Evolution of phenotypic plasticity in response to ethanol between sister species with different ecological histories (*Drosophila melanogaster* and *D. simulans*)

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Running title: Evolution of plasticity to ethanol

Please note: Supplemental Files 1-3 are currently available at: https://github.com/signor-molevol/simulans-ethanol.git

Abstract

The role of phenotypic plasticity in evolution is contentious, in part because different types of plasticity – adaptive, neutral, or non-adaptive, are often not distinguished. Adaptive plasticity is expected to facilitate expansion into new environments, while non-adaptive plasticity will result in a mean phenotype further from the adaptive optimum and/or an increase in variance due to the expression of variation that was neutral and shielded from selection in the prior environment. We explore these patterns here by exposing *Drosophila melanogaster* and *D. simulans* to high ethanol concentrations, with the knowledge that *D. melanogaster* associates with high concentrations of ethanol in nature while *D. simulans* does not. Using changes in gene expression and splicing we find that in *D. simulans* there is a large genotype-specific response to ethanol, orders of magnitude larger than any effect that is not genotype-specific. In *D. melanogaster* the response to ethanol is limited, and it is concordant among different genotypes. This response to ethanol in *D. simulans* is enriched for non-protein coding nested genes that do not have orthologs in *D. melanogaster*, suggesting rapid evolution of transcription. Sequence variation in ethanol-implicated genes is consistent with balancing selection or selection relaxation in *D. simulans*, and they are more divergent from *D. melanogaster* than the genome average. Overall, these patterns are consistent with a maladaptive passive response to ethanol-induced stress in *D. simulans*, while in *D. melanogaster* the reduced response and lack of genotype-specific variation suggests selection for an optimal response.

Keywords: phenotypic plasticity, *Drosophila*, ethanol, alternative splicing

Introduction

Genetic variation occurs when there are differences in a phenotype due to genotypic differences among individuals within a population (Figure 1A). In contrast, plasticity – i.e. environment specific adjustment in phenotype - can occur without genetic variation between individuals and is a change in a given phenotype in response to an external stimulus (Figure 1A). Plasticity is a ubiquitous property of organisms, and it may be adaptive, neutral, or maladaptive with regard to the fitness of a plastic individual (Ghalambor *et al*. 2007; Lande 2009; Marais *et al*. 2013). Genetic variation in plasticity describes variation between genotypes for the response to the environment (Figure 1A). Quantifying genetic variation, plasticity, and genetic variation for plasticity is important for understanding the genotype to phenotype map and adaptation. For example, the evolution of a reduced plastic response can reconfigure the relationship between fitness and the environment, redrawing the genotype to phenotype map. In another example, genetic variation in plasticity can make the evolution of locally adapted populations unpredictable in response to environmental change.

It is often assumed that plasticity is adaptive, however phenotypic plasticity can be adaptive, neutral, or maladaptive. This depends in part on the evolutionary history of exposure to the agent causing plasticity. Were such exposure infrequent, plasticity could arise as a passive consequence of environmental stress. This is likely an important, or perhaps prevalent, form of phenotypic plasticity (Ghalambor *et al*. 2007; 2015). One potential prediction arising from stress inducing passive phenotypic plasticity is that within population variation for performance is expected to be greater, as stress breaks down buffering mechanisms that might normally reduce between individual variation (Joshi & Thompson 1997). Thus under lower stress conditions, populations will exhibit less individual and genetic variation compared to high stress, rare conditions (Ghalambor *et al*. 2007). The novel or rare high stress environment is essentially uncovering cryptic variation that was allowed to neutrally accumulate in lower stress environments where selection was unable to shape the reaction norm (Rutherford 2000).

When a population colonizes a new habitat, this could manifest as a transient increase in genotype by environment interactions, followed by a reduction in genetic variation for plasticity, assuming that there is selection for a single optimal phenotype in response to the environment (Via & Lande 1985; Guntrip & Sibly 1998; Lande 2009; Matzkin 2012; Huang & Agrawal 2016). The maintenance of plasticity, or loss of plasticity, would then depend upon the frequency with which the population continues to experience multiple environments, or the cost of maintaining the plastic response (Joshi & Thompson 1997; Lande 2009; Scoville & Pfrender 2010; Lee *et al*. 2011; Hodgins-Davis *et al*. 2012). Thus if phenotypic plasticity is costly to maintain, or the organism no longer experiences the previous environment, the expectation is that phenotypic plasticity will transition to local adaptation.

In this manuscript we will examine plasticity and genetic variation for plasticity in response to ethanol in *D. simulans* and *D. melanogaster*, two species with divergence adaptive histories with respect to ethanol. *D. melanogaster* and *D. simulans* are thought to have originated in or around Africa (*D. melanogaster*, southern-central Africa, *D. simulans*, Madagascar) followed by an out-of-Africa expansion ~10,000 years ago, however *D. melanogaster* may have colonized Europe and America earlier than *D. simulans* (David and Capy 1988; Lachaise et al. 1988; Baudry et al. 2004; Li and Stephan 2006; Thornton and Andolfatto 2006). *Drosophila simulans* is thought to have colonized the Americas in the last 100 years, creating a large and more recent population bottleneck than in *D. melanogaster* (Sturtevant 1920; Signor *et al.* 2017c).

D. melanogaster and *D. simulans* are cosmopolitan species and human commensals, however while *D. melanogaster* is commonly found inside houses, breweries, and wineries, *D. simulans* is more often observed in orchards and parks (though their niches overlap significantly and they are often found on the same patches) (David & Bocquet 1976; Parsons & King 1977; McKenzie & McKechnie 1979; Parsons & Spence 1981; Dickinson *et al.* 1984; Gibson & Wilks 1988; Thomson *et al.* 1991; Chakir *et al.* 1993; Mercot *et al.* 1994; Joshi & Thompson 1997). *D. melanogaster* is considerably more ethanol tolerant than *D. simulans*, and is found regularly feeding upon and ovipositing in resources with ethanol concentrations greater than 8% (Hoffmann & McKechnie 1991; Fry 2014; Zhu & Fry 2015). At

concentrations of 4% ethanol *D. simulans* shows reductions in survivorship and increased development time relative to *D. melanogaster* (Joshi & Thompson 1997). However, at low concentrations of ethanol (0.5 - 3.0%) adult *D. simulans* show increases in longevity (less so than adult *D. melanogaster*, which increases longevity from 0.5 - 9.0%) (Parsons *et al.* 1979). During multi-generation exposure to ethanol-rich substrates in the lab, *D. simulans* evolves tolerance (Joshi & Thompson 1997; Lefèvre *et al.* 2012). It has been demonstrated that ethanol tolerance comes as a trade-off that reduces the efficacy of processing other biochemical targets, thus it is possible that in *D. simulans* this trade-off is under different selective pressure (Chakraborty & Fry 2016). However, ethanol is by no means a novel resource for *D. simulans*.

In general genetic adaptation and phenotypic plasticity are considered as alternative adaptive strategies in response to the environment (Schlichting & Pigliucci, 1998). For a variety of traits *D. simulans* has been observed to be 'plastic' while *D. melanogaster* is described as being locally adapted (David & Bocquet 1975; Hyytia *et al.* 1985; Watada *et al.* 1986; Singh 1989) . For example, for temperature tolerance *D. simulans* has been shown to exhibit less difference between populations and to produce 'optimal' phenotypes under a wider range of environments (consistent with adaptive plasticity) (Austin & Moehring 2013). This includes less differentiation between populations, in that populations from different temperatures show similar levels of plasticity across temperatures. Population genetic surveys generally corroborate these phenotypic observations, where *D. melanogaster* also shows greater differences between populations, and more evidence of clinal variation than in *D. simulans* (Singh 1989; Weeks *et al.* 2002; Hoffmann & Weeks 2006; Schmidt & Paaby 2008; Machado *et al.* 2015; Sedghifar *et al.* 2016; Bergland *et al.* 2016; Signor *et al.* 2017c). However, *D. simulans* has generally been observed to harbor more within population diversity than *D. melanogaster* (Machado *et al.* 2015; Sedghifar *et al.* 2016; Signor *et al.* 2017c).

Gene expression is both affected by the environment and mediates an organisms response to the environment. It has been shown to be plastic in response to many environmental variables such as heat stress, ethanol, and the presence of predators (Hodgins-Davis & Townsend 2009; Levine *et al*. 2011; Yampolsky *et al*. 2012; Hodgins-Davis *et al*. 2012). While many studies have quantified the effect of the

121 environment on gene expression, far fewer have performed these experiments in multiple genotypes
122 within a common garden to obtain the interaction between genotype and the environment for gene
123 expression (DeBiasse & Kelly 2015). Indeed, most work on gene expression plasticity has not quantified
124 the interaction between genotype and the environment, and has found inconsistent results with regard to
125 theoretical expectations for the maintenance of plasticity (Cheviron *et al*. 2008; McCairns & Bernatchez
126 2009; Hodgins-Davis *et al*. 2012; DeBiasse & Kelly 2015; Heckel *et al*. 2016; Mathur & Schmidt 2016).
127 Furthermore, many plastic responses are time dependent, and if performed in a common garden without
128 sampling across time points may not detect early plastic responses that usher in 'maintenance'
129 phenotypes, or dynamic responses to the environment (Aubin-Horth & Renn 2009; Lewis *et al*. 2014). In
130 addition, alternative splicing has been shown to respond to changes in the environment, and it is thought
131 to diverge more rapidly than gene expression between lineages (Barbosa-Morais *et al*. 2012; Merkin *et al*.
132 2012; Gueroussov & Gonatopoulos-Pournatzis 2015; Jakšić & Schlötterer 2016; Wang *et al*. 2017; Pajoro
133 *et al*. 2017; Singh *et al*. 2017; Calixto *et al*. 2018). As such it may be an important component of
134 plasticity, necessary for adaptation to rapidly changing and heterogeneous environments (Marais *et al*.
135 2013; Preußner *et al*. 2017; Price *et al*. 2018; Calixto *et al*. 2018). Furthermore, it has been previously
136 implicated in the response to ethanol, and in *D. melanogaster* may be a more important component of the
137 response than gene expression changes (Oomizu *et al*. 2003; Newton *et al*. 2004; Pietrzykowski *et al*.
138 2008; Sasabe & Ishiura 2010; Hemby 2012; Zaharieva *et al*. 2012; Robinson & Atkinson 2013; Signor &
139 Nuzhdin 2018). However, alternative splicing is rarely accounted for in studies of plasticity.
140 Overall, we compare plasticity in response to ethanol, including genetic variation for plasticity
141 over time and between genotypes, among *Drosophila* species with different patterns of ethanol utilization.
142 We measure plasticity and variation in expression and splicing in response to ethanol treatment, and we
143 observe that in *D. melanogaster*, which regularly exploits ethanol-rich substrates, there is essentially no
144 genetic variation for plasticity within the tested population, while *D. simulans* exhibits an order of
145 magnitude larger effect of genotype by environment compared to other components of variance. This
146 suggests the following scenario: for *D. simulans* high ethanol concentrations are essentially a novel

