

Evolution of gene expression and expression plasticity in long-term experimental populations of *Drosophila melanogaster* maintained under constant and variable ethanol stress

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Abstract

Gene expression responds to the environment and can also evolve rapidly in response to altered selection regimes. Little is known, however, about the extent to which evolutionary adaptation to a particular type of stress involves changes in the within-generation ('plastic') responses of gene expression to the stress. We used microarrays to quantify gene expression plasticity in response to ethanol in laboratory populations of *Drosophila melanogaster* differing in their history of ethanol exposure. Two populations ('R' populations) were maintained on regular medium, two ('E') were maintained on medium supplemented with ethanol, and two ('M') were maintained in a mixed regime in which half of the population was reared on one medium type, and half on the other, each generation. After more than 300 generations, embryos from each population were collected and exposed to either ethanol or water as a control, and RNA was extracted from the larvae shortly after hatching. Nearly 2000 transcripts showed significant within-generation responses to ethanol exposure. Evolutionary history also affected gene expression: the E and M populations were largely indistinguishable in expression, but differed significantly in expression from the R populations for over 100 transcripts, the majority of which did not show plastic responses. Notably, in no case was the interaction between selection regime and ethanol exposure significant after controlling for multiple comparisons, indicating that adaptation to ethanol in the E and M populations did not involve substantial changes in gene expression plasticity. The results give evidence that expression plasticity evolves considerably more slowly than mean expression.

Keywords: adaptation, alcohol, genetic correlation, genotype–environment interaction

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Introduction

Gene expression has long been known to be highly responsive to the environment; classic examples include the *lac* operon in *E. coli* (Jacob & Monod 1961) and the

heat shock response in a wide range of organisms (Richter *et al.* 2010). In the last decade, microarray technology has allowed the characterization of how genomewide transcript abundance responds to environmental changes in unprecedented detail. Typically, environmental stressors such as heat, starvation or toxin exposure cause hundreds and sometimes thousands of genes to change in expression, often substantially (Lopez-Maury *et al.* 2008; Hodgins-Davis & Townsend 2009; Pancaldi *et al.* 2010; Snell-Rood *et al.* 2010). Some of these gene expression changes increase the organism's ability to tolerate the stress (Richter *et al.* 2010),

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while others may have no effect, or even a negative effect, on fitness (Lopez-Maury *et al.* 2008; Mira *et al.* 2010).

Although gene expression plasticity has been well characterized, little is known about how rapidly it evolves. Laboratory populations selected for stress resistance often show substantial changes in gene expression profiles (Cooper *et al.* 2003; Morozova *et al.* 2007; Sorensen *et al.* 2007; Telonis-Scott *et al.* 2009; Sarup *et al.* 2011; Wertheim *et al.* 2011), but all such studies of which we are aware compared gene expression of selected and control populations in only the 'ancestral', nonstressful environment, and therefore give no information on the extent to which gene expression plasticity evolved. Filling this gap is important, because there are at least two ways in which changes in plasticity might play important roles in the evolution of stress resistance. First, some genes may have their expression perturbed in maladaptive ways by the stressful agent; for such genes, evolution of decreased sensitivity of expression to the stressor would be expected to increase fitness. Second, for genes induced by a stress that help protect against the stress (e.g. detoxifying enzymes, heat shock proteins), the degree of induction might be suboptimal because of 'bet-hedging': in nature, no environmental cue will be perfectly predictive of the continued presence of a particular stress, so that the fittest genotype in the long run will be one that does not completely commit to the presence of the stress (DeWitt *et al.* 1998). Under a predictable laboratory selection regime, in contrast, stronger induction would be favoured.

There are also factors that could constrain or prevent the evolution of gene expression plasticity. Most obviously, genetic variation for plasticity could be limited or absent. Experiments on yeast have revealed substantial variation for expression plasticity among wild isolates grown on different media (Landry *et al.* 2006) or exposed to different temperatures (Eng *et al.* 2010), but similar studies on other organisms appear to be lacking. Even if genetic variation for plasticity is present, evolution of plasticity could be constrained by costs of the mechanisms underlying plastic responses (Van Tienderen 1991; DeWitt *et al.* 1998; Snell-Rood *et al.* 2010), or by adverse pleiotropic effects of changes in plasticity of one gene on the regulation of other genes. Finally, it is possible that many gene expression responses to stressful agents have essentially no effect on fitness; for such genes, exposure to the stress would not exert selection on either mean expression or plasticity of expression.

We examined the evolution of gene expression plasticity in response to ethanol in long-term selection lines of *Drosophila melanogaster* with differing histories of ethanol exposure. Ethanol occurs naturally in the decaying

fruit used by *D. melanogaster* for feeding and breeding (McKenzie & McKechnie 1979; Gibson *et al.* 1981; Merçot *et al.* 1994); it is both an energy source and, at high concentrations, a toxin (Parsons *et al.* 1979). Exposing adult *Drosophila* to ethanol vapour elicits a wide variety of gene expression changes (Morozova *et al.* 2006; Urizar *et al.* 2007; Kong *et al.* 2010), some of which likely underlie the development of tolerance, that is, increased resistance to a second exposure (Scholz *et al.* 2000; Kong *et al.* 2010). The development of tolerance to ethanol also occurs in larvae: larvae that hatch from embryos that developed in contact with ethanol have higher ability to survive on medium supplemented with ethanol than larvae hatching from embryos not exposed to ethanol (Bijlsma-Meeles 1979; Kerver & Rotman 1987; Fry 2001). Unlike adult tolerance, the gene expression correlates of this embryonic/larval tolerance have not been investigated (except for the observation that induction of alcohol dehydrogenase by ethanol exposure of embryos occurs, but is not sufficient to explain the observed tolerance; Bijlsma-Meeles 1979; Bijlsma & Bijlsma-Meeles 1991).

