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ORIGINAL ARTICLE

Hemolymph amino acid variations following behavioral and genetic changes in individual *Drosophila* larvae

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Abstract This study investigated the effect of different sampling environments on hemolymph amino acid content of individual *Drosophila melanogaster* larvae. Hemolymph was collected from individual third instar larvae under cold-anesthetized, awake, and stress conditions. Qualitative and quantitative hemolymph amino acid analyses were performed via capillary electrophoresis with laser-induced fluorescence detection. The hemolymph amino acid concentrations, particularly arginine, glutamate, and taurine, changed significantly depending on the prior-to-sample-collection environments. Hemolymph amino acid analyses of six different *Drosophila* genotypes including two control genotypes and four mutant alleles were also carried out. Two mutant genotypes with over and under expression of a putative cystine-glutamate exchanger subunit were significantly different from each other with respect to their hemolymph glutamate, glycine, lysine, and taurine levels. Hemolymph amino acid analyses of stressed larvae of two control and two mutant genotypes indicated that behavior-related hemolymph chemical changes are also genotype dependent.

Keywords Capillary electrophoresis · Chemical composition · Stress · Anesthetize · Cystine-glutamate exchanger · Third instar larva

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Introduction

Drosophila melanogaster (fruit fly) is a widely used animal model that plays a vital role in investigating pathologies including neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's diseases and several Spinocerebellar ataxias (Shulman et al. 2003; Ghosh and Feany 2004; Wittmann et al. 2001; Manev et al. 2003). The importance of studying neurotransmitters such as glutamate (Glu), glutamine (Gln), glycine (Gly), and taurine (Tau) in fruit flies to better understand disease pathogenesis, treatments, and drug developments has been documented as well (Wittmann et al. 2001; Celotto and Palladino 2005; Marx 2005; Zhang and Broadie 2005). Attaining organism-level chemical information from this vital animal model has been a challenge for decades due to its small size. Recently, Powell et al. (2005) obtained chemical compositions of individual fruit-fly head samples through a homogenization sampling method and later we demonstrated a sampling technique (Piyankarage et al. 2008) that enabled chemical analyses of hemolymph from individual *Drosophila* larvae. In addition, it is desirable to perform chemical measurements of the larvae to establish relationships with their behavior. Such methods would provide a dramatic improvement in attributing chemical and

behavioral changes as a result of genetic manipulation of this animal model.

The chemical analysis of blood provides important information for monitoring, treatment, and management of diseases as well as predicting the possible causes and understanding the underlying neurobiological processes. Variations of amino acids in animal tissues in response to animal behavior and external stimuli have been highlighted (Beis and Newsholme 1975; Milakofsky et al. 1985; Thongkhao-On et al. 2008; Sherman and Gebhart 1974; Isobe-Harima et al. 2008) and recent studies reported correlations between the sexual behavior and the extracellular glutamate levels of fruit flies based on their hemolymph analyses (Augustin et al. 2007; Grosjean et al. 2007; Piyankarage et al. 2008).

One observation from our previous work is that only hemolymph arginine (Arg) and Glu levels were higher when collected from animals placed in mineral oil compared to those collected in a microdissection plate in the open air (Piyankarage et al. 2008). When the larvae were immersed in mineral oil they wriggled vigorously and it was assumed that the hemolymph Arg and Glu level elevations could possibly be a result of this behavior. It was also noted that the hemolymph Glu level reported by Augustin et al. for the dissected-saline-immersed larvae of a control genotype is closer to that of the oil-immersed larvae than to those collected in the open air (Augustin et al. 2007; Piyankarage et al. 2008). These data suggest that the hemolymph amino acid levels may be altered depending on the larval behavior, as a result of the conditions of the sampling technique. The extent to which the sampling environment influences hemolymph chemical composition is unknown. Different sampling techniques have been employed to acquire chemical information from *D. melanogaster* during last five decades (Chen and Hanimann 1965; Chen and Buhler 1969; Ream et al. 2003; Powell et al. 2005; Augustin et al. 2007; Piyankarage et al. 2008). An assessment of the behavioral influences on hemolymph chemical composition based on individual larvae will enable establishment of basal hemolymph chemical levels as well as to better interpret the existing fruit fly chemical information collected through different sampling techniques.

The focus of this study is to relate the effects of sampling environments on hemolymph amino acid levels to provide chemical information regarding physiology of the *D. melanogaster* using individual larvae. In this study the previously described single-larval hemolymph sampling technique is utilized with capillary-electrophoretic analyses of their hemolymph (Piyankarage et al. 2008). Wild-type larvae are treated with five different treatment conditions before collection. Two genetic controls and four mutant alleles with alterations in the *JhI-21* protein were tested to investigate resulting hemolymph chemical changes. *JhI-21* is a putative cystine-glutamate exchange protein highly similar to the genderblind protein which was previously shown to play an important role in regulation of *Drosophila* larval hemolymph amino acid concentrations (Augustin et al. 2007). Larvae of two *JhI-21* mutant genotypes and the two control genotypes were further studied to better understand behavior-related animal-to-animal hemolymph amino acid variations across different genotypes.

