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TECHNICAL REPORT

A new *in vivo* capillary assay of coagulation in invertebrates

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Abstract

Greater understanding of insect clotting requires better tests that can be performed in whole, living insects. We found we could collect hemolymph bleeding from wounded *Drosophila* larvae in microcapillary tubes. The capillary assay showed a difference in the amount of bleeding between feeding and wandering stage third instar larvae, and performed well with clotting mutations. This new *in vivo* coagulation assay will be helpful for studies of coagulation in *Drosophila* and other invertebrates.

Key Words: *Drosophila*; hemolymph; *hemolectin*; hemocytes; phenoloxidase

Introduction

Coagulation prevents excessive fluid loss and contributes to immunity and wound healing in invertebrates (Theopold and Dushay, 2007; Dushay, 2009; Cerenius and Söderhäll, 2011; Loof *et al*., 2011). We are focused on the fruit fly, *Drosophila melanogaster*, with its extensive molecular genetic advantages. Goto *et al*. (2001) first identified *hemolectin* (*hml*) as a gene expressed in hemocytes, and then showed its involvement in coagulation by increased bleeding in *hml* RNAi knockdown larvae (Goto *et al*., 2003). Larval clotting factors, including Hemolectin, were later identified and confirmed with *ex vivo* clotting assays (Karlsson *et al*., 2004; Scherfer *et al.* 2004).

We then sought to study coagulation *in vivo*. Although loss of *hml* completely blocked coagulation as measured by bead aggregation and spaghetti assay (Karlsson *et al*., 2004; Scherfer *et al.,* 2004), unstaged *hml*^{f03374} larvae survived wounding as well as controls (Lesch *et al*., 2007). When larvae were wounded in the feeding stage of the third instar with fewer circulating hemocytes to participate in clotting, *hml*^{f03374} lshowed a small, but significant reduction in survival (Chang *et al*., 2012). However, the great difference between the strong effect of *hml*^{f03374} l on *ex vivo* assays of coagulation and its small effect on wound survival revealed the need for another *in vivo* assay of coagulation.

We have now developed a capillary assay that allows quick and quantitative measure of how much wounded larvae bleed as the clot and other hemostatic mechanisms work inside the animals. Thus, the assay indirectly measures coagulation inside living larvae. As expected, this assay demonstrates that larvae bleed more when wounded in the feeding stage than in the wandering stage of the third instar, and it shows a greater effect of *hml*^{f03374} than was shown by wound survival. Here, we describe the first uses of this assay, which we believe will be a welcome tool for further studies on coagulation in *Drosophila* and other insect and invertebrate species.

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Materials and Methods

Fly stocks

With the exceptions noted below, all fly stocks were obtained from the Bloomington Drosophila Stock Center. The *hml*^{f03374} strain used was the outcrossed strain described in (Chang *et al.*, 2012). Flies were kept on mashed potato, sugar, yeast medium. Larvae were staged by culturing on medium with 0.5 % bromophenol blue (Fletcher and Thummel, 1995). Feeding stage third instar larvae had visibly blue guts, while wandering stage larvae that had stopped eating had paler guts.

Capillary assay

Feeding stage third instar larvae were selected from vials of blue fly food as described in Chang *et al.* (2012). Larvae were washed in deionized water, gently dried for a few seconds on tissue paper, and placed on plastic Petri dishes. These clean dry larvae did not move very much, so they were easily manipulated. Each larva was rolled onto its side and

