

1 **Tissue-specific cis-regulatory divergence implicates a fatty acid**  
2 **elongase necessary for inhibiting interspecies mating in**  
3 ***Drosophila***

4

5 Short title: *eloF* inhibits interspecies mating in *Drosophila*

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14

## 15 Abstract

16 Pheromones known as cuticular hydrocarbons are a major component of reproductive  
17 isolation in *Drosophila*. Individuals from morphologically similar sister species produce  
18 different sets of hydrocarbons that allow potential mates to identify them as a suitable  
19 partner. In order to explore the molecular mechanisms underlying speciation, we  
20 performed RNA-seq in F1 hybrids to measure tissue-specific cis-regulatory divergence  
21 between the sister species *D. simulans* and *D. sechellia*. By focusing on cis-regulatory  
22 changes specific to female oenocytes, we rapidly identified a small number of candidate  
23 genes. We found that one of these, the fatty acid elongase *eloF*, broadly affects both the  
24 complement of hydrocarbons present on *D. sechellia* females and the propensity of *D.*  
25 *simulans* males to mate with those females. In addition, knockdown of *eloF* in the more  
26 distantly related *D. melanogaster* led to a similar shift in hydrocarbons as well as lower  
27 interspecific mate discrimination by *D. simulans* males. Thus, cis-regulatory changes in  
28 *eloF* appear to be a major driver in the sexual isolation of *D. simulans* from multiple  
29 other species. More generally, our RNA-seq approach proved to be far more efficient  
30 than QTL mapping in identifying candidate genes; the same framework can be used to  
31 pinpoint cis-regulatory drivers of divergence in a wide range of traits differing between  
32 any interfertile species.

33

## 34 Introduction

35 Reproductive isolation is a major component of speciation. Postzygotic incompatibilities  
36 leading to hybrid sterility or inviability (also known as Dobzhansky-Muller  
37 incompatibilities) have been especially well-studied, with several examples narrowed  
38 down to specific genes (Watanabe 1979; Sawamura *et al.* 1993; Phadnis *et al.* 2015).  
39 However when the distributions of related species overlap, rejection of interspecific  
40 partners may account for a much larger fraction of reproductive isolation (Coyne and Orr  
41 1997; Quinn *et al.* 2000; Byrne and Rice 2006; Shahandeh *et al.* 2018). This preference  
42 for conspecific mates may be subject to strong selection (Noor 1995; Servedio and Noor  
43 2003; Coyne and Orr 2004), since interspecific hybridization carries significant fitness  
44 costs, including potential inviability or sterility of offspring.

45

46 *Drosophila* has been a key model organism for the study of reproductive isolation,  
47 including the role of mate choice (Coyne and Orr 2004). Courtship in *Drosophila* is a  
48 highly stereotyped procedure, with multiple opportunities for both females and males to  
49 reject interspecific partners (Sokolowski 2001; Lasbleiz *et al.* 2006). This affords the  
50 opportunity for flies to reduce energy expenditure on reproductively fruitless partners.  
51 While female mate choice has been more heavily studied (Spieth 1952; Partridge 1980;  
52 Fowler and Partridge 1989; Greenspan and Ferveur 2000), there is a growing recognition  
53 that choice by males can also be an important factor (Byrne and Rice 2006; Edward and  
54 Chapman 2011; Pischedda *et al.* 2014; Shahandeh *et al.* 2018). In fact, male choice can  
55 be responsible for most reproductive isolation in some cases (Shahandeh *et al.* 2018).  
56 Beyond simply the opportunity cost of devoting time towards courting a heterospecific

57 female, mating itself can be costly for males, with mated male *Drosophila* having  
58 reduced lifespans (Partridge and Farquhar 1981). Simulations have shown that male mate  
59 choice can reinforce speciation under when hybrids are less fit (Servedio 2007).

60

61 *D. simulans* and *D. sechellia* are two closely related sister species, separated by  
62 approximately 250 thousand years (Garrigan *et al.* 2012). The species are believed to  
63 have diverged in allopatry (Kliman *et al.* 2000), though currently their ranges overlap and  
64 hybrids can be found in the wild (Matute and Ayroles 2014). In laboratory conditions, *D.*  
65 *sechellia* males will readily mate with *D. simulans* females, producing sterile male and  
66 fertile female hybrid offspring, while the reciprocal cross is much more difficult  
67 (Lachaise *et al.* 1986). Male mate choice in these species—which accounts for over 70%  
68 of their reproductive isolation (Shahandeh *et al.* 2018)—is mediated by female cuticular  
69 hydrocarbons (CHCs), which are key molecules involved in species recognition that are  
70 produced primarily in specialized cells called oenocytes (Billeter *et al.* 2009).

71

72 In this study, we sought to identify the specific gene(s) responsible for CHC-mediated  
73 behavioral reproductive isolation in *D. simulans* and *D. sechellia*. Thus far, QTL  
74 mapping has been the primary method used to investigate this question. QTLs affecting  
75 CHCs have been mapped, but these contain many CHC-related genes (Coyne *et al.* 1994;  
76 Gleason *et al.* 2005; 2009), and fine-mapping has not been reported. As a complementary  
77 approach, we reasoned that genes responsible for major changes in CHCs may share three  
78 key characteristics: 1) Cis-regulatory divergence in female oenocytes; 2) Female-specific  
79 expression; and 3) Oenocyte-specific expression. Although these are certainly not

80 required—for example, CHC divergence might occur via changes in protein-coding  
81 regions—any genes meeting all three criteria would be excellent candidates.

82

83 Cis-regulatory divergence can be measured genome-wide via high-throughput sequencing  
84 of cDNA (RNA-seq) in interspecific hybrids. Hybrids are required because comparisons  
85 between species involve a combination of both cis- and trans-acting changes; in contrast,  
86 measuring allele-specific expression (ASE) in F1 hybrids neatly controls for potential  
87 trans-acting changes, since each allele experiences the same trans-regulatory environment  
88 within the hybrid nuclei. Thus, differential expression of the two alleles in a hybrid can  
89 only be explained by cis-regulatory divergence.

90

91 To generate genome-wide data covering all three criteria listed above, we performed  
92 RNA-seq in *D. sechellia/simulans* hybrids. To measure female-specificity, we included  
93 samples from both male and female oenocytes, and to measure oenocyte-specificity, we  
94 included samples from male and female fat bodies (an adjacent non-CHC producing  
95 tissue; Lawrence and Johnston 1986). Using this approach, we identified three candidate  
96 genes for drivers of CHC differences between the species. Ablation of these genes  
97 pointed towards a major role of *eloF*, a fatty acid elongase, in the reproductive isolation  
98 of *D. simulans* from both *D. sechellia* as well as the more distantly related *D.*  
99 *melanogaster*.

100

## 101 Results

### 102 Allele-specific expression identifies fatty acid elongases as a major differentiator

### 103 between *D. simulans* and *D. sechellia* female oenocytes

104 We first set out to identify genes with cis-regulatory divergence specific to female  
105 oenocytes. We mated *D. sechellia* males to *D. simulans* females and dissected both  
106 oenocytes and fat bodies from the progeny, pooling approximately 20 individuals from  
107 each sex (Figure 1A). Then, we extracted RNA and constructed RNA-seq libraries, which  
108 we sequenced to approximately 30 million reads per sample (Supplemental Table 1). We  
109 called allele-specific reads for each sample by aligning to a *D. simulans* reference  
110 sequence, and controlled for potential mapping bias by discarding any read that did not  
111 map to the same location if alleles were swapped *in silico* (van de Geijn *et al.* 2015).  
112 Despite the use of a *D. simulans* reference genome, we found a majority of reads were  
113 assigned to *D. sechellia* (Supplemental Table 1), possibly indicating low levels of non-  
114 hybrid *D. sechellia* samples. We estimated the significance of each gene's allele specific  
115 expression (ASE) using a negative-binomial test (Love *et al.* 2014) for deviation from the  
116 average fraction of *D. sechellia* reads in a given sample.

