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Nanoliter Hemolymph Sampling and Analysis of Individual Adult *Drosophila melanogaster*

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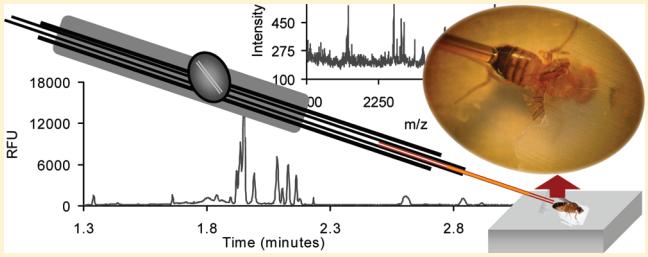
Supporting Information

ABSTRACT: The fruit fly (*Drosophila melanogaster*) is an extensively used and powerful, genetic model organism. However, chemical studies using individual flies have been limited by the animal's small size. Introduced here is a method to sample nanoliter hemolymph volumes from individual adult fruit-flies for chemical analysis. The technique results in an ability to distinguish hemolymph chemical variations with developmental stage, fly sex, and sampling conditions. Also presented is the means for two-point monitoring of hemolymph composition for individual flies.



The *Drosophila melanogaster* is an exceedingly important model system in genetics, neuroscience, and developmental biology. Its use in investigations in both basic science and human pathologies[1−4] is primarily due to its ease of genetic manipulation. As with clinical blood analysis, the measurement of the chemical content of fly hemolymph can provide powerful insights. However, the *Drosophila* hemolymph chemical composition has never been well characterized due to the exceedingly small size of the fly and low hemolymph volumes. Homogenization of populations or individual body parts has been employed to achieve sample volumes large enough for chemical analyses.[5−8] However, homogenization mixes all body fluids and induces biochemical changes that could obscure important chemical phenotypes. New techniques for hemolymph sampling and analysis could also enable analysis of individual flies, which would be extremely valuable for analysis of rare genotypes or following any sort of complex experimental treatment or behavioral selection.

The hemolymph collection from fruit-fly larvae has been reported.[5,6,9,10] During the third instar larval stage, the fly is wormlike and is the largest body-size over the life course of the fly thereby simplifying collection of the hemolymph. However, the study of the larval fly hemolymph has serious problems. For example, previous methods include the use of dissected populations of larvae placed in larger solution volumes to collect leaking hemolymph. Accurate quantitation is complicated due to the unknown total volume of hemolymph from each larva. The previous method presented by us for larvae allowed measurement of the hemolymph (175 ± 84 nL) for accurate quantitation.[11] However, the larval-hemolymph sampling technique, which is suitable for collecting volumes greater than 50 nL, is not capable of collecting hemolymph from the adult fruit flies for accurate analysis. The methods presented here are for hemolymph sampling of individual adult fruit flies that enables accurate hemolymph chemical analysis. This is important, because larval hemolymph chemical information may not be directly correlated to that of adult flies and, consequently, to fly physiology or behavior. For example, larvae are reported to display more robust hemolymph clotting compared to adults.[10,12,13] An improved sampling method would enable monitoring hemolymph of the same fly at a minimum of two different time points. A means to describe compositional changes in the hemolymph for a single fly following an experimental perturbation provides a powerful new tool to study physiological differences resulting from genetic mutations. In addition, the developed technique was used for studying fly hemolymph collected under different conditions for their variations in content.

EXPERIMENTAL SECTION

Chemicals. All chemicals used were of analytical grade or better. HPLC-grade acetone, sodium tetraborate decahydrate, trifluoroacetic acid (TFA), α-cyano-4-hydroxycinnamic acid (CHCA), isopropanol, acetonitrile, histamine dihydrochloride, and α-amino-*n*-butyric acid were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Nitrocellulose protein binding membranes, pore size 0.22 μm, were purchased from Osmonics Inc. (Minnetonka, MN). All other amino acids, fluorescamine, sodium dodecyl sulfate (SDS), and sodium hydroxide were obtained from Fisher Scientific (Itasca, IL). A US Filter Purelab

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Plus purification system (Lowell, MA) was used to obtain ultrafiltered-deionized water for preparing all solutions. Untreated fused-silica capillaries with 100, 75, and 50 μm inner diameter (i.d.) and 360 μm outer diameter (o.d.) were purchased from Biotaq Inc. (Gaithersburg, MD), and 250 μm and 784 μm i.d. Tygon tubes were purchased from Cole-Parmer (Vernon Hills, IL).

