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The Influence of Adh Function on Ethanol Preference and Tolerance in Adult *Drosophila melanogaster*

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Abstract

Preference determines behavioral choices such as choosing among food sources and mates. One preference-affecting chemical is ethanol, which guides insects to fermenting fruits or leaves. Here, we show that adult *Drosophila melanogaster* prefer food containing up to 5% ethanol over food without ethanol and avoid food with high levels (23%) of ethanol. Although female and male flies behaved differently at ethanol-containing food sources, there was no sexual dimorphism in the preference for food containing modest ethanol levels. We also investigated whether *Drosophila* preference, sensitivity and tolerance to ethanol was related to the activity of alcohol dehydrogenase (Adh), the primary ethanol-metabolizing enzyme in *D. melanogaster*. Impaired Adh function reduced ethanol preference in both *D. melanogaster* and a related species, *D. sechellia*. Adh-impaired flies also displayed reduced aversion to high ethanol concentrations, increased sensitivity to the effects of ethanol on postural control, and negative tolerance/sensitization (i.e., a reduction of the increased resistance to ethanol's effects that normally occurs upon repeated exposure). These data strongly indicate a linkage between ethanol-induced behavior and ethanol metabolism in adult fruit flies: Adh deficiency resulted in reduced preference to low ethanol concentrations and reduced aversion to high ones, despite recovery from ethanol being strongly impaired.

Key words: alcohol dehydrogenase, *Drosophila melanogaster*, *Drosophila sechellia*, ethanol preference, ethanol tolerance

Introduction

Ethanol is typically present in nature at very low concentrations. Very small amounts are present in most organisms as a metabolic by-product (Holmes 1994). Higher concentrations (up to 5%) are found in fleshy fruits (Gibson and Oakeshott 1981; Dudley 2002). Animals often show preference for ethanol-enriched food sources. For examples, nymphalid butterflies are attracted to fermenting fruits, whereas ambrosia beetles are attracted to ethanol-containing volatiles from trees (Hill et al. 2001, Ranger et al. 2010). It is thought that in this context, the odor ethanol functions primarily as long-range signal to localize a transient food source (Dierks and Fischer 2008). Adult fruit fly *Drosophila melanogaster* shows a preference to lay their eggs on ethanol-containing media (McKenzie and Parsons 1972). Therefore, ethanol-containing media is the breeding ground for *D. melanogaster* larvae. The preference for ethanol of *D. melanogaster* larvae is dose dependent and is not influenced by changes of the ethanol metabolism, for example, alcohol dehydrogenase (Adh) function. In contrast, ethanol aversion to concentration greater than 10% is negatively linked to Adh function (Gelfand and McDonald 1980; 1983). However, the behavioral preference for food sources of larval and adult *Drosophila* of different species differs (Cooper 1960). To test whether there is a linkage between adult ethanol preference and larval preference, the preference of adult flies has to be analyzed. The odor preference for pure ethanol odors of adult flies has been tested and shows a dose dependence as well (Hoffmann and Parsons 1984). However, pure odors are rarely found in nature. Therefore, we extended our analysis to test whether adult flies show preference for odor mixtures containing food odors and ethanol.

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Adult animals exposed to sufficient ethanol show intoxication. In fruit flies, intoxication causes initial hyperactivity followed by the flies becoming increasingly uncoordinated and sedated (Scholz et al. 2000; Singh and Heberlein 2000; Wolf et al. 2002). As brain and hemolymph ethanol concentrations increase, the flies lose their ability to control posture when challenged (Hoffman and Cohan 1987; Moore et al. 1998), and after long exposure lose control over walking and flying movements (Grell et al. 1968). Inebriated adult flies also show an increase of courtship activity (Lee et al. 2008).

Animals have evolved multiple mechanisms to counteract ethanol's effects. First, ethanol is degraded by Adh, an enzyme present in almost all animals (Holmes 1994). In *D. melanogaster* larvae, 90% of ethanol degradation is due to Adh (Geer et al. 1985), with the rest being metabolized by alternative enzymes such as catalases (Geer et al. 1993). The functional importance of the Adh pathway is well demonstrated by the reduced larval-to-adult survival of *Adh*-null larvae reared on ethanol-containing media (Heinstra et al. 1987). Second, upon repeated exposure, most animals develop tolerance to ethanol as manifested by reduced behavioral changes for equal ethanol consumption. In fruit flies, tolerance results in reduced effects of ethanol on postural control and sedation and depends both on neuronal adaptation and more efficient ethanol metabolism (Scholz et al. 2000). Ethanol tolerance, by whatever mechanism, should reduce ethanol's intoxicating effects and thus help organisms to survive after ethanol ingestion. The importance of metabolic pathways, particularly Adh, in this process, and in modifying ethanol's effects in general, however, is not well understood. For instance, although larvae with reduced Adh activity survive less well on ethanol-containing food, reduced Adh activity does not strongly reduce the preference of adult flies to oviposit on ethanol-containing medium (Siegal and Hartl 1999). In addition, the preference for ethanol-containing food odors of adult flies with impaired or altered Adh function has not been analyzed.

