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Original Article

Methamphetamine causes anorexia in *Drosophila melanogaster*, exhausting metabolic reserves and contributing to mortality

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ABSTRACT — Methamphetamine (MA) appears to produce neurotoxic effects, in part, through disruptions of energy metabolism. A recent study of the whole-body proteome of *Drosophila melanogaster* showed many changes in energy metabolism-related proteins, leading us to hypothesize that MA toxicity may cause whole-body disruptions of energy metabolism. To test this, we monitored the response of energy reserves and other metabolites to MA-exposure with and without the addition of dietary glucose. We also monitored changes in feeding behavior, locomotor activity and respiration rates associated with MA-exposure to investigate how MA affects energy balance. We observed that glycogen and triglyceride levels decreased dramatically within 48 hr of MA-exposure, indicating a strongly negative caloric balance. Behavioral assays revealed that MA-treated flies decreased food consumption by 60-80% and exhibited a 2-fold increase in locomotion. Caloric expenditure decreased with MA-exposure, apparently due to a compensatory decrease in resting metabolism, showing that anorexia was the primary driver of the negative caloric balance. Additionally, we observed that glucose supplementation of MA-containing diet increased glycogen reserves by 44% at 48 hr, leading to a commensurate increase in survivorship. We conclude that dietary sugar supplementation enhances survivorship by partially compensating for decreased caloric intake resulting from MA-induced anorexia. The observation that MA produces similar behavioral changes in *Drosophila* and humans, i.e. increased locomotor activity and anorexia, further supports the use of *Drosophila* as a model organism for the study of the effects of MA.

Key words: Methamphetamine, Toxicity, Feeding behavior, Anorexia, Locomotor activity, Metabolic rate

INTRODUCTION

Methamphetamine (MA) is probably the most widely used synthetic stimulant in the world and appears to be increasing in popularity (EMCDDA, 2009), with some European countries reporting that MA is the most widespread illicit drug after cannabis. In addition to being a psychoactive stimulant, MA also produces numerous toxic side-effects that have important implications for human health, including increased oxidative stress (Cubells *et al*., 1994; Yamamoto and Zhu, 1998), heart failure (Hong *et al*., 1991; Yu *et al*., 2003), neurotoxicity leading to apoptosis (Cubells *et al*., 1994; Yamamoto and Zhu, 1998; Stephans *et al*.,1998; Davidson *et al*., 2001), and alteration of energy metabolism in the brain (Stephans *et al*., 1998; Chan *et al*., 1994; Wang *et al*., 2004; Virmani *et al*., 2002). In many cases, the molecular underpinnings of how MA induces these toxic effects are not completely understood and cannot readily be studied in humans for ethical reasons.

Results from Sun *et al*. (2011) showed that *Drosophila melanogaster* is a useful model system in which to study

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the toxic effects of MA. In a combined proteomic, transcriptomic, and metabolomic study, MA produced similar toxicological effects in *Drosophila* to those observed in humans (Sun *et al*., 2011), suggesting the involvement of evolutionarily conserved pathways. Furthermore, *Drosophila* has many attributes that make it an extremely attractive model organism, including a well characterized and functionally annotated genome (Roy *et al*., 2010; Celniker *et al*., 2009; Adams *et al*., 2000); a large number of genetic tools, such as ability to selectively knock down the expression of the majority of the known genes, using RNAi, in a tissue- and developmental state-specific manner (Dietzl *et al*., 2007); and *Drosophila* is readily maintained in laboratory culture. In addition, researchers have established behavioral bioassays that measure feeding, locomotor activity and aggression in *Drosophila* (Wong *et al*., 2008, 2009; Kume *et al*., 2005; Dankert *et al*., 2009), which are useful for understanding the biological responses to a particular drug/toxin.

The toxic effects of MA are best understood in the brain, where there is compelling evidence that toxicity arises, in part, from the ability of MA to disrupt normal metabolism. Depletion of ATP following MA exposure is correlated to MA-induced neurotoxicity in the striatum and impeding glucose (Glc) metabolism prior to MA-treatment potentiates this toxicity (Chan *et al*., 1994). Moreover, supplementation with substrates or cofactors of energy metabolism alleviates the effects of MA-induced neurotoxicity (Stephans *et al*., 1998; Virmani *et al*., 2002). In human subjects, MA produced a pronounced and long lasting suppression of Glc metabolism in the striatum and thalamus (Wang *et al*., 2004). As thalamic metabolism rebounded with time, corresponding improvements were also observed in neuropsychological function. All of these studies support the role of MA as metabolic toxin in the brain.

Changes in the whole-body proteome observed by Sun *et al*. (2011) suggest that the metabolic changes produced by MA may not be limited to the brain. Carbohydrate transporters and many glycolytic enzymes, including hexokinase, the rate-limiting step of glycolysis, were found to be up-regulated by ~10-fold in whole body homogenates (Sun *et al*., 2011). Furthermore, the addition of dietary carbohydrates increased survivorship of MA exposed flies. While these observations suggest that MA may disrupt normal energy metabolism, they do not elucidate how MA affects energy metabolism, leading us to formulate two competing hypotheses concerning the major driver of the system. One possibility, the "metabolic hypothesis", is that MA directly disrupts metabolism by altering the expression of metabolic genes and/or by interacting directly with metabolic proteins, which is supported by the observed 10-fold increases in carbohydrate transporters and glycolytic proteins (Sun *et al*., 2011). In contrast, according to the "behavior hypothesis," MA changes behaviors, such as feeding behavior and locomotor activity, leading to disruption of the energy balance. The latter hypothesis is supported by observations in humans and other mammals where MA leads to anorexia (Cole, 1967; Evans, 1971; Madden *et al*., 2005) and changes in locomotor activity (Wallace *et al*., 1999). In this study, we monitored the response of energy reserves and other metabolites to MA-treatment to test for changes in energy balance. We also measured behavioral/physiological parameters, such as feeding behavior, locomotor activity and respiration rate, to test the metabolic and behavior hypotheses.

METHODS AND MATERIALS

Fly rearing conditions and virgin collection

Drosophila melanogaster (w^{1118}) stocks attained from Dr. Misha Ludwig (University of Chicago) were reared on Formula 24-4 instant diet (Carolina Biological Supply Co., Burlington, NC, USA) in bottles at 25ºC and 50% relative humidity on a 12 hr photoperiod. All flies used in this study were 5-day old virgin males. To collect virgin flies, adult flies were removed from the bottles on the evening prior to collection, and the following morning the new adults were anesthetized by CO_2. Virgin flies were visually identified and were not anesthetized for longer than 4 min to limit CO_2-induced stress. Virgin males were transferred to 15 ml vials (20 individuals per vial) and matured for 5 d prior to experimental treatments.

Bioassay procedures

Ten 5-day-old virgin male flies were placed in 2.0 ml microcentrifuge tubes containing 400 mg of reconstituted Formula 24-4 instant *Drosophila* diet in the bottom, and the top of each tube was plugged with cotton. Flies were briefly anesthetized with CO_2 to facilitate transfer unless otherwise noted. Flies were placed in one of the four treatments that differed only in the final composition of the diet: (1) Control- diet reconstituted with distilled H_2O; (2) MA- diet supplemented with 0.6% MA; (3) MA+glc- diet supplemented with 0.6% MA and 3% Glc; and (4) Glc- diet supplemented with 3% Glc. Flies were transferred into 2 ml tubes in the morning (2-4 hr after lights on). The tubes containing the flies were kept inside a large plastic pill box, which was closed after 48 hr to prevent excessive desiccation.

Survivorship assays

Flies were handled and maintained according to the guidelines previously outlined in the bioassay procedures. Survivorship, which assessed under a dissecting scope by the presence/absence of movement, was monitored at two-hr intervals from 7 am to 9 pm for seven days.

Trehalose assays

After 12 hr, 24 hr, and 48 hr of treatment using bioassay procedures outlined above, flies were anesthetized with CO_2, transferred to a pre-weighed 1.5 ml tube, weighed to the nearest 0.1 mg, and flash frozen in liquid nitrogen. For the 0 hr time-point, the 5-day-old virgin flies were weighed and flash frozen without being placed in the 2 ml tubes. Flies were stored at -80ºC until metabolites could be extracted.

For the metabolite extraction, 200 µl of ice-cold 100% ethanol were added to the flies, which were then homogenized for 3 min using a sterile pestle driven by a battery-powered pestle motor. The samples were placed in a heating block at 80ºC for 10 min, after which 400 µl of 50% (v/v) methanol was added. The samples were centrifuged at 10,000 *g* for 10 min to sediment insoluble debris. The supernatant from each was transferred to a new tube and was concentrated to dryness using a SpeedVac. Each sample was reconstituted with 50 µl of distilled water. The standard protocol from the spectrophotometric trehalose kit (Megazyme International Ireland Ltd., Wicklow, Ireland, UK), including the borohydride reduction, was scaled down ten-fold to determine trehalose concentrations in a 96-well plate reader assay. The spectrophotometric assay used trehalase to convert trehalose into glucose, which was converted in two successive reactions to 6-phosphogluconate. The conversion of the cofactor for this latter reaction (NADP to NADPH), which was stoichiometric to the amount of trehalose, was measured at 340 nm using a Synergy HT platereader (BioTek Instruments, Winooski, VT, USA). This assay was found to give results similar to HPLC detection of trehalose (Holovati and Acker, 2007). Each sample was run in duplicate and the trehalose concentration was determined from a standard curve on each plate.