Experimental section

Materials

All chemicals used were of analytical grade or better. HPLC-grade acetone, sodium tetraborate decahydrate, cesium chloride, histamine dihydrochloride, and γ-amino-*n*-butyric acid were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). All other amino acids, fluorescamine, sodium dodecyl sulfate (SDS), sodium hydroxide, NaCl, KCl, $MgCl_2$, $CaCl_2$, 2-[Tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid (TES), and sucrose were obtained from Fisher Scientific (Itasca, IL, USA). US Filter Purelab Plus purification system (Lowell, MA, USA) was used to obtain ultrafiltered-deionized water for preparing all solutions. Untreated 50-μm inner diameter fused-silica capillary (360 μm outer diameter) was purchased from Biotaq Inc. (Gaithersburg, MD, USA), and 250-μm and 800 μm inner diameter Tygon tubes were purchased from Cole-Parmer (Vernon Hills, IL, USA).

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. Stock solutions containing 100 mM SDS were prepared by dissolving SDS in 20 mM borate buffer. The optimized MEKC run buffer (pH 9.1) contained 30 mM CsCl and 70 mM SDS in 20 mM borate solution. 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 the 20 mM borate buffer solution, and diluted appropriately with the run buffer to the desired concentrations.

Drosophila melanogaster larvae

All *Drosophila* larvae were reared on standard cornmeal-agar medium in the Department of Biology at UIC. As in our previous work (Piyankarage et al. 2008) wandering third instar stage larvae were carefully selected to study the affect of stress on the hemolymph chemical variations by treating them with five different conditions. Apart from wild-type, six other *D. melanogaster* genotypes were investigated

including two control and four mutant genotypes. For convenience the control genotypes clean excision (P*82) and *W[118]* will be referred to as control genotype 1 and control genotype 2, respectively. The four mutant genotypes include *TubGal4;JhI-21[EP1187]*, the *JhI-21* overexpression mutant, and three *JhI-21* loss-of-function mutants: *JhI-21[KG00977]* (LOF mutant 1), *JhI-21[e02252]* (LOF mutant 2), and *Df(2L)esc-P3-0/JhI-21[KG00977]* (LOF mutant 3). The mutants have variable expression of *JhI-21* that is in the same gene superfamily as the previously studied cystine-glutamate transporter genderblind protein (Augustin et al. 2007; Grosjean et al. 2007; Piyankarage et al. 2008).

Larval preparation methods for sampling

Prior to sampling, all larvae were cleaned with saline and blot dried on Kimwipe®. This cleaning and drying process is considered the control procedure and was followed immediately by sampling as described previously (Piyankarage et al. 2008). There were four experimental groups consisting of additional procedures prior to sampling in order to investigate behavioral effects on observed hemolymph amino acid levels. The four additional treatment methods were (1) immersing the larvae in standard *Drosophila* saline (135 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM TES, and 72 mM sucrose) at room temperature for 2 min followed by blot drying on Kimwipe®, (2) pinching the larvae with a microdissection pin for 50 s, (3) refrigerating the larvae for 25 min at 4°C in a closed pre-cooled 15-mL graduated polystyrene tube (Fisher Scientific, Itasca, IL, USA), and (4) refrigerating the larvae for 25 min at 4°C in a polystyrene tube followed by immersion in cold (10°C) saline for 2 min and blot drying. Samples from at least two larvae were collected for each treatment condition on each sampling day. The 25-min refrigeration period for anesthetization, used in the treatment methods, was selected after testing larval refrigeration time periods such as 5, 10, 15, 20, 25 and 35 min.

Hemolymph sampling, derivatization, and separation

The single larval hemolymph sampling technique used in this study was described previously (Piyankarage et al. 2008). Briefly, a cleaned third instar *Drosophila* larva was pinned to a microdissection dish at its anterior and posterior ends with microdissection pins and was pierced with another microdissection pin. The leaking hemolymph was then pulled into a Tygon-tube sampling probe mounted on a micropositioner by applying a light vacuum. The volume of the hemolymph samples in the 250-μm inner diameter Tygon tube, collected within 30 s from piercing the larval body cavity, was determined by measuring the fluid length. The samples were diluted 250 times with 20-mM borate buffer and were frozen at −20°C until 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 microcentrifuge tube (Fisher Scientific, Itasca, IL, USA). All samples that were collected and diluted as a single batch were also derivatized, and analyzed together.