Sodium tetraborate decahydrate was dissolved in ultrafiltered deionized water to prepare the 20 mM borate run buffer and its pH was adjusted to 9.1 with 0.5 M NaOH. All buffer solutions were sonicated for 5 min prior to use. Fluorescamine was dissolved in acetone to prepare the 15 mg mL^{-1} solution for derivatization. All standard amino acid solutions were prepared in 20 mM borate buffer solution and diluted appropriately with run buffer to the desired concentrations.

Sampling-Probe Construction. The sampling probe, previously described and utilized by us to obtain larval-hemolymph,[11,14] is further developed to enable nanoliter volume fly hemolymph collection. Briefly, a 4 cm long 360 μm outer diameter fused silica capillary piece was connected to a 60 cm long, 250 μm i.d. Tygon-tube piece. The open end of the 250 μm i.d. Tygon-tube was then inserted into an 8 cm long 784 μm i.d. Tygon-tube housing and was connected to a vacuum-pump (Barnant Co., Barrington, IL), a regulator (Squire-Cogswell, Gurnee, IL), and a digital pressure gauge (Vacuubrand DVR 2, Wertheim, Germany). As shown in Figure 1a, the Tygon-tube assembly was mounted on an electrode holder (World Precision Instruments Inc., FL) and was adjusted such that about 3 cm of the fused silica capillary was cleared from one end of the 784 μm i.d. Tygon-tube housing.

The whole sampling probe was mounted on a three-dimensional micropositioner (model Tauruser-B, World Precision Instruments Inc., FL) to facilitate the movement of the sampling probe to focus on the anterior abdominal surface of the fly placed under a dissection microscope (Leica MZ6, Heerbrugg, Switzerland).

Evaluation of Fused Silica Capillary Sampling Probes. A standard amino acid mixture of Arg (1.3 mM), Gln (20.3 mM), Tau (2.6 mM), Glu (0.45 mM), and Asp (0.21 mM) resembling the previously published larval hemolymph[11] was prepared. The standard mixture was then sampled (about 25 nL) with fused silica capillary probe tips with 100, 75, and 50 μm i.d. (360 μm o.d.). The sample-plug lengths were measured and diluted 50-, 75-, 100-, and 125-fold prior to the capillary electrophoretic analysis. The precision of the measurement of each amino acid with the three different inner diameters was compared to assess the error contribution due to sample probe tip i.d.

Fly-Sampling Conditions. Initially groups of flies, in closed, precooled 15 mL graduated polystyrene tubes, were kept in the freezer (at −20 °C) for 5.5, 8.5, 9, and 10 min to estimate their wake up times which were 2, 4.5, 5.5, and 8 min, respectively. Then flies for three different sampling conditions, cold-anesthetized, unanesthetized, and unanesthetized pinched, were prepared according to the anesthetization and corresponding recovery times. The three fly sampling conditions are as illustrated in Figure 1b. In the anesthetized fly sampling condition, a fly in a closed precooled 15 mL graduated polystyrene tube was initially placed in the freezer at −20 °C for 8.5 min (Figure 1b). Then the anesthetized fly was prepared for sampling by adhering it to a stainless steel block with a piece of tape and cutting the wings. This preparation step usually

