

# Chapter 14

## Estimating the Mating Success of Male Butterflies in the Field

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**Abstract** Sexual dimorphism in wing coloration is pervasive in butterflies and has been attributed to the process of sexual selection. However, this view has rarely been tested, partly owing to difficulties in estimating the mating success of males in the field. In the present study, we describe a method for assessing the mating success of male pipevine swallowtail (*Battus philenor*) butterflies, based on the appearance of their reproductive tracts. Laboratory experiments indicated that, in response to mating, components of the males' reproductive tracts become shorter, decrease in mass, and change in appearance, irrespective of age; and these changes persist for at least 2 days. Using these indicators of recent mating, we examined the reproductive tracts of 68 field-caught males and found that the color of the dorsal hindwing, a feature that females use in mate choice, was significantly greener in males that had recently mated than in males that had not.

**Keywords** *Battus philenor* • Ejaculate substance • Male mating success • Ornaments • Sexual selection

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## 14.1 Introduction

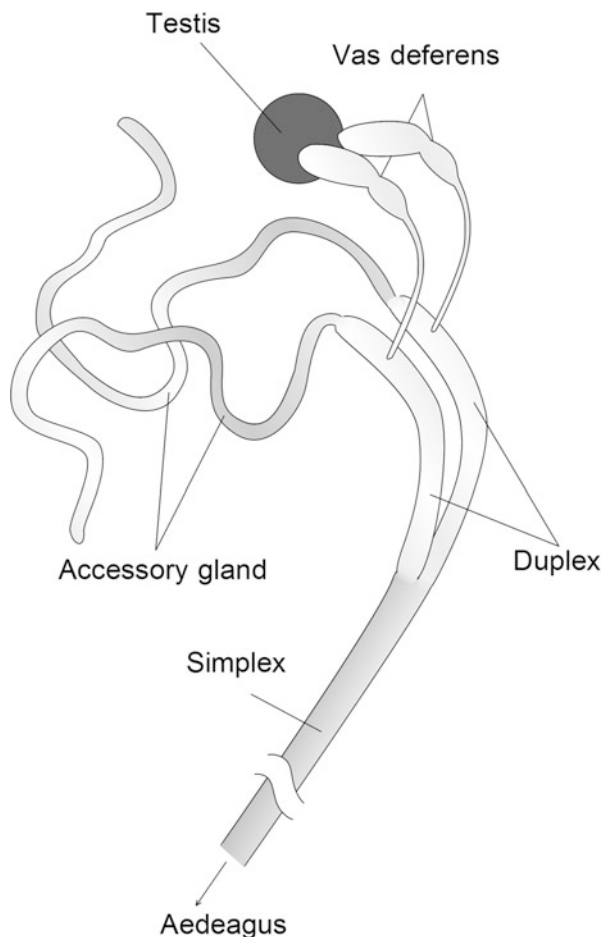
There is a long history of interest in the diversity of butterfly wing pattern and coloration. Starting with work of Darwin (1874) and Wallace (1889), researchers have observed and discussed various issues related to wing pattern, such as inter-specific similarity and variation (Nijhout 1990), ecological relevance (Rutowski 1997), color production mechanisms (Koch et al. 1998), evolutionary and developmental plasticity (Beldade et al. 2002), and genetics (Carroll et al. 1994), as well as intersexual differences. Because male butterflies typically exhibit brighter wing coloration and sometimes exhibit pattern elements that are not found in females, many researchers, including Darwin (1874), have speculated that the coloration of male wings results from female mating preferences associated with exaggerated visual signals (Kemp and Rutowski 2011; but see Allen et al. 2011).

A considerable amount of research has also been motivated by the intersexual variation of butterfly wing coloration; however, relative to other groups of colorful animals, such as birds, fish, and lizards (Blount et al. 2003; Grether et al. 2005; Hill and Montgomerie 1994; Keyser and Hill 1999), relatively little is known about the selective factors that promote the sexual dimorphism of wing color in butterflies (Kemp 2007). This deficit has partly stemmed from the difficulty of setting up the necessary assays of female preference. In addition, since sexual selection ultimately results from biased reproductive success, it is necessary to elucidate the relationship between male traits and reproductive success. However, the highly dispersed, cryptic, and ephemeral nature of butterfly copulation hinders the estimation of male mating success in the field (e.g., Rutowski 1997; Takeuchi 2016).