The home-built capillary electrophoresis system with the laser induced fluorescence detection (CELIF) and the separation procedure for the derivatized hemolymph is the same as previously described by Piyankarage et al. (2008). In brief, untreated 50-μm inner diameter, 50-cm fused-silica capillary with a 1-cm detection window at an effective length of 36-cm was utilized for separation. The derivatized samples or amino acid standards were injected hydrodynamically and separation voltages of 30 and 19 kV were applied with the optimized 20 mM borate and the MEKC run buffers, respectively. The analyte peaks were identified by spiking with 24 different amino acid standards. Quantitation was performed with external calibration curves after verification of statistical similarity of levels determined by the method of standard additions.

Qualitative and quantitative hemolymph chemical analyses of the six *Drosophila* genotypes were followed by a study of hemolymph amino acid variations in response to different pre-sampling environments across different genotypes. For these analyses the larvae of control genotype 1, control genotype 2, *JhI-21* overexpression mutant, and LOF mutant 3 (*Df(2L)esc-P3-0/JhI-21[KG00977]*) were pinched for 50 s with a microdissection pin prior to sampling. During each sampling round at least two larvae from each genotype were utilized.

Data analyses

The raw data were imported to Microsoft Excel to plot electropherograms, box and whisker plots, and to perform quantitation. Statistical comparisons for hemolymph amino acids among different larval behavioral conditions and genotypes 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% ($P < 0.05$). In Tables 1 and 2 different superscripted letters next to the mean values of a given amino acid indicates groups of means that are significantly different. Hemolymph comparisons between the pinched and the control larvae of the four genotypes (*clean excision*, *W[118]*, *TubGal4*, and *KG00977/DF*) were carried out with Microsoft Excel using a two-tailed Student's *t* test with a confidence level of 95% ($P < 0.05$) to determine

Table 1 Average (mM ± SD) hemolymph amino acids concentrations of individual (*n*) wild-type III larvae treated through five different prior-to-sampling conditions

	Larval treatment condition				
	Control (control-treated) ($n = 8$)	In saline[A] ($n = 8$)	Pinched[B] ($n = 8$)	Refrigerated[C] ($n = 10$)	Refrigerated and in cold saline[D] ($n = 10$)
Arg	$1.27^{b} \pm 0.32$	$1.46^{b} \pm 0.43$	$1.95^{a} \pm 0.37$	$1.50^{b} \pm 0.44$	$1.70^{ab} \pm 0.38$
Asp	$0.22^{a} \pm 0.07$	$0.16^{a} \pm 0.08$	$0.18^{a} \pm 0.10$	$0.21^{a} \pm 0.11$	$0.15^{a} \pm 0.09$
Gln	$19.5^{a} \pm 7.4$	$18.6^{a} \pm 5.8$	$19.8^{a} \pm 5.0$	$22.1^{a} \pm 6.3$	$22.7^{a} \pm 7.1$
Glu	$0.50^{b} \pm 0.13$	$0.59^{b} \pm 0.15$	$1.11^{a} \pm 0.19$	$0.65^{b} \pm 0.45$	$0.65^{b} \pm 0.30$
Gly	$3.04^{bc} \pm 0.90$	$3.87^{ab} \pm 0.90$	$2.88^{c} \pm 0.58$	$3.78^{abc} \pm 0.96$	$4.17^{a} \pm 0.93$
Lys	$3.21^{ab} \pm 1.40$	$3.36^{a} \pm 2.53$	$3.41^{a} \pm 2.24$	$2.30^{ab} \pm 0.90$	$1.38^{b} \pm 0.93$
Tau	$1.75^{b} \pm 0.34$	$1.98^{b} \pm 0.40$	$1.78^{b} \pm 0.71$	$2.98^{a} \pm 1.14$	$3.27^{a} \pm 0.50$

Refer Fig. S-1 for the box and whisker plots of these data

Values for a given amino acid followed by different letters are different at a $P < 0.05$. For example, for Arg the first and third groups are different while the fifth group is not different either from the first or third (one way ANOVA)

[A] Larvae were immersed in saline for 2 min

[B] Larvae were pinched for 50 s

[C] Larvae were refrigerated for 25 min

[D] Larvae were refrigerated for 25 min and were then immersed in cold saline (10°C) for 2 min

Table 2 Average (mM ± SD) hemolymph amino acids concentrations of six different *Drosophila* genotypes based on individual larvae (*n*) that were control-treated

