

## A Comparison of the TC10™ Automated Cell Counter and the Hemocytometer for Quantifying Insect Hemocytes

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### Introduction

Hemocytes are an essential component of the invertebrate innate immune system (Strand 2008). Their close similarities to vertebrate white blood cells enable their use as a tractable model for immunological research. The quantification of hemocytes in the hemolymph (invertebrate blood) is one way of assessing the efficacy of the immune response because the absolute number of hemocytes changes during the course of an infection (Bergin et al. 2003, Brayner et al. 2007), and the density of hemocytes in the hemolymph is strongly correlated with the capacity to encapsulate or phagocytose parasites (Prevost and Eslin 1998, Kraaijeveld et al. 2001, Wilson et al. 2003, Costa et al. 2005). Most researchers continue to use cell staining and manual microscopy techniques to enumerate the hemocytes in a given sample. However, given the laborious nature of this method (with a throughput often as low as 6–8 samples/hr), more rapid techniques are required to increase our understanding of the role of hemocytes in invertebrate immunology.

Here, we tested the suitability of Bio-Rad's TC10 automated cell counter, a tool originally developed for assessing mammalian cell counts, for counting insect hemocytes. Hemocyte counts from the model insect species *Spodoptera littoralis* (Lepidoptera: Noctuidae) were compared with those obtained using a traditional tool for counting insect hemocytes: a hemocytometer with improved Neubauer ruling under light microscopy. We then tested the capacity of the TC10 cell counter to distinguish among cell counts in insects fed on artificial diets varying in their protein and carbohydrate contents, which has previously been shown to influence hemocyte counts in this species (Ponton et al. 2011).

### Materials and Methods

Hemolymph samples from 30 sixth instar larvae were collected into a precooled microcentrifuge tube by piercing the cuticle between the first and second prolegs with a 25-gauge BD Microlance needle (Becton, Dickinson and Company). The samples were immediately frozen and

stored at  $-20^{\circ}\text{C}$  until required. For each of the two methods, hemolymph was thawed, diluted 1:1 in phosphate-buffered saline, pH 7.4, and placed on ice. The hemolymph was then stained in a 1:1 ratio with 0.4% trypan blue to distinguish viable from nonviable cells.

In the first method, cell counts were determined using the TC10 automated cell counter in accordance with the manufacturer's instructions (Bio-Rad Laboratories, Inc.). Duplicate 10  $\mu\text{l}$  samples of stained hemolymph were transferred to the two chambers of a Bio-Rad counting slide. The slide was inserted into the automated counter and readings of total and viable cell counts were generated within 30 seconds. In the second method, duplicate 10  $\mu\text{l}$  samples of stained hemolymph were transferred to each side of the chamber in the hemocytometer. Hemocytes were then counted in five nonadjacent squares on the hemocytometer grid under a light microscope, which took approximately 3 min/sample.

The TC10 cell counter was subsequently used to measure the density of hemocytes in the hemolymph of *S. littoralis* larvae fed on artificial diets that varied in their relative amounts of protein and carbohydrate. The methodology was based on previously described experiments (Povey et al. 2009, Cotter et al. 2011). Briefly, a total of 200 larvae on the first day of their final instar were randomly allocated to one of 20 diets that varied in the relative percentages of both soluble protein and digestible carbohydrates, both varying from 2.8 to 52.5%, with the remainder of the diet comprising indigestible cellulose (see Cotter et al. 2011 for a full diet description). After 48 hours, the insects were bled, and 10  $\mu\text{l}$  of hemolymph was added to 10  $\mu\text{l}$  of EDTA/glycerol (50:50) and stored at  $-20^{\circ}\text{C}$  until required. Hemocyte counts were then obtained using the TC10 cell counter as described above.

