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Alpha-ketoglutarate reduces ethanol toxicity in *Drosophila melanogaster* by enhancing alcohol dehydrogenase activity and antioxidant capacity

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ABSTRACT

Ethanol at low concentrations (<4%) can serve as a food source for fruit fly *Drosophila melanogaster*, whereas at higher concentrations it may be toxic. In this work, protective effects of dietary alpha-ketoglutarate (AKG) against ethanol toxicity were studied. Food supplementation with 10-mM AKG alleviated toxic effects of 8% ethanol added to food, and improved fly development. Two-day-old adult flies, reared on diet containing both AKG and ethanol, possessed higher alcohol dehydrogenase (ADH) activity as compared with those reared on control diet or diet with ethanol only. Native gel electrophoresis data suggested that this combination diet might promote post-translational modifications of ADH protein with the formation of a highly active ADH form. The ethanol-containing diet led to significantly higher levels of triacylglycerides stored in adult flies, and this parameter was not altered by AKG supplement. The influence of diet on antioxidant defenses was also assessed. In ethanol-fed flies, catalase activity was higher in males and the levels of low molecular mass thiols were unchanged in both sexes compared to control values. Feeding on a mixture of AKG and ethanol did not affect catalase activity but caused a higher level of low molecular mass thiols compared to ethanol-fed flies. It can be concluded that both a stimulation of some components of antioxidant defense and the increase in ADH activity may be responsible for the protective effects of AKG diet supplementation in combination with ethanol. The results suggest that AKG might be useful as a treatment option to neutralize toxic effects of excessive ethanol intake and to improve the physiological state of *D. melanogaster* and other animals, potentially including humans.

Introduction

Ethanol is a two-carbon alcohol used commonly as a solvent in medications and is the principal ingredient of alcoholic beverages. Its consumption in excessive amounts may cause toxic effects. Numerous experimental studies indicate that acute and long-term consumption of ethanol has adverse effects on many organs and can lead to development of multiple diseases, including those of the liver, pancreas, gastrointestinal tract, and immune system (Adaramoye, Awogbindin, & Okusaga, 2009; Cadirci et al., 2007; Cederbaum, Lu, & Wu, 2009; Das & Vasudevan, 2007). Mammalian models such as rats and mice have been widely used to provide significant insight into the mechanisms of ethanol toxicity (Cederbaum et al., 2009; Hagymási, Blázovics, Lengyel, Kocsis, & Fehér, 2001; Miller-Pinsler & Wells, 2015; Oh, Kim, Chun, & Park, 1998).

In recent years, the fruit fly *Drosophila melanogaster* has been successfully developed as a model to study the effects of ethanol exposure (Devineni & Heberlein, 2013; Logan-Garbisch et al., 2015; McClure, French, & Heberlein, 2011). In natural habitats, these flies feed on rotting fruits and other plant materials that can contain up to 5% ethanol, produced by fermentation of carbohydrates by various yeasts (Devineni & Heberlein, 2013). Ethanol consumed at these low concentrations is efficiently metabolized in *Drosophila* larvae and can be used as an energy source or a substrate for lipid synthesis (Geer, McKechnie, & Langevin, 1986). *Drosophila* adults prefer food supplemented with ethanol, but they did not use ethanol as an energy source (Xu et al., 2012). In *D. melanogaster*,

Abbreviations: ADH, alcohol dehydrogenase; AKG, alpha-ketoglutarate; ALDH, acetaldehyde dehydrogenase; H-SH, high-molecular mass thiol groups; L-SH, low-molecular mass thiol groups; LOOH, lipid peroxides; ROS, reactive oxygen species; TAG, triacylglycerides.

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more than 90% of the dietary ethanol is converted by alcohol dehydrogenase (ADH) to acetaldehyde (Geer, Heinstra, & McKechnie, 1993), and acetaldehyde is then metabolized to acetate by acetaldehyde dehydrogenase (ALDH) (Fry, Donlon, & Saweikis, 2008). Both enzymatic reactions provide NADH production and are essential for resistance of flies to ethanol exposure (Barbancho, Sánchez-Cañete, Dorado, & Pineda, 1987; Fry et al., 2008). Acetate is converted to acetyl-CoA, which can enter the Krebs cycle for energy production or can be used for fatty acid synthesis in the case of significant increase in the NADH/NAD^+ ratio (Das & Vasudevan, 2007; Morozova, Anholt, & Mackay, 2006). As in mammals, catalase and the microsomal oxidation system, involving cytochrome P450, also participate in ethanol detoxification in *Drosophila*. Despite the fact that catalase and cytochrome P450 proteins apparently represent a minor pathway for ethanol degradation in *D. melanogaster* larvae, their role is increased at higher doses of consumed ethanol (Geer et al., 1993; Logan-Garbisch et al., 2015). Whereas ethanol at low concentrations serves as a food source for *Drosophila* larvae, exposure to high concentrations (>4%) was found to cause many toxic effects similar to those seen in mammals (Devineni & Heberlein, 2013; Logan-Garbisch et al., 2015; McClure et al., 2011). Rearing on food containing high concentrations of ethanol delayed development, decreased survival of larvae, and reduced adult body size (Logan-Garbisch et al., 2015; McClure et al., 2011; Ranganathan, Davis, Leeper, & Hood, 1987). In addition, developmental ethanol exposure led to dysregulation of fatty acid metabolism and lipid accumulation and caused oxidative stress in *D. melanogaster* larvae (Logan-Garbisch et al., 2015).

Ethanol exposure is known to lead to development of oxidative stress by increasing the production of reactive oxygen species (ROS) or by reducing antioxidant defense (Das & Vasudevan, 2007; Hagymási et al., 2001; Oh et al., 1998). The development of oxidative stress was proposed to be the most important mechanism underlying the developmental lethality and delay associated with ethanol exposure (Logan-Garbisch et al., 2015; Miller-Pinsler & Wells, 2015). At the same time, expression of antioxidant genes often increases as a result of the increased production of ROS (Cederbaum et al., 2009; Logan-Garbisch et al., 2015; Lushchak, 2014).

Due to the convincing evidence of the involvement of ROS in ethanol toxicity, the utilization of different antioxidant molecules, especially of natural origin, can be one of the effective approaches to minimize harmful effects of ethanol-induced oxidative stress (Adaramoye et al., 2009; Cadirci et al., 2007). Natural alpha-keto acids were identified recently that can act as antioxidants both *in vitro* and *in vivo* (Bayliak, Lylyk, Vytvytska, & Lushchak, 2016; Bayliak et al., 2015; Puntel, Nogueira, & Rocha, 2005). In particular, dietary alpha-ketoglutarate (AKG), which is an intermediate of the Krebs cycle, efficiently protected adult *D. melanogaster* flies against hydrogen peroxide in combined treatments (Bayliak et al., 2015). The administration of AKG also prevented lipid peroxidation in rats under chronic ethanol administration (Velvizhi, Nagalashmi, Essa, Dakshayani, & Subramanian, 2002). In our previous experiments, we showed that the dietary AKG was able to partially alleviate developmental toxicity of ethanol in the *D. melanogaster* w^{1118} strain by increasing the number of total pupae formed; the effects depended on the combinations of concentrations of AKG and ethanol (Shmihel, 2015). In this study, we aimed to examine in detail the possible protective effects of dietary AKG against ethanol-induced toxicity in *D. melanogaster* at both larval and adult stages using fly lines that differ in their sensitivity to these chemicals. To determine whether beneficial effects of AKG could be connected with its antioxidant properties, the oxidative stress markers in adult flies reared on food containing ethanol or a mixture of ethanol and AKG were measured.

