Evolution of 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 contribution of phenotypic plasticity to adaptation is contentious, with contradictory empirical support for its role in evolution. Here I investigate the possibility that phenotype plasticity has contributed to adaptation to a novel resource. If phenotype plasticity contributes to adaptation, it is thought to evolve in a process termed genetic accommodation. Under this model, the initial response to the environment is widely variable due to cryptic genetic variation, which is then refined by selection to a single adaptive response. I examine the role of phenotypic plasticity in adaptation here by comparing two species of *Drosophila* that differ in their adaptation to ethanol (*Drosophila melanogaster* and *D. simulans)*. Both species are human commensals with a recent cosmopolitan expansion, but only *D. melanogaster* is adapted to ethanol exposure. I measure phenotype plasticity in response to ethanol with gene expression and an approach that combines information about expression and alternative splicing. I find evidence for adaptation to ethanol through genetic accommodation, suggesting that the evolution of phenotype plasticity contributed to the ability of *D. melanogaster* to exploit a novel resource. I also find evidence that alternative splicing may be more important for the adaptive response to ethanol than overall changes in exon expression.

Keywords: phenotypic plasticity, *Drosophila*, ethanol, genetic accommodation

Introduction

The contribution of phenotypic plasticity to adaptation remains controversial despite considerable empirical and theoretical investigation. This includes whether phenotypic plasticity itself evolves, and how frequently the evolution of plasticity is important for adaptive evolution. For example, is plasticity adaptive, or does it depend upon the cost (if any) of plasticity, and the reliability of cues that induce plastic phenotypes? If plasticity is adaptive, then selection would proceed by favoring the reaction norm that is most adaptive, a process referred to as genetic accommodation (in contrast to simply fixing a different phenotype, known as genetic assimilation) [1,2]. This could potentially accelerate adaptation or divergence because organisms already possess the ability to form alternative phenotypes, rather than requiring the production of a novel phenotype. This is thought to be an important mechanism of adaptation for invasive species, and potentially to be increasingly important in human modified landscapes and climate.

When exposed to novel environments, the predicted path for evolution by genetic accommodation is one in which initially there is increased variance - or genotype-specific differences – in the reaction norm of a trait, followed by refinement by selection for a single adaptive response [1,3-6]. This is because if a trait only exists as an environmentally induced variant, and is therefore not exposed to selection (or infrequently so) then genetic variation should accumulate in the response of that trait to a novel environment [7-10]. After exposure to the novel environment, this cryptic genetic variation is uncovered, manifesting as greater phenotypic variation [11-14]. As lineages adapt to the novel environment, selection on this variation in reaction norm should result in the loss of variation for this trait as genetic accommodation occurs [15]. As long as the environment is heterogeneous the expectation is that it will remain a plastic trait, rather than evolving to be stably expressed. This would manifest as a transient increase in genotype by environment interactions, followed by reduced genotype by environment interactions but persistent environmental response, a pattern which is consistent evidence of adaptation [16-20].

77 While changes in heritability or variance have been previously shown between populations
78 occupying ancestral and novel habitats, inbred lines have not been used previously to specifically measure
79 genotype by environment interactions in adapted and non-adapted populations. Furthermore, in general
80 the evolution of phenotypic plasticity is investigated with regards to an ancestral and a novel
81 environment, rather than a heterogeneous environment where plasticity is expected to be maintained. In
82 this manuscript I will test the predictions from genetic accommodation using inbred lines in *Drosophila*,
83 an approach which will allow direct quantification of the genotype by environment interaction. I will also
84 investigate a heterogeneous environmental variable, ethanol, for which different species of *Drosophila*
85 have different adaptive histories.

86 *D. simulans* and *D. melanogaster* have divergent adaptive histories with respect to ethanol. *D.*
87 *melanogaster* is considerably more ethanol tolerant than *D. simulans*, and is found regularly feeding upon
88 and ovipositing in resources with ethanol concentrations greater than 8% [21-23]. At concentrations of
89 4% ethanol *D. simulans* shows reductions in survivorship and increased development time relative to *D.*
90 *melanogaster* [24]. Furthermore, *D. melanogaster* and *D. simulans* are both cosmopolitan species and
91 human commensals, however while *D. melanogaster* is commonly found inside houses, breweries, and
92 wineries, *D. simulans* is more often observed in orchards and parks (though their niches overlap
93 significantly and they are often found on the same patches) [24-33]. Therefore, the expectation is that *D.*
94 *simulans* will show a non-adapted phenotype manifested as considerable genotype by environment
95 interaction. *D. melanogaster* will retain an interaction with the environment, but it will not be genotype-
96 specific, which is consistent with adaptation to a heterogeneous environment.

97 The trait I measure to test this prediction is gene expression. Phenotypic plasticity is often
98 achieved by dynamic changes in gene expression across environments, and current evidence suggests that
99 it plays an important role in regulating evolutionarily important phenotypes [34-37]. Gene by
100 environment interactions also appear to be a common attribute of gene expression, and yet while many
101 studies have quantified the effect of the environment on gene expression, far fewer have performed these

experiments in multiple genotypes to obtain the interaction between genotype and the environment for gene expression [38]. Indeed, most work on gene expression plasticity has not quantified the interaction between genotype and the environment [36,38-42].

Short read sequencing fundamentally cannot separate the contributions of alternative splicing and gene expression to changes in abundance of an exon, thus I approach the analysis in a way that includes both phenomenon (gene expression and alternative splicing, see Methods). In addition, because of the potential for alternative splicing to respond more rapidly to environmental differences than gene expression *sensu stricto*, it may be one of the earliest mediators of environmental response. This is because it does not require de novo transcription or protein production. It is thought to diverge more rapidly than gene expression between lineages, and it has been linked to stress responses and changes in transcriptome complexity in response to environmental differences [43-50]. Furthermore, it has been previously implicated in the response to ethanol, and in *D. melanogaster* may be a more important component of the response than gene expression changes [51-57]. Yet it remains the topic of few investigations into environmental response.

Here I investigate plasticity in response to ethanol, including genetic variation for plasticity over time, in *D. simulans* which is not adapted to ethanol use. I will compare these results to previous work on *D. melanogaster*. I find that in *D. simulans* there is an order of magnitude larger effect of genotype by environment compared to *D. melanogaster*, however both species maintain an environmental response of similar magnitude. This suggests the following scenario: In *D. simulans* high ethanol concentrations are essentially a novel environment that reveals cryptic variation for phenotypic plasticity. In *D. melanogaster*, genetic variation in phenotypic plasticity has been removed by selection in favor of a single environmental response through a process of genetic accommodation. This single environmental response involves more alternative splicing than observed in *D. simulans*, suggesting it is an important component of adaptation.

Methods

127 *Fly lines*

128 Six genotypes were used in these experiments. The genotypes used for male flies from *D. simulans* were
129 collected from the Zuma Organic Orchard in Zuma Beach, CA in the winter of 2012 and made nearly
130 isogenic by 15 generations of full sib inbreeding [58,59]. In natural conditions flies will not by homozygous
131 at all loci, thus each inbred genotype was crossed to a white eyed 'tester' strain to create the F1 flies used
132 in the ethanol exposure assays (*D. simulans*, w^{501}, Cornell species stock number 14021-0251.011). Rearing
133 occurred on a standard medium at 25 °C with a 12-h light/12-h dark cycle. In order to control for maternal
134 effects and variation in offspring quality, female parents were collected as virgins, aged one day, and then
135 density matched with male flies (10 per sex). The F1 offspring were then collected as virgins, reared in
136 single sex vials with standardized density (24-30 flies if male, 12-15 if female), and aged for three to four
137 days prior to the assay. A portion of this experiment was intended to analyze behavioral differences in
138 ethanol, thus during the ethanol exposure a female was included as a stimulus, but was not collected for
139 RNA-seq [59,60]. The day prior to the ethanol exposure these females were mated to flies from a standard
140 genotype, such that in the final assay each chamber contained one mated female fly from the y^1w^1 mutant
141 lines and two virgin males from the target F1 cross, of which only the latter were collected for RNA-seq.
142 All procedures used here in the maintenance and raising of *D. simulans* are the same as those used
143 previously in *D. melanogaster*, facilitating easy comparison between studies [59,60].

