TECHNICAL NOTE

Quantifying monarch butterfly larval pigmentation using digital image analysis

Andrew K. Davis*, Bethany Farrey & Sonia Altizer

Department of Environmental Studies, Emory University, 400 Dowman Drive, Atlanta, GA 30322, USA

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Several recent studies have focused on factors governing external coloration in lepidopteran larvae (e.g., Goulson, 1994; Gunn, 1998; Hazel, 2002; Cotter et al., 2004). For example, the larvae of several species reared in cool temperatures become darker than they would under moderate or high temperatures (e.g., Goulson, 1994; Hazel, 2002; Solensky & Larkin, 2003). In addition, larvae can darken when reared at high densities (Goulson & Cory, 1995), and dark larvae have been shown to exhibit greater immune defenses and be more resistant to parasites (Wilson et al., 2001). In fact, the relationship between larval color and immune defenses in insects has received much attention recently and could be caused by simple processes that govern melanic pigmentation and encapsulation of foreign bodies (Wilson et al., 2001).

In studies of lepidopteran larvae and other insects that involve the measurement of pigmentation, researchers have often needed to quantify the amount or degree of dark coloration in groups of larvae. To accomplish this, a range of techniques have been employed, from scoring the level of darkness on a qualitative, categorical scale (e.g., Goulson, 1994; Windig, 1999), to magnifying and manually measuring the width of dark bands on individual body segments (Hazel, 2002). One of the more creative methods recently involved monarch butterfly (*Danaus plexippus* L.) (Lepidoptera: Nymphalidae) larvae, which have alternating bands of black, yellow, and white on their body segments. In this study the authors placed a ruler next to live monarch larvae and counted the number of 1 mm segments that overlapped black bands (Solensky & Larkin, 2003). Despite their utility however, these recent techniques rely on subjective scoring or manual measurements. Here, we describe a method of quantifying larval coloration in monarch butterflies that is both objective and automated, and that could be applied to other studies of insect coloration.

*Correspondence: Tel.: +1 404 727 3262; E-mail: akdavis@emory.edu

The method we describe is based on a technique commonly referred to as 'image analysis', whereby digital images of subjects are obtained and measured via computer software. Modifications of this technique have previously been used to measure blackness in mealworm beetles (Thompson et al., 2002), wing pigmentation in damselflies (Siva-Jothy, 1999), and cuticular melanism in African armyworms (Wilson et al., 2001). We used a version of this technique to measure larval pigmentation while conducting a project that involved the rearing of larvae under varying temperature treatments. We reared monarch larvae derived from eastern North American parents at three different temperatures: 19 °C, 26 °C, and 33 °C to determine, in part, the effect of rearing temperature on larval darkness (i.e., amount of black). We reared a total of 397 larvae from 14 different full-sibling family lines, from hatching to pupation in controlled growth chambers. All larvae were reared in shoebox-sized plastic containers with fiberglass screen lids at densities of no more than 10 per container, and were fed greenhouse raised swamp milkweed, *Asclepias incarnata*. When larvae reached the 5th instar, we placed them individually on a light blue cardboard platform, and photographed them using an Olympus C-3000 Zoom digital camera mounted 25 cm above the platform. We also photographed a subset of 19 larvae three times to test the repeatability of our method, each time moving the larvae into a different position for the photograph. Once all of the images had been obtained, we imported them into Adobe Photoshop version 6 with the Image Processing Tool Kit (IPTK) plug-in installed (Reindeer Graphics, Inc.).

Measuring the amount of black on the image of each larva involved the following four steps: (1) thresholding the image (i.e., creating a black and white image so that all non-black colors became white), (2) removing the blue background to leave only the larva, (3) cropping the antennae from the body (Figure 1), and (4) running the IPTK measurement function, which measures the area (in both pixels and mm^2) of all black vs. white polygons (which

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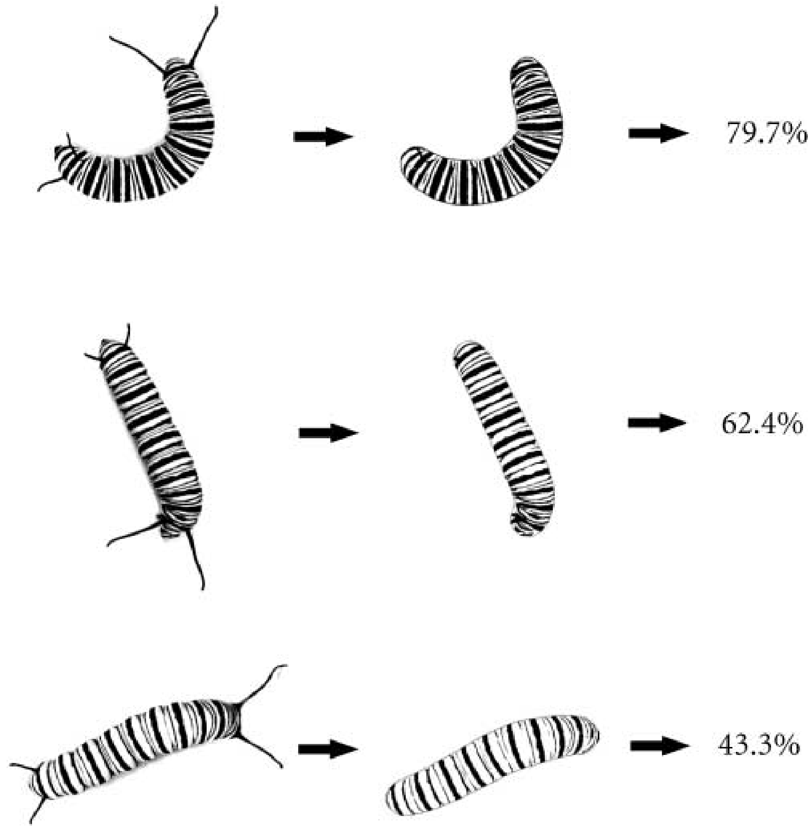