Methods and materials

Flies and rearing conditions

Wild-type Canton S and mutant w^{1118} flies were used in experiments. Stock flies were kindly provided by Bloomington Stock Center (Indiana University, Indiana, USA). All fly cultures were kept at 25 °C, 55–60% humidity in a 12-h dark/light cycle. Parental populations of flies were maintained on yeast-corn-molasses media (Rovenko et al., 2015). For experiments, flies were reared starting from the egg stage on a medium containing 5% sucrose, 5% yeast, 1% agar, and 0.18% methylparaben. This group was used as the control. Experimental cohorts of flies were cultivated on the same food, but supplemented with 8% (w/v) ethanol, 10-mM alpha-ketoglutaric acid disodium salt (AKG) or a mixture of both 8% ethanol and 10-mM AKG. About 100 eggs laid by parental flies within a 6-h time period were put in each 100-mL glass bottle with 15 mL of an experimental diet.

The speed of development of flies on the different experimental diets was assessed by counting once per day the number of pupae formed, starting 96 h after egg deposition. The total number of pupae formed by day 10 was set as 100% and pupation at each day prior to this (days 4–9) was expressed as a percentage of the day-10 value. Developmental survival at egg and larval stages was also assessed as the percentage of eggs that were able to reach the pupa stage.

Newly eclosed flies were transferred into flasks with the respective experimental diets and held for 2 days. Two-day-old flies were mildly anesthetized with carbon dioxide gas, separated by sexes, and then quickly frozen in liquid nitrogen for further biochemical analysis.

Assay of food intake

Food intake was measured as described previously (Lushchak, Rovenko, Gospodaryov, & Lushchak, 2011). Briefly, groups of 15 third instar larvae reared on the control and experimental media were placed for 20 min on respective control and experimental diets (in Petri dishes), which also contained 0.5% FD&C Blue No. 1 dye. After feeding, larvae were homogenized in 50-mM potassium phosphate buffer (KPi, pH 7.0) at a ratio of 1:100 (mg/μL) and centrifuged at room temperature at 13,000 *g* for 15 min. Supernatants were removed and absorbance was measured at 629 nm and compared against a calibration curve built with different concentrations of the dye.

Preparation of homogenates of adult flies for metabolic analyses

Frozen flies were weighed and homogenized using a Potter–Elvehjem glass homogenizer in lysis buffer (50-mM KPi, pH 7.0, 1-mM phenylmethylsulfonyl fluoride, 0.5-mM EDTA) in a 1:10 ratio (mg flies:μL buffer). After centrifugation at 16,000 *g* for 15 min at 4 °C in an Eppendorf 5415 R centrifuge (Hamburg, Germany), the supernatants were collected and used for different assays using a Spekol 211 (Carl Zeiss, Jena, Germany) or SF-46 (LOMO, Leningrad, USSR) spectrophotometer.

Assays of catalase and ADH activities, and protein concentration

Catalase activity was measured by monitoring the disappearance of hydrogen peroxide at 240 nm using the extinction coefficient for hydrogen peroxide of 39.4 M^{-1} cm^{-1} (Aebi, 1984). The activity of catalase was assayed in 2 mL of medium containing 50-mM KPi (pH 7.0), 0.5-mM EDTA, 10-mM H_2O_2, and 3 μL of supernatant.

The activity of ADH was measured by monitoring NAD^+ reduction at 340 nm using the extinction coefficient for NADH of

6220 M^{-1} cm^{-1} (Oudman, van Delden, Kamping, & Bijlsma, 1991). The reaction mixture contained 50-mM glycine-NaOH buffer (pH 9.5), 0.5-mM EDTA, 0.5-mM NAD^+, and 100-mM ethanol and 20 μL of supernatant in a final volume of 1 mL.

Protein concentration was determined by the Coomassie brilliant blue G-250 dye-binding method (Bradford, 1976) with bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) was performed on 3.6% stacking and 7.5% separating gels in standard Tris-glycine buffer (pH 8.3) according to the method of Davis (1964). The supernatants obtained as described above were mixed with glycerol (2:1) and the samples containing 40 μg of total protein were applied to each well. Electrophoresis was performed at 200 V through the stacking gel for 30 min and 180 V through the separating gel for 5 h. Alcohol dehydrogenase bands were detected on the gels according to Jacobson, Murphy, Knopp, and Ortiz (1972). The gels were incubated at 30 °C in darkness in 50 mL of 50-mM M glycine-NaOH buffer, pH 9.5, containing 1% ethanol, 16 mg of NAD^+, 2 mg of N-methylphenazonium methosulfate, and 10 mg of nitro-blue tetrazolium. Dark violet spots were developed within 60 min of incubation. After staining, the gels were immersed in distilled water and photographed immediately. For both Canton S and w^{1118} strains, two independent gel runs were conducted for detecting ADH activity. Relative band intensities were estimated measuring density with TotalLab Quant Software.

Assay of oxidative stress markers

Free thiols were measured spectrophotometrically by the Ellman procedure with 5,5′-dithiobis-2-nitrobenzoic acid at 412 nm (Ellman, 1959). Fly homogenates were prepared, centrifuged, and supernatants saved and then total, low-molecular mass and high-molecular mass thiol contents were measured as was described previously (Lushchak et al., 2011). Briefly, the sum of soluble low- and high-molecular mass thiol-containing compounds was measured in the supernatants. Then, to determine the non-protein low-molecular mass thiols (L-SH), trichloroacetic acid was added to supernatants to a final concentration of 10% followed by centrifugation (10,000 *g*, 15 min, 21 °C); supernatants from this step were used to assay low-molecular mass thiols. High-molecular mass thiol (H-SH) content was calculated by subtraction of the amount of low-molecular mass thiols from total values. Thiol concentrations were expressed as micromoles of SH groups per gram of wet mass (μmol/gwm).

Lipid peroxide (LOOH) content was assayed with xylenol orange (Lushchak et al., 2011). For this, flies were homogenized 1:20 (w:v) in 96% cold (~5 °C) ethanol, centrifuged for 5 min at 16,000 *g* in an Eppendorf 5415 R centrifuge (Hamburg, Germany), and supernatants were used for assay. The content of LOOH was expressed as micromoles of cumene hydroperoxide equivalents per gram of wet mass (μmol/gwm).

TAG assay

Pre-weighed flies were homogenized in chilled 10-mM PBST buffer (10-mM Na_2HPO_4, 2-mM KH_2PO_4, 137-mM NaCl, 2.7-mM KCl, 0.05% Triton X-100, pH 7.4) in a ratio of 1:50 (mg flies:μL buffer). Homogenates were heated for 10 min at 70 °C to denature proteins; after cooling they were used for triacylglyceride (TAG) assay. TAG levels were measured using a Liquick Cor-TG diagnostic kit (PZ Cormay S.A., Łomianki, Poland) following the manufacturer's instructions. Standard TAG solutions in a concentration range from 3 to 30 ĕg/mL were used for calculation of TAG content in flies. TAG levels were expressed as milligrams per gram of wet mass (mg/gwm).

Statistical analysis

Experimental data are expressed as the mean value of 4–8 independent experiments ± the standard error of the mean (SEM). Statistical analysis was performed using a two-tailed Student's *t* test or analysis of variance (ANOVA) followed by the two-tailed Dunnett's test or the *post hoc* Student Newman–Keuls test.