	JhI-21 overexpression mutant ($n = 10$)	Control genotype 1 ($n = 11$)	Control genotype 2 ($n = 11$)	LOF mutant 1 ($n = 9$)	LOF mutant 2 ($n = 9$)	LOF mutant 3 ($n = 11$)
Arg	$1.67^{a} \pm 0.39$	$1.21^{b} \pm 0.27$	$1.71^{a} \pm 0.46$	$1.24^{b} \pm 0.27$	$1.28^{b} \pm 0.37$	$1.45^{ab} \pm 0.22$
Asp	$0.16^{b} \pm 0.07$	$0.34^{a} \pm 0.11$	$0.17^{b} \pm 0.06$	$0.15^{b} \pm 0.04$	$0.18^{b} \pm 0.06$	$0.16^{b} \pm 0.03$
Gln	$18.6^{bc} \pm 5.4$	$19.6^{bc} \pm 2.8$	$16.9^{c} \pm 2.3$	$24.1^{a} \pm 3.5$	$19.0^{bc} \pm 4.2$	$20.4^{ab} \pm 4.6$
Glu	$0.95^{bc} \pm 0.21$	$0.86^{bc} \pm 0.25$	$0.71^{c} \pm 0.20$	$1.10^{ab} \pm 0.45$	$1.30^{a} \pm 0.39$	$1.34^{a} \pm 0.38$
Gly	$2.98^{b} \pm 0.72$	$2.86^{b} \pm 0.79$	$2.68^{b} \pm 0.51$	$3.27^{b} \pm 0.90$	$3.29^{b} \pm 0.84$	$4.25^{a} \pm 0.84$
Lys	$3.01^{c} \pm 1.46$	$4.01^{bc} \pm 0.72$	$4.06^{bc} \pm 1.29$	$4.46^{b} \pm 0.64$	$3.32^{bc} \pm 1.16$	$7.31^{a} \pm 2.41$
Tau	$1.55^{b} \pm 0.38$	$0.85^{c} \pm 0.34$	$1.33^{b} \pm 0.39$	$0.83^{c} \pm 0.33$	$0.79^{c} \pm 0.35$	$1.93^{a} \pm 0.44$

Refer Fig. S-2 for the box and whisker plots of the data presented in this table. Values for a given amino acid followed by different letters are different at $P < 0.05$ (one way ANOVA)

whether the average amino acid levels depended on the two larval treatment conditions. The reported mean values are followed by the corresponding standard deviations (±).

Results and discussion

Selection of larval treatment conditions

In the present study the wild-type larvae were treated with five different prior-to-sampling conditions to investigate any environmentally- or behaviorally related hemolymph content changes. The prior-to-sampling control larval treatment used in this study was the procedure described in our previous study (Piyankarage et al. 2008). In previous work it was noted that some hemolymph amino acids were increased with vigorous larval motion when larvae were immersed in mineral oil for 50 s during hemolymph sampling (Piyankarage et al. 2008). These increases were similar to that observed with a hemolymph sampling technique utilized by Augustin et al. (2007) where larva were immersed in a saline solution for hemolymph collection. These immersions for hemolymph collection may stress the larvae leading to the observed motion that may alter hemolymph chemical content. To study this possibility two pre-sampling conditions were used. In one of the treatments the larvae were immersed in a saline solution for a period of 2 min prior to sampling. Notably, the larval

motion in the saline solution was not as pronounced as compared to that of the mineral oil-immersed larvae. In the other treatment individual larvae were pinched for 50 s with a microdissection pin where vigorous larval wriggling was observed that was similar to that of the mineral oil-immersed larval behavior.

It is equally important to perform sampling on anesthetized larvae to understand their basal hemolymph chemical compositions. Refrigeration has been utilized for anesthetization of insects (Predel et al. 2004; Janisiewicz et al. 1999; Sukontason et al. 2004) and this simple chemical-free method was used for the larval anesthetization. Although the third instar larvae are active and mobile at room temperature, after 20 min of refrigeration at 4°C their mobility was observed to be extremely limited during the tests carried out with different refrigeration periods ranging form 5 to 35 min. The larvae responded to external stimuli such as pinching even after a longer refrigeration time of 35 min indicating that they were alive. Accordingly, the larvae were refrigerated at 4°C for 25 min for anesthetization prior to sampling. The fifth treatment procedure involved immersion of the anesthetized larvae in cold saline solution prior to the sampling for comparison with the non-anesthetized-saline-immersed larvae.

Effects of sampling environments on *Drosophila* behavior and hemolymph composition

The results of the hemolymph qualitative analyses for the pinched, saline immersed, anesthetized, and anesthetized-cold-saline-immersed larval groups were similar to that of the control larval hemolymph in both borate and MEKC buffer separations. Typical electropherograms for derivatized hemolymph of these groups in borate buffer are similar to that of the clean excision genotype shown in Fig. 1a. These results are comparable to the qualitative hemolymph analysis of the wild-type larvae in previous work (Piyankarage et al. 2008) where 9 individual amino acids were resolved and 13 amino acids were identified in 20 mM borate and MEKC buffers, respectively.

