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ULTRAVIOLET COLORATION IN TIGER SWALLOWTAIL BUTTERFLIES (*PAPILIO GLAUCUS* GROUP, PAPILIONIDAE) WITH A METHOD FOR OBJECTIVELY QUANTIFYING ADULT BUTTERFLY WING WEAR

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ABSTRACT. Tiger swallowtail butterflies in the genus *Papilio* have an ability to visually distinguish between a large array of colors including those in the ultraviolet spectrum (UV). However, very little is known about UV reflectance patterns in these butterflies. Using a combination of UV photography and spectral analysis, we here show that several areas on the wings of tiger swallowtails reflect UV light including areas of blue scaling and the anterobasal region of the hind wings. We also discuss how a low level of UV reflectance from the wing membrane itself may be combined with UV photography to quantify wing wear. This technique could be used to objectively place wild-caught individuals into wear classes, approximating their age. Finally, we examine male mating preferences for pristine or artificially aged females and present preliminary results that suggest a strong preference for those that are pristine.

Additional key words: ultraviolet coloration, wear class, *Papilio*, Papilionidae, mate choice

Tiger swallowtail butterflies in the genus *Papilio* have color vision that is among the broadest in the animal kingdom, with an ability to discern color across a spectral range that extends both into the ultraviolet (300 – 400 nm) and the infrared (700–800 nm; Briscoe 2000). Despite this broad visual acuity, surprisingly little is known about the extent of UV reflectance in these butterflies. UV wing coloration is extremely important in a wide variety of other butterfly species, predominately in relation to conspecific recognition and mate selection (Silberglied 1979). Recently, we reported that blue scales on the wings of these butterflies are UV reflective (Aardema & Scriber 2013). Here we expand upon this observation and additionally report a formal way to objectively quantify wing wear of adult *Papilio* butterflies, which may be used to estimate adult age. We also report a preliminary investigation on the implications UV reflectance may have for mating preferences in these butterflies.

MATERIALS AND METHODS

We first assessed ultraviolet coloration in tiger swallowtails by photographing the wings of dead specimens using an XNite330nm UV pass filter attached to a Nikon Coolpix 990 digital camera (3.34 megapixels). This filter has a peak transmission at 330nm of 85% and has 50% transmission at 270nm and 375nm. It also has a small transmission peak of 10% in the infrared at 725nm. With this combination of filter and camera, we photographed the dorsal and ventral sides of a single fore and hind wing from 15 *Papilio glaucus* and *P. canadensis* specimens (3 yellow-morph males of both species, 3 yellow-morph females of both species, and 3 dark-morph female *P. glaucus*). This was done in a darkened room with a circular black light for illumination following techniques previously described (Acorn 2002). We additionally examined the spectral reflectance signature of blue, yellow and black wing areas using an Ocean Optics s2000 miniature fiber optic spectrophotometer. We measured three roughly 1 cm^2 squares of wing, each that had only one solid color (yellow, black or blue) from four pristine Levy County, Florida yellow-morph females (Fig. 1A). Reflectance of color was measured in comparison to a white standard (WS-1 Diffuse Reflectance Standard, Ocean Optics).

During the course of our investigations, we also noticed that scale loss on the wing revealed a low level of UV reflectance from the underlying wing membrane. Such reflectance of the cuticle is not uncommon in insects and led us to consider the possibility of using measures of UV reflectance to quantify scale loss. Wing wear has been used in a large number of studies to approximate the age of individual adult butterflies (e.g. Boggs 1987, Lederhouse & Scriber 1987, Kemp 2000). Therefore, methods to objectively quantify wing wear may be of some value to Lepidopteran researchers.

Starting with a pristine forewing, we artificially removed scales using transparent adhesive tape. This was done by firmly pressing the tape to the wing and then slowly removing it, taking scales off in the process. To remove additional scales, we simply reapplied a new piece of tape to the same area. In this way we sequentially reproduced four wear classes (1–4) following Lederhouse and Scriber (1987). Wear class 1 corresponds to a "fresh" individual whereas wear class 4 is considered "very worn" (no scale loss versus substantial scale loss). Visual examples of the four wear classes can be seen in Figure 2 (column 1). This method appears to reproduce patterns of scale loss that closely resemble those observed in wild-caught specimens (data not shown).