147 stressful environment that reveals cryptic variation for phenotypic plasticity, while *D. melanogaster* does
148 not experience high stress under 15% ethanol and cryptic variation is not revealed. Furthermore, *D.*
149 *melanogaster* does not experience high stress as it is adapted to ethanol and genetic variation has been
150 removed in favor of an optimal, and reduced, plastic response
151

152 Methods

153 *Fly lines*

154 Male flies for *D. melanogaster* originated from six genotypes collected from an orchard in Winters,
155 California in 1998 and made isogenic by 40 generations of full sibling inbreeding (Yang & Nuzhdin
156 2003; Campo *et al*. 2013; Signor *et al*. 2017a; b). Male flies for *D. simulans* came from six genotypes
157 collected from the Zuma Organic Orchard in the winter of 2012 and made isogenic by 15 generations of
158 full sib inbreeding (Signor *et al*. 2017b; c). Residual heterozygosity is similar between these lines of *D.*
159 *melanogaster* and *D. simulans* (Campo *et al*. 2013; Signor *et al*. 2017c). In natural conditions flies will
160 not by homozygous at all loci, thus each inbred genotype was crossed to a white eyed 'tester' strain to
161 create the F1 flies used in the ethanol exposure assays (*D. melanogaster*, w^{1118}, Bloomington stock number
162 3605; *D. simulans*, w^{501}, Cornell species stock number 14021-0251.011). This design allows us to replicate
163 observations of gene expression because the flies are identical twins, while also maintaining
164 heterozygosity such that they more closely resemble wild type flies. Rearing occurred on a standard
165 medium at 25 °C with a 12-h light/12-h dark cycle. Several measures were taken to standardize offspring
166 quality: F1 flies were produced from females of the same age, these females were held at the same density
167 (10 individuals per sex/vial), males used for ethanol exposure assays were collected as virgins and reared
168 in single sex vials, and males used for assays were held at a standard density (24-30 per vial).

169 During the ethanol exposure assay a female was included as a stimulus, but was not collected for
170 RNA-seq (Signor *et al*. 2017a; b). The females were from y^1w^1 mutants for both *D. simulans* and *D.*

171 *melanogaster* (*D. melanogaster*, Bloomington stock number 1495, *D. simulans*, Cornell species stock
172 number 14021-0251.013). Quality of the females was controlled in the same manner as previously
173 described for males, and both males and females were aged three to four days before being used in the
174 ethanol exposure assay.

175 *Experiment setup*

176 Ethanol exposure took place in a circular arena, each of which was part of a larger chamber containing 12
177 arenas each with a diameter of 2.54 cm (VWR cat. no. 89093-496). Prior to the assay the flies were
178 sedated through exposure at 4 °C for ten minutes, to avoid the confounding effects of CO_2 exposure on
179 behavior and gene expression. Once the flies were sedated they were placed in the arenas with a
180 paintbrush (two males and one female per arena) and the chambers were secured with two small pieces of
181 cryogenic tape. It is standard to allow organisms to recover in a new location for ten minutes prior to
182 beginning behavioral assays, which was a portion of the goal of this study (see Signor *et al*. 2017a; b); so
183 the chambers were left upside down for ten minutes while the flies regained consciousness and oriented
184 themselves. The bottom of each chamber contained a standard amount of either grapefruit medium or
185 medium in which 15% of the water has been replaced with ethanol. During ethanol administration the
186 flies were recorded using VideoGrabber (http://code.google.com/p/video-grabber/), and set-up of the
187 assays was facilitated with FlyCapture (PointGrey, Canada). In order to better standardize the
188 transcriptional response, these videos were also used to determine if any male/female pairs mated during
189 the assay or any flies were damaged during setup, and flies in those chambers were not collected for
190 RNA-seq.

191 Assays were conducted for ten, 20, or 30 minutes for three replicates of each of the two conditions.
192 Flies are most active during the hours following dawn, thus to standardize behavior and circadian rhythms
193 all assays were conducted within a two-hour window after dawn. Replicates for both species were
194 conducted randomly under standardized conditions (25 °C, 70% humidity). At the conclusion of the assay
195 the chambers were flash frozen in liquid nitrogen, allowed to freeze through, transferred to dry ice, and all

196 of the males were collected for RNA-seq. For both *D. melanogaster* and *D. simulans* the expectation is
197 that intoxication is occurring through inhalation of ethanol vapors, and evidence of the behavioral effects
198 in both species and the efficacy of this approach have been published previously (Signor *et al*. 2017a; b).

199 *Sample preparation and RNA sequencing*

200 Sample preparation has been described previously, and will be briefly summarized here (Signor & Nuzhdin
201 2018). Flash-frozen flies were freeze dried and ten to 12 heads were placed into a 96-tube plate (Axygen
202 MTS-11-C-R). mRNA purification, cDNA synthesis and library preparation were carried out by RAPiD
203 GENOMICS (http://rapid-genomics.com) using a robot. mRNA was purified using Dynabeads mRNA
204 DIRECT Micro kit (Invitrogen # 61021) with slight modifications. To fragment the RNA mRNA-beads
205 were resuspended in 10 uL 2X first strand buffer (Invitrogen # 18064-014), incubated at 80 C for two
206 minutes and placed on ice, then the supernatant was collected after five minutes on magnetic stand. First
207 strand synthesis was performed using standard protocols for Superscript II (Invitrogen #18640-014) and
208 reverse transcription (25 C 10 min, 42 C for 50 min, 70 C for 15 min, 10 C hold). Second strand synthesis
209 was carried out using standard protocols with DNA Pol I and incubated at 16 C for 2.5 hours. cDNA was
210 purified with 1.8 volume of AMPure XP following manufactures instructions (Beckman Coulter A63880).
211 Illumina RNAseq libraries were prepared by Rapid Genomics (http://rapid-genomics.com/home/) using
212 dual barcodes. Sequencing was performed using the Illumina HiSeq 2500 as both 2×150 bp or 2×50 bp
213 reads. The two run lengths (and runs) were intended to provide extra coverage, and all replicates were
214 sequenced in both runs.

215 *Gene expression analysis*

216 It is common in organisms with alternative splicing for exons from different isoforms of a single gene to
217 overlap with one another, or be shared between all or most isoforms (Figure 1B). Short read data
218 fundamentally cannot resolve these exons to individual isoforms, however, one approach is to quantify each
219 exon separately and decompose exons overlapping between isoforms into those which are shared and

220 unique. When the differences between overlapping exons are less than 10 bp, there is no appreciable
221 amount of information loss in not decomposing overlapping exons, and this approach has been taken in the
222 past (Dalton *et al*. 2013; Graze *et al*. 2014; Newell *et al*. 2016; Fear *et al*. 2016). However, in many cases
223 the differences in exon overlap are much larger than this, so to address this issue we use a classification
224 scheme where reads may be assigned to exons, exonic regions, or exon fragments (Signor & Nuzhdin 2018)
225 (Figure 1B). Here, an *exon* does not overlap any other exons (as opposed to *exonic regions* or *fragments*),
226 and it may be unique if it is found in a single isoform, common if it is shared between some but not all
227 isoforms, or constitutive if it is found in all isoforms of a gene. For example, if a gene has one transcript,
228 all gene regions are unique constitutive exons (Figure 1B). If exons from different isoforms overlap, they
229 are grouped into an *exonic region*. Exonic regions are made up of exons that overlap from different
230 isoforms, thus they are not unique, but they may be common to several isoforms or constitutive and found
231 in all isoforms – they may also be formed of overlapping exons from neighboring genes (Figure 1B). *Exon*
232 *fragments* are classified by decomposing *exonic regions* based on the 5' and 3' positions of the exons within
233 the region. Thus all *exon fragments* are subregions of *exonic regions*. Exon boundaries were determined
234 using the *D. melanogaster* FlyBase 6.17 genome features file and the *D. simulans* 2.02 genome features
235 file. Alignment was performed using BWA-MEM version 0.7.15 and BED files were used to count reads
236 in each region and obtain the length adjusted read count (reads in region divided by the length of region),
237 and the APN (average per nucleotide) (Li 2015).

238 The APN was summed for technical replicates of the same read length then averaged between read
239 lengths to handle the mixture of read lengths for each sample (2×150 bp and 2×50 bp). If the APN was
240 greater than zero in at least half of all samples per condition it was considered detected. While we
241 considered several approaches to normalize coverage counts upper-quartile normalization with log-
242 transformation and median centering within time × treatment × genotype were selected due to better
243 performance of the residuals (Bullard *et al*. 2010; Dillies *et al*. 2013).

244 To test the significance of components of expression variation, the log APN for each exonic region was
245 modeled as

246 $$\Upsilon_{ijkl} = \mu + g_i + t_j + (gt)_{ik} + m_k + (gm)_{ik} + (tm)_{ijk} + \epsilon_{ijkl}$$

247 for the ith genotype (g_i), j^{th} treatment (t_j; j = ethanol or no ethanol), k^{th} time point (m_k; k = 10 min, 20 min, 30
248 min), and l^{th} replicate. For the interaction between treatment and time point, the log APN for each exonic
249 region was modeled as

250 $$\Upsilon_{ij} = \mu + t_j + \epsilon_{ij}$$

251 or the ith condition (time × treatment) and j^{th} replicate. Contrasts to compare treatments within time point
252 (ethanol versus no ethanol, for ten min, 20 min and 30 min) were conducted. Residuals were evaluated for
253 conformation with normality assumptions, and assumptions were met in excess of 95% of the models.

