Gender- and Size-based Variation in Wing Color in Large Milkweed Bugs (*Oncopeltus fasciatus*) in Georgia

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Abstract - Milkweed bugs are aposematically colored, with orange and black on their forewings, and the degree of both colors varies among individuals. Despite the attention given to the warning nature of this color, there has been little research directed at this variation. In this study, the subtle variation in wing colors of one species of milkweed bug, *Oncopeltus fasciatus* (Large Milkweed Bug) was measured to determine if the color variation was related to sex or body size. Fifty-eight bugs were hand-collected at three sites in northeast Georgia, and their wings scanned using a slide scanner and measured digitally using image analysis software. Wings of females were larger than those of males in general, and the color analyses showed statistically significant differences in wing hue between males and females. Females also had darker black wing sections than males, which could be evidence of a sex-related difference in immune function. Regardless of sex, wings of larger bugs had deeper orange color and darker black, which may increase the aposematic contrast. Finally, several differences in wing color were found between sites, suggesting either site-level variation in host-plant quality or relatedness among individuals within sites. This study is the first to quantify in detail the wing colors of milkweed bugs and forms the basis for future research into this little-studied aspect of this insect.

Introduction

The brightly contrasting orange and black aposematic coloration of milkweed bugs has long fascinated researchers (e.g., Berenbaum and Miliczky 1984, Bowdish and Bultman 1993, Prudic et al. 2007). Their toxicity stems from feeding on plants in the *Asclepias* (milkweed) genus, and because they can be easily reared in captivity (Feir 1974), these bugs, especially *Oncopeltus fasciatus* Dallas (Large Milkweed Bug), are ideal subjects for studying questions related to warning coloration. Despite the attention given to warning coloration in this insect, there has been little work focusing on the color itself, which varies from yellow to orange to red in the Large Milkweed Bug (A.K. Davis, pers. observ.). Therefore, basic questions relating to this variation have yet to be addressed, such as whether there are gender- or size-based differences in wing color. Only one study has examined colors in milkweed bug wings, though this was not the main focus of the project (Rodriguez-Clark 2004). Rodriguez-Clark (2004) was the first to assess the color variation of milkweed bugs in a scientific fashion, by visually scoring the dorsal color of bugs on a 5-unit scale as light-yellow, yellow-orange,

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orange, red-orange, or red. While her study focused on the heritability of this trait across generations, it is interesting to note that she found no significant differences in wing color between adult males and females.

The measurement of color in insects and other small animals in research has become easier and more objective in recent years with the availability of digital cameras, scanners, and image analysis software (Davis, in press; Davis and Grayson 2007; Davis et al. 2004, 2005, 2007). This approach to quantifying color, whereby images of subjects are taken under standardized lighting and software is used to assess the subjects' color in the images, allows for extremely subtle differences in color shades between individuals to be measured, which often are not discernable to the naked eye. Further, such minor differences have been shown to be important biologically in other insect species (Davis 2009, Davis et al. 2007). With image analysis software, colors are measured in three quantities: the hue (i.e., the difference between red, blue, green, etc.), saturation (i.e., the degree or intensity of a given color, such as the difference between pink and red), and the brightness of the color. In digital images, all pixels in the image contain this information, and with image analysis, the average pixel values for selected surface areas in the image (i.e., such as a butterfly wing) are calculated. Conveniently, this breakdown of colors into three parts allows each component to be separately compared among individuals. For example, Davis et al. (2007) recently found that the saturation alone of the orange color of *Danaus plexippus* L. (Monarch Butterfly) wings was an important predictor of male mating success, while the hue and brightness components were not important. In addition, hue scores of migrant Monarchs appear to be different from that of breeding and overwintering individuals (Davis 2009).