To resolve this issue, we have investigated a variety of responses to ethanol-containing food odor of fruit flies with and without Adh. Our logic was that, if Adh affected ethanol responses, mutant flies should show reduced preference to ethanol, increased behavioral responses to it, and aversive behavior at lower ethanol concentrations. We therefore analyzed ethanol preference, sensitivity, and tolerance of adult *D. melanogaster* control and Adh-null flies and, to address issues of genetic variability, flies of *D. sechellia*, a sister species of *D. melanogaster* that has naturally low Adh levels. We used an odor trap assay to measure ethanol preference. Flies of both species showed preference to low ethanol concentration and aversion to high ethanol concentration. Ethanol preference correlated positively with Adh activity and ethanol aversion negatively, with Adh-null flies showing no preference (i.e., chose equally between ethanol-free and ethanol-containing food sources). We measured sensitivity and tolerance to ethanol's effects on postural control with an inebriometer (Hoffman and Cohan 1987; Scholz et al. 2000). This assay has been used to select flies over generations to become more resistant to a single ethanol exposure. The increase in resistance correlated with an increase of the Adh^s allele frequency, an allele with reduced Adh activity (Hoffman and Cohan 1987; Garrido and Barbancho 1990). Here, we extend our analysis to plastic behavioral changes of adult flies after an initial ethanol exposure in relationship to Adh function independent of selection. We show that impaired Adh activity correlated with increased ethanol sensitivity and negative tolerance/sensitization. These data show that Adh activity influences preference, sensitivity, and tolerance to ethanol-containing food odors in adult flies.

Material and methods

Experimental animals

Flies of the stock *PZ[+]* (Moore et al. 1998) and Canton S flies were used for initial characterization of ethanol preference. The *PZ[+]* stock carries a PZ[ry^+] transposable element insertion and is used as the control and for calibration for the inebriometer assay (Moore et al. 1998; Scholz et al. 2000).

Flies carrying the spontaneous mutation w^{1118} (Hazelrigg et al. 1984) were obtained from the Heberlein lab. Spontaneously occurring Adh^F alleles with relatively high Adh activity are found in natural populations (Lewis and Gibson 1978). EMS-induced homozygote Adh^{n1} flies do not contain detectable Adh activity (Grell et al. 1968). Both stocks were obtained from the Bloomington stock center. *Drosophila sechellia* 0248.7 was kindly provided by Teun Dekker.

Fly stocks and genetics

Flies were raised on standard cornmeal agar without live yeast at 25 °C under a 16:8 h light:dark cycle at 60–70% relative humidity. For behavioral experiments, 20–30 virgin females were crossed to 15–25 males. After hatching 80 one-to two-day-old male or female flies were collected under CO_2 anesthesia and kept for 2 days at 25 °C for recovery from CO_2 treatment. Flies were not starved prior to the experiment. For most experiments, only male flies were used due to their simpler handling.

Preference assay

Flies were tested for odor preference in a modified trap assay as described by Larsson et al. (2004) (Figure 1). A 1000-mL glass beaker was covered with an exactly fitting plexiglass lid that contained three 3.5 cm diameter holes. Two holes were covered with gauze to allow air exchange, and the third was covered with a foam plug and used to put flies into the beaker. In the beaker, 2 medium-size plastic containers (Greiner

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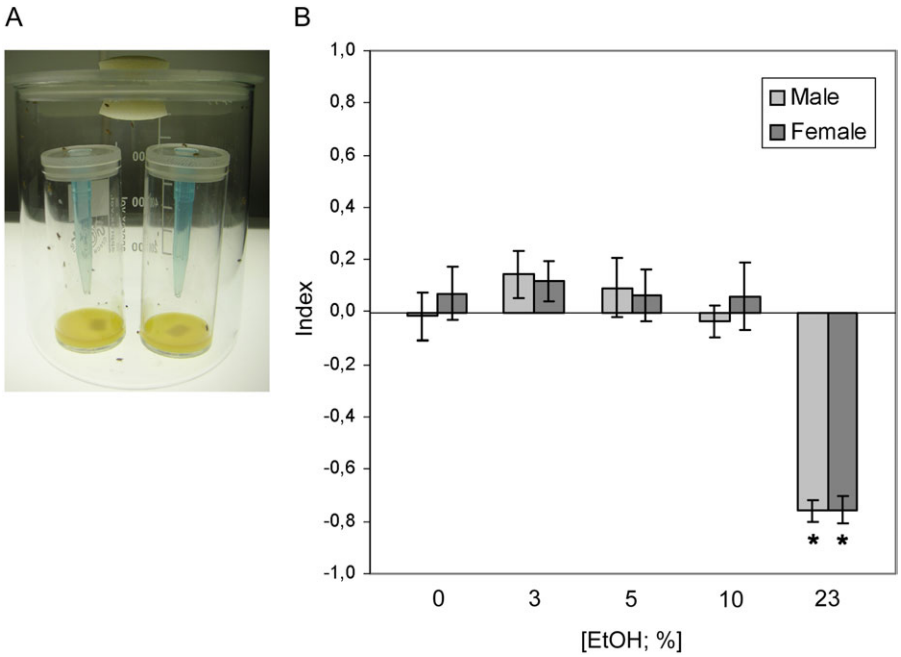


Figure 1 *Drosophila melanogaster* show dose-dependent ethanol preference. **(A)** Eighty flies were inserted into a beaker with 2 trap assays filled with mango–apple juice alone or mango–apple juice with varying ethanol concentrations. The test system was placed onto a light box and flies could chose between the 2 vials for 16 h. The number of flies per vial was counted, and the preference index was calculated (see Materials and methods). An index greater than zero indicates preference, less than zero aversion, and zero no preference. **(B)** Preferences of female and male *D. melanogaster* at 3%, 5%, 10%, and 23% ethanol concentrations. Females and males show significant aversion at 23% (one-sample sign test; $P < 0.0001$ for difference from zero; analysis of variance for difference of preference between different experimental conditions (23% vs. all other concentrations) marked with asterisk; $P < 0.05$).

