A shift to shorter cuticular hydrocarbons causes sexual isolation in the *Drosophila americana* species group

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Abstract

Because sensory signals often evolve rapidly, they could be instrumental in the emergence of reproductive isolation between species. However, pinpointing their specific contribution to isolating barriers, and the mechanisms underlying their divergence, remains challenging. Here we demonstrate sexual isolation due to divergence in chemical signals between members of the *Drosophila americana* group, dissect its underlying phenotypic and genetic mechanisms, and propose a model of its evolutionary history. Mating trials revealed complete sexual isolation between *Drosophila novamexicana* males and *Drosophila americana texana* females, as well as more moderate bi-directional isolation between *D. americana* subspecies. Mating behavior recordings indicate *D. a. texana* males have the highest courtship efficiency and, unlike males of the other two species, are accepted by females of all species. Quantification of cuticular hydrocarbon (CHC) profiles, chemosensory signals that are used for species recognition and mate finding in Drosophila, shows that *D. a. texana* differs from the other two species primarily on the basis of compound carbon chain-length. Moreover, manipulation of male CHC composition via heterospecific perfuming—specifically perfuming *D. novamexicana* males with *D. a. texana* males—abolishes their sexual isolation from *D. a. texana* females. Evaluation of candidate genes for patterns of gene expression reveals a single gene—elongase CG17821—with patterns consistent with a causal role in CHC differences between species. Sequence comparisons indicate *D. novamexicana* and *D. a. americana* share a derived CG17821 truncation mutation that could also contribute to their shared "short" CHC phenotype. We present an evolutionary model for the origin and spread of this allele and the consequences for CHC divergence and sexual isolation in this group.

32 **Introduction**

33 Sensory signals can act as sexual cues that are critical for intraspecific mate evaluation and
34 reproductive success. Moreover, because they are frequently among the most rapidly evolving species
35 differences (Smadja and Butlin 2009, Seddon et al 2013, Wilkins et al 2013), divergence in sensory sexual
36 signals might often contribute to the earliest stages of reproductive isolation, in the form of prezygotic
37 barriers between lineages (Butlin et al 2012, Ritchie 2007). Among all such sexual signals, nowhere is the
38 diversity more evident than those acting as premating traits, including coloration and other visual
39 signals, acoustic signals, and complex pheromone blends. However, convincingly demonstrating the
40 connection between such sensory signal divergence and emerging reproductive isolation can be
41 challenging because it requires, first, identification and demonstration of the direct role of specific
42 signals mediating sexual isolation between species and, second, understanding the specific mechanistic
43 changes that have given rise to lineage differences in this signal.

44 Among insects, sexual signals draw on three primary sensory modalities—visual, auditory, and
45 chemosensory—and most species likely use a combination of all three modalities to identify potential
46 mates. Of these, many insect chemosensory signals primarily consist of cuticular hydrocarbons (CHCs)—
47 a broad group of carbon-chain compounds important for many essential functions including sex
48 pheromone signaling, but also environmental adaptation (Blomquist and Bagneres 2010, Chung and
49 Carrol 2015, Yew and Chung 2017). Unlike many auditory and visual cues, CHCs are produced and
50 received by both males and females—making them potentially important for both male and female
51 mate choice or species recognition. Indeed, studies on *Drosophila* chemical communication—
52 particularly in the *melanogaster* subgroup—have detected clear evidence for sexual isolation based on
53 male choice of female CHCs (Coyne et al. 1995, Billeter et al. 2009), while work in other species has
54 demonstrated female choice of species-specific male CHC profiles (Coyne et al. 2002, Mas and Jallon
55 2005, Curtis et al. 2013, Dyer et al. 2014). Elements of the biochemical pathways of CHC production and

56 the underlying genes are also relatively well understood in *D. melanogaster* (Pardy et al 2018).
57 Accordingly, single genes contributing to species-specific CHC differences have been implicated as
58 causes of reproductive isolation in several cases, including between *D. melanogaster* and *D. simulans*
59 (desatF: Legendre et al. 2008) and *D. serrata* and *D. birchii* (mFAS: Chung et al. 2014). This framework
60 provides an excellent resource for identifying candidate genes for sensory sexual signal variation in
61 other *Drosophila* systems.