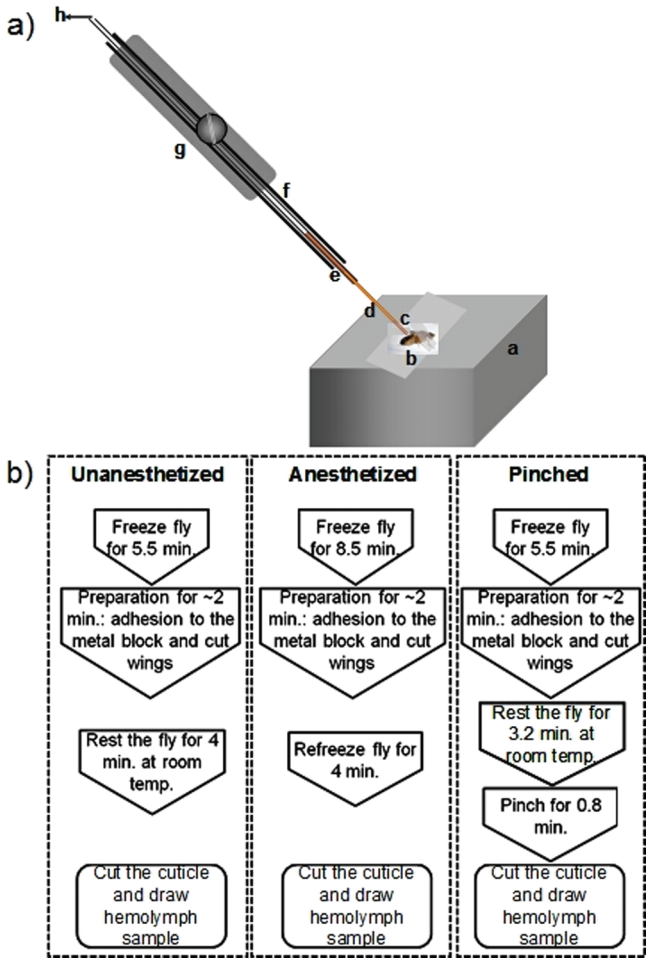

Figure 1. (a) Schematic diagram of fly hemolymph-sampling setup: a, stainless steel block; b, dissected fly on inverted scotch tape; c, hemolymph sample plug inside the fused silica capillary; d, 50 μm i.d. fused silica capillary; e, 250 μm i.d. Tygon tube; f, 784 μm i.d. Tygon tube housing; g, electrode holder; h, to regulator, display, and vacuum pump. (b) Summarized flowchart of fly preparation for the three different fly sampling conditions.

takes about 2 min. The prepared fly was kept at −20 °C for another 4 min prior to sampling to ensure that it is anesthetized during the sampling process. In the unanesthetized sampling condition, the fly was initially kept in the freezer at −20 °C for 5.5 min to limit their locomotive behavior from a cold-shock to aid preparation (Figure 1b). Then the fly was kept at room temperature for 4 min allowing it to wake up before sampling. For the unanesthetized pinched procedure, the flies were incubated at −20 °C for 5.5 min, prepared for 2 min, kept at room temperature for 3 min and 10 s, followed by pinching for 50 s with a microdissection pin at room temperature prior to sampling. Only male fruit flies were utilized when studying the effect of different hemolymph-sampling conditions on fly hemolymph amino acid variations, while both male and females were used in the other parts of the study.

Hemolymph Sampling. The wildtype flies used in this study were based on the *Oregon R* strain reared on standard cornmeal-agar medium and maintained in the Department of Biology at UIC. A piece of tape was secured to a stainless steel block with the adhesive side of the tape exposed. Preparation of the fly for sampling included sticking the immobilized fly to the tape (Figure 1a), careful cutting of $^3/_4$ of each wing with a

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microdissection scissor followed by placing a second piece of tape over the head-thorax area of the fly to hold it down firmly to the steel block during the sampling process. The steel block with the fly was placed under the microscope, and a tiny incision was then made between the second and first tergites on the dorsal side of the fly abdomen with a microdissection scissor. The sampling probe tip was gently pushed against the incision (Figure 1a), and the leaking hemolymph was pulled into the capillary probe-tip with the regulated vacuum pressure. The silica capillary probe tip with a 2 cm long 250 μm Tygon tube piece was pulled from the Tygon tube housing and cut following sample collection. The collected hemolymph volume was determined by measuring the sample plug length in the capillary by aligning it with an electronic digital caliper (World Precision Instruments Inc., FL) under the microscope. The Tygon tube end of the sample-containing capillary-Tygon piece was connected to a 1 mL syringe (Becton Dickinson and Co., Franklin, NJ) to dispense the sample into a 250 μL centrifuge tube (Fisher Scientific, Itasca, IL) that contained the required volume of borate buffer for dilution for the capillary electrophoretic analysis. The diluted sample was mixed well and centrifuged (Clay Adams, NJ) at about 3000 rpm at room temperature for 2 min prior to freezing at −20 °C until analysis.

Two Hemolymph Samples from One Fly. Two male fly groups were utilized for the experiments described in this section where two hemolymph samples were collected from a single fly under two different sampling conditions. The first hemolymph sample from a fly of group 1 was obtained under an unanesthetized condition similar to as illustrated in Figure1b. The fly was initially incubated at −20 °C for 5.5 min, adhered to the stainless steel block (2 min), and kept at room temperature for 4 min prior to collecting the first sample under the unanesthetized condition. After collecting the first sample, the fly adhered to the stainless steel block was returned to the freezer for 8.5 min prior to collecting the second sample under cold-anesthetization. The sampling procedure used for group 2 flies was the reverse of the group 1 procedure. Briefly, the first sample was collected from the group 2 fly under cold-anesthetized conditions as illustrated in Figure1b. Then the fly was kept at room temperature for 8.5 min prior to collecting the second hemolymph sample under the unanesthetized condition. A narrow single incision was made on the fly exoskeleton to gather both hemolymph samples of groups 1 and 2 (n = 7, each group).

Capillary Electrophoresis and MALDI-TOF-MS Analysis. The frozen diluted samples were thawed and were derivatized with 15 mg mL^{-1} fluorescamine solution prior to analyses by mixing equal volumes (4 μL) of fluorescamine solution and amino acid standards or diluted hemolymph in a 250 μL centrifuge tube (Fisher Scientific, Itasca, IL). All samples that were collected and diluted the same day were derivatized and analyzed together.