We used microarrays to measure gene expression in larvae derived from ethanol-exposed and nonexposed embryos in six experimental populations derived from an outbred base population and maintained for over 300 generations (Fry 2001). Two populations ('E populations') had been maintained continuously on medium supplemented with a high level of ethanol (12%, compared to usually not more than 4–5% in decaying fruit; Gibson *et al.* 1981); two ('R') had been maintained on regular medium with only trace amounts of ethanol; and two ('M') had been maintained in a mixed regime in which half the flies in each generation were reared on ethanol-supplemented medium and half on regular medium. Ethanol exposure of embryos caused expression changes in nearly 2000 genes, and the E and M populations diverged from the R populations, but not from each other, in mean expression of over 100 genes. After controlling for multiple comparisons, however, selection regime had no significant effects on gene expression plasticity.

Materials and methods

Experimental populations

The six experimental populations (Fry 2001) were established from an outbred base population derived from flies collected in Raleigh, NC, USA. They have been maintained on 2-week generations at population sizes of >1000 adults each ever since their founding in 1995. Each generation of each population is initiated by allowing 20–30 adults to lay eggs in each of 50 vials

containing either standard cornmeal–molasses–agar medium (all the vials in the R populations and half of the vials in the M populations) or medium supplemented with 12% ethanol (half of the vials in the M populations and all of the vials in the E populations). After 14 days, progeny from the different vials within a population are mixed before being used to set up the next generation.

To make ethanol-supplemented medium, ethanol was added after the medium had cooled below 50 °C, resulting in no more than 10–15% loss due to evaporation, as confirmed by assaying ethanol concentration after the medium had set (J. Zhu and J.D. Fry unpublished). Although the initial recipe for standard medium involved adding about 30 mL/L of ethanol as a vehicle for antifungal agents (Fry 2001), at approximately generation 220 this was reduced to about 10 mL/L; moreover, from the beginning of the experiment, preservative was added while the medium was still 80–90 °C, likely resulting in much evaporation of the ethanol.

The microarray experiments were conducted in 2008, after ~320 generations of selection. Measurements of egg-to-adult survival at this time (J.D. Fry and L.Y. Yampolsky unpublished) gave similar results as those previously reported from generation ~95 (Fry 2001), with E and M population larvae surviving substantially better than R population larvae on medium supplemented with either 12% or 16% ethanol. Survival of all populations is uniformly high on unsupplemented medium (Fry 2001).

RNA extraction and microarray hybridization

To control for environmental effects on gene expression, flies from all populations were reared for two generations on standard medium at 25 °C before being used to lay eggs. To obtain synchronized first-instar larvae for RNA extraction, groups of 50–60 *c.* 1-week-old flies were allowed to lay eggs on apple juice agar medium (100 mL apple juice, 300 mL water, 6 g sucrose, 3.6 g agar) dispensed in 5-mL aliquots into the lids of 35-mm-diameter Petri dishes, such that the surface of the medium was flush with the rim of the lid. A dab of fresh yeast paste was added to the medium to encourage egg production. After 3 h, flies were removed and the yeast paste was gently rinsed off with distilled water, with the majority of eggs remaining embedded in the agar. Each such 'laying cap' was then placed in a 60-mm Petri dish, to which 10 mL of either 18% ethanol or distilled water was added, with the result that the solution just covered (by 1 mm or so) the surface of the agar (18% ethanol was used to result in an approximate final concentration of 12%, taking into account the vol-

ume of laying medium). The dishes were covered, sealed with Parafilm and placed on an orbital shaker at a gentle setting for 15 h. After this period, the caps were removed from the dishes and again rinsed with distilled water, to remove any early hatching larvae. Thereafter, newly hatched larvae were collected at 45-min intervals; if necessary, larvae were stored briefly at –80 °C until enough were obtained for RNA extraction. We verified our earlier finding (Fry 2001) that the ethanol pretreatment, although delaying egg hatch slightly, substantially increases the survival of larvae from all three selection treatments on ethanol-supplemented medium, without affecting embryo viability or subsequent survival on standard medium.

RNA was extracted from groups of 50–200 larvae using the RNeasy mini kit and QIAshredder columns (QIAGEN Inc., Valencia, CA, USA), following the manufacturer's instructions. Extractions were conducted in three blocks on different days, at the same time each day to minimize circadian effects, with one sample per population and pretreatment per block. One RNA sample was lost due to technical error, leaving 35 samples for microarray hybridization.

Sample processing and microarray hybridization was conducted by the Functional Genomics Center at the University of Rochester Medical Center. Total RNA was converted to biotin-labelled, fragmented cDNA with kits from NuGEN Technologies (Ovation Amplification System V2, FL-Ovation cDNA Biotin Module 2) according to the manufacturer's protocols (NuGEN, San Carlos, CA, USA). The cDNA was hybridized overnight to *Drosophila* Genome 2.0 arrays (Affymetrix, Santa Clara, CA, USA) as recommended by NuGEN. Arrays were washed, stained with a fluorescent dye that binds to biotin (streptavidin, R-phycoerythrin conjugate) and scanned as recommended by Affymetrix using an Affymetrix Fluidics Station 450 and Scanner 3000.