### Statistical Analysis

The two sampling methods were first assessed independently for repeatability using a series of linear models in the freeware package R version 2.15.0 (<http://www.R-project.org>) for the relationships between the duplicate hemocyte counts for each sample ( $n = 30$ ). All data (live, dead, and total cell counts) were normalized using a  $\log_{10}$  transformation (count +1) prior to analysis. The linear models were assessed by

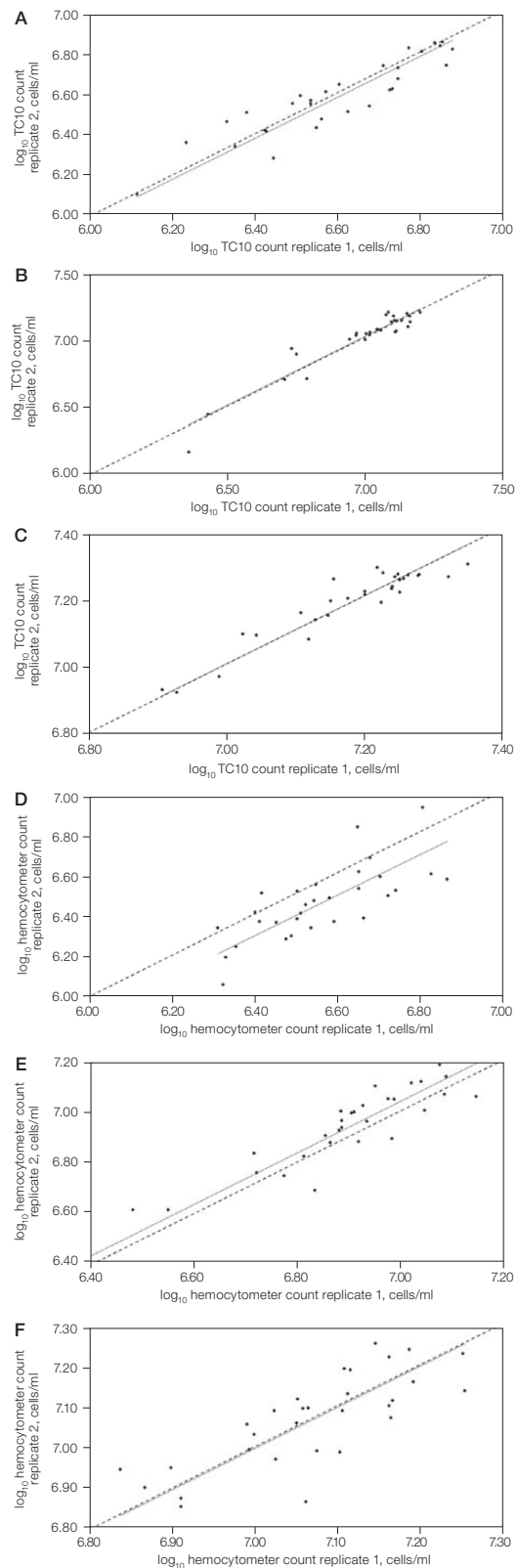
fitting each regression line through the origin. The slope of the regression lines was then tested for deviation from a slope of unity, the expected slope if the paired counts were correlated without bias. The mean numbers of each cell type (live, dead, and total) determined with the two counting methods (hemocytometer vs. TC10 cell counter) were then compared as described above.

The effects of the two digestible macronutrients on live, dead, and total hemocyte counts as measured by the TC10 cell counter were determined using three general linear models in R version 2.15.0. As potential explanatory terms, we tested the total amount of digestible protein in the diet (%P), the total amount of digestible carbohydrate (%C), quadratic functions of the two terms (%P<sup>2</sup> and %C<sup>2</sup>), and an interaction between P and C. The dependent variables (live, dead, and total counts) were first transformed, when appropriate, to conform to the normality assumption, and the minimal model was determined by stepwise deletion using F-tests.