Methamphetamine ELISA

The metabolite extracts prepared for trehalose analysis were analyzed for MA content using a MA direct ELISA kit (Immunanalysis Corporation, Pomona, CA, USA). Reconstituted samples were diluted from 100- to 2,000-fold with 100 mM phosphate buffer, pH 7.0, so that MA concentrations were between 10-50 ng/ml. Plain diet and Glc-treated control samples were diluted 100-fold. A standard curve was run on each plate. In addition, samples from all four treatments were spiked with known amounts of MA standard to test for interference from the metabolite extracts.

Glycogen and total Glc analysis

The treatment of flies for the glycogen analysis only varied from the trehalose assay (see above) with respect to how flies were processed after storage. Following storage at -80ºC, the flies were homogenized in 100 µl of distilled water for 3 min using a battery powered pestle motor. The homogenate was centrifuged at 4ºC for 5 min at 10,000 *g* to sediment debris. Glycogen standards (20 µg/ml, 40 µg/ml, 60 µg/ml, and 80 µg/ml) were treated identically to supernatant from the samples.

To determine total Glc concentrations, Glc-containing compounds, including trehalose and glycogen, were enzymatically hydrolyzed, and the resulting Glc concentration was measured. Trehalose was presumably hydrolyzed by endogenous trehalase; we confirmed that trehalose was not detectable at the end of the incubation and that trehalose was detectable if the homogenate was heat treated at 80ºC following homogenization (data not shown). Fifteen µl of 50 mM acetate buffer, pH 4.5, containing 0.525 units of amyloglucosidase, was added to 3 µl of supernatant from each sample (adapted from Roehrig and Allred (1974)). The total volume was made up to 75 µl with distilled water and the resulting solution was incubated at 55ºC for 20 min. An additional 75 µl of distilled water were added to each sample after incubation. All samples were also incubated with acetate buffer from which amyloglucosidase omitted to permit the calculation of total glycogen. Glc concentrations were determined using a Glc oxidase (GO) assay kit (Sigma, St Louis, MO, USA) (Roehrig and Allred, 1974). The assay was scaled down 20-fold from the kit instructions for a 96-well plate format.

Triglyceride analysis

Flies were prepared and frozen in a manner identical to the trehalose and glycogen assays (see above). After storage at -80ºC, lipids were extracted and quantified according to Al-Anzi and Zinn (2010) with slight modifications. Briefly, flies were crushed in 200 µl of chloroform/methanol (2:1) and were incubated at room temperature for one hour with gentle agitation. The samples were evaporated to dryness in a fume hood and the resulting pellet was resuspended in 250 µl chloroform/methanol per 10 mg fresh mass. Two µl of supernatant from each sample was spotted on a silica gel TLC plate. The plates were run with a mobile phase of hexane and diethyl ether

(4:1) and were visualized by dipping in ceric ammonium molybdate solution (see Al-Anzi *et al*. (2009) for recipe) followed by heating at 90ºC for 15-20 min. Band intensity was quantified using NIH ImageJ software. Two replicate plates (technical replicates) were run for each time-point and each plate contained four biological replicates for each of the four treatments, as well as series of standards (Fig. 1D).

Feeding experiments

Flies were fed Formula 24-4 instant diet reconstituted with a 2.5% solution of blue dye, which is neither assimilated nor degraded, in order to measure gut capacity and gut throughput (adapted from Wong *et al*. (2008)). The bioassay procedures outlined above were used with two exceptions. First, 2.5% (w/v) blue dye #1 was added to the diet in each of the four treatments. Second, in addition to being transferred with CO_2 anesthesia (standard procedure), flies were also transferred into the 2 ml tubes using an aspirator (without anesthesia) to permit comparison between the two methods. After 24 hr, the flies were transferred to pre-weighed clean 1.5 ml microcentrifuge tubes (flies were visually inspected for superficial blue dye, soiled individuals were excluded), were immediately weighed and flash frozen in liquid nitrogen. The flies were ground by hand in 200 µl of distilled water using a blue pestle. The samples were centrifuged for 10 min at 10,000 *g*. After which, the supernatant was removed and filtered through a 0.2 µm filter. An additional 400 µl of distilled water were added to the sample prior to analysis. The absorbance of the blue dye released from the flies, a measure of gut capacity (Wong *et al*., 2008), was measured at 630 nm and the concentration was determined from a standard curve.

The effect of MA and sucrose octaacetate (SOA), a bitter nontoxic tastant, on dietary preference was measured in a two-choice test. Ten flies were starved for 24 hr prior to the experiment with access only to water. The flies

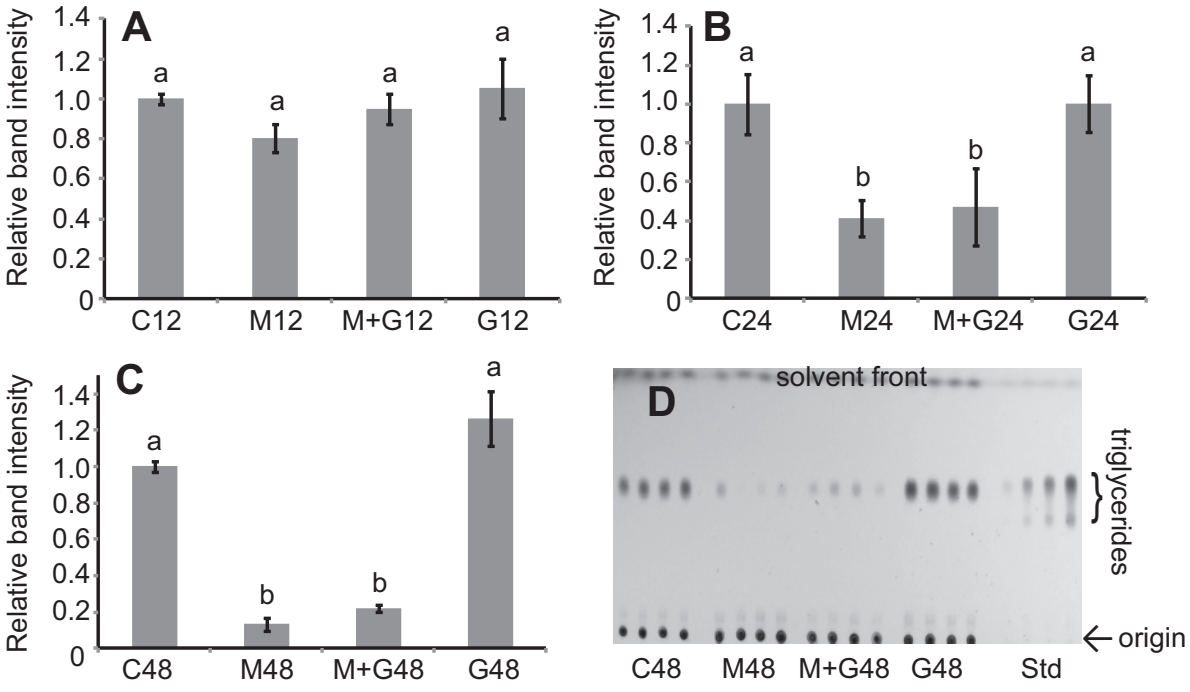

Fig. 1. Triglyceride content as a function of treatment duration. Triglyceride content was measured after 12 hr, 24 hr and 48 hr for ten virgin male 5-day-old *Drosophila* adults that were placed in 2 ml microcentrifuge tubes containing either unadulterated 4-24 instant medium, medium supplemented with 0.6% methamphetamine, medium supplemented with 0.6% methamphetamine and 3% glucose or medium supplemented with 3% glucose (n = 4, ± S.E.M.). Statistical comparisons of mean triglyceride content for 12 hr, 24 hr and 48 hr time-points are presented in A, B and C, respectively. Ordinate axes labels in A, B, C and D use letters to indicate treatment (e.g., C = plain diet control, M = MA-containing diet, M+G = diet supplemented with MA and Glc, and G = Glc supplemented control) and numbers to indicate time. Distinct lowercase letters represent different mean values ($p < 0.05$, Tukey's MCT). D shows a TLC plate with four replicates for each treatment at 48 hr. For the triglyceride standard, 2 µg, 4 µg, 8 µg and 10 µg of triglyceride were spotted from left to right.

were then transferred without anesthesia into an arena (Suppl. Fig. 1) containing one of the following paired diet options: either (i) control diet labeled with 2.5% (w/v) red food coloring and 0.6% MA-containing diet labeled with 2.5% (w/v) blue food coloring or (ii) 1.2% SOA-containing diet labeled with 2.5% (w/v) red food coloring and 0.6% MA-containing diet labeled with 2.5% (w/v) blue food coloring. The flies were placed in the dark during the assay to prevent color from confounding the results. After 90 min, the flies were weighed, flash frozen, and stored at -20°C. The flies were ground by hand in 200 µl of distilled water using a blue pestle. The samples were centrifuged for 10 min at 10,000 *g*. After which, the supernatant was removed and filtered through a 0.2 µm membrane. An additional 50 µl of distilled water was used to rinse the filter membrane. The absorbance of the blue and red dyes in each sample was measured at 630 nm and 500 nm, respectively. The concentration of each was determined from a standard curve.

The effect of MA and sucrose octaacetate (SOA) on short term (6 hr) and long term (24 hr) food consumption was measured in a slightly different method than described above to allow for short term measurements. Thirty mg of dye-labeled diet, which was either unadulterated, or supplemented with 0.6% MA or 1.2% SOA was placed on a polypropylene mesh support inside a clean 2.0 ml microcentrifuge tube (Suppl. Fig. 2). Flies were transferred by aspiration into the tube and were held in there for either 6 hr or 24 hr, at which point they were transferred to pre-weighed clean 1.5 ml microcentrifuge tubes. After which the polypropylene support was removed and the inside of the tube was rinsed with 250 µl of distilled water. For the 24 hr treatment, the rinsate was diluted an additional 40-fold for analysis. The blue dye present in the accumulated excreta on the sides of the tubes was quantified to estimate relative gut throughput/ feeding rates of the treatments. The dye was quantified as described in the previous paragraph.