Raw expression .CEL files were normalized using the gcRMA algorithm (Wu *et al.* 2004) as implemented in the 'Affy' package on Bioconductor (version 2.4) (Gentleman *et al.* 2004). Nonspecific filtering is recommended to increase the power in microarray data analysis (Bourgon *et al.* 2010). We filtered out probesets with low expression and variance by removing those whose mean, interquartile range among arrays and standard deviation among arrays were each less than the median of the respective quantity over all probesets. Because all three measures were strongly right skewed (i.e. median << mean), in practice this removed a cluster of probesets that had both very low mean expression and very low variation in expression. (The vast majority of the removed probesets were called 'not present' on all or almost all arrays by Affymetrix's software). After the

filtering step, 12 236 of the 18 769 original probesets were left for further analysis.

Statistical analysis

For each probeset, we used the 'Mixed' procedure in SAS (Littell *et al.* 1996) to conduct *F*-tests for the fixed effects of selection regime ('selection'), embryo pretreatment ('exposure') and their interaction. The random effects of block and replicate population within selection regime were also included. There are five possible interactions involving the random effects (including interactions between random and fixed effects), but not all of these are likely to be biologically meaningful. In order to determine which of these to include in the analysis of individual probesets, we performed a preliminary analysis on the first eight principal components extracted from the entire data set (with arrays as replicates and probesets as variables), which explained 70% of the total variation among the 35 chips. Three interaction effects—selection \times block, exposure \times replicate population and exposure \times selection \times block—were never significant ($P > 0.05$), and the associated variance components were zero in all but four of 24 cases. These interactions were therefore not included in the analysis of individual probesets. In contrast, the interactions exposure \times block and block \times replicate population were sometimes significant, and the associated variance components were usually nonzero; therefore, these effects were retained. The principal components analysis also revealed that one microarray was an outlier in multivariate space; this array was therefore dropped from further consideration.

For the analysis of individual probesets, we used the 'ddfm = satterth' option in SAS. With this option, denominator mean squares for the fixed effects are calculated using the Satterthwaite approximation, after dropping from the model random effects whose variance component is zero. Nonsignificant random effects with nonzero estimated variance components, no matter how small, are retained. A consequence of this procedure is that denominator degrees of freedom for the *F*-tests for a given effect, and hence statistical power, varied considerably, and to some extent randomly, among probesets (Table S1, Supporting information), depending on which random effects were dropped. In practice, probesets for which the replicate population effect was dropped (about 37% of the total) were about ten times more likely to give rise to a significant effect of selection regime than probesets for which the effect was retained. Although more power could have been obtained by dropping the replicate population effect in all cases in which it was nonsignificant, this would have increased the risk of conflating the

effects of genetic drift with those of selection. Our procedure seems to have suitably guarded against this possibility, because when comparing only the 'E' and 'M' regimes, which show little if any difference in ethanol resistance (J.D. Fry and L.Y. Yampolsky unpublished), only 1.6% and 0.3% of the 12 236 probesets showed a significant effect of selection regime at $P < 0.05$ and $P < 0.01$, respectively, considerably fewer than would be expected by chance.

There are several ways to guard against Type 1 errors when testing multiple hypotheses. One approach is to use a Bonferroni or similar correction to control the family-wise error rate (FWER), defined as the probability of making at least one Type 1 error among all hypotheses tested. However, this procedure is too conservative for genomewide studies (Storey & Tibshirani 2003). Another approach is to control the false discovery rate (FDR), defined as the expected proportion of Type 1 errors among rejected hypotheses (Benjamini & Hochberg 1995; Benjamini & Yekutieli 2001; Storey 2002; Storey & Tibshirani 2003; Storey *et al.* 2004). The *q*-value is a measure of significance in terms of FDR rate. For all our analyses, *q*-values were estimated with the 'qvalue' Bioconductor package (Gentleman *et al.* 2004). The conditional test for overrepresentation of Gene Ontology biological process (BP) terms was used as implemented in the 'GStat' Bioconductor package (version 2.5); we considered *P* values < 0.01 as significant.

One caveat to our conclusions is that, because the probe sequences in the microarrays were based on a single reference genome, it is possible that some of the apparent increases or decreases in expression in the selected lines were due to altered hybridization intensity caused by changes in allele frequencies at SNPs overlapping the probes. Although we did not have resources to confirm the selection responses by real-time PCR, in two other studies of *Drosophila* selection lines that used the same array platform that we used, real-time PCR results were consistent with the microarray results for a high proportion of tested genes (Telonis-Scott *et al.* 2009; Wertheim *et al.* 2011). Moreover, probe mismatches are unlikely to affect conclusions about gene expression plasticity.

Results

Gene expression was affected by ethanol exposure and selection regime, but not their interaction

Gene expression was compared between *Drosophila melanogaster* populations that had been maintained for over 300 generations on either regular medium ('R' populations), medium supplemented with 12% ethanol ('E' populations) or equal proportions of the two medium

types ('M' populations). For each population, RNA was extracted from first-instar larvae that had developed from embryos partly immersed in either 12% ethanol or water, treatments that mimicked the effects of being oviposited into the ethanol-supplemented and ethanol-free media, respectively, used for the maintenance of the experimental populations. For each of the 12 236 transcripts that produced hybridization signals strong enough to be analysed, a mixed-model analysis was used to test the effects of selection regime, ethanol exposure of embryos and the selection regime \times exposure interaction on transcript abundance. In contrast to some previous microarray studies of *Drosophila* experimental populations (Morozova *et al.* 2007; Wertheim *et al.* 2011), our tests for selection regime effects controlled for random divergence between replicate populations caused by genetic drift (see Materials and Methods).