144 *Experimental setup*

145 In this experiment intoxication occurs through inhalation of ethanol vapors, and evidence of the
146 behavioral effects in both species and the efficacy of this approach have been published previously
147 [59,60]. Ethanol exposure took place in a circular arena, each of which was part of a larger chamber
148 containing 12 arenas each with a diameter of 2.54 cm (VWR cat. no. 89093-496) (Figure 1). The bottom
149 of each chamber contained a standard amount of either grapefruit medium or medium in which 15% of
150 the water has been replaced with ethanol. Replicates for both species were conducted randomly under
151 standardized conditions (25 °C, 70% humidity). Prior to the assay the flies were sedated through

152 exposure at 4 °C for ten minutes, to avoid the confounding effects of CO_2, then placed in the chambers
153 which were left then upside down at room temperature for ten minutes while the flies oriented themselves
154 [59-61]. After ten minutes the timing of the assay began, and were conducted for ten, 20, or 30 minutes
155 for three replicates of each of the two conditions (Figure 1). Flies are most active during the hours
156 following dawn, thus to standardize behavior and circadian rhythms all assays were conducted within a
157 two-hour window after dawn. At the conclusion of the assay the chambers were flash frozen in liquid
158 nitrogen, allowed to freeze through, transferred to dry ice, and all of the males were collected for RNA-
159 seq.

160 *Sample preparation and RNA sequencing*

161 Flash-frozen flies were freeze dried and ten to 12 heads were placed into a 96-tube plate (Axygen MTS-11-
162 C-R). mRNA purification, cDNA synthesis and library preparation were carried out by RAPiD
163 GENOMICS (http://rapid-genomics.com) using a robot. mRNA was purified using Dynabeads mRNA
164 DIRECT Micro kit (Invitrogen # 61021) with slight modifications. To fragment the RNA mRNA-beads
165 were resuspended in 10 uL 2X first strand buffer (Invitrogen # 18064-014), incubated at 80 C for two
166 minutes and placed on ice, then the supernatant was collected after five minutes on a magnetic stand. First
167 strand synthesis was performed using standard protocols for Superscript II (Invitrogen #18640-014) and
168 reverse transcription (25 C 10 min, 42 C for 50 min, 70 C for 15 min, 10 C hold). Second strand synthesis
169 was carried out using standard protocols with DNA Pol I and incubated at 16 C for 2.5 hours. cDNA was
170 purified with 1.8 volume of AMPure XP following manufactures instructions (Beckman Coulter A63880).
171 Sequencing was performed using the Illumina HiSeq 2500 as both 2 × 150 bp or 2 × 50 bp reads, resulting
172 in two technical replicates per sample. The two run lengths (and runs) were intended to provide extra
173 coverage, and all replicates were sequenced in both runs.

174 *Exon expression analysis*

175 It is common in organisms with alternative splicing for exons from different isoforms of a single gene to
176 overlap with one another, or be shared between all or most isoforms (Figure 1A). Short read data

177 fundamentally cannot resolve these exons to individual isoforms, however, one approach is to quantify each
178 exon separately and decompose exons overlapping between isoforms into those which are shared and
179 unique. When the differences between overlapping exons are less than 10 bp, there is no appreciable
180 amount of information loss in not decomposing overlapping exons, and this approach has been taken in the
181 past [62-65]. However, in many cases the differences in exon overlap are much larger than this, so to address
182 this issue I use a classification scheme where reads may be assigned to *exons*, *exonic regions*, or *exon*
183 *fragments*, and then compare the abundance of each exon/exonic region/exon fragment in each condition
184 (i.e. the abundance of an exon with and without ethanol) [61] (Figure 1A). Here, an *exon* does not overlap
185 any other exons. If exons from different isoforms overlap, they are grouped into an *exonic region*. *Exon*
186 *fragments* are classified by decomposing *exonic regions* based on the 5' and 3' positions of the exons within
187 the region. Thus, all *exon fragments* are subregions of *exonic regions*. Exon boundaries were determined
188 using the *D. melanogaster* FlyBase 6.17 genome features file and the *D. simulans* 2.02 genome features
189 file. Alignment was performed using BWA-MEM version 0.7.15 (which has been shown to perform better
190 than split read mappers such as STAR [66,67]) and BED files were used to count reads in each region and
191 obtain the length adjusted read count (reads in region divided by the length of region), and the APN (average
192 per nucleotide) [68].

193 The APN was calculated separately for each read length and then combined between read lengths
194 to handle the mixture of read lengths for each sample (2 × 150 bp and 2 × 50 bp). If the APN was greater
195 than zero in at least half of all samples per condition it was considered detected. While I considered several
196 approaches to normalize coverage counts upper-quartile normalization with log-transformation and median
197 centering within time × treatment × genotype were selected due to better performance of the residuals
198 [69,70].

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

201 $$Y_{ijkl} = \mu + g_i + t_j + (gt)_{ik} + m_k + (gm)_{ik} + (tm)_{ijk} + \epsilon_{ijkl}$$

202 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
203 min), and l^{th} replicate (Supplemental File 1-2). For the interaction between treatment and time point, the log
204 APN for each exonic region was modeled as

205 $$\Upsilon_{ij} = \mu + t_j + \epsilon_{ij}$$

206 or the ith condition (time × treatment) and j^{th} replicate. Contrasts to compare treatments within time point
207 (ethanol versus no ethanol, for ten, 20 and 30 minutes) were conducted (Supplemental Files 1-2). Residuals
208 were evaluated for conformation with normality assumptions, and assumptions were met in excess of 95%
209 of the models.

210 In contrast to exon abundance, I evaluated alternative splicing specifically by comparing the
211 abundance of all exons within a gene to each other, as this is more direct evidence of a change in isoform
212 abundance than a change in exon/exonic region/exon fragment abundance; Figure 2A). For example, a
213 change in relative abundance might be detected if the last exon of a gene was the most abundant compared
214 to all other exons/exonic regions/exon fragments without ethanol and the least abundant with ethanol.
215 Exons and exonic regions for each gene and for each sample were ranked and the most expressed region
216 ranked as one, the least expressed region as three and all others as two (Figure 2A). Exon ranks for each
217 gene were modeled as

218 $$\Upsilon_{ijk} = \mu + r_i + t_j + (rt)_{ij} + \epsilon_{ijkl}$$

219 where Υ_{ijk} is the exon rank (1,2,3) of the ith exonic region of the gene, j^{th} condition (time × treatment), and
220 the kth replicate; r_i is the exonic region of the gene; t_j is condition; and $(rt)_{ij}$ is the interaction between
221 exonic region and condition. More traditional GLM approaches can only be taken if their assumptions are
222 met, and in this case they are not due to a lack of normality in the distribution of model residuals.
223 Accordingly, a non-parametric test must be relied upon to look for changes in exon or exonic region
224 representation between exons of a gene and I used a rank test to summarize changes in exon representation
225 (Supplemental File 3). F-tests for the significance of the mean square attributed to the effect tested versus

226 the mean square attributed to error, or the appropriate interaction term, were used. The false discovery rate
227 was controlled using the Benjamini-Hochberg procedure, with a significance cutoff of $\alpha = 0.05$ [71]

228 *GO Analysis*

229 When a gene in *D. simulans* had more than one ortholog in *D. melanogaster* only one ortholog was
230 included for the GO analysis, so as not to inflate the number of genes involved in a given process. This
231 does presume that orthologs will be annotated with the same GO terms, and this is generally the case. For
232 example, in *D. simulans* there is only *AOX4*, while in *D. melanogaster* there is *AOX3* and *AOX4*, but the
233 GO terms for each are the same. However, as *D. simulans* genes are generally not independently
234 annotated, especially those without *D. melanogaster* orthologs, if there was no *D. melanogaster* ortholog
235 the gene was not included in the enrichment analysis. Lists of significant genes were tested for GO
236 enrichment using the PANTHER classification system [72]. They were corrected for multiple testing and
237 a *p*-value of 0.01 was required for significance.

