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

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

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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
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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.

Abstract

The contribution of phenotypic plasticity to adaptation is contentious, with contradictory empirical support for its role in evolution. Phenotype plasticity has been posited as being important for adaptation to novel environments, invasion success, and survival in response to climate change, as it could potentially increase the likelihood of initial persistence. Here we 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. We 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. We measure phenotype plasticity in response to ethanol with gene expression and an approach that combines information about expression and alternative splicing. We 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. We also find evidence that alternative splicing may be more important for the adaptive response to ethanol.

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

67 Introduction

68 The contribution of phenotypic plasticity to adaptation remains controversial despite considerable
69 empirical and theoretical investigation. This includes whether phenotypic plasticity itself evolves, and
70 how frequently the evolution of plasticity is important for adaptive evolution. Recent work has suggested
71 that the evolution of plasticity may be important for adaptation to changing environments (Lande 2009;
72 Scoville and Pfrender 2010; Chevin et al. 2012; Oostra et al. 2018; Li et al. 2018). For example, in a
73 novel environment phenotypic plasticity may increase the probability that a species can persist, which can
74 be followed by adaptation towards local optima (Baldwin 1902; Simpson 1953; (Miner et al. 2005;
75 Ghalambor et al. 2007; Lande 2009; Murren et al. 2015; Velotta et al. 2018). If plasticity itself is
76 adaptive, then selection would proceed by favoring the reaction norm that is most adaptive, a process
77 referred to as genetic accommodation (in contrast to simply fixing a different phenotype, known as
78 genetic assimilation) (Via et al. 1995; West-Eberhard 2005). This could potentially accelerate adaptation
79 or divergence because organisms already possess the ability to form alternative phenotypes, rather than
80 requiring the production of a novel phenotype. This is thought to be an important mechanism of
81 adaptation for invasive species, and potentially to be increasingly important in human modified
82 landscapes and climate.

83 When exposed to novel environments, the predicted path for evolution by genetic accommodation
84 is one in which initially there is increased variance, or genotype-specific differences – in the reaction
85 norm of a trait, followed by refinement by selection for a single adaptive response (West-Eberhard 2005;
86 Ghalambor et al. 2007; Robinson 2013; Schlichting and Wund 2014; Morris et al. 2014). This is because
87 if a trait only exists as an environmentally induced variant, and is therefore not exposed to selection (or
88 infrequently so) then genetic variation should accumulate in the response of that trait to a novel
89 environment (Schlichting 2008; Gibson 2009; Hayden et al. 2011; Paaby and Rockman 2014). After
90 exposure to the novel environment, this cryptic genetic variation is uncovered, manifesting as greater
91 phenotypic variation (Rutherford 2000; Gibson and Dworkin 2004; Hermisson and Wagner 2004;

92 Schlichting 2008). As lineages adapt to the novel environment, selection on this variation in reaction
93 norm should result in the loss of variation for this trait as genetic accommodation occurs (Baldwin 1896;
94 Waddington 1953; West-Eberhard 2003). As long as the environment is heterogeneous the expectation is
95 that it will remain a plastic trait, rather than evolving to be stably expressed. This would manifest as a
96 transient increase in genotype by environment interactions, followed by reduced genotype by
97 environment interactions but persistent environmental response, a pattern which is consistent evidence of
98 adaptation (Via and Lande 1985; Guntrip and Sibly 1998; Lande 2009; Matzkin 2012; Huang and
99 Agrawal 2016).

100 While changes in heritability or variance have been previously shown between populations
101 occupying ancestral and novel habitats, inbred lines have not been used previously to specifically measure
102 genotype by environment interactions in adapted and non-adapted populations. Furthermore, in general
103 the evolution of phenotypic plasticity is investigated with regards to an ancestral and a novel
104 environment, rather than a heterogeneous environment where plasticity is expected to be maintained. In
105 this manuscript we will test the predictions from genetic accommodation using inbred lines in
106 *Drosophila*, an approach which will allow direct quantification of the genotype by environment
107 interaction. We will also investigate a heterogeneous environmental variable, alcohol, for which different
108 species of *Drosophila* have different adaptive histories.

109 *D. simulans* and *D. melanogaster* have divergent adaptive histories with respect to ethanol. *D.*
110 *melanogaster* is considerably more ethanol tolerant than *D. simulans*, and is found regularly feeding upon
111 and ovipositing in resources with ethanol concentrations greater than 8% (Hoffmann and McKechnie
112 1991; Fry 2014; Zhu and Fry 2015). At concentrations of 4% ethanol *D. simulans* shows reductions in
113 survivorship and increased development time relative to *D. melanogaster* (Joshi and Thompson 1997).
114 Furthermore, *D. melanogaster* and *D. simulans* are both cosmopolitan species and human commensals,
115 however while *D. melanogaster* is commonly found inside houses, breweries, and wineries, *D. simulans*
116 is more often observed in orchards and parks (though their niches overlap significantly and they are often

117 found on the same patches) (David and Bocquet 1976; Parsons and King 1977; McKenzie and
118 McKechnie 1979; Parsons and Spence 1981; Dickinson et al. 1984; Gibson and Wilks 1988; Thomson et
119 al. 1991; Chakir et al. 1993; Mercot et al. 1994; Joshi and Thompson 1997). Therefore, the expectation is
120 that *D. simulans* will show a non-adapted phenotype manifested as considerable genotype by environment
121 interaction. *D. melanogaster* will retain an interaction with the environment, but it will not be genotype-
122 specific, which is consistent with adaptation to a heterogeneous environment.