The analysis procedure for the derivatized hemolymph with the laser induced fluorescence detection (CE-LIF) is same as previously described by Piyankarage et al.[11] In brief, separations were performed on untreated 50 μm i.d., 48 cm fused-silica capillary with a 1 cm detection window at an effective length of 34 cm. The derivatized samples or amino acid standards were injected hydrodynamically, and a separation voltage of 30 kV was applied with the 20 mM borate run buffer. The analyte peaks were identified by spiking with 24 different amino acid standards. Quantitation was performed with external calibration curves after verification of the statistical similarity of levels determined by the method of standard additions.

For the MALDI-MS analyses, the collected hemolymph samples were diluted with 1% TFA solution and the blanks contained only 1% TFA. The diluted sample was mixed with a 3 mg mL^{-1} CHCA matrix in acetonitrile and TFA solution (70:30, v/v), followed by depositing 1 μL of the sample-matrix mixture onto the stainless steel MALDI plate (Applied Biosystems, Foster City, CA) for the conventional thin layer method.[13] For the microspotting technique described elsewhere by us,[15] a 50 mg mL^{-1} CHCA matrix mixture was made in a 20 mg mL^{-1} nitrocellulose solution in 1:1 acetone and isopropanol. The CHCA-nitrocelleulose matrix mixture was then deposited on the MALDI plate with a 400 μm pipet tip (Sigma-Aldrich Corp., St. Louis, MO) followed by applying 0.5 μL of the diluted hemolymph sample onto the matrix spot. After 2 min, the excess sample volume was reverse pipetted from the MALDI plate and the sample-applied matrix spot was washed with 5 μL of 1% TFA. Similarly the blanks without the hemolymph were spotted using the conventional and the microspotting techniques. The analyses were performed with the Voyager-DE PRO MALDITOF spectrometer (Applied Biosystems, Foster City, CA) in linear mode.

Data Analysis. The raw data were imported to Microsoft Excel to plot electropherograms and box and whisker plots and to perform quantitation. Statistical comparisons for hemolymph amino acids among different fly sampling conditions were performed with the Statistix software using one-way analyses of variance (ANOVA), and the means were compared by the least significant difference method with a confidence level of 95% (P < 0.05). All other hemolymph comparisons were carried out with Microsoft Excel using a two-tailed Student's t test with a confidence level of 95% (P < 0.05). The reported mean values are followed by the corresponding standard deviations (±).

RESULTS AND DISCUSSION

Nanoliter Volume Measurements with Sampling Probes. Using our techniques, approximately 25 nL of hemolymph can be readily collected from a dorsal abdominal incision of an adult fruit fly. The flies were observed to be alive for about 2 h after the sample collection. This shows that withdrawing about 25 nL of hemolymph can be considered nonlethal. Hence this method was expanded for collecting two hemolymph samples from an individual fly before and after experimental treatments. The limited volume available for collecting two samples from the same fly required that the volume be kept below 10 nL to provide similar sample volumes for subsequent analysis. Consequently the sampling probe design incorporated the capability of accurate collection of volumes below 10 nL. A segment of fused silica capillary with 360 μm o.d. facilitated placement of the capillary aperture over the incision for collection. The hemolymph is directly pulled into the capillary using negative pressure from a rough vacuum pump. The capillary also functions to contain the hemolymph while minimizing evaporation of the collected nanoliter sample. Quantitative determinations of chemical content require the measurement of these nanoliter volumes. Volume is determined by measuring the fluid length in a capillary of known i.d. Alignment of the sample-filled capillary with an electronic digital caliper under the microscope in obtaining the hemolymph-plug length measurement improved the accuracy of the measured volumes. The precision of volume measurements is further improved by using a lower i.d. capillary which

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increases the sample length for a given volume compared to a larger i.d. capillary. For instance, with the smallest measurement of caliper being 0.050 mm, a small volume of 0.1 nL can be accurately measured with a 50 μm i.d. capillary segment (1.96 nL/mm). A standard amino acid mixture model of fly hemolymph was sampled with 50, 75, and 100 μm i.d. capillaries to assess quantitative precision. Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) of the collected samples showed that the 50 μm i.d. capillary gave the most precise amino acid concentrations (see Table 1).

Table 1. Comparison of Fused Silica Capillary Sampling Probe Tips of Three Different i.d. with a Standard Amino Acid Mixture[a]

	100 μm[b] (n = 7)	75 μm[c] (n = 7)	50 μm[d] (n = 7)
average volume (nL)	29.9	24.0	25.2
range (nL)	38−11	35−12	36−12
Arg (RSD %)	13.6	5.6	6.0
Gln (RSD %)	13.3	5.2	4.9
Tau (RSD %)	9.6	8.1	4.8
Glu (RSD %)	14.0	9.8	6.7
Asp (RSD %)	16.1	8.6	8.9

[a]RSD%: percentage relative standard deviation for n measurements. Sample plug volumes per unit length are [b]7.86 nL/mm, [c]4.42 nL/mm, [d]1.96 nL/mm.