Table 1 shows the mean hemolymph amino acid differences seen in response to larval treatments while Supplementary Fig. S-1 shows the observed distributions of the corresponding amino acids for the five conditions. The superscript letters followed by the average concentrations in Table 1 indicate whether the observed concentrations are significantly different ($P < 0.05$) with respect to the pre-sampling condition. A significantly higher level of Arg was observed for the pinched larvae corresponding to a 53% increase over that of controls (Table 1, Fig. S-1). The pinched larvae also showed a significant 122% increase in the Glu level compared to the controls. However, the hemolymph levels of the other five amino acids were similar for both the pinched and control larval groups (Table 1). These results are comparable to the previously reported amino acid variations of the mineral-oil immersed larvae where significant elevations were observed with only Arg and Glu (Piyankarage et al. 2008). Another common observation of both pinched and oil-immersed larvae was the intense wriggling motion. Although other biological explanations are possible, the elevated hemolymph-Arg levels could be due to the larval wriggling as arginine phosphate is the phosphagen of insects (Beis and Newsholme 1975). The behavioral and chemical changes

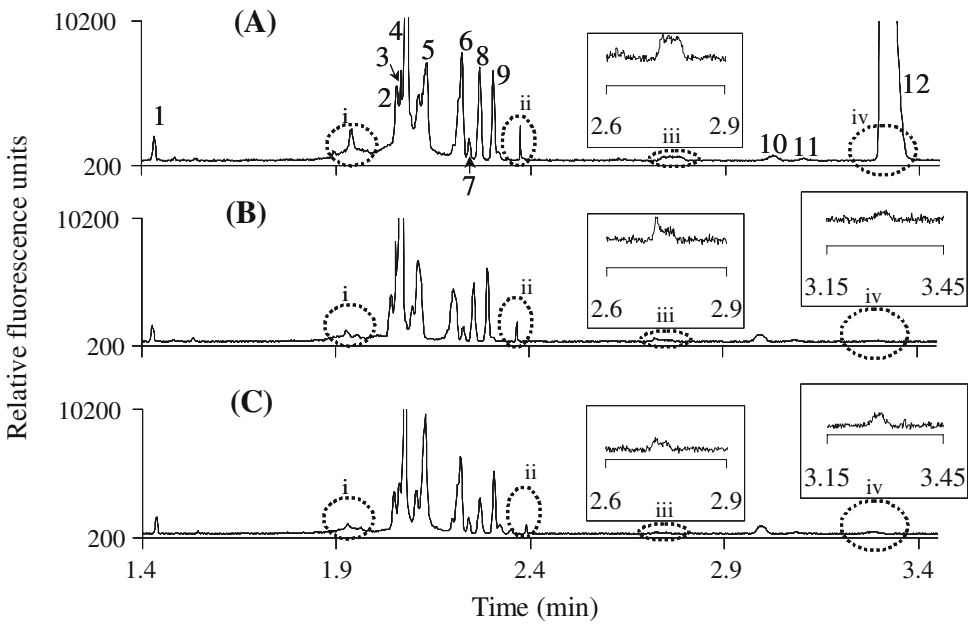

Fig. 1 Electropherograms, time in minutes (min) versus relative fluorescence units, for separation of fluorescamine-labeled amino acids in hemolymph of **a** Control genotype 1, **b** LOF mutant 1, and **c** LOF mutant 2 larvae in 20 mM borate buffer (pH 9.1) at 30 kV in 50 cm bare fused-silica capillary with 50-µm-i.d. and 360-µm-o.d. *1* arginine, *2* tyrosine, *3* histidine, *4* glutamine, *5* asparagine and threonine, *6* alanine and serine, *7* taurine, *8* lysine, *9* glycine, *10* glutamate, *11* aspartate, *12* unknown. The *dotted circles* i, ii, iii, and iv along with the *insets* for iii and iv highlight the differences among the three electropherograms with respect to the unknown peaks

observed for pinched and oil-immersed larvae may also be related to larval stress. This is further supported by the less pronounced locomotor behavior of the saline immersed larvae and their hemolymph Arg and Glu levels being indistinguishable from controls. Under the laboratory conditions the larvae live in their food, which is an aqueous slurry and recent findings on insect respiratory proteins indicate that *D. melanogaster* could survive under hypoxic conditions (Burmester and Hankeln 1999, 2007; Hankeln et al. 2002). This could be the reason for the observed similarities in hemolymph compositions between the control and the short-term saline immersed larvae.

Other effects with different pre-sampling conditions are less clear. Except Tau, all the other investigated hemolymph amino acid levels for the anesthetized larvae are comparable to those of the controls (Table 1). Table 1 and Fig. S-1 show a clear distinction between the Tau levels of the three non-anesthetized (non-refrigerated) and the two anesthetized larval groups, with the anesthetized larvae reporting significantly higher concentrations of 50% over the other animals. This observation with the refrigerated *D. melanogaster* larvae is similar to the published correlations found between the increasing-Tau levels and hypothermia in rats and rabbits (Sgaragli et al. 1975, 1981; Frosini et al. 2003, 2006). An important inhibitory neurotransmitter Gly (Werman et al. 1967, 1968; Wheeler et al. 1999) was found to be 37% higher than control for the anesthetized-cold-saline-immersed larvae. The Gly level of the anesthetized-cold-saline-immersed larvae is also significantly higher than that for the pinched larvae who wriggled intensely. This effect is only seen in the animals that are both anesthetized and stimulated, suggesting the presence of an active mechanism to inhibit activity at lower temperatures. The average hemolymph lysine (Lys) levels for the anesthetized-cold-saline-immersed larvae are significantly lower with a 57% decrease compared to all other experimental groups which are statistically indistinguishable. Possible reasons underlying the observed Lys trends are not clear and will require further investigation. In contrast, the hemolymph Aspartate (Asp) and Gln levels are not affected significantly by the different larval treatment conditions (Table 1).

