)	Hue	36	(1.6)	15	(1.8)
	Saturation	240	(5.0)	179	(29.3)
	Brightness	150	(8.1)	140	(7.9)
Sham (n = 31)	Hue	36	(1.1)	36	(1.7)
	Saturation	238	(6.2)	185	(30.4)
	Brightness	150	(6.3)	142	(9.3)
No manipulation (n = 30)	Hue	35	(2.0)		
	Saturation	238	(14.0)		
	Brightness	148	(7.5)		

Average premanipulation color variables from all males in each treatment are shown first, followed by their postmanipulation average. The effect of adding orange resulted in a generally brick red color. In the sham treatment, we attempted to put the males through the coloring process while leaving the actual color unchanged, although comparison of pre- and postimages revealed that there was a reduction in orange saturation. Values for hue are measured in degrees and represent a position on the color wheel; values for saturation and brightness are on a scale between 1 and 255.

roughly the amount or intensity of color (e.g., the difference between pink and red, or the degree of orange in this case) and is measured on a scale from 1 to 255 with lower values representing deeper orange. Brightness is the overall darkness or brightness of the color (i.e., if it were converted to a black and white image) and is also from 1 to 255. We note here that although any color is technically composed of the hue, saturation, and brightness values combined, we chose to examine the effects of these three variables on mating success separately. Finally, we performed this on both right and left forewings and then calculated the average of the hue, saturation, and brightness of the orange for all individuals, which was used in our analyses.

Experiment 1. Adult monarchs were the offspring of ≈50 monarchs collected as eggs and larvae in Franklin County, MO. We reared larvae in wooden cages (0.5 by 0.3 by 0.6 m; 50–150 larvae per cage) on a mixture of potted *Asclepias curassavica* L. and cut stems of *Asclepias syriaca* L. Larvae developed at room temperature (roughly 24°C) under natural light conditions (roughly photoperiod of 18:6 [L:D] h). On eclosion, adults were placed in glassine envelopes (8.9 cm^2). We checked them for infection by *Ophryocystis elektroscirrha* (Altizer et al. 2000), scanned uninfected adults as described above, and then labeled each with a unique number on the discal cell of both hindwings by using a fine point permanent marker. Adults were fed a 20% honey–water solution to satiation every other day before being released into cages. In the cages, they had continuous access to potted *A. curassavica* or cut stems of *A. syriaca* for oviposition, and sponges saturated with a 20% honey–water solution for feeding.

We released 173 males and an equal number of females into six outdoor mesh cages (1.8 m^3; ≈60 butterflies per cage) in St. Paul, MN, and monitored mating activity for 10 days starting on 10 July 2003. This butterfly density is intermediate between that experienced by wild monarchs in the summer breeding range and the overwintering period (Solensky and Oberhauser 2004). All butterflies were 5 to 10 days old upon release into cages at the start of this experiment. Females were added in response to female mortality or removed in response to male mortality in each cage to maintain an equal sex ratio. Dead males were not replaced. Monarchs typically remain coupled until after dusk, regardless of when the pair couples during the day, so males could mate only once per day and all pairs could be recorded by checking the cage at dusk. We define male mating success as the number of days on which each male coupled with a female (during our 10-d experiment). This 10-d measure of male mating success has been shown to significantly predict lifetime male mating frequency (Solensky and Oberhauser 2004). Although these captive males sometimes mate twice with the same female, which may not substantially contribute to their lifetime reproductive success, the abundance and distribution of wild males make this repeated mating an unlikely occurrence in the wild. Therefore, any factors that positively correlate with 10-d mating frequency among captive males should, if also true of wild monarchs, contribute to lifetime male reproductive success.