Here we report a new technique to assess the recent mating success of males in Lepidoptera that relies on changes that occur in the appearance of internal reproductive organs during mating. In some lepidopteran species, males transfer an ejaculate to females that can account for as much as 15% of the male body mass (e.g., Rutowski et al. 1983; Svård and Wiklund 1989), and in many species, males can produce more than one spermatophore; however, it takes time for males to produce an ejaculate that is comparable in size to the one transferred during the previous mating (Bissoondath and Wiklund 1996; Watanabe and Hirota 1999). Therefore, the internal reproductive organs of mated males might differ in size, contents, or appearance from those of unmated males, at least for a few days after mating.

The typical arrangement of internal reproductive organs in male butterflies includes two fused testes that give rise to a pair of vas deferens, which are secretory ducts, that lead to the duplex, which is a pair of sperm storage organs. Two duplex ducts unite caudally to form the simplex, which is a single duct that leads to the intromittent organ, or aedeagus. Sperm move from the testes into the duplex via the vas deferens (Riemann et al. 1974; LaChance et al. 1977). Due to the arrangement of the reproductive organs in the male body (c.f. Fig. 14.1), the spermatophore materials and accessory substances in the simplex are transferred to the female body during mating before the sperm are transferred (Watanabe and Sato 1993). Thus,

**Fig. 14.1** A schematic representation of the internal reproductive organs of a *B. philenor* male (After Sasaki et al. 2015)



males might not be able to reserve spermatophore materials or accessory substance in the simplex and so the quantity or nature of these materials might be a good indicator of recent mating activity.

The aims of the present study were (1) to document any changes that occur in the appearance of reproductive structures in male pipevine swallowtail (*Battus philenor*) butterflies as a result of mating, as well as the persistence of such changes after mating, in order to develop criteria for identifying males that had recently mated and (2) to examine the reproductive tracts of field-caught *B. philenor* males, assess the variation in their recent mating history, and determine whether their recent mating success was related to their phenotypes. Rutowski and Rajyaguru (2013) have reported that, in a captivity *B. philenor*, females use the dorsal hindwing coloration of males in mate choice.

This issue is mainly reporting previously published results and ideas in Sasaki et al. (2015).

## 14.2 Materials and Methods

### 14.2.1 Source of Animals Used

All specimens were from a population of *B. philenor* that thrives near the confluence of Mesquite Wash and Sycamore Creek in the Mazatzal Mountains, Arizona (33° 43' 50" N, 111° 30' 50" W). Animals used in the mating studies were reared from eggs and early instar larvae collected in the field from early June to mid-July in 2011. All larvae were reared in a walk-in environmental chamber, programmed for 14 h of light at 30 °C and 10 h of dark at 24 °C with relative humidity held constant at 55%, and were fed ad libitum on cuttings of the local larval food plant, *Aristolochia watsonii*. On the day of eclosion, males were weighed, their forewing length measured, and given an individual number. Sexes were kept separately in small flight cages (~1 m<sup>3</sup>) at room temperature (~24 °C) and individually fed 20% sucrose solution for about 20 min each day.

### 14.2.2 Examination of Reproductive Tracts of Virgin and Mated Males

To examine the effect of mating on the appearance of the male's reproductive tract, we hand-paired males with 0–3-day-old virgin females using the method of Watanabe and Hirota (1999). Then, each male was dissected and his reproductive tract examined to assess changes in the appearance of simplex with age and with mating experience. We divided males into three experimental groups: (1) males that never mated and dissected on the day of eclosion or 3 or 6 days after eclosion; (2) males that mated 1, 3, or 5 days after eclosion and dissected immediately after the mating; and (3) males mated 1 day after eclosion and dissected right after the mating or 1, 2, 3, or 5 days after mating.

Before dissection, each male was immobilized by gently pinching their thorax. Each male's abdomen was then removed from the body and placed in a petri dish filled with fresh insect Ringer's solution. The reproductive organs including the simplex and duplex were carefully removed from his abdomen. To describe the simplex of each male, we measured its length, appearance, and mass. We first imaged each simplex after removing any fat bodies attached to it and then recorded its appearance with a digital camera attached to a microscope. After capturing images, each simplex was separated from the attached duplex and aedeagus. Wet mass of each simplex was then determined to the nearest 0.01 mg.