Capillary segments with 50 μm i.d. hold 1.96 nL/mm and provide 5−9% RSD via CE-LIF determinations. Hence, 360 μm o.d. capillaries with 50 μm i.d. were selected as the nanovolume hemolymph sampling probe tips (Figure 1a).

Fly Hemolymph Sample Volumes and Qualitative Amino Acid Analysis. With the 50 μm i.d. capillary probe-tip, hemolymph was collected from 18 adult flies at volumes ranging from 8 to 45 nL with an average of 24.8 (±11.1) nL. Sampled volumes track relative body size. Male flies are $^1/_3$ smaller than females and, collection from male flies led to sample volumes (19.1 ± 46% nL) that were 1.6 times less than those from females (31.3 ± 31% nL). Male fly hemolymph samples are also 9 times lower than from larvae (175 ± 48% nL). This significantly low hemolymph sample volume from individual flies required the modification of previously developed larval sampling probes for this small volume compatibility.

Qualitatively, adult hemolymph amino acid electropherogram profiles are similar to those for larvae,[11] but a couple of differences are also seen. Figure 2 is a representative electropherogram of 100-fold diluted adult fly hemolymph

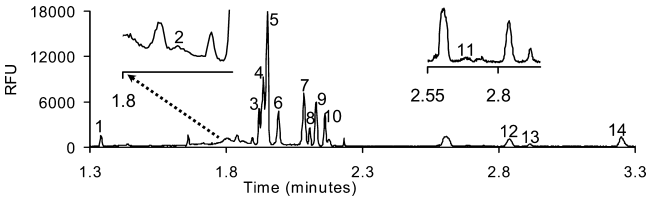

Figure 2. Electropherogram for separation of fluorescamine-labeled amino acids of 100-fold diluted wildtype adult fruit fly hemolymph in 20 mM borate buffer (pH 9.1) at 30 kV in 48 cm bare fused-silica capillary with 50 μm i.d. and 360 μm o.d.: (1) arginine, (2) citrulline, (3) tyrosine, (4) histidine, (5) glutamine, (6) asparagine and threonine, (7) alanine and serine, (8) taurine, (9) lysine, (10) glycine, (11) cystine, (12) glutamate, (13) aspartate, (14) unknown.

derivatized with the primary amine reactive fluorophore, fluorescamine. The amino acids citrulline and cystine are found in measurable levels in adult hemolymph in addition to the nine amino acids resolved previously from third instar larval hemolymph.[11,14] Also, an unknown hemolymph component (peak 14) was dramatically smaller for the adult flies.[11,14] Quantitative determinations showed significantly (P < 0.05) higher levels of glutamate (Glu) and lower levels of glutamine (Gln), glycine (Gly), and taurine (Tau) compared to that previously seen with larvae (Table S-1 in the Supporting Information).[11]

Quantitative Amino Acid Analysis of Hemolymph. Direct measurement of adult hemolymph content from individuals demonstrates information not available via previous methodologies. A collection of hemolymph amino acid composition assays from individual flies is used to describe population distributions of amino acid concentrations. Clear differences between male and female flies are exposed. A box and whisker plot in Figure 3 compares the hemolymph amino

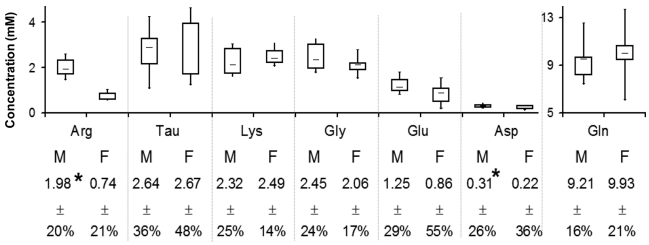

Figure 3. Box and whisker plots showing the variations of seven hemolymph amino acids in male (M, n = 9) and female (F, n = 9) fruit flies. The top and the bottom of the box show the upper and the lower quartiles, respectively, and the horizontal line in the middle indicates the median of the corresponding distribution while the minimum and maximum observed values are the bars connected to the box. The average levels with the percentage standard deviations are provided below the horizontal axis. *At P < 0.05, male and female are significantly different.

acid concentration distributions for adults of both sexes. The arginine (Arg) and aspartate (Asp) concentration distributions for males are significantly (P < 0.05) higher than that of females, 167% and 41%, respectively. As arginine phosphate is used as a phosphogen in insects,[16] arginine may be generated during activity and these results may reflect a wider distribution of activity among the sampled male flies. The female-hemolymph Glu and Tau concentrations show the widest distributions with RSDs over 45%, although median levels are similar between the sexes. The source of this variation is not known.

