Total cysteine and glutathione determination in hemolymph of
individual adult *D. melanogaster*

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Abstract

Determination of thiols, glutathione (GSH) and cysteine (Cys) are important due to their roles in oxidative stress and aging. Oxidants such as soluble O₂ and H₂O₂ promote oxidation of thiols to disulfide (-S-S-) bonded dimers affecting quantitation accuracy. The method presented here reduces disulfide-bonded species followed by fluorescence labelling of the 29.5 (±18.2) nL hemolymph volumes of individual adult *D. Melanogaster*. The availability of only tens of nanoliter (nL) samples that are also highly volume variant requires efficient sample handling to improve thiol measurements while minimizing sample dilution. The optimized method presented here utilizes defined lengths of capillaries to meter tris (2-carboxyethyl) phosphine reducing
reagent and monobromobimane derivatizing reagent volumes enabling Cys and GSH quantitation with only 20-fold dilution. The nL assay developed here was optimized with respect to reagent concentrations, sample dilution, reaction times and temperatures. Separation and identification of the nL thiol mixtures were obtained with capillary electrophoresis-laser induced fluorescence. To demonstrate the capability of this method total Cys and total GSH were measured in the hemolymph collected from individual adult *D. Melanogaster*. The thiol measurements were used to compare a mutant fly strain with a non-functional cystine-glutamate transporter (xCT) to its background control. The mutant fly, *genderblind* (*gb*), carries a non-functional gene for a protein similar to mammalian xCT whose function is not fully understood. Average concentrations obtained for mutant and control flies are 2.19 (±0.22) and 1.94 (±0.34) mM Cys and 2.14 (±0.60) and 2.08 (±0.71) mM GSH, respectively, and are not significantly different (p>0.05). Statistical analysis showed significant differences in total GSH of males and females independent of the xCT mutation. Overall, the method demonstrates an approach for effective chemical characterization of thiols in nL sample volumes.

**Keywords** capillary electrophoresis, cystine-glutamate transporter, genderblind, tris(2-carboxyethyl)phosphine hydrochloride, monobromobimane, thiol label

**Abbreviations** cysteine (Cys), cystine (Cyt), glutathione (GSH), oxidized glutathione (GSSG), cystine-glutamate transporter (xCT), *Drosophila Melanogaster* (*D. Melanogaster*), genderblind (*gb*), tris (2-carboxyethyl) phosphine (TCEP), dithiothrietol (DTT), monobromobimane (mBBr), capillary electrophoresis-laser induced fluorescence (CE-LIF), inner diameter (id), outer diameter (od).
1. Introduction

Detection of thiols is significant due to their roles in many biological processes but their chemical determinations are challenged by air oxidized disulfide formation affecting solution stability [1–3]. Glutathione (GSH) is the most abundant non-protein thiol playing an important role as an antioxidant [4] and cysteine (Cys) is the major low molecular weight thiol in plasma [5]. Electrochemical measurements of thiols are limited by stability of these compounds in stored solutions, electrode stability and calibration. Fluorescence based determinations, require labelling of these non-natively fluorescent amino acids. For thiol-containing amino acids, both primary amine and sulfur moieties can be labelled. However, most common approaches label sulfur groups of GSH and Cys using alkylating agents like iodoacetates and malemides [6–8]. Primary amine labelling is not preferred, due to fluorescence signal quenching from the presence of a disulfide bond in oxidized forms. The disulfide-bonded dimeric forms can be reduced prior to labelling for accurate thiol determinations. Dithiothrietol (DTT) and tris (2-carboxyethyl) phosphine (TCEP) are the most commonly used reducing agents for disulfides [9–11]. Physiological thiol determinations and their ratios to disulfides are a common measure of oxidative stress and aging [12].

*D. Melanogaster* offers great advantages over other biological models due to fast generation turnover, inexpensive maintenance, ease of genetic manipulations and significant homology to the human genome [13]. The fast generation time and ease of genetic manipulations in this fly model are advantageous for determining the function of poorly understood proteins. Genderblind (gb) is a Drosophila protein with 43% amino acid identity to human xCT [14–17], suggesting that gb functions similar to xCT. xCT is the catalytic subunit of system xc-, a type of membrane
transporter hypothesized to play a role in maintaining extracellular glutamate levels [14,16,18]. The glutamate efflux via xCT is associated with a cystine (Cyt) influx, which is thought to be important for intercellular GSH synthesis and also believed to maintain Cys/Cyt homeostasis [19–21]. While glutamate measurements in \( gb \) mutants with reduced genderblind expression have been well established, so far no information has been obtained for thiol content in \( gb \) mutants [14,16]. Though the de-novo pathway to GSH synthesis requires Cys, glutamate and glycine as precursors, glutamate seems to play a regulatory role in GSH synthesis via the Cyt uptake mechanism of xCT [22].

