Efficacy of D-Glucose-$^{13}C_6$ as a Label for Amino Acid Identification and Quantification in Mediterranean Fruit Flies (Diptera: Tephritidae)[1]

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Abstract The efficacy of D-glucose labeled with $^{13}C_6$ (D-glucose-$^{13}C_6$) in the study of amino acid metabolism in insects was determined by incorporating either D-glucose-$^{13}C_6$ or unlabeled D-glucose into larval and adult diets of the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae). Feeding treatments were: (a) labeled glucose diet for larvae + unlabeled glucose diet for adults; (b) unlabeled glucose diet for larvae + labeled glucose diet for adults; (c) labeled glucose diet for larvae + labeled glucose diet for adults; and (d) unlabeled glucose diet for larvae + unlabeled glucose diet for adults. Amino acid content of extracts from unmated adults and mated adults was determined with gas chromatography-mass spectrometry (GC-MS). L-alanine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glycine, L-isoleucine, L-leucine, L-methionine, L-proline, L-phenylalanine, L-serine, L-threonine, L-tyrosine, and L-valine were identified from the insect extracts. In the treatment that fed unlabeled glucose larval and adult diets (control), amino acid content was lower after mating than before mating except for L-cysteine (e.g., 13-fold increase). Amino acid content in insects fed D-glucose-$^{13}C_6$ also decreased after mating except for L-valine, L-leucine, L-tyrosine, and L-isoleucine, all of which remained comparatively equal before and after mating, and L-threonine which increased almost 12-fold after mating. Only trace amounts of L-tyrosine and L-isoleucine were detected before and after mating in those fed labeled diets. These results suggest that stable isotopes can be used as labels for insect metabolic studies.

Key Words amino acids, Mediterranean fruit fly, GC-MS, stable isotopes, D-glucose-$^{13}C_6$

Stable isotopes have been used as labeling tools in biological studies of a variety of insects including Lepidoptera (Hood-Nowotny et al. 2016) and the Mediterranean fruit fly (medfly), *Ceratitus capitata* (Wiedenmann) (Diptera: Tephritidae). Stable isotopes are noninvasive and effective markers in biological systems and the various types, for example C, H, O, N, and S, are not species-specific. They have been used successfully to study food resources, dispersal, mating, and resource

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allocation (Al-Khshemawee et al. 2018, 2019; Becker 2008; Helinski et al. 2007; Hood-Nowotny and Knols 2007, Ponsard et al. 2004).

Amino acids have important roles in insect physiology, including energy metabolism and protein synthesis (Oppenoorth et al. 1985). Insects store proteins ingested or synthesized during larval stages for use in reproduction and metamorphosis (Okuse 2007). Unbalanced protein losses have been implicated in the ephemeral nature of the adult life stage.

The study reported herein was undertaken (a) to determine if consumption of D-glucose labeled with $^{13}C_6$ (D-glucose-$^{13}C_6$) affects the amino acid content of the insect, and (b) to use D-glucose-$^{13}C_6$ to elucidate the amino acid profile before and after mating. The medfly was chosen as the subject of the study.

The medfly is a major pest of fruit and vegetable crops worldwide (Al-Khshemawee et al. 2017). Investigations on its management have included pesticides, exclusion, attractants, and sterile insect technique (Enkerlin 2005). A greater comprehension of this pest's physiology, including amino acid and protein metabolism, is needed. The efficacy of D-glucose-$^{13}C_6$ in amino acid metabolism studies in the medfly was thus undertaken.

Materials and Methods

Medflies used in this study were obtained from a colony maintained in the Post-Harvest Biosecurity and Food Safety Laboratory at Murdoch University (Perth, Western Australia) at a temperature of 23 $\pm$ 2°C, relative humidity 75 $\pm$ 5%, and a light:dark cycle of 12:12 h. This colony was established from a colony at the Department of Agriculture and Food, Western Australia, Perth. Briefly, medfly pupae were placed in screen cages (40 $\times$ 40 $\times$ 40 cm), each containing adult medfly food of crystalline sugar and yeast hydrolysate at a ratio of 4:1 dissolved in 50 ml water. About 10–12 d after adult emergence from pupae and subsequent mating, female adults were allowed to oviposit through a mesh substrate, and eggs then dropped into a tray of water. Eggs were collected from the water daily, and larvae hatching from the eggs were placed on a carrot media larval diet (Tanaka et al. 1969) until pupation.