123 The trait we measure to test this prediction is gene expression. Phenotypic plasticity is often
124 achieved by dynamic changes in gene expression across environments, and current evidence suggests that
125 it plays an important role in regulating evolutionarily important phenotypes. (Hodgins-Davis and
126 Townsend 2009; Levine et al. 2011; Yampolsky et al. 2012; Hodgins-Davis et al. 2012). Gene by
127 environment interactions also appear to be a common attribute of gene expression, and yet while many
128 studies have quantified the effect of the environment on gene expression, far fewer have performed these
129 experiments in multiple genotypes to obtain the interaction between genotype and the environment for
130 gene expression (DeBiasse and Kelly 2015). Indeed, most work on gene expression plasticity has not
131 quantified the interaction between genotype and the environment (Cheviron et al. 2008; McCairns and
132 Bernatchez 2009; Hodgins-Davis et al. 2012; DeBiasse and Kelly 2015; Mathur and Schmidt 2016;
133 Heckel et al. 2016).

134 Short read sequencing fundamentally cannot separate the contributions of alternative splicing and
135 gene expression to changes in abundance of an exon, thus we approach our analysis in a way that includes
136 both phenomenon (gene expression and alternative splicing, see Methods). In addition, because of the
137 potential for alternative splicing to respond more rapidly to environmental differences than gene
138 expression *sensu stricto*, it may be one of the earliest mediators of environmental response. This is
139 because it does not require de novo transcription or protein production. It is thought to diverge more
140 rapidly than gene expression between lineages, and it has been linked to stress responses and changes in
141 transcriptome complexity in response to environmental differences (Merkin et al. 2012; Barbosa-Morais
142 et al. 2012; Gueroussov and Gonatopoulos-Pournatzis 2015; Jakšić and Schlötterer 2016; Singh et al.

143 2017; Wang et al. 2017; Pajoro et al. 2017; Calixto et al. 2018). Furthermore, it has been previously
144 implicated in the response to ethanol, and in *D. melanogaster* may be a more important component of the
145 response than gene expression changes (Oomizu et al. 2003; Newton et al. 2004; Pietrzykowski et al.
146 2008; Sasabe and Ishiura 2010; Hemby 2012; Zaharieva et al. 2012; Robinson and Atkinson 2013). Yet it
147 remains the topic of few investigations into environmental response.

148 Overall, we compare plasticity in response to ethanol, including genetic variation for plasticity
149 over time and between genotypes, among *Drosophila* with different patterns of ethanol utilization. We
150 measure plasticity and variation in expression and splicing in response to ethanol treatment. We address
151 three questions from theoretical work on phenotypic plasticity: Is cryptic genetic variation uncovered in
152 the non-adapted species (*D. simulans*)? Do we observe maintenance of an environmental response and
153 removal of genotype by environment interactions in the adapted species (*D. melanogaster*)? What
154 contribution does alternative splicing have to adaptation? We find that in *D. melanogaster*, which
155 regularly exploits ethanol-rich substrates, there is maintenance of an environmental response of similar
156 magnitude to *D. simulans*. However, there is essentially no genetic variation for plasticity within the
157 tested *D. melanogaster* population, while *D. simulans* exhibits a much larger effect of genotype by
158 environment compared to other components of variance (cryptic variation). This suggests the following
159 scenario: In *D. simulans* high ethanol concentrations are essentially a novel environment that reveals
160 cryptic variation for phenotypic plasticity. In *D. melanogaster*, genetic variation in phenotypic plasticity
161 has been removed by selection in favor of a single environmental response through a process of genetic
162 accommodation. This single environmental response involves more alternative splicing than observed in
163 *D. simulans*, suggesting it is an important component of adaptation.