Besides increasing understanding of the genderblind protein function, GSH and Cyt measurements in \( D. Melanogaster \) are also important as GSH and Cyt play significant roles as antioxidants in cellular homeostasis, oxidative stress and aging-related life span [1,4,22–25]. Due to the analytical challenges of fly hemolymph collection, traditional approaches employ homogenization and dilution of 25 whole flies to generate one sample in \( \mu \)L volumes for analysis [24,25]. Chemical determinations from such measurements would not virtuously reflect hemolymph or blood compositions of a single fly. Sampling and chemical content determinations from hemolymph of an individual \( D. Melanogaster \) has been well established by us [16,18]. While sampling from individual \( D. Melanogaster \) provides advantages over averaging a group of flies, handling the nanoliter hemolymph volumes towards chemical measurements can be arduous. Previously, we were able to quantitate mM concentrations of 7 amino acids in these tens of nL hemolymph samples with dilution processes that did not hinder identification [18]. However, similar sample dilution and amine group labelling methods did not provide consistent Cys, Cyt and GSH determinations.
In the work presented here, reduction and derivatization of Cys and GSH were performed with TCEP, a thiol reductant, and monobromobimane (mBBr), a thiol specific dye. The primary difficulty of these measurements is the availability of low nanoliter, highly variant sample volumes. Defined lengths of fused-silica capillary were used to meter nL reagent volumes, enabling only a 20-fold dilution for these submicroliter scale reactions. To ensure accurate quantitation, the method is also optimized for reaction time, temperature and concentrations. The nL assay developed here was used to compare total Cys and total GSH levels in hemolymph of male and female; control and gb mutants using individual *D. Melanogaster* samples.

2. Experimental

2.1. Reagents and solutions

L-Cyt, L-GSH oxidized, sodium tetraborate decahydrate, tris (2-carboxyethyl) phosphine hydrochloride (TCEP), monobromobimane (MBBr), dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MI). Fused silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ). Tygon tubes were purchased from Cole-Parmer (Vernon Hills, IL).

A 20 mM borate stock solution was prepared with sodium tetraborate decahydrate and filtered through a Millex GP 0.22 μm filter (Sigma-Aldrich) prior to its use as capillary electrophoresis run buffer. Stock solutions of standard amino acids were prepared in deionized water and further diluted appropriately with the capillary electrophoresis run buffer to the desired concentrations.
A 1 mM stock solution of Cyt standard was prepared in deionized water and stirred for 48 hr to solubilize sparingly soluble Cyt. A 10 mM stock solution of TCEP and its dilutions were made in borate buffer. A stock solution of 20 mM mBBr was made in DMSO and further dilutions were made in capillary electrophoresis run buffer. All solutions were prepared using deionized water from 18.3 MΩ ultra filtered water (US filter, Lowell, MA). The TCEP and mBBr solutions were freshly prepared from stock on the day of the experiment.

2.2. Hemolymph sample collection

Wildtype (WT) ‘Oregon R’ and genderblind (gb [CG070905]) mutant Drosophila strains were reared on standard cornmeal-agar medium and maintained in the Department of Biological Sciences at UIC. The unanesthetized sampling condition as described by Piyankarage et al. [18] has been employed for Drosophila sampling. Briefly, the flies were immobilized at −20 °C for 5.5 min in a 15 mL graduated polystyrene tube. Cold immobilized flies were then affixed on to the stainless steel block with a piece of tape with the adhesive side of the tape exposed. A 4-cm long 50/360 μm (id/od) fused silica capillary was used as a sampling probe. The sampling probe tip was gently pushed against an incision made between second and first tergites and the leaking hemolymph was collected via capillary action.

