A NON-DESTRUCTIVE, AUTOMATED METHOD OF COUNTING SPORES OF OPHRYOCYSTIS ELEKTROSCIRRHA (NEOGREGARINORIDA: OPHRYOCYSTIDAE) IN INFECTED MONARCH BUTTERFLIES (LEPIDOPTERA: NYMPHALIDAE)

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Monarch butterflies (*Danaus plexippus*) are parasitized by the neogregarine protozoan parasite *Ophryocystis elektroscirrha* in varying levels throughout North America. In the eastern population, less than 8% of individuals are heavily infected, whereas in the western population, approximately 30% are heavily infected. Moreover, more than 70% of monarchs from the non-migratory South Florida population are heavily infected (Altizer et al. 2000). In Florida, the related queen butterfly (*D. gilippus*) is also susceptible to this parasite (McLaughlin & Myers 1970; Leong et al. 1997). The life cycle of *O. elektroscirrha* is such that its spores are transmitted by ingestion by larvae, vegetative reproduction occurs in the larvae and pupae, and the adult monarch emerges with spores covering the surface of its abdomen (McLaughlin & Myers 1970). Depending on the stage of larval growth at the time of exposure, the age and number of spores, infected adults can harbor from 200 to 974,000 spores on their abdomens (Leong et al. 1997). Furthermore, depending on the parasite loads, infected adults have decreased survival to eclosion, are subsequently smaller as adults, and have shorter adult lifespans (Leong et al. 1992; Altizer & Oberhauser 1999).

The susceptibility of monarchs to this parasite and the ease at which monarchs can be reared and inoculated with its spores has provided researchers with a model system for examining host-parasite relationships (e.g., Leong et al. 1992; Altizer et al. 1997; Altizer & Oberhauser 1999; Altizer 2001). In most of these studies, researchers are required to estimate the degree of infection in individual monarchs, which is inferred by the number of spores present on its abdomen, where they are known to be most numerous (Leong et al. 1992). To sample and count these spores, two general methods have been employed most commonly in the past. In the first method, the abdomens of adult specimens are removed, placed in a vial with water and a wetting agent, shaken on a vortexer, and the number of spores per volume of suspension is counted with a haemocytometer (Leong et al. 1992; Leong et al. 1997). Viewing and counting the spores in the haemocytometer is effective because the spores are dispersed throughout the solution, facilitating counting of individual spores. However, this method requires that monarchs be dead prior to quantifying parasite loads.

A non-destructive, simpler method is widely used by butterfly breeders and researchers to detect and remove parasitized individuals in their captive stock. It involves pressing a small piece of transparent tape over the abdomen of the live monarch, pulling it off to remove a section of abdominal scales, placing it on a microscope slide and viewing it under low power (e.g., Altizer et al. 2000; Altizer 2001). Spores of *O. elektroscirrha* are readily seen and counted among the butterfly scales at 50× or greater magnification. Unfortunately, when actual counts of individual spores are required, this method is tedious because there can be hundreds of thousands of spores that must be counted manually. Furthermore, since the tape removes many scales with the spores, the resulting slide contains dense clusters of butterfly scales that make it hard to discern the parasite spores.

Advances in photographic and computer technology during the past 15 years have led to the development of image analysis techniques. These techniques involve taking a picture of one or more objects, then transferring the picture to a computer, where digital measurements are made of the objects in the picture with special graphical software. This technique has many applications, spanning a multitude of disciplines. We used a variation of this technique to develop a method to quantify spores of *O. elektroscirrha* on adult monarch butterflies in a recent study, and we describe this method in the present paper. This method is objective, non-destructive, and once the samples are obtained, the counting process can be fully automated.