In order to properly interpret population-averaged hemolymph content data from the last 5 decades,[5,6,8−10] experiments were performed to determine how hemolymph content is affected by sampling conditions. Our previous work with larvae sampled under different conditions demonstrated several significant hemolymph amino acid concentration differences.[14] For instance, the pinched larvae showed significant Glu and Arg levels in the hemolymph which were related to the larval stress and activity.[11,14] Hence, it is interesting to study the fly hemolymph collected under similar pinched conditions. Cold anesthetization (at −4 to −20 °C) is the most common anesthetization method and is assumed to be the least physiologically traumatic method compared to others such as exposure to CO_2 or diethyl either. Therefore, investigating fly

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hemolymph collected under cold-anesthetized and unanesthetized conditions would provide important insights of adult flies. Only male flies were utilized in studying the hemolymph chemical variations under different sampling conditions due to the differences between the male and female flies in hemolymph contents and volumes discussed in previous sections.

Table 2 shows hemolymph amino acid levels of flies collected during three different sampling conditions. The first group of flies was cold-anesthetized; the second group was unanesthetized; the third group was unanesthetized and pinched similar to our previous studies with larvae.[14] Pinched flies display 31% higher hemolymph Arg concentrations compared to non-pinched groups, and this is in line with larval studies.[11,14] Elevated hemolymph Arg may be the result of the notably increased movements during the pinching stress. The increased Arg may be related to observed activity and released as a result of the cleavage of arginine phosphate.[16] The pinched fly group also showed the highest hemolymph Tau level in contrast with the previous larval study.[14] Hemolymph biochemistry may, therefore, be substantially different between larvae and adults.

Table 2. Average Hemolymph Amino Acid Concentrations (mM ± Standard Deviation) of Individual (n) Wild-Type III Flies Treated through Three Different Conditions Prior to Sampling

	cold-anesthetized[a] (n = 9)	unanesthetized[b] (n = 9)	unanesthetized and pinched[c] (n = 9)
averge volume (nL)	19.8 ± 8.9	17.4 ± 6.3	15.4 ± 7.7
Arg	1.98[b] ± 0.40[d]	2.04[b] ± 0.33[d]	2.67[a] ± 0.75[d]
Gln	9.21 ± 1.53	10.7 ± 3.2	12.5 ± 5.1
Glu	1.25[a] ± 0.36[d]	0.69[b] ± 0.32[d]	0.91[b] ± 0.27[d]
Gly	2.45 ± 0.60	2.53 ± 0.71	2.56 ± 0.58
Lys	2.32 ± 0.59	2.05 ± 0.46	2.04 ± 0.73
Tau	2.64[b] ± 0.98[d]	2.83[ab] ± 0.41[d]	3.41[a] ± 0.78[d]

[a]8.5 min in freezer, 2 min preparation followed by 4 min in freezer prior to sampling. [b]5.5 min in freezer, 2 min preparation, and 4 min waiting for flies to wakeup prior to sampling. [c]5.5 min in freezer, 2 min preparation, 3 min 10 s waiting followed by 50 s pinching prior to sampling. [d]Values for a given amino acid followed by different letters are different at a $P < 0.05$, e.g., for Tau the first and third groups are different while the second group is not different either from the first or third (one way ANOVA).

The hemolymph-Glu level for the cold-anesthetized fly group is significantly elevated, while the pinched and unanesthetized groups show the second highest and the lowest Glu levels, respectively. Previous studies have shown that the stress level of the animals correlate with the variation of amino acid levels such as Glu,[17,18] which also is a neurotransmitter. Our previous work also suggests that elevated hemolymph Glu levels could also be linked with stress in fruit-fly larvae.[11,14] While further study is needed including sampling during alternative anesthetization techniques, these data suggest that cold-anesthetization for the fly appears to generate a substantial physiological response and must be considered when interpreting data from cold-anesthetized flies. Among the three tested sampling conditions, the least affected hemolymph amino acid levels are shown for the unanesthetized condition. Analysis of hemolymph collected under both unanesthetized and anesthetized conditions from the same fly would be the ideal experiment, and the temporal sampling capability of the newly introduced technique enables one to perform this experiment.