117

118 Even at a stringent cutoff, we identified 239 genes with significant (negative binomial q-  
119 value < 0.001) ASE in female oenocytes. This is not surprising, since various *Drosophila*  
120 interspecific hybrids have also yielded large numbers of genes with strong ASE. Of the  
121 239 significant genes, 27 have been annotated with the Gene Ontology term “Fatty acid  
122 biosynthetic process” (GO:0006633) (Supplemental Table 3). Therefore we concluded  
123 that, even when combined with GO annotations, ASE in female oenocytes was

124 insufficient to identify a manageable number of candidate genes involved in CHC  
125 differences and speciation.  
126  
127 We reasoned that in addition to ASE, genes important to female CHC differences  
128 between *D. simulans* and *D. sechellia* would likely be expressed specifically in female  
129 oenocytes (Figure 1B and C). To identify candidate genes, we looked for genes that had  
130 significantly higher expression in the female oenocytes compared to both male oenocytes  
131 and female fat bodies (Sleuth q-value<0.001 for both comparisons; (Pimentel *et al.*  
132 2017)). Only six genes passed these cutoffs. Reassuringly, one of these was *desatF* (also  
133 known as *Fad2*), a fatty-acid desaturase which is known to be expressed in *D. sechellia*  
134 female oenocytes, but not in males or in *D. simulans* (Shirangi *et al.* 2009).  
135  
136 Among the six candidate genes, the only enriched molecular function Gene Ontology  
137 terms were related to “fatty acid elongase activity” (GO:0009922 and its parent GO  
138 terms), which describe the three genes *eloF*, *CG8534*, and *bond* (in all cases, we use the  
139 names of the *D. melanogaster* orthologs) (Boyle *et al.* 2004). All three of these have  
140 ELO family domains (Szafer-Glusman *et al.* 2008). Both *eloF* and *CG8534* were *D.*  
141 *sechellia*-biased, while *bond* was *D. simulans*-biased. We further detected a weak signal  
142 for *FASN3*, a putative acyl transferase (Table 1). No other gene that is both oenocyte- and  
143 species-specific in its expression has an annotated Gene Ontology term or protein domain  
144 that is clearly related to CHC production (Table 1).

145

146 Compared to the female oenocytes, male oenocytes had a much weaker signal of ASE  
147 among genes with sex- and oenocyte-specific expression (Supplemental Figure 2). Given  
148 the overall weaker signal in male oenocytes, we chose to focus on changes in female  
149 CHC production that might drive speciation.

150

151 Male fat bodies had over 80 genes with tissue- and species-specific expression  
152 (Supplemental Figure 2A). Gene ontology analysis of these male fat body genes  
153 highlighted several significant GO terms, including “oxidation-reduction process”  
154 ( $p=2.9 \times 10^{-7}$ ) and “catalytic activity” ( $p=6.7 \times 10^{-10}$ ) (Boyle *et al.* 2004), but no candidate  
155 genes with obvious roles in pheromone production or mating activity were present.  
156 However, these genes may be useful for future studies of regulatory evolution in fat  
157 bodies, which could affect traits including metabolism and mating behavior (Lazareva *et*  
158 *al.* 2007).

159

160 **eloF has widespread effects on the hydrocarbon profile of *D. sechellia* and *D.***  
161 ***melanogaster***

162 To explore the role of our candidate genes on CHC profiles of these species, we  
163 performed gas chromatography coupled to mass spectrometry (GCMS). Consistent with  
164 previous measurements of hydrocarbon profiles of *Drosophila*, we found that wildtype *D.*  
165 *simulans* has more short-chain hydrocarbons than *D. sechellia* (Figure 2A; (Jallon and  
166 David 1987)). In particular, *D. sechellia* has almost no 23-carbon CHCs, while the  
167 predominant *D. simulans* hydrocarbon is 7-tricosene, a 23-carbon monoene. Indeed, there  
168 was only one hydrocarbon shorter than 26 carbons with a greater representation in *D.*



169 *sechellia* than *D. simulans*, the 25-carbon pentacosadiene (~2 fold higher in *D. sechellia*).

170 There were no CHCs longer than 26 carbons that were more abundant in *D. simulans*

171 than *D. sechellia*.

172

173 To explore the effects of our candidate genes on CHC profiles, we studied the phenotypic

174 effects of their RNAi knockdowns in *D. melanogaster*. We did not pursue *desatF*, which

175 already has a well-established role in *Drosophila* speciation (Legendre *et al.* 2008; Fang

176 *et al.* 2009; Shirangi *et al.* 2009), or *FASN3*, which is essential for viability (Chung and

177 Carroll 2015). For the remaining three CHC-related candidates, we created RNAi

178 knockdowns in *D. melanogaster* females for each of these genes specifically in oenocytes

179 by crossing PromE(800)-gal4 males with UAS-shRNA females from the TRiP project

180 (Billeter *et al.* 2009; Perkins *et al.* 2015), then screened the CHC profiles of the progeny

181 by GCMS. As negative controls, we crossed PromE(800)-gal4 males with females of

182 Bloomington stock #32186, which carries 10 copies of UAS-driven mCD8-tagged GFP.

183

184 Of our three candidate genes, we found that one (*CG8534*) was essential for viability. Its

185 highest expression is in the 3<sup>rd</sup>-4<sup>th</sup> day of pupation (Graveley *et al.* 2011), so it may be

186 involved in development. Attempts to delay induction of *gal4*-driven RNAi by incubating

187 larvae at 18°C were not successful in rescuing females.

188

189 Knockdown of our second candidate (*bond*) in females led to ~60% increases in levels of

190 pentacosadiene (a 25 carbon hydrocarbon) and ~60% decrease in levels of

191 heptacosadiene (27 carbon) (Supplemental Figure 3). However other hydrocarbons were  
192 not significantly affected.

193

194 We observed the most pronounced effects for RNAi knockdown of our third candidate,  
195 *eloF*. We found that female flies with *eloF* knocked down have significantly fewer long-  
196 chain CHCs and more short-chain CHCs than wildtype flies (>3-fold change between  
197 CHCs with longer vs. shorter than 26 carbons; Figure 2B), consistent with previous work  
198 (Chertemps *et al.* 2007). Interestingly, *eloF* also had the strongest ASE among the six  
199 candidate genes (79-fold higher expression from *D. sechellia* alleles).

200

201 To examine the effect of *eloF* on CHCs in *D. sechellia*, we used CRISPR/Cas9 genome  
202 editing to create two independent lines of *D. sechellia* with *eloF* knocked out and  
203 replaced with P3-*RFP*. As expected, nearly all of the CHCs whose levels changed after  
204 *eloF* knockdown in *D. melanogaster* show a similar difference in *D. sechellia* (Figure  
205 2C). Thus, we conclude that the molecular substrates and products of *eloF* are  
206 substantially similar between *D. melanogaster* and *sechellia*.

207

208 We noticed that there was a strong correlation between the changes observed between the  
209 sister species *D. simulans* and *D. sechellia* and the changes between wild-type and *eloF*  
210 depleted females from both *D. melanogaster* and *D. sechellia* (Figure 2D and  
211 Supplemental Figure 4). Consistent with *eloF*'s role as a fatty acid elongase, much of this  
212 variation consisted in broad differences in overall length of the hydrocarbons. To  
213 visualize entire CHC profiles, we performed principal components analysis, which

214 showed that 94% of the total variation was captured by the first two components. The  
215 first principal component of variation separated *D. simulans* from both *D. melanogaster*  
216 and *D. sechellia* (Figure 2E). While knockdown or knockout of *eloF* did not completely  
217 transform the profiles of either species to *D. simulans*, it did make the profiles  
218 significantly closer. Thus, we concluded that one or more of the products of *eloF* may be  
219 acting as an anti-aphrodisiac to *D. simulans* males (or, alternatively, one of the substrates  
220 may be an aphrodisiac).

221

222 Notably, several previous studies have mapped quantitative trait loci (QTLs) that include  
223 *eloF*. For example, *eloF* is located within QTLs affecting CHC differences and mate  
224 discrimination between *D. simulans* and *D. sechellia* (Gleason *et al.* 2005; 2009), as well  
225 as a QTL for copulation frequency between *D. simulans* males and *D. mauritiana*  
226 females (Moehring *et al.* 2004). However in all of these studies, the QTLs also contained  
227 hundreds of other genes (including many other elongases). Therefore, although *eloF* is an  
228 excellent candidate gene, its role in reproductive isolation has not been explored.