D-glucose-$^{13}C_6$ (CAS 110187-42-3, Sigma-Aldrich, St. Louis, MO) was used as the isotope label. Medfly larvae and adults were fed diets containing the labeling compound. To expose larvae, 100 medfly eggs were placed on a 25-g block of carrot media in 9-mm sterile Petri dishes. As the eggs began to hatch, 100 mg of D-glucose-$^{13}C_6$ was added to the carrot media in each Petri dish, thus allowing larvae to consume the isotope with the larval diet media (Neto et al. 2012). For adults, 100 mg of D-glucose-$^{13}C_6$ was mixed with 1 g of 99.5% sucrose (Sigma-Aldrich) and suspended in 15 ml of water, on which adults imbibed and consumed the label. Feeding treatments were established as follows: (a) labeled glucose diet for larvae + unlabeled glucose diet for adults; (b) unlabeled glucose diet for larvae + labeled glucose diet for adults; (c) labeled glucose diet for larvae + labeled glucose diet for adults; and (d) unlabeled glucose diet for larvae + unlabeled glucose diet for adults.

Samples of adult medflies were obtained at time of mating and after 3 d of mating activity. A total of 50 mg of medfly adults was collected from each of the four feeding treatment regimens for the two different sampling intervals. Samples were first

homogenized and then centrifuged at 1.957 × G for 2 min in 1 ml of methanol/water/ribitol (20:2:1). Samples were evaporated with 3 to 4 h of exposure to nitrogen and then freeze-dried for 16 h. These extracts were transferred individually into 200-µl glass inserts within 2-ml analytical vials.

The amino acid profiles for each extract were obtained by GC-MS analysis using an auto-sampler G4513A (CN16220044), GC-MS 7890E gas chromatograph equipped with a detector (5977E) mass spectrometer and an HP-5MS column (30 m, 0.25 µm, 0.25-µm film thickness, catalog number 95051) (Agilent Technologies, Santa Clara, CA). The carrier gas was helium (99.999%) and the operational conditions for the GC-MS were as follows: injector port temperature 250°C; initial oven temperature 50°C, increased to 250°C at a rate of 5°C/min; MS Quad 150°C; MS source 230°C; pressure 0.747 kg/cm^2. The flow rate was 1.2 ml/min, and the splitless injection was 20 ml/min for 1.5 min. The total equipment run time was 45 min. A 10-µl syringe was used for addition of the internal standard to the sample before injection. Between additional steps, the syringes were washed with methanol and n-hexane before each injection. Amino acid standards were acquired from Sigma-Aldrich.

Statistical analysis. Principal component analysis (PCA) with the correlation matrix method was used for statistical analysis using MetaboAnalyst 3.0 (Xia and Wishart 2017), a comprehensive online tool for metabolomics analysis and interpretation. Fifteen principal components (PCs) were estimated from the factors represented in the correlation matrix. Data were normalized by a pooled sample from the group. Principal component analysis, ANOVA, biplot, and correlations were used to analyze the data (Chong and Xia 2018).

Results and Discussion

Of the metabolic compounds identified in the *C. capitata* adults, 15 peaks were those of amino acids, namely L-alanine, ammonium chloride, L-aspartic acid, L-cysteine, L-glutamic acid, glycine, L-isoleucine, L-leucine, L-methionine, L-proline, L-phenylalanine, L-serine, L-threonine, L-tyrosine, and L-valine (Table 1). The amount of each amino acid is expressed by the area under each peak of the GC-MS plots, with variance indicated by standard deviation (Table 1) or percentage (Fig. 1). Based on nonoverlapping standard deviations of the mean areas under the peaks, the amount of most of the amino acids detected in *C. capitata* adults decreased after mating (Table 1). Some exceptions will be noted.

Weckwerth et al. (2004) effectively used correlation analysis to detect sugar synthase in metabolic networks of plants. Using a similar approach with the same correlation analysis, we found that several amino acids increased following labeling while sugar levels decreased, which can explain the relationship between amino acids and sugars in *C. capitata* labeled and not labeled with D-glucose-$^{13}C_6$ (Table 1). Multivariate PCA of the normalized raw peaks showed a measure of separation among five biological samples. Based on the data of a set of metabolites, or using PCA in relation to the overall variance of the experiments, the treatment showed metabolic changes during mating. Plotting the principal components of the samples from the five samples should reveal a treatment-dependent clustering of samples in a 5-dimensional space. When PCA analysis was applied to the peak areas from

Table 1. Mean ± SD* area of amino acid peaks resulting from GC-MS analysis of *C. capitata* adults not labeled and labeled with D-glucose-$^{13}C_6$, before and after mating.