The degree of melanism (i.e., blackness) in insects is also an area where image analysis can be utilized to objectively compare individuals, especially in insects (Davis et al. 2005). Besides having three color components, all pixels in digital images also have a "density" value, which is the brightness value when the color information is removed and the image converted to a greyscale form. Thus, the degree of "blackness" of a selected surface (on a wing section, for example) can be scored as the mean density value of all pixels in the selection. Similar systems have been used previously to measure melanization levels in butterfly wings (Davis et al. 2005, Ellers and Boggs 2003) and beetle elytra (Thompson et al. 2002).

The current study is an examination of the natural variation in wing color of Large Milkweed Bugs using an image analysis approach. Wild adults were collected from three different locations in northeast Georgia, and both their orange and black colors were assessed by scanning their wings with a flatbed scanner and using image analysis software. The possible variation in orange color and melanism were then compared among sexes, as well as in relation to body size.

Methods

Collecting specimens

Adult Large Milkweed Bugs were collected by hand from *Asclepias incarnata* L. (Swamp Milkweed) plants at three sites each separated by 5 km around the city of Athens, Clarke County, GA during one week in August, 2006. At each site, there were between 5–15 plants and at least 15 individuals per site were collected. All bugs were placed in plastic containers for transport back to the lab, where they were killed by freezing. The sex of each bug was identified following Rodriguez-Clark (2004), using the caudal median point on the posterior margin of the 4th abdominal sternite, and the triangular cleft pygidium.

Scanning wings

Before scanning, all bugs were held at room temperature for 20 minutes to thaw. For each specimen, the left and right forewings were removed and placed with the dorsal side down on an HP Scanjet 4670 see-thru vertical scanner with a 35-mm slide scanner adapter. The slide scanner adapter was ideal for scanning the wings because it 1) is designed to scan small items, and 2) illuminated the background, which standardized the amount of lighting for every scan. The wings were thusly scanned at 1200 dpi, and the image saved (Fig. 1). After all wings were scanned, a standard metric ruler was scanned with the same settings to calibrate the image analysis software.

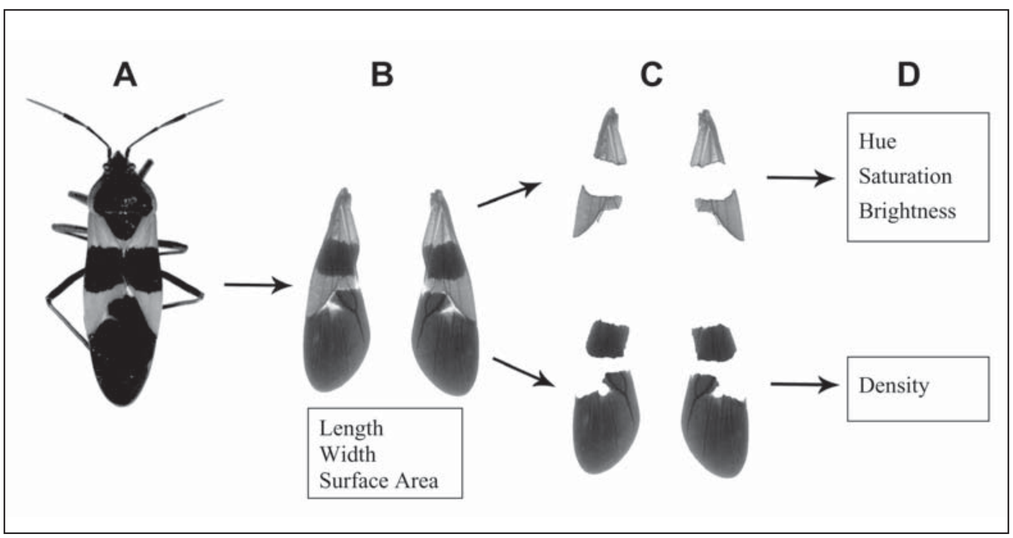

Figure 1. Diagram showing Large Milkweed Bug forewing characters measured. Forewings from *Oncopeltus faciatus* (Large Milkweed Bug) (A) were removed from the body and scanned face down (B), and their lengths, widths, and surface areas measured with image analysis software (see methods). Next the orange and black sections of the wings were digitally isolated (C), and color measure routines were used to obtain average hue, saturation, and brightness values of orange sections and average density values for black sections (D).