Metabolomic analysis

Between 95 and 110 flies were placed in vial containing Formula 24-4 media supplemented with 3% natural abundance Glc. After 24 hr, the experimental flies were transferred to a new vial of media containing 0.6% MA and 3% [1,2-$^{13}C_2$]Glc or containing just 3% [1,2-$^{13}C_2$]Glc for the control flies. After 24 hr on the media supplemented with ^{13}C-labeled Glc (+/- 0.6% MA), the flies were anesthetized with CO_2, consolidated into a 1.5 ml microcentrifuge tube, weighed to the nearest tenth of a milligram, and flash frozen with liquid nitrogen. Metabolites were extracted immediately after flash freezing.

For the metabolite extraction, 500 µl of ice-cold 100% ethanol were added to the flies and then homogenized for 5 min using a sterile blue pestle driven by a battery-powered pestle motor. After homogenization, an additional 500 µl of ethanol were added and mixed thoroughly. From the sample, 500 µl were transferred to a new 1.5 ml tube, and both tubes were placed in a heating block at 80ºC for 10 min, after which 1 ml of 50% (v/v) methanol was added to each. The samples were centrifuged at 13,000 x g for 10 min to sediment insoluble debris. The supernatant from each was transferred to a new tube. Samples were stored at -80ºC until they could be further processed.

Metabolite profiling of the extracts was conducted using GC-MS (Gas Chromatography-Mass Spectroscopy) as described previously by Rupassara (2008) with the following modifications. Samples of 2-ml extracts, 10 reps each for the treatment and control, were vacuum-dried (SpeedVac, ThermoFinnigan, Waltham, MA, USA). Ten microliters of internal standard (10 mg Melissic acid in 1 ml pyridine) were added to each of the dried extracts and vacuum-dried again. The dried samples were derivatized with 50 µl of methoxamine hydrochloride (20 mg/ml in pyridine) for 1 hr at 50°C, followed by the addition of 100 µl N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA+1% TMCS: trimethylchlorosilane) and further derivatization at 50°C for 30 min. GC-MS analysis of 2 µl of each derivatized sample was performed using 6890N GC and a 5973N mass detector (Agilent Technologies, Santa Clara, CA, USA). Samples were analyzed under electron impact ionization. The GC-MS interface temperature was 310°C, and the ion source was kept at 220°C. The data system used was Chem Station (Agilent Technologies) with Amdis software used for chromatographic peak deconvolution and spectral extraction. The spectral identification and analysis were done as reported by Rupassara (2008).

Locomotion experiments

Flies were individually placed in 6.5 cm x 3 mm glass tubes (length x inner diameter, transferred with CO_2 anesthesia or by aspirator for comparison), containing 24-4 instant diet +/- 0.6% MA. To assess activity, the number of times that each fly crossed the infrared beam was recorded at 1 min intervals using a Trikinetics (Waltham, MA, USA) *Drosophila* activity monitoring system (Kume *et al*., 2005). Accumulated activity was logged for 16 individuals per treatment for up to 48 hr.

Respirometry experiments

Rates of CO_2 release by respiration were assessed over both short (min) and long (hr) timescales. Short-

term respiration rates were measured using a closed gas exchange system. This comprised a machined aluminum sample chamber that had a nickel/Teflon coating to prevent unnecessary ad/absorption of CO_2 and water vapor, and a LI-840 CO_2/H_2O Analyzer (LI-COR, Lincoln, NE, USA) with a 0.5 l min^{-1} pump to mix the gas within the system. For twenty four hours prior to the measurements, fifteen flies were held under humidity controlled conditions in a nylon mesh bag (5 cm x 2.5 cm x 1 cm, L x W x H) containing 15 mg of 24-4 instant media +/- 0.6% MA. Rates of CO_2 release by 5-6 replicate batches of flies from each treatment were measured at 25°C over a period of two min. A negative control was also tested demonstrating that there was no significant CO_2 release from mesh bags containing food that was fed upon by flies, but not flies themselves.

Long-term rates of respiration were determined by holding ten flies in a 2-ml glass vial containing 30 mg of instant diet +/- the addition of 0.6% MA and measuring the increase in [CO_2] at 21.5°C over 5-6 hr. Flies were acclimatized in the glass vials for 18 hr prior to measurements with the vial opening plugged with cotton. During measurements the vial was then sealed by a gas impermeable cap with a rubber septum (National Scientific Company, Rockwood, TN, USA). Respiration rates were calculated by comparing the [CO_2] in 6 replicate vials from the MA and control treatments to the [CO_2] in negative control vials (from which the flies were removed immediately before sealing the vial) at the end of the 5-6 hr incubation period. [CO_2] was measured by injecting three 10 µl aliquots of gas from each sample vial into the closed gas exchange system described above. After the respirometry experiments, flies were homogenized in 300 µl of 50 mM Tris buffer, pH 7.4, and soluble protein content of each sample was analyzed with a Coomassie plus (Bradford) assay kit (Pierce Biotechnology, Rockford, IL, USA).

Statistical analyses

Typically, data were analyzed separately by time-point using a one-way ANOVA. Afterwards, if statistical significance was observed, individual means were compared in a pair-wise manner using Tukey's MCT. In instances where experiments were performed both with and without CO_2 anesthesia, data were analyzed using a two-way ANOVA, which permitted simultaneous testing of effects the transfer method and treatment. In experiments with only two treatments and one time point, such as the respirometry experiments, the data were analyzed by the Student's t-test. Because the locomotor activity data were counts, a heterogeneous variance was assumed, i.e. we expected larger numbers to have larger variance. A square root transformation did not fix the problem, thus we used a t-test based on unequal variance, and the Cochran and Satterthwaite approximate degrees of freedom with unequal confidence intervals were calculated and significance determined.

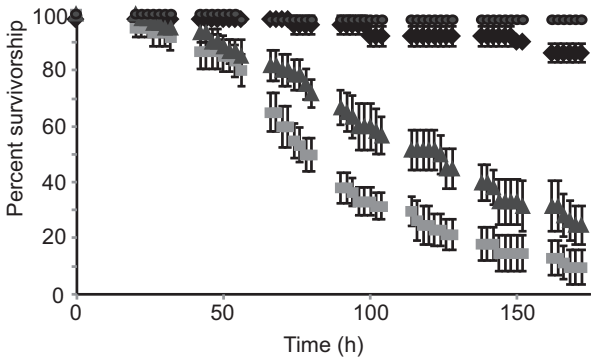

Fig. 2. Glucose affects ability to survive methamphetamine exposure. Ten virgin male 5-day-old *Drosophila* adults were placed in 2 ml microcentrifuge tubes containing either unadulterated 4-24 instant medium (black diamonds), medium supplemented with 0.6% methamphetamine (light grey squares), medium supplemented with 0.6% methamphetamine and 3% glucose (dark grey triangles) or medium supplemented with 3% glucose (black circles). The mean survivorship (± S.E.M., n = 6) is presented for each time point.

RESULTS

Supplementation with dietary glucose forestalled MA-induced mortality

The LT_{50} for MA-exposed flies was 80.5 hr (77.4 hr-83.6 hr, 95% CI) (Fig. 2). Supplementation of the MA-containing diet with 3% Glc increased the LT_{50} to 116.9 hr (112.5 hr- 121.9 hr, 95% CI) ($p < 0.05$). The LT_{50} for the plain diet and Glc-treated controls was not determined because survivorship was greater than 80% after one week.

Carbohydrates reserves decreased steadily with MA-exposure and were buffered by addition dietary glucose

Total Glc showed a continual decrease with increasing duration of MA-exposure (Fig. 3). At 12 hr, MA-treatment reduced total Glc/mg fresh mass (total Glc includes free Glc, in addition to Glc liberated from enzymatic hydrolysis of glycogen and trehalose) by 27% relative to the control ($p < 0.0001$). By 24 hr, MA-treatment decreased total