Results and discussion

Dietary AKG reduces developmental toxicity of ethanol on D. melanogaster *by improving food intake*

Ethanol at concentrations higher than 4–5% becomes toxic for *D. melanogaster*, with tolerance of ethanol depending on fly strain and the concentrations used (Barbancho et al., 1987; McClure et al., 2011). In this study, we examined the protective ability of dietary AKG against ethanol-induced larval toxicity in two strains of *D. melanogaster*, namely Canton S and w^{1118}, which were found previously to differ in sensitivity to diverse toxicants (Bayliak et al., 2015; Lozinsky et al., 2013). The concentrations of ethanol and AKG used in this study were those that were found earlier to reduce and restore the pupation rate of the w^{1118} strain, respectively (Shmihel, 2015). Fig. 1 demonstrates the effect on development, assessed by pupation speed, when fruit fly larvae were raised on media supplemented with 8% ethanol alone or with 8% ethanol and 10-mM AKG, as compared with controls. In both Canton S and w^{1118} strains, consumption of food containing ethanol delayed pupation (Fig. 1A, B) and reduced the total number of pupae formed (Fig. 1C). The delay in the transition from larvae to pupae was more pronounced in the w^{1118} strain (Fig. 1B) with the median time to pupation for larvae raised on diets with 8% ethanol being 16% and 24% higher in Canton S and w^{1118} strains, respectively, compared to controls (Fig. 1D). The total percentage of larvae that pupated did not significantly differ between the control cultures of the two strains (96 ± 3% in Canton S versus 92 ± 4% in w^{1118}) and decreased similarly in both strains on food supplemented with 8% ethanol (Fig. 1C). Accordingly, the total yield of pupae from ethanol-fed larvae was 58 ± 3% and 62 ± 3% of laid eggs in Canton S and w^{1118} strains, respectively. Our data confirmed developmental ethanol toxicity in flies as previously reported (Logan-Garbisch et al., 2015; McClure et al., 2011; Ranganathan et al., 1987). It should be noted that the number of pupae reflects ethanol toxicity only on egg and larval stages but not on the pupal stage. The mortality also occurs during the metamorphosis of pupae and the number of emerged flies can be lower than the number of total pupae formed (McClure et al., 2011). Food supplementation with a mixture of 8% ethanol and 10-mM AKG significantly reduced larval mortality and enhanced the total number of pupae formed to 73 ± 6% and 80 ± 7% in Canton S and w^{1118} strains, respectively. However, AKG did not change the pupation delay seen for w^{1118} ethanol-exposed larvae, and the median time to pupation was similar in w^{1118} larvae fed on ethanol and mixture of ethanol and AKG (Fig. 1D). In Canton S larvae, a trend of improved pupation time in the presence of AKG was observed (Fig. 1D).

D. melanogaster larvae spent more time on the ethanol-containing half of the agar plate compared to the other side without ethanol (Depiereux et al., 1985). Assuming that such ethanol preference could affect the feeding behavior of larvae, we measured food intake by third instar larvae on the control diet and the diets supplemented with 8% ethanol or with 8% ethanol and 10-

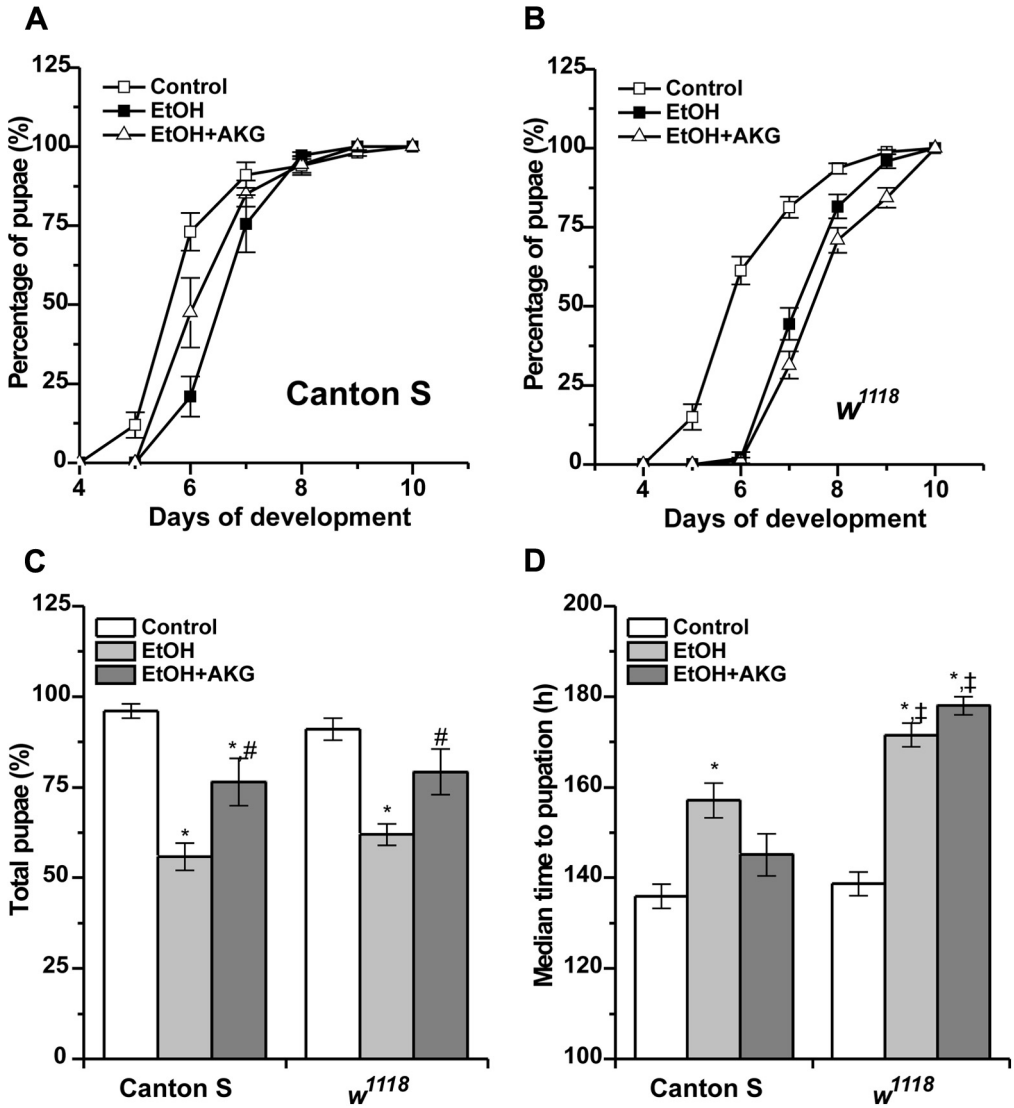

Fig. 1. Developmental pattern of *D. melanogaster* flies reared on yeast-sucrose medium or on diets supplemented with 8% ethanol or 8% ethanol plus 10-mM AKG. Graphs show percentage of larvae that pupated over time for Canton S (A) and w^{1118} (B) strains, percentage of eggs pupated (C), and median time of fly development to pupation (D). Data are means ± SEM, $n = 5–8$. *Significantly different from the corresponding control group; #significantly different from the ethanol-fed group; ‡significantly different from the corresponding Canton S group with $p < 0.05$ as determined by ANOVA and the *post hoc* Student Newman–Keuls test.

mM AKG. Canton S and w^{1118} larvae consumed 48% and 28% less food when raised on a diet containing 8% ethanol as compared with controls (Fig. 2). However, the addition of 10-mM AKG to ethanol-containing diet raised food intake by larvae back to control levels (Fig. 2). Lower consumption of food containing ethanol suggests that ethanol at the 8% concentration is not preferable as a food source for the fly lines studied, likely due to toxicity of ethanol at these levels. Other studies demonstrated that the preference of larvae for ethanol-rich food depends on their rate of ethanol metabolism (Depiereux et al., 1985). Furthermore, the lower feeding rate on diets containing ethanol can be considered as an adaptive response of larvae to these stressful conditions. It is established that if there are toxic compounds in the food, larvae can slow their feeding rates and at the same time increase efficiency of food use (Mueller & Barter, 2015). The higher food intake on the diets containing a mixture of ethanol and AKG, as compared with diets with ethanol alone, can be regarded as an additional confirmation of the ability of AKG to diminish the toxicity of ethanol. Comparison of the food consumption and pupation rate in the two strains reveals a rather interesting fact. On diets with ethanol and ethanol plus AKG, Canton S larvae pupated ~2-fold faster and consumed 32% less food compared to w^{1118} larvae. Thus, we propose that the higher food consumption and resulting greater ethanol intake by w^{1118} larvae could be at least partially responsible for the lower pupation rate compared with Canton S larvae. This supports the previously reported extreme toxicity of high ethanol for developmental stages in *Drosophila* (Logan-Garbisch et al., 2015; McClure et al., 2011).