238 *Functional class enrichment*

239 I tested the significant sets of exons and exonic regions for enrichment with non-protein coding genes.
240 Multigene exonic regions were not included, meaning exons that belong to more than one gene, as they
241 often do not correspond to the same functional class of gene. Every test of functional class enrichment
242 compared the frequency of a given subcategory among all exons and exonic regions detected in the
243 dataset compared to the frequency within a significant list of exons and exonic regions. A χ^2 test was
244 performed in R to test the significance of the enrichment of each of these categories.

245 <u>Results</u>

246 *Exon expression and isoform usage*

247 It is difficult to decouple alternative isoform usage from gene expression, given that many exons are
248 shared between isoforms or overlap other exons. To infer isoforms from short read data, one must rely
249 upon unique junctions or regions of individual isoforms and extrapolate to shared regions. This requires

250 accurate isoform annotation (knowing that any given exon/junction is found in combination with other
251 exons/junctions) and in general can be very noisy. Accordingly, I subscribe to a simpler but more robust
252 approach and summarize the abundance of different exons and exonic regions separately [61,62,64,65]. I
253 detail overall abundance of exons, exonic regions, and exon fragments, and changes in the relative
254 abundance of exons within a gene (Figure 2 A-C; Figure 3 A&B).

255 *Changes in exon expression in D. simulans*

256 Table 1 summarizes the number of exons, exonic regions, and exon fragments which alter their
257 expression in response to ethanol for *D. simulans*, and a full list of genes is available in the Supplemental
258 File 1 & 2. Compared to previous work in *D. melanogaster*, for treatment seven exons and exonic regions
259 were shared between species: *Drat*, *cabut*, *CG11741*, *CG32512*, *CG4607*, *Pinocchio*, and *sugarbabe*
260 (Table 1; Figure 2 A-C, [61]). For the interaction between ethanol and time three genes were shared
261 between exons and exonic regions for these species, *Drat*, *cabut*, and *CG43366*. At ten and twenty
262 minutes *cabut* is shared between exons and exonic regions in *D. melanogaster* and *D. simulans*. At 30
263 minutes *Drat*, *CG32103*, *sugarbabe*, *cabut*, *Pinocchio*, *CG32512*, and *CG4607* are shared between
264 species. This suggests that *Drat*, *cabut*, *sugarbabe*, *Pinocchio*, *CG32512*, and *CG407* are important for
265 the response to ethanol, as they are shared between species for multiple components of variance and have
266 been implicated in the response to ethanol previously [73-75].

267 *D. simulans* is enriched for several GO terms in response to interactions with genotype, including
268 several terms relating to cilia, such as the axonemal dynein complex and ciliary part (Table 2). GO terms
269 were also enriched for the sensory perception of taste and detection of chemical stimulus. Many
270 components of variance in *D. simulans* are not enriched for GO terms due in part to the frequency with
271 which non-protein coding genes were implicated – for example in response to ethanol 15% of the
272 implicated genes do not have a *D. melanogaster* ortholog, and of those only two are protein coding.

273 *Changes in the relative abundance of exons within a gene in D. simulans*

274 In response to treatment in *D. simulans* 54 genes showed changes in the relative abundance of their
275 exons/exonic regions, and 94 genes change the relative abundance of their exons for the interaction
276 between treatment and time (Figure 3B, for a full list see Supplemental File 3). For the interaction
277 between treatment and time *lola*, *Mhc*, and *Prm* were shared between species. Changes in relative
278 abundance were not enriched for any GO terms in *D. simulans*, with the exception of peroxiredoxin
279 activity in response to ethanol, albeit slightly above the more conservative cut-off used in my other tests
280 (p = .017). Peroxiredoxin activity has been associated with protection against alcohol induced liver
281 damage [76,77].

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

283 In *D. simulans* many exons and exonic regions are significant for components of variance that interact
284 with genotype: 1457 for the interaction between genotype, treatment and time, and 486 for the interaction
285 between genotype and treatment, compared to two and three exons and exonic regions respectively in *D.*
286 *melanogaster* (Table 1; Figure 2 A-C). For example, for the interaction between genotype, treatment and
287 time .1% as many exons were implicated in *D. melanogaster* as in *D. simulans*. For other components of
288 variance, including genotype (suggesting that this is not due to differences in overall polymorphism), the
289 number of exons and exonic regions implicated in expression differences is comparable between species.
290 For example, for genotype 87% as many exons were implicated in *D. melanogaster* as in *D. simulans*; for
291 the interaction between ethanol and time it is 97%; to highlight the scale of the difference.

292 *In D. simulans interactions with genotype are enriched for nested non-protein coding genes*

293 A large number of the genes implicated in differences due to interaction with genotype were non-protein
294 coding genes. The number of non-protein coding genes that were significant for ethanol, ethanol by
295 genotype, and ethanol by genotype by time, were more than would be expected by chance in *D. simulans*
296 ($\chi^2 = 49.598, p < 0.0005; \chi^2 = 235.21, p < 2.2\ x\ 10^{-16}; \chi^2 = 727.17, p < 2.2\ x\ 10^{-16}$). These are
297 generally long non-coding RNAs (lncRNAs) as the shortest is annotated at 713 bp, and the majority are
298 over 4,000 bp (long non-coding RNAs being any non-protein coding genes over 200 nt), with some

299 pseudogenes. *D. melanogaster* is not enriched for non-protein coding genes in response to ethanol,
300 ethanol by time, ethanol by genotype, ethanol by genotype by time, ten or 20 minutes, exons or exonic
301 regions expressed only in one environment, or genes implicated in changes in relative abundance [61].
302 However, at 30 minutes *D. melanogaster* is enriched for non-protein coding genes, though this concerns
303 far fewer genes than in *D. simulans* ($\chi^2 = 12.831, p = 0.005$).

304 Many of the non-protein coding genes implicated in interactions with genotype are nested in the
305 introns of other genes, thus I sought to determine if it was more than expected by chance. First, I must
306 determine the overall frequency of gene nesting, using the criteria that an exon nested in an intron must
307 overlap the intron by at least 80 bp or 10%. I found that in *D. melanogaster* 9.2% of exons were nested
308 within introns, while in *D. simulans* 9.7% were nested, similar to what has been previously reported [78].
309 This was reflected in the data, where for both *D. melanogaster* and *D. simulans* 6.8% of exons and exonic
310 regions were nested within introns (lower because multi-gene exons were excluded). However, among
311 significant exons and exonic regions in *D. melanogaster* 18.6% were nested, while in *D. simulans* 33.5%
312 were nested [61]. This is a significant enrichment of exons that are nested within other introns, for both *D.*
313 *melanogaster* ($\chi^2 = 12.344, p < .0004$) and *D. simulans* ($\chi^2 = 1721.1, p < 2.2\ x\ 10^{-16}$). In addition,
314 compared to the total number of nested genes that are noncoding within the dataset, the number that are
315 significant for the response to ethanol is enriched for nested, non-protein coding genes in *D. simulans*
316 ($\chi^2 = 237.49, p < 2.2\ x\ 10^{-16}$), but not in *D. melanogaster*. Of these nested non-protein coding genes,
317 83% are on the opposite strand as their parental genes, suggesting that these genes are regulated
318 independently of their parental genes (and their expression is uncorrelated, see Supplemental File 4).
319 Overall *D. melanogaster* has more annotated non-protein coding genes (2963) than *D. simulans* (1675),
320 and similarly more exons from noncoding genes are nested within introns (1772 *D. melanogaster*, 1066
321 *D. simulans*). The importance of nested non-protein coding genes for the response to the environment, or
322 potentially delays in splicing that are specific to certain components of variance, is unclear.