62 Here we use the *Drosophila americana* group to investigate how species variation in CHC
63 profiles contributes to reproductive isolation and to identify the underlying chemical and genetic
64 changes that may comprise this variation. This group—consisting of two subspecies *Drosophila*
65 *americana americana* and *Drosophila americana texana* and their sister species *Drosophila*
66 *novamexicana*—is a member of the *virilis* clade found across North America. These species are closely
67 related (MRCA ~0.5 mya, Morales-Hojas et al. 2008), and have been shown to occupy distinct
68 geographic and environmental habitats (Davis and Moyle 2019). Classic studies have shown some
69 evidence for premating isolation (Spieth 1951) as well as differences in CHC composition between males
70 (Bartelt at al. 1986), however the contribution of hydrocarbons to premating isolation has not been
71 assessed. With lines from each of the three lineages *D. novamexicana*, *D. a. americana*, and *D. a. texana*
72 (referred to here as species, for simplicity), here we use a combination of mating and behavioral studies,
73 chemical analysis and manipulation, and gene expression and molecular sequence variation analyses to
74 assess the role of CHCs in reproductive isolation. Together, our data suggest that evolution of a male
75 sexual signal—an overall shift in the relative abundance of longer versus shorter cuticular hydrocarbons,
76 due to novel mutation in an elongase gene—has produced complete premating isolation between
77 derived males and females from species that retain the ancestral trait and preference, as proposed in
78 classical models (Kaneshiro 1976, 1980) of the evolution of asymmetric sexual isolation.
79

Results

D. a. texana *females discriminate against heterospecific males in mating trials*

We found clear evidence of moderate to strong sexual isolation between *D. a. texana* and the other two species (Table 1). Mating rate (average proportion of females mated, in 4x4 mating trials) ranged from very high in conspecific crosses and some heterospecific crosses, to <50% in heterospecific combinations of female *D. a. texana* with each of the other species. Notably, *D. novamexicana* males were never successful in mating with *D. a. texana* females, indicating complete sexual isolation in this cross direction between these two species. In contrast, mating rate in the reciprocal cross was 70%. Other pairings with mating rates at or below 50% were both reciprocal directions between *D. a. americana* and *D. a. texana*. Across all cross combinations in the 4x4 mating trials, we found that female identity (Kruskal-Wallis test: $\chi^2(2) = 8.99$, $P = 0.012$), but not male identity (Kruskal-Wallis test: $\chi^2(2) = 0.80$, $P = 0.67$), significantly affected success. Post-hoc comparisons confirmed no pairwise differences in mean mating rate between any male species (not shown). In contrast, *D. a. texana* female copulation rate was significantly lower on average than both *D. a. americana* (*P-adj* = 0.025) and *D. novamexicana* (*P-adj* = 0.042), whereas *D. a. americana* and *D. novamexicana* females did not differ (*P-adj* = 0.15). Together, these results indicate that *D. a. texana* females discriminate against heterospecific males more strongly than do females of the other species, with the greatest discrimination against *D. novamexicana* males. In addition, while we observe clear asymmetric sexual isolation between *D. a. texana* and *D. novamexicana*, premating isolation between the two *D. americana* subspecies is bi-directional and more moderate, indicating the specific mechanisms of isolation might differ among pairs of species within this group (see discussion).

Table 1: Copulation rates and latency for each cross in unperfumed 4x4 and 1x1 mating trials.