In total, the results of developmental and behavior experiments demonstrate that dietary AKG can partially alleviate toxic effects of ethanol on the development of *D. melanogaster* and thereby promote increased food intake by larvae and increased numbers of pupae formed.

Alpha-ketoglutarate partially prevents ethanol-induced protein decrease and body mass reduction in adult flies

The body masses of 2-day-old males developed on ethanol or ethanol plus AKG diets did not differ from those of corresponding controls (Table 1). However, ethanol-fed Canton S and w^{1118} females

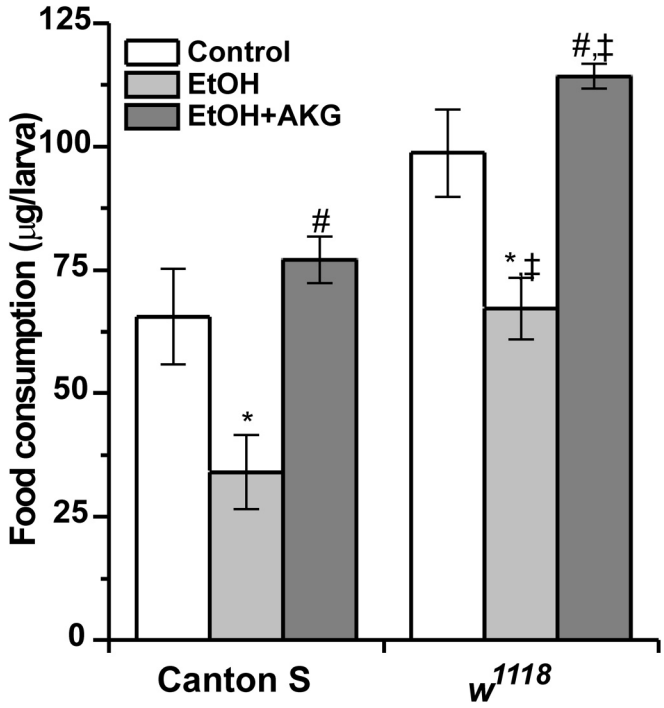

Fig. 2. Food consumption by third instar larvae reared on the control diet or on the diet supplemented with 8% ethanol or a mixture of 8% ethanol plus 10-mM AKG. Data are means ± SEM, $n = 4–5$. *Significantly different from the corresponding control group; #significantly different from the ethanol-fed group; ‡significantly different from the corresponding Canton S group with $p < 0.05$ as determined by ANOVA and the *post hoc* Student Newman–Keuls test.

had 18% and 13% lower body masses, respectively, than control counterparts. The diet with a mixture of ethanol and AKG suppressed this effect, and promoted by 9% a lesser reduction in body mass in Canton S females and returned body mass values in w^{1118} females to control ones. Rearing on ethanol-containing food did not affect the levels of water-soluble protein in adult Canton S flies but decreased this parameter by ~20% in w^{1118} males and females (Table 1). The protein levels in fly bodies of both strains did not differ from respective control values when food contained the mixture of ethanol and AKG. Thus, AKG supplementation of ethanol-containing food partially prevented the reduction in body mass in adult females of both strains and the decrease in protein levels in w^{1118} females. The ability of AKG to prevent protein loss under different pathological states and stresses is well documented, and it may be at least partially due to capacity of AKG to serve as an amino acid precursor (Yao et al., 2012). It seems that the decrease in body mass observed in ethanol-reared females is not necessarily dependent on changes in protein levels, at least in Canton S strain. In addition to this, despite the fact that ethanol-fed w^{1118} males had lower protein levels, their body mass did not differ from the control group. The reduced body mass of adult flies reared on high ethanol concentrations was observed earlier and it was suggested to be connected with a decrease in cell proliferation in the developing fly larvae and with a slowing of metamorphosis (McClure et al., 2011). At the same time, AKG has been shown to be able to maintain the proliferation activity of embryonic stem cells (Carey, Finley, Cross, Allis, & Thompson, 2015). Therefore, one may suggest that this mechanism is one of the preventive actions that dietary AKG has that limits body mass reduction in adult females grown on ethanol-containing diet.

Ethanol exposure does not affect ADH activity, while combined treatment with AKG and ethanol increases enzyme activity in 2-day-old flies

To explore molecular mechanisms of the protective effects of AKG against ethanol toxicity in *D. melanogaster*, selected biochemical parameters were determined in 2-day-old flies reared on the experimental media. First, we measured the activity of alcohol dehydrogenase (ADH), a key enzyme in ethanol metabolism, which oxidizes over 90% of ethanol consumed in *D. melanogaster* (Geer et al., 1993). First, we noticed that control males of the Canton S and w^{1118} lines had 1.4- and 1.5-fold higher ADH activity than the respective control females (Fig. 3A, B). This is consistent with previous reports that demonstrated sex differences in ADH activity (Malherbe, Kamping, van Delden, & van de Zande, 2005) and a lower rate of ethanol metabolism in females as compared with males (Devineni & Heberlein, 2012). The supplementation of food with AKG or ethanol alone did not affect ADH activity in either males or females of both strains, whereas diet containing a mixture of ethanol + AKG promoted higher ADH activity by 1.5-fold in Canton S males and by 1.4-fold in w^{1118} males and females as compared with respective control cohorts.

It was found previously that ethanol exposure induced *Adh* gene transcription and increased ADH enzyme activity in larvae but not in adult flies (Geer et al., 1993; Malherbe et al., 2005). This is connected with the fact that the *Adh* gene is temporally regulated by two distinct promoters, the proximal (or larval) one, which is induced by ethanol, and the distal (or adult) one, which is insensitive to ethanol (Geer et al., 1993; Heinstra, 1993). These facts can explain the similar ADH activity in control and ethanol-fed flies in our experiments. At the same time, the higher ADH activity in flies reared on food supplemented with ethanol and AKG can be considered as a protective mechanism against toxic effects of ethanol in adult flies. This idea is well supported by the fact that in many cases, *D. melanogaster* flies with higher ADH activity were more resistant to ethanol toxicity (Barbancho et al., 1987; Malherbe et al., 2005; Ranganathan et al., 1987). On the other hand, it is not always advantageous to enhance the activity of this enzyme under

Table 1
Body mass and water-soluble protein level in 2-day-old *D. melanogaster* Canton S and w^{1118} flies reared on control food or on diets supplemented with ethanol or a mixture of ethanol and AKG.

Conditions	Canton S				w^{1118}			
	Body mass, μg		Protein, μg/mg wet mass		Body mass, μg		Protein, μg/mg wet mass	
	Males	Females	Males	Females	Males	Females	Males	Females
Control	807 ± 33	1398 ± 66	38.3 ± 2.4	38.3 ± 1.7	768 ± 21	1332 ± 48	41.4 ± 2.9	40.0 ± 1.4
Ethanol	737 ± 17	1145 ± 21*	35.0 ± 2.5	38.8 ± 3.2	736 ± 27	1171 ± 30*	32.0 ± 1.7*	30.5 ± 1.7*
Ethanol + AKG	728 ± 21	1269 ± 29*,†	32.9 ± 1.6	37.5 ± 4.5	763 ± 37	1349 ± 68†	35.2 ± 3.2	39.3 ± 3.8†

Data are presented as means ± SEM, $n = 5–8$.
* Significantly different from the corresponding control group.
† Significantly different from the ethanol-fed group with $p < 0.05$ as determined by ANOVA and the *post hoc* Student Newman–Keuls test.