Measuring wing characteristics

The Fovea Pro image analysis software (Reindeer Graphics, Inc.) was used to measure all forewings in this study, and image analysis methods generally followed Davis et al. (2005) and Davis et al. (2007). Basic wing-size traits were first measured for the left and right wings, including length (mm), width (mm), and surface area (mm^2). Then the left-right average of each variable was calculated for each individual to use in analyses. For the analyses of color traits (below), the wing area variable was used as the index of body size.

Color features of the wings were measured as follows: all non-black areas of the wings were selected first (Fig. 1C), and the Fovea Pro color measure routine was initiated, which returned the average hue, saturation, and brightness score for all pixels in the selection (typically over 10,000 pixels; Figure 1D). The same process was performed on the black selections, but in this case the mean "density" values of all pixels were used. Density is a computer-based, numerical value reflecting the degree of "blackness" of a selection, and is useful as an index of "melanism" in insects (Davis et al. 2005). In both cases, the orange and black values on the left wings were measured first, then the right wings. Then the average value of each was calculated to use in analyses, as was done with the size measurements. The nature of the computer color-scoring was such that hue values were scored on a scale from 0–360, while all other scores (saturation, brightness, and density [melanism]) were on a scale from 0–255. Note that in the melanism score, lower values represent darker, more intense black colors (Davis et al. 2005).

Data analysis

Basic comparisons of wing size (wing length, width, area) between males and females were made using Student's *t*-tests. Analysis of covariance was used to determine which variables related to wing color. Specifically, the hue, saturation, brightness, and melanism were examined separately, and in all cases, the independent variables included sex, site, and wing area as a covariate. All two-way interactions were initially included in each model, but were removed if found non-significant. All analyses were conducted using Statistica 6.1 software (Statistica 2003).

Results

General results

A total of 58 adult Large Milkweed Bugs were collected and measured, of which 26 (44.8%) were female and 32 (55.2%) were male (Table 1). Basic comparisons of wing features between sexes revealed that female wings were significantly larger than male wings in length, width, and total area (Student's *t*-test, $P < 0.001$ for all; Table 1). Moreover, this trend was consistent across all three sites.

Orange color results

In the analysis of orange hue scores, there was no support initially for any of the two-way interaction terms in the ANCOVA model ($P > 0.05$). Results from a simplified model with main effects only revealed no significant effect of site ($F_{2,53} = 1.56$, $P = 0.220$), but a significant effect of sex ($F_{1,53} = 8.49$, $P = 0.005$) and wing area ($F_{1,53} = 4.56$, $P = 0.037$). The effect of sex was such that females tended to have higher hue scores (Fig. 2A), or in other words, were more yellow than males. The relationship with wing size indicates that individuals with larger wings, regardless of sex, tended to have lower hue scores, or were more orange, although this

Table 1. Summary of wing size measurements by sex and site. All sites were within 5 km of Athens, GA and were plots of *Asclepias incarnata* (Swamp Milkweed). All individuals were captured during one week in August, 2006. For all measurements, the average of the left and right wings was used for each individual. Average wing values for each sex and site are shown, with standard errors in parentheses. Asterisk denotes results of statistical comparisons (*t*-tests) of traits of all females with all males.

Site	Sex	Wing length (mm)		Wing width (mm)		Wing area (mm^2)	
1	M	8.92	(0.23)	3.07	(0.06)	19.10	(0.82)
1	F	10.68	(0.66)	3.59	(0.24)	27.19	(3.30)
2	M	10.26	(0.18)	3.46	(0.06)	24.25	(0.71)
2	F	12.59	(0.20)	4.15	(0.07)	35.12	(1.08)
3	M	9.50	(0.39)	3.19	(0.11)	21.20	(1.50)
3	F	10.93	(0.25)	3.57	(0.07)	26.96	(1.06)
All sites	M	9.60	(0.17)	3.26	(0.05)	21.68	(0.65)
All sites	F	11.44*	(0.26)	3.78*	(0.09)	29.85*	(1.26)

*$P < 0.001$.