In the first analysis, with all three selection treatments (R, E and M) distinguished, levels of 1912 transcripts were significantly (false discovery rate $q < 0.05$) affected by ethanol exposure; 129 of these were significant using a more stringent Bonferroni correction. In addition, 42 transcripts showed a significant effect of selection regime, including eight after Bonferroni correction. In contrast, the interaction between ethanol exposure and selection regime was never significant, with the lowest $q = 0.41$. Therefore, gene expression was influenced by both ethanol exposure of embryos and selection regime, but the magnitude of transcriptional responses to ethanol (i.e. gene expression plasticity) showed comparatively little effect of selection history.

For each of the 42 transcripts for which the main effect of selection regime was significant, we performed pairwise comparisons between the E, M and R regimes. Differences between the E and M populations were

significant at $P < 0.05$ in only two instances, essentially what would be expected by chance, and neither difference was significant after a sequential Bonferroni correction (Rice 1989). In contrast, after sequential Bonferroni correction, the E and M populations differed significantly from the R populations in 41/42 and 42/42 cases, respectively. Therefore, even though the M populations were maintained on a regime intermediate between those of the E and R populations, they were statistically indistinguishable in gene expression from the E populations. This suggests that selection on gene expression on regular food was weak compared to selection on ethanol-supplemented food; otherwise, genes evolving expression differences between the E and R populations would have been expected to have evolved intermediate expression in the M populations.

To gain more power to detect effects of selection regime, as well as possible interactions between selection regime and ethanol exposure, we pooled the E and M populations into a single treatment ('selected') with four replicate populations, and repeated the mixed-model analysis for each transcript. This increased by approximately threefold the number of transcripts with significant effects of selection regime, from 42 to 125, and slightly increased the number with significant effects of ethanol exposure (Table 1; detailed results for each probeset are given in Table S1, Supporting information).

As before, after controlling for multiple comparisons, no transcripts showed significant interactions between selection regime and ethanol exposure (lowest $q = 0.13$). The failure to reject the null hypothesis of no interactions does not, of course, mean that the null hypothesis is true, particularly given the reduced statistical power resulting from the need to correct for multiple comparisons. Nonetheless, the discrepancy between the number of transcripts showing significant ($q < 0.05$) main effects

Table 1 Number of probesets showing significant (FDR $q < 0.05$) main effects of selection regime (E and M populations vs. R populations) and/or ethanol exposure (embryonic development in the presence of 12% ethanol vs. water) on expression. No probesets showed significant interaction ($q < 0.05$) between the main effects; thus, to the first approximation, expression responses to ethanol were similar between the selected and unselected populations, and expression differences between the selection regimes were similar whether measured in ethanol-exposed or unexposed larvae

	Effect of ethanol exposure			Row total
	Significantly increased expression	No significant change	Significantly decreased expression	
Effect of selection regime				
Significantly higher expression in selected lines	4	42	30	76
No significant difference	961	10 164	986	12 111
Significantly lower expression in selected lines	4	41	4	49
Column total	969	10 247	1020	12 236

FDR, false discovery rate.

of selection regime (125) and the number showing significant regime by exposure interactions (0) gives evidence that the evolutionary history of ethanol exposure affected mean expression considerably more than plasticity of expression.

Genes upregulated by ethanol exposure

The 969 transcripts significantly upregulated by embryonic ethanol exposure (see Table S1, Supporting information) include many coding for enzymes involved in the metabolism of ethanol, either directly or indirectly (Fig. 1). These include *alcohol dehydrogenase* (*Adh*), *aldehyde dehydrogenase* (*Aldh*) and *acetyl-coA synthase* (*AcCoAS*, actually a 'synthetase'; Nelson & Cox 2008), which catalyse the three steps by which ethanol enters central metabolism (Fig. 1). *Adh* is known to be inducible by ethanol in larvae (Geer *et al.* 1988; Kapoun *et al.* 1990), and two of three microarray studies of adults have found *AcCoAS* to be inducible by ethanol (Urizar *et al.* 2007; Kong *et al.* 2010). (Oddly, although the gene ontology biological process term 'alcohol catabolic process' was significantly overrepresented among the upregulated genes, neither *Adh*, *Aldh* or *AcCoAS* is associated with this term in current annotations, providing a cautionary tale against unquestioning reliance on GO annotations!). The biological roles of the other enzymes in Fig. 1, as well as those of other enzymes upregulated by ethanol exposure, are discussed in Data S1 (Supporting information).

In addition to metabolic enzymes, many genes with nonmetabolic functions were upregulated by ethanol exposure. Nonmetabolic GO biological process categories overrepresented among upregulated genes included cardiac cell differentiation, regulation of metal ion transport, regulation of microtubule cytoskeleton organization, establishment or maintenance of cell polarity, and cell surface receptor-linked signal transduction. All three probesets for the *Hsp70* gene cluster also showed approximately twofold upregulation by ethanol, consistent with experiments on adults (Morozova *et al.* 2006; Kong *et al.* 2010), albeit much smaller than the up to 50-fold upregulation reported by Kong *et al.* (2010).

Kong *et al.* (2010) tabulated 17 genes that were upregulated by exposing adult *Drosophila* to ethanol vapour in each of three independent studies. Nine of these were upregulated in this study (see Data S1, Supporting information), in spite of the different stage and method of ethanol exposure. These nine will be termed 'robustly upregulated genes'.

