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TECHNICAL NOTE

A simple, computer-assisted method for obtaining and counting hemocytes in milkweed bugs

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Introduction

Insect hemocytes have been the focus of researchers for decades (e.g., Jones, 1962; Ravindranath, 1978; Hall, 1983; Ribeiro & Brehelin, 2006). Most research has sought to increase our understanding of what factors influence hemocytes numbers, especially in response to various stressors (e.g., Silva et al., 2002; Andrade et al., 2003; Mochiah et al., 2003). Moreover, the recent increase in questions related to invertebrate ecological immunology (Rolff & Siva-Jothy, 2003) have further stimulated interest in these factors. To carry out such studies, it is often necessary for the researcher to quantify the numbers of hemocytes in their study subjects, and several techniques have been traditionally employed for this purpose. One method relies on estimates of overall hemocyte volume after centrifugation of hemolymph in hematocrit tubes (Clark & Jones, 1980). Electronic particle counters have been used to count marine arthropod hemocytes (Stewart et al., 1967). Liquid nitrogen fixation has been used to compare the numbers of hemocytes in tissue sections to counts made of hemolymph from severed appendages (Feir & O'Connor, 1969). However, the most common method by far involves placing a diluted amount of hemolymph in a hemocytometer and counting the number of cells that fall randomly within specified areas of the hemocytometer grid (e.g., Feir, 1964; Armitage & Siva-Jothy, 2005). The hemocytometer technique is simple and provides data that can be compared between species or within a species. However, this method is also tedious, especially if large numbers of individuals are examined in a study, and because many hundreds of cells must often be quantified for each. In this article, I describe a computer-assisted (i.e., using image analysis software) technique for counting hemocytes in milkweed bugs that is both objective and automated and that yields data within seconds.

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Materials and methods

Study specimens

I conducted this research on a set of larval milkweed bug, *Oncopeltus fasciatus* (Dallas) (Homoptera: Lygaeidae), that I had collected as early instars in an *Asclepias incarnata* L. (Asclepiadaceae) patch in northeast Georgia in August 2006 and that had been reared in the laboratory on fresh *A. incarnata* seeds. I sampled all fifth instars within this laboratory group on 28 August 2006, as described below. Because all individuals were field collected, I could not know if the larvae had hatched synchronously, and given that the duration of the fifth-instar stage is approximately 7 days in this species (Feir, 1974), the individuals in this set therefore represented a mix of variously aged individuals.

Obtaining hemolymph samples

For each milkweed bug, one antenna was severed midway and the bug held upside down to allow a small bead of hemolymph to form (Figure 1). The insect was then lowered onto a glass slide so that the hemolymph bead was transferred to the glass. The slide was then placed in a humid chamber for 15 min to allow the hemolymph bead to spread out and the hemocytes within it to settle to the bottom. The slide was then viewed ×100 under a light microscope fitted with a trinocular head and a Canon Powershot G6 (Canon, Newport News, VA, USA) digital camera. The lighting and diaphragm on the microscope were set so that the hemocytes appeared as dark green spots, which stood out on the light background. The hemolymph bead appeared as a circle within the field of view, with hemocytes easily observable within the circle (Figure 2). Finally, I captured a digital image of the entire field of view to use for the hemocyte counting (below).

Counting hemocytes

When all hemolymph samples and images were obtained, I imported each image into Adobe Photoshop with Fovea