229

### 230 **Expression of *eloF* is sufficient for species discrimination by *D. simulans* males**

231 To determine whether the change in *eloF* expression (and concomitant CHC changes)  
232 could be responsible for sexual isolation between the species, we performed mate choice  
233 assays. We placed single *D. simulans* males in a chamber with a single female and  
234 recorded video in well-lit conditions for 30 minutes. We noted the time of the first  
235 instance of various copulatory behaviors, including tapping, male wing song, and licking  
236 (Figure 3A-C). With the exception of licking, these behaviors are not subject to rejection

237 by females (the mating chambers are small enough that females are effectively unable to  
238 escape, while tapping is very rapid and wing song does not involve contact), and thus  
239 primarily represent choice by the males.

240

241 We first tested whether *eloF* might drive the behavioral isolation of *D. simulans* and *D.*  
242 *sechellia*, and so tested *D. sechellia* females with *D. simulans* males. As expected, *D.*  
243 *simulans* males courted wild-type *D. sechellia* females at a significantly lower rate than  
244 *D. simulans* females. Remarkably, *D. simulans* males courted *eloF*- *D. sechellia* females  
245 at the same rate as conspecific females (Figure 3D). We observed no significant  
246 difference in the courtship rate between the two independently generated *D. sechellia*  
247 knockout lines.

248

249 We then asked whether *eloF* might also mediate mate discrimination between *D.*  
250 *melanogaster* and *D. simulans*. As expected, when *D. simulans* males were presented  
251 with wildtype *D. melanogaster* females they rarely proceeded to courtship (Figure 3D  
252 and Supplemental Figure 5A). However, when we knocked down *eloF* expression in *D.*  
253 *melanogaster* females using oenocyte-specific RNAi, males courted them at rates only  
254 slightly lower than conspecifics.

255

256 The choice by males seems to be nearly binary. In the rare cases when *D. simulans* males  
257 did court wild-type *D. melanogaster* females, they did so approximately as quickly as  
258 they did for *D. simulans* females (Figure 3E and Supplemental Figure 5B). In none of the  
259 comparisons was there a significant difference in time between first contact between the

260 flies and any of the steps in courtship at a nominal (i.e. without correcting for multiple  
261 testing)  $\alpha=0.01$  level.

262

## 263 Discussion

264 Sexual selection in *Drosophila* has been studied for over one hundred years, with  
265 chemical odorants quickly being noticed as a primary signal (Sturtevant 1915), although  
266 the study of the evolution of these odorants came only after gas chromatography allowed  
267 the separation of different components (Hedin *et al.* 1972). Early work in the field sought  
268 to identify differences in CHC profiles between species and their effects on mating  
269 (Pechine *et al.* 1985; Jallon and David 1987; Cobb and Jallon 1990), and more recent  
270 genetic approaches have allowed for mapping of QTLs affecting these CHC differences  
271 (Moehring *et al.* 2004; Gleason *et al.* 2005; 2009). However, pinpointing the genes  
272 responsible for these changes is still quite difficult (Shirangi *et al.* 2009).

273

274 In this study, we have found that RNA-seq in F1 hybrids is a rapid, efficient means of  
275 identifying genes potentially involved in phenotypic divergence. Neither comparisons of  
276 expression across tissues nor of ASE within a single tissue was able to sufficiently  
277 narrow the list of candidate genes (Figure 1C); however, the combination of these  
278 orthogonal filters, together with gene annotations, allowed us to focus on only three  
279 excellent candidate genes. This can be compared with the most widely used alternative  
280 for studying the genetic basis of phenotypic divergence, QTL mapping. In QTL mapping,  
281 hundreds of progeny from genetic crosses must be genotyped and phenotyped, requiring  
282 years of effort even for rapidly reproducing species such as *Drosophilids*. Moreover, this

283 effort leads to QTLs that typically span over a hundred genes, since resolution is limited  
284 by infrequent recombinations. Therefore, follow-up studies to test specific genes are  
285 often prohibitive. We envision that our approach of intersecting filters based only on  
286 RNA-seq in F1s may be widely applicable to other tissue-specific, sex-specific, stage-  
287 specific, or condition-specific traits that differ between interfertile populations or species.  
288

289 Consistent with other recent observations (Shahandeh *et al.* 2018), we found that CHC  
290 differences between the species seem to be the major source of sexual isolation between  
291 *D. simulans* males and females from both *D. sechellia* and *D. melanogaster*, and we also  
292 showed that ablating *eloF* alleviates nearly all of the isolation from both *D. sechellia* and  
293 *D. melanogaster*. The magnitude of this effect is comparable to the reduction in barriers  
294 between *D. simulans* males and *D. melanogaster* females by ablating oenocytes entirely,  
295 a much more radical intervention (*eloF* appears to represent ~85% of the barrier in this  
296 study, compared to ~100% in Billeter *et al.* 2009).

297  
298 One important caveat is that this isolation is observed under forced-choice laboratory  
299 conditions. Providing the choice between conspecifics and heterospecifics has been  
300 shown to increase isolation, while rates of hybridization in the wild have been strikingly  
301 higher than laboratory predictions (Coyne *et al.* 2005; Llopart *et al.* 2005).

302  
303 Our identification of *eloF* as the necessary for *D. simulans* isolation is buttressed by  
304 understanding its role in the biochemical pathways of CHC synthesis but does not  
305 entirely depend on that foreknowledge. It is important that we were able to design our

306 experiments knowing that the CHC biochemical pathway takes place almost completely  
307 in the oenocytes (Wicker-Thomas *et al.* 2015). However, having identified the candidate  
308 genes using RNA-seq, previous work investigating CHC synthesis allowed us to  
309 hypothesize why the candidates lead to different CHC profiles (Coyne 1996; Ferveur *et*  
310 *al.* 1997; Coyne *et al.* 1999; Labeur *et al.* 2002; Chertemps *et al.* 2007; Legendre *et al.*  
311 2008). An interesting direction for future work would be to measure the effects of  
312 knocking out other genes in this pathway on CHC profiles and reproductive isolation.  
313  
314 Because *eloF* affects so many CHCs, it is not clear which CHC(s) act as the  
315 discriminative signal. The 27-carbon CHC 7,11-heptacosadiene has been shown to be  
316 involved in male *D. melanogaster* and *D. simulans* preference (Antony *et al.* 1985;  
317 Billeter *et al.* 2009), although other CHCs could also contribute. Further, the identity of  
318 the male receptor is unknown, although Gr32a seems to be the major chemoreceptor in *D.*  
319 *melanogaster* responsible for species recognition (Fan *et al.* 2013). While reagents in  
320 non-*melanogaster* Drosophilids are now available (Stern *et al.*, 2017), screening multiple  
321 gustatory receptors in *D. simulans* is not yet as straightforward as an RNAi experiment in  
322 *D. melanogaster*.  
323  
324 However, even without knowing the specific causal CHCs we can hypothesize a  
325 parsimonious evolutionary scenario to explain our observations. *D. sechellia* and *D.*  
326 *melanogaster* both express *eloF* in female oenocytes; therefore this is likely to be the  
327 ancestral state for these species, with the 79-fold lower *eloF* expression in *D. simulans*  
328 being a derived change specific to this species. Our experiments show that *D. simulans*

329 males prefer mates lacking *eloF*, suggesting that male preferences have co-evolved with  
330 CHC profiles in *D. simulans*. An intriguing question for future work will be whether the  
331 gene(s) responsible for this co-evolved male preference could be identified with a similar  
332 tissue-specific ASE approach as demonstrated here.