bio-one tubes, 68 mL volume; normally used for fly maintenance) were filled with 1 mL apple–mango juice (Alnatura). For all experiments, we used apple–mango juice (see Results). The vials were covered with a plexiglass lid that contained a pipette tip (MultiFit pipette tips no. 81-10020 from peqlab). The tip of the pipette tip was cut to increase the size of the opening so that only one fly at a time could climb into the vial. The beaker filled with flies was set on a cold light source in a 25 °C incubator with 60% humidity for 16 h overnight. To determine the time points of entry into the vial and to observe whether flies would leave the vials, we filmed the flies for 16 h (data not shown). After 5 h, the first flies started to enter the vials, and once flies had entered a vial they did not leave it within 16 h of the experiment. The next day, the flies in each vial were counted. Experiments were evaluated only if at least 70 of the 80 flies had entered the 2 vials. The preference index equaled the number of flies that were in the ethanol- and juice-containing vial, minus the number of flies in the juice-only vial, divided by the number of flies in both vials. Values greater than zero indicate preference for ethanol-containing liquids and values less than zero indicate aversion. If flies did not favor one odorant stimulus over the other, they would randomly distribute to both vials and the preference index would be zero. We repeated the same experiment with different sets of flies the number of times indicated by *N*.

Ethanol tolerance

Three- to four-day-old male flies were tested for ethanol tolerance development in the inebriometer as previously described (Scholz et al. 2000) (Figure 4A); in each experiment 100 flies were used. The assay consisted of a 122-cm long column filled with a mixture of 50:45 ethanol-saturated air:water-saturated air. Flies were inserted into the top of the column. Sober flies stayed there. Intoxicated flies exhibited increased locomotor activity followed by loss of postural control, at which time they fall through the column. The number of flies at the bottom of the column was counted by passing a light beam. The number of flies eluting from the columns was quantified by determining the mean elution time (MET) the population spent in the column (Figure 3B). The initial exposure to ethanol measured the sensitivity of the population to ethanol. To measure tolerance, flies were collected and allowed to recover from ethanol treatment at 25 °C in a humidified environment. After 4 h, wild-type flies normally have metabolized all absorbed ethanol and were reintroduced into the column (Scholz et al. 2000). On second exposure, wild-type flies were more resistant to the effect of ethanol on postural control. The degree of tolerance development was described as percent tolerance and calculated as $100 \times ([MET2 - MET1]/MET1)$.

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Statistical analysis

All data were normally distributed. In all figures, error bars are standard errors of the mean. To evaluate differences between 2 experimental groups, we used paired Student's *t*-test. Whether the choice of the flies for a given vial was random was determined by testing whether the preference score differed from zero with a one-sample sign test. A full-factorial analysis of variance was used to assess differences between more than 2 experimental groups. For post hoc comparison, Tukey honest significant difference tests were used. All statistical analyses were done in STATISTICA for Windows (Version 7.1.; www.statsoft.com).

Results

Ethanol preference in adult flies

Female *D. melanogaster* flies prefer to lay their eggs on ethanol-containing media (Richmond and Gerking 1979). However, it is unknown how attractive different ethanol concentrations are for adult flies and whether males and females show equal preferences. To analyze ethanol preference, flies were offered 2 vials, one containing only apple-mango juice and the other apple-mango juice with 0%, 5%, 10%, and 23% ethanol. A trap assay was used to prevent flies from climbing out of a chosen vial (Figure 1A). We used apple–mango juice because plain odors are not normally present in nature, blends of odors are more attractive than single odors, and mango is highly attractive (Zhu et al. 2003). We choose our ethanol concentrations because olfactometer experiments have suggested that the attraction of pure ethanol vapor increases linearly from a threshold of 0.25% to a maximum response at 8% (Fuyama 1976) with aversive responses beginning at 10% (Hoffmann and Parsons 1984). *PZ[+]*-control flies showed a dose-dependent preference for ethanol-containing solutions with solutions containing 3% and 5% ethanol tending to be attractive (although this tendency did not reach statistical significance) and solutions containing 23% being significantly aversive (Figure 1B). Male and female flies did not differ in ethanol preference. *Drosophila melanogaster* thus prefers low levels of ethanol vapor and shows no sexual dimorphism for this behavior at this level of resolution. We used only male flies in subsequent experiments.

Genetic factors alter ethanol aversion

To analyze the effect of different genetic backgrounds on ethanol preference, we repeated the same experiments with Canton S, *PZ[+]*, and $white^{1118}$ flies (Figure 2). Canton S flies showed significant preference for 5% ethanol and significant aversion for 23% ethanol (Figure 2A). $white^{1118}$ flies behaved similarly (data not shown). To directly compare the preferences of control, *PZ[+]*, and $white^{1118}$ flies and to analyze ethanol preference at higher resolution, the experiments for 5% and 23% ethanol were repeated (Figure 2B,C). All strains showed a significant and equal preference (0.23 ± 0.08 for wild type (wt); 0.30 ± 0.08 for *PZ[+]*; 0.27 ± 0.07 for $white^{1118}$) for 5% ethanol (Figure 2B). However, they significantly differed in aversion to 23% ethanol (–0.88 ± 0.03 for wt and 0.8 ± 0.03 for *PZ[+]* vs. –0.065 ± 0.07 for $white^{1118}$, $P < 0.05$; Figure 2C). Canton S did not differ in their ethanol preference from *PZ[+]* flies (Student's *t*-test with $P > 0.05$) and therefore we used *PZ[+]*as controls in subsequent experiments. Flies of all genetic lines showed preference to