2.3. Nanoliter reagent handling

After hemolymph sample collection, a 4-cm long 250 μm (id) tygon tubing was connected to one end of the capillary for handling nL volumes. The volumes collected in these fused silica capillaries were determined by measuring the plug length after aligning it with a digital calliper.
(World Precision Instruments Inc., FL) under a dissecting microscope. A bench top centrifuge was then used to dispense solutions form the capillaries into a centrifuge tube. During centrifugation the tygon tube connector was used to suspend the capillary tip well above the bottom of the centrifuge tube to allow fluid to collect at the bottom and avoiding re-filling into the capillary. Measured lengths of 150/360 μm (id/od) fused silica capillaries were used to collect TCEP and mBBr solutions. Sample centrifuge vials were sealed with parafilm and placed on a 5°C ice bath to maintain reaction conditions.

2.4. Capillary Electrophoresis-Laser Induced Fluorescence

The TCEP reduced and mBBr derivatized samples were separated and analysed using capillary electrophoresis with-laser-induced fluorescence detection (CE-LIF). The CE instrument used for these studies was built in-house and its specifications have been previously discussed in detail [16,26]. A 20 s gravity injection at a 15 cm displacement was used to inject samples to provide a calculated estimate of 6.8 nL of the treated sample. The applied potential was 27 kV with a 20 mM borate run buffer. The separation and detection for the mBBr Cys and GSH derivatives were complete by 4 min. The data acquisition was performed by a custom Lab View (National Instruments, Austin, TX) program.

2.5. Data analysis

Each sample or standard was analyzed in triplicate. Analyte peaks were identified by spiking with TCEP-reduced and mBBr-labelled amino acid standards. Peak heights from individual trials were converted to concentrations for analysis by a standard calibration curve. Microsoft Excel was used to plot electropherograms, perform calibration regressions and statistical analyses.
Students’ $t$-tests and F-tests were performed at the 95% confidence level to identify differences between groups.

3. Results and Discussion

The sulfhydryl group of thiols undergo aerobic oxidation to form symmetric and asymmetric disulfides eventually leading to a mixture of oxidized species [3]. Identification of Cys and GSH (red) forms in solutions or body fluids containing thiols and proteins would lead to inaccuracy if aerobic oxidation or its effects are not addressed. DTT and TCEP are the most common and effective thiol reductants used in reducing disulfide bonds [10]. Earlier studies using DTT and TCEP show TCEP is more effective than DTT in reducing Cyt, GSSG and many proteins [10,11,27,28]. The thiol group of DTT could potentially react with thiol specific dyes, requiring removal of excess DTT before labelling. In our preliminary studies DTT and TCEP were studied and TCEP gave higher and more consistent signals upon reaction than DTT (data not shown). Due to the various advantages of using TCEP in terms of stability, efficiency and redundant reagent removal, the Cyt and GSSG standard solutions and hemolymph of individual adult $D. \text{Melanogaster}$ were subject to TCEP reduction. A thiol specific fluorescent dye, mBBr, was used to label the reduced disulfides [29]. The alkylating properties of mBBr are similar to iodoacetamides, where the bromine is displaced by thiol [30]. The proposed method presented here involves 3 steps after sample collection (i) reduction (ii) labelling and (iii) separation and analysis using CE-LIF. The concentrations of TCEP and mBBr, along with the reaction times for reduction and labelling were varied to find optimal conditions. The optimized conditions were further used to study total Cys and total GSH (red) in hemolymph of individual adult $D. \text{Melanogaster}$. 

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3.1. Reduction with TCEP

Effective labelling of Cys and GSH residues requires reduction of the disulfide bonds. The effect of an increasing ratio of TCEP to thiol was explored by adding concentrations between 10 μM to 1000 μM to standard solutions of 25 μM Cyt. The reduced Cyt was derivatized with 100 μM mBBr and separated and quantified using CE-LIF (Figure 1A). As the added TCEP concentration increased, the fluorescent signal of mBBr Cys derivative reached a maxima followed by a diminishing signal with >100 μM TCEP concentrations. The rise in fluorescence signal up to 100 μM TCEP is likely evidence for increasing Cys formation from Cyt. The decrease in fluorescence beyond 100 μM suggests signal inhibition. A disadvantage associated with reductants is their interference with the subsequent reaction with a thiol-reactive dye. mBBr, the derivatizing agent used here is similar to class of iodoacetamides in terms of its alkylating property. While DTT is commonly stated to react with iodoacetamides and malemides, TCEP also showed a decrease in labelling efficiency probably due to inhibition of mBBr reaction with thiols preventing formation of labelled product. An inhibition in fluorescence labelling was also noted by other groups [10,28] when using high TCEP concentrations. Subsequent use of higher concentration Cys and GSH standards for calibration curves for hemolymph samples avoid this signal loss due to TCEP.