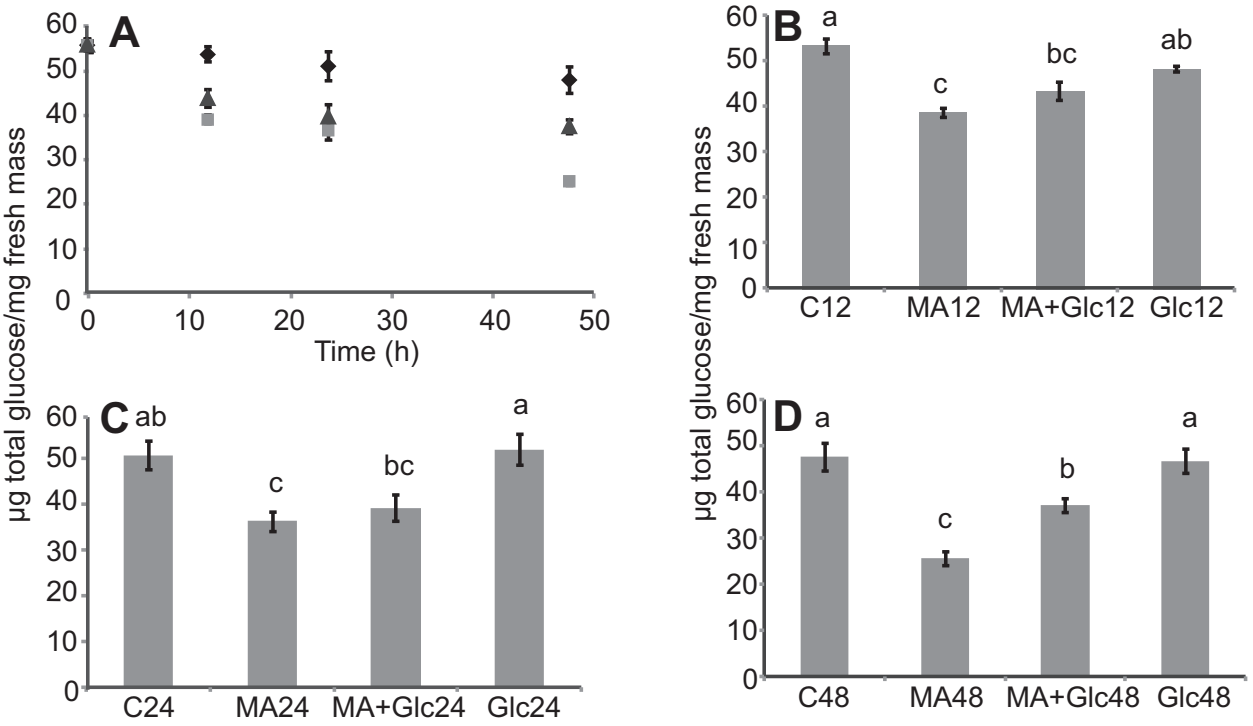

Fig. 3. Total glucose levels (~total carbohydrates) as a function of treatment duration. (A) Mean total glucose content was measured after 12 hr, 24 hr and 48 hr for ten virgin male 5-day-old *Drosophila* adults that were placed in 2 ml microcentrifuge tubes containing either unadulterated 4-24 instant medium (black diamonds), medium supplemented with 0.6% methamphetamine (light grey squares), medium supplemented with 0.6% methamphetamine and 3% glucose (dark grey triangles) or medium supplemented with 3% glucose (data not shown in A) (n = 4, ± S.E.M.). Statistical comparisons of mean total Glc levels for 12 hr, 24 hr and 48 hr time-points are presented in B, C and D, respectively. Ordinate axes labels in B, C and D use letters to indicate treatment (e.g., C = plain diet control, M = MA-containing diet, M+G = diet supplemented with MA and Glc, and G = Glc supplemented control) and numbers to indicate time. Distinct lowercase letters represent different mean values ($p < 0.05$, Tukey's MCT).

Glc by over 28% ($p = 0.02$). After 48 hr, MA-treatment reduced total Glc levels by 46% ($p < 0.0001$).

The MA+Glc-treatment increased total Glc reserves ($p = 0.0154$) relative to the MA-treated flies at 48 hr (Fig. 3D), showing that dietary carbohydrates are able to buffer carbohydrate reserves in the presence of MA. While the same trend in the data was observed at 12 hr and 24 hr (Figs. 3B,C), that is to say MA+Glc-treated flies exhibited higher carbohydrate reserves than those treated with MA alone, the differences between the MA-treatment and MA+Glc-treatment were not statistically significant.

Changes in glycogen levels with MA-exposure mirrored those observed in total Glc levels. Glycogen levels showed steady declines over time in the MA-treated flies relative to the control, decreasing by ~22% ($p = 0.0324$), 31% ($p = 0.0309$), 48% ($p = 0.0002$) after 12 hr, 24 hr, and 48 hr, respectively (Fig. 4). When Glc was added to the MA-treatment, glycogen reserves were increased by ~44% at 48 hr ($p = 0.049$) relative to MA-treatment alone (Fig. 4D).

Trehalose levels decreased, but stabilized with continuing MA-exposure

Trehalose levels trended lower after 12 hr of MA-treatment, but did not decrease significantly relative to the control ($p = 0.1427$) (Fig. 5B). Interestingly, the MA+Glc-treatment increased the amount of trehalose relative to flies treated with MA alone ($p = 0.0214$). However, trehalose levels in the MA+glc treatment were statistically the same as those in the plain diet and Glc-treated controls ($p = 0.6889$ and $p = 0.9633$, respectively).

At 24 hr of treatment, the MA-treatment decreased trehalose by 22% relative to the control ($p = 0.0002$)

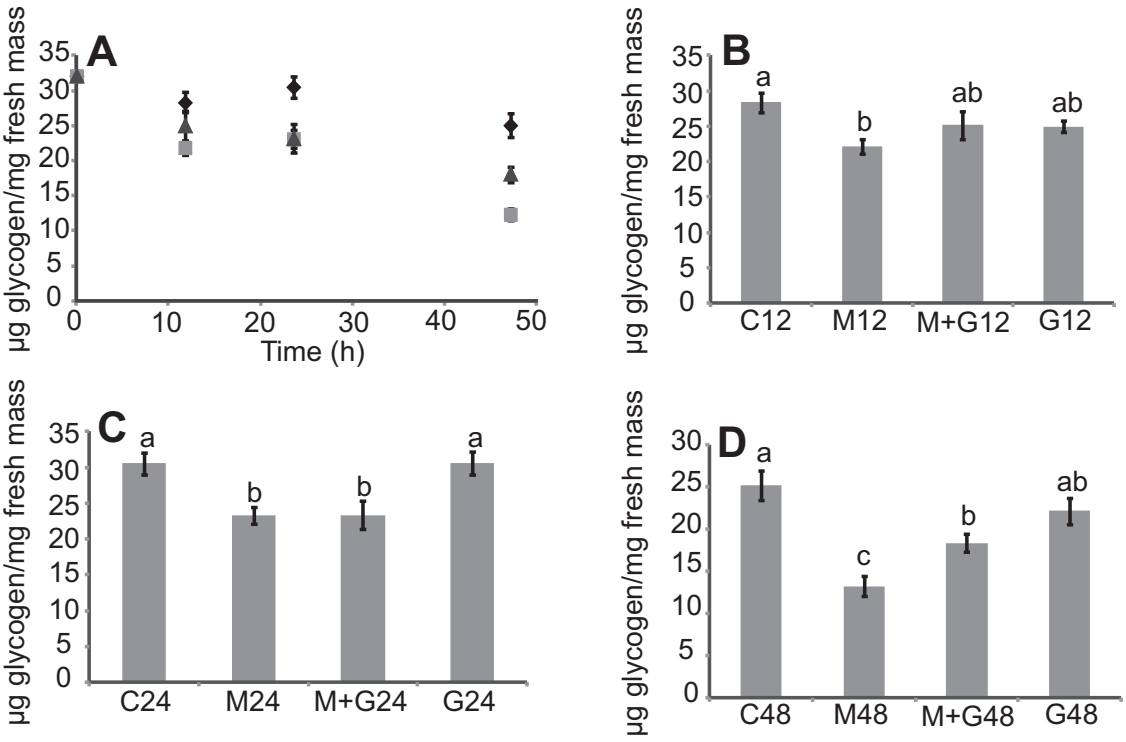

Fig. 4. Glycogen as a function of treatment duration. (A) Glycogen content was measured after 12 hr, 24 hr and 48 hr for ten virgin male 5-day-old *D. melanogaster* adults that were placed in 2 ml microcentrifuge tubes containing either unadulterated 4-24 instant medium (black diamonds), medium supplemented with 0.6% methamphetamine (light grey squares), medium supplemented with 0.6% methamphetamine and 3% glucose (dark grey triangles) or medium supplemented with 3% glucose (data not shown in A) (n = 4, ± S.E.M.). Statistical comparisons of mean glycogen levels for 12 hr, 24 hr and 48 hr time-points are presented in B, C and D, respectively. Ordinate axes labels in B, C and D use letters to indicate treatment (e.g., C= plain diet control, M = MA-containing diet, M+G = diet supplemented with MA and Glc, and G = Glc supplemented control) and numbers to indicate time. Distinct lowercase letters represent different mean values ($p < 0.05$, Tukey's MCT).

(Fig. 5C). The MA+Glc-treatment no longer increased trehalose levels relative to MA-treated flies ($p = 0.8360$) as it had done at 12 hr. In addition, trehalose levels in both the MA- and MA+Glc-treatments were lower than those of either of the control treatments ($p < 0.0044$). After 48 hr a similar pattern was observed. MA-treatment reduced trehalose levels by 25% ($p = 0.0060$) relative to the control and no difference was observed between the MA- and MA+Glc-treatments ($p = 0.2126$) (Fig. 5D). However, trehalose levels in the MA+glc-treatment trended higher and were not significantly lower than the Glc-treated control ($p = 0.0570$).

Triglyceride reserves are rapidly depleted with MA-exposure

Triglyceride levels decreased dramatically with MA-exposure. At 12 hr of MA-exposure, there was no significant difference among any of the treatments ($p = 0.2965$, F-value = 1.38, d.f. = 3, one-way ANOVA), even though triglyceride levels in MA-treated flies trended ~20% lower (Fig. 1A) than those in the control. However, at 24 hr triglycerides in MA-treated flies decreased by more than 50% relative to the untreated control ($p = 0.0033$) (Fig. 1B), and a similar decrease was observed in MA+Glc-treatment. At 48 hr, we observed that triglyceride levels decreased in the MA-treated flies by 87% ($p < 0.0001$) (Fig. 1C). No significant difference was observed in the triglyceride levels of the MA-treated and the MA+Glc treated flies; however, triglyceride levels in MA+Glc-treated flies trended higher at all time-points.