333

334 Another open question regards the sequence changes that have led to the expression  
335 differences of *eloF*. It seems significant that both a nearby coding gene (*CG8534*, also a  
336 fatty acid elongase) and a non-coding RNA (*CR44035*, of unknown function) share a  
337 similar pattern of female oenocyte-specific ASE. Neither of the genes bordering these 3  
338 genes share this pattern, suggesting the existence of a species-variable topologically  
339 associated domain that is transcriptionally active in *D. sechellia* but not *D. simulans*. The  
340 transcription factor Doublesex has been implicated in the evolution of other *Drosophila*  
341 species' CHC profiles (Shirangi *et al.* 2009), but searches for clear changes in canonical  
342 or non-canonical Doublesex binding sites have been fruitless in the species pair in this  
343 work. Further, the set of fixed changes is too large to easily test just a small set of  
344 candidates—in the noncoding region around *eloF* and *CG8534*, there are 136 SNPs and  
345 10 indels (comprising 67 bases) where *D. simulans* has a derived allele differing from  
346 both *D. sechellia* and *D. melanogaster* (thus matching the parsimonious evolutionary  
347 scenario described above), in addition to several nonsynonymous changes in *eloF*  
348 (Supplemental Figure 6). An association study of *eloF* expression or CHC profiles in a  
349 panel of sequenced *D. simulans* may provide more targeted hypotheses, but only if the  
350 causal variant(s) are segregating within *D. simulans*, which seems unlikely given the  
351 major effect they would have on CHCs that are essential for mate choice.



352

353 Unlike previous observations that CHC changes can affect desiccation resistance (Chung  
354 *et al.* 2014; Ferveur *et al.* 2018), our preliminary tests of *eloF*'s effects on desiccation did  
355 not yield a strong effect (data not shown). These studies examined flies from widely  
356 varying ecological niches (Australian desert/jungle and France/Zimbabwe), whereas *D.*  
357 *simulans* and *D. sechellia* have overlapping ranges (Matute and Ayroles 2014). Thus, we  
358 would not expect strong pressure for differences in tolerance to desiccation.

359

360 Evolution of elongase expression may be involved in other insect speciation events as  
361 well. For instance, QTL studies between the jewel wasps *Nasonia vitripennis* and *N.*  
362 *giraulti* have implicated an elongase in CHC changes between those species (Niehuis *et*  
363 *al.* 2011). Furthermore, our analysis of CHC profiles in stingless bees shows at least two  
364 speciation events that show broad changes in the length of CHC backbones, which may  
365 be explained by divergence in elongase activity (Supplemental Figure 7; Nunes *et al.*,  
366 2017). Therefore, we hypothesize elongases may represent a general mechanism  
367 contributing to many cases of reproductive isolation in diverse insects.

## 368 **Materials and Methods**

### 369 **RNA extraction and sequencing**

370 Oenocyte and fat body dissections were performed as described in Krupp and Levine  
371 (2010). The oenocytes and fat body of 10-day-old *D. simulans*/*D. sechellia* hybrid flies  
372 were isolated separately from the dorsal abdominal segments of both adult male and  
373 female abdomens. Each tissue sample represented the pooled material collected from 20  
374 flies. Hybrid flies were reared in a 12hr light:12 hr dark cycle and tissues dissected at

375 equal time intervals across a 24hr period. Immediately following dissection tissues were  
376 placed into cell lysis buffer to aid in preserving the integrity of the RNA. Total RNA was  
377 isolated using the RNeasy Micro kit (Qiagen).

378

379 We prepared libraries from the RNA using the NextFLEX RNA-seq library preparation  
380 kit (BioO Scientific, Austin, TX), and sequenced the libraries using 101bp paired end  
381 reads on an Illumina HiSeq 2000.

382

383 We created a corrected *D. simulans* genome by using bowtie2 version 2.2.5 with  
384 arguments `--very-sensitive` to map genomic DNA reads from *D. simulans* and *D.*  
385 *sechellia* to the FlyBase 2.01 *D. simulans* reference genome (Hu *et al.* 2013; Coolon *et*  
386 *al.* 2014). Polymorphisms were called using GATK (`HaplotypeCaller --`  
387 `genotyping_mode DISCOVERY -fixMisencodedQuals -stand_emit_conf 10 -`  
388 `stand_call_conf 30`) (DePristo *et al.* 2011), then the ~34,000 SNPs that were fixed in  
389 both *D. simulans* and *D. sechellia* were replaced with the consensus sequence (this step  
390 was more important for creating a *simulans/sechellia* version of the *D. melanogaster*  
391 genome for Supplemental Figure N). RNA-seq reads were mapped to the reference  
392 genome using STAR with arguments `--outFilterMultimapNmax 1 --`  
393 `outSAMattributes MD NH --clip5pNbases 6 --sjdbGTFfile` (Dobin *et al.* 2013).  
394 Following the WASP pipeline, duplicate reads were discarded randomly, then filtered  
395 based on whether reads with the alleles swapped *in silico* to create artificial transcripts  
396 from the other species mapped to the same position (van de Geijn *et al.* 2015). Reads  
397 were assigned to a species only if both paired ends mapped unambiguously to one  
398 species, and allele-specific expression negative binomial p-values were calculated from

399 aligned read counts using DESeq2 with model `~Replicate + AlignsToSpecies` (Love  
400 *et al.* 2014). Default DESeq settings were used to correct for multiple hypothesis testing.  
401 Transcript abundances were estimated using kallisto with default arguments (Bray *et al.*  
402 2016). We used sleuth to identify differentially expressed genes between samples with  
403 matched sex and tissue type (Pimentel *et al.* 2017).

404

#### 405 **Fly rearing**

406 For RNAi flies, virgin females of the shRNA driver were isolated within 18 hours of  
407 eclosion, then kept isolated from males for 3 days on standard cornmeal media to ensure  
408 virgin status. We used Bloomington Stock IDs 34676 (*bond*), 53947 (*eloF*), 53299  
409 (*CG8534*), and 32186 (GFP control). We combined approximately 25 UAS-shRNA  
410 females with approximately 10 Gal4 driver males. Adults were moved to fresh vials  
411 every 3 days to ensure separation of the parents and the Gal4+UAS offspring.

412

413 Knockout *D. sechellia* flies were created using CRISPR/Cas9 mediated editing. We  
414 designed guides to cut at the 55<sup>th</sup> nucleotide downstream of the ATG and the 114<sup>th</sup>  
415 nucleotide upstream of the stop codon of *GM23846* (the *D. sechellia* ortholog of *eloF*).  
416 We used sense oligos CTTCGCAGCGATCCATGGGTCCCCA (gene 5'-ward cut site)  
417 and CTTCGATCCGCATCCGTAGGTCAA (gene 3'-ward cut site). Embryos were  
418 injected (WellGenetics, Taipei, Taiwan) with both guides and a dsDNA donor containing  
419 ~1000bp homology arms and RFP driven by 3 P3 promoters and flanked by LoxP sites.  
420 Embryos were from the *D. sechellia* genome strain #14021-0248.25.

421

422 All flies, either RNAi or CRISPR edited were separated by sex within 18 hours of  
423 eclosion, then kept isolated for 5-7 days to ensure virgin status. Any vials with larvae  
424 after 5 days were discarded. Since the PromE(800)-gal4 construct is balanced with  
425 Tm3.5b, we selected straight-winged flies as RNAi positive.

426

#### 427 **Gas chromatography–mass spectrometry**

428 We performed GCMS by anesthetizing 5 females at 4°C for 3-5 minutes, then washing  
429 them for 5 minutes with 50µL of hexane spiked with 10mg/mL of n-hexane as a standard.  
430 Spectra were obtained using an Agilent (HP) 7890/5975 single quadrupole GC-MS  
431 instrument with a split ratio of 1:20, injector temperature of 280°C, and an oven  
432 temperature program of 35°C hold for 3.75min, 20°C/min ramp from 35°C to 320°C, and  
433 a 320°C hold for 7 min. We collected spectra for at least 3 sets of 5 flies for each  
434 genotype. Identities of different hydrocarbon peaks were inferred by inspecting the  
435 singly-ionized mass spectrum bin.

436

#### 437 **Mating assays**

438 We performed mating assays by anesthetizing separate vials of males and females at 4°C  
439 for 3-5 minutes, then used a paintbrush to transfer one male and one female to each well  
440 of the mating chamber. The mating chamber was 3D printed from acrylic plastic and has  
441 18 separate 2cm diameter x 5mm circular wells, with a removable clear plastic lid. We  
442 allowed flies to acclimate at room temperature and ambient light for 10-15 minutes, then  
443 recorded 30m of video with bright lights, which we found were required for *D. simulans*  
444 males to initiate courtship. The mating light was a 75W, 14” circular fluorescent bulb  
445 placed approximately 30cm above the mating chamber. Video of mating assays was

446 recorded using a Dino-Lite digital microscope, then analyzed by two separate graders  
447 (PAC and NMK), who recorded the time of first contact by the male, the time of the male  
448 first following the female, the time of the first wing song by the male, and the time of  
449 first licking by the male of the female's abdomen (Sokolowski 2001). Graders were  
450 blinded to the fly identities in each video.