254 To evaluate whether there was evidence for splicing differences among times or treatments, exons
255 and exonic regions for each gene and for each sample were ranked and the most expressed region ranked
256 as one, the least expressed region as three and all others as two. Exon ranks for each gene were modeled as

257 $$\Upsilon_{ijk} = \mu + r_i + t_j + (rt)_{ij} + \epsilon_{ijkl}$$

258 where Υ_{ijk} is the exon rank (1,2,3) of the ith exonic region of the gene, j^{th} condition (time × treatment), and
259 the kth replicate; r_i is the exonic region of the gene; t_j is condition; and $(rt)_{ij}$ is the interaction between
260 exonic region and condition. We took this approach, rather than what may be viewed as a more traditional
261 GLM approach, due to a lack of normality in the distribution of model residuals. Accordingly, a non-
262 parametric test must be relied upon to look for changes in exon or exonic region representation between
263 exons of a gene and we used of a rank test to summarize changes in exon representation (Supplemental File
264 3). F-tests for the significance of the mean square attributed to the effect tested versus the mean square
265 attributed to error, or the appropriate interaction term, were used. The false discovery rate was controlled

266 using the Benjamini-Hochberg procedure, with a significance cutoff of $\alpha = 0.05$ (Benjamini & Hochberg
267 1995).

268 *GO Analysis*

269 When a gene in *D. simulans* had more than one ortholog in *D. melanogaster* only one ortholog was
270 included for the GO analysis, so as not to inflate the number of genes involved in a given process. This
271 does presume that orthologs will be annotated with the same GO terms, and this is generally the case. For
272 example, in *D. simulans* there is only *AOX4*, while in *D. melanogaster* there is *AOX3* and *AOX4*, but the
273 GO terms for each are the same. However, as *D. simulans* genes are generally not independently
274 annotated, especially those without *D. melanogaster* orthologs, if there was no *D. melanogaster* ortholog
275 the gene was not included in the enrichment analysis. This is a significant fraction of the overall genes
276 that were involved in the response to treatment, treatment by genotype, and genotype by treatment by
277 time, however there is no viable alternative. Lists of significant genes were tested for GO enrichment
278 using the PANTHER classification system (Mi *et al.* 2017). They were corrected for multiple testing and
279 a *p*-value of 0.01 was required for significance.

280 *Functional class enrichment*

281 To test for functional class enrichment multigene exonic regions were not included. Every test of
282 functional class enrichment compared the frequency of a given subcategory among all exons and exonic
283 regions detected in the dataset compared to the frequency within a significant list of exons and exonic
284 regions. A χ^2 test was performed in R to test the significance of the enrichment of each of these
285 categories.

286 *D. melanogaster polymorphism and divergence*

287 If the response to ethanol has been under selection in either *D. simulans* or *D. melanogaster*, the genes
288 which respond to ethanol exposure may show indicative population genetic signatures. To compare

289 polymorphism between the significant sets of genes and genome-wide averages we calculated Tajima's *D*
290 genome-wide for the source populations of *D. melanogaster* and *D. simulans*. We obtained the VCF files
291 from the Winters population sequenced by (Campo *et al.* 2013), which includes all six of the genotypes
292 assayed here as well as 29 other inbred lines sampled from the same population at the same time. The
293 coordinates of the genes implicated for exons, exonic regions, and exon fragments were converted from
294 the current assembly coordinates (v6) to those used in the previous study (v5). For *D. simulans* we used
295 data previously obtained from 170 individuals from this population (Signor *et al.* 2017c). To obtain
296 estimates that were consistent with our dataset (which is gene regions) we extracted regions from
297 genome-wide VCFs that corresponded to genes as annotated in the latest assembly. Note that we are
298 considering gene regions here rather than trying to include regulatory regions for a number of reasons: 1)
299 Gene regions includes introns, which will include some regulatory regions and splice sites. 2) The
300 location of regulatory regions for these genes is not well established, much less so between species where
301 we do not know if they may have shifted locations. This approach is more conservative than including
302 arbitrary amounts of putative upstream regulatory regions. 3) If there has been recent selection on a
303 regulatory region it may still be linked to the gene region and show the same differences in polymorphism
304 frequency. We excluded regions of reduced recombination near the centromeres, either 1 MB or more if
305 there were significant reductions in diversity for a broader region, calculated as extended negative
306 Tajima's *D* or values of $\pi < ½$ the chromosomal average (Sedghifar *et al.* 2016; Signor *et al.* 2017c).
307 SNPs within these coordinates were separated using bedtools intersect (v2.26.0). We excluded regions
308 from the fourth chromosome and unassembled scaffolds for the analysis of population genetic differences.
309 In both species this is a trivial number of significant regions. Alternate SNPs that were present in > 99%
310 of the mapping population were excluded, as were SNPs with more than 10% missing data. Tajima's *D*
311 was calculated in windows of 1 KB using VCFtools v1.12a. Windows of Tajima's *D* that overhung the
312 ends of genes were included in the analysis.

313 We calculated per-gene D_{XY} for the significant exons, exonic regions, and genes with significant
314 changes in rank abundance, to determine if there were any unusual patterns of divergence for these

315 subsets. To calculate divergence comparable regions of the genome need to be identified, and there has
316 been considerable evolution of transcription start sites between *D. melanogaster* and *D. simulans* (Main *et*
317 *al.* 2013). Comparing regions annotated as the gene regions in each species introduces large and
318 unexpected gaps in the start of the alignments. While the evolution of transcription start sites is of
319 interest, if unaccounted for it will artificially inflate estimates of D_{XY}. Thus for genes implicated in
320 differences in *D. melanogaster* the regions annotated as genes were blasted using ncbi blastn (v2.4.0) to
321 the *D. simulans* assembly and then back to the *D. melanogaster* assembly and used for divergence
322 statistics, and vice versa for *D. simulans* genes. This means that for different genes small non-coding
323 regions may be included depending upon the direction of evolution of transcription start sites, or small
324 portions of coding regions may be excluded. Furthermore, for genes from *D. simulans* with more than one
325 ortholog in *D. melanogaster* the top blast hit was used, and while there may have been orthologous
326 sequence in *D. melanogaster* (or *D. simulans*) if there was no annotated ortholog it was excluded from the
327 analysis. For example, some of the non-protein coding genes from *D. simulans* have very conserved
328 BLAST hits in *D. melanogaster* that are not annotated as containing genes. The sequences were aligned
329 using the R package DECIPHER (Wright 2016) and if necessary they were reverse complimented using
330 Biostrings (Pages *et al.* 2017, for example, in the region on 3R in *D. melanogaster* that is an inversion).
331 Following alignment the *D. simulans* and *D. melanogaster* reads were output separately in aligned fasta
332 format and D_{XY} was calculated using an R script courtesy of Dr. Emily Delaney, which incorporates
333 commands from the R packages ape and pegas (Supplemetnal file 4, Paradis *et al.* 2004; Paradis 2010)) .
334 This was done for every orthologous gene in the genome from *D. melanogaster* and *D. simulans*, and
335 each significant subset from each component of variance, to compare distributions of divergence.
336

337 Results

338 *Gene expression and isoform usage*

339 It is difficult to decouple alternative isoform usage from gene expression, given that many exons are
340 shared between isoforms or overlap other exons. To infer isoforms from short read data, one must rely
341 upon unique junctions or regions of individual isoforms and extrapolate to shared regions. This requires
342 accurate isoform annotation (knowing that any given exon/junction is found in combination with other
343 exons/junctions) and in general can be very noisy. Accordingly, we subscribe to a simpler but more robust
344 approach and summarize the abundance of different exons and exonic regions separately. We detail
345 overall abundance of exons, exonic regions, and exon fragments, and changes in the rank abundance of
346 exons within a gene (Figure 1 C&D). As illustrated in Figure 1 C&D, these approaches summarize
347 different features of expression and splicing – for example differences in the expression level of exon
348 fragments from *cabut* suggest both that there are overall expression differences for the gene between
349 environments, and that in both *D. simulans* and *D. melanogaster* one of the alternative isoforms is more
350 abundant with ethanol (Figure 1C). While exons within *Drat* change their expression in response to
351 ethanol by time, and between environments at 30 minutes, that doesn't capture the fact that the third exon
352 is most abundant in ethanol while the first is most abundant without it, suggesting differences in isoform
353 abundance that may also belong to unannotated isoforms (Figure 1D). In the following sections we will
354 first summarize changes in exon, exonic region, and exon fragment abundance followed by differences in
355 rank abundance between environments.

356 *Exons, exonic regions, and exon fragments*

357 1994 exons, 406 exonic regions, and 608 exon fragments changed their expression in response to
358 genotype in *D. simulans*, while in *D. melanogaster* 1445 exons, 631 exonic regions, and 1135 exon
359 fragments altered their representation (Table 1). Note that the results for *D. melanogaster* are summarized
360 in (Signor & Nuzhdin 2018), but are included here for comparison. 76 exons, 23 exonic regions, and 18
361 exon fragments changed expression in response to ethanol in *D. simulans*, compared to 15 exons, 13
362 exonic regions, and 21 exon fragments in *D. melanogaster* (Table 1; Signor & Nuzhdin 2018). Seven
363 exons and exonic regions were shared between species for treatment, *Drat*, *cabut*, *CG11741*, *CG32512*,

364 *CG4607*, *Pino*, and *sugarbabe.* For all discussion of shared genes, the particular exon or exonic region
365 may or may not be the same, as well as the direction or nature of the change in expression. The
366 complexity of this comparison is shown in Figure 1C, where in *D. melanogaster cabut* has three
367 annotated transcripts and four exon fragments (one exonic region), while in *D. simulans cabut* there are
368 two transcripts, one exon, and two exon fragments (one exonic region). In addition, in *D. melanogaster*
369 *cabut* is nested within the gene *ush*, while in *D. simulans* it is not nested. In *D. melanogaster* an area
370 annotated as an intron in *D. simulans* is unique to its third isoform, and this is more frequent with ethanol
371 than without; in *D. simulans* there is one unique exon fragment belonging to one isoform which is more
372 frequent with ethanol than without, suggesting that isoform is more common in ethanol environments
373 (Figure 1C). In *D. melanogaster* the exon fragment which is unique in *D. simulans* is not unique, and only
374 increased frequency of its unique third isoform can be inferred (Figure 1C). The interaction between
375 genotype and treatment was significant for 387 exons, 98 exonic regions, and 81 exon fragments in *D.*
376 *simulans* and three exons, no exonic regions, and no fragments in *D. melanogaster* (Table 1; Figure 2
377 A&B, Signor & Nuzhdin 2018). No fusions or fragments were shared between *D. simulans* and *D.*
378 *melanogaster*.