Hemolymph analyses for six *D. melanogaster* genotypes

The recent discovery of *genderblind* (*gb*) mutants and the corresponding changes in extracellular glutamate concentrations and differential Glu receptor tissue localization highlights the importance of understanding the hemolymph chemical information of the transgenic animal model, fruit fly (Augustin et al. 2007; Grosjean et al. 2007). These *gb* mutants show significantly lower extracellular Glu levels and higher synaptic Glu receptor abundance (Grosjean et al. 2007; Piyankarage et al. 2008). Four mutant genotypes based on the *JhI-21* protein, which is proposed to function similarly to the genderblind protein, were studied. The *JhI-21* mRNA levels of the *JhI-21* overexpression mutant were significantly increased several fold compared to the control genotypes. The three *JhI-21* loss-of-function mutants, on the other hand, showed significantly lower *JhI-21* mRNA, compared to controls. LOF mutant 3 showed the lowest expression of *JhI-21*. Figure 1a is a typical electropherogram for the derivatized hemolymph of control genotype 1 larva and its identified amino acid peak pattern is similar to that of the other five investigated genotypes. The peaks of the electropherograms for the six genotypes were confirmed by spiking with 24 different amino acid standards to demonstrate qualitative differences between the genotypes. The large unknown peak 12 that migrated after Asp was observed for the control genotype 1 (Fig. 1a) and control genotype 2 and also for genotypes of *JhI-21* overexpression mutant and LOF mutant 2. However, this large unknown peak is extremely small for LOF mutant 1 (Fig. 1b) and LOF mutant 3 (Fig. 1c) hemolymph. The dotted circles in Fig. 1 also show the other differences among the control genotype 1, LOF mutant 1, and LOF mutant 3 genotypes. These unknown peaks could be due to the peptides or proteins of hemolymph reported elsewhere (Vierstraete et al. 2003, 2004; Predel et al. 2004; Chen and Hanimann 1965; Chen and Buhler 1969).

Table 2 lists the observed mean hemolymph amino acid levels and Fig. S-2 again presents the data as a box and whisker plot to demonstrate the variance about the observed means. The average Glu levels for LOF mutant 2 and LOF mutant 3 were significantly higher with respect to the two control genotypes and the *JhI-21* overexpression mutant genotype. This result is in contrast to the previously observed Glu levels between the wild-type and *gb* mutants (Augustin et al. 2007). Also the hemolymph-Glu level for LOF mutant 1, the other *JhI-21* loss-of-function mutant genotype, did not differ from either the *JhI-21* overexpression mutant or control genotype 1 larvae, although it is higher with respect to the control genotype 2. Accordingly, the *JhI-21* mutations do not appear to affect hemolymph Glu levels in a manner similar to genderblind mutations in spite of both *JhI-21* and genderblind proteins belonging to the same gene superfamily. These data suggest that the effect of genetic variations on observed hemolymph content is complex and that *JhI-21* and genderblind do not function the same way, despite high protein similarity. Notably, the LOF mutant 3 larvae that showed the lowest expression of *JhI-21* mRNA does lead to the most chemically distinct of the *JhI-21* loss-of-function mutant (LOF 1, 2, and 3 mutant) genotypes with significantly elevated Gly, Lys, and reduced Tau levels.

Perhaps the most surprising result of this series of experiments is the observation that the two control genotypes display a number of significant differences from each other. These differences include their hemolymph Arg, Asp, and Tau levels (Table 2). While there is a good degree of congruence in the observed hemolymph composition, functionally equivalent genotypes would ideally be identical. The source of content variations may represent the genetic background or noise. Given the importance of experimental controls and the need for optimal signal-to-noise for any measurement, these results merit further study.

Physical perturbation of different genotypes to probe larval physiology

These studies assessed the variations of hemolymph amino acid concentrations of different genotypes with and without a pinch stimulus. These experiments are based on the results from the different pre-sampling conditions suggesting that a pinching was a significant perturbation and that there were significant hemolymph composition differences across several genotypes. In all cases Arg and Glu levels of the pinched (stressed) larvae were significantly elevated compared to the control-treated larvae (Tables 2, 3). While there were no clear behavioral differences observed among the genotypes, quantitative assessment of hemolymph amino acid composition did show some variance among them. Table 3 shows the observed mean hemolymph amino acid levels for the pinched larvae of these four genotypes. The pinched *JhI-21* overexpression mutant larvae showed a 100% Arg increase over control-treated larvae, while for the other three genotypes the Arg increase due to pinching was less than 53%. Hemolymph Glu was observed to increase only by 85% for the stressed control genotype 1, while for the other stressed genotypes the change was over 100%. This astounding increase in hemolymph Glu, irrespective of the genotypes, may reflect more glutamate spillover to hemolymph due to the neuronal response to the pinch stimulus.