323 <u>Discussion</u>

The prediction from theory on genetic accommodation was that in *D. simulans* there would be abundant cryptic genetic variation in response to ethanol exposure, while in *D. melanogaster* there would be a response to the environment that was not genotype-specific. This is because *D. simulans* does not exploit ethanol-rich resources, thus environmentally induced variants are not exposed to selection and accumulate as cryptic genetic variation. This manifests as greater variation between genotypes in the novel environment [11-14]. In *D. melanogaster* ethanol is used as a resource, however the environment is patchy and therefore response to ethanol is expected to be selected upon for the optimal response, but to remain plastic. This would manifest as an environmental response, but without extensive genotype-specific variation for that response [16-20]. The use of inbred lines is a unique opportunity to assess these predictions, as the contribution of interactions with genotype can be directly assessed.

Here I show that these expectations are met in *D. melanogaster* and *D. simulans*. In *D. melanogaster* adaptation to ethanol has occurred through genetic accommodation – selection on plasticity to reduce genetic variation and produce a single plastic response. This has facilitated expansion to a novel resource in *D. melanogaster*, ethanol rich substrates. In *D. simulans* this is not the case, and lack of selection in ethanol environments has allowed for the accumulation of cryptic genetic variation for phenotype plasticity manifested as extensive genotype by environment interactions. Joshi and Thompson (1997) found that in *D. simulans* there was a reduction in variation between families for the phenotypic response to ethanol substrate after selection for tolerance to ethanol. This is consistent with the observed scenario in D. *melanogaster* and *D. simulans* – adaptation to a novel environment results in a reduction in variation for environmental response and while retaining a single response to the environment. This is also consistent with genetic accommodation as a mechanism of adaptation to ethanol, where initial responses to environmental differences are a mix of adaptive and maladaptive, which are subsequently honed by selection.

Previous literature on the response to novel environments is equivocal concerning the expectation that plasticity evolves from a starting point of increased genetic variance due to cryptic genetic variation.

349 For example, early estimates of heritability found reduced expression of genetic variation under 'stressful'
350 conditions – though stressful does not necessarily imply novel [79,80]. Work in *Drosophila* has been
351 similarly ambiguous – in *D. mojavensis* a similar reduction in genotype by environment interactions was
352 detected in adaptation to a novel cactus host, however in *D. melanogaster* differences in genotype by
353 environment interactions were not detected between cold-adapted and non-cold adapted populations
354 [16,42]. These differences may be due to difficulty in defining novelty, and in equating novelty with
355 stress. As posited by Chevin and Hoffman (2017), it may be that genetic variance increased in novel
356 environments, but that favorable conditions are rare – meaning that 'stressful' environments are
357 commonly experienced resulting in a reduction in genetic variation.

358 The abundance of lncRNAs which are involved in genotype by environment interactions in *D.*
359 *simulans* is perhaps not surprising, given that they have been implicated previously in dynamic responses
360 [81]. It is also possible they are more frequently involved because lncRNAs are often less conserved, as
361 there is no requirement for the maintenance of ORFs and codon synonymy [82-85]. If neutral variation
362 was allowed to accumulate without selection, and then uncovered in a novel environment (cryptic genetic
363 variation), preferential accumulation within less constrained sequences would be expected.

364 Alternative splicing has the potential to be an important component of adaptation and response to
365 the environment, as it does not require de novo transcription or protein production. In *D. melanogaster* it
366 is a more important component of the response to ethanol than in *D. simulans*, as evidenced by
367 differences in the frequency of significant changes in relative abundance of exons (nearly twice as many
368 in *D. melanogaster* [61]). The response to ethanol in *D. melanogaster* is adaptive, thus it can be deduced
369 that alternative splicing is important for adaptation to ethanol. Alternatively, the fact that it is less
370 important in *D. simulans* suggests that perhaps there is selection against the accumulation of cryptic
371 genetic variation for splicing patterns. This is not surprising – previous studies have linked alternative
372 splicing to the response to the environment [43-50], as well as important adaptive differences [43,86-88].

373 Furthermore, it has been previously implicated in the response to ethanol, and in *D. melanogaster* may be
374 a more important component of the response than gene expression changes [51-57].

375 In general, the GO terms implicated in this study relate to known effects of ethanol, for example,
376 alcohol-induced ciliary dysfunction is a known consequence of alcohol exposure [89,90]. Furthermore,
377 the genes implicated in both species (*Drat*, *cabut*, *sugarbabe*, *Pinocchio*, *CG32512*, and *CG407*) have
378 generally been implicated in the response to ethanol in other studies. *cabut* has been previously observed
379 as being upregulated in response to ethanol, and it is in general responsive to changes in metabolic
380 conditions [75]; [91,92]. *sugarbabe* and *cabut* are both downstream of the *Mondo-Mlx* sugar sensing
381 pathway [93], which has been linked to severe obesity, high circulating triglycerides, and tumorigenesis
382 [94-97]. *Drat* has been implicated in ethanol-related cell death [98]. Specifically with regard to other
383 studies of ethanol exposure in *Drosophila* overlap is low between significant datasets, however this may
384 in large part be due to differences in methodology [61].

385 Inferring that gene expression differences are adaptive or non-adaptive remains a major challenge
386 in the study of gene expression reaction norms, given the lack of direct correlation between gene
387 expression phenotypes and organismal phenotypes. However, the patterns observed in *D. simulans*
388 suggest that abundant genotype by environment interactions have accumulated neutrally and become
389 uncovered in response to a novel environment. In contrast, in *D. melanogaster* this ethanol environment is
390 not novel and variation in plasticity has been selected out in favor of an adaptive phenotypic response.
391 lncRNAs are preferentially differentially expressed in *D. simulans* in response to ethanol likely because
392 they are less constrained and can accumulate more neutral variation. This is an excellent illustration of
393 genetic accommodation, where phenotypic plasticity has encouraged adaptation to a novel resource
394 within a patchy environment.

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400
401 Data Accessibility
402 *D. melanogaster* sequence data have been submitted to Bioproject: accession number PRJNA482662. *D.*
403 *simulans* sequence data will be made available upon acceptance.
404
405 Competing interests
406 I declare that I have no competing interests.
407
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410
411
412

D. simulans

	G	ETOH	G x ETOH	ETOH x T	G x ETOH x T	10	20	30
Exons	1994	76	387	24	1158	9	3	29
Exonic Regions	406	23	98	7	299	3	1	4
Fragments	608	18	81	6	225	4	2	6

413

414 **Table 1**: A summary of the exons, exonic regions, and exon fragments that are significantly differently
415 expressed for each component of variance. Abbreviations are as follows: (G) Genotype, (ETOH)
416 Ethanol, and (T) Time. Ethanol in this case is the environment.