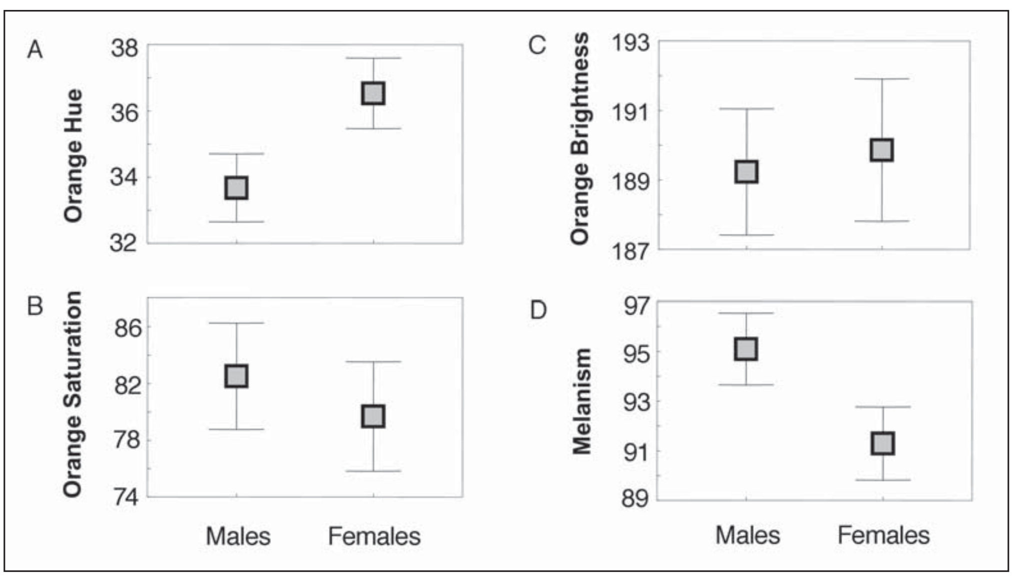

Figure 2. Comparison of male and female color scores (hue, saturation, brightness, and melanism [pixel density]). Shown are the mean values of all 32 males and 26 females, with standard error bars. Lower melanism scores represent darker shades of black.

relationship was not strong in direct comparisons of the two variables (r = -0.19, P = 0.163; Fig. 3A).

None of the two-way interactions were significant ($P > 0.05$) in the analysis of orange saturation. In the model with main effects only, all three effects were significant, although the differences in all cases were slight. Males tended to have higher saturation scores than females ($F_{1,53}$ = 7.94, P = 0.007; Fig. 2B), meaning they were a deeper orange color, and there was a trend of higher scores with increasing size of Large Milkweed Bugs ($F_{1,53}$ = 7.66, P = 0.008; Fig. 3B), so that bugs with larger wings tended to be deeper orange as well. There was also differences in saturation between sites ($F_{2,53}$ = 5.17, P = 0.009).

In the analysis of wing brightness, there were multiple significant interactions and main effects, although their interpretation is difficult. The interaction of sex*site ($F_{2,48}$ = 4.10, P = 0.023) as well as visual inspection of the categorized graph suggests a lack of consistency in the difference between males and females for this trait. There was a similar lack of consistency in the trends between brightness and wing area (i.e., in the wing area*site interaction, $F_{2,48}$ = 4.26, P = 0.020). The significant interaction between sex and wing area ($F_{1,48}$ = 4.23, P = 0.045) was such that while in both sexes individuals with larger wings tended to have lower brightness scores, the slope of the trend line was steeper in males than females (males: -1.02, females: -0.27). Finally, there was a significant main effect of site ($F_{2,48}$ = 4.50, P = 0.016) on brightness scores.