### ***14.2.3 Estimation of Recent Mating Success of Field-Caught Male***

Sixty-eight wild males were collected from 16 July to 1 August 2011 in the morning near Sunflower, Arizona. Each captured male was scored as to his wing wear as an indicator of his age. Age-class was scored on the scale (I (least worn) to V (most worn)) described by Watanabe et al. (1986). The forewing length of each male was measured from the wing base to the wing tip. All males were dissected on the day of capture. To assess recent mating success of males, the mass, length, and transparency differences of each male's simplex were measured.

### ***14.2.4 Spectral Analyses of Iridescent Wing Areas***

In preparation for spectral measurements, the left hindwing of each butterfly was removed from the thorax and mounted dorsal side up on black card stock with spray adhesive. Reflectance spectra were collected from these wings using techniques described in Rutowski et al. (2010). Reflectance relative to a magnesium oxide white standard was measured between 300 and 700 nm from the wings. Because the reflectance spectra of these iridescent wing surfaces are unimodal, we extracted three color parameters, intensity, hue, and chroma, to describe and analyze the properties of the wing reflectance (Montgomerie 2008).

## **14.3 Results**

### ***14.3.1 Virgin Male Reproductive Tract***

For virgin males, the simplex mass adjusted for forewing length ( $(\text{simplex mass})^{1/3}/\text{forewing length}$ ), simplex length adjusted for forewing length ( $\text{simplex length}/\text{forewing length}$ ), and the transparency difference of simplex was approximately 0.5, 1.3, and 1.3, respectively, and these did not change with male age [mass (ANOVA,  $F_{2,21}=1.554$ ,  $p = 0.237$ ); length (ANOVA,  $F_{2,21}=2.276$ ,  $p = 0.130$ ); transparency difference (ANOVA,  $F_{2,21}=0.475$ ,  $p = 0.629$ )]. Consequently, simplex of virgin males did not change in appearance with time since eclosion.

### ***14.3.2 Reproductive Tract of Males Immediately After Mating***

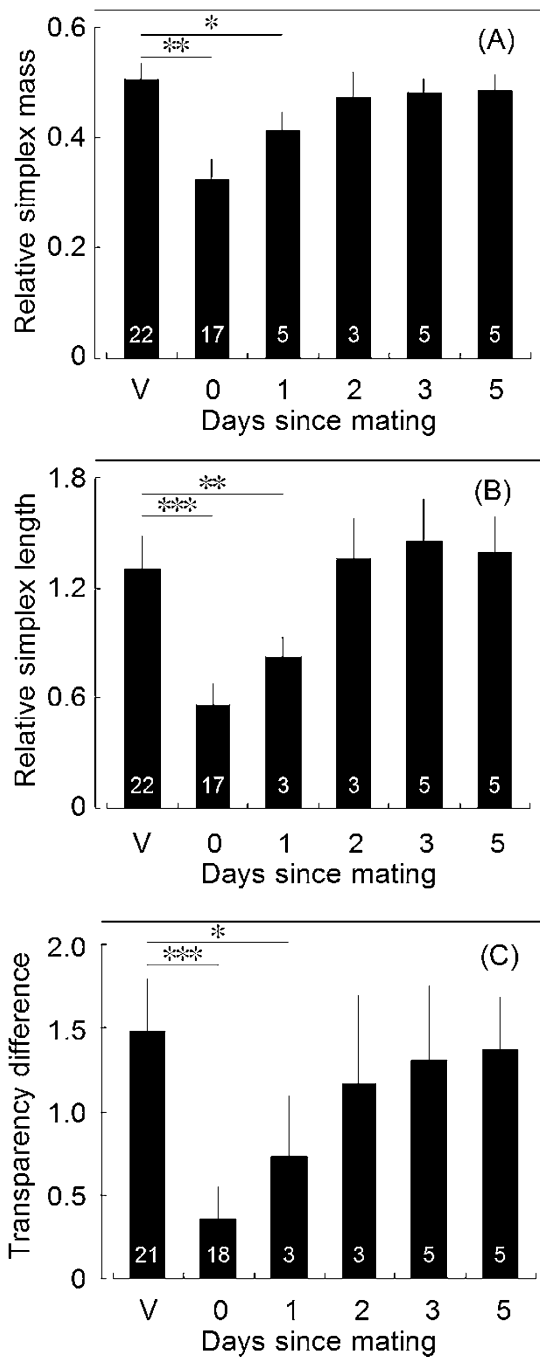
For males, immediately after the termination of copulation, the simplex mass adjusted for forewing length, the simplex length adjusted for forewing length, and the transparency difference were approximately 0.32, 0.5, and 0.3, respectively, and these did not vary with the age of the male at mating [mass (ANOVA,  $F_{2,16}=0.027$ ,  $p = 0.974$ ); length (ANOVA,  $F_{2,16} = 1.331$ ,  $p = 0.296$ ); transparency difference (ANOVA,  $F_{2,16}=0.170$ ,  $p = 0.845$ )]. Although the simplex of males just after the termination of copulation was different in appearance from that of virgin males, these were not affected by age at mating.