Experiment 2. Adult monarchs were reared from eggs laid by a single female obtained from the "Monarch Lab" at the University of Minnesota. Larvae were reared and adults processed as in experiment 1, with the exception of slightly colder rearing temperatures (≈20°C). The adult male monarchs were scanned using the same methods as in experiment 1. Males were randomly assigned to one of three treatments. In treatment one (n = 32), we added orange to all four wings by using an orange Sharpie marker. Treatment two (n = 31) was a sham treatment in which we colored all four wings with a yellow Sharpie marker to impose the coloring procedure without visibly altering the orange wing coloration. Treatment three (n = 30) was a control in which butterflies remained unmanipulated. To elucidate the effects of the treatments, we rescanned all manipulated males (before the mating trials) and recalculated the orange color variables of their forewings as described previously. The average values for each treatment are shown in Table 1. Our first treatment resulted in a lowering of the hue and saturation of the orange. The effect of this was that males had a generally brick red color. In the sham treatment, we attempted to put the males through the "coloring" process but leave the actual color un-

Table 2. Univariate results of weighted linear regression analysis of forewing characteristics on male mating success in experiments 1 (n = 174) and 2 (n = 93)

Dependent variable	Exp 1			Exp 2		
	β	t	Significance	β	t	Significance
Wing area	0.24	3.05	0.003	0.32	1.99	0.054
Orange hue	−0.03	−0.36	0.722	−0.01	−0.08	0.939
Orange saturation	−0.42	−4.91	0.000	−0.71	−3.92	0.000
Orange brightness	−0.09	−1.09	0.279	0.21	1.35	0.185

The dependent variable in each test was the number of matings per days alive (see Materials and Methods). In both experiments, the analyses were weighted by the number of days the males were alive.

changed, although comparison of pre- and postimages revealed that there was a reduction in orange saturation. This resulted in males from this treatment looking slightly drabber or yellower than unmanipulated males. Despite that this color alteration was not our intention, we included the males from this treatment in our mating trials to see whether this unintentional alteration affected mating success.

For the mating trials we released the males from each treatment along with an equal number of females ranging from 6 to 10 d old into each of four outdoor mesh cages (1.8 m^3; ≈60 butterflies per cage) in Wooster, OH. Each cage contained at least eight males of each of the three color treatments in addition to eight males subjected to a different treatment not discussed here. We monitored mating frequency for 10 d beginning on 22 June 2005, as in experiment 1.

Data Analysis. Although each experiment lasted only 10 d, not all males survived the 10-d period. However, many of these males had mated at various frequencies before dying, and we did not want to exclude these data. We therefore divided the number of matings by the number of days alive for all males, and we used this variable (matings per day) as our unit of mating success. Using this variable, we tested for relationships between mating success and our wing variables by using linear regression, with the analysis weighted by the number of days the males survived. This approach allowed us to include data from all males, with the data from males that survived longer factoring more into the analysis. The independent variables were wing area, orange hue, orange saturation and orange brightness scores. For experiment 2, we compared the manipulated wing colorations and mating frequencies of males in the three color treatments with one-way analysis of variance (ANOVA). All variables were normally distributed in both experiments, and significance was accepted when $P < 0.05$.

Results

Experiment 1. The average number of matings per day for the 174 males in experiment 1 was 0.48 (SD = 0.30), or roughly five matings for a 10-d period. In this experiment, we found that larger males mated more often than smaller males ($t = 3.05$, $P = 0.003$) (Table 2). We also found a highly significant relationship between mating success and orange saturation ($t = -4.91$, $P < 0.001$), such that males with lower orange saturation scores mated more often.

Experiment 2. The average number of matings per day in this experiment was 0.28 (SD = 0.30), or approximately three matings for a 10-d period. We found no significant variation in mating success (matings per day) among our three treatment groups (one-way ANOVA: $F_{2, 93} = 0.384$; $P = 0.682$). Because we did not detect an effect of our color manipulations on mating frequency, we then asked whether the original (before manipulation) wing coloration affected the mating success of these males. We thus pooled all three treatment groups and performed the same analysis as in experiment 1 (weighted linear regression), by using the same variables (size and three color variables) obtained during the initial scans of these males, and with the analysis weighted by the number of days the males survived. Interestingly, results of this analysis were similar to that of experiment 1. Wing size was a nearly significant predictor of mating success in this experiment ($t = 1.99$, $P = 0.054$) (Table 2) and positively correlated with mating frequency. Furthermore, orange saturation was again related to mating success, with less saturated males mating more frequently ($t = -3.92$, $P < 0.001$).

