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Effects of a low dose of ethanol on mating success of *Drosophila melanogaster* males: implications for the evolution of ethanol resistance?

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Abstract

Ethanol occurs naturally in the decaying fruit in which many species of *Drosophila* (Diptera: Drosophilidae) breed, potentially generating selection for resistance to its toxic and sedating effects. Studies measuring mortality of flies exposed to a range of ethanol concentrations have shown that within *Drosophila melanogaster* Meigen, populations from temperate regions are more ethanol resistant than ancestral tropical African populations. The high ethanol resistance of temperate *D. melanogaster* presents a puzzle, however, because breeding and feeding sites in the wild seldom contain enough ethanol to kill even more ethanol-sensitive Afrotropical genotypes. We hypothesize that the ethanol concentrations encountered by temperate populations, though usually sub-lethal, are nonetheless high enough to reduce fitness in other ways, potentially generating indirect selection for genotypes that can survive exposure to unnaturally high ethanol concentrations. As a first step in testing this hypothesis, we compared the effects of a sub-lethal dose of ethanol, comparable to that obtainable from fermenting fruit, on the mating success of males from one European and one Afrotropical population. Ethanol significantly reduced mating success of males from the Afrotropical population, but had no effect on that of males from the European population. We also show that when flies are placed on medium with a realistic concentration of ethanol, considerably more ethanol is absorbed through vapor than through feeding, suggesting that courting males may be unable to avoid being exposed to ethanol. We hypothesize that the higher resistance of temperate populations to being killed by high, unnatural ethanol concentrations may have evolved in part as a correlated response to selection for behavioral insensitivity to natural concentrations.

Abbreviated abstract

Ethanol occurs in decaying fruit on which *Drosophila melanogaster* (Diptera: Drosophilidae) feed, but are fruit ethanol concentrations high enough to adversely affect fitness? A sub-lethal dose of ethanol, comparable to one flies could obtain from fruit, reduced mating success of males from a more ethanol-sensitive tropical population, but had no effect on that of males from a relatively ethanol-resistant temperate population. This higher resistance may have evolved in part as a correlated response to selection for behavioral insensitivity to natural concentrations.

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Keywords

adaptation; courtship; genetic variation; geographic variation; sexual selection; sub-lethal effects; toxin resistance; Diptera; Drosophilidae

Introduction

Animals that feed on fermenting fruit or nectar can be exposed to physiologically significant concentrations of ethanol (Gibson et al., 1981; Fitzgerald et al., 1990; Wiens et al., 2008), potentially generating selection to adapt to ethanol's toxic and sedating effects. This has apparently happened in the genus *Drosophila* (Diptera: Drosophilidae), in which fruit-breeding species are more resistant to ethanol-induced mortality, and have higher constitutive activity of the enzyme alcohol dehydrogenase, than species that do not normally breed in fruit (Merçot et al., 1994). Adaptation to ethanol has gone to an extreme in *Drosophila melanogaster* Meigen, particularly in populations inhabiting the temperate zone. In a standard assay, the concentration of ethanol needed to cause 50% mortality over 2 days (LC₅₀) was 2-5% for most fruit-breeding *Drosophila* species tested (Merçot et al., 1994), but was 12-20% for temperate *D. melanogaster* strains (David & Bocquet, 1975; David et al., 1986; Merçot et al., 1994; Parkash et al., 1999). Tropical populations of this species have an intermediate level of ethanol resistance, with LC₅₀'s in the 6-10% range.

One puzzling feature of the high ethanol resistance of temperate *D. melanogaster* is that these flies appear to be adapted to ethanol concentrations that they would seldom encounter in the wild. In a study in temperate Australia (Gibson et al., 1981; see also McKechnie & Morgan, 1982; Oakeshott et al., 1982), about 60% of fruits from which *D. melanogaster* were reared had little or no ethanol, about 20% had ethanol concentrations between 1 and 2%, and most of the rest had concentrations between 2 and 4%. Ethanol concentrations of 4% or lower, however, cause little mortality even of more ethanol-sensitive tropical strains, and may even increase some components of fitness (Parsons et al., 1979). Unfortunately, no studies of which we are aware have reported ethanol concentrations of breeding sites of tropical *D. melanogaster* (see Discussion). Thus, we are left with an apparent puzzle: how could the non-lethal ethanol concentrations found in the breeding sites of temperate *D. melanogaster* have resulted in the evolution of resistance to high, unnatural concentrations?