For each of the four classes we photographed UV reflectance using the same methods described above. We quantified the amount of UV reflectance using two distinct measures with the freely available program ImageJ (Abramoff et al. 2004). For each UV picture we first used ImageJ to convert it to an 8-bit, black and white image. We then enhanced the contrast of the image using a pixel saturation point of 0.4%. Our first quantification of wing wear based on UV reflectance used the average gray scale measure of the pixels found along a straight line stretching from the wing base to the tip. The gray scale ranges from 0 (black) to 255 (white). Our second measure of wing wear was based on the total area of UV reflectance. To calculate this, we first converted each picture to a binary image. This resulted in the UV reflecting areas becoming black regions whereas the remaining areas were white (Fig 2, column 4). Using ImageJ we measured the total area of the UV reflecting regions. The presence of a ruler in each photograph allowed us to calibrate the scale for each picture.

Finally, we postulated that these butterflies themselves may potentially utilize the UV reflectance that comes with increasing wing wear to assess reproductive potential in members of the opposite sex. Specifically, we predicted that younger looking individuals with less scale loss would be preferred over older looking individuals, independent of mating status. In tiger swallowtail butterflies older males are more likely to have previously mated and thus have a smaller spermatophore to pass on to a female (Lederhouse et al. 1990). Correspondingly, older females have also likely mated and will have fewer eggs available for fertilization. To examine the potential influence of scale loss and UV reflectance on mate selection, we conducted two-choice male preference trails in two populations of tiger swallowtail. The first was in northern Michigan (*P. canadensis*) and the second was in central Florida (P. *glaucus*). In both populations, methods followed those described in Aardema and Scriber (2013). Briefly, we prepared pairs of females, one that's wings were left pristine and another one that had ~50% of her scales removed haphazardly from both sides of the wing by us using transparent adhesive tape as described above. We handled both artificially aged and pristine females for a similar amount of time to reduce potential handling affects. Within each pairing, the females were of approximately the same size as measured by forewing length from base to tip (± 1 mm). A wooden dowel was placed horizontally through the top of a 2 m long vertical stake so that equal halves stuck out in opposite directions. This allowed us to tether females approximately one meter apart by a fine thread at the ends of the dowel. We used between four and six female pairs placed a minimum of eight meters from one another in areas where there were abundant nectar plants.

Males flying by these females would alter their flight path and engage them if interested. Typically this involved repeatedly circling the females on their tethers. Upon contact with a male the females would generally become enlivened and flutter rapidly at the ends of their strings, spiraling in tight circles. At this point the male would either land on one of the females and initiate copulation or in some cases fly off. In most instances the male engaged both females of a pair before copulation was initiated. While they were tethered, we checked each pair of females at least once every five minutes. After we observed a mating being initiated or else found a copulation in progress, we would gently remove the male from the female. We retained all males. For further details see Aardema and Scriber (2013).

Our null hypothesis was that there would be no preference for one treatment over another and that mating frequencies would be approximately 50/50. Alternatively, if either pristine females or artificially aged females were preferred we would expect a statistically significant deviation from 50/50. To evaluate male mating preferences we used a χ^2 goodness-of-fit test.

RESULTS

Our ultraviolet photography of wing coloration revealed distinct areas that reflected UV light and other areas that appear to be entirely UV absorbent (Fig 1, A & B). Most of the UV reflective areas occur in blue regions of the wings in both species and sexes. The anterobasal area of the hind wing also shows a high degree of UV reflectance. Black and yellow colored areas were UV absorbent. All our photographs revealed