Collecting More than One Sample from a Fly. A more powerful tool to relate hemolymph chemical composition to fly physiology and behavior would provide the ability to determine relative content differences from a single fly before and after an experimental perturbation. A study was then performed to demonstrate collection of two nanoliter-volume hemolymph samples from the same fly at two different time points. The experimental demonstration employed two groups of male flies where samples were collected from both unanesthetized and cold-anesthetized flies. In group 1, flies (n = 7) were unanesthetized for the collection of the first sample (10.1 ± 3.9 nL) followed by cold-anesthetization prior to collection of the second sample (9.2 ± 4.6 nL). In group 2 (n = 7), the first sample (6.4 ± 2.0 nL) was collected under cold-anesthetized conditions and the second sample (8.3 ± 5.7 nL) was collected under unanesthetized conditions, after allowing the fly to recover from anesthetization.

Although the technique of collecting two samples from a single fly is designed to be less invasive, the physiological condition of a fly may be different before and after collecting the first sample. Hence, in order to understand the effects of the two sampling conditions on hemolymph amino acids, two fly groups were used for the sampling where the sequence of the two sampling conditions used in group 1 is switched in group 2. If a significant difference in hemolymph amino acid content is observed in group 1, a significant difference reverse to that with the switched conditions should be observed in group 2. This

Table 3. Hemolymph Amino Acid Comparison of Two Samples Collected under Two Different Conditions from the Same Fly

	group 1 (n = 7)		group 2 (n = 7)	
	unanesthetized[a]	cold-anesthetized[b]	cold-anesthetized[c]	unanesthetized[d]
volume (nL)	10.1 ± 3.9	9.2 ± 4.6	6.4 ± 2.0	8.3 ± 5.7
Arg (mM)	1.7	2.5[ae]	1.6	2.5[be]
Gln (mM)	11	16[ae]	10	16[be]
Glu (mM)	2.0	2.8[ae]	1.8	2.6[be]
Gly (mM)	2.0	3.1[ae]	2.2	3.1[be]
Lys (mM)	2.5	3.0[ae]	2.2	2.9[be]
Tau (mM)	0.9	1.4[ae]	1.1	1.4[be]

[a]5.5 min in freezer, 2 min preparation followed by 4 min waiting for flies to wakeup prior to first sample. [b]8.5 min in freezer since the first sampling and prior to second sample. [c]8.5 min in freezer, 2 min preparation followed by 4 min in freezer prior to first sample. [d]8.5 min at room temperature since the first sampling and prior to second sample, *. [e]Superscripts **a,b** show a significant difference at $P < 0.05$ within groups 1 and 2, respectively.

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approach enables assessment of effects of the two sampling conditions while controlling for physiological effects of sampling condition order.

Table 3 shows no significant difference between the average volumes of the first and the second samples within group 1 (between unanesthetized and cold-anesthetized) or group 2 (between cold-anesthetized and unanesthetized). However, irrespective of the group, the second sample always shows significantly higher amino acid levels (Table 3). Individual fly amino acid concentrations for the first and the second samples of both groups 1 and 2 displayed in Figures S-1 and S-2 in the Supporting Information similarly follow this trend. After collecting the first sample, a fraction of the remaining hemolymph in the fly can be evaporated through the incision and this could be the main reason for the significant amino acid increase observed for the second sample in both groups. Interestingly, the increase of hemolymph amino acid concentration within groups 1 and 2 ranged from 20% to 60% and 30−55%, respectively. This indicates the increases may also include amino acid variations due to reasons other than evaporation. The impact of the sampling procedure is not well understood. To control for the large background increase in absolute amino acid concentrations, the relative increase between the first and second samples was calculated by subtraction. The following equation was used for calculating the relative average increase for each amino acid within groups 1 and 2.

$$\text{average increase} = \frac{\sum_{n=1}^{n} \{([\text{2nd sample}] - [\text{1st sample}])\}}{n}$$

The amino acid concentrations for the first and the second samples from individual flies in both groups 1 and 2 are shown in Figures S-1 and S-2 in the Supporting Information. For each amino acid, the concentration of the first sample is subtracted from the second, within each group. The concentration increases for the given amino acid is then averaged for the n number of flies of that group. Sample-one-to-sample-two average increases for each amino acid are plotted for groups 1 and 2 in Figure 4. The trends in the average sample-to-sample increases exactly match those between singly sampled cold-anesthetized and unanesthetized groups shown in Table 2. For instance, the average Arg increase between first and second samples for both groups 1 and 2 are nearly the same, as are averaged Arg levels for the cold-anesthetized and unanesthetized groups in Table 2. For Glu, Table 2 shows that singly sampled, cold-anesthetized flies have significantly ($P < 0.05$) higher hemolymph glutamate concentrations compared to unanesthetized flies. Figure 4 similarly shows a significantly ($P < 0.05$) larger increase in hemolymph Glu concentrations between the first and second samples collected from unanesthetized flies that are then cold-anesthetized (group 1) compared to the reverse procedure (group 2). In groups 1 and 2, the effects of sampling conditions on hemolymph are added and subtracted, respectively, to and from the background amino acid increase. It is not surprising then that a significant difference in Glu levels between sampling conditions also are seen between groups 1 and 2 (Figure 4). These data strongly suggests that, in spite of a large background change in amino acid concentration, this two sample method allows the use of an experimental design where a control and experimental sample can be collected from the same fly.