We hypothesize that a partial answer to this question may lie in the effects of ethanol on behavior. It is self-evident to anyone who has observed alcohol consumption in humans that the dose of ethanol required to affect behavior is far lower than that required to cause life-threatening poisoning, an observation that has been borne out in laboratory studies of species ranging from *Drosophila* to humans (Pohorecky, 1977; Frye & Breese, 1981; Erickson & Kochhar, 1985; Smoothy & Berry, 1985; Waller et al., 1986; Parr et al., 2001; Fillmore, 2003). Doses of ethanol that cause mild sedation or incoordination, without being directly life-threatening, could reduce fitness of flies in the wild in a variety of ways, such as by impairing predator avoidance, oviposition, or courtship. If ethanol levels in decaying fruit are sufficient to impair behavior, selection would favor genotypes whose behavior is relatively insensitive to ethanol. Furthermore, it is easy to imagine that the same type of

genetic changes that would confer resistance to the behavioral effects of low doses of ethanol, e.g., those increasing activity of ethanol-detoxifying enzymes, or rendering neuronal signaling pathways less sensitive to perturbation by ethanol, would also increase resistance to poisoning by higher doses.

As a first step in testing this hypothesis, it is necessary to verify that the behavior of *D. melanogaster* can be adversely affected by a non-lethal dose of ethanol, such as one that could be received from decaying fruit, and that genotypes from the wild vary in their susceptibility to such an effect. We focus on male courtship behavior because its complexity makes it seem likely to be affected by ethanol, and the fitness consequences of impaired courtship – reduced mating success – can be readily quantified. Courtship in *D. melanogaster* consists of a series of stereotypical steps including orienting, walking, tapping, singing, licking, and attempting copulation; males must often carry out several series of these steps before mating is achieved (Bastock & Manning, 1955). In the wild, courtship and mating take place on decaying fruit (Markow, 1988), where males could be exposed to ethanol vapors, even if they do not feed.

We used a simple method to expose flies to a non-lethal, non-incapacitating dose of ethanol vapor during courtship and mating. Our study had three objectives: (1) to investigate the effects of ethanol exposure on mating success of males from two *D. melanogaster* populations, one European and one African; (2) to determine whether the internal ethanol concentrations of flies in the mating assays were similar to those of flies on medium with ethanol concentrations in the range of natural breeding sites; and (3) to compare the amount of ethanol absorbed through vapor and through feeding when flies are placed on ethanol-supplemented medium.

Materials and methods

Strains and rearing conditions

Flies were reared in shell vials on standard maize meal-molasses-Brewer's yeast-agar medium and handled under light CO₂ anesthesia. We used temperate and tropical isofemale lines established from field collections made in 2004. The temperate lines (kindly provided by C. Schlötterer and colleagues) were collected in a forested area near Vienna, Austria (48°N). The tropical lines (kindly provided by J. Pool) were collected in a village in Cameroon (8°N). Before being used for experiments, the lines were maintained by mass transfer on 3-week generations at 21°C. For at least two generations prior to experiments, the lines were reared at a more controlled density (eight females and six males per vial) on 2-week generations at 25°C. Experiments were conducted under continuous lighting in a walk-in environmental chamber at 25°C and 60-70% r.h.