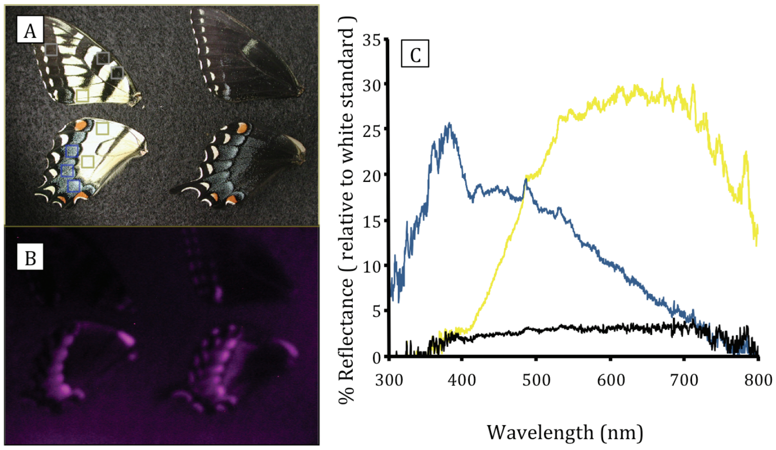
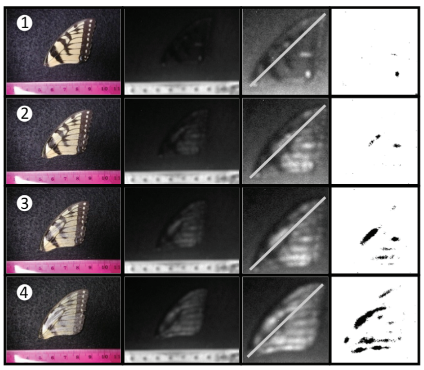


FIGURE 1



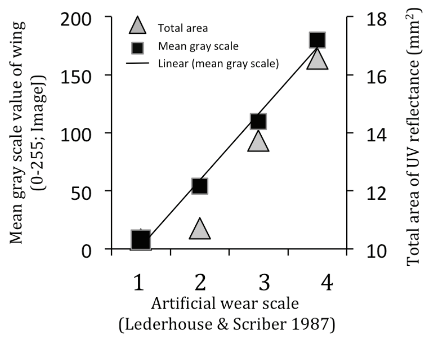


FIGURE 2

FIGURE 3

FIG. 1. Examples from our photographic and spectral examination of ultraviolet reflectance in tiger swallowtail butterflies. All examined butterflies showed very similar patterns (photographic: n=15, spectral: n=4). **A**: Representative visual image of the dorsal side of yellow morph (left) and dark morph (right) female P. *glaucus*. The nine squares on the yellow-morph female's wing represent the approximate locations of our sampling for the spectral analysis. Gray squares correspond to 'black' regions, gold squares correspond to 'yellow' regions, and blue squares correspond to 'blue' regions. **B**: The same wings as in 'A' but photographed using a UV pass filter to reveal UV coloration. Areas that reflected UV light appear purple whereas areas that absorbed UV light appear black. **C**: An example of the reflectance spectrum patterns of blue, yellow and black coloration from a single yellow morph, P. *glaucus* female. This figure shows a clear UV signature (300–400 nm) for blue coloration and virtually no UV reflectance for yellow and black coloration.

FIG. 2. Examples of wings classified as wear class 1–4 (rows from top to bottom). From left to right, Column 1: Visual images of wings showing increasing scale loss. Column 2: UV images of wings showing increasing scale loss. Column 3: UV images enhanced for contrast using ImageJ to estimate the mean gray scale along a linear transect from wing base to tip. Column 4: UV pictures converted to binary images to estimate the total area of scale loss based on UV reflectance.

FIG. 3. Correlation between subjective wear class (1–4) and two measures of wing wear based on UV reflectance. The left Y-axis shows the mean gray scale estimate along a line drawn from the base of the wing to the tip (after image was enhanced for contrast). The right Y-axis shows the total area of UV reflectance (after binary conversion). Both measures correlate strongly with wing wear (Mean gray scale R^2 = 0.991; Total area R^2 = 0.926).

very similar patterns across the 15 individual specimens we examined. In agreement with our photographic results, our spectral analysis revealed a strong UV signature for blue coloration, but virtually no UV reflectance for yellow or black coloration (Figure 1, C). Again, these results were very consistent across samples.