Discussion

The consistency of patterns observed in both experiments presented here provides strong evidence that certain aspects of wing color (and wing size) in male monarch butterflies are important indicators of future mating success. To our knowledge, this is the first experimental evidence of a correlation between male reproductive success and wing color in monarchs. Interestingly, only the saturation of orange, not the hue or brightness, seemed to be the important factor for mating success in both experiments. Males with a deeper shade of orange mated significantly more often than those with lighter shades of orange.

The cause of the relationship between orange wing coloration and male mating success remains unclear. Because the original wing color was correlated with male mating frequency in experiment 2, but color manipulations had no effect, we can surmise that the relationship between wing coloration and male mating success could stem from a correlation between orange coloration and some component of male mating ability, and not from female preference for males with more saturated orange wing coloration (because there was no effect of color manipulation). In an early study, Brower (1963) used paint to manipulate the wing

coloration of related female *Danaus gilippus* (Cramer) and found no effect of these color manipulations on female mating success, although the presence of paint (not color) reduced mating success. He concluded that scent plays a more important role than color in danaid courtship (Brower 1963), which is consistent with the observations reported here. Although female choice for male coloration is not supported by these data, they do not rule out female choice for other male characteristics. It is possible that some unidentified male trait is favored during the wrestling phase of the mating attempt, and the degree of orange saturation on male wings simply correlates with this trait. Previous research has demonstrated a genetic component to both male mating success (Solensky and Oberhauser 2004) and adult wing coloration in monarch butterflies (Davis et al. 2005), so the correlation between these two traits may stem from either genetic linkage or pleiotropic effects.

The results we gathered in these experiments stimulate further questions about the factors influencing color variation in butterflies in the first place. Early work in the 1970s demonstrated that the yellow coloration in monarch larvae and pupal cases is the result of carotenoids that are sequestered in the diet of the larvae (Rothschild et al. 1978), and that many other species of butterflies have pigments formed by carotenoids (Feltwell and Rothschild 1974). These studies also showed that larvae of several species that have diets rich in carotenoids later become adults with brighter yellow wing scales and that even nonyellow parts of butterfly wings were affected by variations in dietary carotenoids. Although similar research on adult monarchs has not been conducted, these early studies do serve to emphasize the general relationship between diet and adult coloration in butterflies. Interestingly, this relationship is paralleled in birds, as males of many species of birds are well known to sequester carotenoids from their diet to synthesize brightly colored plumage (e.g., Hill et al. 1994). Bright plumage is thought to be a signal of fitness in males, and males with brighter plumage are preferred by females because of this (Hill 1990, Hill et al. 1999). Given this relationship between carotenoids, diet, and color in male birds, it would not be unreasonable to think that some of the variation we uncovered in orange wing color among male monarch butterflies might be attributable to variations in either their diet or carotenoid-sequestering ability. This idea deserves further study. For example, follow-up experiments might include testing whether larval nutrition or parasite infection influences adult color, or whether variation in dietary carotenoids leads to variation in orange wing colors, which might influence larval host plant preferences.

In addition to our color variables, we found that wing size was an important predictor of mating success in monarch butterflies, with larger males mating more frequently. Interestingly, despite the many previous studies on monarch mating, no relationships between size and male mating success have been found (Oberhauser and Frey 1997, Falco 1998, Solensky and Oberhauser 2004). The difference may result from our more comprehensive measure of wing size; we used digital image analysis to calculate total wing area, rather than manually measuring wing length, which is the traditional method used for assessing size. Combined with the fact that we found an additional suite of color characters that relate to mating success in monarch butterflies, this affirms the promise of this digital technology in addressing similar questions in monarchs or other organisms that involve difficult-to-measure parameters. Indeed, the variation in orange color we observed here was subtle enough to be almost undetectable to human observers by anything but digital image analysis techniques. Nevertheless, the consistency in our results from two separate experiments suggests that even this subtle variation is important for mating success in monarch butterflies. The same could be said for male wing size, which we found to be important, despite previous findings. Moreover, the data presented here provide an important starting point for future research on the subject of monarch coloration.

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