To obtain samples of the spores, we first held the adult butterfly by the base of its wings so that its abdomen was exposed. Next, we used a standard cotton swab to gently swipe one side of the abdomen with the tip of the swab from the posterior end forward, so that at least 4 abdominal segments were swiped (usually segments 4-8). We repeated this 4 times using the same swab in the same place on the abdomen. The end result was a small accumulation of abdominal scales and spores on the tip of the swab. Next, we lightly dabbed the swab tip on a dry, standard micro-
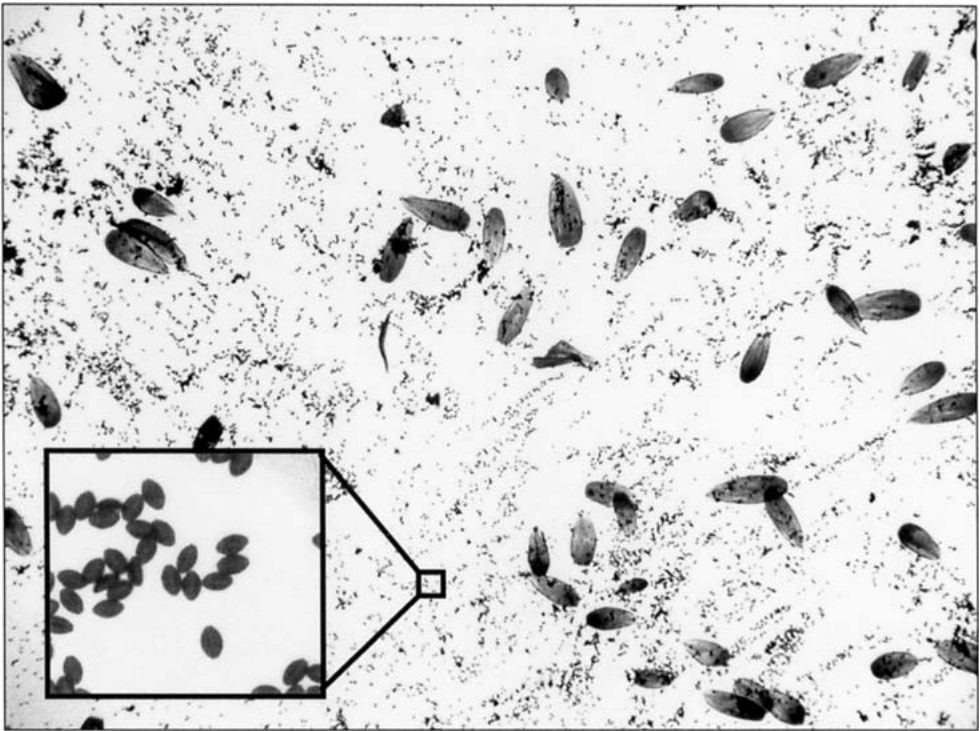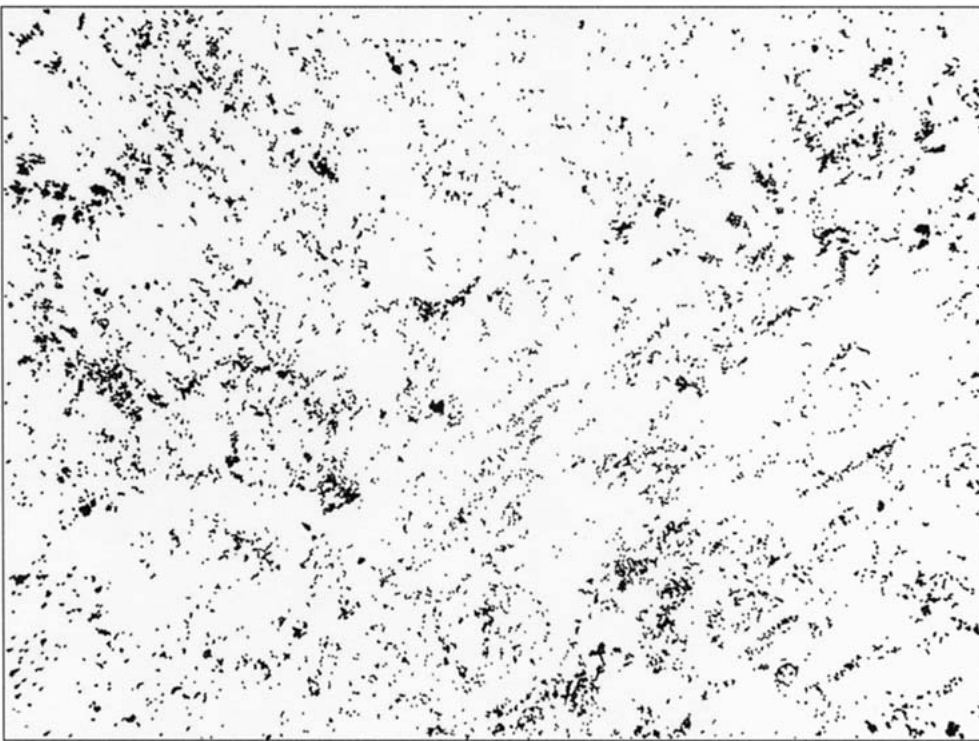
Fig. 1. (A) Image of *O. elektroscirrha* spores from a heavily infected monarch butterfly (40×) before image processing. Insert shows spores at 400×. (B) Same image after digitally deleting the scales to leave only spores for automated image analysis counting. Approximately 3,600 spores were counted in this sample.

scope slide 4 times, leaving a small circle of scales (and spores) which adhere to the dry slide. Then, we viewed the slide under 40× magnification with a light microscope equipped with a trinocular head and digital camera (Olympus C-3000 Digital Zoom). We positioned the field of view so that it was centered on the entire cluster of scales. In infected individuals, spores of *O. elektroscirrha* can be seen readily among the scales (Fig. 1A). We photographed this image with our digital camera at 3 megapixel resolution. This process was performed twice for each butterfly, once for each abdominal side, to reduce the chances of a poor swipe with the swab. Two images per butterfly were thus obtained.