164

165 <u>Methods</u>

166 *Fly lines*

167 Six genotypes from each species were used in these experiments. The genotypes used for male flies from
168 *D. melanogaster* were collected from an orchard in Winters, California in 1998 and made isogenic by 40
169 generations of full sibling inbreeding (Yang and Nuzhdin 2003; Campo et al. 2013; Signor et al. 2017b,c).
170 The genotypes used for male flies from *D. simulans* were collected from the Zuma Organic Orchard in the
171 winter of 2012 and made isogenic by 15 generations of full sib inbreeding (Signor et al. 2017a,b).
172 Residual heterozygosity is similar between these lines of *D. melanogaster* and *D. simulans* (Campo et al.
173 2013; Signor et al. 2017a). In natural conditions flies will not by homozygous at all loci, thus each inbred
174 genotype was crossed to a white eyed 'tester' strain to create the F1 flies used in the ethanol exposure
175 assays (*D. melanogaster*, w^{1118}, Bloomington stock number 3605; *D. simulans*, w^{501}, Cornell species stock
176 number 14021-0251.011). Rearing occurred on a standard medium at 25 °C with a 12-h light/12-h dark
177 cycle. In order to control for maternal effects and variation in offspring quality, female parents were
178 collected as virgins, aged one day, and then density matched with male flies (10 per sex). The F1
179 offspring were then collected as virgins, reared in single sex vials with standardized density (24-30 flies if
180 male, 12-15 if female), and aged for three to four days prior to the ethanol exposure assay.

181 A portion of this experiment was intended to analyze behavioral differences in ethanol, thus during
182 the ethanol exposure a female was included as a stimulus, but was not collected for RNA-seq (Signor et al.
183 2017b,c). The females were from y^1w^1 mutants for both *D. simulans* and *D. melanogaster* (*D. melanogaster*,
184 Bloomington stock number 1495, *D. simulans*, Cornell species stock number 14021-0251.013). The day
185 prior to the ethanol exposure these females were mated to flies from a standard genotype, such that in the
186 final assay each chamber contained one mated female fly from the y^1w^1 mutant lines and two virgin males
187 from the target F1 cross, of which only the latter were collected for RNA-seq.

188 *Experimental setup*

189 In this experiment intoxication occurs through inhalation of ethanol vapors, and evidence of the
190 behavioral effects in both species and the efficacy of this approach have been published previously
191 (Signor et al. 2017b,c). Ethanol exposure took place in a circular arena, each of which was part of a

192 larger chamber containing 12 arenas each with a diameter of 2.54 cm (VWR cat. no. 89093-496). The
193 bottom of each chamber contained a standard amount of either grapefruit medium or medium in which
194 15% of the water has been replaced with ethanol. Replicates for both species were conducted randomly
195 under standardized conditions (25 °C, 70% humidity). Prior to the assay the flies were sedated through
196 exposure at 4 °C for ten minutes, to avoid the confounding effects of CO_2, then placed in the chambers
197 which were left then upside down at room temperature for ten minutes while the flies oriented themselves
198 (see Signor et al. 2017a; b; Signor & Nuzhdin 2018). After ten minutes the timing of the assay began, and
199 were conducted for ten, 20, or 30 minutes for three replicates of each of the two conditions. Flies are most
200 active during the hours following dawn, thus to standardize behavior and circadian rhythms all assays
201 were conducted within a two-hour window after dawn. At the conclusion of the assay the chambers were
202 flash frozen in liquid nitrogen, allowed to freeze through, transferred to dry ice, and all of the males were
203 collected for RNA-seq.

204 *Sample preparation and RNA sequencing*

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

217 in two technical replicates per sample. The two run lengths (and runs) were intended to provide extra
218 coverage, and all replicates were sequenced in both runs.

219 *Exon expression analysis*

220 It is common in organisms with alternative splicing for exons from different isoforms of a single gene to
221 overlap with one another, or be shared between all or most isoforms (Figure 1A). Short read data
222 fundamentally cannot resolve these exons to individual isoforms, however, one approach is to quantify each
223 exon separately and decompose exons overlapping between isoforms into those which are shared and
224 unique. When the differences between overlapping exons are less than 10 bp, there is no appreciable
225 amount of information loss in not decomposing overlapping exons, and this approach has been taken in the
226 past (Dalton et al. 2013; Graze et al. 2014; Fear et al. 2016; Newell et al. 2016). However, in many cases
227 the differences in exon overlap are much larger than this, so to address this issue we use a classification
228 scheme where reads may be assigned to *exons*, *exonic regions*, or *exon fragments*, and then compare the
229 abundance of each exon/exonic region/exon fragment in each condition (i.e. the abundance of an exon with
230 and without ethanol) (Signor and Nuzhdin 2018) (Figure 1A). Here, an *exon* does not overlap any other
231 exons. If exons from different isoforms overlap, they are grouped into an *exonic region*. *Exon fragments*
232 are classified by decomposing *exonic regions* based on the 5' and 3' positions of the exons within the region.
233 Thus, all *exon fragments* are subregions of *exonic regions*. Exon boundaries were determined using the *D.*
234 *melanogaster* FlyBase 6.17 genome features file and the *D. simulans* 2.02 genome features file. Alignment
235 was performed using BWA-MEM version 0.7.15 (which has been shown to perform better than split read
236 mappers such as STAR (Newman et al. 2018)) and BED files were used to count reads in each region and
237 obtain the length adjusted read count (reads in region divided by the length of region), and the APN (average
238 per nucleotide) (Li 2015).