3.2. Derivatization of Cys and GSH (red) with mBBr

After studying the effect of increasing TCEP concentration, the concentration of labelling reagent, mBBr was varied from 80, 250, 500 to 1000 μM to gain efficient labelling of the
Figure 1 (A) The effect of increasing TCEP concentration while maintaining constant cystine shows an increase followed by a decrease in the fluorescence intensity; (B) The effect of increasing mBBr concentration for derivatization of TCEP reduced glutathione shows an increase in signal; (C) Separation of TCEP reduced and mBBr derivatized cysteine and glutathione standard shows (i) mBBr reagent peak, (ii) hydrolysis products of mBBr (iii) mBBr cysteine derivative (green arrow) and (iv) mBBr glutathione derivative (orange arrow). Capillary electrophoresis separation conditions include 20 mM borate as run buffer (pH 9), a 50 cm long, 50/360 µm (id/od) capillary dimension with 35 cm effective separation length and 540 Vcm⁻¹ of electric field strength.
reduced forms (Figure 1B). A thiol group displaces the bromine group of mBBr to generate an mBBr thiol derivative. As the mBBr concentration was increased, an increase in the fluorescence signal was observed, suggesting labelling of the free monomeric thiols. Though the increasing mBBr concentration provided higher signal compared to its lower concentrations, there is deterioration in peak resolution and identification. A typical electropherogram when using 1000 μM mBBr concentration is shown in Figure 1C. The separation shows (i) an intense mBBr reagent peak followed by (ii) hydrolysis products of mBBr, (iii) mBBr Cys derivative and (iv) mBBr GSH derivative. Others reported the intense mBBr peak at 1 min as tetramethylbimane (Me₄B), which is a part of mBBr synthesis [31,32]. With increasing mBBr concentration, there is an increase in mBBr hydrolysis products and Me₄B peak area. The broadness of Me₄B peak is important for Cys identification, as the Cys peak is unresolved from Me₄B peak tailing. When using high mBBr concentrations, the identification of fluorescently tagged GSH is relatively easy as its migration time is well resolved from hydrolysis products of mBBr.

3.3. Maintaining mole ratios and constant dilution of hemolymph

Our previous studies of hemolymph amino acid composition from individual D. Melanogaster found mM concentrations in the nL volumes of hemolymph [16,18]. Unfortunately the small size of the fly provides only 10’s of nL of hemolymph for sample collection. Previous quantitation of amino acids in these nL sample volumes relied on a high degree of dilution (>150 fold) prior to derivatization to enable facile sample handling. Assuming mM concentrations of Cys and GSH in D. Melanogaster hemolymph, the assay was initiated following the previous approach by adding borate buffer to the collected hemolymph. To a 500 nL aliquot of diluted hemolymph, 500 nL each of TCEP and mBBr were added. The added concentrations were 1.25 mM TCEP
and 2 mM mBBr, as these levels showed no signal inhibition and minimal hydrolysis products of mBBr. The capillary electrophoretic separation of *D. Melanogaster* hemolymph employing this approach is shown in Figure 2A. There are no peaks for Cys while there was positive peak identification for GSH in the diluted hemolymph sample. The inability to identify Cys could have been due to the sample dilution.

The addition of 500 nL volumes of each of TCEP and mBBr to undiluted hemolymph resulted in peaks for both Cys and GSH (red) (Figure 2B). In doing so, the overall sample dilution was reduced from 145- to 16-fold. The effect of the dilution is also likely seen in the migration time of the glutathione peak in the two electropherograms in Figure 2. The longer migration time shift for glutathione in the less-diluted hemolymph sample may be the result of increase protein level in the sample that is decreasing electroosmotic flow. Evidence to support this hypothesis is the uneven baseline seen between the mBBr hydrolysis products and Cys that may be due to labelled proteins that have labelled cys residues.