Free amino acids trended towards decreased concentrations with MA-exposure

All of the free amino acids observed in metabolomic analysis, including glutamine, glycine, alanine, glutamic acid, aspartic acid, and serine, trended toward a decreased

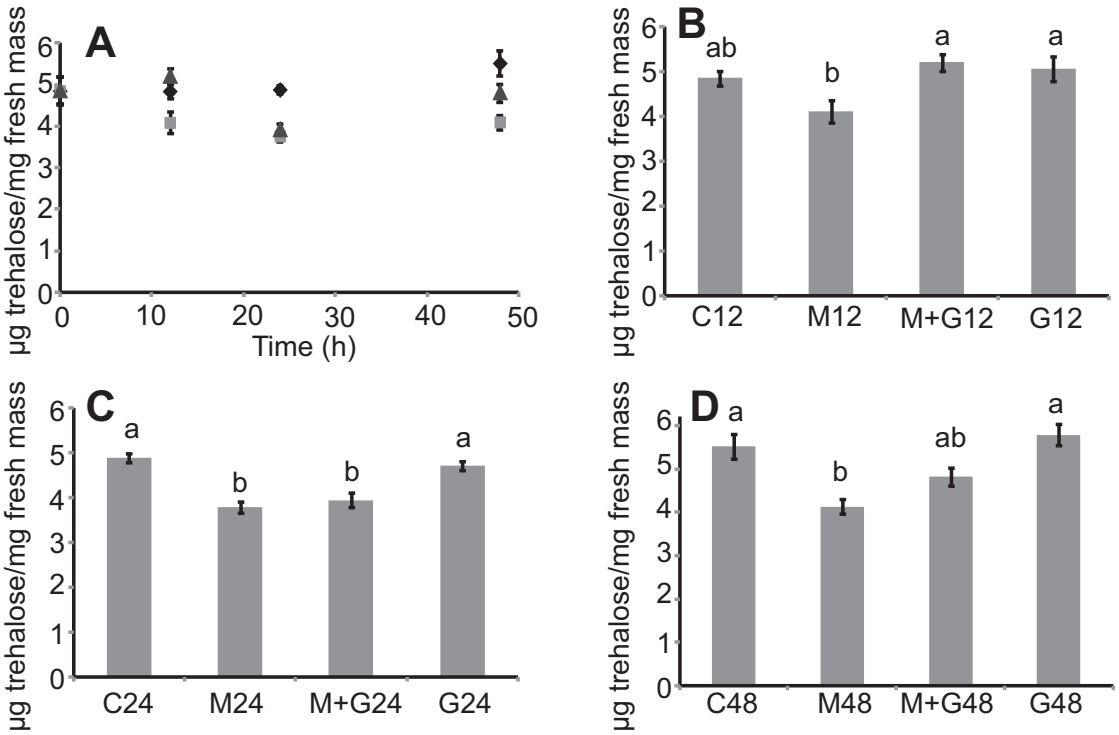

Fig. 5. Trehalose as a function of treatment duration. (A) Trehalose content was measured after 12 hr, 24 hr and 48 hr for ten virgin male 5-day-old *Drosophila* adults that were placed in 2 ml microcentrifuge tubes containing either unadulterated 4-24 instant medium (black diamonds), medium supplemented with 0.6% methamphetamine (light grey squares), medium supplemented with 0.6% methamphetamine and 3% glucose (dark grey triangles) or medium supplemented with 3% glucose (data not shown in A) (n = 4, ± S.E.M.). Statistical comparisons of mean trehalose levels for 12 hr, 24 hr and 48 hr time-points are presented in B, C and D, respectively. Ordinate axes labels in B, C and D use letters to indicate treatment (e.g., C= plain diet control, M = MA-containing diet, M+G = diet supplemented with MA and Glc, and G = Glc supplemented control) and numbers to indicate time. Distinct lowercase letters represent different mean values ($p < 0.05$, Tukey's MCT).

concentration in MA-exposed flies with the exception of serine (Table 1). However, only two of these, glutamine and glycine, were found to decrease significantly with MA exposure (Table 1).

Dietary and endogenous carbohydrates showed opposing trends

Three additional carbohydrates/ sugar alcohols detected in metabolomic analysis were found to change significantly with MA-exposure (Table 1). Both endogenous compounds, namely glycerol 3-phosphate and trehalose, were found to decrease. Glycerol 3-phosphate, a precursor for lipid biosynthesis, was reduced in the MA+Glc-treated flies by more than 50% relative to the Glc-treated control ($p = 0.0034$). Trehalose also decreased with MA-exposure ($p = 0.02$), confirming the results of the spectrophotometric assays (Fig. 5C). A diet-derived carbohydrate, [1,2-$^{13}C_2$]Glc, increased in the MA+Glc-treated flies by 65% ($p = 0.0002$) relative to the Glc-treated control (Fig. 6). Thus, endogenous carbohydrates decreased with MA-exposure, while those derived from the diet increased.

Locomotor and feeding behaviors change in response to MA

Locomotor activity increased with MA-treatment from 6 hr-12 hr ($p = 0.0018$), 12 hr-24 hr ($p = 0.0029$) and 24 hr-48 hr ($p = 0.0004$) after the onset of treatment (Fig. 7A). However, locomotor activity did not increase during the first 6 hr ($p = 0.52$) time bin, indicating that MA administered in the diet requires several hours to increase locomotor activity. Note that MA-treated flies maintained the same circadian rhythm as the controls, but were generally more active. Fig. 7B shows flies transferred into the

Table 1. The mean relative abundance (arbitrary units, n = 10) of selected metabolites detected by GC-MS for MA+Glc- and Glc-treated *Drosophila* after 24 hr of treatment

	MA+Glc treatment		Glc control treatment		
	Mean	SEM	Mean	SEM	p-value
Glycerol 3-phosphate	49.58	5.72	108.95	16.63	0.0034*[FDR]
Glycine	20.07	1.85	34.90	4.48	0.0067*[FDR]
Glutamine	20.02	4.53	66.14	14.48	0.0071*[FDR]
Trehalose	766.24	130.24	1310.66	169.19	0.020*[FDR]
Sucrose	670.03	81.21	443.86	66.86	0.045*
Glucose 6-phosphate	12.99	1.09	16.23	1.67	0.12[NS]
Alanine	278.46	36.58	384.52	56.64	0.13[NS]
Glutamic acid	158.35	16.38	205.48	24.79	0.13[NS]
Succinic acid	12.78	2.29	21.86	5.53	0.15[NS]
Methionine or IAA	1.21	0.14	1.72	0.32	0.16[NS]
Lactic acid	35.21	4.37	26.73	4.43	0.19[NS]
Fructose 6-phosphate	10.59	1.42	13.30	1.47	0.20[NS]
Aspartic acid	46.00	7.38	63.75	11.40	0.21[NS]
Ribose 5-phosphate	0.38	0.08	0.55	0.12	0.23[NS]
Fructose	102.38	15.36	80.43	12.35	0.28[NS]
Serine	7.48	0.70	6.40	0.87	0.34[NS]

* Metabolites differed significantly between the two treatments according to Student's t-test ($p < 0.05$).
[FDR] Metabolites differed significantly between the two treatments with a false discovery rate (FDR) of 5%.
[NS] Metabolites did not differ significantly between treatments (Student's t-test ($p > 0.05$)).

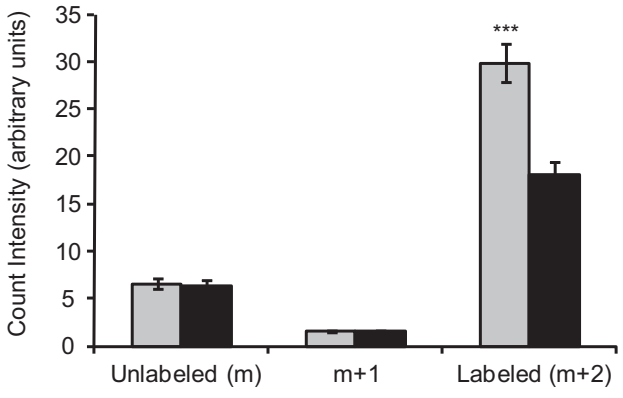

Fig. 6. Glucose isotopic distribution. Mean relative abundance of glucose isotopomers (± S.E.M.) detected by GC-MS for MA+[1,2-$^{13}C_2$]Glc- (grey bars) and [1,2-$^{13}C_2$]Glc-treated (black bars) *Drosophila* after 24 hr of treatment. The first x-axis label (from left to right) indicates unlabeled/natural abundance Glc with a mass of m. m+1 indicates glucose that contains a single ^{13}C and m+2 indicates the distribution of doubly ^{13}C-labeled Glc, which was almost exclusively obtained from the fly diet. Asterisks indicate that MA+Glc-treated is different than control ($p = 0.0002$, Student's t-test, n = 10).

locomotor assay without CO_2 anesthesia act qualitatively the same as those transferred with anesthesia.

In a separate locomotor activity experiment, the presence of MA in the diet increased locomotor activity regardless of the presence of Glc. When compared to the MA-treatment, MA+Glc-treated flies were statistically the same across all time bins ($p > 0.2594$) (Suppl. Fig. 3). Furthermore, both the MA+Glc- and Glc-treatments showed significantly more activity that the control flies at from 6 hr-12 hr ($p < 0.0414$), 12 hr-24 hr ($p < 0.0407$) and 24 hr-48 hr ($p < 0.0089$) after the onset of treatment. In both cases, locomotor activity in the first time bin did not differ from the control ($p > 0.4428$).