239 The APN was calculated separately for each read length and then combined between read lengths
240 to handle the mixture of read lengths for each sample (2 × 150 bp and 2 × 50 bp). If the APN was greater
241 than zero in at least half of all samples per condition it was considered detected. While we considered

242 several approaches to normalize coverage counts upper-quartile normalization with log-transformation and
243 median centering within time × treatment × genotype were selected due to better performance of the
244 residuals (Bullard et al. 2010; Dillies et al. 2013).

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

247 $$\Upsilon_{ijkl} = \mu + g_i + t_j + (gt)_{ik} + m_k + (gm)_{ik} + (tm)_{ijk} + \epsilon_{ijkl}$$

248 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
249 min), and l^{th} replicate (Supplemental File 1-2). For the interaction between treatment and time point, the log
250 APN for each exonic region was modeled as

251 $$\Upsilon_{ij} = \mu + t_j + \epsilon_{ij}$$

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

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

264 $$\Upsilon_{ijk} = \mu + r_i + t_j + (rt)_{ij} + \epsilon_{ijkl}$$

265 where Y_{ijk} is the exon rank (1,2,3) of the ith exonic region of the gene, j^{th} condition (time × treatment), and
266 the kth replicate; r_i is the exonic region of the gene; t_j is condition; and $(rt)_{ij}$ is the interaction between
267 exonic region and condition. More traditional GLM approaches can only be taken if their assumptions are
268 met, and in this case they are not due to a lack of normality in the distribution of model residuals.
269 Accordingly, a non-parametric test must be relied upon to look for changes in exon or exonic region
270 representation between exons of a gene and we used a rank test to summarize changes in exon representation
271 (Supplemental File 3). F-tests for the significance of the mean square attributed to the effect tested versus
272 the mean square attributed to error, or the appropriate interaction term, were used. The false discovery rate
273 was controlled using the Benjamini-Hochberg procedure, with a significance cutoff of $\alpha = 0.05$ (Benjamini
274 & Hochberg 1995).

275 *GO Analysis*

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

285 *Functional class enrichment*

286 We tested the significant sets of exons and exonic regions for enrichment with non-protein coding genes.
287 Multigene exonic regions were not included, meaning exons that belong to more than one gene, as they
288 often do not correspond to the same functional class of gene. Every test of functional class enrichment

289 compared the frequency of a given subcategory among all exons and exonic regions detected in the
290 dataset compared to the frequency within a significant list of exons and exonic regions. A χ^2 test was
291 performed in R to test the significance of the enrichment of each of these categories.

292 Results

293 *Exon expression and isoform usage*

294 It is difficult to decouple alternative isoform usage from gene expression, given that many exons are
295 shared between isoforms or overlap other exons. To infer isoforms from short read data, one must rely
296 upon unique junctions or regions of individual isoforms and extrapolate to shared regions. This requires
297 accurate isoform annotation (knowing that any given exon/junction is found in combination with other
298 exons/junctions) and in general can be very noisy. Accordingly, we subscribe to a simpler but more robust
299 approach and summarize the abundance of different exons and exonic regions separately (Dalton et al.
300 2013; Graze et al. 2014; Newell et al. 2016; Signor & Nuzhdin 2018). We detail overall abundance of
301 exons, exonic regions, and exon fragments, and changes in the relative abundance of exons within a gene
302 (Figure 1 A-C; Figure 2 A&B).

303 *Changes in exon expression in D. melanogaster and D. simulans*

304 Table 1 summarizes the number of exons, exonic regions, and exon fragments which alter their
305 expression in response to ethanol, but note that the table for *D. melanogaster* is reproduced from Signor
306 & Nuzhdin 2018. For treatment seven exons and exonic regions were shared between species: *Drat*,
307 *cabut*, *CG11741*, *CG32512*, *CG4607*, *Pinocchio*, and *sugarbabe* (Table 1; Figure 1 A-C, Signor &
308 Nuzhdin 2018). For the interaction between ethanol and time three genes were shared between exons and
309 exonic regions for these species, *Drat*, *cabut*, and *CG43366*. At ten and twenty minutes *cabut* is shared
310 between exons and exonic regions in *D. melanogaster* and *D. simulans*. At 30 minutes *Drat*, *CG32103*,
311 *sugarbabe*, *cabut*, *Pinocchio*, *CG32512*, and *CG4607* are shared between species. This suggests that
312 *Drat*, *cabut*, *sugarbabe*, *Pinocchio*, *CG32512*, and *CG407* are important for the response to ethanol, as

313 they are shared between species for multiple components of variance and have been implicated in the
314 response to ethanol previously (Morozova et al. 2006; Kong et al. 2010; Awofala et al. 2012).