451

## 452 **Data Availability**

453 Sequencing data has been deposited at the Gene Expression Omnibus under access  
454 number GSE114478. An interactive tool to explore the RNA-seq dataset is available at  
455 <http://combsfraser-oenocytes.appspot.com/>.

456

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## References

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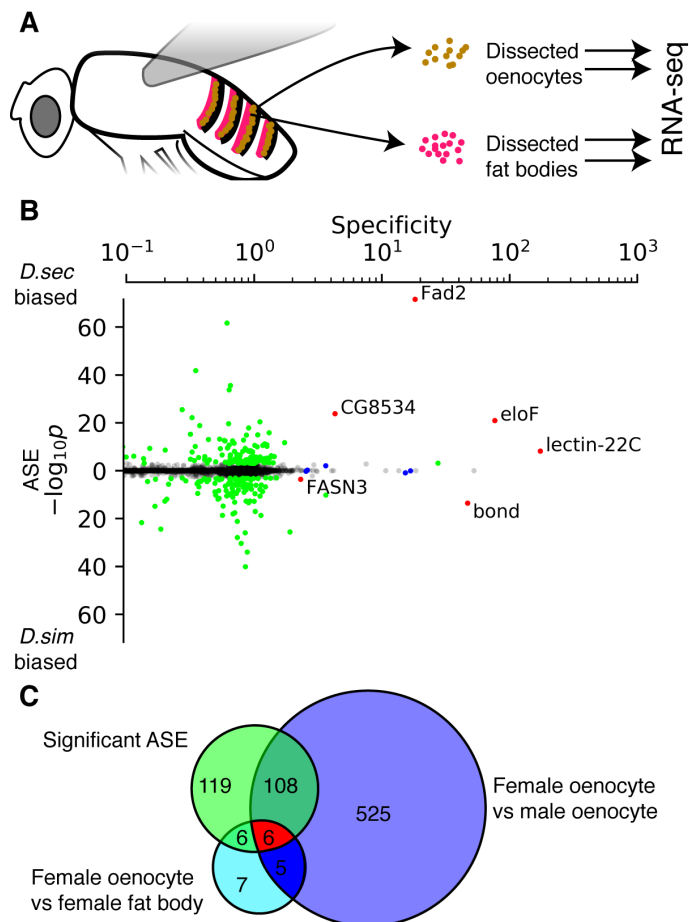
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631

632 **Figure 1: RNA-seq of oenocytes and fat bodies from hybrid *D. simulans* x *D. sechellia***  
633 **flies reveals a strong cis-regulatory component of CHC production.**  
634



635 **A)** We dissected oenocytes (blue dots) and fat bodies (green regions) from hybrid *D. simulans* x  
636 *D. sechellia* males and females and performed RNA-sequencing.

637 **B)** Genes are plotted by specificity of expression to female oenocytes (x-axis; mean of female  
638 oenocyte expression divided by maximum expression in female fat bodies, male oenocytes, and  
639 female oenocytes) and allele-specific expression p-value (y-axis). Green dots indicate genes with  
640 significant ASE compared to the distribution of reads in the female oenocytes, blue dots indicate  
641 those that have significantly higher expression in female oenocytes compared to female fat  
642 bodies and male oenocytes, and red dots indicate genes with both tissue-specific and species-  
643 specific expression.

644 **C)** Overlap of genes with ASE in female oenocytes (green circle), and differential expression in  
645 female oenocytes compared to other tissues (blue and cyan circles).  
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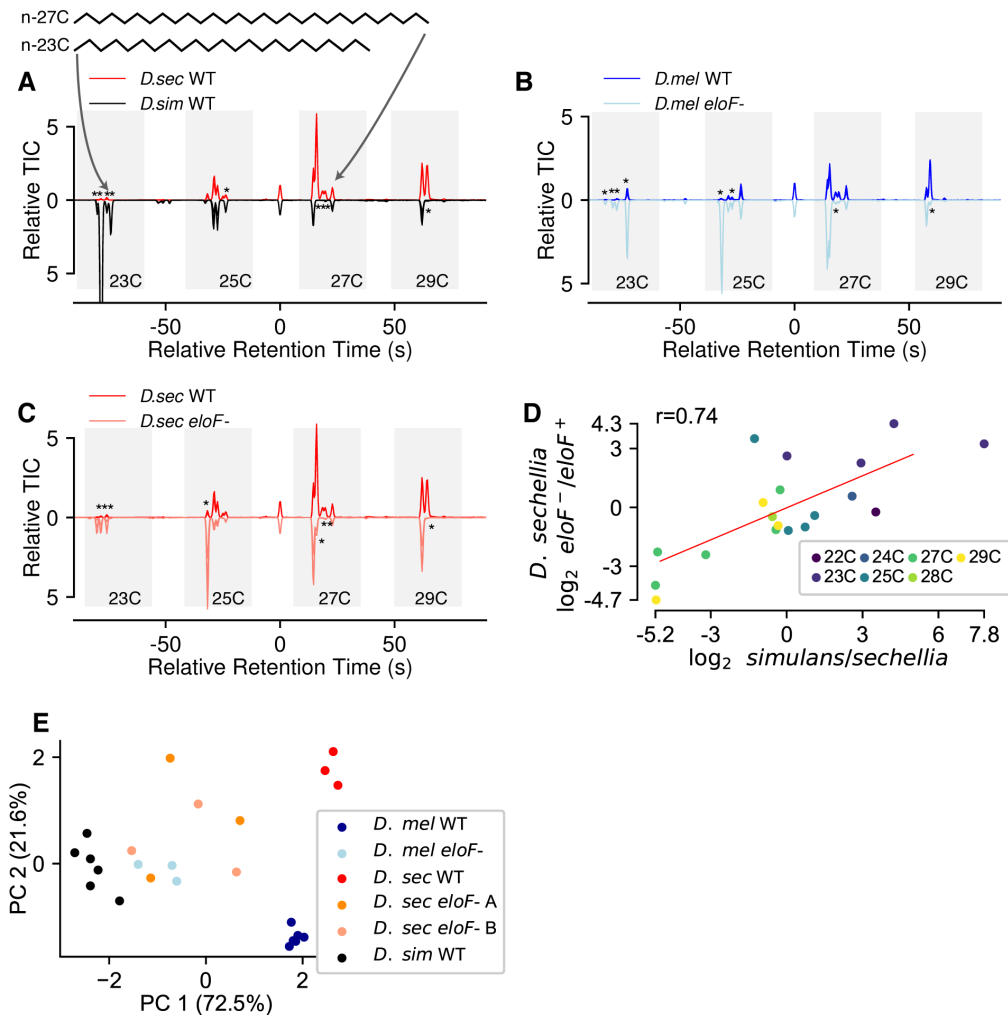
647 **Table 1: Genes with female oenocyte- and species-specific expression**  
648

<i>Gene</i>	<i>Female Oenocyte Specificity (oenocyte/female Sleuth q-value)</i>	<i>% D. sechellia reads in female oenocytes (negative binomial p-value)</i>	<i>GO term(s)</i>	<i>Protein Domain(s)</i>
<i>eloF</i>	76.6 (4.5e-9/2.3e-5)	98.75% (1.2e-21)	fatty acid elongase activity	ELO family
<i>Fad2</i>	18.2 (4.5e-9/2.5e-5)	95.5% (2.5e-72)	<i>Catalysis of an oxidation-reduction (redox) reaction in which hydrogen or electrons are transferred from each of two donors...</i>	Fatty acid desaturase type I, conserved site; Fatty acid desaturase domain; Acyl-CoA desaturase
<i>CG8534</i>	4.3 (8.2e-4/3.6e-5)	93.75% (1.6e-24)	<i>fatty acid elongase activity</i>	ELO family
<i>FASN3</i>	2.31 (2.7e-4/3.8e-5)	60.5% (.00026)	<i>3-hydroxypalmitoyl-[acyl-carrier-protein] dehydratase activity; 3-oxoacyl-[acyl-carrier-protein] reductase (NADPH) activity; 3-oxoacyl-[acyl-carrier-protein] synthase activity; [acyl-carrier-protein] S-acetyltransferase activity; [acyl-carrier-protein] S-malonyltransferase activity; enoyl-[acyl-carrier-protein] reductase (NADPH, B-specific) activity; myristoyl-[acyl-carrier-protein] hydrolase activity; oleoyl-[acyl-carrier-protein] hydrolase activity; palmitoyl-[acyl-carrier-protein] hydrolase activity; phosphopantetheine binding</i>	Ketoacyl synthase (N-terminal, C-terminal, and C-terminal extension), Acyl transferase, Polyketide synthase, Alcohol dehydrogenase C-terminal
<i>lectin-22C</i>	173.9 (3.0e-4/2.0e-4)	85.75% (6.7e-09)	<i>galactose binding</i>	C-type lectin-like/link domain superfamily
<i>bond</i>	47.0 (7.8e-4/6.3e-5)	25.5% (2.8e-14)	fatty acid elongase activity	ELO family