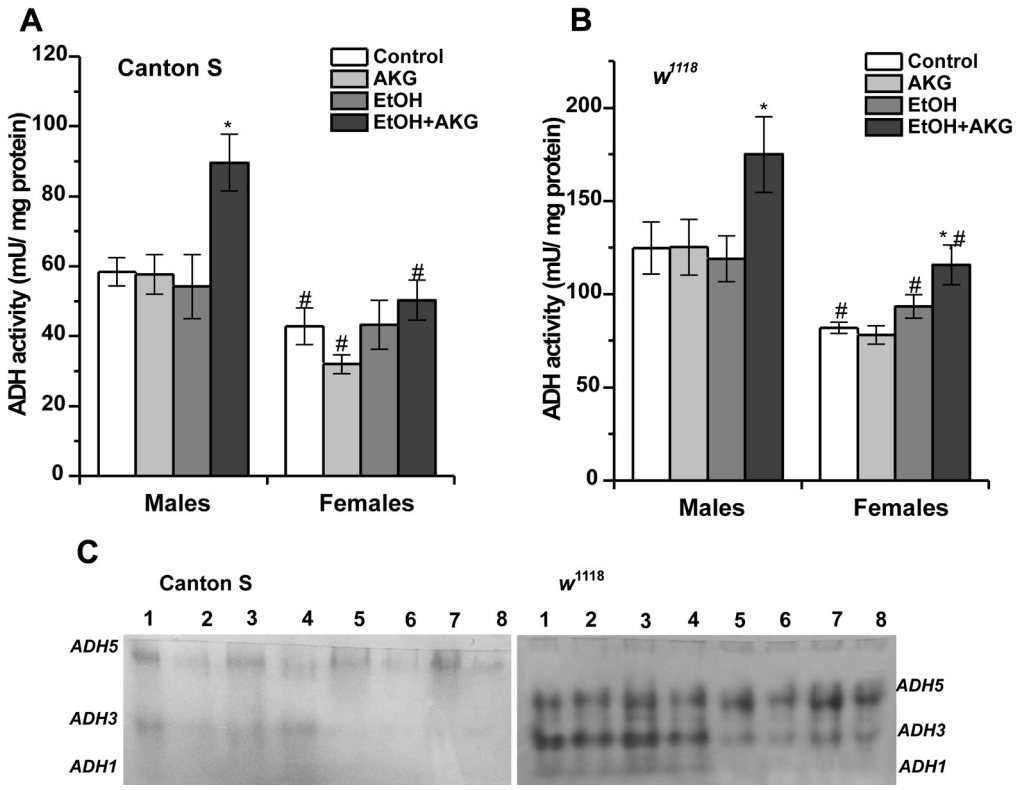

Fig. 3. Activity (A, B) and electrophoretic patterns (C) of ADH in 2-day-old male and female *D. melanogaster* flies reared on the control diet or on the diet supplemented with 10-mM AKG, 8% ethanol, or both ethanol plus AKG. Lanes as denoted: 1 – male, control; 2 – female, control; 3 – male, AKG; 4 – female, AKG; 5 – male, ethanol; 6 – female, ethanol; 7 – male, AKG + ethanol; 8 – female, AKG + ethanol. Data are means ± SEM, $n = 5$–7. *Significantly different from the corresponding control group using Dunnett's test; #significantly different from the corresponding values for males using Student's t test, $p < 0.05$.

ethanol exposure due to a possible increase in the levels of toxic intermediates, especially acetaldehyde. The ADH enzyme works in tandem with acetaldehyde dehydrogenase (ALDH), which catalyzes oxidation of acetaldehyde to acetate, which can be easily metabolized further. Therefore, to avoid toxic effects of acetaldehyde, the activity of ALDH can also be induced in adult flies (Fry et al., 2008; Malherbe et al., 2005). Taking this into account, we propose that higher ADH activity could be accompanied by higher ALDH activity in flies reared on diets containing ethanol and AKG. Mechanisms leading to increased ADH activity in adult flies raised on ethanol plus AKG diet seem to be connected with post-translational regulation of ADH activity rather than with changes in *Adh* gene transcription. It was shown previously that the high activity of ADH protein in adult flies, especially in ethanol-selected lines, was not necessarily associated with a high level of *Adh* gene transcripts (Malherbe et al., 2005). We observed a similar discrepancy between the activity of enzymes (Bayliak, Semchyshyn, & Lushchak, 2007) and the levels of corresponding mRNA and protein synthesis in yeast *Saccharomyces cerevisiae* that was also observed by others authors (Godon et al., 1998). This is related to the fact that enzyme activity is regulated not only at the transcriptional level.

To check our assumption, we conducted gel electrophoresis of ADH in crude extracts from the flies reared on control food or food supplemented with AKG, ethanol, or both (Fig. 3C). Two and three electrophoretic bands of ADH were clearly detected in Canton S and w^{1118} strains, respectively. Under all feeding regimens tested, the intensities of ADH bands were greater in males as compared to females, paralleling the results for enzyme activity (Fig. 3A, B). Diet with AKG alone did not affect ADH electrophoregrams (lane 3 male & lane 4 female) as compared with controls (lane 1 male & lane 2 female), whereas diets with ethanol alone (lane 5 male & lane 6 female) or a mixture of AKG and ethanol (lane 7 male & lane 8 female) caused a redistribution of the intensities of the different ADH bands with a prominent decrease in the relative amount of ADH activity in the faster running bands (ADH3, ADH1). There was no significant difference observed between ADH electrophoregrams for Canton S females reared on ethanol and ethanol plus AKG diets, while w^{1118} females and Canton S and w^{1118} males reared on ethanol plus AKG diet had more intensive ADH bands than ethanol-fed ones.

The ADH enzyme is a homodimer encoded by a single gene, *Adh*, which is present in natural populations in two major allele forms, called "fast" (F) and "slow" (S) because of their electrophoretic mobilities (Devineni & Heberlein, 2013; Heinstra, 1993). The ADH allozymes differ in a single amino acid that affects their enzymatic activity: ADH^F shows higher enzymatic activity than does ADH^S (Geer et al., 1993; Heinstra, 1993; Malherbe et al., 2005). Post-translational modifications of ADH^S and ADH^F form three conformational patterns for each (ADH1, ADH3, and ADH5), which differ in electrophoretic mobility and catalytic activity (Heinstra, Scharloo, & Thörig, 1986; Jacobson et al., 1972). These electrophoretic forms were proposed to be the result of the binding of one or more negatively charged NAD-carbonyl compounds to the ADH protein: (*i*) ADH5 is bound with NAD^+ only and possesses the highest catalytic activity and simultaneously the slowest electrophoretic mobility; (*ii*) ADH3 has a molecule of NAD^+ bound in one monomer and bound NAD-carbonyl compound in another monomer; this ADH form has lower enzymatic activity compared to ADH5; (*iii*) ADH1 protein has two molecules of NAD-carbonyl bound and possesses the lowest catalytic activity with the fastest electrophoretic mobility; this ADH isoform is present in small amounts in young flies under normal conditions and is not always

detectable on gel electrophoresis (Heinstra et al., 1986; Jacobson et al., 1972). Furthermore, it was shown that it was possible to convert one isoform to another using NAD^+ and acetone (Jacobson et al., 1972), or β-keto metabolites and ethanol (Heinstra et al., 1986). Taken together we propose the following explanation of our results. ADH activity was approximately equally represented in ADH3 and ADH5 forms in control flies (Fig. 3C). Under equal amounts of protein loaded per well, the ADH1 form was not detected in Canton S flies, but trace amounts were found in w^{1118} flies. The diets with ethanol or a mixture of AKG and ethanol led to a conversion between enzyme forms with the formation of mostly the ADH5 configuration. As a result, the ADH3 band in the Canton S strain and the ADH1 band in the w^{1118} flies were virtually invisible. Despite the domination of the ADH5 form in flies fed on ethanol (lanes 5 & 6), the intensity of the ADH5 band was lower than total intensity of ADH5 and ADH3 forms in control flies of both the Canton S and w^{1118} strains. This may suggest that ethanol exposure leads not only to a conversion between ADH isozymes, but perhaps also to a decrease in the level of active ADH protein, possibly due to inactivation of the protein from by-products of ethanol metabolism. Furthermore, the lower content of ADH protein seems not to affect the overall enzymatic activity because ADH activity was unchanged in ethanol-reared flies using the spectrophotometric assay (Fig. 3A, B). This is consistent with previous data that reported that the total ADH activity measured after electrophoresis is often a poor predictor of total ADH activity *in vivo* (Heinstra et al., 1986). At the same time, Canton S and w^{1118} males and w^{1118} females reared on AKG and ethanol diet showed 20%, 32%, and 42% higher intensity bands of ADH5, respectively, than those from the ethanol-fed flies. It is in accordance with higher ADH activity in these flies compared to bands in ethanol-fed individuals. Therefore, one may suppose that the diet with AKG and ethanol mixture promotes stability and post-translational modifications of the ADH protein with the formation of the highly active ADH5 form. This could be one of the defensive mechanisms of AKG providing higher resistance of the flies to the toxic effects of ethanol.