### ***14.3.3 Changes in the Male's Reproductive Tract with Time Since Mating***

Although the simplex of males just after the termination of copulation was short, it lengthened and refilled again as time passed since mating. During this period, the color of the simplex turned from yellow to colorless, and the amount granular substances increased in the basal end of the tube. Statistically, the mass, length, and transparency difference of simplex all changed with time between mating and dissection (Fig. 14.2; mass: ANOVA:  $F_{5,61} = 59.202$ ,  $p < 0.001$ ; length: ANOVA:  $F_{5,55} = 42.770$ ,  $p < 0.001$ ; transparency difference: ANOVA:  $F_{5,55} = 17.139$ ,  $p < 0.001$ ). After copulation, simplex mass (A) and length (B) dropped to half their precopulatory values, but returned to precopulatory values in about 2 days. The transparency difference decreased with mating but also returned to pre-mating values within about 2 days (C).

Using the results of these analyses, we developed criteria for assessing whether a male's reproductive tract showed evidence of recent mating. The distribution of simplex mass adjusted for forewing length of males within 1 day after mating was 0.251–0.453, whereas that of virgin males was 0.465–0.565, with no overlap in these ranges. The observed ranges of simplex length adjusted for forewing length and the transparency difference of males within 1 day after mating and virgin males also did not overlap (length, 0.401–0.940 vs 1.084–1.628; transparency difference, 0.013–0.964 vs 1.105–2.148). So, we set the lower end of the ranges of values for simplex characteristics of virgin males as the value below which would indicate that the male had recently mated. That is, a field-caught male that had a simplex of less than 0.46 in mass, less than 1.0 in length, or less than 1.0 in transparency difference was taken as indicating that the male had recently mated.

**Fig. 14.2** Simplex mass (a), length (b), and transparency difference (c) of simplex for virgin males (V, 0, 3, 6 days old) and for mated males (1, 3, 5 days old) dissected at various number of days after mating (mean±S.D.) (After Sasaki et al. 2015). \*, \*\* and \*\*\* represent  $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.001$  in Tukey's HSD test, respectively



### 14.3.4 Mating Success of Field-Caught Males

All field-caught males were evaluated and placed in groups based on which of the three criteria for recent mating they met and which they did not (Table 14.1). For the eight possible groups and to maximize contrasts any group that met two or more of the criteria (Groups E to H) we labeled as showing strong evidence of recent mating. However, because there were no individuals in Group G, we regarded males in Group E, F, and H as recently mated males. Of the 68 males in the list, 12 showed this strong evidence of having mated recently. We also confidently labeled as not recently mated, males that met none of the criteria (Group A).

Using GLM with binomial errors and a logit link function, we compared the phenotypic characteristics of those that had recently mated (Groups E, F, and H) with those that had not (Group A). The characters included in the analysis were the intensity and hue of the iridescent area of male dorsal hindwing and age-class. Chroma was not included as an independent variable in the analysis because there were significant correlations between chroma and all other characteristics (Table 14.2). As shown in Table 14.3, while intensity was not related to their recent mating success, hue and age-class significantly affect their recent mating success. Recently mated males were older and had a higher hue value (were greener) than males that had not recently mated (Figs. 14.3 and 14.4).