315 *GO enrichment analysis of differentially expressed exons in D. melanogaster and D. simulans*

316 *D. simulans* is enriched for several GO terms in response to interactions with genotype, including several
317 terms relating to cilia, such as the axonemal dynein complex and ciliary part (Table 2). GO terms were
318 also enriched for the sensory perception of taste and detection of chemical stimulus. Many components of
319 variance in *D. simulans* are not enriched for GO terms due in part to the frequency with which non-
320 protein coding genes were implicated – for example in response to ethanol 15% of the implicated genes
321 do not have a *D. melanogaster* ortholog, and of those only two are protein coding . In *D. melanogaster*
322 exons and exonic regions were not significantly enriched for any category of genes. In general, the small
323 number of genes implicated for many categories precludes any conclusion of enrichment.

324 *Changes in the relative abundance of exons within a gene in D. melanogaster and D. simulans*

325 In response to treatment in *D. simulans* 54 genes showed changes in the relative abundance of their
326 constitutive exons/exonic regions, and 94 genes change the relative abundance of their exons for the
327 interaction between treatment and time (Figure 2B). In *D. melanogaster* 71 genes changed the relative
328 abundance of their exons in response to ethanol and 145 changed for treatment by time (Signor and
329 Nuzhdin 2018). For the interaction between treatment and time *lola*, *Mhc*, and *Prm* were shared between
330 species.

331 *GO enrichment analysis of genes with changes in relative abundance in D. melanogaster and D. simulans*

332 Changes in relative abundance were not enriched for any GO terms in *D. simulans*, with the exception of
333 peroxiredoxin activity in response to ethanol, albeit slightly above the more conservative cut-off used in
334 our other tests (p = .017). Peroxiredoxin activity has been associated with protection against alcohol
335 induced liver damage (Bae et al. 2011; Chattopadhyay et al. 2015). In *D. melanogaster* genes implicated
336 in changes in relative abundance in response to ethanol by time were enriched for cellular components

337 actin cytoskeleton, again somewhat above our more conservative cutoff ($p = 0.018$) (Signor and Nuzhdin
338 2018).
339
340 *In D. simulans genotype-specific reactions to the environment are abundant*
341 In *D. simulans* many exons and exonic regions are significant for components of variance that interact
342 with genotype: 1457 for the interaction between genotype, treatment and time, and 486 for the interaction
343 between genotype and treatment, compared to two and three exons and exonic regions respectively in *D.*
344 *melanogaster* (Table 1; Figure 1 A-C). For other components of variance, the number of exons and
345 exonic regions implicated in expression differences in roughly comparable between species.
346
347 *In D. simulans interactions with genotype are enriched for nested non-protein coding genes*
348 A large number of the genes implicated in differences due to interaction with genotype were non-protein
349 coding genes. The number of non-protein coding genes that were significant for ethanol, ethanol by
350 genotype, and ethanol by genotype by time, were more than would be expected by chance in *D. simulans*
351 ($\chi^2 = 49.598, p < 0.0005; \chi^2 = 235.21, p < 2.2\ x\ 10^{-16}; \chi^2 = 727.17, p < 2.2\ x\ 10^{-16}$). These are
352 generally long non-coding RNAs (lncRNAs) as the shortest is annotated at 713 bp, and the majority are
353 over 4,000 bp (long non-coding RNAs being any non-protein coding genes over 200 nt), with some
354 pseudogenes. *D. melanogaster* is not enriched for non-protein coding genes in response to ethanol,
355 ethanol by time, ethanol by genotype, ethanol by genotype by time, ten or 20 minutes, exons or exonic
356 regions expressed only in one environment, or genes implicated in changes in relative abundance.
357 However, at 30 minutes *D. melanogaster* is enriched for non-protein coding genes, though this concerns
358 far fewer genes than in *D. simulans* ($\chi^2 = 12.831, p = 0.005$).
359 Many of the non-protein coding genes implicated in interactions with genotype are nested in the
360 introns of other genes, thus we sought to determine if it was more than expected by chance. First we must
361 determine the overall frequency of gene nesting, using the criteria that an exon nested in an intron must
362 overlap the intron by at least 80 bp or 10%. We found that in *D. melanogaster* 9.2% of exons were nested