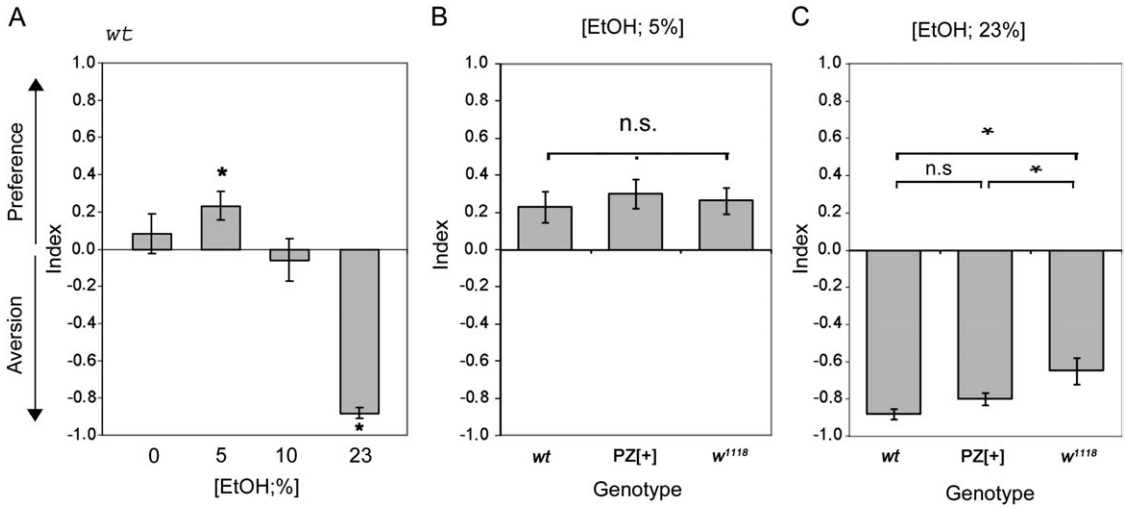


Figure 2 Genetic factors influence preference. **(A)** Wild-type Canton S flies were tested for their preference of ethanol-containing solutions. Asterisks show significant differences from zero after One-sample sign test ($N = 12–15$ per condition; $P < 0.05$). **(B)** Wild-type flies *PZ[+]* and w^{1118} significantly preferred 5% ethanol (one-sample sign test; $P < 0.05$; $N = 17$ for the control, $N = 26$ for *PZ[+]* and $N = 23$ for w^{1118}). The degree of preference did not differ between strains. **(C)** Wild-type, *PZ[+]* and w^{1118} flies showed significant aversion for 23% ethanol (one-sample sign test; $P < 0.001$; $N = 18$ for the control, $N = 20$ for *PZ [+]* and 15 for w^{1118}). The degree of aversion differed significantly (unpaired Student's *t*-test with equal variance, $P < 0.05$; asterisk).

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low ethanol concentration and aversion to high concentration. Because *white*^{1118} flies show reduced aversion, we conclude that the aversion can be modified by genetic factors.

Influence of Adh function on ethanol preference

To investigate the influence of Adh function on ethanol preference, we tested mutants with altered Adh activity for preference to 5% and 23% ethanol (Figure 3). Homozygote Adh^{n1} flies with a null Adh allele showed no preference for 5% ethanol (Figure 3A). This behavior clearly differed from homozygote Adh^{F} flies but not from heterozygote Adh^{n1}/Adh^{F} flies, in which Adh activity is only reduced (Adh^{F}, 0.25 ± 0.05; Adh^{n1}/Adh^{F}, 0.22 ± 0.06; Adh^{n1}, 0.07 ± 0.04; $P < 0.05$; Figure 3A). Homozygote Adh^{n1} flies showed a reduction of around 73% of aversion toward 23% ethanol compared with homozygote Adh^{F} flies and heterozygote Adh^{n1}/Adh^{F} flies (Adh^{F}, –0.79 ± 0.06; Adh^{n1}/Adh^{F}, –0.77 ± 0.07; Adh^{n1}/Adh^{n1}, –0.21 ± 0.07; Figure 3B). Heterozygote Adh^{n1}/Adh^{F} flies showed similar behavior as homozygote Adh^{F} flies toward low concentrations of ethanol (5%), suggesting that reducing Adh activity has no influence on ethanol preference. However, complete loss of Adh function correlated with both reduced preference and reduced aversion. The reduced aversion to high ethanol concentration of flies with impaired Adh function is surprising because impaired Adh function should decrease survival of offspring raised on ethanol-containing food.

Influence of Adh function on ethanol tolerance

Animals can generally develop both chronic (induced by prolonged exposure to low ethanol concentrations) and acute (defined by the animals appearing more intoxicated at a given ethanol concentration on the rising part of the blood alcohol curve than at the same concentration on the descending part of the curve) tolerances to ethanol (Kalant et al. 1971). Flies can also develop both chronic tolerance to ethanol (Berger et al. 2004) and a rapidly developing tolerance demonstrated by increased resistance to intoxication upon subsequent exposure to ethanol after an initial ethanol intoxication (although in this case, after 4 h the flies had completely metabolized the ethanol from the initial exposure) (Scholz et al. 2000). In *Adh* mutants, the defect in ethanol metabolism leads to a prolonged presence of ethanol in the animal. To analyze the consequences of this defect on ethanol-induced behavior, we tested flies with altered Adh activity for ethanol sensitivity and tolerance.