379 24 exons, seven exonic regions, and six exon fragments were significantly different in response to
380 the interaction between ethanol and time in *D. simulans*, compared to 22 exons, eight exonic regions, and
381 12 fragments in *D. melanogaster* (Table 1; Signor & Nuzhdin 2018). Three genes were shared between
382 exons and exonic regions for these species, *Drat*, *cabut*, and *CG43366*. In *D. simulans* 1158 exons, 299
383 exonic regions, and 225 exon fragments were significantly different for the interaction between genotype,
384 ethanol, and time, while in *D. melanogaster* no exons, two exonic regions, and four exon fragments were
385 significantly different (Table 1; Figure 2 A&B, Signor & Nuzhdin 2018). No exon fusions or fragments
386 were shared between species for this component of variance. At ten minutes nine exons, three exonic
387 regions, and four exon fragments are significantly different between treatments, compared to two exons,
388 one exonic region, and three exon fragments in *D. melanogaster* (Table 1; Figure 2A). *cabut* is shared

389 between exons and exonic regions in *D. melanogaster* and *D. simulans*. At 20 minutes three exons, one
390 exonic region, and two exon fragments were different in *D. simulans*, two of which belong to *cabut* in
391 both exons/exonic regions and exon fragments. In *D. melanogaster* one exon, four exonic regions, and
392 five exonic fragments are different at this time point, and the gene *cabut* is shared between species for
393 both categories. At 30 minutes 29 exons, four exonic regions, and six fragments in *D. simulans* were
394 different between treatments, all of which have *D. melanogaster* orthologs (Table 1). In *D. melanogaster*
395 46 exons, 24 exonic regions, and 20 exon fragments were significantly different between treatments at 30
396 minutes (Signor & Nuzhdin 2018). Among exons and exonic regions *Drat*, *CG32103*, *sugarbabe*, *cabut*,
397 *Pinocchio*, *CG32512*, and *CG4607* are shared between species. Among exon fragments *cabut* and
398 *Pinocchio* are shared.

399 <u>*Shared and unique exons, exonic regions, and fragments*</u>

400 Comparisons between *D. simulans* and *D. melanogaster* are shown in Figure 2C for the three components
401 of variance in which enough genes are implicated in *D. melanogaster* to make the frequency of different
402 categories meaningful. When an exon is unique/constitutive it belongs to the only annotated transcript for
403 that gene, and as such no exonic regions are unique/constitutive. However, the combined counts are
404 shown for the common and constitutive categories. However, in *D. melanogaster* constitutive exons and
405 exonic regions are much more common than in *D. simulans*, where unique/constitutive exons are
406 overwhelmingly implicated. This could potentially be explained by differences in annotation, for example
407 if fewer genes have multiple transcripts annotated in *D. simulans*. While this does appear to be the case
408 (Supplemental Figure 1), it is unclear if it is enough to explain the discrepancy between species. It is also
409 possible that in response to the environment genes with alternative splicing are more important in *D.*
410 *melanogaster* compared to *D. simulans*. The proportion of genes in each category in *D. simulans* is
411 consistent between components of variance, including ethanol by genotype and ethanol by genotype by
412 time, again suggesting the possibility that differences in annotation are responsible. If annotation
413 differences are responsible this does not affect the overall results, for example the number of exons

414 implicated, but may make comparison between species for the number of unique versus constitutive
415 differences not meaningful. It also underlines the importance of not relying upon isoform annotation
416 when trying to understand differences in expression and alternative splicing.

417 *GO enrichment analysis*

418 We report here only the results of enrichment for exons and exonic regions (Table 2). In *D. simulans* the
419 response to treatment, treatment by time, and the three time points were not enriched for any GO terms.
420 Much of the lack of enrichment is likely due to annotation issues – for example 9% of the exons
421 implicated in treatment over time could not be resolved to a single gene, and of the remaining genes 14%
422 do not have a *D. melanogaster* ortholog. Of those with annotated orthologs, 20% do not have any gene
423 ontology terms associated with them. A summary of significant GO terms for the remaining components
424 of variance is shown in Table 2. In *D. melanogaster* exons and exonic regions were not significantly
425 enriched for any category of genes. In general, the small number of genes implicated for many categories
426 precludes any conclusion of enrichment.

427 *Changes in rank abundance*

428 In response to treatment 54 genes showed changes in rank abundance, and 94 genes change the rank
429 abundance of their exons for the interaction between treatment and time. In *D. melanogaster* 71 genes
430 changed the rank abundance of their exons in response to ethanol and 145 changed for treatment by time
431 (Signor & Nuzhdin 2018). No genes were shared between species. For the interaction between treatment
432 and time *lola*, *Mhc*, and *Prm* were shared between species. Figure 1D illustrates an example of a change
433 in rank abundance for the gene *Drat* in *D. simulans*, where with ethanol the third exon is most abundant,
434 and without ethanol the first exon is most abundant. While differences in exon abundance summarized
435 above capture some of this variation, as *Drat* is significant for some exons for ethanol by time and 30
436 minutes, the change in rank abundance highlights potential differences in exon inclusion between
437 environments.

438 *GO enrichment analysis*

439 Changes in rank abundance were not enriched for any GO terms in *D. simulans*, with the exception of
440 peroxiredoxin activity in response to ethanol, albeit slightly above the more conservative cut-off used in
441 our other tests (p = .017). Peroxiredoxin activity has been associated with protection against alcohol
442 induced liver damage (Bae *et al.* 2011; Chattopadhyay *et al.* 2015). In *D. melanogaster* genes implicated
443 in changes in rank abundance in response to ethanol by time were enriched for cellular components actin
444 cytoskeleton, again somewhat above our more conservative cutoff (p = 0.018) (Signor & Nuzhdin 2018).

445

446 *In D. simulans genotype-specific reactions to the environment are abundant*

447 In *D. melanogaster* components of variance for interaction terms have very few significant genes, with
448 the largest category being exons and exonic regions that respond to ethanol and that are different at 30
449 minutes. *D. simulans* is roughly comparable for the majority of these categories. However, many more
450 exons and exonic regions are significant for components of variance that interact with genotype: 1457 for
451 the interaction between genotype, treatment and time, and 486 for the interaction between genotype and
452 treatment, compared to two and three exons and exonic regions respectively in *D. melanogaster* (Table 1;
453 Figure 2 A&B). This suggests that in *D. simulans* interactions with genotype are a far more important
454 component of the response to ethanol than in *D. melanogaster*. It is also worth noting that in *D. simulans*
455 the response to ethanol (plasticity) is three times as large (28 exons and exonic regions in *D.*
456 *melanogaster* compared to 99), though this is many order of magnitudes less of a difference than for
457 genetic variation for plasticity.

458

459 *Genotype-specific responses are enriched for non-coding genes in D. simulans*

460 A large number of non-protein coding genes were implicated in gene expression changes in *D. simulans*
461 in these analyses, so we applied a χ^2 test to understand if our gene lists were enriched for this functional
462 category. The number of non-protein coding genes that were significant for ethanol, ethanol by genotype,
463 and ethanol by genotype by time, were more than would be expected by chance in *D. simulans* (13, $\chi =$

464 $49.598, p < 0.0005$; $68, \chi = 235.21, p < 2.2\ x\ 10^{-16}$; 196, $\chi = 727.17, p < 2.2\ x\ 10^{-16}$). Other than
465 the pseudogenes, these are long non-coding RNAs (lncRNAs) as the shortest is annotated at 713 bp, and
466 the majority are over 4,000 bp (long non-coding RNAs being any non-protein coding genes over 200 nt).
467 The interaction between treatment and time, and the differences in expression at ten, 20, and 30 minutes
468 were not enriched for non-protein coding genes, nor were changes in rank abundance.
469 *D. melanogaster* is not enriched for non-protein coding genes in response to ethanol, ethanol by
470 time, ethanol by genotype, ethanol by genotype by time, ten or 20 minutes, exons or exonic regions
471 expressed only in one environment, or genes implicated in changes in rank abundance. However, at 30
472 minutes *D. melanogaster* is enriched for non-protein coding genes, though this concerns far fewer genes
473 than in *D. simulans* (5, $\chi = 12.831, p = 0.005$).
474
475 *Non-coding genes that respond to the environment in D. simulans do not have D. melanogaster orthologs*
476 Given that a considerable number of non-protein coding genes were implicated in this analysis for *D.*
477 *simulans*, we were suspicious that this may be an annotation issue in *D. melanogaster*. Using the exons
478 associated with every transcript from each of these non-protein coding genes as a reference we did not
479 find that *D. melanogaster* reads mapped to these exons across a range of relaxed mapping parameters
480 allowing for mismatches and gaps (using bwa mem, from defaults to -B 2 -O 3; using both the *D.*
481 *simulans* exons or existing homologous regions in *D. melanogaster*). In several cases, such as *CG30377*,
482 an annotated protein coding gene in *D. melanogaster* is annotated as a pseudogene in *D. simulans*, with
483 no noted relationship between them. Therefore there may be a small number of cases in which an ortholog
484 exists but is not recognized, but in general these non-protein coding genes appear to be unique to *D.*
485 *simulans*. It has been noted previously that lncRNAs can share high sequence similarity between related
486 species, but be expressed in only one (Ulitsky 2016). Determining the orthology of these genes and the
487 dynamics of non-protein coding gene evolution on the *Drosophila* phylogeny is an interesting question
488 that will require additional future research.
489

490 *The response to the environment is enriched for nested non-protein coding genes in D. simulans*

491 In analyzing the non-protein coding genes identified in *D. simulans* we noted that many of them were
492 nested in the introns of other genes. We investigated the frequency with which nested genes were
493 implicated in our analysis, and nested non-protein coding genes, to understand the possibility that
494 differences in expression could also be changes in intron retention in the parental genes (as 'nestedness'
495 generally refers to exons or entire genes found within the introns of other genes). Using the criteria that an
496 exon nested in an intron must overlap the intron by at least 80 bp or 10%, we found that in *D.*
497 *melanogaster* 9.2% of exons were nested within introns, while in *D. simulans* 9.7% were nested, similar
498 to what has been previously reported (Lee & Chang 2013). This was reflected in our data, where for both
499 *D. melanogaster* and *D. simulans* 6.8% of exons and exonic regions were nested within introns (lower
500 because multi-gene exons were excluded). However, among significant exons and exonic regions in *D.*
501 *melanogaster* 18.6% were nested, while in *D. simulans* 33.5% were nested. This is a significant
502 enrichment of exons that are nested within other introns, for both *D. melanogaster* ($\chi = 12.344, p <$
503 $.0004$) and *D. simulans* ($\chi = 1721.1, p < 2.2\ x\ 10^{-16}$).