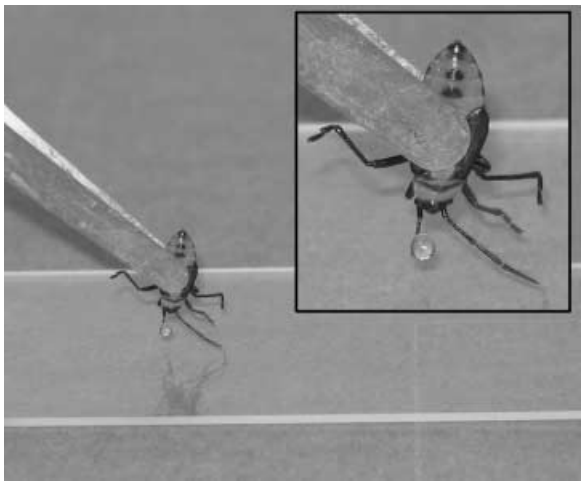

Figure 1 Photograph of the hemolymph collection procedure from *Oncopeltus fasciatus*. The hemolymph bead from a severed antenna is touched directly to a microscope slide, and the slide placed in a humid chamber for 15 min to allow the hemocytes to settle to the bottom.

Pro (Reindeer Graphics Inc., Asheville, NC, USA) software installed. The image analysis methods that follow were modified from those used to count parasite spores in digital images (Davis et al., 2004). First, the scale of the images was calibrated from a picture taken earlier of a stage micrometer. Then for each image I selected the outline of the hemolymph circle and used a Fovea Pro measuring routine to measure its area (in mm^2). The image was then thresholded to black and white and the area outside the circle removed so that only the hemocytes (which were now black) remained. I then ran the Fovea Pro 'count objects' routine, which returns the number of black objects in the image. Furthermore, both the circle area and hemocyte counts were automatically saved to a text file during the process, eliminating any possible transcribing errors. Finally, I recorded these steps in a Photoshop action so that the entire sequence could be automated and run at the push of a button for the subsequent images.

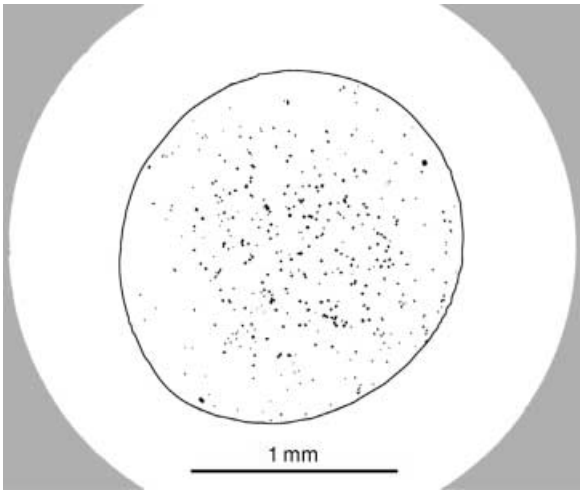

Figure 2 Black and white (i.e., thresholded) image of a typical *Oncopeltus fasciatus* hemolymph 'circle' as seen ×100 under a light microscope. Image analysis software calculates the area of the circle and the number of spots (hemocytes). There were 382 hemocytes in this sample.

Results and discussion

With the method described here there was a significant positive relationship between the area of the hemolymph circle and the number of hemocytes counted (Pearson correlation: n = 94, r = 0.422, P<0.001; Figure 3). This was to be expected because a larger volume of hemolymph would naturally hold more hemocytes. I therefore divided all hemocyte counts by the area of the hemolymph circle in the digital picture to account for variations in hemolymph volume. This resulted in a normally distributed array of values for the 94 bugs sampled with each value being the number of hemocytes per mm^2 on the microscope slide (Figure 4).

Hemocytes and bug size

As a general check of these data, I compared the data gathered here to those of Feir (1964). In that study, a general decrease in hemocyte numbers was observed throughout the 7-day fifth-instar stage. While I did not know the age of