Genes downregulated by ethanol exposure

Two striking patterns were evident among the 1020 transcripts downregulated by ethanol exposure. First, expression of genes involved in information transfer was repressed by ethanol: significantly overrepresented GO biological process categories among downregulated genes included ribosome biogenesis, RNA processing, DNA replication and tRNA aminoacylation for protein

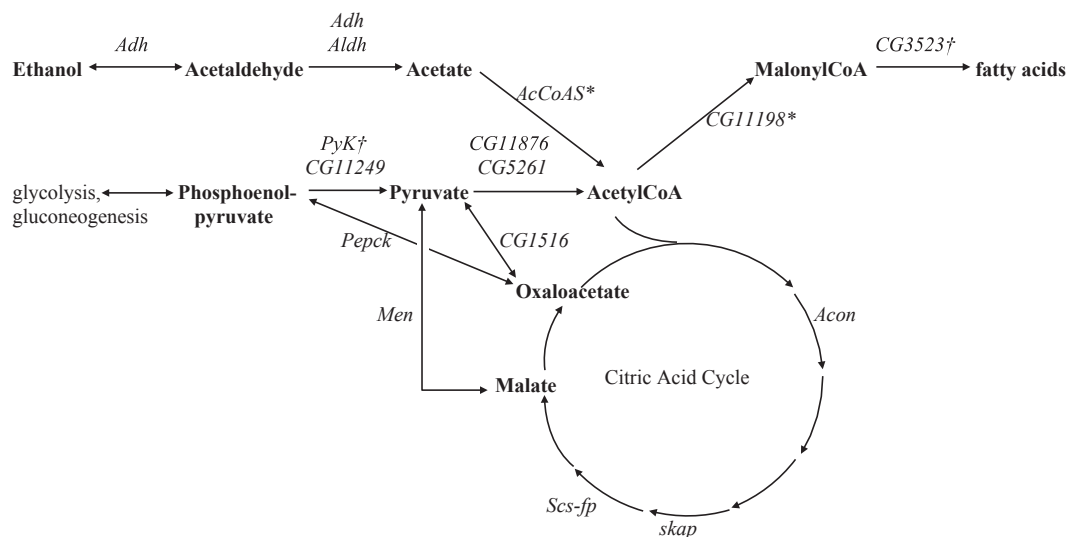


Fig. 1 Some of the enzyme genes significantly upregulated by ethanol exposure. See text and Data S1 (Supporting information) for full explanation. *Twofold or greater upregulation; †0.05 < q < 0.10 (all others q < 0.05). *AcCoAS*, *Acetyl-coA synth[et]ase*; *Acon*, *Aconitase*; *Adh*, *Alcohol dehydrogenase*; *Aldh*, *Aldehyde dehydrogenase*; *CG1516*, *Pyruvate carboxylase activity*; *CG3523*, *Fatty acid synthase activity*; *CG5261*, *Component of pyruvate dehydrogenase complex*; *CG11198*, *Acetyl-coA carboxylase activity*; *CG11249*, *Pyruvate kinase activity*; *CG11876*, *Component of pyruvate dehydrogenase complex*; *Men*, *Malic enzyme*; *Pepck*, *Phosphoenolpyruvate carboxykinase*; *PyK*, *Pyruvate kinase*; *SCS-fp* (=SdhA), *Succinate dehydrogenase A*; *skap*, *Succinyl-coA synthetase activity*.

translation. Second, the most strongly downregulated genes were heavily weighted towards apparent digestive enzymes. In particular, of the 24 genes showing >4-fold repression by ethanol (i.e. ethanol-exposed expression/control expression <0.25), 17 show larval expression primarily in the midgut, the main site of food digestion and absorption, as determined using the FlyAtlas database of gene expression (Chintapalli *et al.* 2007); of these 17, 14 have annotated molecular functions consistent with digestion (see Data S1, Supporting information).

The well-characterized downregulation of genes involved in growth that occurs in response to starvation and other forms of stress is known to be partly mediated by reduced insulin/TOR signalling (Grewal 2009). Supporting a role for this pathway in the transcriptional responses to ethanol, at least four transcriptional targets of insulin/TOR signalling showed altered transcript abundance in response to ethanol, in each case consistent with reduced activity of the pathway: the rRNA transcription initiation factor *Tif-IA* (Grewal 2009; downregulated at $q < 0.05$); *l(2)efl* and *Hsp68* (Biteau *et al.* 2011; upregulated at $q < 0.0001$ and $q < 0.07$, respectively); and the translational suppressor *Thor* (Grewal 2009; upregulated at $q = 0.093$).

Evolutionary changes in gene expression

Expression level of 76 transcripts was significantly higher in the E and M populations than in the R populations, while the reverse was true for 49 transcripts (Table 1). For simplicity, we will refer to these as 'upselected' and 'downselected' transcripts, respectively. Genes showing expression differences between the selection regimes were not significantly enriched for GO biological process categories with obvious relationships to alcohol resistance, such as alcohol catabolism, resistance to stress or resistance to toxins. Nonetheless, three genes that evolved lower expression in the selected lines, *lid*, *capa* and *Takr99D*, have documented or plausible connections to ethanol resistance (see Data S1, Supporting information). Moreover, because nearly half of the up- and downselected genes (42% and 55%, respectively) are not currently associated with any GO biological process term, and many others have functions inferred only by homology, it is possible that some play heretofore uncharacterized roles in ethanol resistance.