		Not Labeled		Labeled	
Amino Acid	**Standard**	**Before Mating**	**After Mating**	**Before Mating**	**After Mating**
L-Valine	12.151 ± 9.707	8.035 ± 2.431	0.057 ± 0.023	2.445 ± 0.721	2.221 ± 1.076
L-Alanine	97.477 ± 84.897	1.223 ± 0.338	0.065 ± 0.032	71.788 ± 20.185	22.635 ± 3.7081
L-Glycine	5.885 ± 5.771	0.164 ± 0.284	0.002 ± 0.008	1.948 ± 0.469	0.579 ± 0.561
L-Proline	25.372 ± 28.854	2.456 ± 2.099	0.001 ± 0.004	48.209 ± 15.892	6.764 ± 2.503
L-Serine	27.150 ± 23.526	1.991 ± 1.716	0.003 ± 0.009	1.617 ± 0.838	0.310 ± 0.253
L-Leucine	104.095 ± 9.285	48.74 ± 17.38	50.82 ± 9.948	129.496 ± 32.71	128.355 ± 51.809
L-Threonine	36.959 ± 36.923	76.60 ± 18.72	8.879 ± 3.151	3.839 ± 1.753	45.850 ± 10.677
Ammonium chloride	168.375 ± 50.060	1.859 ± 0.924	0.007 ± 0.003	1.486 ± 0.357	0.006 ± 0.002
L-Methionine	34.677 ± 30.378	0.022 ± 0.001	0.001 ± 0.005	0.009 ± 0.002	0.002 ± 0.001
L-Aspartic acid	24.718 ± 21.712	0.327 ± 0.125	0.004 ± 0.006	0.284 ± 0.073	0.000 ± 0.000
L-Phenylalanine	17.986 ± 15.941	8.731 ± 4.636	0.003 ± 0.008	1.631 ± 0.758	0.002 ± 0.001
L-Glutamic acid	51.408 ± 44.599	0.053 ± 0.024	0.005 ± 0.003	0.231 ± 0.056	0.008 ± 0.006
L-Cystine	109.315 ± 9.225	0.034 ± 0.001	0.445 ± 0.191	0.246 ± 0.073	0.002 ± 0.001
L-Tyrosine	62.489 ± 73.421	2.720 ± 1.649	0.006 ± 0.002	0.005 ± 0.003	0.003 ± 0.001
L-Isoleucine	50.904 ± 8.845	2.720 ± 1.649	0.973 ± 0.816	0.005 ± 0.003	0.001 ± 0.001

* 1 unit = GC area of 10^5 ($n = 3$).