**Table 14.1** A summary of the state of reproductive tract components of 68 males caught in the field

| Criterion               | Group |    |   |   |   |   |   |   |
|-------------------------|-------|----|---|---|---|---|---|---|
|                         | A     | B  | C | D | E | F | G | H |
| Simplex mass            | –     | –  | – | + | – | + | + | + |
| Simplex length          | –     | –  | + | – | + | – | + | + |
| Transparency difference | –     | +  | – | – | + | + | – | + |
| <i>N</i>                | 23    | 30 | 2 | 1 | 2 | 5 | 0 | 5 |

After Sasaki et al. (2015)

A plus sign means that the state of that component met the criteria we set for indicating the male recently mated. A minus sign means it did not meet the criteria. In later analysis, any group that met two or more of the criteria (Groups E to H) and males that met none of the criteria (Group A) were used as recently mated males and as not recently mated males, respectively

**Table 14.2** Spearman correlation coefficients for the relationship between male age and the various color parameters for the dorsal hindwing coloration

| Measure      | 1      | 2       | 3     | 4 |
|--------------|--------|---------|-------|---|
| 1. Intensity | –      |         |       |   |
| 2. Chroma    | 0.584* | –       |       |   |
| 3. Hue       | –0.087 | –0.383* | –     |   |
| 4. Age-class | –0.102 | –0.581* | 0.211 | – |

After Sasaki et al. (2015)

Significant correlation between intensity and chroma, chroma and hue, and chroma and age-class were found

\**p* < 0.01



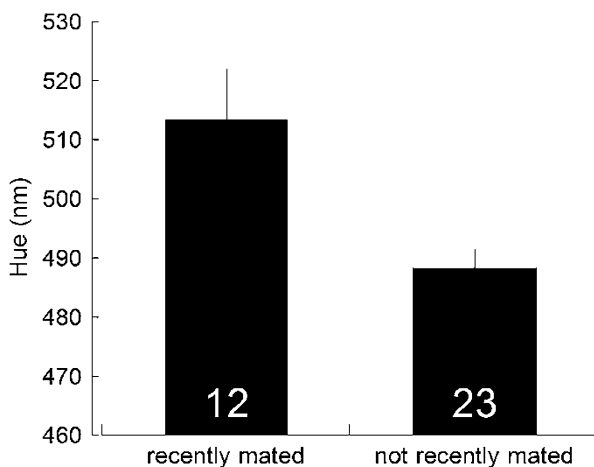
**Table 14.3** Results of an ANOVA (GLM) of factors that contribute to variation in recent mating success of wild males

| Effect    | df | Deviance |
|-----------|----|----------|
|           | 34 | 45.004   |
| Intensity | 1  | 0.669    |
| Hue       | 1  | 4.744**  |
| Age       | 1  | 1.760*   |

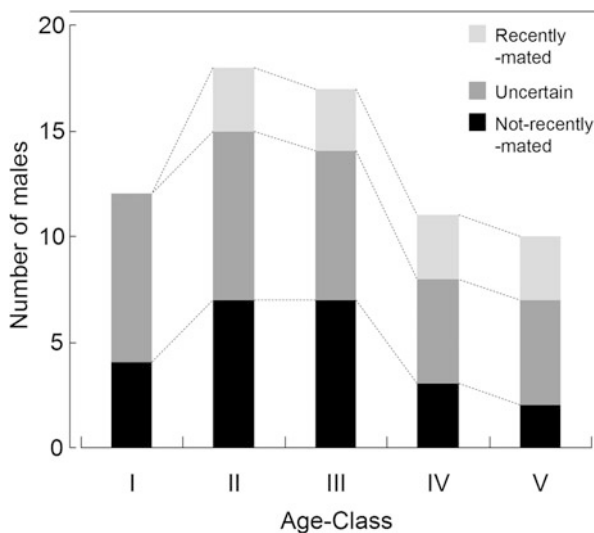
After Sasaki et al. (2015)

Effect of hue and age-class on male recent mating success were significant. \* and \*\* represent  $p < 0.05$  and  $p < 0.01$ , respectively

**Fig. 14.3** The hue (wavelength of maximum reflectance) of the dorsal hindwing for field-caught males that met the criteria for evidence of having recently mated and those males that did not meet the criteria ( $\pm$ S.E.) (After Sasaki et al. 2015)



**Fig. 14.4** Change with age-class in the number of clearly recently mated males (*light-gray bar*), clearly not recently mated males (*black bar*), and males of uncertain recent mating history (*dark-gray bar*) for field-caught males (After Sasaki et al. 2015)