363 within introns, while in *D. simulans* 9.7% were nested, similar to what has been previously reported (Lee
364 and Chang 2013). This was reflected in our data, where for both *D. melanogaster* and *D. simulans* 6.8%
365 of exons and exonic regions were nested within introns (lower because multi-gene exons were excluded).
366 However, among significant exons and exonic regions in *D. melanogaster* 18.6% were nested, while in *D.*
367 *simulans* 33.5% were nested. This is a significant enrichment of exons that are nested within other
368 introns, for both *D. melanogaster* ($\chi^2 = 12.344, p < .0004$) and *D. simulans* ($\chi^2 = 1721.1, p <$
369 $2.2\ x\ 10^{-16}$). In addition, compared to the total number of nested genes that are noncoding within the
370 dataset, the number that are significant for the response to ethanol is enriched for nested, non-protein
371 coding genes in *D. simulans* ($\chi^2 = 237.49, p < 2.2\ x\ 10^{-16}$), but not in *D. melanogaster*. Of these
372 nested non-protein coding genes, 83% are on the opposite strand as their parental genes, suggesting that
373 these genes are regulated independently of their parental genes (and their expression is uncorrelated, see
374 Supplemental File 4). Overall *D. melanogaster* has more annotated non-protein coding genes (2963) than
375 *D. simulans* (1675), and similarly more exons from noncoding genes are nested within introns (1772 *D.*
376 *melanogaster*, 1066 *D. simulans*). The importance of nested non-protein coding genes for the response to
377 the environment, or potentially delays in splicing that are specific to certain components of variance, is
378 unclear.
379
380 Discussion
381 The prediction from theory on genetic accommodation was that in *D. simulans* there would be abundant
382 cryptic genetic variation in response to ethanol exposure, while in *D. melanogaster* there would be a
383 response to the environment that was not genotype-specific. This is because *D. simulans* does not exploit
384 ethanol-rich resources, thus environmentally induced variants are not exposed to selection and accumulate
385 as cryptic genetic variation. This manifests as greater variation between genotypes in the novel
386 environment (Rutherford 2000; Gibson and Dworkin 2004; Hermisson and Wagner 2004; Schlichting
387 2008). In *D. melanogaster* ethanol is used as a resource, however the environment is patchy and therefore
388 response to ethanol is expected to be selected upon for the optimal response, but to remain plastic. This

389 would manifest as an environmental response, but without extensive genotype-specific variation for that
390 response (Via and Lande 1985; Guntrip and Sibly 1998; Lande 2009; Matzkin 2012; Huang and Agrawal
391 2016). The use of inbred lines is a unique opportunity to assess these predictions, as the contribution of
392 interactions with genotype can be directly assessed.

393 Here we show that these expectations are met in *D. melanogaster* and *D. simulans*. In *D.*
394 *melanogaster* adaptation to ethanol has occurred through genetic accommodation – selection on plasticity
395 to reduce genetic variation and produce a single plastic response. This has facilitated expansion to a novel
396 resource in *D. melanogaster*, ethanol rich substrates. In *D. simulans* this is not the case, and lack of
397 selection in ethanol environments has allowed for the accumulation of cryptic genetic variation for
398 phenotype plasticity manifested as extensive genotype by environment interactions. Joshi and Thompson
399 (1997) found that in *D. simulans* there was a reduction in variation between families for the phenotypic
400 response to ethanol substrate after selection for tolerance to ethanol. This is consistent with the observed
401 scenario in D. *melanogaster* and *D. simulans* – adaptation to a novel environment results in a reduction in
402 variation for environmental response and while retaining a single response to the environment. This is
403 also consistent with genetic accommodation as a mechanism of adaptation to ethanol, where initial
404 responses to environmental differences are a mix of adaptive and maladaptive, which are subsequently
405 honed by selection.

406 Previous literature on the response to novel environments is equivocal concerning the expectation
407 that plasticity evolves from a starting point of increased genetic variance due to cryptic genetic variation.
408 For example, early estimates of heritability found reduced expression of genetic variation under 'stressful'
409 conditions – though stressful does not necessarily imply novel (Charmantier and Garant 2005; Chevin and
410 Hoffmann 2017). Work in *Drosophila* has been similarly ambiguous – in *D. mojavensis* a similar
411 reduction in genotype by environment interactions was detected in adaptation to a novel cactus host,
412 however in *D. melanogaster* differences in genotype by environment interactions were not detected
413 between cold-adapted and non-cold adapted populations (Matzkin 2012; Heckel et al. 2016). These
414 differences may be due to difficulty in defining novelty, and in equating novelty with stress. As posited

415 by Chevin and Hoffman (2017), it may be that genetic variance increased in novel environments, but that
416 favorable conditions are rare – meaning that 'stressful' environments are commonly experienced resulting
417 in a reduction in genetic variation.

418 The abundance of lncRNAs which are involved in genotype by environment interactions in *D.*
419 *simulans* is perhaps not surprising, given that they have been implicated previously in dynamic responses
420 (Valadkhan & Valencia-Hipólito 2016). It is also possible they are more frequently involved because
421 lncRNAs are often less conserved, as there is no requirement for the maintenance of ORFs and codon
422 synonymy (Chodroff et al. 2010; Ulitsky et al. 2011; Ulitsky 2016; Quinn et al. 2016). If neutral variation
423 was allowed to accumulate without selection, and then uncovered in a novel environment (cryptic genetic
424 variation), preferential accumulation within less constrained sequences would be expected.