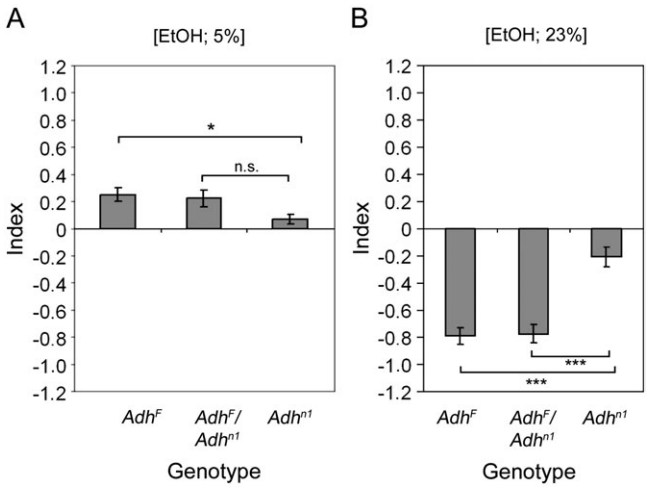


Figure 3 *Adh* mutants showed differences in ethanol preference. **(A)** Adh^{n1} flies differed significantly from Adh^{F}, but not Adh^{n1}/Adh^{F}, flies in their preference for 5% ethanol (N = 32–36 per condition; $P < 0.05$). **(B)** Adh^{n1} flies differed significantly from Adh^{F} and Adh^{n1}/Adh^{F} flies in their preference for 23% ethanol (N = 12–15 per condition; $P < 0.001$).

The effect of ethanol on fly postural control was measured in an inebriometer (Hoffman and Cohan 1987; Figure 4A). In brief (for a complete description, see Materials and methods), this device works as follows: flies are inserted into a column perfused with a constant mixture of ethanol and air. Intoxicated flies fall to the bottom of the column where they are counted by crossing a light barrier. The average time spent in the column (MET) defines the sensitivity of the flies to the effects of ethanol on postural control (Figure 4B). After a 4 h recovery period control, flies are sober again and when reexposed to ethanol are more resistant to its effects (i.e., have developed tolerance) (Figure 4B; Scholz et al. 2000). Homozygous Adh^{F} flies (normal Adh activity) were used as controls (Figure 4C). Heterozygous Adh^{n1}/Adh^{F} (reduced Adh function) and homozygous Adh^{n1} (no Adh activity) flies were tested for ethanol sensitivity and tolerance (Figure 4C,D). Adh^{n1} flies were significantly more sensitive to ethanol than Adh^{n1}/Adh^{F} or Adh^{F} flies (Adh^{n1}, 22.4 ± 0.6 min; Adh^{n1}/Adh^{F}, 26.8 ± 0.6 min; Adh^{F}/Adh^{F}, 25.5 ± 1 min; $N = 8$; $P < 0.05$). Adh^{n1}/Adh^{F} flies did not differ from Adh^{F} flies (Figure 4C). Control and Adh^{n1}/Adh^{F} flies developed comparable levels of ethanol tolerance. Adh^{n1} flies, alternatively, did not develop tolerance but instead showed increased sensitivity to ethanol (i.e., developed negative tolerance) (Adh^{F}/Adh^{F}, 19.7 ± 0.8%, Adh^{n1}/Adh^{F}, 12.3 ± 1.6%; Adh^{n1}, –88.2 ± 6.3%; $N = 8$; $P < 0.001$) (Figure 4D). These results show that reducing Adh activity does not change immediate responses to ethanol or development of ethanol tolerance but complete loss of Adh activity does. The failure to develop tolerance is consistent with the idea that these flies do not recover from the initial ethanol exposure and still have considerable amounts of ethanol in their bodies.

Ethanol preference in *D. sechellia*

To investigate possible effects of genetic background on ethanol responses, we measured ethanol responses in *D. sechellia*, a species genetically very similar to *D. melanogaster*, but in which Adh activity is reduced by about 80% (Mercot 1994; Lachaise and Silvain 2004). We first showed that male and