		4x4 mating trials	1x1 mating trials	
Female species	**Male species**	**Mating rate[a] (mean ± SE)**	**Mating rate[a] (mean)**	**Copulation latency (mean ± SE)[b]**
D. novamexicana	*D. novamexicana*	0.90 ± 0.06	1.00	69.89 ± 22.73
	D. a. americana	0.69 ± 0.12	0.60	147.48 ± 27.00
	D. a. texana	0.71 ± 0.18	0.60	83.63 ± 39.70
D. a. americana	*D. novamexicana*	1.00 ± 0	0.20	158.33 ± 21.67
	D. a. americana	1.00 ± 0	0.60	123.46 ± 29.01
	D. a. texana	0.50 ± 0.18	0.60	99.42 ± 34.74
D. a. texana	*D. novamexicana*	0.00 ± 0	0.00	180 ± 0
	D. a. americana	0.40 ± 0.14	0.60	99.42 ± 34.74
	D. a. texana	0.85 ± 0.06	0.80	50.32 ± 33.07

[a] n = 5, rate is a proportion out of maximum possible matings (4 or 1)
[b] n = 5, units are in minutes. Unmated trials are scored as a latency of 180 min—the maximum time observed.

D. a. texana *males have greater courtship efficiency and mating success*

We video-recorded single-pair matings for each cross combination to evaluate species differences in courtship strategies and whether these differed in heterospecific pairings. Three male courtship behaviors were quantified from these 1x1 matings—display rate, tapping rate, and licking rate. All three behaviors showed similar patterns of among-species variation, when each was evaluated for the effect of male species, female species, or their interaction, using 2-way ANOVAs. Males significantly differed with respect to display-rate ($F(2) = 3.76$, $P = 0.03$) and tap-rate ($F(2) = 4.76$, $P = 0.017$), but only marginally for lick-rate ($F(2) = 2.67$, $P = 0.083$). For all three behaviors, this difference is due to *D. a. texana* males exhibiting higher rates (Tukey HSD post-hoc tests; Figure 1, supplementary Table S1). In contrast, we detected no female identity effects on male behavioral rate (display-rate: $F(2) = 1.83$, $P = 0.18$; tap-rate $F(2) = 0.58$, $P = 0.56$; lick-rate: $F(2) = 0.43$, $P = 0.65$), or any female x male interactions (display-rate: $F(2) = 0.72$, $P = 0.58$; tap-rate $F(2) = 0.45$, $P = 0.77$; lick-rate: $F(2) = 0.65$, $P = 0.63$). Because behavior rates are dependent on copulation latency, and not simply the total number of behavioral events, these rates approximate the efficiency with which each courtship behavior results in a mating. Taken together, our results indicate that *D. a. texana* males have greater courtship efficiency (in terms

of display and tap rates), and that males do not significantly vary these behavioral rates depending on female species identity.

The single pair mating assays broadly reiterated the patterns of moderate to strong sexual isolation we observed for *D. a. texana* in 4x4 trials. As with our 4x4 mating assay, individual pairs of *D. novamexicana* males never mated with *D. a. texana* females, while mating rate between the two *D. americana* subspecies was moderately reduced in both directions. A logistic regression assessing how mating rate varied based on male identity, female identity, and their interaction showed a significant interaction effect only (males: $\chi^2(2) = 2.97$, $P = 0.28$; females: $\chi^2(2) = 2.52$, $P = 0.23$; males*females: $\chi^2(4) = 12.67$, $P = 0.013$)—a difference from 4x4-mating trials where female species identity was the primary predictor of mating rate. This variation between the assays appears to be driven by female *D. a. americana* accepting fewer *D. novamexicana* males in 1x1 trials (20%) compared to 4x4 trials (100%, Table 1), an observation that suggests male density within mating trials might affect copulation success in this particular species combination. Finally, copulation latency differed marginally based on both male identity (ANOVA: $F(2) = 3.14$, $P = 0.055$) and the male by female interaction ($F(4) = 2.48$, $P = 0.062$), but did not differ based on female species identity ($F(2) = 0.68$, $P = 0.5147$). Post-hoc tests suggest that the marginal male species effect is likely due to lower copulation latency in male *D. a. texana* relative to *D. novamexicana* males (Tukey HSD; Nov-Tex: *P-adjust* = 0.07; Ame-Tex: *P-adjust* = 0.23; Nov-Ame: *P-adjust* = 0.81).