Both measures of UV reflectance were strongly correlated with our wear class categories (Fig. 3). For our test sample, the mean gray scale gave a slightly higher correlation between the wear classes than did the total area of UV reflectance (mean gray scale $R^2 = 0.991$; total area $R^2 = 0.926$), but both were highly significant ($p<0.001$). How much variance would be observed around these points remains to be determined.

In our assessment of male mating preferences for pristine or artificially aged females, we observed 38 copulations in total, 30 in Michigan and eight in Florida (Table 1). While the χ^2 has been shown to work reasonably well even with small expected values, it is generally agreed that the average expected value across categories should be at least 5 (Roscoe & Byars 1971). Therefore, we only statistically examined mating preferences for the Michigan population and for both populations combined. This assessment of male preference for artificially aged or pristine females revealed a clear choice for pristine females (Table 1). In both Michigan and Florida at least 75% of all initiated copulations were with a pristine female rather than an artificially aged female.

TABLE 1. The results of our two-choice mating trials comparing male preferences for pristine and aged females (no scale loss vs. wings artificially aged to category 4).

Population (Species)	# pristine female pairings	# aged female pairings	p value[1]
Michigan (*P. canadensis*)	24	6	0.001
Florida (*P. glaucus*)	7	1	NA
ALL	**31**	**7**	**<0.001**

[1] These p values are based on 2-tailed χ^2 test with 1 degree of freedom and expected frequencies for both categories of 50%.

DISCUSSION

We have shown that tiger swallowtail butterflies have extensive UV reflectance on their wings, predominately in areas that are blue as well as the anterobasal region. However, it should be noted that UV images such as those described here only capture reflectance in a small part of the UV spectrum (Rutowski & Macedonia 2008). It is possible that our description of UV coloration in tiger swallowtails may be rather different from the way these insects view one another, in addition to how other butterfly species, predators, etc. view them.

Another point to consider is that tiger swallowtail females generally have significantly more blue coloration on their wings and therefore more UV reflectance than males (Aardema & Scriber 2013). Unlike in many other Lepidopteran systems, this coloration does not seem to be utilized for conspecific recognition or mate choice (Aardema & Scriber 2013). It is possible that UV and blue coloration relates to enhanced mimicry of the pipevine swallowtail (*Battus philenor*) by dark-morph *P. glaucus* females. However, *B. philenor* have wings that reflect almost no UV light (Aardema & Scriber, unpublished data). More testing will be needed to determine if the ultraviolet coloration of tiger swallowtail butterflies has ecological or behavioral significance.

Beyond the basic observations of UV in these butterflies, we have proposed that techniques similar to those described here could be used to objectively quantify wing wear in butterflies. Such methods are in contrast to many systems currently employed which use some form of subjective wear class. These systems may be affected by observer biases or differences between observers. Interestingly, our observation of increasing UV reflectance with wear in tiger swallowtails is the opposite pattern to that observed in *Colias eurytheme*. In this species the scales have evolved to reflect a maximal amount of UV, and this reflectance declines with increased wing wear (Kemp 2006). Recently it was shown that UV coloration in butterflies could evolve very rapidly (Wasik 2014). Therefore, it is not surprising that different species exhibit differences in how their UV coloration is affected by wear. Regardless of whether UV coloration increases or decreases, when changes in UV reflectance correlate with wing wear, these patterns could be used to objectively quantify the wing wear of individuals, and correspondingly age. However, species-specific calibrations would likely be in order.

Finally, the results of our mate-choice study lend preliminary support to our hypothesis that older looking individuals are less desirable as potential mates. However, we were only able to examine male mate choice for worn or fresh appearing females. It remains to be determined whether females also discriminate among males based on wing wear and/or the exposure of the underlying wing membrane with corresponding increases in UV reflectance. Furthermore, whether UV reflectance is the actual cue that indicates an individual's age to conspecifics of the opposite sex requires additional research to determine. Ultimately, these results should be viewed as preliminary and follow-up research is warranted.

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