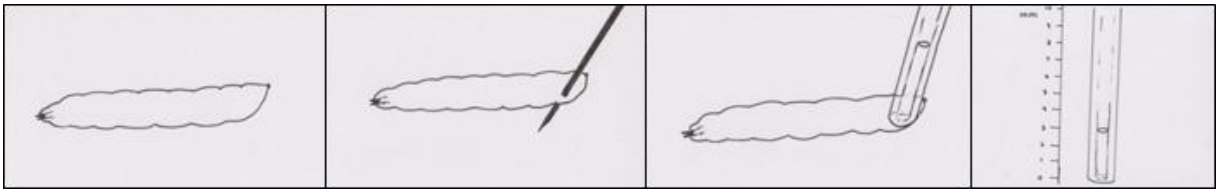

Fig. 1 Feeding stage larvae were selected, washed in deionized water, and gently dried. Larvae were wounded in the lateral caudal-most region with a fine tungsten needle. A 1 µl microcapillary tube was then used to collect the hemolymph as the animal bled. Finally, the amount of hemolymph in the tube was measured.

held with a paintbrush while a fine tungsten needle was used to wound the larva in the lateral caudal region, not striking the gut. Immediately after wounding, the needle was removed and a 1µl microcapillary tube (Drummond Scientific number 1-000-0010) was placed over the wound to collect the hemolymph as larvae bled. Some larvae moved at this point, but it was not difficult to hold the capillary over the wound and follow the larva. Hemolymph sometimes pooled around larvae, and this too was collected with the capillary tube. The capillary tube was held over the wound until the larvae stopped bleeding. This took less than 20 sec., and we could see when hemolymph stopped moving up the capillary tube and no more hemolymph pooled around the larvae. The amount of hemolymph in the tubes was measured in mm. A schematic drawing of the method is shown in Figure 1. To be clear; coagulation was not monitored in the capillary tubes. Rather, the tubes were used to measure how much hemolymph bled from wounded larvae.

Results

We found we could collect hemolymph bled from wounded larvae with microcapillary tubes as described in Materials and Methods and shown schematically in Figure 1.

We first tested the assay using wild type larvae wounded in feeding or wandering stages of the third instar. More hemocytes circulate in the wandering stage than in feeding stage larvae, so coagulation is likely to be greater and/or faster in the wandering stage. Capillary assay results confirmed that larvae wounded in the wandering stage bleed less than when wounded in the feeding stage. (Fig. 2). Then we tested whether the capillary assay would provide a larger signal for effects of the *hmlf*^{03374} mutation than displayed by wound survival. As shown in Figure 3, *hmlf*^{03374} mutant larvae bled significantly more than controls, and this difference was of greater magnitude than the slight difference found in