Also, it is interesting to note the contrasting hemolymph chemical differences observed between the *JhI-21* overexpression mutant and the LOF mutant 3 larvae when they were pinched or stressed. The stress-related-hemolymph Gly and Tau variations for these two mutant genotypes are observed to be opposite to each other. For *JhI-21* overexpression mutant larvae Gly and Tau levels increased significantly with stress compared to the control-treated larvae while hemolymph levels of those two amino acids decreased significantly with stress for the LOF mutant 3 larvae (Tables 2, 3). The complementarity of these responses suggests that the variance in the expression of functional *JhI-21* leads to variable neuronal effects in response to pinching. Also, the *JhI-21* overexpression mutant larvae display a significant change in six out of seven hemolymph amino acids assessed in this pinch study compared to the four of seven of the LOF mutant 3 larvae (Tables 2, 3) with respect to their control-treated group. The greater variation in amino acids of the stressed *JhI-21* overexpression mutant stocks may reflect a greater overall perturbation due to the mutation and may be related the relative increase in difficulty in maintaining these fly stocks.

As with the basal levels the two genetic controls also showed differences in response to pinching. For instance, the significant increase of Gln and Tau in the stressed control genotype 2 larvae is not seen for the larvae of stressed control genotype 1 (Tables 2, 3). The pinched larvae of control genotype 1 also showed a significant decrease of 62% in its Asp level compared to its control-treated group. This is not observed either with the control genotype 2 (Tables 2, 3) or with the wild-type larvae (Table 1). In contrast, the percentage increase in Gln and Tau for the pinched larvae of control genotype 2 is comparable to that of the pinched *JhI-21* overexpression mutant larvae (Tables 2, 3). The Glu concentrations of these

Table 3 Average (mM ± SD) hemolymph amino acids concentrations of pinched individual (*n*) *Drosophila* larvae of four different genotypes

	JhI-21 overexpression mutant ($n = 9$)	Control genotype 1 ($n = 9$)	Control genotype 2 ($n = 10$)	LOF mutant 3 ($n = 9$)
Arg	3.35* ± 0.90 (100%)	1.85* ± 0.50 (53%)	2.31* ± 0.48 (35%)	2.19* ± 0.49 (51%)
Asp	0.19 ± 0.07	0.13◆ ± 0.03	0.16 ± 0.05	0.16 ± 0.04
Gln	24.9* ± 6.4	19.5 ± 4.2	22.5* ± 6.2	18.7 ± 3.6
Glu	1.99* ± 1.09 (109%)	1.59* ± 0.72 (85%)	1.48* ± 0.68 (108%)	2.76* ± 0.76 (106%)
Gly	5.23* ± 1.78	2.33 ± 0.43	2.71 ± 0.43	2.83◆ ± 0.58
Lys	34.8* ± 13.2	4.48 ± 1.26	4.56 ± 1.08	5.61 ± 2.04
Tau	2.77* ± 0.60	1.07 ± 0.25	2.20* ± 0.55	1.34◆ ± 0.34

Given in parenthesis are the percentage increase from the control Arg and Glu levels for each genotype

* For a given genotype the amino acid concentration of the pinched is significantly higher from its control-treated at $P < 0.05$

◆ For a given genotype the amino acid concentration of the pinched is significantly lower from its control-treated at $P < 0.05$