417

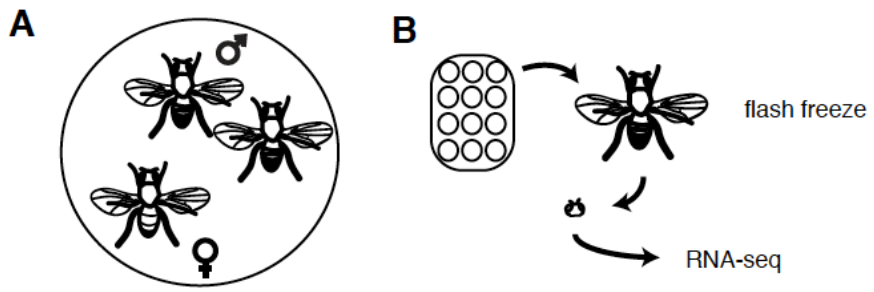
D. simulans

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

418

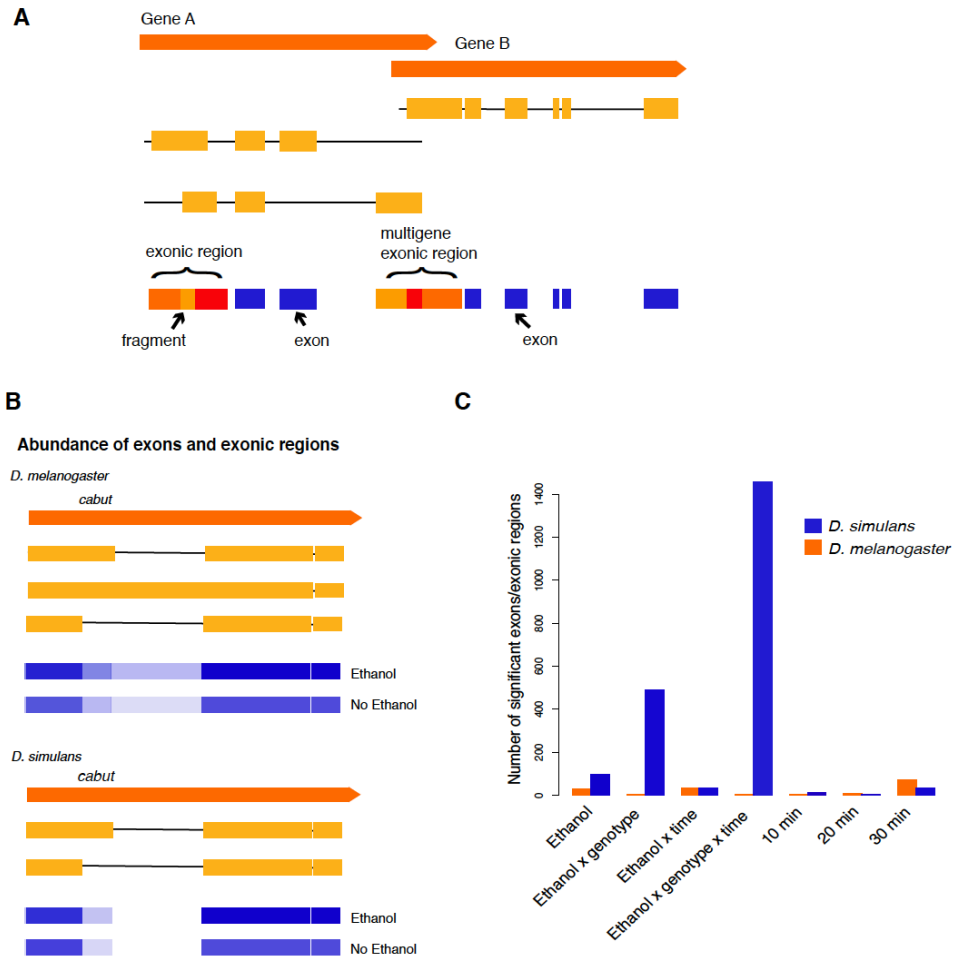
419 **Table 2**: A summary of the significant GO enrichment terms for *D. simulans*. The abbreviations are the
420 same as in Table 1. *p*-values are listed adjacent to the significant term, the significance cutoff is $p < .01$.

421



422

423 Figure 1: Experimental setup. A. This is an illustration of the social environment that each *Drosophila*
424 male was exposed to during the experiment. Each chamber contained two male flies and one female fly.
425 Within a plate there were twelve chambers, and the males from a single plate where pooled to create a
426 sequencing library. B. Males from each of the twelve chambers were collected and flash frozen after
427 either 10 minutes, 20 minutes, or 30 minutes. After flash freezing their heads were isolated for RNA-seq.



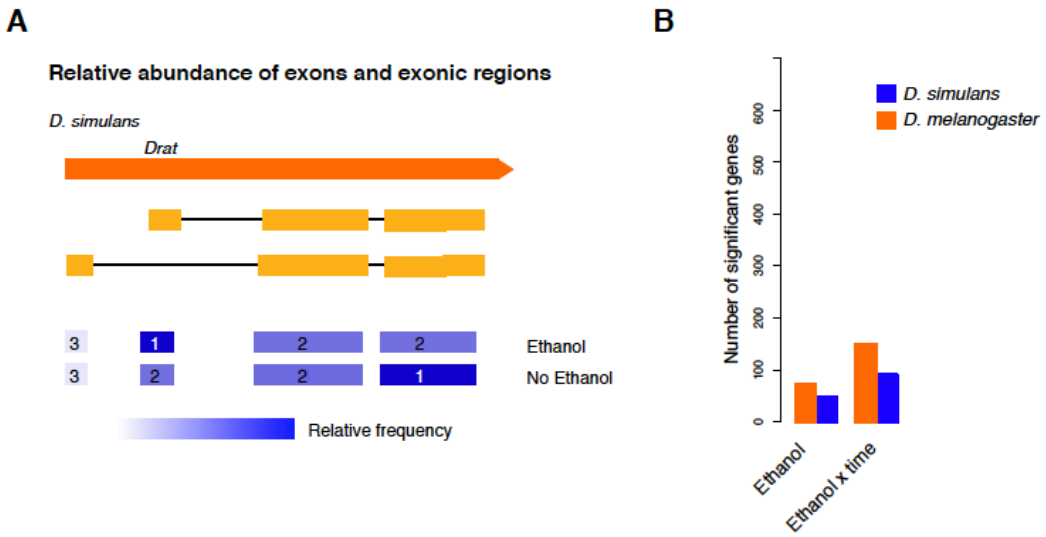
428

429

430

431 **Figure 1**: **A**. An illustration of the classification scheme for exons, exonic regions, and exon fragments.
432 Exons either do not overlap other exons in different isoforms (exons, shown in blue), or are fused regions
433 consisting of a set of overlapping exons (exonic regions, shown in shades of orange and red). Exonic
434 regions can be decomposed into exon fragments, depending upon their overlap between different
435 isoforms. **B.** An example of the information about gene expression and isoform abundance that can be
436 inferred from measures of differential exon abundance. *cabut* is a relatively simple example, having few
437 isoforms and exons, and yet it is still a complicated inter-species comparison given that *D. melanogaster*
438 has more annotated isoforms that *D. simulans*. Below each gene model the frequency of different exons
439 and exon fragments in each environment is shown, with the most frequent in dark blue. In *D. simulans* the
440 first of two isoforms shown has an exon fragment that is more abundant in the presence of ethanol,
441 suggesting that that isoform is more abundant. In *D. melanogaster* only the second of the three isoforms
442 has a unique fragment, which is also more abundant in ethanol and suggests differential isoform usage. **C.**
443 The number of exons or exonic regions that are significant for each component of variance in *D.*
444 *melanogaster* and *D. simulans*. Many more exons and exonic regions are significant for *D. simulans* than
445 *D. melanogaster*, but only for components of variance that contain an interaction with genotype

446



447

448

449 **Figure 2: A.** An illustration of a change in the relative abundance of the gene *Drat*. Only the example of

450 *D. simulans* is shown. With and without ethanol different exons are the most abundant compared to the

451 other exons within the gene. As one of these exons is not shared among all isoforms, this suggests a

452 change in isoform usage between environments. **B.** The number of genes that have a significant change in

453 relative abundance for each component of variance in *D. melanogaster* and *D. simulans*.