Experiment 1: Mating success in the presence of ethanol

To determine whether non-lethal amounts of ethanol might differentially affect fitness of the temperate and tropical lines by impairing behavior, we assayed competitive mating success of a random sample of three lines from each population in the presence and absence of a low ethanol concentration. For each replicate, a male from one of the isofemale lines was placed

in a vial containing a piece of cotton moistened with 1 ml 3% sucrose, together with a male from a marked 'Cy/Pm' competitor stock (Fry et al., 1999). Here, 'Cy' and 'Pm' refer to the dominant second chromosome markers Curly (wings) and 'Plum' (variegated brown eyes). Because the markers are carried on different homologues (as indicated by the '/'), one or the other are transmitted to all progeny of Cy/Pm males, making such progeny readily identifiable. After the males were added, a second cotton plug was inserted and pushed to the middle of the vial. After 1 day of recovery from anesthesia, 400 µl 10% ethanol or 400 µl distilled water (as a control) was added to the middle plug, after which the vial was stoppered with a cork. In a separate experiment, this ethanol concentration was found to cause no mortality of males over a 3-day period. Four h later, the middle plug was quickly removed and, while tapping down the vial to prevent escape of the males, a wild-type virgin female from a highly ethanol-resistant strain (the 'HE1' selected population of Fry et al., 2004) was added without anesthesia. The plug was then replaced and the vial recorked. After 12 h, the males were discarded and females were placed singly in regular food vials for egg laying. Eleven days later, paternity was determined by the phenotype of the offspring.

The experiment was performed in two blocks, with 50 vials set up for each line-treatment-block combination. About 10% of vials failed to produce offspring (usually because the female died without laying eggs), and about 5% of the remainder contained both genetically marked and wild-type offspring, indicating that both males had mated. This left an average of 43 (range 40-49) vials per line-treatment combination for analysis (i.e., with single inseminations). The proportion of vials excluded because of lack of progeny or multiple mating did not vary significantly among treatments, lines, or blocks ($P>0.25$).

Experiment 2: Internal ethanol concentrations of flies

To determine whether flies in the mating success assays (experiment 1) experienced internal ethanol concentrations that could be experienced by flies in the wild, we compared internal ethanol concentrations of flies exposed to ethanol in the same manner as in the mating assays with those of flies placed on medium with ethanol concentrations spanning the range of wild breeding sites. Because measuring fly ethanol concentrations in numerous genotype-treatment combinations would have been impractical, we used a single population formed by hybridizing 10 Vienna and 10 Cameroon isofemale lines for this work. The population had been maintained for approximately 75 generations at a population size of $>1\ 000$ before use.

To prepare ethanol-supplemented medium, ethanol was added to the medium mixture after allowing it to cool until just before setting (roughly 50°C). Fifty ml of medium with nominal ethanol concentrations (not accounting for evaporation; see below) of either 0, 4, or 8% was transferred to 180-ml polypropylene bottles, and allowed to set for 2.5 h at room temperature. Approximately 80 3- to 4-day-old flies of a given sex were transferred to each bottle without anesthesia, and the bottles were covered with gas-permeable cardboard lids. After 12 h, flies from each bottle were transferred to four 1.5-ml microcentrifuge tubes in sets of ca. 20, frozen in liquid nitrogen, and vortexed gently to separate the heads from the bodies. Heads were isolated by passing through a sieve and used for analysis of ethanol concentrations. Heads were similarly isolated from males and females exposed to ethanol vapor or water in the same manner as in the mating assays (20-22 flies per vial).

Heads were ground in groups of ca. 70 (i.e., all heads originating from one bottle, or from four replicate vials in the vapor treatments) in 100 μ l 50 mM Tris-HCl buffer (pH 7.5). The homogenate was centrifuged at 15 500 *g* for 2 min, and 40 μ l supernatant was used for the ethanol assay. We used a spectrophotometric assay based on measuring the amount of NAD⁺ + reduced to NADH in the presence of yeast alcohol dehydrogenase (Genzyme Diagnostics, Charlottetown, PE, Canada), following the manufacturer's instructions. A sample of the supernatant was also measured for total protein using the Lowry method (Lowry et al., 1951; Fry et al., 2004); normalized ethanol concentrations were expressed as micromole ethanol per gram of protein.