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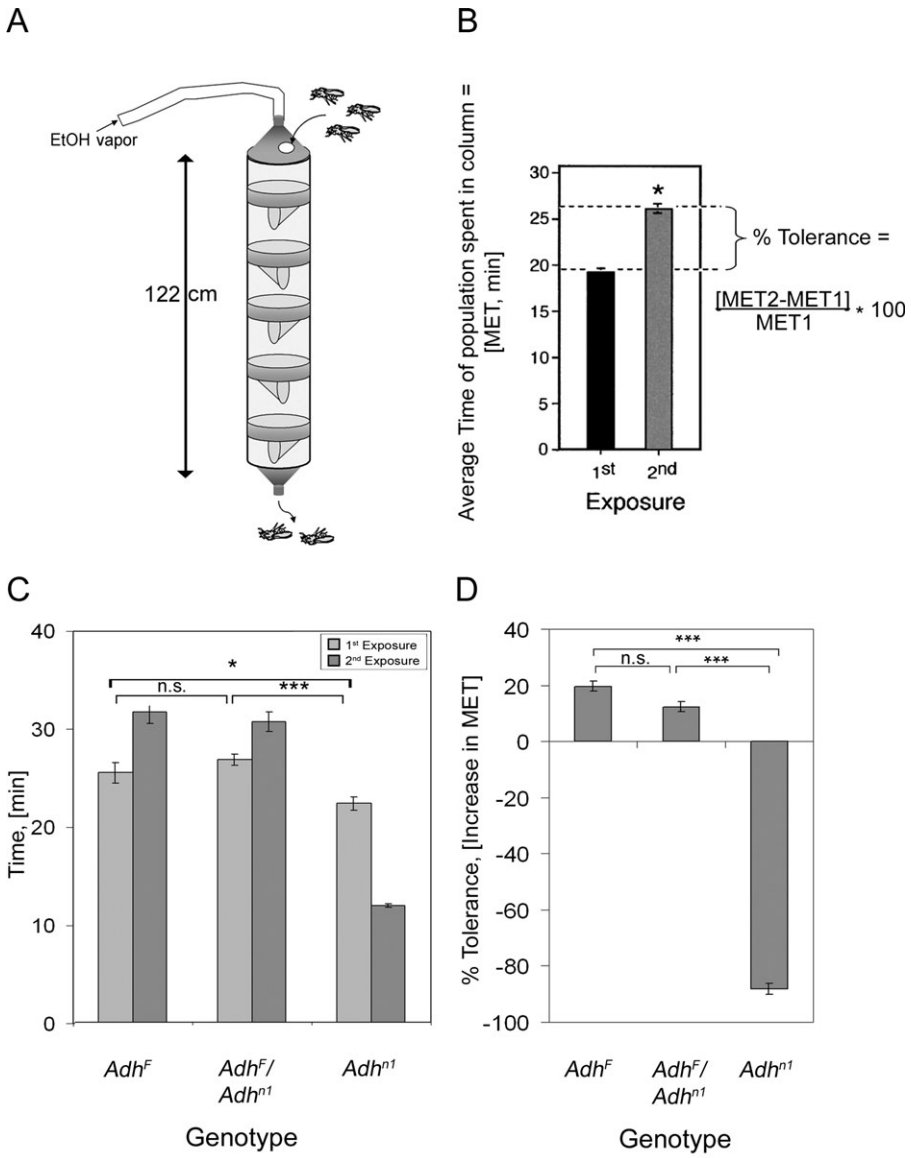


Figure 4 *Adh* mutants had altered ethanol tolerance. **(A)** Hundred flies were inserted into the top of a column filled with a constant mixture of ethanol and humidified air. Intoxicated flies lose their postural control and then elute (fall) from the column (Scholz et al. 2000). **(B)** To quantify this behavior, the mean time the flies spent in the column (MET) is determined. MET1 (black column) is the behavior of the population at the initial ethanol exposure, MET2 (gray column) the behavior at a second exposure 4 h later. The relative difference between MET2 and MET1 defines the amount of tolerance that developed during this period. In **(C)** and **(D)**, light gray bars are MET1 and dark gray bars are MET2. Homozygous Adh^{n1} mutant flies were significantly more sensitive to ethanol than Adh^{n1}/Adh^{F} and Adh^{F} flies (C) and developed negative tolerance (i.e., became more sensitive to ethanol on subsequent exposure) (D). $N = 8$. One asterisk indicates P values <0.05 and 3 asterisks <0.001.

female *D. sechellia* had a dose-dependent ethanol preference. Because no difference between males and females was observed, we only used males in subsequent experiments. *Drosophila sechellia* differed from *D. melanogaster* in several ways: it did not prefer 5% ethanol (Figure 5A), showed significantly reduced (about 51%) aversion to high ethanol concentrations (Figure 5B), was significantly more sensitive to ethanol (*PZ(+)*, 22.9 ± 1.1 min; *D. sechellia*, 16.9 ± 0.6 min; $N = 8$; $P < 0.001$; Figure 5C), and showed negative tolerance (*PZ(+)*; 16.1 ± 1.9%; *D. sechellia*, –17 ± 4.2; $N = 8$; $P < 0.001$; Figure 5D). Interestingly, the reduced aversion still differed from random choice suggesting that they still can sense higher ethanol concentration (One-sample sign test; $P < 0.0001$). These phenotypic differences are all similar to those observed in Adh^{n1} flies. Taken together, these results suggest that loss of Adh function correlates with reduced aversion to high ethanol concentrations, increased sensitivity to ethanol, and negative tolerance in adult flies.