It should be noted that ADH activity and intensity of ADH bands were significantly higher in w^{1118} (~2-fold) flies than in Canton S counterparts (Fig. 3). This might suggest that these *D. melanogaster* strains have different ADH alleles. The w^{1118} strain seems to have an ADH allozyme with higher catalytic activity and faster electrophoretic migration as seen from Fig. 3B and C. In *D. melanogaster*, strains with the more enzymatically active fast electromorph tend to have higher ethanol resistance than those with the slow electromorph (Heinstra, 1993; Malherbe et al., 2005). In our experiments, a comparison of ADH activity and developmental time on ethanol-containing medium in Canton S and w^{1118} strains showed an inverse relation between these parameters: the Canton S strain had lower enzymatic activity but pupated faster, while w^{1118} flies with higher ADH activity pupated more slowly on ethanol-containing diet. These results confirm that *Adh* may not be the only gene responsible for variation in ethanol resistance within and among *Drosophila*.

Alpha-ketoglutarate does not influence ethanol-induced TAG level increase in 2-day-old females in combined treatment

Similar to results from mammalian studies, ethanol exposure increases the activities of lipogenic enzymes such as glycerol-3-phosphate dehydrogenase and fatty acid synthetase, as well as levels of free fatty acids and triacylglycerides (TAG) in *D. melanogaster* larvae (Geer et al., 1986, 1993; Logan-Garbisch et al., 2015; Morozova et al., 2006). We determined TAG levels in 2-day-old females reared on the diet with ethanol alone or in combination with AKG. Females fed on ethanol diet had higher TAG contents in their bodies, as compared with respective controls: TAG levels were 1.6-fold higher in both ethanol-fed Canton S and w^{1118} females, compared with their corresponding controls (Fig. 4). The rearing on ethanol plus AKG diets did not affect TAG content in females of both strains. We can conclude that higher levels of storage lipids in ethanol-fed adult flies could result from the intensification of synthesis of fatty acids and eventually TAG as was reported previously for larvae. Active oxidation of ethanol by ADH and subsequent oxidation of acetaldehyde to acetate can lead to a significant increase in the NADH/NAD^+ ratio. Acetate in the form of acetyl-CoA is metabolized via the Krebs cycle, but the increase in NADH level leads to Krebs cycle inhibition, redirection of acetyl-CoA for synthesis of fatty acids and, eventually, to accumulation of storage lipids (Das & Vasudevan, 2007; Morozova et al., 2006). NADH at high concentrations can also inhibit β-oxidation of fatty acids; therefore, this mechanism might contribute to increased TAG levels in ethanol-fed flies. Consequently, the conversion of ethanol to non-toxic products such as lipids appears to be an important mode of ethanol detoxification for adult flies, as was also shown for *D. melanogaster* larvae (Geer et al., 1986). Our results also suggest that protective effects of dietary AKG are not connected with changes in the accumulation of storage lipids in ethanol-fed flies.

Alpha-ketoglutarate induces antioxidant defense in ethanol-reared flies

Ethanol exposure is known to lead to oxidative stress development by increasing production of ROS and/or by decreasing the capacity of the endogenous antioxidant system. Ethanol may lead to increased ROS production in multiple ways such as by (*i*) increased activity of the respiratory chain as a result of intensification of Krebs cycle activity, (*ii*) stimulating activity of cytochrome P450s that

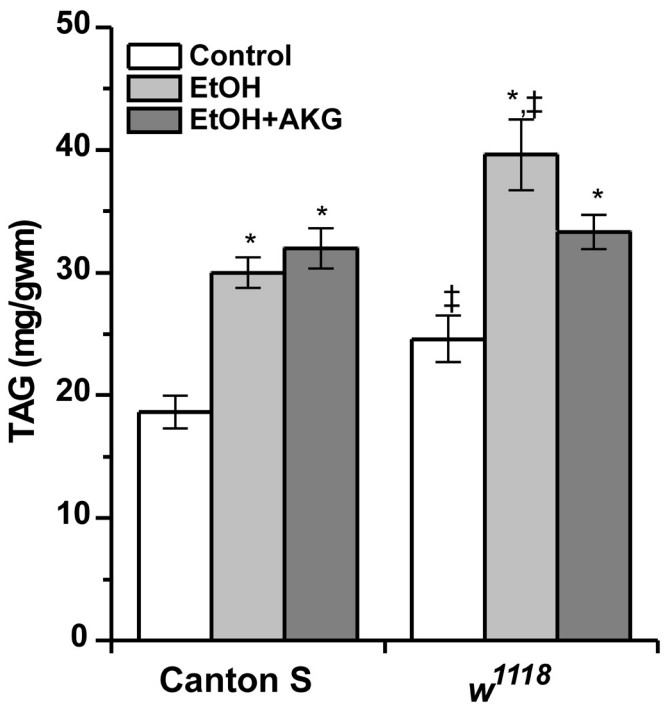

Fig. 4. Levels of TAG in 2-day-old *D. melanogaster* Canton S and w^{1118} females reared on the control diet or on the diet supplemented with 8% ethanol or a mixture of 8% ethanol plus 10-mM AKG. Data are means ± SEM, $n = 5–8$. *Significantly different from the corresponding control group using the *post hoc* Student Newman–Keuls test; ‡significantly different from the corresponding Canton S group using Student's *t* test, $p < 0.05$.

detoxify ethanol, which leads to increased ROS production, (*iii*) depletion of glutathione levels and decreased activity of antioxidant enzymes, (*iv*) direct production of an ethanol-derived radical (1-hydroxyethyl radical) (Cederbaum et al., 2009; Das & Vasudevan, 2007; Oh et al., 1998).

Dietary AKG was shown previously to possess antioxidant properties (Bayliak et al., 2015, 2016; Puntel et al., 2005). Furthermore, this keto acid demonstrated the ability to prevent ethanol-induced lipid peroxidation and to decrease antioxidant enzyme activities in rats (Velvizhi et al., 2002). Based on this, we propose that the protective effects of AKG observed here may also be associated with AKG-mediated antioxidant activity, not just with AKG effects on ADH activity. To test the idea that AKG can reduce the intensity of oxidative stress in ethanol-treated flies, we measured the activity of catalase (Fig. 5A, B) and levels of oxidative stress markers in 2-day-old flies (Fig. 5C, Table 2), which emerged from pupae raised on a diet with 8% ethanol or with a mixture of 8% ethanol and 10-mM AKG (see Fig. 1C).