However, the hemolymph volumes collected from individual adult *D. Melanogaster* were found to be variable at 29.5(±18.2 nL) for N=20. The high RSD (61%) associated with hemolymph sample collection is likely due to the differences in sizes of the flies. Male flies are one third the size of females and have proportionally less hemolymph. Further, there are size variations within same sex flies. Additionally, the collection methods for these low nanoliter volume samples may be a factor. The variance in sample volume would introduce dramatically different dilutions if reagent volumes were not varied. As hemolymph sample dilution appeared to play a significant role in Cys and GSH identification, a method was developed to maintain a
Figure 2: Separation of an individual adult *D. Melanogaster* hemolymph when treated with (A) borate buffer, 500nL TCEP, 500nL mBBr resulted in 145-fold sample dilution; (B) 500nL TCEP, 500nL mBBr resulted in 16-fold sample dilution, shows (i) mBBr reagent peak, (ii) mBBr hydrolysis product, (iii) mBBr cysteine derivative and (iv) mBBr glutathione derivative peaks. Separation conditions are as mentioned in Figure 1.
20-fold dilution of hemolymph samples. Such a method required addition of 100-600 nL volumes of TCEP and mBBr to the tens of nL hemolymph samples. To access these volumes reliably and accurately, TCEP or mBBr solutions were filled in defined lengths and diameters of 150/360 μm (id/od) fused silica capillary via capillary action. The ability to handle nL reagent volumes in terms of collection and dispensing using defined lengths of fused silica capillary has been previously illustrated by us [26].

Once the hemolymph was collected in a 50/360 μm (id/od) fused silica capillary, its length was measured, by visual alignment with a vernier caliper under microscope (Figure 3A) and spun down for collection in the sample vial. The recorded length of sample was entered into a spreadsheet that would generate the precise volume collected and the capillary lengths needed to deliver the calculated volumes of TCEP and mBBr for a final 20-fold sample dilution. These capillary lengths, ranging from 5 to 25-mm, were cut and one end was inserted into a tygon tubing to handle the dispenser capillary. The open end of the capillary was dipped in to reagent vials to collect fluid via capillary action. TCEP solution from the silica capillary dispenser was spun down in to the sample vial containing hemolymph. Similarly 100s of nL volumes of mBBr solution were collected and dispensed to the sample vial.

Standard Cyt sample volumes of 20, 40 and 60 nL were generated using defined capillary lengths to simulate varying hemolymph sample volume and demonstrate method reproducibility. Lengths of 50/360 μm and 150/360 μm (id/od) capillary used to dispense standard samples and reagent solutions, respectively, as illustrated in Figure 3B. Adding varying volumes of TCEP and mBBr to the varying hemolymph volumes helps maintain a 20-fold sample dilution and constant
Figure 3 (A) Image of plug length measurement in a 50/360 (id/od) μm capillary under microscope; (B) Table showing lengths and volumes of 50/360 μm and 150/360 μm (id/od) fused silica capillaries used to collect nL sample and reagent volumes respectively; (C) Fluorescence signal from 20, 40 and 60 nL cystine standard solutions treated with 190, 380 and 570 nL reagent volumes respectively show no t-test significant difference (p≤0.05) among them suggesting maintenance of constant dilution and thiol: TCEP: mBBr ratio.
thiol: TCEP: mBBr ratio therefore providing similar fluorescence intensities for similar concentrations. Here 20, 40 and 60 nL of 50 µM Cyt solutions were 20 times diluted after reagent additions resulting in a final concentration of 2.5 µM Cyt. The three different sample volumes showed similar fluorescence intensity that was not statistically different suggesting that there was constant dilution for samples of the same concentration (Figure 3C). This demonstrates the applicability of this method for Cys and GSH analysis in varying volumes of fruit fly hemolymph. Using this optimized method, limits of detection (S/N= 3) for the total amount of thiol collected in a hemolymph sample is 0.62 µmoles for Cys and 0.36 µmoles for GSH at 5.65 and 8.52 % RSD, respectively. The linear regression of the calibration curves were y = 19.6 [Cys] – 334 (R^2 = 0.9813) and y = 66.6 [GSH] – 92.5 (R^2 = 0.9924) for standard curves over 50-300 µM.

3.4. Reaction Kinetics

An optimized reaction time for both TCEP reduction and mBBr derivatization is crucial for the determination of Cys and GSH (red). Reduction of a standard solution of GSH with TCEP was explored for 60 nL standards at 1, 3, and 5 min (Figure 4A). For reduction of structurally simple disulfide bonds in water, concentrations between (0.2-10) mM TCEP showed reduction was complete within 5 min [11]. Considering the exclusive hemolymph collections and their nL sample sizes a 3-5 min TCEP reduction time seemed appropriate to avoid any evaporation effects.