In the first experiment, there were two samples per sex in each of five treatments (medium with 0, 4, or 8% ethanol, ethanol vapor, and water vapor), repeated over three temporal blocks. To determine to what extent flies in the bottle treatments absorbed ethanol through vapor, as opposed to through feeding, we conducted a second experiment, using only the 4 and 8% ethanol food treatments, in which half of the bottles received quinine (1.5 g/l) in addition to ethanol. This quinine concentration was found to prevent flies from feeding in a preliminary experiment using dyed medium (see Zhu & Fry, 2015, for methods); dye was visible in the abdomens of most flies on normal medium, but none was seen in flies placed on medium with quinine. Sample sizes per sex and treatment (two ethanol concentrations, with and without quinine) were the same as in the first experiment.

Because ethanol evaporates rapidly from fly medium (Hageman et al., 1990), we also estimated the actual ethanol concentrations of medium in a set of bottles, just before addition of flies, and just after their removal. Five g of food medium from each test bottle was diluted in 45 ml distilled water and centrifuged at 1 900 *g* for 5 min. 100 μ l supernatant was diluted in 900 μ l 50 mM Tris-HCl buffer and centrifuged at 15 500 *g* for 2 min. For each sample, 40 μ l supernatant was used for the ethanol assay.

Statistical analysis

Data for the mating success trials were analyzed using the GLIMMIX (generalized linear mixed model) procedure in SAS (SAS Institute, Cary, NC, USA), with the logit link function, with success or failure of the wild-type male to mate as the binary response variable. (An alternative analysis, using arcsin *x*-transformed mating proportions in the MIXED procedure, gave similar results). Region (Vienna or Cameroon), ethanol treatment, and their interaction were fitted as fixed effects, and line within region and block were fitted as random effects. All five possible interactions involving the random effects, including interactions with the fixed effects, were also included; of these, all but the region*block interaction produced variance component estimates of zero, and were consequently dropped from the final model. F-tests of the fixed effects were generated using the Satterthwaite option; tests of the random effects, based on residual pseudo-likelihoods, were produced by the Covtest statement. For graphical presentation (only), the proportions of matings by wild-type flies with binomial standard errors are shown for each line-treatment combination, based on pooled data from the two blocks (all effects involving block were non-significant).

The data from the first experiment on internal ethanol concentrations was used to estimate the medium (and by extension, fruit) ethanol concentration that would produce the same

internal ethanol concentration as in the mating assay vials, for each sex separately. The first step was to estimate B , the regression slope of internal ethanol concentration against nominal (i.e., without accounting for evaporation) ethanol concentration of medium (0, 4, or 8%). This was done using the MIXED procedure in SAS, with block and the block*concentration interaction as random effects. Because B is the amount by which fly internal ethanol concentration increases per every 1% increase in the nominal ethanol concentration of medium, we multiplied it by the ratio (nominal concentration) / (actual concentration), using the estimates of the actual medium ethanol concentrations obtained as described above. The result, B' , is an estimate of the increase in internal ethanol concentration per 1% increase in actual medium concentration. Finally, if we let M be the internal ethanol concentration observed in flies placed in mating assay vials, the medium ethanol concentration needed to reproduce this concentration can be estimated as M/B' .

A previous study (Fry, 2014), in which flies were exposed to ethanol in a similar manner as in the mating assays, showed that Cameroon lines accumulated 2-3 $\times$ more internal ethanol than Vienna lines, most likely due to the greater alcohol and aldehyde dehydrogenase activities of the latter (Fry, 2014). If it is assumed that the ratio of internal ethanol concentrations between Cameroon and Vienna flies would not be affected by the exposure method, both M and B' in the Cameroon lines would be increased by the same factor relative to their values in the Vienna lines, resulting in no difference in the M/B' ratio. Thus, the fact we used a hybrid population for the ethanol concentration experiment should not affect the conclusions, unless for some reason the M/B' ratio would have been different for the two populations. In this case, the hybrid population would presumably give an intermediate estimate of M/B' compared to the pure populations.