In the control groups, Canton S and w^{1118} males had 1.4- and 1.5-fold higher catalase activities than respective females (Fig. 3A, B). This is consistent with our previous reports, which demonstrated a sex difference in catalase activity in *D. melanogaster* (Lushchak et al., 2011; Rovenko et al., 2015). The food supplemented with ethanol did not affect catalase activity in experimental female groups, whereas ethanol-fed males of both strains had 1.25-fold higher catalase activity than their respective controls. When food was supplemented with the mixture of AKG and ethanol, catalase activity was unaltered compared with controls. However, w^{1118} males grown on the ethanol + AKG diet showed 1.5-fold higher catalase activity compared to controls. The levels of lipid peroxides (LOOH), which represent the intensity of ROS-promoted lipid peroxidation, were measured only in females of both Canton S and w^{1118} strains (Fig. 3C). Females of both strains reared on the diets containing ethanol or ethanol + AKG had ~50% lower LOOH levels as compared with their respective control groups. The levels of high (H-SH) and low (L-SH) molecular mass thiols were similar in control groups of both strains and both sexes (Table 2). The diet with ethanol promoted lower H-SH levels by 40% and 50% in w^{1118} males and females, respectively, compared with controls. In ethanol-fed Canton S flies, only a tendency to lower H-SH levels was observed. On a diet containing a mixture of AKG and ethanol, levels of H-SH were 40% and 55% lower in Canton S and w^{1118} males,

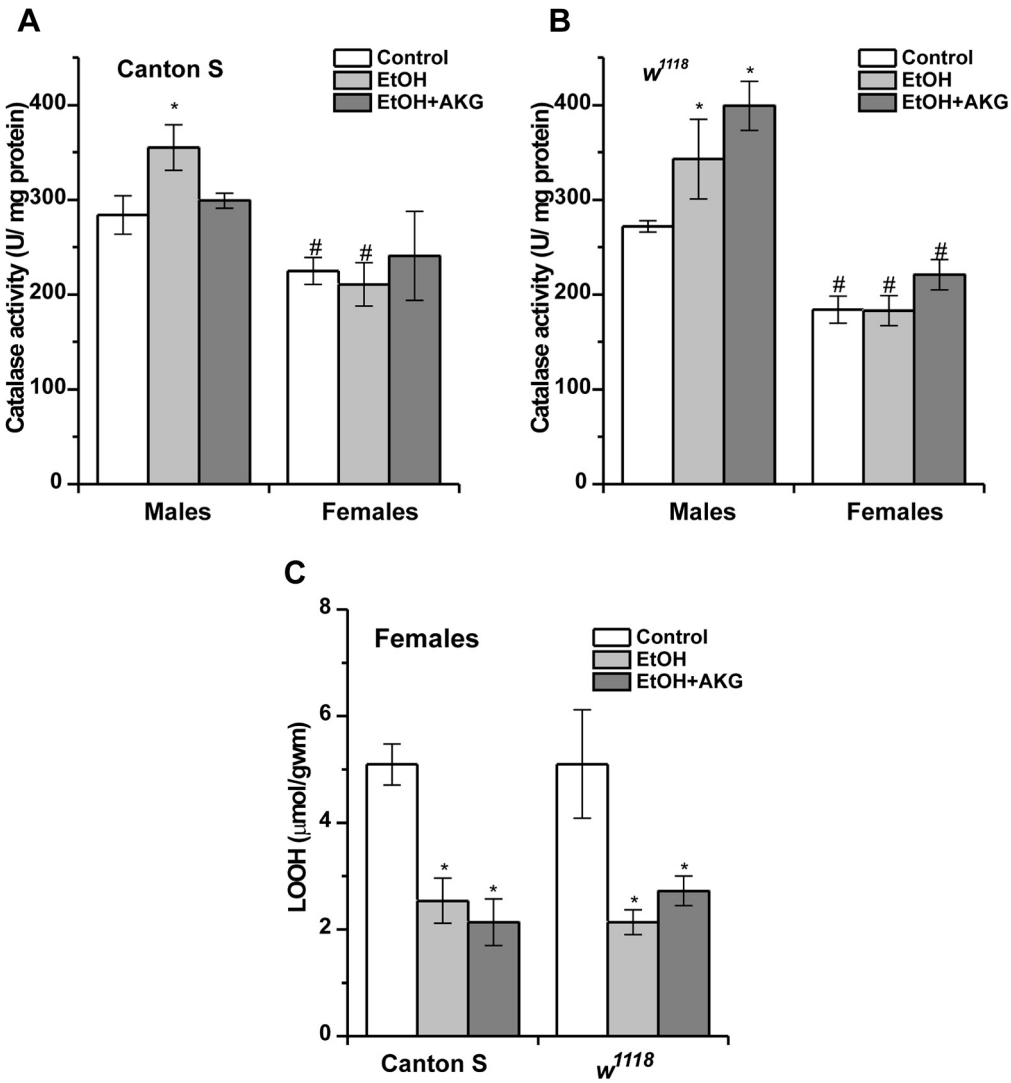

Fig. 5. Catalase activity in Canton S (A) and w^{1118} (B) male and female flies as well as LOOH levels (C) in female flies. All flies were 2-day-old *D. melanogaster* reared on the control food or on the food supplemented with 8% ethanol or 8% ethanol plus 10-mM AKG. Data are means ± SEM, $n = 4–7$. *Significantly different from the corresponding control values using Dunnett's test; #significantly different from the corresponding values for males using Student's *t* test, $p < 0.05$.

Table 2
Levels of high- and low-molecular mass thiols in 2-day-old *D. melanogaster* flies reared on control food or on diets supplemented with ethanol or a mixture of ethanol and AKG.

Conditions	Canton S				w^{1118}			
	H-SH, μmol/g wet mass		L-SH, μmol/g wet mass		H-SH, μmol/g wet mass		L-SH, μmol/g wet mass	
	Males	Females	Males	Females	Males	Females	Males	Females
Control	4.76 ± 0.68	5.2 ± 0.45	0.83 ± 0.07	0.98 ± 0.06	5.33 ± 0.12	5.6 ± 0.62	0.96 ± 0.18	0.74 ± 0.10
Ethanol	3.13 ± 0.77	3.53 ± 0.57	0.81 ± 0.11	0.82 ± 0.09	3.24 ± 0.41*	2.8 ± 0.33*	0.92 ± 0.12	1.01 ± 0.09
Ethanol + AKG	2.15 ± 0.67*	4.23 ± 1.05	1.47 ± 0.16*,†	1.35 ± 0.21*,†	3.12 ± 0.4*	4.24 ± 0.46	0.97 ± 0.04	1.61 ± 0.30*,†

Data are presented as means ± SEM, $n = 4–7$.
* Significantly different from the corresponding control group.
† Significantly different from the ethanol-fed group with $p < 0.05$ as determined by ANOVA and the *post hoc* Student Newman–Keuls test.

respectively, and did not differ from control values in females. L-SH levels in ethanol-fed flies did not differ from respective control values, but the combined diet led to 1.8-, 1.4- and 2.2-fold higher levels of L-SH in Canton S males and females, and w^{1118} females, respectively.