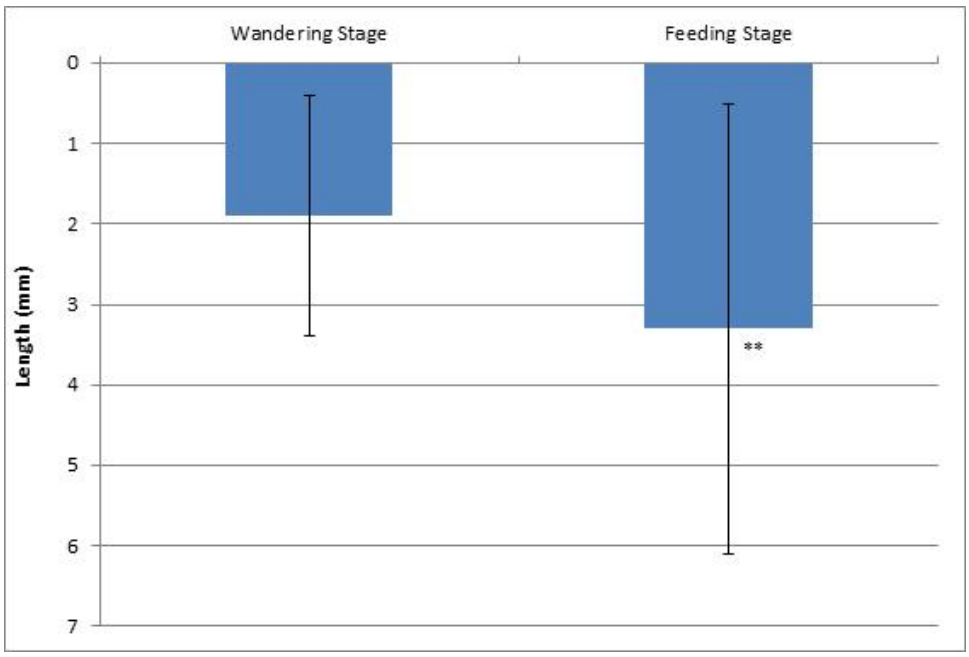

Fig. 2 Capillary assay results for feeding and wandering stages of third instar larvae. Wandering stage larvae bled significantly less ($p < 0.01$ by Student's t-test). Error bars indicate standard deviation, and n = 30 for each group.

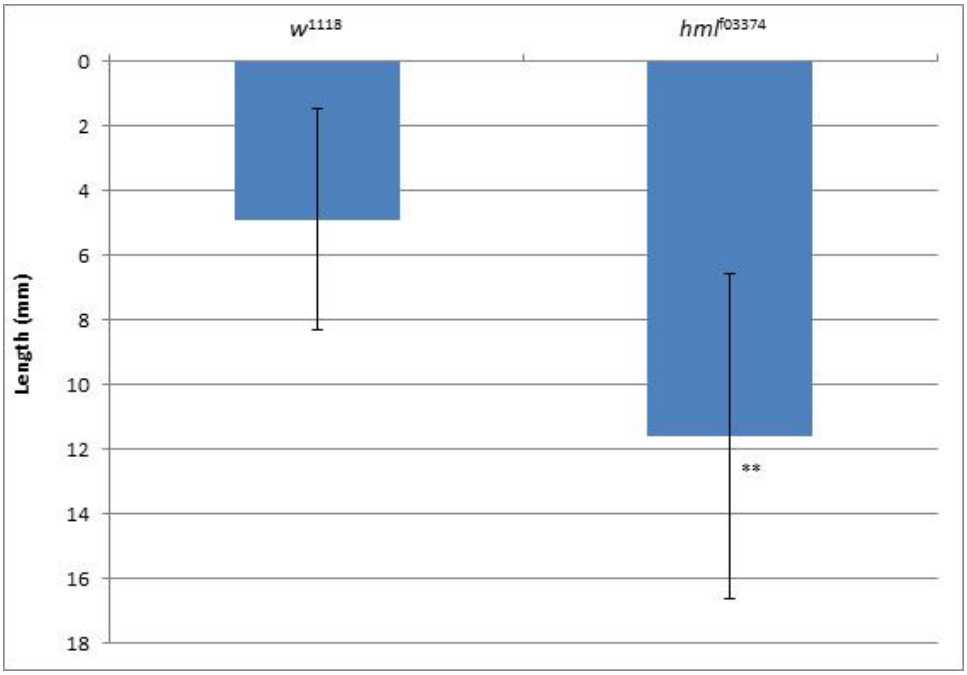

Fig. 3 Capillary assay results for *hml*^{f03374} and w^{1118} wildtype. As expected for a strong coagulation mutant, more hemolymph was collected from *hml*^{f03374} ($p < 0.01$ by Student's t-test). Error bars indicate standard deviation, and n = 30 for each group.

wound survival (Chang *et al.*, 2012). These results confirmed the capillary assay as a valid and useful measure of coagulation.

Next, we tested the effects of removing hemocytes completely. In *Drosophila*, apoptosis can be induced by the expression of *grim* (Chen *et al.*, 1996). We employed the UAS GAL4 system (Brand and Perrimon, 1993) and drove the expression of *grim* in hemocytes by setting crosses to generate *hml*Δ*GAL4UAS::GFP>>UAS::grim* larvae (Charroux and Royet, 2009). GFP-expressing hemocytes fluoresce in *hml*Δ*GAL4UAS::GFP* larvae (Fig. 4A). In contrast, this fluorescence is not visible in larvae bearing both *hml*Δ*GAL4* and *UAS::grim*, indicating the loss of hemocytes by apoptosis. Capillary assays performed on these larvae showed increased bleeding comparable to *hmlf*^{03374} mutant larvae (Fig. 4B). The bleeding defect of larvae lacking hemocytes reinforced the value of the capillary assay for measuring coagulation *in vivo*.