Similarly reaction times for derivatization with mBBr were also varied 60 nL standards for 0.5, 1, 3, 5 and 7 mins to obtain maximal labelling (Figure 4B). The derivatization was observed to
Figure 4 (A) The effect of TCEP reduction time at 1, 3 and 5 min and (B) The effect of mBBr derivatization time on fluorescence of mBBr glutathione derivative at 0.5, 1, 3, 5 and 7 min.
be maximal at 5 min. Others reported 3 min derivatization time for Cys and GSH determinations in nL rat caudate nucleus microdialysates [6] and 5 min derivatization time for GSH in single erythrocytes [33]. This contrasts with a 30 min reaction in the dark for derivatizing molecularly more complicated thiols in foliage samples [31] and a 5-30 min labelling time used in the study of chemically fixed rat pituitary slices [34].

To further enhance labelling, the reaction temperature was roughly optimized using 5°C, 29°C (room temperature) and a 60°C warm condition at 3 min reaction times each for reduction with TCEP and derivatization with mBBr (Figure 5 Inset). The separation of mBBr thiol derivatives at 60°C led to complex electropherograms with a growth of new hydrolysis product peaks as shown in Figure 5. In addition to increases in hydrolysis product formation, the mBBr solution at 60°C showed the formation of a light yellow color suggesting mBBr to be unstable at these conditions. At 5°C less hydrolysis product formation was observed which is in line with reports that TCEP was found to be reasonably stable at 4°C [10]. It is not unreasonable to assume that both the hydrolysis product formation and the labelling reaction would proceed more slowly at cold temperature conditions, so the reduction and derivatization reactions were each increased to 5 min. The 5 min reaction at 5°C showed a statistically significant increase in signal compared to 3 min reaction at all temperatures tested (Figure 5 Inset). The 5 min reaction time at 5°C was chosen for further studies.

3.5. Total Cys and total GSH (red) identification in hemolymph of individual adult D. Melanogaster

To demonstrate the suitability of the method developed, total Cys and total GSH analysis was
Figure 5: Separation of glutathione standard solutions at (A) 60°C, (B) 29°C and (C) 5°C showing (i) mBBr reagent peak, (ii) mBBr hydrolysis product and (iii) mBBr glutathione derivative. New peaks (*) were observed due to mBBr degradation at 60°C. Inset: Showing effect of temperature at 3 min reaction times for both TCEP reduction and mBBr derivatization at 5°C, 29°C and 60°C and compared to 5 min reaction times for both TCEP reduction and mBBr derivatization at 5°C. Separation conditions are as mentioned in Figure 1.
performed in nL hemolymph samples from an individual adult *D. Melanogaster*. The developed assay was performed in both wildtype and *gb* mutant male and female flies. *gb* mutants lack ‘genderblind’, a protein with high sequence similarity to mammalian xCT. xCT is a transmembrane protein that exchanges intracellular glutamate for extracellular Cyt. Cyt is thought to be important for GSH production and protection from oxidative stress [35]. Previous work has shown that *gb* mutant flies have reduced extracellular glutamate, as expected [16]. But Cyt and GSH levels have not been measured. Using the proposed method, the average concentration of total Cys and total GSH in *D. Melanogaster* hemolymph were measured to be 2.19 (±0.36) mM and 2.23 (±1.41) mM, respectively. The mM concentrations are in accordance with previous primary amine labelled amino acid measurements in individual *D. Melanogaster* [16,18]. The primary amine labelling by fluorescamine did not provide consistent Cys/Cyt or GSH peak identifications probably due to quenching from disulphide-bonded forms and the >150 sample dilution. The thiol specific labelling assay developed here required collection, handling and treatment of nL hemolymph volumes unlike traditional µL volumes obtained in tissue homogenization studies. To date there is no study reporting GSH concentrations in individual *D. Melanogaster* hemolymph as insect and fly studies commonly employ homogenates of 25 flies to generate one sample [24,25]. The tissue homogenization studies cannot be compared to our hemolymph concentrations, as they carry added Cys and GSH from the differing tissues.