425 Alternative splicing has the potential to be an important component of adaptation and response to
426 the environment, as it does not require de novo transcription or protein production. In *D. melanogaster* it
427 is a more important component of the response to ethanol than in *D. simulans*, as evidenced by
428 differences in the frequency of significant changes in relative abundance of exons. The response to
429 ethanol in *D. melanogaster* is adaptive, thus it can be deduced that alternative splicing is important for
430 adaptation to ethanol. Alternatively, the fact that it is less important in *D. simulans* suggests that perhaps
431 there is selection against the accumulation of cryptic genetic variation for splicing patterns. This is not
432 surprising – previous studies have linked alternative splicing to the response to the environment (Merkin
433 et al. 2012; Barbosa-Morais et al. 2012; Gueroussov and Gonatopoulos-Pournatzis 2015; Jakšić and
434 Schlötterer 2016; Singh et al. 2017; Wang et al. 2017; Pajoro et al. 2017; Calixto et al. 2018), as well as
435 important adaptive differences (Singh et al. 2017; Dong et al. 2017; Evantal et al. 2018; Shi et al. 2019).
436 Furthermore, it has been previously implicated in the response to ethanol, and in *D. melanogaster* may be
437 a more important component of the response than gene expression changes (Oomizu et al. 2003; Newton
438 et al. 2004; Pietrzykowski et al. 2008; Sasabe and Ishiura 2010; Hemby 2012; Zaharieva et al. 2012;
439 Robinson and Atkinson 2013).

440 In general, the GO terms implicated in this study relate to known effects of ethanol, for example,
441 alcohol-induced ciliary dysfunction is a known consequence of alcohol exposure (Yang et al. 2015; Price
442 et al. 2018). Furthermore, the genes implicated in both species (*Drat*, *cabut*, *sugarbabe*, *Pinocchio*,
443 *CG32512*, and *CG407*) have generally been implicated in the response to ethanol in other studies. *cabut*
444 has been previously observed as being upregulated in response to ethanol, and it is in general responsive
445 to changes in metabolic conditions (Awofala et al. 2012; Havula and Hietakangas 2012; Bartok et al.
446 2015). *sugarbabe* and *cabut* are both downstream of the *Mondo-Mlx* sugar sensing pathway (Mattila et al.
447 2015), which has been linked to severe obesity, high circulating triglycerides, and tumorigenesis (Kooner
448 et al. 2008; Kathiresan et al. 2008; Herman et al. 2012; Carroll et al. 2015). *Drat* has been implicated in
449 ethanol-related cell death, though it is not as well studied as *cabut* (Chen et al. 2012). Specifically with
450 regard to other studies of ethanol exposure in *Drosophila* overlap is low between significant datasets,
451 however this may in large part be due to differences in methodology (Signor and Nuzhdin 2018).
452 Previous studies used targeted qPCR (which requires candidate genes) (Morozova et al. 2011), and
453 microarrays (Kong et al. 2010), as well as different assays and exposure lengths (inebriometer
454 (Morozova 2011, 2015), booz-o-mat (Kong et al. 2010). What we are primarily interested in here is the
455 overall pattern rather than the individual genes.
456 Inferring that gene expression differences are adaptive or non-adaptive remains a major challenge
457 in the study of gene expression reaction norms, given the lack of direct correlation between gene
458 expression phenotypes and organismal phenotypes. However, the patterns observed in *D. simulans*
459 suggest that abundant genotype by environment interactions have accumulated neutrally and become
460 uncovered in response to a novel environment. In contrast, in *D. melanogaster* this ethanol environment is
461 not novel and variation in plasticity has been selected out in favor of an adaptive phenotypic response.
462 lncRNAs are preferentially differentially expressed in *D. simulans* in response to ethanol likely because
463 they are less constrained and can accumulate more neutral variation. This is an excellent illustration of
464 genetic accommodation, where phenotypic plasticity has encouraged adaptation to a novel resource
465 within a patchy environment.

466
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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

*D. melanogaster**

	G	ETOH	G x ETOH	ETOH x T	G x ETOH x T	10	20	30
Exons	1445	15	3	22	0	2	1	46
Exonic Regions	631	13	0	8	2	1	4	24
Exon Fragments	1135	21	0	12	4	3	5	20