649  
650 Genes with significant tissue-specific (sleuth q-value <0.001 in comparisons both between the  
651 two female tissues, and between the two oenocyte samples) and species-specific expression  
652 (negative binomial p-value < .001). Specificity is the ratio of the mean expression in female  
653 oenocytes to the highest expression among male oenocytes, female fat bodies, and male fat  
654 bodies. Gene ontology (GO) terms are annotated molecular function terms (see Supplemental  
655 Table 2 for citations). GO terms without experimental evidence are in italics. Protein domains  
656 are InterPro annotated protein domains/motifs as listed on FlyBase v2017\_06 (Finn *et al.* 2017;  
657 Gramates *et al.* 2017).

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**Figure 2: *eloF*- flies have an overall shorter CHC complement**



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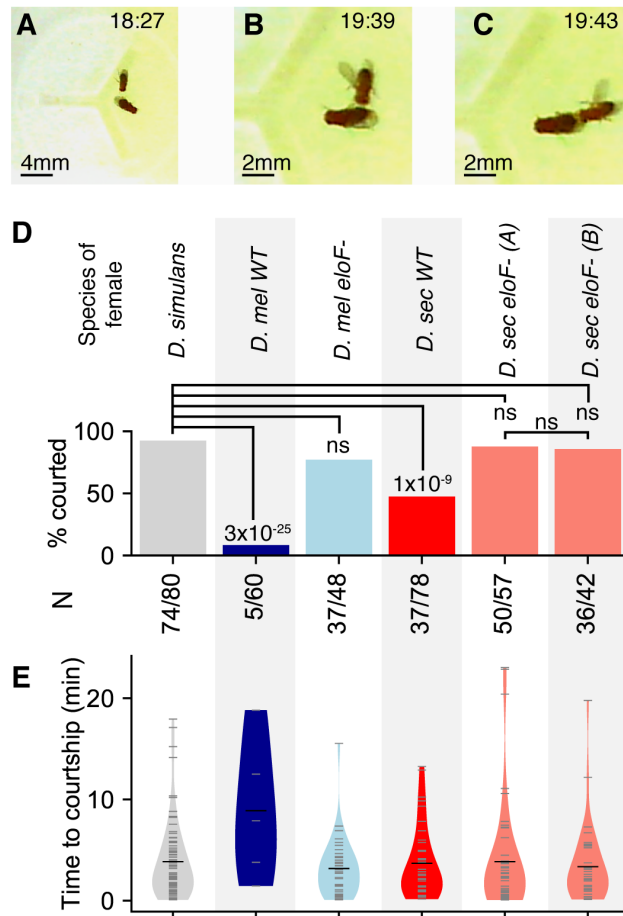
661 A) Total ion chromatographs of the hydrocarbon profile of wild-type *D. sechellia* (top)  
662 and *D. simulans* (bottom). Retention time and abundance is relative to the n-hexacosane  
663 (26C) normalization peak. Grey regions indicate number of carbons in CHC backbone.  
664 CHCs with more than a 3-fold change marked with asterisks at the location of the peak in  
665 the genotype with lower production.

666 B-C) Total ion chromatographs of the hydrocarbon profile of wild-type (top) and *eloF*-  
667 (bottom) *D. melanogaster* (A) and *D. sechellia* (B).

668 D) Average log<sub>2</sub> fold changes of the measured compounds between *D. simulans* and *D.*  
669 *sechellia* vs log<sub>2</sub> fold changes between wild-type and knockout of *eloF* in *D. sechellia*.  
670 Points are colored by the number of carbons in the backbone.

671 E) Principal components analysis of wild-type and *eloF*- *D. melanogaster*, *simulans*, and  
672 *sechellia*. Principal components were calculated for the wild-type data, then *eloF*- data  
673 projected onto the same coordinates.

674 **Figure 3: *D. simulans* males court interspecific *eloF*- females at significantly higher**  
675 **rates**



676  
677 A-C) We recorded between 42 and 80 pairs of single *D. simulans* males courting single  
678 females of the indicated genotype. We recorded the time between male's first tapping the  
679 female (and ostensibly sampling the female CHCs) and either singing behavior or licking  
680 of the female's posterior prior to copulation.  
681 D) Female flies bearing a functional copy of *eloF* (*D. melanogaster* WT and *D. simulans*  
682 WT) were courted by *D. simulans* males at significantly lower rates than *D. simulans*  
683 conspecific females and interspecific females without *eloF*. We performed the indicated  
684 Fisher's exact tests for differences in courtship rate (as measured by rate of proceeding to  
685 precopulatory licking), with Bonferroni-corrected *p*-values above each bar when  
686 significant.

687 E) Violin plots of the delay between first contact between males and females and  
688 initiation of courtship. Black lines indicate mean time to courtship. Gray ticks indicate  
689 the underlying data. Although the *D. simulans* males were slower to court *D.*  
690 *melanogaster* WT females, this represents only 5 cases of courtship (out of 60 trials), and  
691 no comparisons were significant by t-test at even a nominal  $p=0.05$  cutoff.

692 **Supplemental Information**

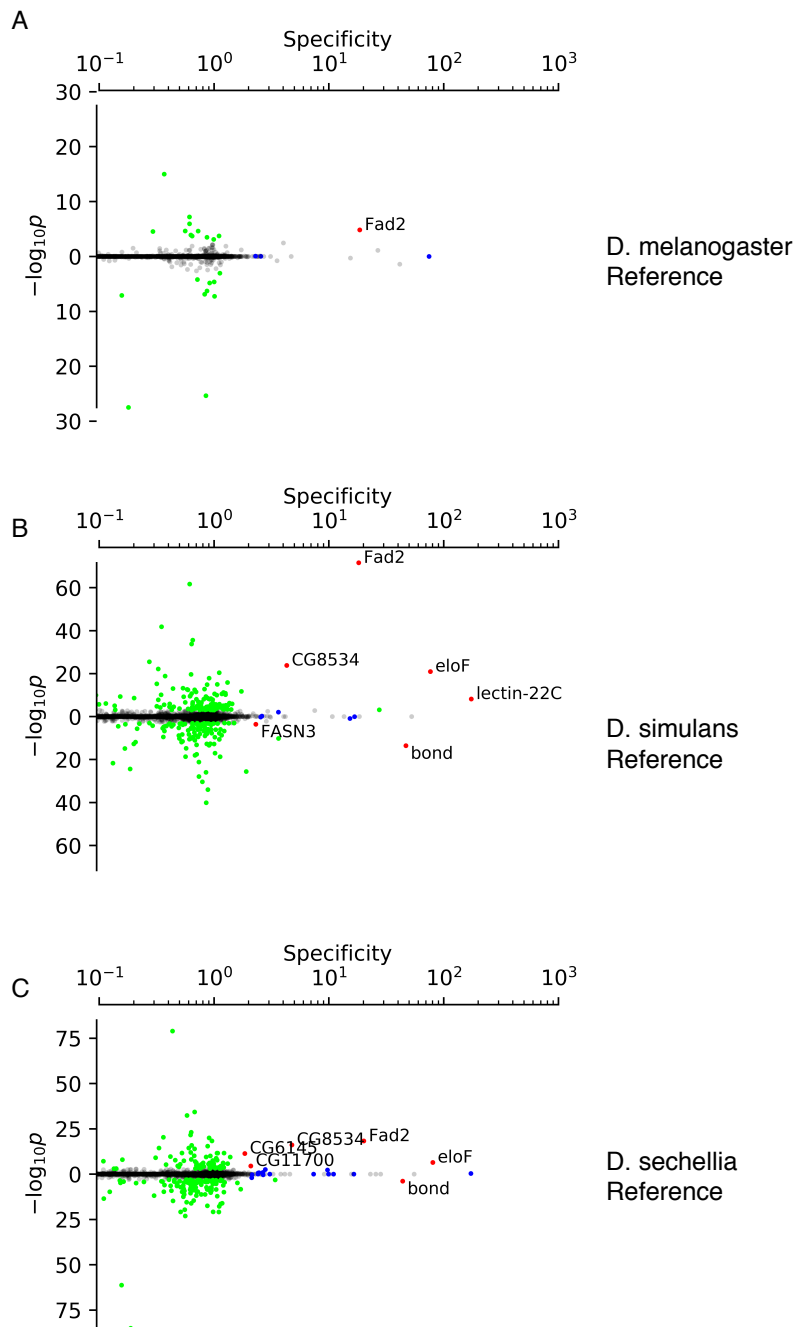
693 **Supplemental Table 1: RNA sequencing library details**