504 The remaining question then is whether nested genes are more likely to be non-protein coding, or
505 whether the dataset is enriched for both. Indeed, compared to the total number of nested genes that are
506 noncoding within the dataset, the number that are significant for the response to ethanol is enriched for
507 nested, non-protein coding genes in *D. simulans* ($\chi = 237.49, p < 2.2\ x\ 10^{-16}$), but not in *D.*
508 *melanogaster*. Overall *D. melanogaster* has more annotated non-protein coding genes (2963) than *D.*
509 *simulans* (1675), and similarly more exons from noncoding genes are nested within introns (1772 *D.*
510 *melanogaster*, 1066 *D. simulans*), suggesting that this pattern is not reflective of annotation issues
511 between the two species. However, the question remains as to why nested genes are so much more likely
512 to be non-protein coding than non-nested genes – for example in the total dataset of *D. simulans* 9.7% of
513 exons are nested, while of the annotated non-coding genes in *D. simulans* 63% are nested. Furthermore
514 the importance of nested non-protein coding genes for the response to the environment, or potentially
515 delays in splicing that are specific to certain components of variance, is unclear.

516

517 *Nested non-protein coding genes involved in the response to ethanol are regulated independently of their*
518 *parental gene*

519 In *D. simulans* 328 significant nested genes (375 nested exons and exonic regions) are on the opposite
520 strand as their parental gene, 75% of the total significant nested genes. Of non-protein coding genes, this
521 bias is stronger, with 83% having the opposite strandedness as their parental gene. In *D. melanogaster* 13
522 significant genes are nested (16 total exons and exonic regions) and of these nine are on the opposite
523 strand as their parent gene (69%). Only one non-protein coding gene shares strandedness with its parental
524 gene, *CR44660*/*drp1*. It has been observed that many nested non-protein coding genes require expression
525 of the parental gene, share strandedness, and depend on splicing out of the parental intron for activation
526 (miRNAs, (Boivin *et al.* 2018). In both *D. simulans* and *D. melanogaster*, however, we do not observe
527 non-protein coding genes as being more likely to share strandedness with their parental gene among our
528 significant nested exons and exonic regions. This suggests that whatever the reason for the observed
529 enrichment in nested non-protein coding genes, it is likely not because the parental genes are being
530 expressed and the nested non-protein coding genes are being spliced out of the introns. We could not test
531 the *D. melanogaster* dataset for correlation between the expression of parental and nested genes as it is
532 too small. However, in *D. simulans* genes that were both non-coding and shared strandedness with their
533 parental gene had highly correlated expression differences between treatments (0.83, 14 genes). All other
534 categories (i.e. nested non-protein coding, opposite strand) were essentially uncorrelated (0.09-0.27). We
535 note that of the parental genes only three are also represented as significant in the main dataset, *sima*,
536 *mira*, and *bru-3* (significant in response to ethanol or genotype by ethanol). Thus it is unlikely that overall
537 the observed differences in expression are due to changes in the expression of the parental gene, as this
538 would likely result in differences in expression detected at both loci.

539

540 *Polymorphism and divergence in D. melanogaster and D. simulans*

541 We were interested to determine if the patterns of polymorphism and divergence in the genes implicated
542 in the response to ethanol suggested that they were evolving under a particular selection regime or were
543 unusually diverged compared to background levels of polymorphism or divergence. If the genes
544 implicated in expression differences in *D. melanogaster* had unusually low Tajima's *D*, for example, this
545 could suggest that directional selection for adaptation to ethanol is responsible for the observed
546 expression differences. We compare polymorphism in genes with expression differences in D. simulans
547 in both *D. simulans* and *D. melanogaster*, because it is possible that it is those same genes that were under
548 selection for adaptation to ethanol in *D. melanogaster*. This might help to clarify whether the observed
549 expression differences are adaptive or show evidence of adaptation. For both polymorphism and
550 divergence in the following sections we will compare the genome-wide background distribution of
551 polymorphism and divergence to differences in expression and rank abundance as a whole, rather than by
552 components of variance, for two reasons. Firstly, for the majority of categories other than genotype
553 interaction terms in *D. simulans*, the number of independent genes on each list is ~50% confounding any
554 attempt to separate out components of variance. Second, in *D. melanogaster* there are too few genes that
555 are significant for many components of variance and they cannot be considered separately.
556 *Polymorphism*
557 We calculated Tajima's *D* in windows of 1 kb for both all annotated gene regions in the genome and the
558 subset of genes implicated in differences in ethanol response in both species, and consider anything in the
559 top ±2.5% to be an outlier. Outliers for *D. simulans* and *D. melanogaster*, separated between the X and
560 the autosome, are shown in Table 3, and the percentage of outliers for each category are graphed in Figure
561 3A.
562
563 ***D. melanogaster***
564 For changes in abundance of exons and exonic regions on the X and autosome, *D. melanogaster* is not
565 enriched for outliers of Tajima's *D* but there are more outliers than expected in *D. simulans*
566 (Autosomes: $\chi = 5.26, p < 0.02$, X: $\chi = 29.361, p < 0.0005$). In *D. melanogaster* these intervals

567 correspond to 27 genes, while in *D. simulans* they cover a total of 25, including both the X and
568 autosomes. Between the two lists there is considerable overlap in the location of outlier intervals, at
569 *CG43366*, *AOX4*, *dsb*, *fumble*, *CG31875*, *Mical, Ada2b, forked, CG1986,* and *CG42749.*
570 For genes implicated in changes in rank abundance in *D. melanogaster*, there is also no
571 significant enrichment for outliers of Tajima's *D* relative to the genome-wide frequency in *D.*
572 *melanogaster* or *D. simulans.* In *D. melanogaster* on the autosome these intervals cover 59 genes, while
573 in *D. simulans* they cover 56. *14-3-3ε*, *AcCoAs*, *boi*, *CG31522*, *CG34398*, *eff*, *elF4EHP*, *fru*, *grh*, *kay*,
574 *lds*, *lola*, *mRpL21*, *osp*, *siz*, *Syp*, *Tep2*, and *tweek* are shared in containing outlier intervals for Tajima's *D*
575 in both *D. simulans* and *D. melanogaster*. In *D. simulans* on the X chromosome these intervals cover
576 four genes, while in *D. melanogaster* they cover nine, and two are in common between the species
577 (*CG34417*, *Ten-a*).
578
579 ***D. simulans***
580 Many more genes were implicated in the response to ethanol in *D. simulans*, and for exons and exonic
581 regions that changed in abundance there was an excess of outliers for Tajima's *D* for the autosomes and
582 the X chromosome (Table 3; Figure 3; Autosomes: $\chi = 9.41, p = 0.0022$, X: $X = 14.03, p = 0.00018$).
583 These intervals correspond to 428 unique genes, and many outlier intervals overlap the same gene region.
584 For example, every 1 kb interval that overlaps the gene *Cubilin* is an outlier, with an average of -2.41.
585 Polymorphisms in the human ortholog of *Cubilin*, *CUBN*, have been associated with lifetime rates of
586 heavy drinking (Hamidovic *et al.* 2013). It is worth noting here that the outliers that were significantly
587 enriched in the *D. melanogaster* dataset for *D. simulans* were all in the positive direction, while these are
588 both positive and negative. Given the tendency towards positive Tajima's *D* in this population of *D.*
589 *simulans*, negative values of Tajima's *D* may be a more suggestive measure of selection, either purifying
590 or directional (Signor *et al.* 2017c). In *D. melanogaster* the genes that were implicated in changes in the
591 expression of exons and exonic in response to ethanol in *D. simulans* were less enriched for outliers on
592 the X chromosome than expected, and not enriched on the autosomes (X: $\chi = 6.38, p = .0012$).

593 For genes implicated in changes in rank abundance in *D. simulans* there was a significant
594 enrichment compared to genome-wide frequency for both the autosomes and the X (Autosomes: $\chi =$
595 $19.78, p < 8.7\ x\ 10^{-6}$, X: $\chi = 458.73, p < 2.2\ x\ 10^{-16}$). This includes intervals in 44 unique genes
596 including 16 intervals in the gene *Mondo*. In *D. melanogaster* this did not constitute an enrichment
597 relative to genome-wide. Outlier intervals that occur within shared genes between the two species include
598 *CG7881*, *lola*, *mbf1* (a stress response gene), *Mhc*, *PI31*, and *Pka-1* (which has been implicated in the
599 response to ethanol previously (Chen *et al.* 2008)). Note that for changes in the abundance of exons and
600 exonic regions in *D. simulans*, if regions of low recombination are excluded from the candidate regions
601 the enrichment is no longer significant, but there is no change among the other tests.
602
603 *Divergence*
604 We calculated D_{XY} for orthologous genes on the autosomes (10,435 genes) and the X (1,943 genes)
605 between *D. melanogaster* and *D. simulans* (Figure 3B). D_{XY} was similar to that previously reported, with
606 an average of 0.52 on the autosomes and 0.55 on the X (compared to 0.048 on the autosomes, 0.054 on
607 the X (Nolte *et al.* 2013)). For the subsets of genes implicated in expression differences the mean was
608 0.047 on the autosomes and 0.057 on the X for expression differences in *D. melanogaster* and 0.053 on
609 the autosomes and 0.06 on the X in *D. simulans*. The genes implicated in changes in rank abundance in *D.*
610 *melanogaster* had a mean D_{XY} of 0.047 on the autosomes and 0.05 on the X. In *D. simulans* this was
611 0.049 on the autosomes and 0.059 on the X. To determine if the distribution of D_{XY} for any of the
612 significant subsets of genes differed compared to the genome-wide distribution we used a Kolmogorov-
613 Smirnov two-sample test, separately for genes on the X and the autosomes. In *D. melanogaster* exons and
614 exonic regions that changed abundance in response to ethanol were not significantly different on the
615 autosomes or the X. However, exons and exonic regions with significant differences in rank abundance
616 did differ in distribution on the autosomes, but not the X (Autosomes: D = 0.14, p = 0.002). The genes
617 implicated in expression differences on the autosomes in *D. simulans* differed significantly from the