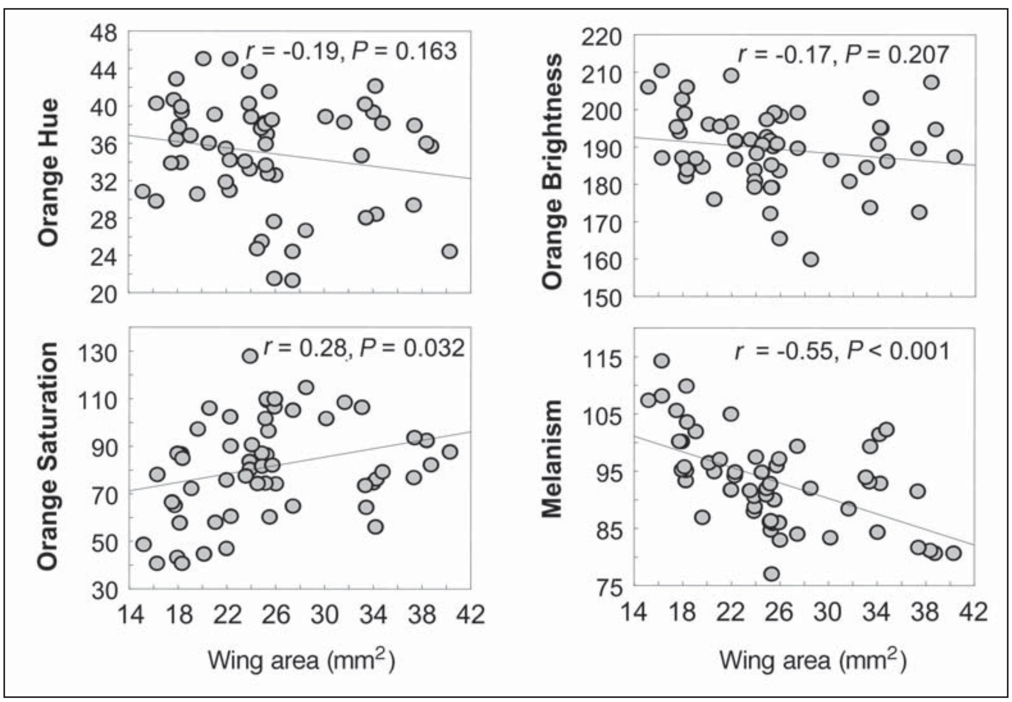

Figure 3. Relationships between Large Milkweed Bug wing size (average left and right wing area) and all wing color variables in this study (hue, saturation, brightness, and melanism [pixel density]). Lower melanism scores represent darker shades of black.

Melanism results

Interactions of site*sex and site*wing area were nonsignificant in the initial model, but the interaction of sex*wing area was significant ($F_{1,52}$ = 6.94, P = 0.011). The main effect of sex approached significance ($F_{1,52}$ = 3.80, P = 0.056; Fig. 2D), and there was a strong negative relationship with wing area ($F_{1,52}$ = 30.96, P < 0.001; Fig. 3D) such that individuals with larger wings tended to have darker black wing sections. The interaction of sex*wing area again showed that the slope of the negative relationship with wing area was steeper for males than for females (males: -1.5, females: -0.5). Individuals with larger wings had darker black wing sections, but the magnitude of this relationship depended on the sex. Finally, there was a significant effect of site on melanization scores ($F_{2,52}$ = 3.59, P = 0.035).