All flies, regardless of treatment, spent most of their time on the sides of the 2 ml tube and MA(+Glc)-treated flies were no more likely to spend time on the food surface than the control flies (data not shown), suggesting that the amount of blue dye in the excreta accumulated on the side of the tube is proportional to the excretion rate. Dietary treatment (presence or absence of MA) was the most important determinant of feeding behavior ($p < 0.0001$, two-way ANOVA) (Fig. 8). The amount of excreta accumulated over 24 hr, a proxy for feeding rate, significantly decreased ($p < 0.0001$, Tukey's MCT) in

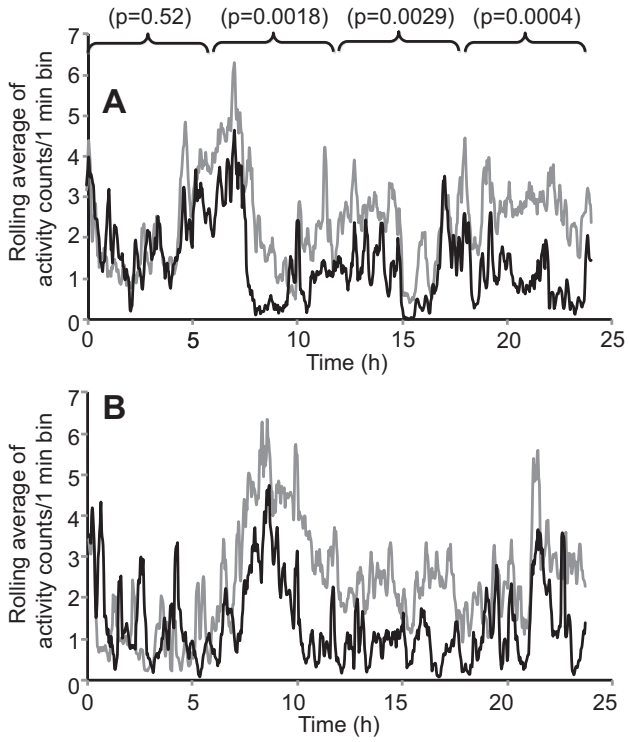

Fig. 7. Locomotor activity as a function of treatment duration. The locomotor activity of five-day-old virgin male *Drosophila* adults was monitored after the transfer of a single fly with CO_2 anesthesia (A) or by aspirator (B) to a 3 mm tube. Each tube contained either unadulterated diet (black trace) or diet supplemented with 0.6% MA (light grey trace) (n = 16). The rolling average of five time bins is presented to reduce variability. For A, the activity data was divided into four six-hour time bins and a t-test based on unequal variance was used to calculate p-values (located at the top of the panel).

both MA- and MA+Glc-treated flies relative to either control (Fig. 8C). However, the quantity of food contained in the gut did not change with treatment ($p = 0.3988$, ANOVA) (Fig. 8A).

When flies were transferred without anesthesia, similar qualitative trends were observed in accumulated excreta; however, the method of transfer (i.e., CO_2 anesthesia vs. aspiration) did significantly affect feeding behavior (two-way ANOVA, $p = 0.0329$). Similar to results attained with CO_2 anesthesia, accumulated excreta decreased for MA-treated flies when compared to that of the plain diet controls ($p = 0.0025$, Tukey's MCT) (Fig. 8D). In addition, accumulated excreta for MA+Glc-treated flies decreased relative to the plain diet control ($p = 0.0115$) and trended lower relative Glc-treated control ($p = 0.0587$). The most striking difference between the two transfer methods was that accumulated excreta trended higher by 70% and 40% for Glc and plain diet controls transferred without anesthesia, respectively, indicating that CO_2 anesthesia suppressed feeding behavior. No interaction was observed between the method of transfer and the dietary treatment (two-way ANOVA, $p = 0.2436$).

When measured following transfer of flies without anesthesia, the quantity of food contained in the gut was found to increase significantly for flies co-treated with MA and Glc relative to those treated with MA alone ($p = 0.0366$) (Fig. 8B). The same trend was observed following CO_2 anesthesia, but the difference was not significant (Fig. 8A).

Drosophila preferred not to eat diet containing MA and SOA. When given the choice between control diet and diet supplemented with 0.6% MA, flies consumed 14-fold more of the former than the latter (Student's t-test, $p < 0.0001$) (Fig. 9A). In contrast, when given the choice between MA- or SOA-supplemented diet flies consumed similar amounts of each (Student's t-test, $p = 0.62$) (Fig. 9B). In the first 6 hr, aversion to these compounds led to decreased consumption of food containing MA or SOA when compared to the control ($p < 0.0251$, Tukey's MCT) (Fig. 9C) with consumption of both compounds being the same ($p = 0.8921$, Tukey's MCT). However, by 24 hr consumption of food adulterated with SOA, increased and was no longer different from the control ($p = 0.1367$, Tukey's MCT) (Fig. 9D). In contrast, consumption of MA-containing food remained suppressed by more than 60% relative to the control ($p = 0.0004$) and by more than 50% relative to SOA-containing diet ($p = 0.0076$).

Levels of MA were higher in flies' bodies when Glc was added

MA levels, measured in whole-body metabolite extracts, were significantly higher for MA+Glc-treated flies relative those measured on MA-treated flies (Suppl. Fig. 4) (two-way ANOVA, $p = 0.0065$). However, MA concentrations in the flies' bodies did not change with the duration of exposure (two-way ANOVA, $p = 0.1066$), nor was any time by treatment interaction observed (two-way ANOVA, $p = 0.1360$).

MA-exposure decreased rates of respiration

Respiration rates per unit fresh weight trended lower with MA exposure when assessed in short-term incubations (-13%, $p = 0.066$, Student's t-test, control n = 6, MA treatment n = 5). Since we expected MA-treatment to increase respiration due to increased locomotor activity, we decided to pursue long-term incubation to avoid any

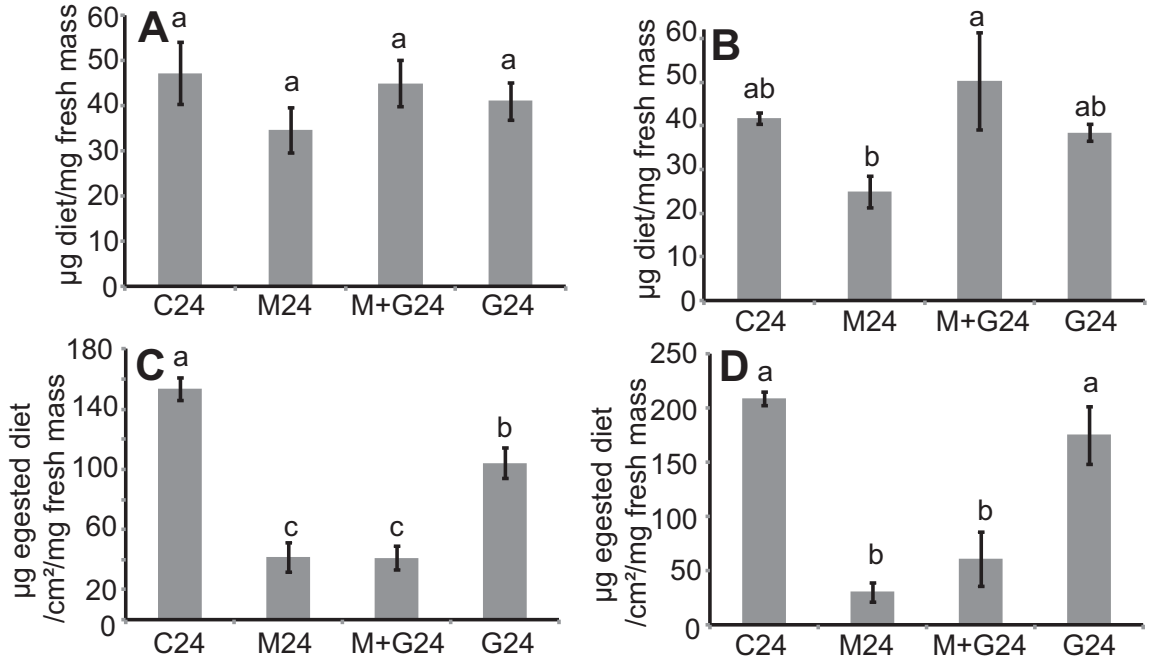

Fig. 8. MA-treatment and transfer method affect feeding behavior. The mean gut contents of ten virgin male 5-day-old *Drosophila* adults was measured 24 hr after placement in 2 ml microcentrifuge tubes containing media supplemented with 2.5% blue dye. There were four treatments: plain medium (C24), medium supplemented with 0.6% methamphetamine (M24), medium supplemented with 0.6% methamphetamine and 3% glucose (M+G24) or medium supplemented with 3% glucose (G24) (n = 5, ± S.E.M.). Flies were transferred to the 2 ml tubes both with (A) and without (B) the use of CO_2 anesthesia. The relative amount of egested dye was also measured for each of the four treatments with (C) and without (D) the use of CO_2 anesthesia. Distinct lowercase letters represent different mean values ($p < 0.05$, Tukey's MCT).

handling-induced perturbations of behavior that could lead to spurious results. In the long-term incubations, respiration rates decreased from 3.7 ± 0.2 ml CO_2 g fresh mass^{-1} h^{-1} (mean ± S.E.M.) for the control flies to 2.9 ± 0.1 ml CO_2 g fresh mass^{-1} h^{-1} (-20%, p = 0.0047, Student's t-test, n = 6) with MA-treatment. Furthermore, when CO_2 production was normalized to total soluble protein, MA-treatment similarly decreased rates of whole-body respiration from the control level of 178 ± 11 ml CO_2 g^{-1} h^{-1} to 145 ± 6 ml CO_2 g^{-1} h^{-1} (-18%, p = 0.029, Student's t-test, n = 6). Similar results in both the short-term and long-term incubations gave us confidence that the results were not an artifact of altered behavior during the measurement process.