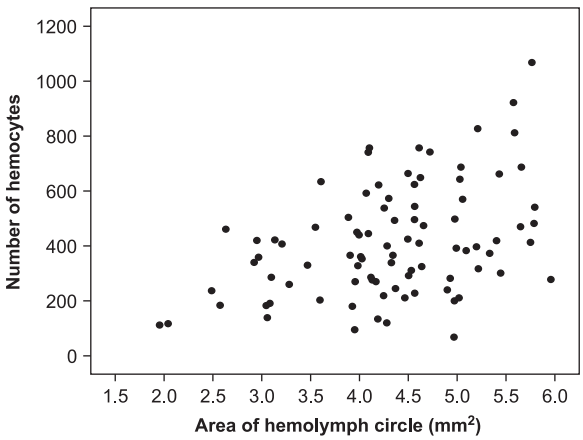

Figure 3 Relationship between the size of the hemolymph circle and number of hemocytes counted in *Oncopeltus fasciatus*. Both variables were significantly correlated (Pearson correlation: n = 94, r = 0.422, P<0.001).

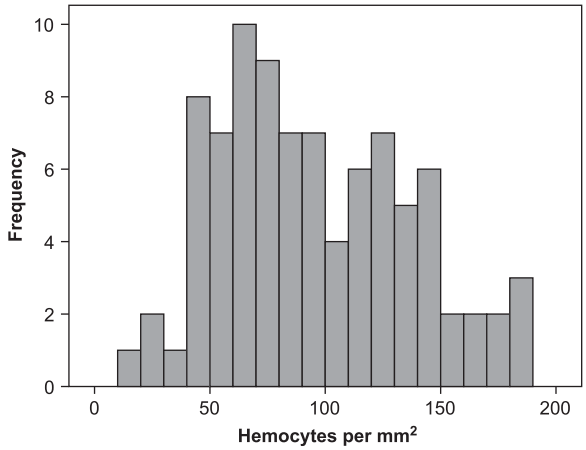

Figure 4 Histogram showing the number of hemocytes per mm^2 of 94 fifth-instar *Oncopeltus fasciatus*.

my bugs, I was able to measure their body length, which could be considered a proxy for age. I found a significant negative relationship between hemocyte counts and body length at fifth instar (Pearson correlation: $n = 94$, $r = -0.218$, $P = 0.040$; Figure 5). This relationship is what would be expected based on the results of Feir (1964).

Conclusion

I describe here a technique for obtaining and counting insect hemocytes that is simple and nearly fully automated. Indeed, the main advantage that this method offers over hemocytometer counts is its increased efficiency. For example, once the hemolymph samples and their images were obtained in this study (which is the limiting factor with this method), I was able to generate data for each individual bug at a rate of 1 every 10 s, and the hemocyte counts for all 94 bugs were obtained within 15 min. This efficiency is impressive given that many images contained over 600 hemocytes (Figure 3). Furthermore, the direct transfer of hemolymph from the insect to the slide (as opposed to transfer via pipettes or capillary tubes) may also increase efficiency, although this would be difficult to do with insect species that have rapidly coagulating hemolymph. Given the recent attention to insect immunity by researchers, this method may prove valuable in future studies that rely on hemocyte counts.

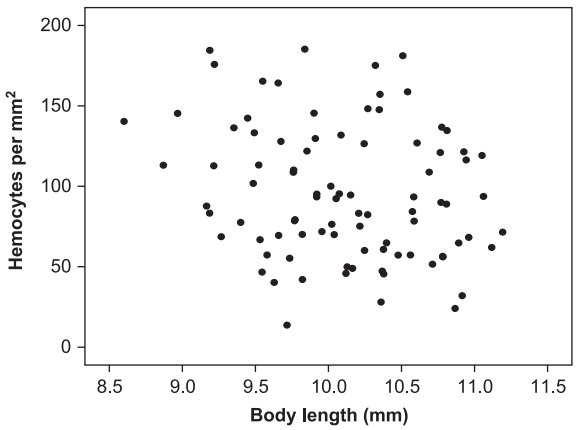

Figure 5 Relationship between *Oncopeltus fasciatus* size and its hemocyte count (number of cells per mm^2). Both variables were significantly negatively related (Pearson correlation: $n = 94$, $r = -0.218$, $P = 0.040$).

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