The experiment with quinine was analyzed with a four-way factorial mixed model, with sex, medium ethanol concentration (4 or 8%), and presence/absence of quinine as fixed effects, and block and all possible interactions involving block as random effects. F-tests for the fixed effects were generated using the Satterthwaite option.

Results

Experiment 1: Mating success in the presence of ethanol

As an initial test of whether there might be a positive correlation between ability to survive exposure to high, unnatural ethanol levels vs. male mating success at more natural, non-lethal levels, we measured male mating success of lines collected from one temperate location (near Vienna, Austria) and one tropical location (a village in Cameroon). Exposure to a non-incapacitating dose of ethanol significantly reduced competitive mating success of the Cameroon lines, but not the Vienna lines (Figure 1, Table 1). The mean ($\pm$ SE) proportion of inseminations by Cameroon males, estimated using least square means from GLIMMIX, was 0.61 ± 0.06 in the water treatment and 0.43 ± 0.06 in the ethanol treatment. In contrast, for Vienna males, the corresponding proportions were 0.75 ± 0.09 and 0.75 ± 0.09 . As Latter & Sved (1994) pointed out regarding competitive fitness assays, however, relative fitness is proportional to the ratio [experimental strain fitness/competitor strain fitness], rather than [experimental / (competitor + experimental)]; the latter will underestimate fitness differences between experimental strains. Using the more appropriate

ratio measure, ethanol decreased the relative mating success of Cameroon males by 52% (from 1.56 to 0.75), and had no effect on the relative mating success of Vienna males. In spite of this large effect on mating success, the ethanol concentration used caused no mortality of males even when they were exposed over a much longer, 3-day period (survival over 3 days was at least 99% regardless of whether ethanol was present).

Although the random effect of line within region was significant ($P < 0.001$), indicating that there was variation within populations in mating success averaged across treatments (Figure 1), the interaction between ethanol treatment and line within region was non-significant ($P > 0.5$), giving no evidence for within-population variation in sensitivity of mating success to ethanol.

Experiment 2: Internal ethanol concentrations of flies

To determine whether flies feeding on medium with ethanol concentrations in the range of documented breeding sites of wild flies would experience doses of ethanol similar to those experienced by flies in our mating assays, we measured internal ethanol concentrations in heads of flies from different ethanol treatments. Females and males exposed to ethanol in the manner of the mating assays had internal ethanol concentrations of 309 ± 34 and 160 ± 20 μM ethanol/g protein, respectively (Figure 2); flies from the water control had ethanol levels indistinguishable from zero. Based on the slope of the relationship between fly and medium ethanol concentrations, internal ethanol concentrations of flies housed in bottles with ethanol-supplemented medium increased by 66 ± 19 $\mu\text{M g}^{-1}$ in females and 27 ± 6 $\mu\text{M g}^{-1}$ in males for every 1% increase in the nominal ethanol concentration of the food. The relatively large standard errors resulted from significant variation in the slopes among blocks, possibly caused by differences in the extent to which ethanol had evaporated from the medium. Analysis of ethanol concentrations of the food itself indicated that actual concentrations were about 12% lower than the nominal concentrations (data not shown), probably due to evaporation. Taking the above information into account (see Materials and methods, Statistical analysis), the internal ethanol concentrations of flies in the mating assays were roughly equivalent to what would be expected in females and males on media containing 4.1 and 5.2% ethanol, respectively.

To determine to what extent flies in the bottle treatments absorbed ethanol through vapor, as opposed to feeding, we conducted a second experiment in which half of the bottles received the feeding deterrent quinine in addition to ethanol. Addition of quinine to ethanol-supplemented medium significantly reduced internal ethanol concentrations of flies. For flies housed in bottles with quinine-supplemented food, average ethanol concentrations were 19% lower in females, and 18% lower in males, than in flies housed in bottles with food without quinine (Figure 3, Table 2). These effects, although significant, are relatively slight, indicating that the majority of ethanol was absorbed as vapor, rather than through ingestion. In both experiments, females accumulated significantly more internal ethanol than males (Figure 2, Figure 3, Table 2).