Notably absent from the list of genes upselected at $q < 0.05$ were *Adh*, *Aldh* and *Gpdh*, which play critical roles in ethanol detoxification (David *et al.* 1976; Geer *et al.* 1993; Fry & Saweikis 2006; Eanes *et al.* 2009), and appear to contribute to natural variation in ethanol

resistance (Cavener & Clegg 1978; Geer *et al.* 1993; Merçot *et al.* 1994; Fry *et al.* 2008). Because of the previous evidence for the importance of these genes, we considered it appropriate to use a less stringent comparisonwise $P < 0.05$ criterion for them. At this level, all three showed significant increases in expression in the selected populations. The strongest effect was shown by *Gpdh*, for which each of four probesets showed expression increases ranging from 17% to 28% ($0.004 \leq P \leq 0.045$). The single probesets for *Aldh* and *Adh* showed expression increases of 17% ($P = 0.035$) and 5% ($P = 0.048$), respectively.

Relationship between evolutionary and plastic responses

Only eight (16%) of the downselected transcripts also showed plastic responses to ethanol ($q < 0.05$), evenly divided between up- and downregulation; downselected transcripts were in fact no more or less likely to be up- or downregulated by ethanol exposure than those showing no evolutionary change in expression (Table 1). In contrast, 30 (40%) of the upselected transcripts were downregulated by ethanol exposure, while only four (5%) were upregulated (Table 1). The 30 downregulated, upselected genes included five involved in DNA replication and six with functions related to protein synthesis (translation, tRNA processing, mRNA processing or protein amino acid glycosylation). At a broader scale, without regard to the identity of individual genes, the Gene Ontology biological process categories 'DNA replication' and 'tRNA aminoacylation for protein translation' were significantly overrepresented in *both* the lists of all 1020 downregulated genes and of all 76 upselected genes. A possible explanation (see Data S1, Supporting information) is that downregulation of information-transfer genes by ethanol reduced fitness under the conditions of the E and M populations, and selection favoured increased expression to compensate.

Nonetheless, overall regulatory and evolutionary responses to ethanol bore only a loose relationship to one another. For example, none of the nine 'robustly upregulated genes' or 14 strongly downregulated digestive enzymes discussed above showed evidence for evolutionary responses to ethanol ($q > 0.40$ and $P > 0.05$ for each).

Discussion

Limited evolution of gene expression plasticity

Ethanol exposure during embryonic development had profound effects on gene expression in hatchling larvae,

significantly altering transcript abundance of nearly 2000 genes after correction for multiple comparisons. A smaller but still substantial number of genes showed significant evolutionary divergence in mean expression between ethanol-selected and control populations. In contrast, after controlling for multiple comparisons, we detected no statistically significant interactions between ethanol exposure and selection regime, meaning that there was comparatively little evolutionary change in gene expression plasticity.

There are three nonmutually exclusive possible explanations for why adaptation to ethanol involved changes in mean expression, but relatively little change in plasticity. The simplest is that there was no selection for plasticity to change. Alternatively, the base population may have contained little genetic variation for plasticity. Finally, even if such variation was present, it may have been unable to contribute to selection responses due to adverse side effects of alleles altering plasticity. We consider each of these explanations in turn.

Selection for altered plasticity would not have occurred for genes whose expression had little effect on fitness in the experimental populations, or whose expression was already at the joint optimum, such that changes in expression in either the presence or absence of ethanol would reduce fitness under the respective conditions. Although this may have been the case for many genes, it cannot have been the case for all genes: otherwise, no evolution of mean expression would have occurred. To determine whether divergence in mean expression, without divergence in plasticity, is consistent with a purely selectionist scenario, without invoking constraints on the evolution of plasticity such as limited genetic variation, we follow Falconer (1952) and Via & Lande (1985) and regard expression in each environment as a separate 'trait'. If we let X and Y be the expression of a genotype in the ethanol-absent and ethanol-present environments, respectively, then the genotype's plasticity can be defined as $Y - X$. Plasticity is most free to evolve when X and Y both have high additive genetic variance, but the genetic correlation between them is close to zero. In this case, selection in a given environment could change a population's mean expression in that environment with little or no correlated response in the other environment. Such a change would necessarily cause plasticity to be altered relative to the ancestral population. Applying this logic to the E and R populations, unless a gene's plasticity changed to an equal extent relative to the base population in both treatments (e.g. by an increase in Y in the E populations and an equivalent decrease in X in the R populations), then plasticity would end up differing between treatments, regardless of starting conditions. The possibility of equal and opposite changes in gene expression in the

two environments seems unlikely, because selection was likely to have been stronger in the ethanol-present environment. Moreover, under the equal and opposite change scenario, the M populations, in which selection acted on both X and Y , would have been expected to diverge in plasticity from both the E and R populations. This is best illustrated by an example: if selection in the E and R populations had favoured, respectively, an increase of Y by one unit and a decrease of X by one unit, causing changes in plasticity of +1 in each regime, then both changes would have been expected to have been favoured in the M populations, resulting in a net change in plasticity of >1 unit.

A simpler explanation for the limited evolutionary change in expression plasticity is that cross-environment genetic correlations in expression were generally too high to permit independent evolution in the two environments, so that correlated responses in gene expression were approximately equal to direct responses. (For now, we will assume that a cross-environment genetic correlation close to one implies both that genetic variation for plasticity is nearly absent and that correlated responses will equal direct responses; neither needs be the case if genetic variances are unequal in the two environments, a possibility that we will consider below). We are unaware of any studies that have directly assessed genetic variation for genomewide expression plasticity in *Drosophila*, but studies of yeast revealed substantial variation for expression plasticity among wild isolates grown on different media (Landry *et al.* 2006) or exposed to different temperatures (Eng *et al.* 2010). Similar studies on *Drosophila* would be valuable. QTL affecting expression plasticity have also been mapped in crosses between strains of yeast (Smith & Kruglyak 2008) and *C. elegans* (Li *et al.* 2006). None of these studies, however, investigated whether genetically variable populations allowed to adapt to different conditions showed changes in plasticity.