DISCUSSION

Our study clearly demonstrates that MA has profound effects on energy metabolism in *Drosophila*. Triglycerides and glycogen are the two predominant energy storage molecules in animals and both decreased steadily with continued MA-exposure over a 48 hr period (Figs. 1 and 4), suggesting that MA administered in the diet resulted in a negative caloric balance. The large decrease in observed levels of glycerol 3-phosphate (Table 1), an essential precursor in triglyceride biosynthesis, in conjunction with decreased levels of triglycerides suggests that lipid biosynthesis was dramatically reduced, supporting the case for a caloric deficit.

Moreover, the observation that trehalose, the major blood sugar in insects (Bedford, 1977; Wyatt, 1961), decreased with MA-exposure (Fig. 5, Table 1) is consistent with the response to starvation in other insects (Wyatt, 1961; Jones *et al*., 1981; Duchateau and Florkin, 1959). The stabilization of trehalose levels after 12 hr of MA exposure (Fig. 5), when compared to the continual decrease of glycogen levels (Fig. 4), suggests that trehalose levels in the hemolymph were actively regulated at the expense of the major storage carbohydrate, which is consistent with the physiologically important role of tre-

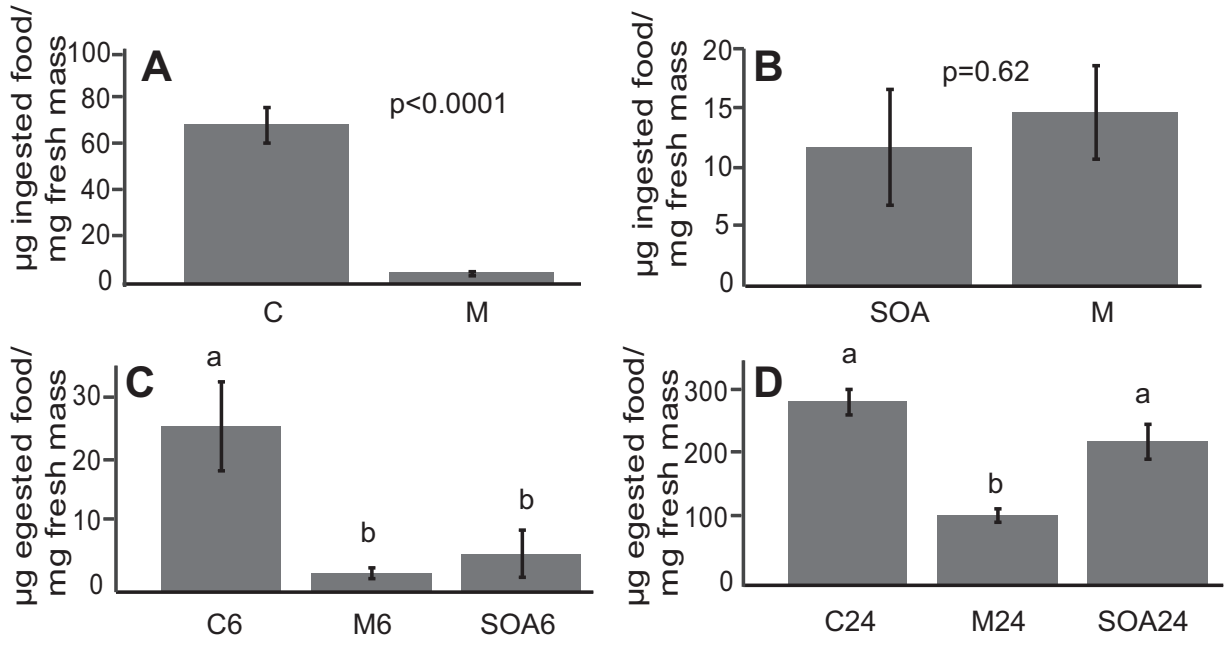

Fig. 9. Aversive compounds and food consumption. In two-choice experiments, flies were given the choice between red-dye labeled control diet and blue dye-labeled diet containing 0.6% MA (C) or red dye-labeled diet containing 1.2% SOA and blue dye-labeled diet containing 0.6% MA (D). Flies were allowed to feed for 90 min, after which they were homogenized and dye was quantified. (n = 5, ± S.E.M.). In addition, the amount of blue dye egested by virgin male 5-day-old *Drosophila* adults measured 6 hr and 24 hr (A and B, respectively, n = 5, ± S.E.M.) after placement in 2 ml microcentrifuge tubes containing one of the following: plain medium + 2.5% blue dye (C), medium supplemented with 0.6% methamphetamine and 2.5% blue dye (M) or medium supplemented with 1.2% sucrose octaacetate and 2.5% blue dye (SOA). Distinct lowercase letters represent different mean values ($p < 0.05$, Tukey's MCT).The number component of each column label indicates the treatment time (e.g., M6 indicates 6 hr of exposure to MA-containing diet) (n = 5, ± S.E.M.).

halose as a blood sugar (Lee and Park, 2004). Decreased blood sugar has also been associated with MA-exposure in mammalian models for the study of MA (Mcmahon *et al*., 1971). Finally, Sun *et al*. (2011) previously reported that MA-exposure decreased trehalose levels in *Drosophila*.

Decreased concentrations of glutamine and glycine (Table 1) may also reflect a negative caloric state. Tissue-specific decreases in free amino acids have been shown to occur during short-term starvation in thoracic muscles of the tsetse fly (*Glossina swynnertoni*) (Bursell, 1960), and in human muscles (Hammarqvist *et al*., 2005). In fact, the amino acid proline can be utilized as an energy source to power insect flight muscle [37, 38] (Brosemer and Veerbhadrappa, 1965; Sacktor and Wormser-Shavit, 1966) and glutamine can be directly oxidized by cells from the fall armyworm (*Spodoptera frugiperda*) in culture (Neermann and Wagner, 1996). However, studies that monitored free amino acids in the hemolymph of insects have shown that concentrations remain constant or increase with starvation (Wyatt, 1961; Po-Chedley, 1958), presumably to buffer energy metabolism. In this study, since we evaluated whole body extracts, we observed the net change for amino acids contained in both the intracellular and extracellular compartments. Our observation that free amino acid concentrations decreased (Table 1) is consistent with others' observations that most free amino acids are intracellular (Hanzal *et al*., 1992; Bjerke and Zachariassen, 1997) and this pool of amino acids typically decreases with starvation (Bursell, 1960; Hammarqvist *et al*., 2005). However, since glutamine and glycine have important roles outside of energy metabolism, including protein biosynthesis, osmotic regulation (Woodring and Blakeney, 1980), and maintaining reduced glutathione pools (glutamine specific) (Roth *et al*., 2002), the observed changes may reflect

changes other than energy status.

The negative caloric balance in MA-exposed flies appears to be driven predominantly by anorexia, supporting the "behavior hypothesis". We observed that amount of excreta accumulated in 24 hr, a proxy for the amount of food consumed, decreased by ~60-80% when MA was added to the diet (Figs. 8, 9D), indicating a 60-80% reduction in caloric intake. We can reject the metabolic hypothesis because if metabolic changes were causing the observed depletion of glycogen and triglycerides, then we would expect to observe an increase, not a decrease, in metabolic rate, assuming that metabolism at the organismal level remains aerobic. Perhaps a more parsimonious explanation for the over-expression of glycolytic proteins observed by Sun *et al*. (2011) is that glycolytic proteins, are highly susceptible to carbonylation arising from drug-induced oxidative stress (England *et al*., 2004). Thus, these proteins are up-regulated to compensate for decreased enzymatic activity resulting from carbonylation. Additionally, the observed over-expression of carbohydrate transporters observed by Sun *et al*. (2011) probably arises from anorexia-induced starvation, since reduced levels of carbohydrates lead to the up-regulation of carbohydrate transporters (Ferraris and Diamond, 1992; Nagamatsu *et al*., 1994).

Our data indicate that bitter taste of MA, per se, did not cause a prolonged anorexia, since flies habituated to bitterness. Naïve flies strongly preferred to eat unadulterated diet over that containing MA (Fig. 9A), which is reported to have a bitter taste (Scibelli *et al*., 2011), revealing that flies can detect MA and find it aversive. When given the choice between SOA, a bitter non-toxic tastant, and MA, flies showed no preference for either compound and generally ate much less than when unadulterated diet was available (Figs. 9A, B). In the short term, the presence of either MA or SOA in the diet led to decreased food consumption (Fig. 9C). However, flies readily habituated to bitterness, leading to increased consumption of SOA-containing food over the first 24 hr (Fig. 9D). In contrast, flies did not habituate to MA and continued to exhibit pronounced anorexia.

MA-induced anorexia could be attributed to a conditioned aversion to MA resulting from post-ingestive toxic effects of the drug and/or may arise from the ability of MA to suppress appetite. The method of drug administration (dietary) does not allow us to independently evaluate these two possibilities and both are supported by the literature. For instance, Aravich and Sclafani (1980) concluded that rats lost weight when bitter quinine was incorporated in their diet, not because of the bitterness of the compound, but rather because of its toxicity. In fact, rats learned to prefer SOA over bitter quinine even though naïve rats preferred the latter. In support of the second possibility, appetite suppression is a commonly observed side effect of MA in humans and in mammals regardless of the method of administration (Cole, 1967; Evans, 1971; Madden *et al*., 2005).