Finally, we tested *Bc* larvae lacking phenoloxidase. This enzyme is known for its role in invertebrate immune defense (Cerenius *et al.* 2008), although the importance of phenoloxidase in *Drosophila* immune defense has been contested (Leclerc *et al.,* 2006). The mature clot is melanized by phenoloxidase, and the enzyme is thought to play a late role in coagulation (Bidla *et al.,* 2007), as well as in subsequent wound healing (Galko and Krasnow, 2004). While loss of phenoloxidase in *Bc* mutant larvae reduced wound survival (Chang *et al.*, 2012), its effect on bleeding specifically was not tested at that time. The capillary assay showed that *Bc* mutant larvae bled more than controls, but much less than *hml* mutant larvae (Fig 5).

Discussion

In vivo studies are needed to learn more about coagulation in *Drosophila* and other insects. Wound survival has been tried, but it is not specific to coagulation, as survival is also affected by other still-poorly understood hemostatic processes, immune defense, and wound healing. In addition, the high wound survival rate of strong clotting mutant *hmlf*^{03374} showed survival alone was a poor measure of coagulation. We developed a capillary assay to better monitor coagulation *in vivo*. This assay showed that *Drosophila* larvae bleed more after wounding in the feeding stage of the third instar than in the wandering stage, in line with the change in numbers of hemocytes circulating in the different larval stages. The capillary assay also showed greater magnitude effects of *hmlf*^{03374} on bleeding, more consistent with tests of coagulation *ex vivo* than its slight effect on wound survival.

Hemolymph coagulation requires both humoral and cellular factors. We tested *hml*ΔGAL4 *UAS::GFP*>>UAS::*grim* larvae lacking hemocytes to measure the limits of the capillary bleeding assay. We do not argue that all hemocytes were completely removed from these larvae: only the loss of *hml*-expressing cells as demonstrated by absence of GFP fluorescence. Regardless, the similar amounts of bleeding by these larvae and *hmlf*^{03374} mutants is