454

455 1. West-Eberhard, M. J. 2005 Developmental plasticity and the origin of species differences.
456 *Proc. Nat. Acad. Sci. USA* **102**, 6543–6549.

457 2. Via, S., Gomulkiewicz, R., De Jong, G., Scheiner, S. M., Schlichting, C. D. & Van
458 Tienderen, P. H. 1995 Adaptive phenotypic plasticity: consensus and controversy. *Trends in*
459 *Ecol. Evol.* **10**, 212–217.

460 3. Robinson, B. W. 2013 Evolution of growth by genetic accommodation in Icelandic
461 freshwater stickleback. *Proc. R. Soc. B* **280**, 20132197–20132197.

462 4. Schlichting, C. D. & Wund, M. A. 2014 Phenotype plasticity and epigenetic marking: an
463 assessment for evidence of genetic accommodation. *Evolution* **68**, 656–672.

464 5. Ghalambor, C. K., McKAY, J. K., CARROLL, S. P. & Reznick, D. N. 2007 Adaptive
465 versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in
466 new environments. *Funct. Ecol.* **21**, 394–407.

467 6. Morris, M. R. J., Richard, R., Leder, E. H., Barrett, R. D. H., Aubin-Horth, N. & Rogers, S.
468 M. 2014 Gene expression plasticity evolves in response to colonization of freshwater lakes
469 in threespine stickleback. *Mol. Ecol.* **23**, 3226–3240.

470 7. Schlichting, C. D. 2008 Hidden reaction norms, cryptic genetic variation, and evolvability.
471 *Ann. N. Y. Acad. Sci.* **1133**, 187–203.

472 8. Gibson, G. 2009 Decanalization and the origin of complex disease. *Nat. Rev. Genet.* **10**,
473 134–140.

474 9. Paaby, A. B. & Rockman, M. V. 2014 Cryptic genetic variation: evolution's hidden
475 substrate. *Nat. Rev. Genet.* **15**, 247–258.

476 10. Hayden, E. J., Ferrada, E. & Wagner, A. 2011 Cryptic genetic variation promotes rapid
477 evolutionary adaptation in an RNA enzyme. *Nature* **474**, 92–95.

478 11. Rutherford, S. L. 2000 From genotype to phenotype: buffering mechanisms and the storage
479 of genetic information. *BioEssays* **22**, 1095–1105.

480 12. Gibson, G. & Dworkin, I. 2004 Uncovering cryptic genetic variation. *Nat. Rev. Genet.* **5**,
481 681–690.

482 13. Hermisson, J. & Wagner, G. P. 2004 The population genetic theory of hidden variation and
483 genetic robustness. *Genetics* **168**, 2271–2284.

484 14. Le Rouzic, A., Carlborg, O. 2008. Evolutionary potential of hidden genetic variation.
485 *Trends Ecol. Evol.* **1**, 33-37.

486 15. Baldwin, J. M. 1896 A new factor in evolution. *The American Naturalist* **30**, 441–451.

487 16. Matzkin, L. M. 2012 Population transcriptomics of cactus host shifts in Drosophila
488 mojavensis. *Mol. Ecol.* **21**, 2428–2439.

489 17. Lande, R. 2009 Adaptation to an extraordinary environment by evolution of phenotypic
490 plasticity and genetic assimilation. *J. Evol. Biol.* **22**, 1435–1446.

491 18. Huang, Y. & Agrawal, A. F. 2016 Experimental evolution of gene expression and plasticity
492 in alternative selective regimes. *PLoS Genet.* **12**, e1006336.

493 19. Guntrip, J. & Sibly, R. M. 1998 Phenotypic plasticity, genotype-by-environment interaction
494 and the analysis of generalism and specialization in Callosobruchus maculatus. *Heredity* **81**,
495 198–204.

496 20. Via, S. & Lande, R. 1985 Genotype-environment interaction and the evolution of
497 phenotypic plasticity. *Evolution* **39**, 505–522.

498 21. Hoffmann, A. A. & McKechnie, S. W. 1991 Heritable variation in resource utilization and
499 response in a winery population of *Drosophila melanogaster*. *Evolution* **45**, 1000-1015.

500 22. Fry, J. D. 2014 Mechanisms of naturally evolved ethanol resistance in *Drosophila*
501 *melanogaster*. *J. Exp. Biol.* **217**, 3996–4003.

502 23. Zhu, J. & Fry, J. D. 2015 Preference for ethanol in feeding and oviposition in temperate and
503 tropical populations of *Drosophila* melanogaster. *Ent. Exp. App.* **155**, 64-70.

504 24. Joshi, A. & Thompson, J. N. 1997 Adaptation and specialization in a two-resource
505 environment in *Drosophila* species. *Evolution* **51**, 846.

506 25. Gibson, J. B. & Wilks, A. V. 1988 The alcohol dehydrogenase polymorphism of *Drosophila*
507 *melanogaster* in relation to environmental ethanol, ethanol tolerance and alcohol
508 dehydrogenase activity. *Heredity* **60 (Pt 3)**, 403–414.

509 26. Mercot, H., Defaye, D., Capy, P., Pla, E. & David, J. R. 1994 Alcohol tolerance, ADH
510 activity, and ecological niche of *Drosophila* species. *Evolution* **3**, 746–757.

511 27. Chakir, M., Peridy, O., Capy, P., Pla, E. & David, J. R. 1993 Adaptation to alcoholic
512 fermentation in *Drosophila*: A parallel selection imposed by environmental ethanol and
513 acetic acid. *Proc. Nat. Acad. Sci. USA* **90**, 3621–3625.

514 28. Thomson, M. S., Jacobson, J. W. & Laurie, C. C. 1991 Comparison of alcohol
515 dehydrogenase expression in *Drosophila melanogaster* and *D. simulans*. *Mol. Biol. Evol.* **8**,
516 31–48.

517 29. Parsons, P. A. & Spence, G. E. 1981 Ethanol utilization: threshold differences among three
518 *Drosophila* species. *Am. Nat.* **117**, 568–571.

519 30. McKenzie, J. A. & McKechnie, S. W. 1979 A comparative study of resource utilization in
520 natural populations of *Drosophila melanogaster* and *D. simulans*. *Oecologia* **40**.

521 31. Dickinson, W. J., Rowan, R. G. & Brennan, M. D. 1984 Regulatory gene evolution:
522 adaptive differences in expression of *alcohol dehydrogenase* in *Drosophila melanogaster*
523 and *Drosophila simulans*. *Heredity* **52 (Pt 2)**, 215–225.

524 32. Parsons, P. A. & King, S. B. 1977 Ethanol: Larval discrimination between two *Drosophila*
525 sibling species. *Experientia* **33**, 898–899.

526 33. David, J. & Bocquet, C. 1976 Compared toxicities of different alcohols for two *Drosophila*
527 sibling species: *D. melanogaster* and *D. simulans*. *Comp. Biochem. Physiol. C, Comp.*
528 *Pharmacol.* **54**, 71–74.

529 34. Yampolsky, L. Y., Glazko, G. V. & Fry, J. D. 2012 Evolution of gene expression and
530 expression plasticity in long-term experimental populations of Drosophila melanogaster
531 maintained under constant and variable ethanol stress. *Mol. Ecol.* **21**, 4287–4299.

532 35. Levine, M. T., Eckert, M. L. & Begun, D. J. 2011 Whole-genome expression plasticity
533 across tropical and temperate Drosophila melanogaster populations from Eastern Australia.
534 *Mol. Biol. Evol.* **28**, 249–256.

535 36. Hodgins-Davis, A., Adomas, A. B., Warringer, J. & Townsend, J. P. 2012 Abundant gene-
536 by-environment interactions in gene expression reaction norms to copper within
537 Saccharomyces cerevisiae. *Genome Biol. Evol.* **4**, 1061–1079.