Figure 1 Digital photographs of three typical 5th instar monarch butterfly larvae before (reduced to greyscale) and after image analysis and the percentage of black measured for each larva.

represented the black and white body segments) on the resulting image. Before measurements were initiated, the program was first calibrated with an image of a standard ruler obtained using the same camera setup. The total area and percentage of the entire body encompassed by black was then calculated based on the area of black vs. white polygons obtained by the program. Finally, we automated the entire routine so that the program could process and measure each image automatically. While the processing time could vary with the speed of the computer, our

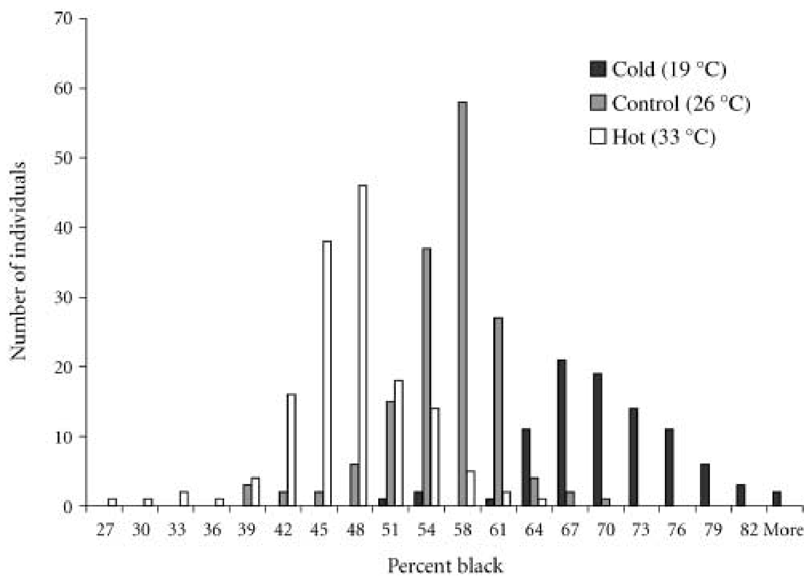

Figure 2 Frequency distribution of all black percentages (%) measured with the image analysis technique across all treatments.

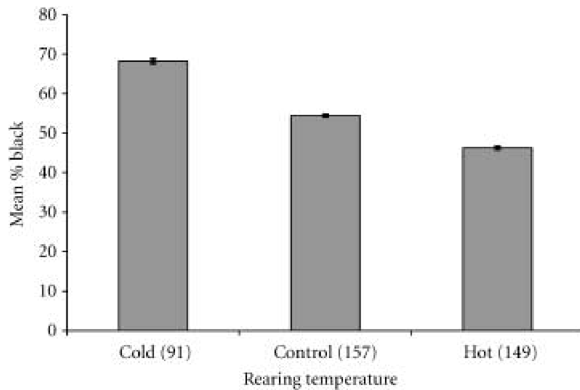

Figure 3 Effects of rearing temperature on larval blackness in monarch butterflies. Larvae were reared simultaneously in cold (19 °C), control (26 °C), or hot (33 °C) temperatures. Sample sizes are shown in parentheses. Error bars show standard errors.

computer completed the measurement of the 397 larval images from our experiment in approximately 1 h.

The percentage of black calculated with this technique ranged from 27 to 85% for all larvae in our temperature experiment (Figure 2). Furthermore, the results of our experiment showed that, as expected, temperature had a significant effect on larval pigmentation (one-way ANOVA; $F_{2,394} = 494.7$, $P<0.001$; Figure 3). These results are consistent with those of Solensky & Larkin (2003), who used a manual method to estimate black pigmentation in monarch larvae reared under different temperatures. In addition, the image analysis method we used was highly repeatable; we found no significant difference in the percentage of black between the three pictures of the subset of 19 larvae (repeated measures ANOVA: $F = 0.407$, d.f. = 18, $P = 0.532$). Furthermore, pair-wise correlations between the three picture sets revealed Pearson correlation coefficients of 0.97, 0.95, and 0.99, which were all highly significant ($P<0.001$).

We conclude that this technique provided us with an objective, repeatable, and automated method for estimating the degree of dark coloration in monarch butterfly larvae. Furthermore, the method is flexible. For example, the routines we used in Photoshop could be easily modified to measure the percentage of yellow or white on monarch larvae. Finally, because the method involved obtaining digital images of larvae, using this technique created a permanent archive of all larvae in our experiment that can be subsequently examined for other characteristics at any time in the future.

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