### Results and Discussion

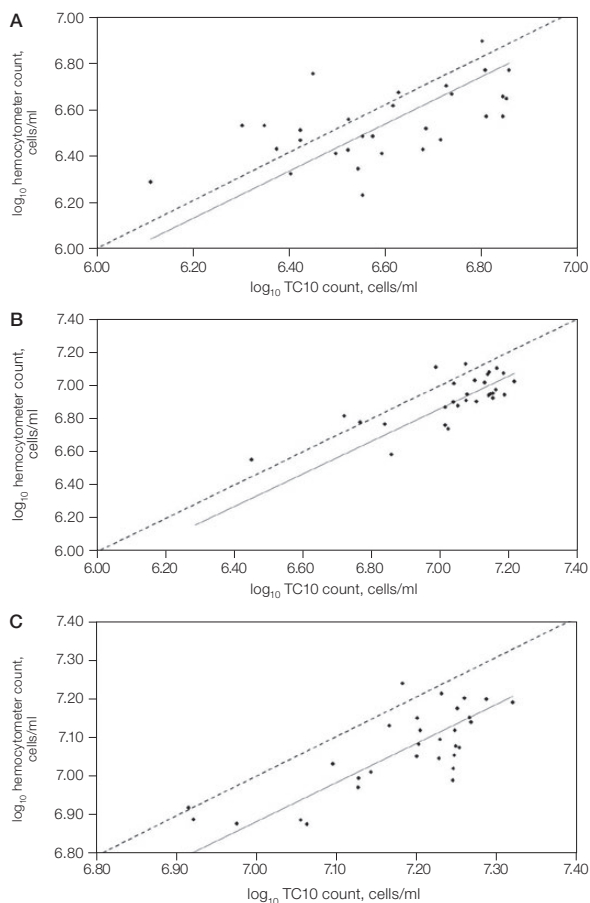
There was a high level of repeatability for the TC10 cell counter method, as indicated by a strong correlation between counts for the paired samples (Figure 1A–C; live cell count: slope,  $b \pm SE = 0.997 \pm 0.002$ ,  $r^2 = 0.99$ ; dead:  $b \pm SE = 1.001 \pm 0.002$ ,  $r^2 = 0.99$ ; total:  $b \pm SE = 1.000 \pm 0.001$ ,  $r^2 = 1.00$ ); the slopes of all the regression lines did not differ significantly from unity ( $P > 0.05$ ). The hemocytometer also showed a good correlation between sample replicates (Figure 1D–F; live cell count: slope,  $b \pm SE = 0.984 \pm 0.003$ ,  $r^2 = 0.99$ ; dead:  $b \pm SE = 1.006 \pm 0.002$ ,  $r^2 = 0.99$ ; total:  $b \pm SE = 0.999 \pm 0.002$ ,  $r^2 = 0.99$ ); however, for both live and dead counts there was evidence of deviation from a slope of one (live:  $t_{29} = 5.43$ ,  $P < 0.001$ ; dead:  $t_{29} = 5.3$ ,  $P < 0.001$ ), indicating that the hemocytometer method was slightly less reliable for counting insect hemocytes than the TC10 cell counter. In particular, there was a slight reduction in the number of live cells in the second replicate count compared with the first, which may be attributable to the length of time required to complete the second reading (up to 20 min between samples) but which did not affect the total number of cells.

When compared across methods, the mean counts of live, dead, and total cells were also strongly correlated (Figure 2A–C; live cell count: slope,  $b \pm SE = 0.987 \pm 0.004$ ,  $r^2 = 0.99$ ; dead:  $b \pm SE = 0.980 \pm 0.004$ ,  $r^2 = 0.99$ ; total:  $b \pm SE = 0.984 \pm 0.002$ ,  $r^2 = 0.99$ ), demonstrating that the Bio-Rad method is a suitable replacement for the hemocytometer for estimating hemocyte counts in an insect hemolymph sample. However, it should be noted that the slopes were significantly different from unity for all three cell counts ( $t_{29} > 3.25$ ,  $P < 0.003$ ), which was due to the consistently lower cell counts obtained with the hemocytometer compared with the TC10 automated cell counter.