538 37. Hodgins-Davis, A. & Townsend, J. P. 2009 Evolving gene expression: from G to E to G×E.
539 *Trends in Ecol. Evol.* **24**, 649–658.

540 38. DeBiasse, M. B. & Kelly, M. W. 2015 Plastic and Evolved Responses to Global Change:
541 What Can We Learn from Comparative Transcriptomics? *J. Heredity* **107**, 71–81.

542 39. Cheviron, Z. A., Whitehead, A. & Brumfield, R. T. 2008 Transcriptomic variation and
543 plasticity in rufous-collared sparrows (Zonotrichia capensis) along an altitudinal gradient.
544 *Mol. Ecol.* **17**, 4556–4569.

545 40. McCairns, R. J. S. & Bernatchez, L. 2009 Adaptive divergence between freshwater and
546 marine sticklebacks: insights into the role of phenotypic plasticity from an integrated
547 analysis of candidate gene expression *Evolution* **64**, 1029–1047.

548 41. Mathur, V. & Schmidt, P. S. 2016 Adaptive patterns of phenotypic plasticity in laboratory
549 and field environments in Drosophila melanogaster. *Evolution* **71**, 465–474.

550 42. Heckel, von, K., Stephan, W. & Hutter, S. 2016 Canalization of gene expression is a major
551 signature of regulatory cold adaptation in temperate Drosophila melanogaster. *BMC*
552 *Genomics*, 1–14.

553 43. Singh, P., Börger, C., More, H. & Sturmbauer, C. 2017 The role of alternative splicing and
554 differential gene expression in cichlid adaptive radiation. *Genome Biol. Evol.* **9**, 2764–2781.

555 44. Gueroussov, S. & Gonatopoulos-Pournatzis, T. 2015 An alternative splicing event amplifies
556 evolutionary differences between vertebrates. *Science* **349**, 868-873.

557 45. Jakšić, A. M. & Schlötterer, C. 2016 The interplay of temperature and genotype on patterns
558 of alternative splicing in Drosophila melanogaster. *Genetics* **204**, 115-25.

559 46. Merkin, J., Russell, C., Chen, P. & Burge, C. B. 2012 Evolutionary dynamics of gene and
560 isoform regulation in Mammalian tissues. *Science* **338**, 1593–1599.

561 47. Barbosa-Morais, N. L. et al. 2012 The evolutionary landscape of alternative splicing in
562 vertebrate species. *Science* **338**, 1587–1593.

563 48. Wang, G., Weng, L., Li, M. & Xiao, H. 2017 Response of gene expression and alternative
564 splicing to distinct growth environments in tomato. *IJMS* **18**, 475–19.

565 49. Pajoro, A., Severing, E., Angenent, G. C. & Immink, R. G. H. 2017 Histone H3 lysine 36
566 methylation affects temperature-induced alternative splicing and flowering in plants.
567 *Genome Biology* **18**, 102.

568 50. Calixto, C. P. G. et al. 2018 Rapid and dynamic alternative splicing impacts the Arabidopsis
569 cold response transcriptome. *The Plant Cell* **40**, 1424-1444.

570 51. Robinson, B. G. & Atkinson, N. S. 2013 Is alcoholism learned? Insights from the fruit fly.
571 *Curr. Opin. Neurobiol.* **23**, 529–534.

572 52. Sasabe, T. & Ishiura, S. 2010 Alcoholism and alternative splicing of candidate genes.
573 *IJERPH* **7**, 1448–1466.

574 53. Oomizu, S., Boyadjieva, N. & Sarkar, D. K. 2003 Ethanol and estradiol modulate alternative
575 splicing of dopamine D2 receptor messenger RNA and abolish the inhibitory action of
576 Bromocriptine on Prolactin release from the pituitary gland. *Alcohol Clin. Exp. R.* **27**, 975–
577 980.

578 54. Hemby, S. E. 2012 Alternative splicing of AMPA subunits in prefrontal cortical fields of
579 cynomolgus monkeys following chronic ethanol self-administration. *Front. Psychiatry* **3**,
580 72.

581 55. Zaharieva, E., Chipman, J. K. & Soller, M. 2012 Alternative splicing interference by
582 xenobiotics. *Toxicology* **296**, 1–12.

583 56. Newton, P. M., Tully, K., McMahon, T., Connolly, J., Dadgar, J., Treistman, S. N. &
584 Messing, R. O. 2004 Chronic ethanol exposure induces an N-type calcium channel splice
585 variant with altered channel kinetics. *FEBS Letters* **579**, 671–676.

586 57. Pietrzykowski, A. Z., Friesen, R. M., Martin, G. E., Puig, S. I., Nowak, C. L., Wynne, P. M.,
587 Siegelmann, H. T. & Treistman, S. N. 2008 Posttranscriptional regulation of BK channel
588 splice variant stability by miR-9 underlies neuroadaptation to alcohol. *Neuron* **59**, 274–287.

589 58. Signor, S. A., New, F. N. & Nuzhdin, S. 2017 A large panel of Drosophila simulans reveals
590 an abundance of common variants. *Genome Biol. Evol.* **10**, 189–206.

591 59. Signor, S. A., Abbasi, M., Marjoram, P. & Nuzhdin, S. V. 2017 Conservation of social
592 effects (Ψ) between two species of Drosophila despite reversal of sexual dimorphism. *Ecol.*
593 *Evol.* **7**, 10031–10041.

594 60. Signor, S. A., Abbasi, M., Marjoram, P. & Nuzhdin, S. V. 2017 Social effects for
595 locomotion vary between environments in Drosophila melanogaster females. *Evolution* **71**,
596 1765–1775.

597 61. Signor, S. & Nuzhdin, S. 2018 Dynamic changes in gene expression and alternative splicing
598 mediate the response to acute alcohol exposure in Drosophila melanogaster. *Heredity* **121**,
599 342-360.

600 62. Dalton, J. E., Fear, J. M., Knott, S., Baker, B. S., McIntyre, L. M. & Arbeitman, M. N. 2013
601 Male-specific Fruitless isoforms have different regulatory roles conferred by distinct zinc
602 finger DNA binding domains. *BMC Genomics* **14**, 659.

603 63. Fear, J. M., León-Novelo, L. G., Morse, A. M., Gerken, A. R., Van Lehmann, K., Tower, J.,
604 Nuzhdin, S. V. & McIntyre, L. M. 2016 Buffering of genetic regulatory networks in
605 Drosophila melanogaster. *Genetics* **203**, 1177–1190.

606 64. Newell, N. R., New, F. N., Dalton, J. E., Genes, L. M. G., Genomes2016 2016 Neurons that
607 underlie Drosophila melanogaster reproductive behaviors: detection of a large male-bias in
608 gene expression in fruitless-expressing neurons. *G3* **6**, 2455–2465.

609 65. Graze, R. M., McIntyre, L. M., Morse, A. M., Boyd, B. M., Nuzhdin, S. V. & Wayne, M. L.
610 2014 What the X has to do with it: Differences in regulatory variability between the sexes in
611 Drosophila simulans. *Genome Biol. Evol.* **6**, 818–829.

612 66. Dobin, A., Davis, C., Schlesigner, F., Drenkow, J., Zaleski, C., Sonali, J., Batut, P.,
613 Chaisson, M., Gingeras, T. 2013 STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*
614 **29**, 25-21.

615 67. Newman, J. R. B., Conesa, A., Mika, M., New, F. N., Onengut-Gumuscu, S., Atkinson, M.
616 A., Rich, S. S., McIntyre, L. M. & Concannon, P. 2017 Disease-specific biases in alternative
617 splicing and tissue-specific dysregulation revealed by multitissue profiling of lymphocyte
618 gene expression in type 1 diabetes. *Genome Res*. **27**, 1807-1815.