We hypothesized that MA-induced increases in locomotor activity that we observed in *Drosophila* (Fig. 7) would worsen the caloric deficit by increasing caloric expenditure. Increased locomotor activity is also a known side effect of MA consumption in mammals (Wallace *et al*., 1999) and in humans where MA is taken, in part, for its stimulant effects (National Institutes on Drug Abuse, 2010). However, the metabolic contribution of increased locomotion in *Drosophila* was probably small since flies were largely restricted to walking in the bioassays, and the contribution of walking to the overall metabolic rate is modest. In fact, walking at an average speed is predicted to increase the metabolic rate in *Drosophila* by only 5-10% over routine metabolism (Berrigan and Partridge, 1997). Therefore, the increased locomotor activity observed in the MA-treated flies might be expected to increase the metabolic rate by roughly 5-10% over that of the control flies. In contrast to our hypothesis, we observed that the overall metabolic rate for MA-treated flies tended to decrease, indicating that MA decreased the resting metabolic rate. The decreased metabolic rate may be related to MA-induced starvation, since starvation is known to decrease the relative metabolic rate in insects by 10-90% when compared to non-starved individuals (Scriber and Slansky, 1981). Importantly, our data are consistent with the overall the caloric balance being strongly negative, since caloric intake decreased by 60-80%, but the compensatory decrease in resting metabolism was only 10-20%.

With the discovery that MA-induced mortality appeared to be mediated by behavioral changes, we decided to perform key behavioral assays with and without CO_2 anesthesia (flies were routinely anesthetized with CO_2 to facilitate handling during experimental setup) because acute CO_2 exposure has been shown to produce behavioral effects lasting at least 24 hr (Barron, 2000). We wanted to ensure that treatment (MA exposure), not transfer method (CO_2 anesthesia vs. aspiration), was the primary driver of the observed behavioral changes. A two-way ANOVA revealed both transfer method and treatment affect feeding behavior, with treatment being the primary driver of differences in food consumption ($p < 0.0001$). The primary effect of the transfer method was that CO_2 anesthesia reduced the feeding rate and/or delayed onset of feeding (Figs. 8C, D). However, in all the feeding and

locomotor experiments, no interactions between transfer method and treatment were observed and similar qualitative trends whether flies were transferred with or without anesthesia (Figs. 7, 8), indicating that MA is responsible for increased locomotor activity and the decreased food consumption.

The exhaustion of carbohydrate reserves, resulting from anorexia, appears to have contributed to MA-induced mortality in *Drosophila*. When the diet was supplemented with 3% glucose, total carbohydrate levels and glycogen reserves at 48 hr (Figs. 3D and 4D) were increased by 49% and 44%, respectively, showing that Glc supplementation forestalls the exhaustion of carbohydrate reserves. Furthermore, we observed a commensurate response in survivorship, with the LT_{50} for MA-treatment increasing from 80.5 hr (77.4 hr- 83.6 hr, 95% CI) to 116.9 hr (112.5 hr- 121.9 hr, 95% CI) with Glc supplementation, corresponding to an increase in survivorship of 45%.

Our results suggest that supplementation with 3% Glc helped to prevent MA-induced depletion of carbohydrate reserves by increasing the nutrient/carbohydrate density of the *Drosophila* diet. In the dye consumption experiments, flies excreted ~20 to 30% less dye when the control diet was supplemented with Glc (Figs. 8C, D), indicating that a smaller quantity of the Glc-supplemented food was able to meet the flies caloric needs. However, a corresponding decrease in food consumption was not observed for Meth-containing diet, leading us to conclude that MA+Glc treated flies consumed more calories/carbohydrates than MA-treated flies.

In addition, Glc supplementation may have increased the consumption MA-containing diet. When MA-containing diet was supplemented with Glc, flies tended to exhibit increased gut capacity compared to MA-treated flies (Figs. 8A, B), suggesting that Glc may have partially masked the bitterness of MA and led to increased crop filling. In insects, the presence of carbohydrates can mask taste of certain unpalatable plant compounds (Glendinning, 2002), suggesting that it is plausible that sugar could mask the taste of MA. Further support for increased crop filling is provided by the observation that MA+Glc treated flies contained higher levels of MA than flies treated with MA alone (two-way ANOVA, $p = 0.0065$) (Suppl. Fig. 4).

The results of the metabolomic experiments also suggest that Glc increased crop filling in the presence of MA. The crop is a collapsible portion of the foregut in which diet is stored. Once food leaves the crop, dietary carbohydrates are rapidly absorbed by the midgut, transported into the hemolymph, and metabolized throughout the body (Lee and Park, 2004). Accordingly, concentrations of dietary sugars are likely to be many-fold higher in food contained in the crop than in the rest of the body. Thus, any increase in crop volume would presumably lead to an overall increase in abundance of dietary carbohydrates. Therefore the observation that two diet-derived carbohydrates, namely [1,2-$^{13}C_2$]Glc and sucrose, increased by 66% ($p = 0.0002$) (Fig. 6) and 50% ($p = 0.045$) (Table 1), respectively, in MA+Glc-exposed flies compared to the Glc controls suggests that more food was stored in the crop when flies were fed MA.

Furthermore, we observed in feeding experiments that total gut capacity trended 10-30% higher, although not significantly so, in MA+Glc-treated flies relative to the Glc-treated controls (Figs. 8A, B). Given that crop volume is highly variable, whereas the remainder of the gut volume is relatively stable, most of the increase in gut volume can likely be attributed to an increased crop volume. Since the crop represents only a fraction of the total gut volume, the magnitude of change in crop volume would be much greater than that observed in overall gut capacity. For instance, if we assume that the crop is completely collapsed in MA-treated flies transferred without CO_2 (Fig 8B), then the increase in crop volume would be estimated to be between ~25-66% for the MA+Glc-treated flies relative to Glc-treated flies. Even though Glc appeared to contribute to increased crop filling, virtually no difference in accumulated excreta was observed for flies transferred with anesthesia (Fig. 8C), suggesting that the contribution of increased consumption of diet (MA+Glc-relative to MA-containing diet) to increased survivorship was minor in comparison to the effect of the increased caloric density of the diet.

The observation that levels of MA were significantly higher in MA+Glc treated flies relative to those fed diet containing MA alone (Suppl. Fig. 4) is consistent with our hypothesis that the addition of Glc led to increased crop filling. Thus, the increased levels of MA observed in the bodies of the MA+Glc-treated flies, were likely not physiologically relevant since the additional MA was contained in the crop (physiological dead space). Furthermore, our data do not support increased gut throughput (Fig. 8C), further supporting our assertion that the increased levels of MA measured in MA+Glc-treated flies were physiologically irrelevant. This interpretation of the data also explains why apparently higher levels of MA did not lead to increased toxicity and mortality.

Even though our data support the hypothesis that depletion of carbohydrate reserves plays an important role in MA-induced mortality, MA is known to produce numerous toxic side effects that also likely contribute to

MA-induced mortality at the organismal level, including increased oxidative stress and mitochondrial dysfunction (Davidson *et al*., 2001). The quantity of MA extracted from treated flies, which ranged from 14 ng/mg to 112 ng/mg (= mg/kg) (Suppl. Fig. 1)) would suggest that additional toxic effects of MA are likely. These levels of MA were up to ~11-fold greater on a per mass basis than a dose administered to mice that produced acute effects (10 mg/kg) (Fantegrossi *et al*., 2008). However, it is difficult to know how much of the MA is physiologically relevant, since it is unclear what proportion of the MA is present in the crop versus in physiological fluids, and this ratio may change over time as detoxification proteins are up-regulated and the degree of crop filling changes. Furthermore, using an identical experimental design, Sun *et al*. (2011) observed that MA led to transcriptomic and proteomic changes in *Drosophila* that were consistent with the known effects of MA in humans and other model organisms. For instance, transcripts of detoxification proteins, including cytochrome P450s and glutathione S-transferases, were differentially regulated by ten-fold (Sun *et al*., 2011). In addition, changes in the proteome suggested that MA disrupted the normal regulation of calcium and iron homeostasis, muscle homeostasis and oxidative status (Sun *et al*., 2011).

The results of our Glc supplementation experiments extend the work of Sun *et al*. (2011), which found that dietary sucrose and trehalose enhanced survivorship to MA-exposure. Not only does our study add another carbohydrate to the list, strengthening the argument that any well-metabolized carbohydrate will likely enhance survivorship to MA, but it also provides a probable mechanism through which the observed life extension is achieved: increased nutrient/carbohydrate density of diet helps to forestall the exhaustion of carbohydrate reserves. This hypothesis also explains the observation that sorbitol, a poorly metabolized sugar alcohol, did not allow the flies to survive MA longer (Sun *et al*., 2011), since supplementing with sorbitol did not significantly increase the nutrient density of the diet.

Our results show that MA administered in the diet caused behavioral and metabolic changes in *Drosophila*. MA increased locomotor activity 2-fold and decreased food consumption by up to 80%, resulting in a highly negative caloric balance. MA-induced starvation likely led to a compensatory decrease in resting metabolism; however, this decrease was not sufficient to overcome the large caloric deficit, resulting in a rapid depletion of energy reserves. Dietary Glc supplementation buffered carbohydrate reserves and led to a commensurate increase in survivorship. However, the relationship between dietary carbohydrates and the toxicity of MA remains unclear. For instance, do carbohydrates enhance survivorship solely by forestalling the depletion of energy reserves? Or do carbohydrates actively mitigate the toxicity of MA, perhaps by participating in the pentose phosphate pathway and thereby help to combat oxidative stress by generating NADPH and reduced glutathione? If the latter is true, this offers an intriguing explanation for why human MA addicts frequently drink large quantities of sugary drinks. The observation that MA produces similar behavioral changes in *Drosophila* and humans, i.e. increased locomotor activity and anorexia, further supports the use of *Drosophila* as a model organism for the study of biological effects of MA.

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