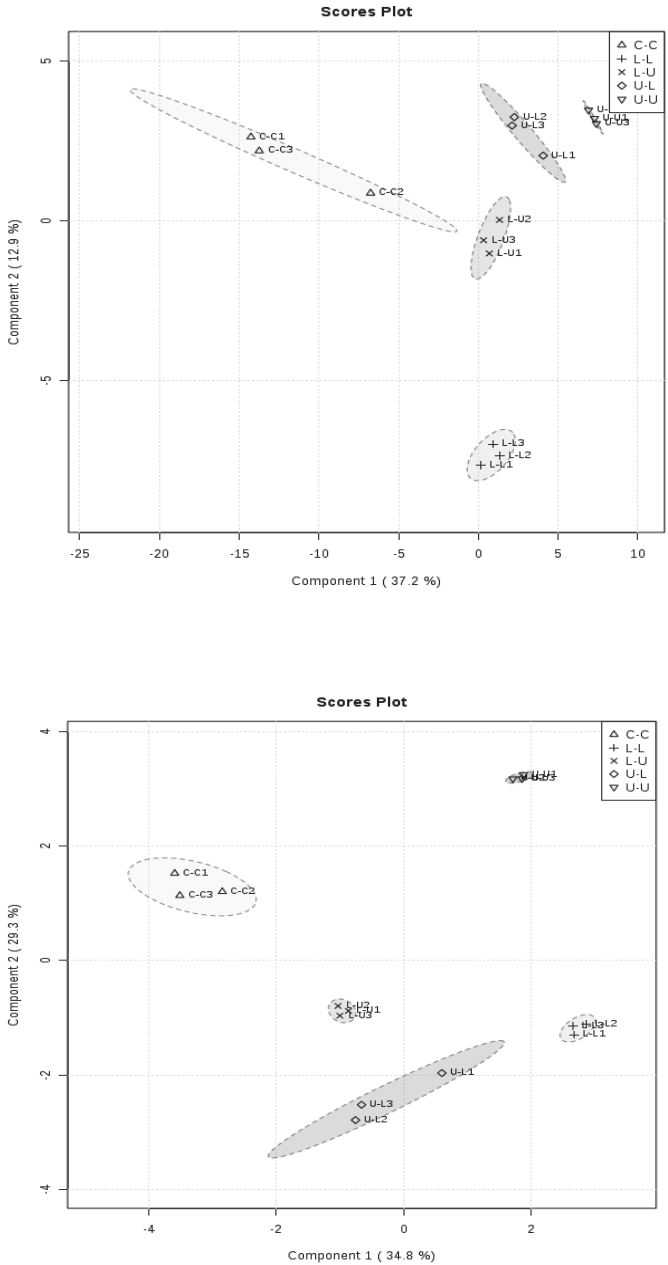
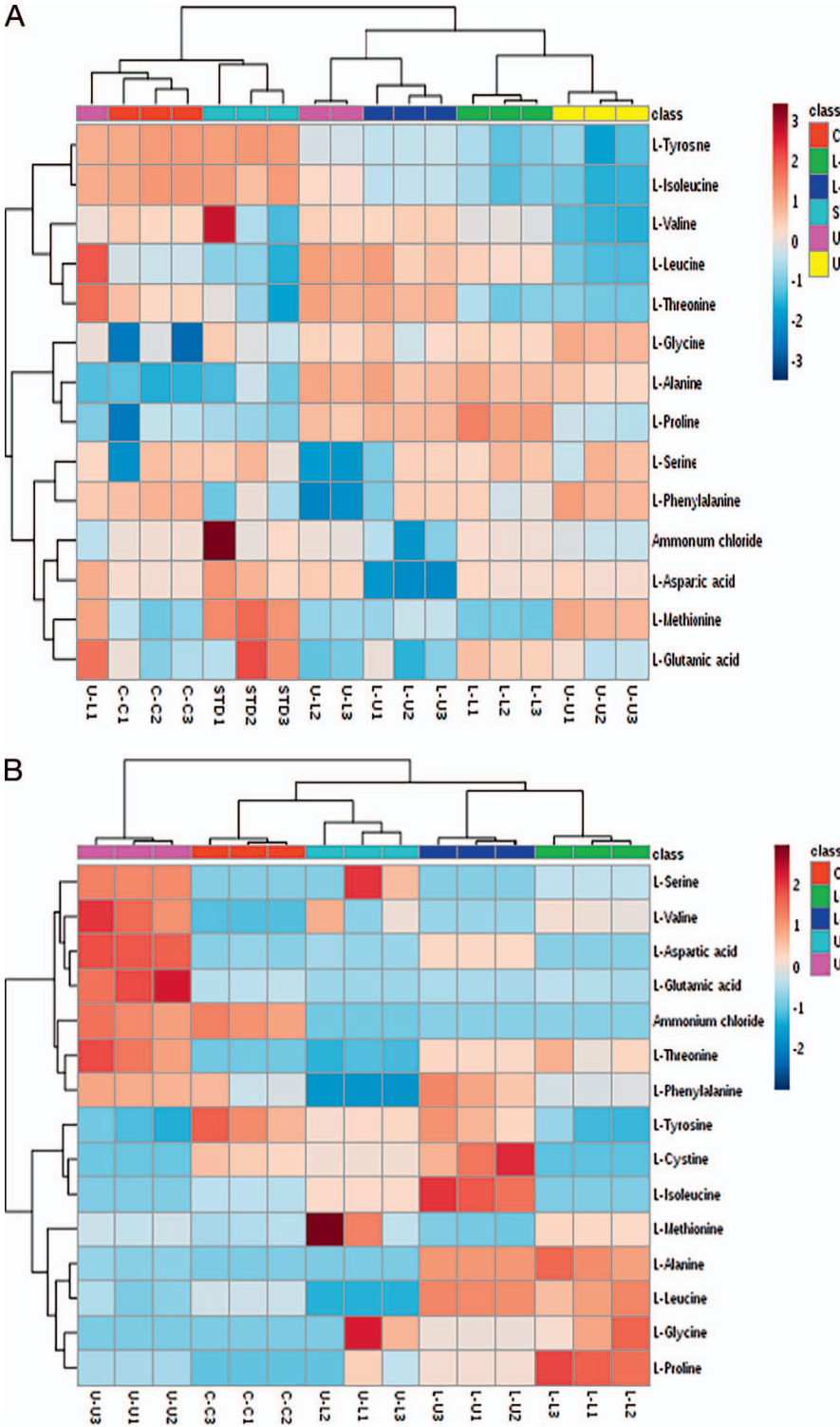