If genetic variances differ between environments, a situation termed 'scale effects', it is possible for genetic variation for plasticity to be present (in more formal terms, for genotype-environment interaction to be present) without implying $r_g < 1$, and hence without allowing independent evolution in the two environments (Yamada 1962; Fry 1992). More important, with scale effects, it is theoretically possible for r_g to be substantially less than one, but for the correlated response to equal the direct response, resulting in no change in plasticity. This is because the ratio of the correlated response of trait Y to the direct response of trait X , CR_y/R_x , is given by the 'genetic regression' of Y on X , which can be written as $r_g \sigma_{g,y}/\sigma_{g,x}$ (Falconer & Mackay 1996). Here, $\sigma_{g,x}$ and $\sigma_{g,y}$ are the square roots of the

additive genetic variances of traits X and Y , and r_g is the additive genetic correlation between the traits. Thus, if $\sigma_{g,y}/\sigma_{g,x} = 1/r_g$, the changes in the two traits will be the same, and plasticity will be unaffected. This seems unlikely as a general explanation for the lack of evolution of gene expression plasticity in our study, however, because it would require that, among genes whose expression evolved in response to ethanol, there was an inverse relationship between r_g and $\sigma_{g,y}/\sigma_{g,x}$. It seems more parsimonious to conclude that for most genes whose expression evolved in response to selection regime, r_g was close to 1 and $\sigma_{g,y}$ and $\sigma_{g,x}$ were similar in magnitude.

An additional possibility is that, to the extent that genetic variation for plasticity was present in our base population, alleles altering plasticity were prevented from contributing to selection responses due to adverse pleiotropic effects. (A related possibility, that evolution of plasticity was constrained by fitness costs of the mechanisms to maintain plasticity, seems less likely as a general explanation, because such costs would not have prevented plasticity from decreasing). One possible source of such adverse pleiotropic effects is suggested by the QTL mapping studies of yeast and *C. elegans* discussed above, which gave evidence that, relative to 'mean expression' QTL, plasticity QTL are enriched for trans-regulators that affect the expression of multiple genes simultaneously (Li *et al.* 2006; Smith & Kruglyak 2008). If this is the case in general, then selection to alter plasticity of a given gene could often be constrained by adverse pleiotropic effects on the regulation of other genes (Denver *et al.* 2005; Emerson *et al.* 2010).

We did find one piece of evidence that gene expression plasticity evolved in response to ethanol: those genes showing the most evidence (albeit not formally significant after correcting for multiple comparisons) for exposure \times selection regime interactions tended to show greater transcriptional responses to ethanol exposure in the unselected populations than in the selected populations, a pattern illustrated in Fig. 2. One interpretation of this result is that selection directly favoured 'canalization' (Waddington 1942) of expression of individual genes in the selected populations. While this may have been true to some extent, there is an alternative explanation. We previously showed that adults from the selected populations evolved higher activity of the ethanol-metabolizing enzymes alcohol dehydrogenase and aldehyde dehydrogenase (Fry *et al.* 2004). This was caused at least in part by increased frequency of the *Adh-Fast* and *Aldh-Phe* alleles (Table S2, Supporting information), both of which have amino acid substitutions that increase catalytic rate (Choudhary & Laurie 1991; Fry *et al.* 2008; M. Chakraborty and J.D. Fry

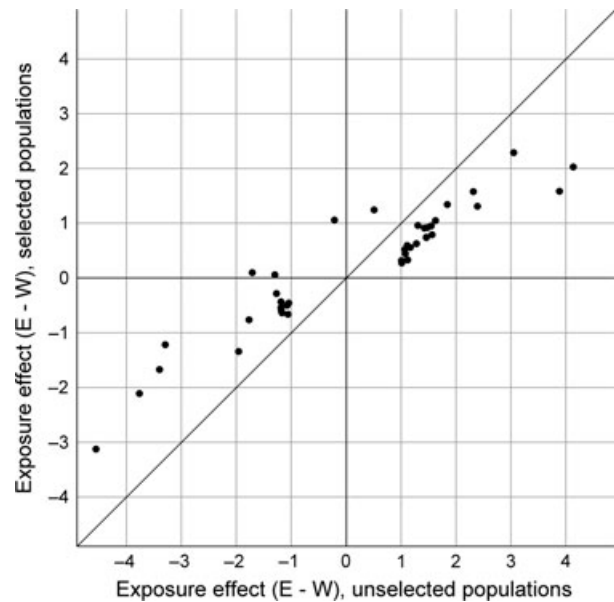


Fig. 2 Difference in expression between ethanol-exposed and control (water-exposed) larvae for probesets showing the most evidence of exposure \times selection regime interaction (albeit not significant after controlling for multiple comparisons). Probesets included are those for which interaction was significant at comparisonwise $P < 0.01$ and for which ethanol exposure resulted in a twofold or greater change in expression in either the selected (E and M) populations, unselected (R) populations, or both. The axes are in \log_2 scales (e.g. 2 = a fourfold expression difference). The diagonal line represents equal responses; note that in the vast majority of cases, the exposure response was greater in the unselected populations. (Although this pattern is visually striking, a statistical test of the pattern assuming independence of the points would not be appropriate, because expression values of different genes are correlated across samples).

unpublished); moreover, both of these genes, as well as *Gpdh*, another enzyme playing a key role in ethanol metabolism, showed evidence of higher expression in larvae of the selected lines (see Results). Thus, although we did not measure enzyme activity in embryos or larvae, we would expect ethanol-detoxifying ability of these stages to have been higher in the E and M populations than in the R populations, resulting in lower internal concentrations of ethanol and the toxic intermediate acetaldehyde to trigger gene expression changes. This, rather than changes in the regulatory machinery of individual genes, seems to us to be the most parsimonious explanation of the apparent 'damping' of gene expression responses to ethanol exposure in the selected populations shown in Fig. 2.