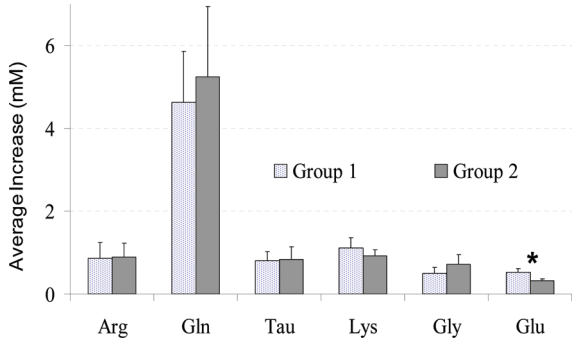

Figure 4. Comparison of average amino acid increase of group1 (unanesthetized to cold-anesthetized, $n = 7$) and group 2 (cold-anesthetized to unanesthetized, $n = 7$). *At $P < 0.05$, groups 1 and 2 are significantly different.

Hemolymph Protein Analysis. Similar to clinical blood analysis, it is also desirable to profile hemolymph protein content. However, hemolymph protein analysis of individual adult flies has not been reported previously and this could be attributed to the fly hemolymph sampling challenges. Using the novel fly hemolymph sampling technique, the possibility of hemolymph protein analysis in individual flies is demonstrated here as preliminary results. Figure 5 is a representative MALDI-TOF mass spectrum of 100-fold diluted adult hemolymph obtained after spotting with the microspot sample preparation technique.[15]

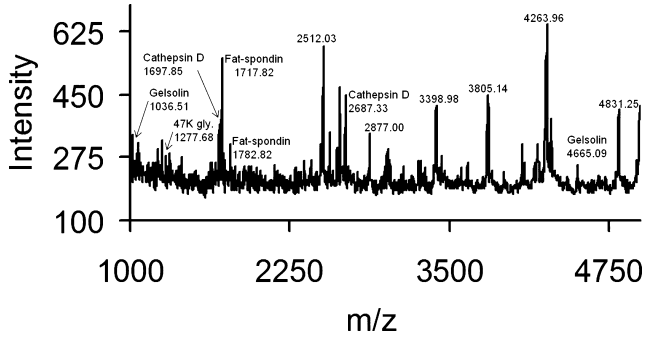

Figure 5. Representative linear MALDI-mass spectrum for 100-fold diluted adult fruit fly hemolymph sample.

The microspot technique concentrates proteins and peptides onto a spatially restricted matrix area to increase the number of observed peptides, improves signal-to-noise ratios, and provides more reliable measurements compared to conventional thin layer sample preparation (data not shown). Results show peptides that appear to match those previously reported from a study of a trypsinized *Drosophila* larval hemolymph clot.[10] Tentative assignments can be made for peptides of Cathepsin D (1697.8744, 2687.3556 m/z), Fat-spondin (1717.8180, 1782.8115), Gelsolin (1036.5309, 4665.0871), and 47K glycoprotein (1277.6736) (Figure 5). These peptide fragments were repeatedly observed for the analyzed adult hemolymph while the blanks without the hemolymph showed no peaks. While these preliminary data illustrate the capability in analyzing hemolymph proteins of individual flies, LC−MS/MS or MALDI-TOF analysis of hemolymph with a protein database search is required for detailed fly hemolymph protein analysis.

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CONCLUSIONS

The results demonstrate the ability to analyze the chemical content of hemolymph from adult rather than larval fruit flies. These results point toward several new directions in scientific research with the fruit fly model. The first is that this is a low-cost and relatively simple means to collect and analyze nanoliter volumes of hemolymph from individual fruit flies. The possibility of exploring *in vivo* chemical effects of specific fly mutations related to clinical diseases will expand the utility of this model. The ability to collect and analyze *adult* hemolymph better relates to the physiology of the mature animal and would be expected to be unaffected by overriding developmental effects in the fly. Moreover, the ability to work with adults and larvae allows the description of chemical content changes over the life course of the fruit fly to better understand normal and disease developmental processes. Perhaps most significantly, the ability to monitor hemolymph composition from the same subject opens the door to a pre- and post-test sample collection design that can be used with the fruit fly model. Using the same individual as its control improves the ability to determine experimental effects and differences in effects between individuals over populations. These results are a step toward filling an important gap in chemical information from this commonly used and historically significant model organism.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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