Discussion

Low-dose ethanol exposure halved the competitive mating success of *D. melanogaster* lines from a tropical African population, but had no effect on that of lines from a European population. The higher resistance of European flies to behavioral perturbation by a sub-lethal dose of ethanol parallels their higher resistance to being killed by unnaturally high ethanol concentrations (David & Bocquet, 1975; David et al., 1986; Fry, 2014). Here, we consider the relevance of our findings to understanding the evolution of ethanol resistance in *D. melanogaster*, in particular the ability of temperate populations to survive exposure to ethanol concentrations that they would not normally encounter in the wild.

Ethanol concentrations of *D. melanogaster* breeding sites, even those in wineries, rarely exceed 4% (Gibson et al., 1981; McKechnie & Morgan, 1982; Oakeshott et al., 1982). Thus, ethanol concentrations of around 4% or less, which cause little or no mortality (David et al., 1986; Fry, 2014), must somehow have selected for the observed ability of most temperate *D. melanogaster* populations to survive prolonged exposure to much higher ethanol concentrations (David & Bocquet, 1975; David et al., 1986; Merçot et al., 1994; Parkash et al., 1999; Fry, 2014). We propose a partial solution to this puzzle, which we call the 'behavioral impairment' hypothesis: ethanol at concentrations found in fermenting fruit perturbs behavior in ways that reduce fitness, and selection for resistance to such behavioral perturbation has resulted in the evolution of resistance to being killed by unnatural, high ethanol concentrations. We verified two prerequisites of this hypothesis by showing that exposure to a sub-lethal ethanol dose can impair a behavior important for fitness, and that genotypes from the wild vary in sensitivity to this effect.

Our ethanol concentration experiment showed that males in the mating assays developed internal ethanol concentrations roughly equivalent to what they would obtain from feeding on fruit with 5.2% ethanol, assuming that flies feeding on (or inside) a fruit with a given ethanol concentration would develop similar levels of internal ethanol as flies on medium with the same concentration. Although 5.2% is at the upper end of the distribution of ethanol concentrations of fruit breeding sites (Gibson et al., 1981), the large (50%) reduction in mating success of the African lines suggests that these lines would have been affected by somewhat lower concentrations. Moreover, although ethanol did not reduce the mating success of the European males, this does not mean that their mating behavior was unaffected by ethanol, only that the magnitude of such an effect was similar between the experimental and competitor males. (The competitor strain, like virtually all *D. melanogaster* laboratory stocks, was also of temperate origin). In one-on-one mating assays, our low-dose ethanol exposure regime did not reduce the probability of successful mating, but did increase the average time until mating took place, even in European flies (J Zhu & J Fry, unpubl. data). This suggests that males exposed to ethanol court females less vigorously, and/or females resist initial mating attempts by males exposed to ethanol, possibilities that would be interesting to investigate. Although we did not detect variation in sensitivity of mating success to ethanol among the temperate lines, the sample size was small, and a larger study might reveal such variation.

We also made the unexpected discovery that flies placed in bottles with ethanol-supplemented medium absorbed 4× as much ethanol through vapor than through feeding. Although it is well known that flies absorb ethanol vapor rapidly (Moore et al., 1998; Singh & Heberlein, 2000), the relative contributions of absorption vs. ingestion have not, to our knowledge, previously been quantified. The relative contributions of the routes might be different in flies feeding on fruits than in flies in our medium bottles, but vapor still probably makes a considerable contribution in the former case, especially given that flies often congregate in small pockets within decaying fruits, rather than feeding on the outside (J Fry & J Zhu, pers. obs.). This suggests that courting males will be unable to avoid being exposed to ethanol. Another implication of the importance of ethanol vapor is that ethanol in fruits might protect *D. melanogaster* against predators (Milan et al., 2012), particularly generalist predators, which appear to be considerably more sensitive to ethanol than *D. melanogaster* (cf. Bouletreau & David, 1981; Milan et al., 2012).