Fig. 1. Plots of amino acids before and after mating of *C. capitata*, with principal component percentages representing the variance of each component. Data are expressed by the peak area of amino acids. (Key: L-U, labeled in larval stage + unlabeled in adult stage; U-L, unlabeled in larval stage + labeled in adult stage; L-L, labeled in larval stage + labeled in adult stage; U-U, unlabeled in larval stage + unlabeled in the adult stage.)



labeled and unlabeled samples at different stages, the principal component plot showed a clustering effect along the first principal component, PC1 (Fig. 1).

The PC1 for the amino acid compounds included L-alanine, ammonium chloride, L-aspartic acid, L-cysteine, L-glutamic acid, glycine, L-isoleucine, L-leucine, L-methionine, L-proline, L-phenylalanine, L-serine, L-threonine, L-tyrosine, and L-valine and accounted for 49.2% of the variance and, therefore, contributed most to the variation across the samples. ANOVA confirmed 15 identifiable elevated amino acids, some of which were less abundant during mating. The greatest increase and decrease observed in the labeled adults were with threonine and proline, which elevated 10- and 15-fold, respectively. Alanine was significantly reduced in the labeled adults after mating. We also observed a linear correlation of L-leucine and L-isoleucine (Fig. 2), which might be explained in that L-valine, L-isoleucine, and leucine are produced in the same biosynthetic pathway, with the exception that the first enzymatic step uses different precursors for each amino acid (Hagelstein et al. 1997).

The amino acid profile we observed in *C. capitata* deviated from what might be considered an ideal profile for insects and is likely attributable to feeding on an artificial diet, as noted by Brodbeck (1987). Chen (1966) also noted that profound changes in protein metabolism occur during various developmental periods of insects. Embryogenesis, larval development and growth, molting, and metamorphosis are accompanied by characteristic variations in the patterns of peptides, amino acids, and proteins. Indeed, metabolic studies have revealed changes in amino acid and other metabolites in response to different conditions and stimuli (Malmendal et al. 2006; Michaud and Denlinger 2007). For example, the amounts of specific amino acids extracted from the sharpshooter *Homalodisca coagulata* (Say) were essentially independent of the levels in the xylem fluids of the host plant (Andersen et al. 1989). Glutamic acid increases have also been observed as a reaction to low temperature in the cricket *Teleogryllus emma* (Ohmachi & Matsuura) (Tomeba et al. 1988). Osanai and Yonezawa (1986) reported changes in the amino acids in the silkworm, *Bombyx mori* L., during embryogenesis, as well as alanine accumulation and its conversion to proline during diapause, an amino acid that is elevated in association with diapause in insects. Yi and Adams (2000) studied the effect of pyriproxyfen and photoperiod on free amino acid concentrations and proteins in the hemolymph of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). Given these examples, it is conceivable, therefore, that our observation of changes in amino acid concentration before and after mating of *C. capitata* results from physiological and metabolic changes associated with reproduction.

In conclusion, an accurate and robust GC-MS method was developed for the automated quantitative analysis of amino acids in labeled treatments of the

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Fig. 2. Heatmap representation of relative amino acid content extracted from *C. capitata* (A) during and (B) after mating. Key: L-U, labeled in larval stage + unlabeled in adult stage; U-L, unlabeled in larval stage + labeled in adult stage; L-L, labeled in larval stage + labeled in adult stage; U-U, unlabeled in larvae stage + unlabeled in adult stage.

Mediterranean fruit fly, *C. capitata*. Based on these results, 15 amino acids can be reliably detected using D-glucose-$^{13}C_6$ as a label. Our results further demonstrate that changes in the quantity rather than the composition of amino acids occurs with mating in *C. capitata*.

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