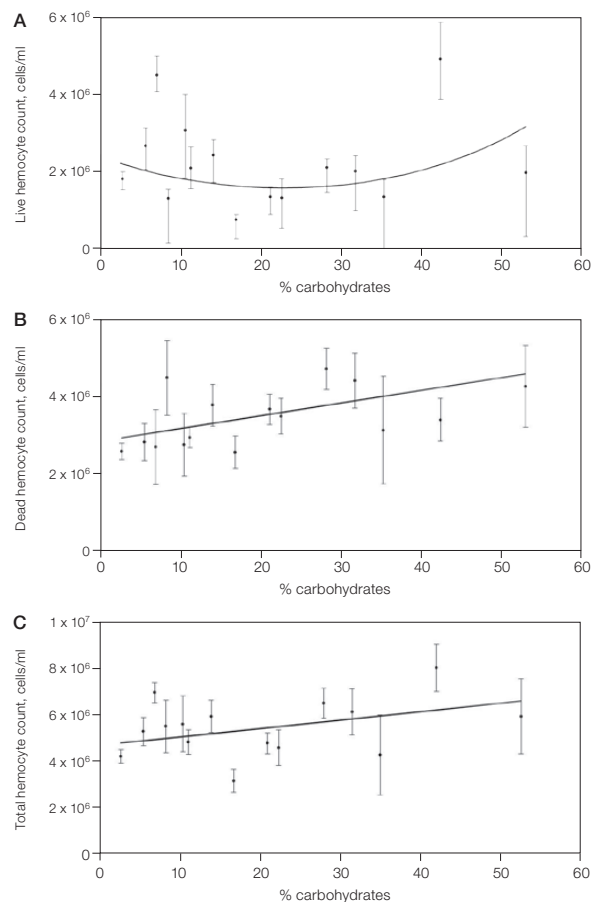


**Fig. 1. Repeatability of the two methods for counting hemocyte density in hemolymph from *S. littoralis* larvae.** Live, dead, and total cell counts, respectively, are displayed in **A–C**, TC10 automated cell counter, and **D–F**, hemocytometer. The dashed lines indicate a slope of unity and the solid lines are the fitted regressions forced through the origin. All six regression analyses were statistically significant ( $P < 0.001$ ).

To determine whether the TC10 cell counter was capable of distinguishing between hemocyte counts obtained from different experimental treatments, we used the cell counter to estimate hemocyte densities in larvae fed on diets varying in the percentages of both digestible carbohydrate and soluble protein. We found that for both dead and total cell counts, the percentage of carbohydrate in the diet had a significant positive effect on the density of hemocytes in the caterpillar hemolymph (dead:  $F_{1,144} = 7.71, P = 0.006$ ; total:  $F_{1,144} = 4.65, P = 0.03$ ); more digestible carbohydrate in the diet yielded more hemocytes (Figure 3B–C). In contrast, the amount of protein in the diet did not affect the total number of hemocytes or the number that were dead ( $P > 0.05$ ). The number of live blood cells depended on both the amount of digestible carbohydrate in the diet and the amount of protein, with the density of hemocytes best described by a linear function of the percentage protein (%P:  $F_{1,142} = 8.50, P = 0.004$ ) and a quadratic function of the percentage carbohydrate (%C:  $F_{1,142} = 4.75, P = 0.031$ ; %C<sup>2</sup>:  $F_{1,142} = 6.06, P = 0.015$ ), leading to a nonlinear relationship between percentage carbohydrate and live cell counts (Figure 3A).



**Fig. 2. Comparison between the TC10 automated cell counter and a hemocytometer for the mean hemocyte counts taken from *S. littoralis* larvae. A, live cell counts; B, dead cell counts; C, total cell counts. The dashed line indicates a slope of unity. The solid line is the fitted regression forced through the origin; all regressions were statistically significant ( $P < 0.001$ ).**



**Fig. 3. The effect of percentage digestible carbohydrate on the hemocyte counts of *S. littoralis* larvae measured with the TC10 automated cell counter. Data shown are the means for each percentage dietary carbohydrate tested  $\pm 1$  SE. Solid lines are the mean predicted lines, at the mean percentage protein, for A, live cell counts; B, dead cell counts; and C, total cell counts.**

### Conclusions

We demonstrated for the first time that Bio-Rad's TC10 automated cell counter provides a quick, easy, and accurate method for estimating the number of insect hemocytes in a sample and one that is comparable to the more laborious hemocytometer method. Sample processing rate was increased from approximately 6–8 duplicate samples/hr for the hemocytometer to 20–30 samples/hr for the TC10 cell counter, thus allowing for increased sample sizes, and therefore, greater statistical power than available previously and greater reliability in assessing live hemocytes. We also showed that the TC10 automated cell counter is capable of distinguishing between counts in samples obtained from insects under different experimental conditions. In this instance, larger sample sizes than in previous experiments of this kind (Ponton et al. 2011) were used because of the easier and faster processing times, and more useful information was obtained (for example, objective differential counts of live and dead cells). These findings provide a useful step forward in efforts to advance our understanding of the role of hemocytes in insect immunity.

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