618 genome-wide distribution, but not on the X (Autosomes: D = 0.061, p = 0.005). However, those
619 implicated in changes in rank abundance did not, on the autosomes or the X. A difference in distribution
620 does not indicate the direction of effect, and in fact in *D. melanogaster* the mean D_{XY} for changes in rank
621 abundance is lower than genome-wide, while for expression differences in *D. simulans* it is slightly
622 higher. This suggests that the genes implicated in changes in rank abundance in response to ethanol in *D.*
623 *melanogaster* are somewhat more conserved than expected based on genome-wide distributions, while
624 those implicated in expression differences in *D. simulans* are slightly less conserved. Note that because
625 we can only include genes with orthologs here this does not include any of the abundant non-protein
626 coding genes implicated in *D. simulans*, likely to be among the more rapidly evolving genes.
627 Population genetic patterns – or a lack thereof- among genes that respond to ethanol are difficult
628 to interpret, in part because plastic responses may be due to a single regulatory change at an upstream
629 gene. As the number of regulatory (or coding) changes involved in the plastic response are not known, it
630 is difficult to interpret a lack of enrichment, as it is possible, for example, that only one of the genes
631 whose expression changed could be under selection. Furthermore, it is not known whether the response to
632 ethanol is adaptive – while it is presumed to be so in *D. melanogaster* due to its relative fitness on ethanol
633 compared to *D. simulans*, functional links between gene expression differences and the phenotype were
634 not established.
635
636 Discussion
637 In this study both *D. melanogaster* and *D. simulans* have a plastic response to the environment, but *D.*
638 *melanogaster* is lacking in genetic variation for plasticity. Given the observed patterns, it appears most
639 likely that for *D. simulans* 15% ethanol is a novel environment, and that the marked G x E is a passive
640 stress response. This maladaptive plasticity would allow for the expression of previously cryptic variation
641 that had accumulated in the absence of selection. In *D. melanogaster* this environment may not be
642 stressful or novel, resulting in past selection removing genetic variation for plasticity that in *D. simulans*
643 was allowed to neutrally accumulate. In this sense *D. melanogaster* may be more 'locally adapted', as it

644 has evolved a reduced plastic response compared to *D. simulans*. However, it could also be that because
645 *D. melanogaster* colonized the Americas a few thousand years prior to *D. simulans*, *D. simulans* has not
646 yet reached an optimal level of plasticity (a nonequilibrium situation). Also, the frequency with which
647 they encounter ethanol rich environments may vary for *D. simulans* and *D. melanogaster*, resulting in
648 different selection pressures for developing the optimal level of plasticity – particularly if there is a trade-
649 off with traits that are beneficial to the semi-domestic habitat of *D. simulans*. Trade-offs among
650 genetically correlated traits may mean that a genotype with optimal phenotypic plasticity in one
651 environment is constrained from evolving the optimal phenotype in another environment, resulting in the
652 maintenance of variation for phenotypic plasticity.

653 Joshi and Thompson (1997) found that in *D. simulans* there was greater between family variation
654 for the phenotypic response to ethanol substrate prior to selection for tolerance to ethanol. Compared to
655 *D. melanogaster* and control populations, after selection in an ethanol environment the greatest change
656 was a reduction in variation for plasticity between *D. simulans* families. Joshi and Thompson (1997)
657 quantified phenotypes such as development time, which are not easily generalizable to expression
658 patterns, but it is suggestive. However, in studies of other phenotypes in presumably adapted and non-
659 adapted populations of *D. melanogaster*, no increase in genotype by environment response were observed
660 in non-adapted populations (Heckel *et al.* 2016). This could be due to differences in the degree of stress or
661 novelty of different environments, or it is possible that the response in *D. simulans* is due to something
662 other than stress induced maladaptive plasticity.

663 The abundance of lncRNAs which are involved in genotype by environment interactions
664 in *D. simulans* is perhaps not surprising, given that they have been implicated previously in the response
665 to stress (Valadkhan & Valencia-Hipólito 2016). It is also possible they are more frequently involved
666 because lncRNAs are often less conserved, as there is no requirement for the maintenance of ORFs and
667 codon synonymy (Chodroff *et al.* 2010; Ulitsky *et al.* 2011; Quinn *et al.* 2016; Ulitsky 2016). It has been
668 observed previously that transcription evolves more quickly than sequences, and lncRNA are commonly
669 homologous to non-transcribed sequences in other species, however these species are typically more

670 diverged than *D. melanogaster* and *D. simulans* (Main *et al.* 2013; Ulitsky 2016). If neutral variation was
671 allowed to accumulate without selection, and then uncovered in a stressful environment, preferential
672 accumulation within less constrained sequences would be expected. *D. melanogaster* is also enriched for
673 lncRNAs for the response to ethanol at 30 minutes, which could be explained by increasing ethanol stress
674 over time. While it is possible that lncRNAs are indicative of stress-induced maladaptive plasticity this
675 cannot be separated from a more general involvement in the stress response, which would not necessarily
676 occur as a result of maladaptive plasticity.

677 Why the lncRNAs are preferentially nested in *D. simulans* is less easily envisioned, though four
678 potential scenarios for an increase in nested lncRNAs are depicted in Figure 4. The simplest explanation
679 is only that there has been a change in the expression of nested lncRNAs, and perhaps nested lncRNAs
680 are more commonly involved in less essential processes than other lncRNAs and are therefore less
681 constrained. It is also possible that ethanol causes a change in the intron stability of the spliced transcript,
682 causing increased (or decreased) detection of the nested lncRNA (Figure 4). It is also possible that a
683 change in the processing of the parental gene occurred, causing a change in the number of reads mapping
684 to unspliced introns, or that the parental gene simply changed expression. These latter two explanations
685 would predict correlation between the parental gene expression and nested gene expression, which was
686 only observed for the very small fraction of lncRNAs that shared strandedness with their parental gene.
687 Thus it is more likely that either nested lncRNAs are less constrained, or ethanol alters the stability of
688 introns during or after the process of splicing.

689 The population genetic patterns observed in these two species are not easily interpreted. *D.*
690 *melanogaster* does not show any increase in outliers for Tajima's *D* in any direction for either changes in
691 rank abundance or expression (with the exception of fewer than expected on the X chromosome for *D.*
692 *simulans* genes implicated in expression differences). *D. simulans* is an outlier in the positive direction for
693 *D. melanogaster* genes implicated in differential expression, which could indicate that the genes involved
694 in expression differences in *D. melanogaster* are subject to relaxed purifying or balancing selection in *D.*
695 *simulans*. As these are all in the positive direction, it is worth noting that genome-wide *D. simulans* is

696 biased towards positive values of Tajima's *D*, likely due to recent population contraction (Signor *et al.*
697 2017c). While they are still more positive than expected due to background levels of Tajima's *D*, caution
698 is also warranted in interpreting these patterns as due to selection. Among genes with expression
699 differences and changes in rank abundance in *D. simulans*, there is an enrichment of outliers for
700 expression differences on the autosome and X. This is due in large part to negative Tajima's *D* outliers,
701 which given genome-wide patterns is overall more suggestive of selection, in this case directional
702 selection. It may be that *D. simulans* phenotypic plasticity is currently not in equilibrium, and there is
703 selection for an optimal phenotypic response. This can be true whether or not the observed response is
704 due to maladaptive plasticity – diversity of the passive stress response to ethanol implies that some
705 responses will be more beneficial than others, and there may be selection against the less adaptive stress
706 responses.

707 Between species divergence (D_{XY}) suggests that in *D. melanogaster* the genes involved in
708 changes in rank abundance are less diverged than expected compared to background levels of divergence.
709 In *D. simulans* the genes implicated in expression differences for exons and exonic regions are more
710 diverged than expected based on background levels of divergence, which combined with being outliers
711 for largely negative Tajima's *D* could indicate that they have been important for adaptation in *D.*
712 *simulans*. However, given that the number of nucleotide differences involved in the response to the
713 environment is unknown – for example all the observed patterns could be due to a single *trans* variant, it
714 is difficult to interpret the results of Tajima's *D* and D_{XY} in terms of selection.

715 Inferring that gene expression differences are adaptive or non-adaptive remains a major challenge
716 in the study of gene expression reaction norms, given the lack of direct correlation between gene
717 expression phenotypes and organismal phenotypes. However, the patterns observed in *D. simulans* do
718 suggest maladaptive plasticity in response to ethanol exposure. In this scenario abundant genotype by
719 environment interactions are expected to have accumulated neutrally and become uncovered in response
720 to environment stress. In contrast, in *D. melanogaster* this ethanol environment is not novel and
721 maladaptive plasticity has been selected out in favor of an adaptive phenotypic response. lncRNAs are

preferentially differentially expressed in *D. simulans* in response to ethanol either because they are less constrained and can accumulate more neutral variation, or because they are involved in the general stress response. It is also possible that environmental heterogeneity has caused *D. simulans* to maintain balanced polymorphisms for plasticity in a way that has not occurred in *D. melanogaster*, though we believe there is less evidence in favor of this interpretation. In the future comparing African, non-ethanol adapted populations of *D. melanogaster* to cosmopolitan populations may be a way of discerning between these hypotheses.

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Data Accessibility

D. melanogaster sequence data have been submitted to Bioproject: accession number PRJNA482662. *D. simulans* sequence data will be made available upon acceptance.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

S.S. performed the experiments, analyzed the dataset, and wrote the manuscript. S.V.N. conceived of the experiment and assisted in writing the manuscript.