These results show that the diet with ethanol promotes similar changes in oxidative stress parameters in both *D. melanogaster* strains, in particular decreased high molecular mass thiols and lipid peroxide levels, with unchanged L-SH levels but higher catalase activity. Various studies have demonstrated increases in markers of ROS-promoted oxidative modification of lipids and proteins as well as alterations in the levels of endogenous antioxidants in the liver and brain of rodents that were exposed to ethanol (Adaramoye et al., 2009; Cadirci et al., 2007; Cederbaum et al., 2009). In general, variable effects of ethanol treatment were demonstrated on antioxidant enzyme activities, with reports of decrease, no effect, or increase in the activities in rodents (Adaramoye et al., 2009; Cadirci et al., 2007; Hagymási et al., 2001; Oh et al., 1998). Such variable results might be associated with induction of oxidative stress of differing intensities (Lushchak, 2014). Regarding *D. melanogaster*, ethanol exposure was shown to induce changes in antioxidant gene expression in *D. melanogaster* larvae. In particular, upregulation of catalase and superoxide dismutase genes was observed indicating a cellular response to higher levels of ROS (Logan-Garbisch et al., 2015). Catalase is known to play a dual role in the protection against ethanol toxicity: catalase reduces ethanol-associated oxidative stress due to its action as a H_2O_2-scavenging enzyme and catalase can also convert ethanol to acetaldehyde in a modified form of its reaction: $CH_3CH_2OH + H_2O_2 \rightarrow CH_3CHO + 2H_2O$ (Das & Vasudevan, 2007; Miller-Pinsler & Wells, 2015). Catalase may be responsible for up to 5% of ethanol degradation in *D. melanogaster* larvae (Geer et al., 1993). Thus, the higher catalase activity in ethanol-reared males can be considered as an adaptive mechanism that helps to neutralize toxic levels of ethanol. The difference in catalase behavior in ethanol-fed males vs. females (Fig. 5A, B) may be partly connected with a different regulation of catalase gene expression in the two genders: males express two isoforms of catalase (CG6871, a typical peroxisomal catalase, and G9314, testes-specific catalase isoform), whereas females express only one isoform (CG6871) (Faust, Verma, Peng, & McNew, 2012). Moreover, in *Drosophila*, catalase activity was found to be more changeable in males than in females, under dietary influences (Rovenko et al., 2015). The diet with ethanol + AKG did not cause substantial changes in catalase activity in adult flies as compared to flies raised on ethanol-diet; only in Canton S males was the enzyme activity slightly lower (by 15%) and thereby it did not differ from control values. Hence, in general, it seems that the protective effects of AKG were not connected with effects on catalase activity. Moreover, AKG can partly substitute for catalase function due to its ability to react nonenzymatically with H_2O_2 (Bayliak et al., 2016).

A decrease in content of free thiol groups is one of the most prominent markers of ethanol-initiated oxidative stress, and this is mostly attributed to a depletion of GSH levels (Adaramoye et al., 2009; Das & Vasudevan, 2007; Hagymási et al., 2001; Oh et al., 1998). Low molecular mass thiols are represented mainly by GSH (Lushchak, 2012), but we did not observe changes in this pool in ethanol-reared flies. Changes occurred only in the high molecular mass thiols that mainly reflect the content of free SH groups in proteins. Upon ethanol exposure, protein thiols were shown to undergo oxidation or acetylation by acetaldehyde, an intermediate of ethanol metabolism, forming inactive cross-linked proteins (Das & Vasudevan, 2007; Tyulina, Prokopieva, Boldyrev, & Johnson, 2006). In ethanol-fed flies, the SH groups in proteins, but not in glutathione, seem to be the preferential target for attack by ROS or ethanol metabolites, with significant decreases seen in H-SH content in w^{1118} and similar trends in Canton S flies. The increased L-SH level in flies reared on diet containing a mixture of AKG and ethanol suggests that these flies had a higher adaptive capacity to cope with oxidative stress than those fed on ethanol only. Given that the ability of AKG to serve as a glutamate precursor for glutathione biosynthesis has been shown previously in human erythrocytes (Lushchak, 2012; Whillier, Garcia, Chapman, Kuchel, & Raftos, 2011), we propose that the elevated AKG available from the diet could be partially used to increase glutathione biosynthesis resulting in the increased L-SH levels seen in the ethanol + AKG groups of flies in our experiments. In addition, the antioxidant action of AKG could also be involved in the protection against ethanol-induced oxidation of free thiol groups in both low- and high-molecular mass compounds.

In most cases, ethanol exposure causes an increase in lipid oxidation resulting in the accumulation of toxic end products or cross-linked adducts with other biomolecules (Cadirci et al., 2007; Cederbaum et al., 2009; Das & Vasudevan, 2007). We detected a significant decrease in LOOH levels in flies exposed to ethanol alone or in combination with AKG compared to control flies. Lipid hydroperoxides are so-called secondary products of lipid peroxidation that result from interactions of primary products of lipid peroxidation with new molecules of unsaturated fatty acids. Other oxidants such as Fe^{3+} ions are capable of initiating a new free-radical chain oxidation of LOOH. The formed peroxyl radical ($LOO^{\bullet}$) can undergo further oxidation and decomposition with formation of end products of lipid peroxidation such as malondialdehyde and 4-hydroxynonenal (Lushchak, Semchyshyn, & Lushchak, 2012). An increase in malondialdehyde levels is the mostly commonly detected indicator of lipid oxidation in ethanol-exposed animal models (Adaramoye et al., 2009; Cadirci et al., 2007; Cederbaum et al., 2009). Thus, we can propose two possible mechanisms for the lower LOOH levels found in ethanol-reared flies. First, the rate of oxidation of lipid hydroperoxides in these flies could be significantly higher than in controls that decreased their accumulation. On the other hand, the synthesis of unsaturated fatty acids could be lowered in adult flies maintained on the ethanol diet, but this mechanism is less likely to be involved due to the previously observed increase in unsaturation of fatty acids in *D. melanogaster*

larvae maintained on ethanol-containing diet (Geer et al., 1986). The addition of AKG to ethanol-containing food did not influence ethanol-induced decrease in LOOH level in adult flies. Hence, the protective effects of AKG were not realized at this stage of lipid oxidation. It was shown previously that exogenous AKG protected lipids against oxidation and reduced malondialdehyde formation *in vitro* (Puntel et al., 2005) and *in vivo* (Velvizhi et al., 2002). This ability of AKG was connected with its antioxidant properties.

In general, it can be postulated that flies reared on ethanol plus AKG-supplemented food starting from the egg stage may have induced effective mechanisms to metabolize ethanol and to protect against ethanol-induced oxidative stress, thereby minimizing its toxic effects and enhancing survival at the larval stage even though they were not fully protected against oxidative damage. The combined administration of AKG and ethanol enhances ADH activity and provides the increase in antioxidant potential.

Conclusions

The present study demonstrates that supplementation of food with alpha-ketoglutarate partly alleviates the toxic effects of ethanol in high concentrations on *D. melanogaster* development, allowing increased numbers of pupae to form. Adult flies, reared on the diet containing AKG plus ethanol, have higher ADH activity as compared with those reared on the control diet or the ethanol diet. The native electrophoresis data suggest that the ethanol + AKG diet promotes post-translational modifications of ADH protein with the formation of a highly active ADH form. This can be considered as one of the molecular mechanisms providing higher resistance of the flies to toxic effects of ethanol. It should be noted that in the absence of AKG, *D. melanogaster* flies with lower ADH activity pupated faster on the ethanol diet than flies with higher activity of this enzyme. This indicates that ADH is not the only protein responsible for ethanol resistance in *Drosophila*. As expected, the ethanol-containing diet caused a significant increase in TAG levels in adult flies, which was not improved by the presence of AKG in the diet. Fly rearing on the diet with ethanol plus AKG resulted in higher catalase activity in males and higher levels of low molecular mass thiols in both sexes, as compared with controls. At the same time, ethanol-fed flies had lower L-SH levels than control flies. This suggests that AKG can improve redox status of flies under ethanol exposure and thereby can partly prevent development of ethanol-induced oxidative stress. Thus, it can be concluded that the protective action of AKG against ethanol toxicity is realized through stimulating both ADH and antioxidant activities.

Abusive ethanol consumption causes serious problems in modern society. Our results support the promising utilization of AKG as a treatment to neutralize short-term and long-term effects of consumption of ethanol and alcohol drinks. In turn, the fruit fly *D. melanogaster* is a useful model for *in vivo* studies of the adverse effects of different toxicants and to analyze potential protective antidotes. Particularly given that ethanol can cause deleterious changes in the nervous system, especially at the embryonic stage, studies using antidotes like AKG and *Drosophila* as the model may give insights into which neurobehavioral and biochemical changes appear during development, with age or with long-term poisoning, and allow determination of the efficiency of AKG in the prevention of these perturbations.

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