Our finding of relatively limited evolution of gene expression plasticity has interesting parallels in two experiments, one on *Drosophila* (Scheiner & Lyman 1991) and one on *Bicyclus* butterflies (Wijngaarden *et al.*

2002), in which workers selected for altered phenotypic plasticity of morphological traits with respect to temperature, with only limited success. Nonetheless, neither these results nor ours indicate that selection is unlikely to alter plasticity in natural populations over timescales of thousands or more generations. Indeed, in a comparison of gene expression responses to cadmium between cadmium-adapted and nonadapted natural populations of the soil arthropod *Orchesella cincta*, Roelofs *et al.* (2009) found hundreds of genes showing population \times exposure interaction in expression. Moreover, we have compared gene expression of African and European *D. melanogaster* natural populations using similar methods and sample sizes as described here, and identified nearly 2000 transcripts showing significant ($q < 0.05$) population \times environment interaction for expression (J.D. Fry and G.V. Glazko unpublished). The considerable differences in expression plasticity between the European and African populations, in contrast to the slight differences between our selected and control populations, give a sobering reminder that even a relatively long-running laboratory selection experiment cannot predict the results of a few thousand, let alone a few million, years of evolution in wild populations.

Relationship between gene expression plasticity and responses to selection

The direction and magnitude of the response of a gene's expression to selection might be expected to depend on whether the gene's plastic response to ethanol was adaptive. A 'naïve' adaptationist expectation is that short-term expression changes in response to a stress help protect against the stress, but not as much as would greater expression changes. Such seemingly suboptimal responses would be expected due to environmental unpredictability and limits on information (DeWitt *et al.* 1998): in nature, no environmental cue will be perfectly predictive of the continued presence of a particular stress, so that the fittest genotype in the long run will be one that does not completely 'commit' to the presence of the stress. In contrast, embryos developing in contact with ethanol in our E and M populations were in a highly predictable environment: upon hatching, the larvae were guaranteed to have no choice but to feed on ethanol-supplemented food. Under the adaptationist scenario, therefore, given that the E and M populations showed no evidence of evolving enhanced plastic responses to ethanol (if anything, the reverse was true; Fig. 2), we would have expected mean gene expression in these populations to have evolved largely in the same direction as the plastic responses. Our results refute this expectation: most

genes showing plastic responses did not show evolutionary responses, and *vice versa* (Table 1). Moreover, the main exceptions were genes that were downregulated by ethanol exposure but evolved higher expression in the selected lines, the opposite of the 'adaptationist' prediction. (We hypothesize that excessive downregulation of these genes, many of which are involved in DNA synthesis, gene expression or protein synthesis, may have delayed development on ethanol-supplemented food and that selection in the E and M populations favoured higher baseline expression of the genes to compensate; see Data S1, Supporting information for details).

In contrast to our failure to find support for the above 'adaptationist' prediction, Roelofs *et al.* (2009) found that many genes upregulated (downregulated) by cadmium in the nonadapted *O. cincta* population were constitutively upregulated (downregulated) in the cadmium-adapted population. In contrast, laboratory experiments in which *Drosophila* populations were allowed to adapt to cold (Telonis-Scott *et al.* 2009) or parasitoid attack (Wertheim *et al.* 2011) found only slight overlap between genes showing evolutionary changes in expression and those showing short-term transcriptional response to the stress. Similar results were found for natural *Daphnia* genotypes with and without a history of exposure to high water temperatures (L.Y. Yampolsky, J.K. Colbourne and M.E. Pfrender in preparation).

There was one set of genes in our study for which the adaptationist prediction appeared to hold: those encoding the three key enzymes in ethanol catabolism ADH, ALDH and GPDH. All three genes were significantly ($q < 0.05$) upregulated by ethanol exposure, and evolved significantly higher expression in the selected lines, albeit only at the comparisonwise $P < 0.05$ level. Moreover, as noted above, at both *Adh* and *Aldh*, amino acid variants conferring faster catalytic rates increased in frequency in the selected lines. For these genes, therefore, the initial degree of induction by ethanol may not have been sufficient to cope with the high ethanol concentrations experienced by the selected lines, and higher baseline enzyme activity appears to have been favoured.

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J.D.F. established the experimental populations and designed the experiments, with input from L.Y.; L.Y. performed the RNA extractions; G.G. and J.D.F. analysed the data; J.D.F. wrote the manuscript with assistance from L.Y. and G.G.

Data accessibility

The microarray data have been deposited in the Gene Expression Omnibus database, accession no.GSE38036 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38036>).

Supporting information

Additional supporting information may be found in the online version of this article.

Data S1 (1) Notes on enzymes upregulated by embryonic ethanol exposure; (2) Genes upregulated by ethanol exposure in common between this study and three previous studies of expression responses to ethanol vapour in adult flies; (3) Apparent digestive enzymes downregulated by ethanol

exposure; (4) Genes with known or suspected effects on ethanol resistance among those responding to selection; (5) Possible consequences of downregulation of growth-related genes by ethanol.

Table S1 Summary of analysis results for each of the 12 236 expressed probesets.

Table S2 *Adh* and *Aldh* allele frequencies in the experimental populations.

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