D. simulans

	G	ETOH	ETOH x G	ETOH x T	ETOH x G x T	10	20	30
Exons								
Unique	96	6	16	2	75	1	0	6
Unique/Constitutive	1547	58	324	19	943	5	1	16
Common	52	2	3	0	19	0	0	1
Constitutive	299	10	44	3	121	3	2	6
Sum	**1994**	**76**	**387**	**24**	**1158**	**9**	**3**	**29**
Exonic Regions								
Common	119	9	22	1	76	1	0	2
Constitutive	287	14	76	6	223	2	1	2
Sum	**406**	**23**	**98**	**7**	**299**	**3**	**1**	**4**
Fragments								
Unique	101	2	5	2	26	1	1	2
Unique/Constitutive	169	7	45	3	93	0	0	1
Common	147	6	6	1	32	1	0	2
Constitutive	191	3	25	0	74	2	1	1
Sum	**608**	**18**	**81**	**6**	**225**	**4**	**2**	**6**

*D. melanogaster**

	G	ETOH	G x ETOH	ETOH x T	G x ETOH x T	10	20	30
Exons								
Unique	79	3	0	0	0	0	0	4
Unique/Constitutive	630	1	1	12	0	1	0	17
Common	89	2	2	3	0	0	0	4
Constitutive	647	9	0	7	0	1	1	21
Sum	**1445**	**15**	**3**	**22**	**0**	**2**	**1**	**46**
Exonic Regions								
Common	84	2	0	1	0	0	1	2
Constitutive	547	11	0	7	2	1	3	22
Sum	**631**	**13**	**0**	**8**	**2**	**1**	**4**	**24**
Exon Fragments								
Unique	191	6	0	5	1	0	1	10
Unique/Constitutive	99	0	0	1	1	0	0	2
Common	301	4	0	1	1	1	1	7
Constitutive	544	11	0	5	1	2	3	1
Sum	**1135**	**21**	**0**	**12**	**4**	**3**	**5**	**20**

**reproduced from Signor and Nuzhdin 2018*

984 **Table 1**: A summary of the exons, exonic regions, and exon fragments that are significantly differently
985 expressed for each component of variance. Abbreviations are as follows: (G) Genotype, (ETOH)
986 Ethanol, and (T) Time.

987

		Biological Process		Cellular Component		Molecular Function	
D. simulans	Ethanol x genotype						
				axonemal dynein complex	3×10^{-3}	dynein light chain binding	3.3×10^{-3}
	Ethanol x genotype x time						
		sensory perception of taste detection of chemical stimulus	6.6×10^{-3} 8.2×10^{-3}	nucleplasm cytoskeletal part ciliary part	6.5×10^{-3} 8.4×10^{-3} 4.6×10^{-3}	RNA binding	7.8×10^{-3}

988

989 **Table 2**: A summary of the significant GO enrichment terms for *D. simulans*. Two components of
990 variance (genotype x time; genotype x ethanol x time) have significant terms, the cutoff is $p < .01$.
991

	Threshold	Abundance: # of outliers	Rank abundance: # of outliers		Threshold	Abundance: # of outliers	Rank abundance: # of outliers
***D. melanogaster* candidate gene regions**				***D. simulans* candidate gene regions**			
D. melanogaster				***D. melanogaster***			
X-linked	-1.49	5	10	X-linked	-1.49	**13**	11
	2.29	4	9		2.29	**35**	19
Autosomal	-1.5	18	68	Autosomal	-1.5	191	1
	1.9	13	49		1.9	225	4
D. simulans				***D. simulans***			
X-linked	-1.89	**0**	10	X-linked	-1.89	77	77
	3.25	**15**	6		3.25	**46**	**4**
Autosomal	-1.09	**0**	37	Autosomal	-1.09	**318**	**72**
	3.06	**35**	67		3.06	**267**	**15**

992
993 **Table 3**: A summary of the threshold values for Tajima's *D* and the number of intervals in each species
994 which fall beyond this cutoff. X-linked and autosomal genes are shown separately, outlier categories
995 which are significantly enriched are shown in bold. The same numbers are shown as percentages of the
996 total number of intervals in Figure 4.
997

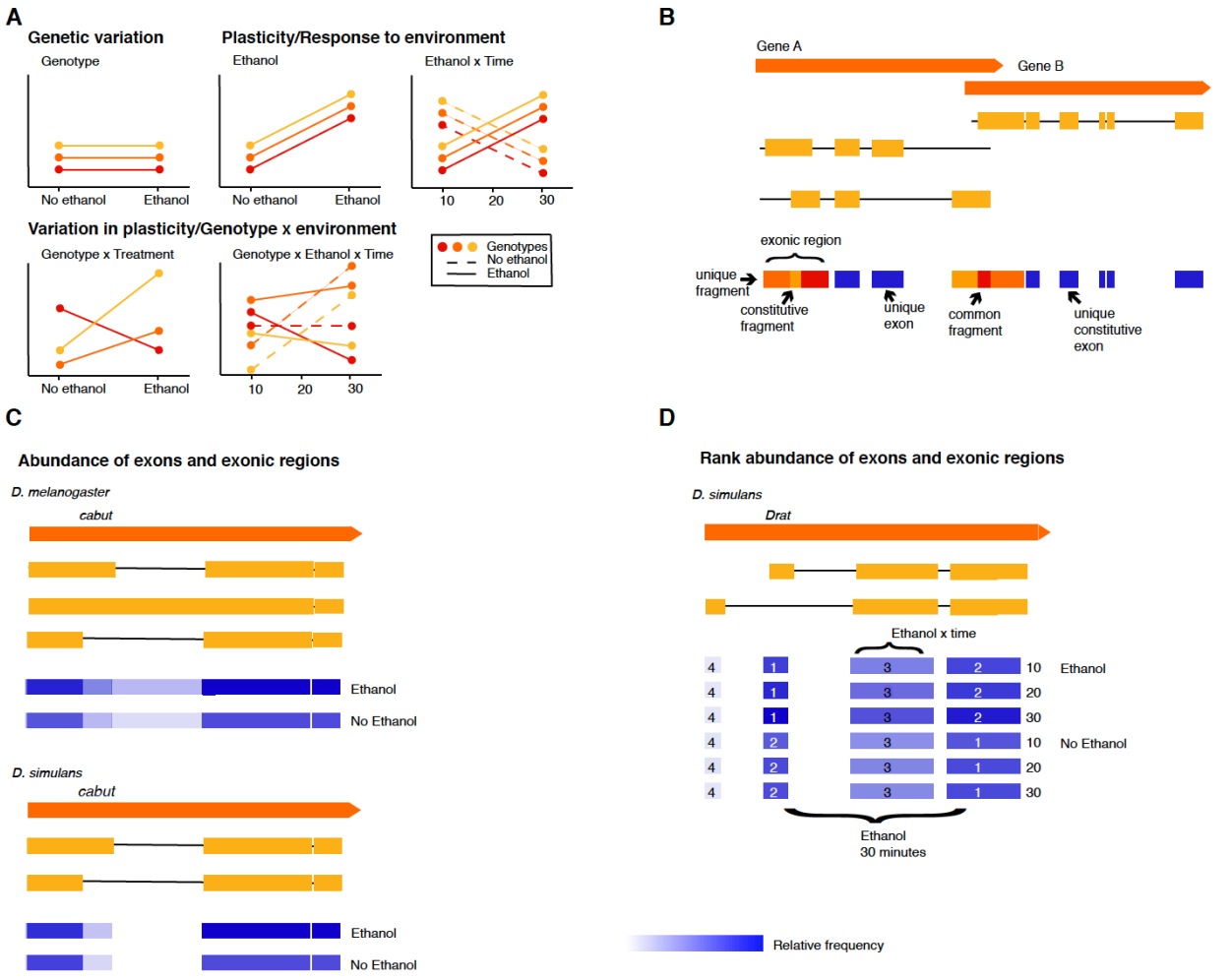

Figure 1: **A**. An illustration, for a single gene, of hypothetical gene expression changes for different genotypes with different main and interaction effects. For main effect genotype, no difference is seen in expression between environments but different genotypes have different expression levels. In response to the environment (ethanol), gene expression level changes between environments but for every genotype it changes in the same way. Ethanol by time is complicated by the fact that the x-axis is now time, and there are separate lines for ethanol (solid) and non-ethanol (dashed) environment. Here again there is a component of time for gene expression changes, but every genotype changes in the same manner. For genotype by environment interactions in the first panel different genotypes respond to ethanol differently, and in the second panel different genotypes respond to ethanol differently over time. **B**. An illustration of the classification scheme for exons, exonic regions, and exon fragments. Exons either do not overlap other exons in different isoforms (exons, shown in blue), or are fused regions consisting of a set of overlapping exons (exonic regions, shown in shades of orange and red). Exonic regions can be decomposed into exon fragments, depending upon their overlap between different isoforms. Exons may be unique (found in a single isoform), common (found in some isoforms but not all), or constitutive (present in all isoforms). **C.** An example of the information about gene expression and isoform abundance that can be inferred from measures of differential exon abundance. *cabut* is a relatively simple example, having few isoforms and exons, and yet it is still a complicated inter-species comparison given that *D. melanogaster* has more annotated isoforms that *D. simulans*. Below each gene model the frequency of different exons and exon fragments in each environment is shown, with the most frequent in dark blue. In

D. simulans the first of two isoforms shown has an exon fragment that is more abundant in the presence of ethanol, suggesting that that isoform is more abundant. In *D. melanogaster* only the second of the three isoforms has a unique fragment, which is also more abundant in ethanol and suggests differential isoform usage. **D.** An illustration of a change in the rank abundance of a the gene *Drat*. Only the example of *D. simulans* is shown. *Drat* has two annotated isoforms in *D. simulans*, with two constitutive exons and two exons unique to each of the two isoforms. With ethanol, the exon unique to the first annotated transcript is the most abundant, while without ethanol the first constitutive exon is most abundant. In addition, the second constitutive exon is significant for changes in abundance for the interaction between treatment and time, and the two constitutive exons and exon unique to the first transcript have significant differences in expression for ethanol, and between environments at 30 minutes. The dynamic changes in the expression of different exons are captured in different ways by these three approaches to quantifying changes in expression and splicing, where even within one gene there can be four complicated effects on expression characterized by two approaches.