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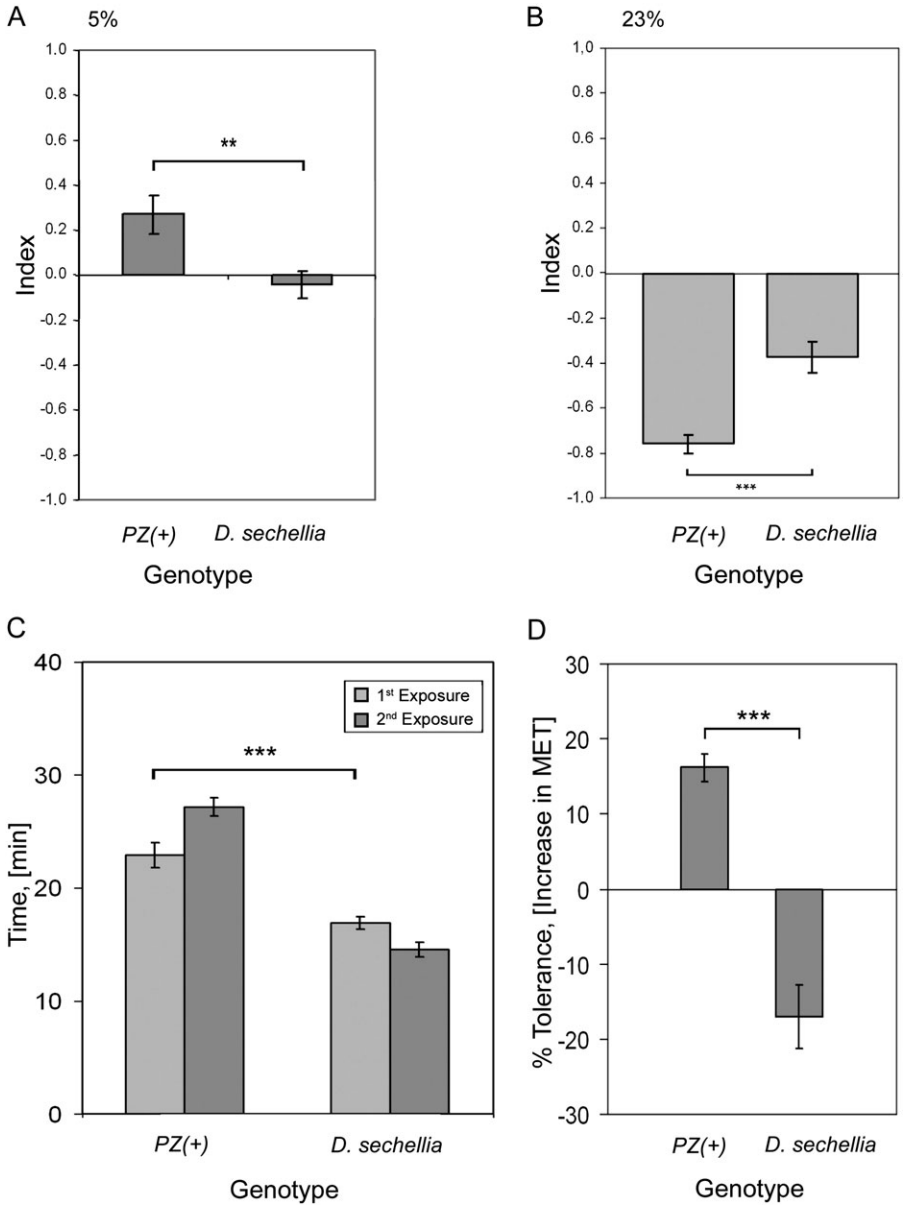


Figure 5 *Drosophila sechellia* showed reduced ethanol aversion and increased negative ethanol tolerance. **(A)** Male *D. melanogaster* flies preferred 5% ethanol but male *D. sechellia* flies did not ($N = 21–22$; $P < 0.01$). **(B)** Aversion to 23% ethanol was significantly reduced in comparison with the control strain ($N = 22–24$; $P < 0.001$). **(C)** *Drosophila sechellia* showed a significantly increased first MET (light gray bars) and reduced second MET (dark gray bars) when measured in the inebriometer. **(D)** In addition, their ability to develop ethanol tolerance was significantly impaired ($N = 8$). Asterisks mark $P < 0.001$.

Discussion

Adult *D. melanogaster* flies showed a dose-dependent ethanol preference, preferring apple-mango juice containing up to 5% ethanol. It has been reported previously that flies prefer up to 16% pure ethanol vapor (Fuyama 1976; Hoffmann and Parsons 1984). We could not detect preference above 5% ethanol, suggesting that perception of pure ethanol vapor and an odor mixture containing ethanol differs. The concentration causing preference in our experiments is, however, similar to concentrations found in nature (Gibson and Oakeshott 1981), suggesting our data are relevant to the behavior of animals in natural environments. Male and female flies showed identical preferences. On one level this is not surprising because for both sexes ethanol is an olfactory cue to localize a transient food source and, because it attracts other adult flies, a place to mate (Dudley 2002; Reaume and Sokolowski 2006). However, because only for female flies ethanol indicate an oviposition site (McKenzie and Parsons 1972), more detailed analysis may reveal subtle gender-based differences.

A possible concern with these data is that they are due to history or learning during the odor trap assay. However,

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examination of the films of the flies during the 16 h choice period showed that no flies entered and then left a vial to enter the other. The flies thus had no chance to compare the contents of the 2 vials. Our data are thus most likely due to inherent olfactory preferences about food sources containing varying ethanol concentrations.

The variability of the ethanol preference was relatively high and only around 55% of flies chose ethanol-containing solutions. This variability and low preference would enable a fly to continue to respond to other environmental stimuli and suggests that low ethanol concentrations modulate already present responses to odors. In contrast, high ethanol-containing vapors caused strong (about 95% of animals avoided 23% ethanol) aversion with low variance in *D. melanogaster*. This suggests that high ethanol concentrations may be directly perceived as putative danger stimuli as opposed to acting by modulating other already present odor preferences.

The dose dependency of ethanol preference and aversion suggests that ethanol may be perceived and/or evaluated differently at different concentrations, that is, that low ethanol concentrations are perceived as a different sensual modality than high concentration. The olfactory system can distinguish between odor quality and intensity, and intensity can be mediated by olfactory receptor-independent mechanisms (DasGupta and Waddell 2008). That aversion and preference could be under different genetic control is supported by our finding that w^{1118} mutants showed reduced aversive behavior while preference was unaltered. This is further supported by the finding that the attraction to low concentration of vinegar is mediated by different olfactory neurons than the aversion to high concentrations (Semmelhack and Wang 2009). Differential control of odor quality and intensity could explain why different odor intensities form separate memory traces in *D. melanogaster* (Masek and Heisenberg 2008). It is also, of course, possible that this behavioral separation is mediated via independent sensory mechanisms; for instance, low odor concentration may be mainly perceived by the olfactory system, whereas high levels are sensed by the tracheal system. The tracheal system normally regulates very precisely the minimizing of water loss and respiratory gas exchange (Lehmann 2001).