TISSUE	SEX	REPLICATE	TOTAL PAIRED END READS	UNIQUE DEDUPLICATED DE-BIASED READS (%)	READS ASSIGNABLE TO PARENT (% OF MAPPED)	D. SEHELLIA READS (% OF ASSIGNABLE)
Fat body	Female	1	29,958,590	3,099,853 (10.3%)	1,166,014 (37.6%)	683,982 (58.7%)
Fat body	Female	2	26,693,093	4,156,810 (15.6%)	1,610,951 (38.8%)	919,575 (57.1%)
Fat body	Male	1	30,135,650	2,671,563 (8.9%)	917,125 (34.3%)	502,862 (54.8%)
Fat body	Male	2	36,340,739	4,283,897 (11.8%)	1,726,324 (40.3%)	910,829 (52.8%)
Oenocyte	Female	1	48,560,642	5,189,309 (10.7%)	1,711,402 (33.0%)	994,986 (58.1%)
Oenocyte	Female	2	41,310,971	4,295,068 (10.4%)	1,541,471 (35.9%)	832,732 (54.0%)
Oenocyte	Male	1	26,683,030	2,540,477 (9.5%)	808,148 (31.8%)	408,929 (50.6%)
Oenocyte	Male	2	53,693,579	4,324,451 (8.1%)	1,478,937 (34.2%)	790,366 (53.4%)

694

695 Unique deduplicated mapped reads indicates reads that pass the WASP pipeline (van de Geijn *et al.* 2015), i.e. that map to a single  
 696 position and map to the same position when alleles are swapped. Overall mapping rates (including multimappers) are typically around  
 697 65%.

698 **Supplemental Figure 1: Primary candidate genes are robust to the choice of reference**  
699 **genome**

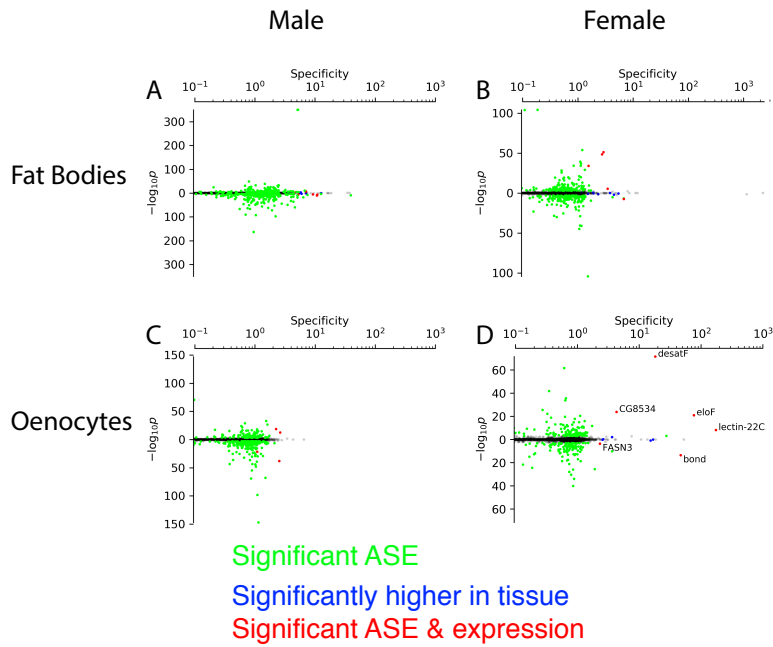


700  
701 Specificity-ASE plot as in Figure 1B, but with reads mapped to the (A) *D. melanogaster*,  
702 (B—same as Fig. 1B) *D. simulans*, or (C) *D. sechellia* genome.



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## Supplemental Figure 2: Male oenocytes do not show strong tissue-specific expression



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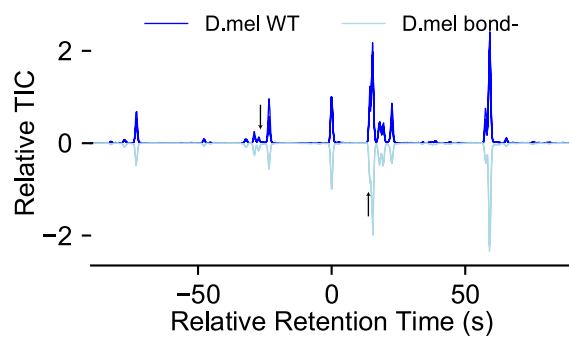
A-C) ASE and tissue specificity as in Figure 1B, except for male fat bodies (A), female fat bodies (B), male oenocytes (C), and female oenocytes (D—same as Fig. 1B).

710 **Supplemental Table 2: References for Gene Ontology Terms in Table 1**  
 711

<i>Gene</i>	<i>GO Term</i>	<i>GO Term ID</i>	<i>Direct evidence?</i>	<i>Reference</i>
<i>eloF</i>	fatty acid elongase activity	GO:0009922	Y	(Chertemps <i>et al.</i> 2007)
<i>desatF</i>	stearoyl-CoA 9-desaturase activity	GO:0004768	N	(Finn <i>et al.</i> 2017; Gramates <i>et al.</i> 2017)
<i>CG8534</i>	fatty acid elongase activity	GO:0009922	N	(Szafer-Glusman <i>et al.</i> 2008)
<i>FASN3</i>	3-oxoacyl-[acyl-carrier-protein] synthase activity	GO:0004315	N	(Finn <i>et al.</i> 2017; Gramates <i>et al.</i> 2017)
<i>lectin-22C</i>	Galactose binding; Carbohydrate binding	GO:0005534; GO:0030246	N	(Theopold <i>et al.</i> 1999; Tanji <i>et al.</i> 2006)
<i>bond</i>	fatty acid elongase activity	GO:0009922	Y	(Szafer-Glusman <i>et al.</i> 2008)

712  
 713 Summary of FlyBase-curated function gene ontology codes and evidence for genes with  
 714 female oenocyte-specific and allele-specific expression (Gramates *et al.* 2017). Direct  
 715 evidence indicates evidence codes of “Inferred from Direct Assay”, “Inferred from  
 716 Genetic Interaction”, and “Inferred from Physical Interaction”.

717 **Supplemental Figure 3: GCMS of *bond-* *D. melanogaster* females**

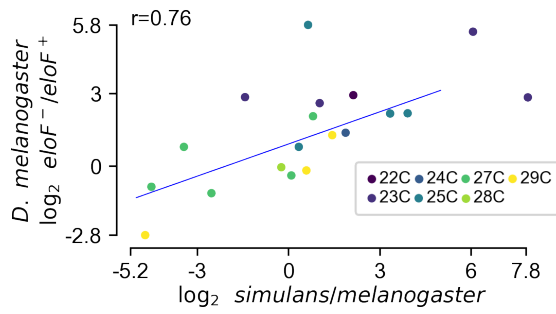


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719 Arrows indicate hydrocarbons with ~60% change in levels.