Discussion

As this study was the first direct quantification of variation in wing color of Large Milkweed Bugs, the questions addressed were inherently basic, such as are wings of males and females differently colored, and is there a relationship with body size? As for the first question, the data gathered indicate that males and females of this species do differ in wing color—the wings of males tend to be more orange, whereas wings of females are more yellow. At the same time, females have darker black wing sections than males. These results then represent the first confirmation of sexual dichromatism in this species, which is in contrast to Rodriguez-Clark (2004), who found no sex-based variation in wing color of this species (using manual color-scoring). However, the functional significance of this sexual dimorphism is not clear at this time. One possibility is that the greater melanism levels in females is associated with differences between the sexes in immune function, which is linked with cuticular melanism levels in insects (Wilson et al. 2001). While it is not known if the sexes differ in immunity in this species, this question may be a direction for future research; male and female immune parameters such as hemocyte numbers (Feir 1964) could be compared, especially since new methods of counting hemocytes in milkweed bugs have recently been developed. These new methods also use image analysis (Davis 2007). This idea becomes especially important when one considers that the sex-related differences in wing melanism found here parallel those found in Monarch Butterflies (where females are also darker than males), and in that species, females have recently been shown to have higher concentrations of hemocytes (Lindsey and Altizer 2009).

There were relationships between wing size (used here as a proxy of body size) and certain color traits of milkweed bugs, but in some cases, the strength of the relationships depended on the sex. In general, individuals with larger wings tended to have deeper orange and darker black wing sections. These relationships could be interpreted in light of the aposematic function of the wing colors. Darker black and deeper orange colors may be associated with large size, with the result that the aposematic contrast of these colors is more apparent in the larger individuals (Prudic et al. 2007). On the other hand, the ability to synthesize pigment may be tied with larval growth, in that the larvae with optimal food resources may grow larger and produce more intense pigmentation than those with poor resources. Evidence in support of this idea comes from the similarities in wing color traits observed within sites (discussed further below). Research in certain other insect species (*Bembecinus quinquespinosus* Say [Digger Wasps]) also uncovered links between body size and color, though the functional significance of the relationships in that species remain unclear as well (O'Neill and Evans 1983, O'Neill et al. 1989).

While not one of the main objectives of this study, there were several statistical differences in wing colors found between the three sites where Large Milkweed Bugs were collected. This result was surprising and may need to be verified with additional data. However, it indicates that milkweed bugs can have similar coloration within sites, which would support the idea that there is some site-level variation in larval food resources. While all Large Milkweed Bugs were collected from the same host plant species, *Ascepias incarnata*, local variation could exist in the quality of these plants, which is then reflected in the degree of pigmentation on the Large Milkweed Bugs at the sites. There is also the possibility that the Large Milkweed Bugs at each site were related to one another. Indeed, it has been shown that wing colors are heritable (Abbott 1968, Rodriguez-Clark 2004). In any case, this result speaks to the need to ensure high genetic diversity or at least to account for the collection site as a variable in future analyses.

This study represents an important first step into an area of research ripe with questions that until now have not been easily addressed because of technological limitations. With the color quantification methods now available to researchers (similar to those outlined in this study), many questions could now be readily addressed with Large Milkweed Bugs. These questions could relate to those already addressed in other species, such as linkages between external melanism and immune function (Wilson et al. 2001), melanism and population variation (Davis et al. 2005), or color and mating success (Davis et al. 2007). Another idea would be to examine wing color variations in relation to migratory propensity, an issue well-studied within milkweed bugs (Dingle 1981, Dingle et al. 1980). This idea has been examined already in Monarch Butterflies, and indeed there was evidence found that wings of migrants are differently colored than those of breeding individuals (Davis 2009).

On a related note, the approach used to quantify Large Milkweed Bug wing colors in this study should be adaptable to the study of other insect species as well. Most wings can be scanned flat with a standard flatbed scanner, or with a slide scanner, and the scanner emits a standardized level of light on the subject (but this must be specified beforehand by the user), so that the variations among individuals in shades of black or hues of orange (or yellow, blue, etc.) can be measured on-screen with the image analysis software

(Davis, in press; Davis et al. 2005, 2007; Lindsey and Altizer 2009). For insects with curved surfaces (beetle elytra, for example), digital photographs can also work, providing that individuals are photographed under standardized lighting (Davis et al. 2004, Todd and Davis 2007). Whatever the species under study, the results of the current study will hopefully provide a framework on which to build.

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