In *D. melanogaster*, reducing Adh function by 50% did not alter ethanol preference, but flies with a complete loss of Adh chose randomly between a complex odor with and without 5% ethanol. These data are consistent with either the flies being unable to distinguish between the 2 odor mixtures or being able to prefer the 2 mixtures equally. Fifty percent of the Adh-null flies still choose by chance ethanol-containing media even though they are unable to detoxify ethanol by Adh. In Adh-minus flies, small (3–5%) amounts of ethanol are likely metabolized by alternative pathways, for example, catalases (Geer et al. 1993), and thus loss of preference does not require complete abolition of ethanol metabolism. A lower bound for how much Adh-mediated ethanol metabolism is required to maintain preference is provided by the *D. sechellia* data, in which a reduction of around 80% of Adh was associated with loss of preference. However, oviposition preference for ethanol-containing medium is still present even in Adh-null *D. melanogaster* (Joseph et al. 2009), data also supported by detailed analysis of *D. melanogaster* strains with interspecific *Adh* gene transfer showing that flies with reduced Adh activity continue to prefer oviposition on ethanol-containing media (Siegal and Hartl 1999). Taken together, these data suggest that flies with reduced Adh activity are capable of sensing ethanol vapor, and hence, the lack of preference for ethanol-containing food sources in Adh-null flies is due to the flies equally preferring the 2 food sources.

In addition to altered ethanol preference, flies without Adh activity, or with reduced Adh activity in case of *D. sechellia*, showed reduced aversion to high ethanol concentrations. This means that ethanol is not evaluated/perceived as negatively as in control strains. This could be due to at least 2 reasons. First, adult and larvae of *Adh*-null mutants might never encountered ethanol in their environment and do not evaluate it as a negative stimulus. Alternatively, it could be that it is not ethanol but instead acetaldehyde (the principle metabolic product of ethanol by Adh) that is aversive in control flies and larvae, a hypothesis consistent with studies in rats suggesting that changes of monoamine levels in the nucleus accumbens caused by acetaldehyde increases are negatively reinforcing (Ward et al. 1997). In this case, the failure of high ethanol levels to be aversive for *Adh*-null flies would be due to acetaldehyde never reaching high levels in them during development or previous encounters with ethanol-containing food sources.

Our data also showed that altered Adh levels affect fly responses to ethanol exposure. In control flies, ethanol exposure for 20 min results in multiple behavior changes including loss of postural control (Moore et al. 1998), an initial induction of locomotor activity, and a subsequent reduction of locomotion (Scholz et al. 2000; Wolf et al. 2002). *Adh*-mutant *D. melanogaster* and *D. sechellia* with reduced Adh function showed increased ethanol sensitivity. In *Adh* mutants more ethanol is found 5 min after the start of ethanol exposure than in wild-type flies (Wolf et al. 2002). Thus, the increased sensitivity of Adh mutants presumably is due to higher levels of ethanol in their hemolymph.

Altered Adh levels also affect fly responses to repeated ethanol exposure. Control files metabolize ingested ethanol and regain control behaviors within 4 h (Scholz et al. 2000). Experiments measuring ethanol concentrations show that in *Adh* mutants ethanol levels remain high (data not shown), and thus the remaining ethanol-metabolizing pathways cannot compensate for the loss of Adh. However, even these flies show recovery of at least locomotion in 4 h, presumably due to acquisition of acute tolerance. The mutant flies nonetheless show a pronounced increased sensitivity to subsequent exposure to ethanol. These data show that ethanol metabolism influences ethanol long-term tolerance in the fruit fly. In combination with the unchanged

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preference of the mutant flies for ethanol but the increased sensitivity and decreased development of tolerance, these data suggest that Adh mutants should have a strongly reduced chance of survival in nature compared to control flies.

These observations raise interesting questions about the role of Adh in evolution. In nature, the presence of ethanol is correlated with sugar, and thus such food sources would likely be calorie rich. However, as food continues to ferment ethanol levels reach toxic levels. Control *D. melanogaster* larvae have relatively high Adh levels, are thus able to continue to take advantage of these food sources at even high ethanol levels, and show increased survival on ethanol-containing food sources. Adh-null larvae lack this advantage (indeed, show reduced survival on ethanol-containing medium, Heinstra et al. 1987), which may explain why in natural populations flies carrying *Adh*-null alleles (whose larvae can only metabolize ethanol using relatively inefficient alternative pathways) are present only at low frequencies (Voelker et al. 1980). It is thus striking that, despite the decreased-larval resistance to ethanol, *Adh*-mutant females show an increased preference to oviposit on ethanol-containing food.

In summary, we have shown that in fruit flies, ethanol metabolism influences preference, sensitivity and tolerance to ethanol. Aversion to high ethanol concentrations correlated negatively with Adh function, which would reduce offspring survival by promoting egg laying on high ethanol-containing media. Given the wide intra- and inter-species variation in Adh, Adh levels are not determined only by the effects of ethanol on individual offspring survival.

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