**reproduced from Signor and Nuzhdin 2018*

724

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

728

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}		

729

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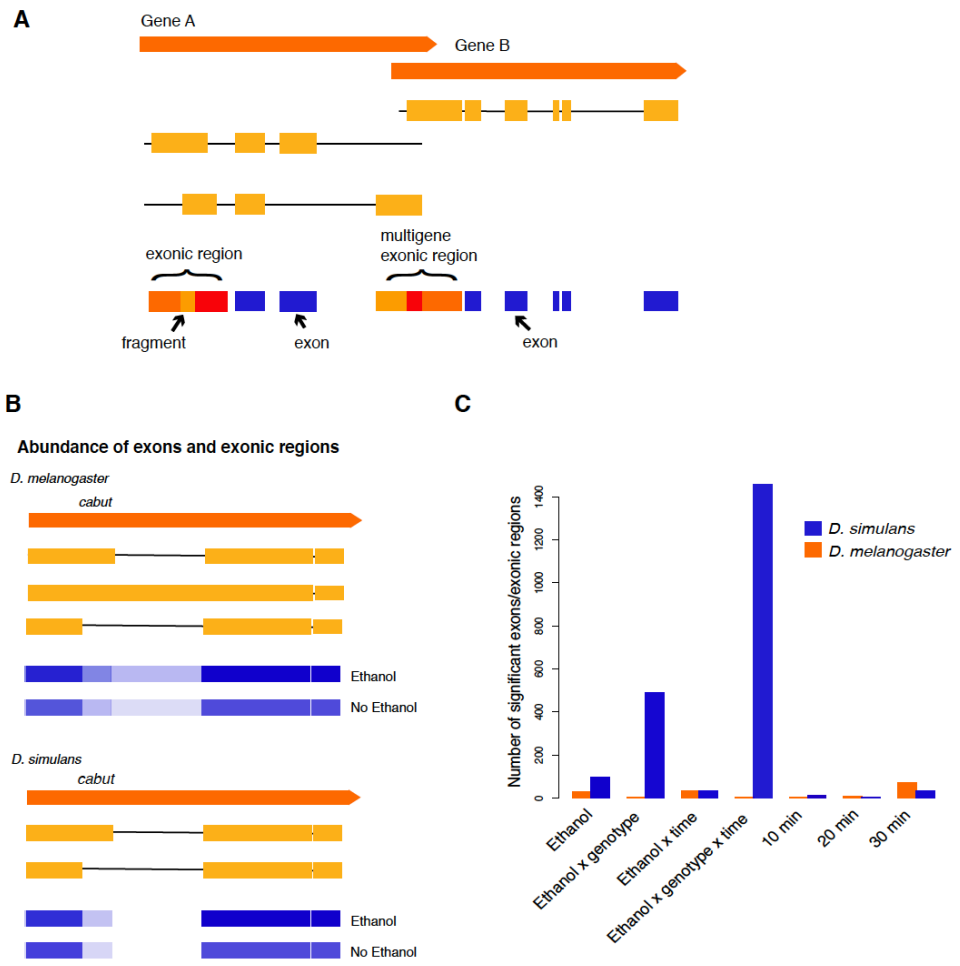

Figure 1: **A**. An illustration of the classification scheme for exons, exonic regions, and exon fragments. Exons either do not overlap other exons in different isoforms (exons, shown in blue), or are fused regions consisting of a set of overlapping exons (exonic regions, shown in shades of orange and red). Exonic regions can be decomposed into exon fragments, depending upon their overlap between different isoforms. **B.** 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. **C.** The number of exons or exonic regions that are significant for each component of variance in *D. melanogaster* and *D. simulans*. 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

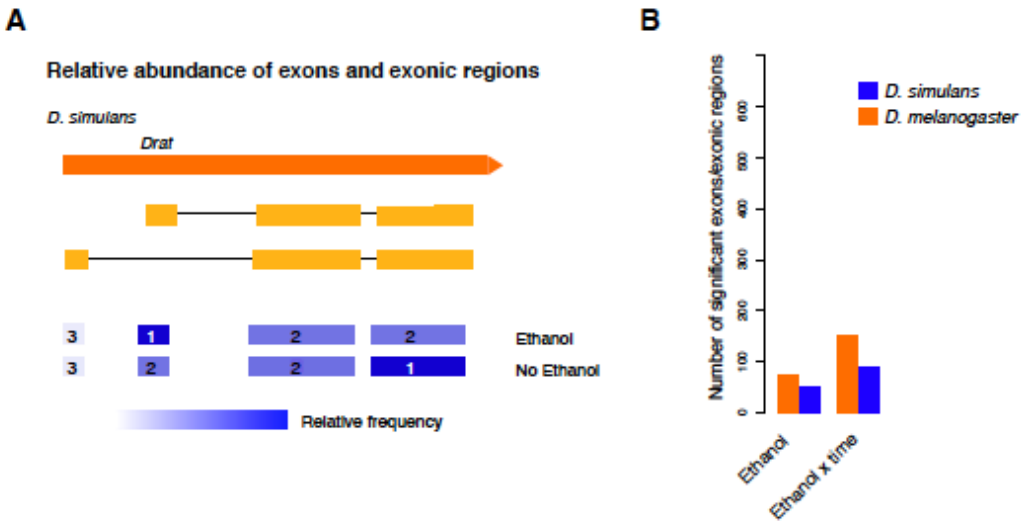

Figure 2: A. An illustration of a change in the relative abundance of the gene *Drat*. Only the example of *D. simulans* is shown. With and without ethanol different exons are the most abundant compared to the other exons within the gene. As one of these exons is not shared among all isoforms, this suggests a change in isoform usage between environments. **B.** The number of genes that have a significant change in relative abundance for each component of variance in *D. melanogaster* and *D. simulans*.