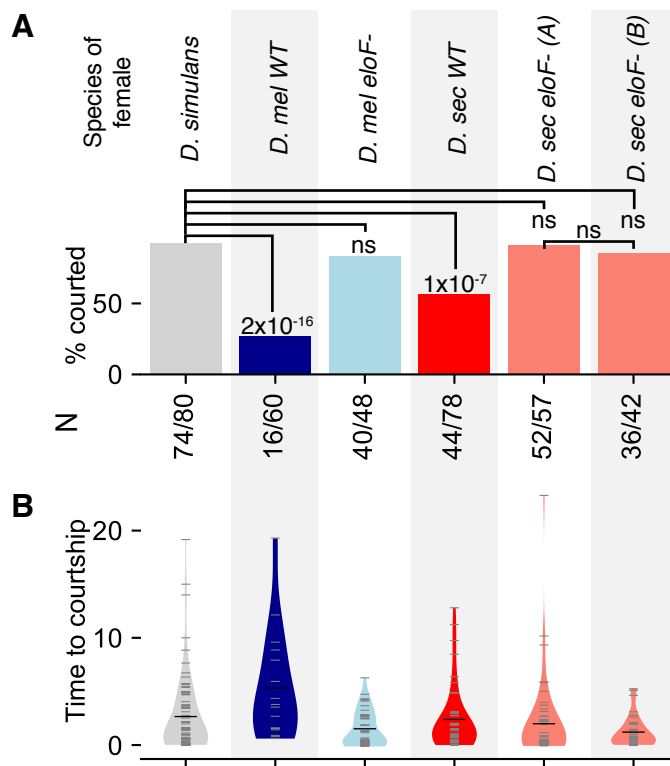
720

721 **Supplemental Figure 4: Pairwise comparison of *D. melanogaster* WT vs *eloF***  
722 **knockdown and *D. melanogaster* WT vs *D. simulans* WT**



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724 Log fold change between the indicated comparisons for average area under the total ion  
725 chromatogram curve of each of 19 different CHC peaks.

726 Supplemental Figure 5: *D. simulans* males perform wing song for *eloF*- females at  
727 significantly higher rates than *eloF*+ females



728 Courtship rate (A) and delay until initiation of wing song (B), as in Figure 3D-E, except  
729 using time to initiation of wing song instead of pre-copulatory licking.  
730

731 **Supplemental Figure 6: Protein alignments of the *D. melanogaster*, *simulans*, and**  
 732 ***sechellia* versions of *eloF* show few *D. simulans*-specific changes**

```

Dmel   MFAPIDPVKIPVVSNPWITMGTLLIGYLLFVLKLGPKIMEHRKPFHLNGVIRIYNIFQILY  60
Dsim   MFAPIDPVKIPVFSDPWITMATLSGYLLFVLKLGPKIMENRKPFHLSGVIRVYNIFQILY  60
Dsec   MFAPIDPVKIPVFSDPWVPMVTLSTGYLLFVLKLGPKIMENRKPFHLSGVIRVYNIFQILY  60
      ***** .:***: * ** *****:***** .****:*****

Dmel   NGLILVLGVHFLFVLKAYQISCIVSLPMDHKYKDRERLICITLYLVNKFVDLVETIFFVLR  120
Dsim   NGLILVLGVHFLFVLKAYQISCIVSLPMDHKYKDRERLICILYMLNKFVDLVETIFFVLR  120
Dsec   NGLILVLGIHFLFVLKAYQISCIVSLPMDHKYKDRERLICILYMLNKFVDLVETIFFVLR  120
      *****:*****:*****:***** **.:*****

Dmel   KKDRQISFLHVFHHFAMAFFGYLYYCFHGYGGVAFPPQCLLNTAVHVMYAYYYLSSISKE  180
Dsim   KKDRQISFLHVFHHFAMAF LGYLYYFHYGGVAFPPQCLLNTAVHVMYAYYYLSSISQE  180
Dsec   KKDRQISFLHVFHHFAMAF LGYLYYFHYGGVAFPPQCLLNTAVHVMYAYYYLSSISQE  180
      *****:*****:***** *****:*****:*****:*****
      ELO family signature

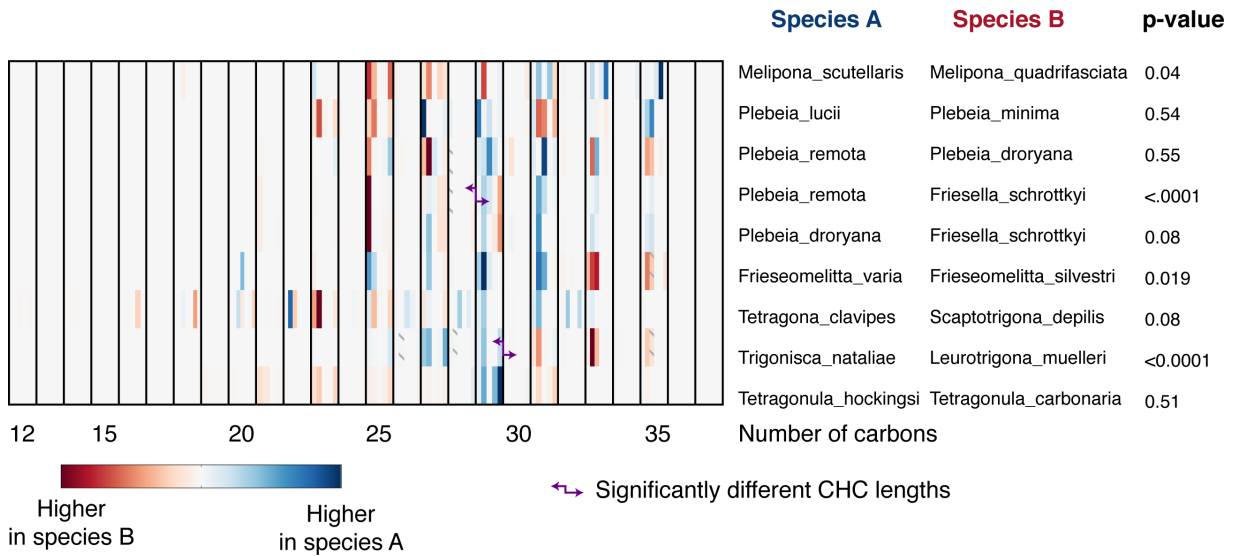
Dmel   VQRSLWwKKYITIAQLVQFAIILLHCTITLAQPNCVNRPLTYGCGSLSAFFAVIFSQFY  240
Dsim   LQRSLWwKKYITIAQLVQFGIILLHCTITLAQPDCVNRPLTYGCGSLSAFFAVIFSQFY  240
Dsec   LQRSLWwKKYITIAQLVQFGIILLHCTITLAQPDCVNRPLTYGCGSLSAFFAVIFSQFY  240
      .*****:*****:*****:*****:*****:*****

Dmel   YHNYIKPGKKSQKQKN---- 257
Dsim   YHNYIKPGEKSSKQSAIHKNL 261
Dsec   FQNYIKPGKKSQKQSAIH--- 258
      :*****:***:*.
    
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We aligned the coding sequences of *eloF* and its orthologs in *D. simulans* and *D. sechellia* using Clustal Omega (Sievers, *et al.* 2012). Red boxes indicate where the *D. simulans* does not match at least one of the other species. The blue box is the ELO family signature PS01188 from ProSite (Sigrist, *et al.* 2012).

740 **Supplemental Figure 7: Hydrocarbon length is connected to speciation in some**  
 741 **stingless bees.**  
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We examined the changes in hydrocarbon profiles of stingless bee queens between recently diverged species pairs, as measured in Nunes *et al*, 2017. To determine whether there was a change in overall hydrocarbon length, we looked for a critical CHC length that maximized the sum of the squares of CHCs shorter than the critical length plus the sum of the squares of CHCs longer than the critical length. To calculate p-values, we randomized the order of CHCs (while keeping CHCs with the same number of carbons together), performing 10,000 permutations. After Bonferroni correction, we found a significant divergence in CHC length between *P. remota* and *F. schrottkyi*, with *P. remota* having more CHCs with 29 or more carbons, and between *T. nataliae* and *L. muelleri*, with *L. muelleri* having more CHCs with 30 or more carbons.