In *Drosophila* and other species in which males invest little more than sperm in the progeny, lifetime reproductive success is likely to be more strongly dependent on the number of mates in males than in females (Bateman, 1948; Clutton-Brock, 2017). As a result, the sensitivity of mating behavior to ethanol could result in males in the wild being under stronger selection for ethanol resistance than females. This could explain several differences that have been observed between males and females in their responses to ethanol. Males take longer than females to be sedated when placed in a vapor stream containing a moderate concentration of ethanol, and recover more rapidly when removed from the ethanol stream (Devineni & Heberlein, 2012; De Nobrega & Lyons, 2016). Both differences appear to be at least in part due to the observed faster metabolic elimination of ethanol by males (Devineni & Heberlein, 2012; Fry, 2014, Figure 2), which in turn likely results from their higher activities of the ethanol-metabolizing enzymes alcohol dehydrogenase (Pipkin & Hewitt, 1972; Geer et al., 1988; Fry et al., 2004) and aldehyde dehydrogenase (Fry et al., 2004). Furthermore, when given the choice of feeding on ethanol-supplemented (2 or 4%) or normal medium, males show stronger aversion to ethanol than females (Zhu & Fry, 2015), as expected if male fitness is more negatively affected by ethanol than female fitness.

Although our results provide an important first step in testing the behavioral impairment hypothesis, additional experiments are needed to verify the hypothesis. We studied only one temperate and one tropical population; the sensitivity of mating success to low ethanol doses needs to be measured in more populations to confirm that the temperate-tropical difference we observed is general. Assuming that this is the case, additional work will be needed to verify that the genetic differences responsible for the higher resistance of temperate populations to behavioral perturbation by sub-lethal ethanol doses also contribute to their higher resistance to being killed by high ethanol doses. One way this could be done would be to select a tropical population for increased mating success in the presence of a low ethanol dose and determine whether increased resistance to ethanol-induced mortality evolves as a correlated response. In a preliminary experiment, a population selected in this manner evolved higher ethanol resistance than three unselected populations (Zhu, 2014); because the selection treatment was unreplicated, however, genetic drift cannot be ruled out as an explanation.

Another unanswered question is why selection for ethanol resistance, whatever its precise basis, is apparently stronger in temperate than in tropical populations. Unfortunately, we know of no published estimates of ethanol concentrations of breeding sites of tropical or subtropical *D. melanogaster* populations, or of those of other fruit-breeding *Drosophila* species. In tropical West Africa, *D. melanogaster* has been reared from fruits of 25 native and cultivated plant species (Lachaise et al., 1988), including relatively sweet (and therefore potentially high-alcohol producing) fruits like mango, papaya, banana, and guava. Although it is unclear how much alcohol these and other tropical fruits contain when *D. melanogaster* breeds in them, there is one report of naturally fermenting palm fruits with ethanol concentrations approaching 10% (Dudley, 2004), indicating that substantial natural fermentation can occur in the tropics. Undecayed, and hence ethanol-free, fruit might be more consistently available in the tropics than in temperate regions due to the continuous growing season, but *D. melanogaster* females cannot puncture undamaged fruit to oviposit, and are specifically attracted to odors associated with overripe fruits (Zhu et al., 2003), including those produced by fermentative yeasts (Becher et al., 2012; indeed, the latter authors showed that whereas *D. melanogaster* larvae develop well on pure baker's yeast, or on fruit inoculated with yeast, larval survival is low and development prolonged on fruit lacking yeast). We suspect that the reason that temperate *D. melanogaster* populations are more ethanol-resistant than tropical populations is not that more ethanol is present in fruits in temperate regions, but that breeding in ethanol-rich fruits is more advantageous there. In support of this possibility, although both temperate and tropical populations exhibit preference in oviposition and larval feeding for ethanol-supplemented over normal medium, this preference is stronger in temperate than in tropical populations (Parsons, 1977; Parsons, 1980; Zhu & Fry, 2015). Hypotheses for why breeding in ethanol-rich resources might be more advantageous for temperate than for tropical populations have been advanced by Eanes (1999) and Zhu & Fry (2015).