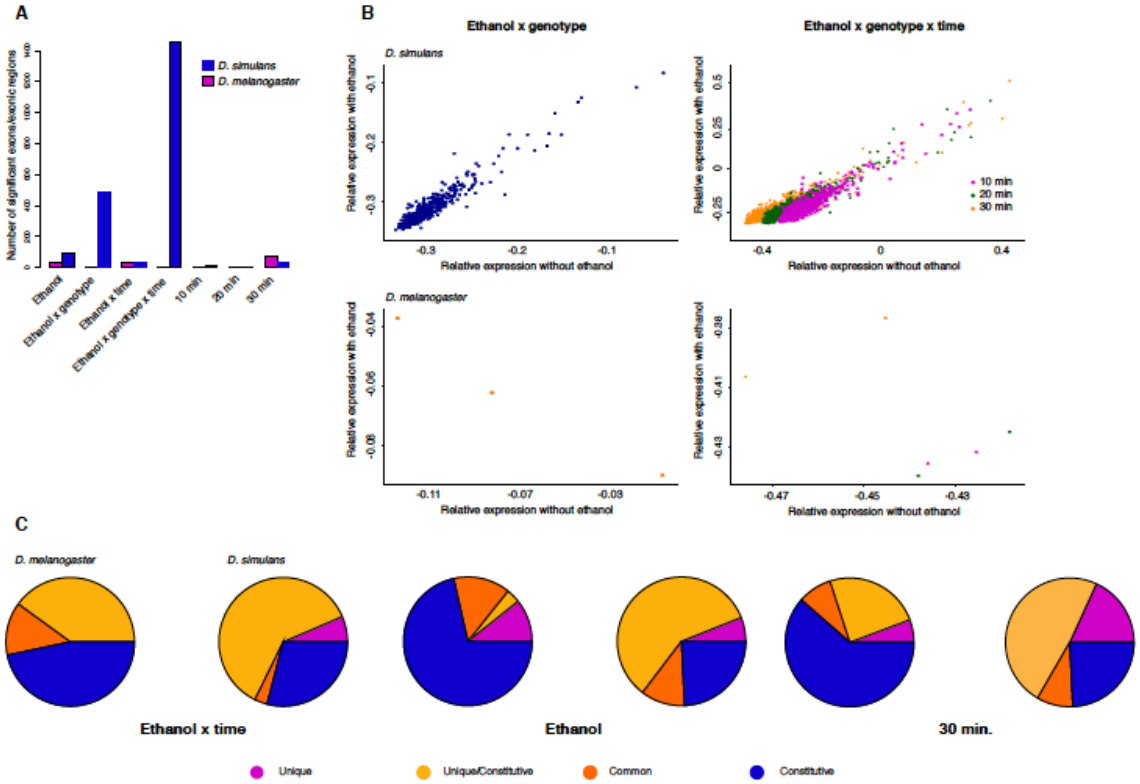

Figure 2: A. Components of variance for *D. melanogaster* and *D. simulans*. The number of significant exons and exonic regions for each component of variance are shown. Please note that in cases where so few exons and exonic regions are significant that no color can be seen, *D. melanogaster* is always on the left. Many more exons and exonic regions are significant for *D. simulans* than *D. melanogaster*, but only for components of variance that contain an interaction with genotype. **B.** *D. melanogaster* and *D. simulans* differ markedly in their genotype-specific reactions to ethanol, thus shown here is the relative expression in ethanol (*y*-axis) against the relative expression without ethanol (*x*-axis) for all genes with an interaction term with genotype. Too few genes are significant in *D. melanogaster* to make anything but a qualitative comparison, but the graphs serve to illustrate the magnitude and nature of this contrast. **C**. The proportion of exons and exonic regions that are either unique, unique/constitutive, common, or constitutive for the three components of variance for which a reasonable number of genes are significant in *D. melanogaster*. Note that while species names are only indicated for the first component of variance, *D. melanogaster* is on the left for every comparison. *D. melanogaster* has far more 'constitutive' exons and exonic regions compared to *D. simulans*, where unique/constitutive is more common. This is likely

due to differences in the annotation of isoforms between the species, where *D. melanogaster* has more isoforms per gene annotated. It is also possible that alternatively spliced genes are more important for expression differences in *D. melanogaster*.

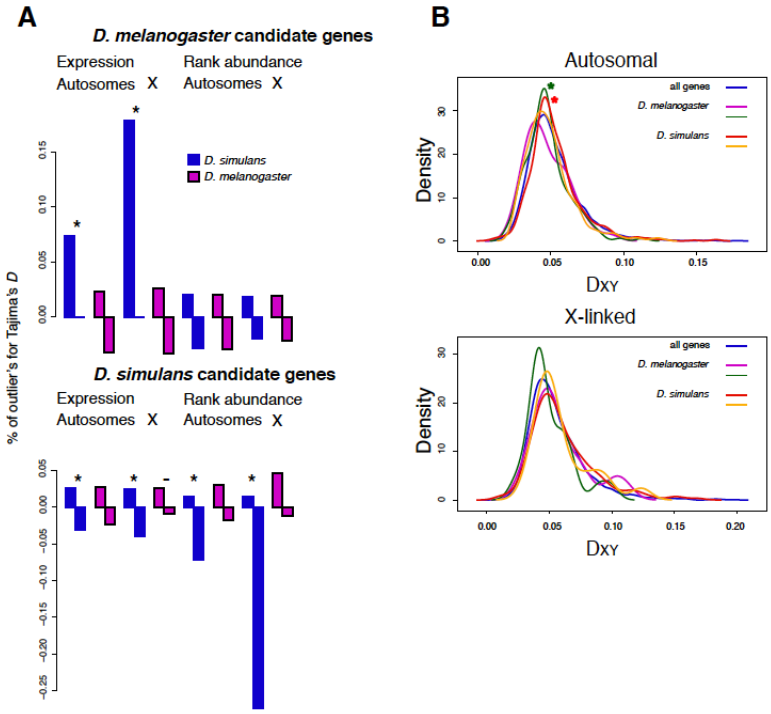

Figure 3: **A.** The percentage of intervals that were considered outliers in *D. melanogaster* and *D. simulans*, split between the autosomes and the X. The percentage of intervals that are outliers are shown separately for positive and negative, with the percent negative shown as a negative number. Note that the percentage is not actually negative but rather serves to illustrate the distinction between positive and negative. Enrichment for outlier intervals was determined as a composite of positive and negative intervals. Significant enrichment is indicated by a star, significant under-enrichment is illustrated with a minus sign. *D. melanogaster* is always on the left. The genome-wide cutoff for outliers was ±2.5%, thus enrichment is anything significantly more than or less than 5% for the positive and negative intervals together. **B**. The distribution of D_{xy} for *D. simulans* and *D. melanogaster*, compared to the genome-wide background distribution of D_{xy}. The genes are split between the autosomes (top) and X-linked (bottom), and the genome-wide background distribution is shown in blue. For *D. melanogaster* and *D. simulans* the color corresponding to changes in the abundance of exons or exonic regions is shown first, while that corresponding to changes in rank abundance is shown second , i.e. *D. melanogaster* pink – changes in overall abundance; green – changes in rank abundance.

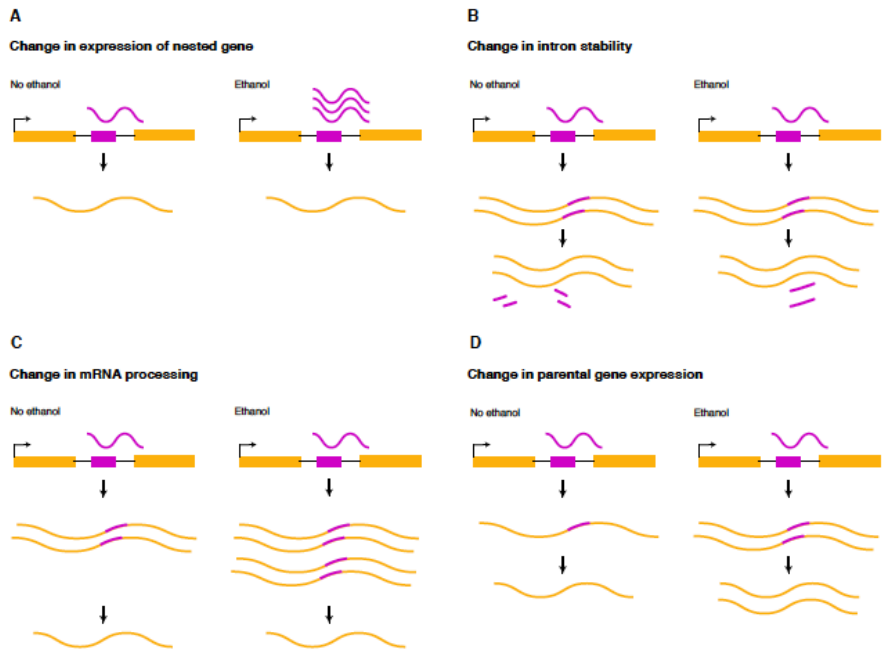

Figure 4: An illustration of the possible explanations for an enrichment in nested genes, and nested non-protein coding genes, in *D. simulans*. Note that in each illustration there are many possible details for each scenario – for example gene expression could increase or decrease in the presence of ethanol, but we illustrate here an increase in scenario **A.** This does not assume that this particular change is more likely, only that illustrating both an increase and a decrease will not aid in the readers comprehension. A. In the first scenario the nested gene changes its expression without any relationship with the expression of its parental gene, as shown by the number of squiggly lines representing RNA. Generic exons are represented by colored squares, introns by lines. The parental gene is shown in mustard yellow, and the nested gene in magenta, with RNA belonging to either in matching colors **B.** It is possible that ethanol alters the stability of introns after they are spliced out of the maturing transcript from the parental gene, potentially altering the expression level of the non-protein coding gene within the intron. Shown here and in the remaining scenarios is an extra step, with an unspliced RNA transcript from the parental gene preceding a spliced transcript. We do not attempt to accurately depict the process or machinery of splicing, only to illustrate that while expression of the nested gene may occur independently, some expression may also occur from introns as they are spliced out of the parental gene. **C.** Expression of the nested gene could also be affected if there were changes in the number of unspliced transcripts, for example through ethanol-induced alterations in the rate of splicing. **D.** The expression of the nested gene could also appear to change, or actually change through changes in the expression of the parental gene. Note that **C-D** would all predict some amount of correlation between parental gene expression and nested gene expression – such that if a change in nested gene expression is detected a change in parental gene expression would generally be expected as well.