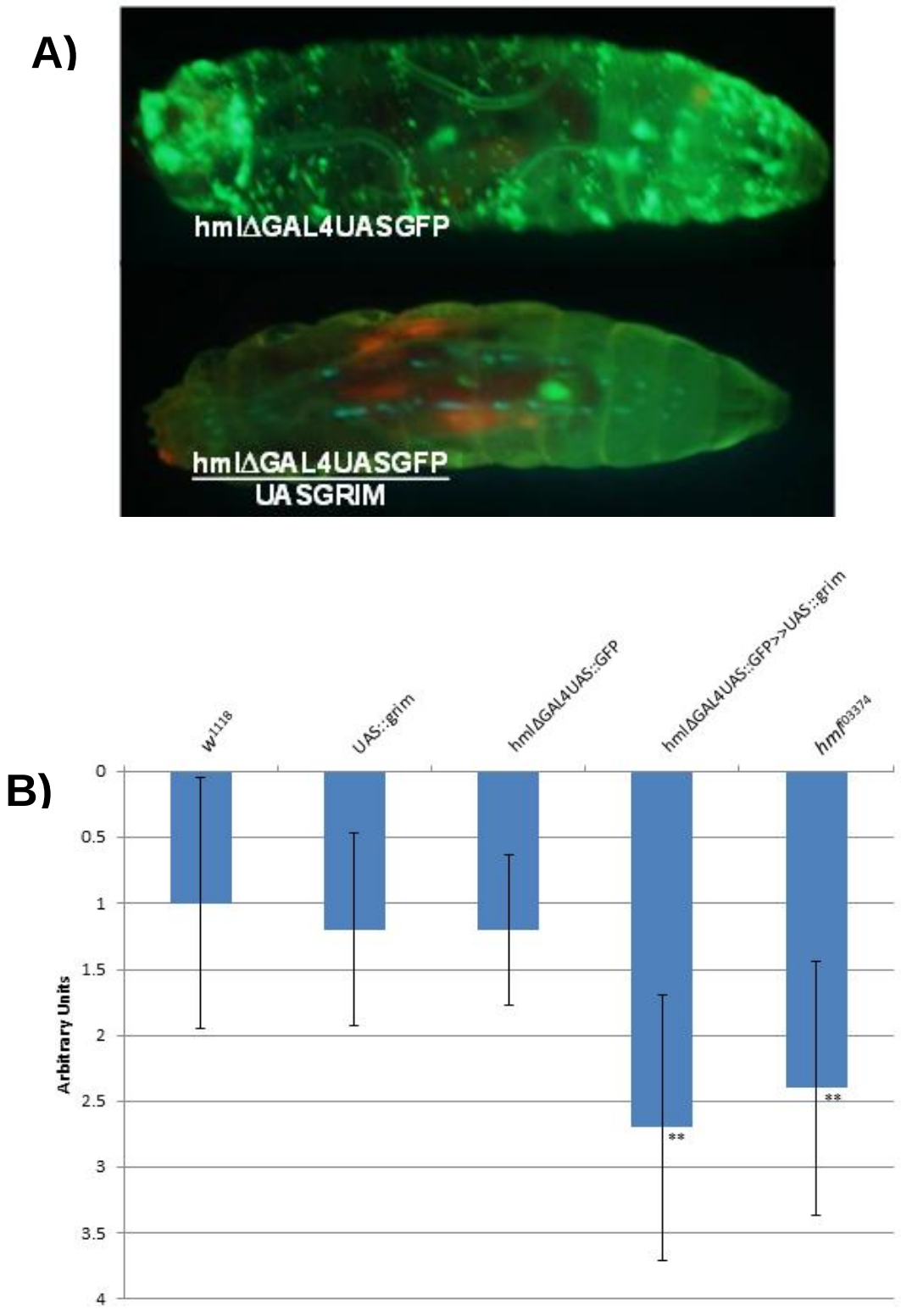

Fig. 4 A) Green Fluorescent Protein-expressing hemocytes can be seen in *hml*Δ GAL4UAS::GFP larvae (top). Apoptosis was induced by expressing the *grim* gene using the binary GAL4 UAS system. The hemocytes were killed and the fluorescence lost in larvae expressing the *grim* cell death gene in *hml*Δ GAL4 UASGFP>>UAS::*grim* larvae (bottom). **B**) Capillary assay results for larvae expressing *hml*Δ GAL4 UASGFP>>UAS::*grim*. The mean of the capillary measurements of w^{1118} was set to 1, and the capillary values collected for the other genotypes were set relative to this. The expression of the *grim* cell death gene led to the death of hemocytes and an increase in bleeding. The values for *hml*Δ GAL4 UASGFP>>UAS::*grim* and hml^{f03374} were statistically indistinguishable. The differences between the controls, w^{1118}, *hml*Δ GAL4 UASGFP, and UAS::*grim,* were also not statistically significant. The *hml*Δ GAL4 UASGFP>> UAS::*grim* and hml^{f03374} larvae bled significantly more than w^{1118} ($p < 0.01$ by Student's *t*-test), indicated by asterisks. Error bars indicate standard deviation, and n = 30 for each group.