## 14.4 Discussion

### 14.4.1 *Assessing the Mating History of Male Butterflies in the Field*

The most convincing demonstrations of the evolutionary significance of mating preferences are those in which the results of manipulative experiments are matched by observations in the wild or in wild-caught populations (Kemp 2007). In butterflies, many laboratory experiments have demonstrated the occurrence of female preference for particular male traits, including wing coloration (Krebs and West 1988; Robertson and Monteiro 2005; Andersson et al. 2007). However, the conclusions of these studies have rarely been validated against data obtained in more natural field-based settings (Kemp and Rutowski 2011). In other insects, the comparison of traits from copulating males and unattached males in the field has been extensively used to make inferences about population mating biology (Flecker et al. 1988; Harari et al. 1999; Alcock and Kemp 2006). However, this strategy has not been used in butterflies, except in cases of extremely high density, owing to the difficulty of observing butterfly mating in the field (Kemp and Rutowski 2011). Therefore, to determine the effect of female preference on the evolution of traits in male butterflies, it was necessary to establish an alternative method for evaluating male mating success.

Tsubaki and Matsumoto (1998) estimated the mating frequency of male *Luehdorfia japonica* by assessing the degree of scale loss from males' claspers. In this species, males consume scales and use them to form a mating plug on the female abdomen during copulation. Kazuma (1987) reported that the degree of scale loss in laboratory-reared males increased with repeated hand pairing. The degree of scale loss was scored on a scale of 0 (slight scale loss) to 3 (almost all scales were lost), and each stage corresponded to 0, 1, 2, and 3 or more matings. Since mating frequency must have a strong relationship to lifetime reproductive success, this method may provide an accurate estimation of reproductive success in wild males. However, because the mating-related loss of scales is not the rule among lepidopterans, this method is not applicable to all species.

In the present study, we reported a new technique for assessing male mating success that relies on changes that occur in the appearance of internal reproductive organs during mating. Since the internal reproductive organs of butterflies are not much different between species and it is common for males to ejaculate spermatophores and accessory substances during mating (e.g., Drummond 1984), this method can likely be applied to any butterfly species. In fact, it is known that the status of the male simplex just after mating is also different from that before mating in *Papilio xuthus* (Sasaki, personal observation), *P. machaon* (Sasaki, personal observation), *Byasa alcinous* (Sasaki, personal observation), and *Eurema hecabe* (Konagaya, personal communication). In addition, this new strategy can be used at

both middle- or low-density mating sites, since it can detect the occurrence of male mating within a few days, and even though the technique only distinguishes between males that have recently mated and those that have not, it still holds promise for examining variation in male mating success and, thereby, investigating traits that correlate with mating success and the intensity of sexual selection.

We note that, when using this method, there might be some traits that are not suitable for investigating the relationship with mating history. For example, it is impossible, in principle, to investigate the relationship between recent mating success and spermatophore production capacity, which is closely related to the reproductive success of both male and female butterflies.

#### **14.4.2 Phenotypic Correlates of Mating Success in Male *B. philenor* in the Field**

Our results indicate that males that exhibit signs of having recently mated differ from those that do not, in that they are older and have a greener coloration. This could mean that age and coloration are important determinants of male mating success and under selection, either in the context of (1) female choice, in which females prefer older and greener males, or (2) male competition, in which older and greener males are, for some reason, more effective competitors. In general, the results of the present study support the prediction that male coloration and mating success are related. However, Rutowski and Rajyaguru (2013) reported that the dorsal hindwings of successfully mated *B. philenor* males possessed more chromatic iridescence than those that failed to mate, rather than a different hue, as reported here.

Such differences between field and laboratory study have also been reported by previous studies. For example, in-copula males of *Eurema hecabe* were reportedly older than their free-flying counterparts and possessed significantly less bright markings, while brighter males were preferred by females in the laboratory (Kemp 2008). In this case, the difference was caused by the existence of newly emerged females that could not reject mating, and since the density of individuals at the study site was high and activity is centered at localized breeding sites, males could profitably locate such females.

The reasons for difference the color parameters correlated with the mating success of male *B. philenor* have not yet been clarified. It is possible that the data reported here about the specifics of male age and color associated with recent mating success were affected by the several significant correlations among color parameters and between coloration and age. We made efforts to control for these correlations by excluding variables, such as chroma, from our analysis, but in the end, it is difficult to make conclusions with confidence about the reasons for the

connections between male color, age, and male mating success suggested by our data set. In addition, we have not controlled or taken into consideration several other variables that might affect male mating history, such as body size, population density, time during the breeding season, and weather. To convincingly identify factors that determine male mating success in *B. philenor*, the experimental manipulation of candidate variables is needed.

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