Although we have found some initial evidence for the behavioral impairment hypothesis, we do not mean to suggest that the high ethanol resistance of temperate *D. melanogaster* populations is likely to be entirely explained by the hypothesis. There is some evidence, for example, that ethanol concentrations as low as 1.5-3.0% can slow larval development (Oakeshott, 1976; but see Parsons et al., 1979); selection for faster development or higher fecundity on such concentrations might result in resistance to being killed by higher concentrations (although the one relevant experiment we know of failed to find such an effect; Oakeshott et al., 1985). Perhaps more important, breeding sites of temperate *D. melanogaster* occasionally contain ethanol concentrations from 4-7%, and rarely even higher (McKenzie & McKechnie, 1979; Gibson et al., 1981). At 5-7% ethanol, adult and larval mortality of tropical strains can be significant (David et al., 1986; Fry, 2014), and some mortality of temperate strains may also occur, depending on the methodology used (e.g., McKenzie & Parsons, 1972; Parsons et al., 1979; but see David et al., 1986; Milan et al., 2012; Fry, 2014). Recall that although our ethanol-exposure treatment was roughly equivalent to placing flies on food with 4-5% ethanol, no mortality occurred, even of the tropical strains.

In conclusion, our results support the view that the sub-lethal effects of a toxin, particularly effects on behavior, may sometimes be important agents of selection on insect populations.

Although the ecological and behavioral effects of sub-lethal toxin exposures have long been recognized (Desneux et al., 2007), more attention should be paid to the evolutionary consequences of such exposures.

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Figure 1.

Mean ($\pm$ SE) male mating success of *Drosophila melanogaster* isofemale lines from Vienna (V8, V17, and V18) and Cameroon (C23, C41, and C44), in the presence or absence of a non-lethal concentration of ethanol. Males from each line competed for mating with males from a genetically-marked stock. See Table 1 for statistical analysis.

Figure 2.

Mean ($\pm$ SE) ethanol concentrations in *Drosophila melanogaster* fly heads (μ M ethanol/g protein). “Medium”: flies allowed to feed on medium with 0, 4, or 8% ethanol (not accounting for evaporation). “Vapor”: flies exposed to ethanol vapor (‘E’) or water (‘C’).

Figure 3.

Mean ($\pm$ SE) ethanol concentrations in heads of *Drosophila melanogaster* flies placed on ethanol-supplemented medium either with or without quinine, a feeding deterrent. See Table 2 for statistical analysis.

Table 1

Generalized linear mixed-model analysis of male mating success of *Drosophila melanogaster* isofemale lines from Vienna and Cameroon, in the presence or absence of ethanol (see Figure 1)

		d.f.	F	P
Both populations	Population	1,3.96	3.89	0.12
	Ethanol treatment	1,1026	6.71	0.010
	Population*ethanol treatment	1,1026	7.58	0.006
Vienna only	Ethanol treatment	1,512	0.01	0.92
Cameroon only	Ethanol treatment	1,514	16.40	<0.001

Table 2

Linear mixed-model analysis of log₁₀-transformed ethanol concentration in *Drosophila melanogaster* fly heads in the presence of 4 or 8% ethanol, with or without quinine in food (see Figure 3)

	d.f.	F	P
Ethanol	1,4	17.95	0.013
Quinine	1,32	14.14	0.0007
Sex	1,4	72.18	0.001
Ethanol*quinine	1,32	0.23	0.63
Ethanol*sex	1,4	0.02	0.9
Quinine*sex	1,32	0.03	0.85
Ethanol*quinine*sex	1,32	0.00	0.99