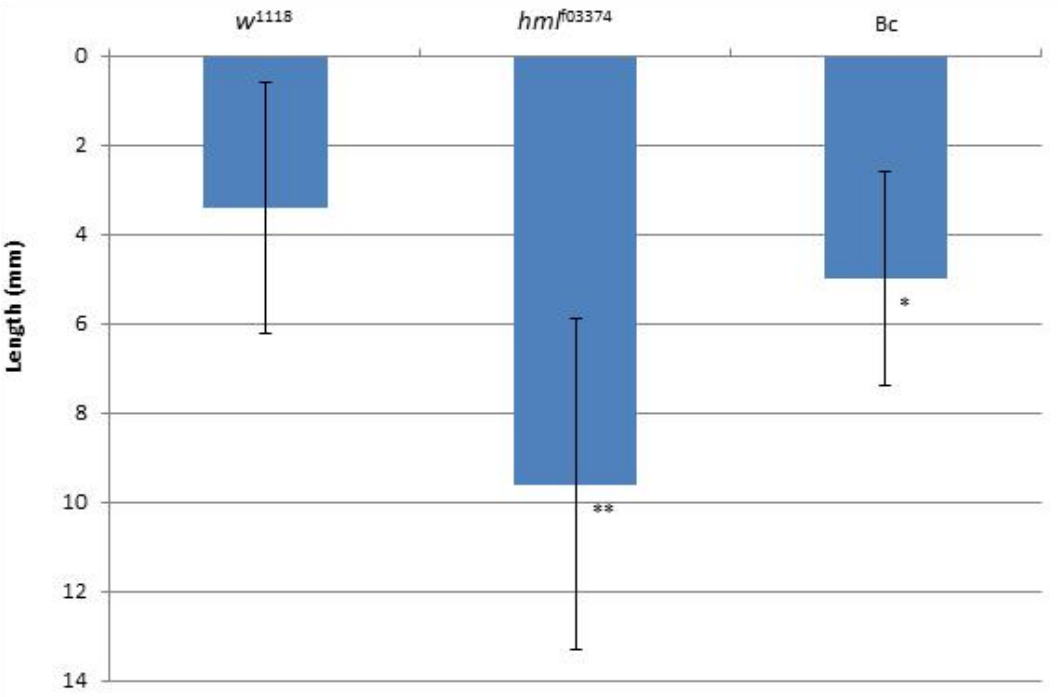

Fig. 5 Capillary assay results for *Bc* larvae lacking phenoloxidase. *Bc* larvae bled significantly more than controls ($p < 0.05$ by Student's *t*-test), but less than *hml*^{f03374} mutants, whose greater difference from controls ($p < 0.01$ by Student's *t*-test) is indicated by double asterisks.

consistent with Hemolectin being a major hemocyte coagulation factor, since loss of hemocytes did not markedly increase bleeding compared to loss of *hml*.

Finally, the capillary bleeding assay showed that wounded *Bc* larvae bled more than wildtype, but less than *hml*^{f03374} mutants. This too is consistent with current models of *Drosophila* coagulation, and suggests that much of the effect of *Bc* on wound survival is due to loss of phenoloxidase and defects in plug formation and wound healing, similar to the effects of comparable phenoloxidase blocking mutation *lz*^{15} (Galko and Krasnow, 2004).

The capillary assay has advantages over wound survival as a means to measure coagulation *in vivo*. The capillary assay yields results in one day, unlike the wounding assay, which takes twice as long. Also, whereas wound survival may be affected by a variety of processes that may add to or obscure the effects of mutations on clotting, capillary assay results are more specific to coagulation and hemostasis. This was highlighted by our study of *hml*^{f03374} mutants. This mutation that abolished coagulation in *ex vivo* assays only reduced larval wound survival by 5 %. In contrast, the capillary assay showed that *hml*^{f03374} mutant larvae bled twice as much as wildtype. The capillary assay thus provides a robust *in vivo* measure of coagulation that is more specific, easier, and faster to perform than wound survival. This assay should be easy to adapt to other small invertebrates.

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