619 68. Li, H. 2015 Aligning sequence reads, clone sequences and assembly contigs with BWA-
620 MEM. *arXiv*, 1–3.

621 69. Bullard, J. H., Purdom, E., Hansen, K. D. & Dudoit, S. 2010 Evaluation of statistical
622 methods for normalization and differential expression in mRNA-Seq experiments. *BMC*
623 *Bioinformatics* **11**, 94–13.

624 70. Dillies, M. A. et al. 2013 A comprehensive evaluation of normalization methods for
625 Illumina high-throughput RNA sequencing data analysis. *Brief. Bioinf*. **14**, 671–683.

626 71. Benjamini, Y., Hochberg, Y.1995 Controlling the false discovery rate: a practical and
627 powerful approach to multiple testing. *J. R. Stat. Soc., B* **57**, 289–300.

628 72. Mi, H., Huang, X., Muruganujan, A., Tang, H., Mills, C., Kang, D. & Thomas, P. D. 2017
629 PANTHER version 11: expanded annotation data from Gene Ontology and Reactome
630 pathways, and data analysis tool enhancements. *Nuc. Acids Res*. **45**, D183–D189.

631 73. Morozova, T., Anholt, R. & Mackay, T. F. 2006 Transcriptional response to alcohol
632 exposure in *Drosophila melanogaster*. *Genome Biol*. **7**, R95.

633 74. Kong, E. C., Allouche, L., Chapot, P. A., Vranizan, K., Moore, M. S., Heberlein, U. &
634 Wolf, F. W. 2010 Ethanol-regulated genes that contribute to ethanol sensitivity and rapid
635 tolerance in Drosophila. *Alcohol Clin. Exp. R*. **34**, 302–316.

636 75. Awofala, A. A., Davies, J. A. & Jones, S. 2012 Functional roles for redox genes in ethanol
637 sensitivity in Drosophila. *Funct. Integr. Genomics* **12**, 305–315.

638 76. Bae, S. H., Sung, S. H., Cho, E. J., Lee, S. K., Lee, H. E., Woo, H. A., Yu, D.-Y., Kil, I. S.
639 & Rhee, S. G. 2011 Concerted action of sulfiredoxin and peroxiredoxin I protects against
640 alcohol-induced oxidative injury in mouse liver. *Hepatology* **53**, 945–953.

641 77. Chattopadhyay, A. et al. 2015 Fortilin potentiates the peroxidase activity of Peroxiredoxin-1
642 and protects against alcohol-induced liver damage in mice. *Sci. Rep*., 1–16.

643 78. Lee, Y. C. G. & Chang, H. H. 2013 The evolution and functional significance of nested
644 gene structures in Drosophila melanogaster. *Genome Biol. Evol*. **5**, 1978–1985.

645 79. Chevin, L.-M. & Hoffmann, A. A. 2017 Evolution of phenotypic plasticity in extreme
646 environments. *Philos. Trans. R. Soc. B* **372**, 20160138–12.

647 80. Charmantier, A. & Garant, D. 2005 Environmental quality and evolutionary potential:
648 lessons from wild populations. *Proc. R. Soc. B* **272**, 1415–1425.

649 81. Valadkhan, S. & Valencia-Hipólito, A. 2016 lncRNAs in Stress Response. In *Long Non-*
650 *coding RNAs in Human Disease*, 203–236. Springer International Publishing.

651 82. Chodroff, R. A., Goodstadt, L., Sirey, T. M., Oliver, P. L., Davies, K. E., Green, E. D.,
652 Molnár, Z. & Ponting, C. P. 2010 Long noncoding RNA genes: conservation of sequence
653 and brain expression among diverse amniotes. *Genome Biology* **11**, R72.

654 83. Ulitsky, I. 2016 Evolution to the rescue: using comparative genomics to understand long
655 non-coding RNAs. *Nat. Rev. Genet.* **17**, 601–614.

656 84. Ulitsky, I., Shkumatava, A., Jan, C. H., Sive, H. & Bartel, D. P. 2011 Conserved function of
657 lincRNAs in vertebrate embryonic development despite rapid sequence evolution. *Cell* **147**,
658 1537–1550.

659 85. Quinn, J. J., Zhang, Q. C., Georgiev, P., Ilik, I. A., Akhtar, A. & Chang, H. Y. 2016 Rapid
660 evolutionary turnover underlies conserved lncRNA-genome interactions. *Genes Dev.* **30**,
661 191–207.

662 86. Evantal, N., Anduaga, A. M., Bartok, O., Patop, I. L., Weiss, R. & Kadener, S. 2018
663 Thermosensitive alternative splicing senses and mediates temperature adaptation in
664 Drosophila. *bioRxiv* 1–63.

665 87. Shi, Y. et al. 2019 Alternative splicing coupled to nonsense-mediated mRNA decay
666 contributes to the high-altitude adaptation of maca (Lepidium meyenii). *Gene* **694**, 7–18.

667 88. Dong, W.-X., Ding, J.-L., Gao, Y., Peng, Y.-J., Feng, M.-G. & Ying, S.-H. 2017
668 Transcriptomic insights into the alternative splicing-mediated adaptation of the
669 entomopathogenic fungus Beauveria bassianato host niches: autophagy-related gene 8 as an
670 example. *Environ Microbiol* **19**, 4126–4139.

671 89. Yang, F., Pavlik, J., Fox, L., Scarbrough, C., Sale, W. S., Sisson, J. H. & Wirschell, M.
672 2015 Alcohol-induced ciliary dysfunction targets the outer dynein arm. *Am. J. Physiol.-*
673 *Lung Cell. Mol. Physiol.* **308**, L569–L576.

674 90. Price, M., Yang, F., H Sisson, J. & Wirschell, M. 2018 *Ciliary dynein dysfunction caused by*
675 *chronic alcohol exposure*. In: Dyneins, 402-417. Academic Press.

676 91. Bartok, O. et al. 2015 The transcription factor Cabut coordinates energy metabolism and the
677 circadian clock in response to sugar sensing. *The EMBO Journal* **34**, 1538–1553.

678 92. Havula, E. & Hietakangas, V. 2012 Glucose sensing by ChREBP/MondoA–Mlx
679 transcription factors. *Seminars in Cell & Developmental Biology* **23**, 640–647.

680 93. Mattila, J. et al. 2015 Mondo-Mlx mediates organismal sugar sensing through the Gli-
681 similar transcription factor Sugarbabe. *Cell Reports* **13**, 350–364.

682 94. Carroll, P. A. et al. 2015 Deregulated Myc requires MondoA/Mlx for metabolic
683 reprogramming and tumorigenesis. *Cancer Cell* **27**, 271–285.

684 95. Kooner, J. S. et al. 2008 Genome-wide scan identifies variation in MLXIPL associated with
685 plasma triglycerides. *Nature Genet.* **40**, 149–151.

686 96. Kathiresan, S. et al. 2008 Six new loci associated with blood low-density lipoprotein
687 cholesterol, high-density lipoprotein cholesterol or triglycerides in humans. *Nature Genet*.
688 **40**, 189–197.

689 97. Herman, M. A., Peroni, O. D., Villoria, J., Schön, M. R., Abumrad, N. A., Blüher, M.,
690 Klein, S. & Kahn, B. B. 2012 A novel ChREBP isoform in adipose tissue regulates systemic
691 glucose metabolism. *Nature* **484**, 333–338.

692 98. Chen, P., Tu, X., Akdemir, F., Chew, S. K., Rothenfluh, A. & Abrams, J. M. 2012 Effectors
693 of alcohol-induced cell killing in Drosophila. *Cell Death Differ*. **19**, 1655–1663.

694