The total Cys and total GSH concentrations in controls and *gb* mutants of both sexes are plotted in Figure 6A and the groups showing significant statistical differences when compared are represented in Figure 6B. Students’ *t*-test for total Cys and total GSH was evaluated to
Figure 6: Column graph representation of total cysteine (Cys) and total glutathione (GSH) in hemolymph of individual adult *D. Melanogaster* in males and females of control, *gb* and pooled (control + *gb*) groups (N=10 each group). †(N=9 for *gb* males). (A) Comparisons for control and *gb* mutant using students *t*-test showed no significant difference in total cysteine and total GSH. (B) Comparisons for males and females in control, *gb* mutant and pooled (control + *gb*) groups using students *t*-test *p*<0.05 for total glutathione and pooled (control + *gb*) groups for total cysteine. *Significantly different at *p*<0.05. (C) Box and whisker plots of data showing population distributions. Error bars represent measurement range; top and bottom of the box are the upper and lower quartile range, respectively; and the color change between the top and bottom of box is the median measurement. F-test of standard deviations show no significant population variation, *p*>0.05.
compare control and gb mutants of both sexes and pooled (male + female) groups (Figure 6). These comparisons showed no significant differences between controls and gb mutants before or after sex pooling. In this regard, our GSH comparisons between control and gb mutant flies are similar to comparisons between control and xCT−/− mice (xCT mutant) which showed no difference in genotype for GSH levels [7].

Previous comparisons among control and gb mutants in individual D. Melanogaster used pooled larvae undifferentiated for male and female [16]. In this study males and females of control, gb mutant and pooled (controls + gb mutants) groups were compared for total Cys and total GSH content. No significant difference was observed between males and females for total Cys while total GSH comparisons among males and females showed significant differences among all the 3 groups (Figure 6B) demonstrating a clear difference between sexes. GSH, a major antioxidant, plays a significant role in age related processes by inhibiting free radical reactions [23]. Also Cys, a monomer of Cyt and a building block for GSH is presumed to play important roles in aging and oxidative stress [1,22]. Recent studies in lifespan of Drosophila showed effect of genetics, diet, and mitochondrial DNA on lifespan, besides the long-established observation that females live longer than males [36–38]. The possible sources of enhanced female longevity is not fully known but, this clear sex-dependent increased concentration of GSH, an established anti-oxidant species that impacts aging, is worthy of further study.

An advantage of the method developed here is the ability to determine Cys and GSH concentrations in individual flies. Figure 6C are the box and whisker plots comparing Cys and GSH population variations for male and female control and gb mutants. It is notable that there
are no significant variations in the population distributions of Cys or GSH. The data here show that not only are average Cys control and mutant levels not different but also the variation about the median is indistinguishable. It is interesting to note that the differences between the mean GSH levels for males and female flies do not manifest as different distributions between male and female flies for either the controls, gb mutants or pooled.

The apparent lack of a decrement of total Cys between control and gb mutant flies is surprising. The impact on glutamate, a tightly regulated neurotransmitter, has been established both with populations of flies [14] and at the individual larva level [16] by us. Two interesting interpretations of these results include the possibility that the genderblind protein, while homologous to mammalian xCT, is somewhat different in function. Therefore there is a lack of concordance with the previously measured glutamate effects. However, it is also possible that the xCT protein is not strongly associated with the homeostatic regulation of GSH via import of Cyt. Further study of this system is warranted.

While this method adds the capability of thiol determinations from individual adult fly hemolymph samples excluding contributions from other tissues, the results provide total thiols content in reduced form only. Total reduced thiol determinations alone may not provide a complete picture of xCT function and any role in oxidative stress related conditions, however the method proposed here can help devise any further nL method developments for simultaneous measurement of Cys, Cyt, GSH and GSSG.
4. Conclusion

The assay method developed here provides Cys and GSH determinations with nL hemolymph volumes from individual adult *D. Melanogaster*. The use of defined lengths of capillaries to add sample reagents for analysis provided for a consistent sample dilution of 20-fold and the ability to handle these low and highly variant sample volumes. Method optimization for reagent concentrations, reaction times and temperatures allowed the successful measurement of total Cys and GSH. Statistical comparison between *D. Melanogaster* groups showed significant GSH differences between male and female fly hemolymph. The results show this method is applicable to volume limited, nanoliter assay of thiols and is likely generalizable to other categories of composition determinations in nL sample volumes while minimizing sample dilution.

5. References