To count the spores in the images, we used Adobe Photoshop with the Image Processing Tool Kit (Reindeer Graphics, Inc.) plugin installed. Before we began the counting procedure, we first calibrated the software by digitally drawing a line of a known distance on an image of a haemocytometer grid (which is 1 mm^2). Once the pixel-to-distance ratio in this image was saved, it was then possible to obtain correct sizes for any objects in all images thereafter. For each image of spores and scales, we then performed a series of steps to remove the butterfly scales from the image based on their size, and threshold the image (i.e., change the image to black and white) so that all remaining objects (spores) were black on a white background (Fig. 1B). It was then a simple matter to have the computer measure the area encompassed by black objects (spores) in the image. Because we previously found that the average area of an individual spore is approximately 45 µm^2, we divided the total spore area we obtained by the known individual spore area to arrive at the approximate number of spores in the image. In Fig. 1B, we obtained an estimate of 3,600 spores.

For greater accuracy, we calculated the average of the two counts (from separate swabs of the left and right sides of the abdomen) obtained for each butterfly. This resulted in an estimate of spore load that is comparable to manual counting from tape samples. As a test of this point, we sampled and counted spores on a set of 31 infected specimens by both the image analysis method and the tape method (i.e., by manually counting the number of spores on 1cm^2 tape samples at low power to the nearest 100 spores). Both methods were significantly positively correlated (e.g., for log-transformed values, r = 0.76, N = 31, *P* < 0.001; Fig. 2). Further, because we obtained two samples (from the left and right side of the abdomen) from each butterfly, we compared the repeatability of our method by testing whether the samples obtained from each side differed. The number of spores obtained from the left side of all butterflies differed from that obtained from the right side by an average of 478 (± 334, SD). There was a significant positive correlation between the
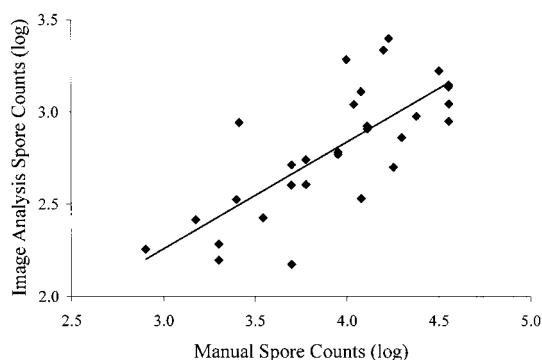

Fig. 2. Plot of manual spore counts of 31 adult monarch butterflies made from the manual (tape) method against spore counts obtained by the image analysis method for the same individuals (both counts are log-transformed). Counts obtained from both methods were significantly correlated (r = 0.76, *P* < 0.001). Least squares regression line shown (y = 0.5195 + 0.5795x).

counts obtained from the left and right sides for each individual monarch (Pearson correlation, N = 31, r = 0.65, *P* < 0.001).

In terms of assessing parasite loads on infected, live monarchs, this 'swab method' combines both the non-destructiveness of the tape method and the clear images of spores in the haemocytometer method. Furthermore, this method produces a fast, non-destructive, quantitative estimate of infection levels of individual butterflies. The only drawback of the method that we can perceive is its relative inability to detect minute spore loads (i.e., less than 100 spores on the abdomen). However, the advantages of the method may outweigh this one weakness. One unique advantage is the fact that for each butterfly sampled with this method, a permanent image of its spore load is created that can be saved and archived. Moreover, if image analysis software is not available to the researcher, the images can be easily emailed to persons capable of performing the counting procedure. Finally, with automation routines common to many software programs such as Adobe Photoshop, the counting procedure can be set up to run on multiple images either in the background of the desktop, or while the computer is not being used. The speed of the procedure will vary with the speed of computer used. In 30 minutes our software can obtain counts of spores on 50-60 images, with typical counts ranging from 300 to 12,000 spores in infected butterflies.

SUMMARY

Monarch butterflies are susceptible to the protozoan parasite, *O. elektroscirrha*, and they have been the subject of study by researchers interested in host-parasite interactions (e.g., Leong et

al. 1997; Altizer et al. 2000; Altizer 2001). As such, methods have been developed to sample individual butterflies for the presence and severity of this parasite, but past methods are either destructive or tedious with respect to providing quantitative (non-categorical) measures of actual parasite loads. We developed a novel method for sampling and counting *O. elektroscirrha* spores on monarch abdomens based on image analysis techniques. This method is simple, non-destructive, and objective, and will provide a useful and effective tool for future researchers of this parasite.

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