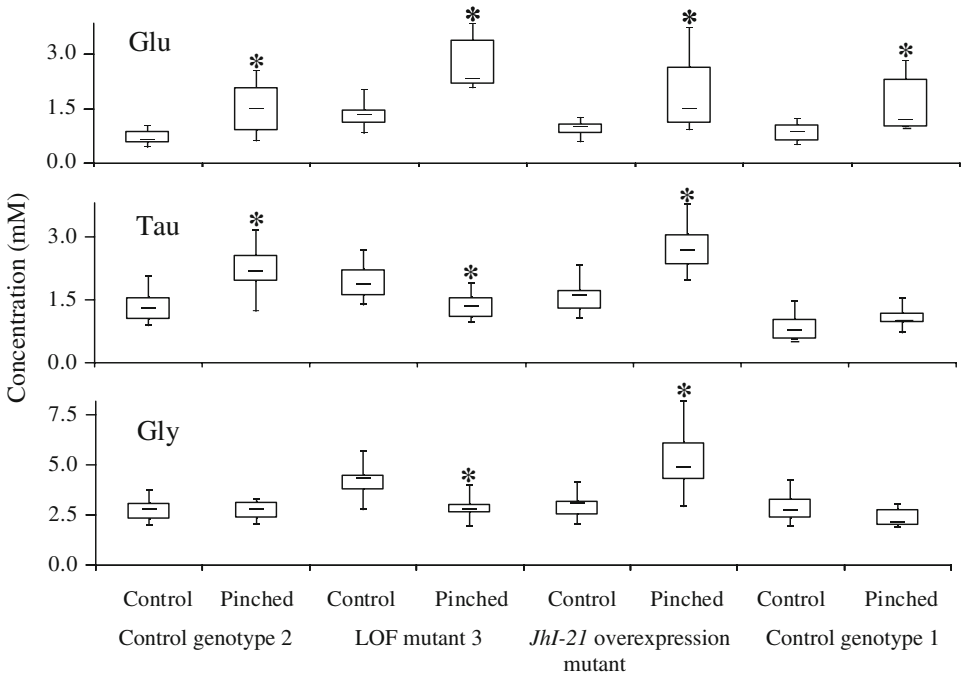

Fig. 2 *Box and whisker plots* showing the hemolymph variations of glutamate, taurine, and glycine for the larvae of four different genotypes those were treated through the control (control-treated) and pinched conditions prior to sampling. The number of larvae analyzed under each condition from each genotype is as given in Tables 2 and 3. 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. *For a given genotype the amino acid concentration of the pinched is significantly different from control-treated larvae at $P < 0.05$

stressed controls mirror previous results published by Augustin et al. for the control genotype 1 (Tables 1, 3). Augustin et al. immersed dissected larvae in a saline solution for about 5 min to obtain a hemolymph sample that was analyzed by HPLC (Augustin et al. 2007). The longer immersion period of 5 min for the already dissected larvae could have contributed to the elevated hemolymph Glu levels. However, this immersion approach complicates quantitation as the absolute volume of the collected hemolymph was not known.

Glu, Tau, and Gly distribution in stressed and non-stressed (control) larvae of four genotypes

A primary advantage of using our sampling method is the quantitation of amino acids from individual larvae that can be used to calculate statistical distributions for amino acid content of individual genotypes. There are several findings uncovered from these studies beyond the trends for the mean amino acid levels observed for the four different genotypes studied following the pinching. Figure 2 shows the box and whisker plots of hemolymph levels of Glu, Tau, and Gly for the four genotypes under both control and pre-sampling pinch conditions. The Glu levels of the pinched larvae are distributed over broader concentration ranges compared to those of the control-treated (Fig. 2) for all four genotypes. This larger variability may be a result of individual larval differences in response to the stress. Additionally this may reflect the differences in execution of the pinch stimulus, which was performed manually. There are also individual genotypic differences that suggest the relationship between the genetic variations and observed chemical content are linked in ways that are not completely understood. For example, the Glu distributions for the two larval groups of control genotype 2, the control-treated and the pinched, are different from those of the other three genotypes. The median Glu level for the control-treated larvae of control genotype 2 is closer to the lower quartile while for the stressed control genotype 2 the Glu median is at the middle of the box (Fig. 2). The positions of the Glu medians of the control-treated LOF mutant 3, *JhI-21* overexpression mutant, and control genotype 1 are closer to the upper quartiles while the pinched of these genotypes display medians closer to the lower quartiles. This trend is also seen with the Tau medians. Gly distributions for the pinched LOF mutant 3 and the pinched *JhI-21* overexpression mutant larvae are significantly different, with the latter showing the broadest and the former showing the narrowest concentration ranges. While the significance of the variance is not entirely clear, the medians for Gly in these non-pinched mutants are toward the top quartile contrasting the corresponding pinched group medians (Fig. 2). The pinched control genotype 1 larvae showed a narrow distribution for Tau levels compared to its control-treated larvae as well as with respect to the larvae of the other three genotypes (Fig. 2).

Conclusion

The findings of this study show that pre-sampling conditions of *D. melanogaster* larvae significantly alter their

hemolymph amino acid levels. Further, the use of a pinch stress highlights differences between genotypes that are not clearly evident otherwise. Some pinch-related hemolymph chemical changes such as for Arg and Glu were irrespective of the studied genotypes while other changes were genotype specific. The chemical analyses of hemolymph from *JhI-21* mutants suggest there are significant physiological differences compared to *gb* mutants although both genderblind and *JhI-21* proteins are of the same gene superfamily. Strikingly, basal and pinch-related hemolymph chemical differences were also observed between the two investigated control genotypes. Because of the role of controls in any experimental design, characterizing and determining the sources of these differences between "equivalent" genotypes is fundamentally important. Further work is also needed to precisely identify the physiological responses that lead to these hemolymph composition changes of individual amino acids. Work is also needed with other methods of chemical analysis to assess hemolymph protein, metabolite or ionic composition. The studies here demonstrate a broader application of collecting hemolymph from individual fruit-fly larvae to probe the physiological changes that result from genetic manipulations. The chemical information obtained in these methods can be used to more completely characterize fruit-fly models of human disease potentially identifying therapeutic targets and improving treatments.

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