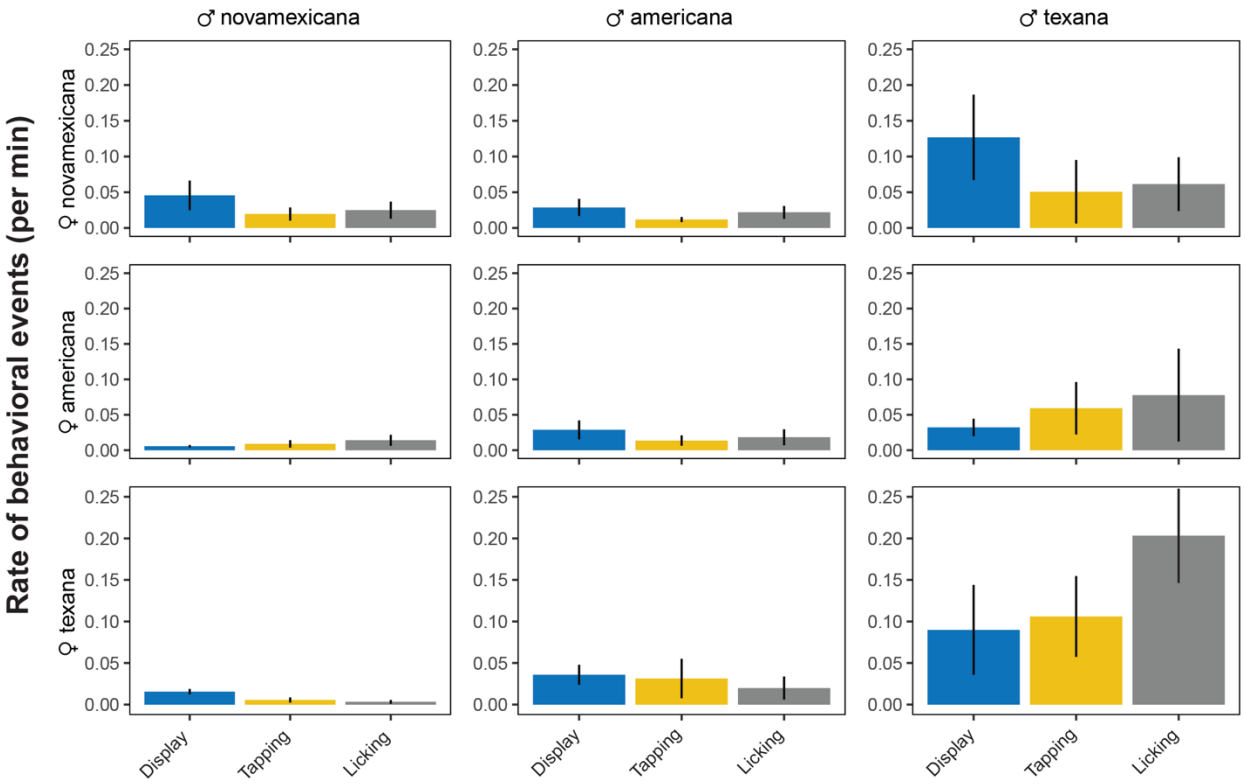


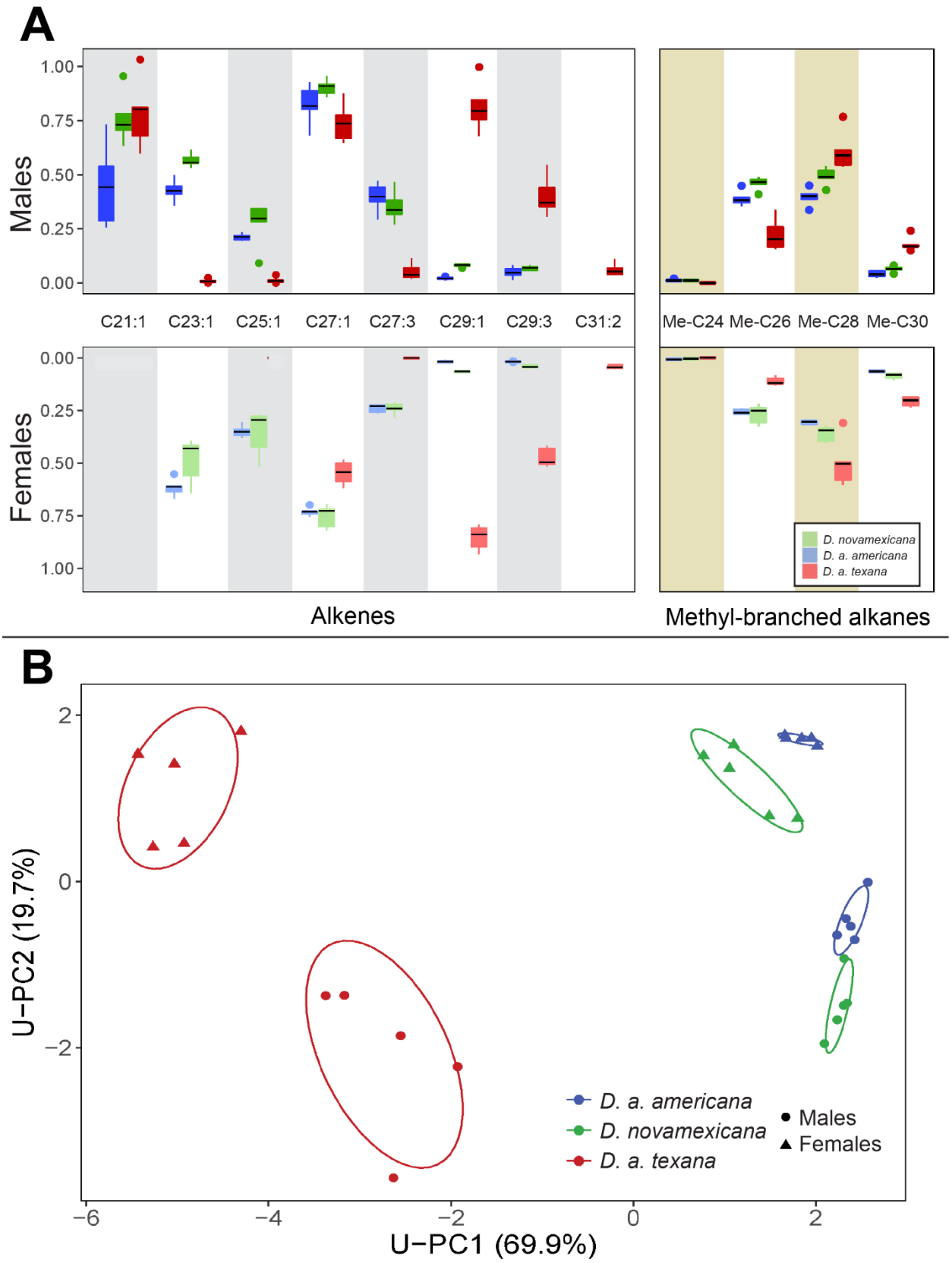
Figure 1: Male courtship behavior rates in 1x1 mating assays across all cross types. Males (columns) show significant differences in display rate and tap rate, and marginal differences in lick rate. Rates did not differ based on female identity (rows) or male x female interaction (see results). Bar and whiskers indicate mean and standard error (SE).

Species and sexes differ in CHC composition

We found that both species and sexes within species showed different and distinctive CHC profiles, with the largest differences detected between *D. a. texana* and the other two species (Figure 2). Across all samples (*n*=5 replicate samples for each identity) we detected 8 alkene compounds (2 of which are sex-specific) and 4 methyl-branched alkane compounds present in at least one of the species. A principal component analysis of profiles from unmanipulated flies (unmanipulated-PCA or 'U-PCA') summarizing the primary axes of CHC profile composition found that 95.3% of compound variation across all samples was explained by the first three principal components (U-PC1 to 3). Of these, CHC composition varied significantly for both species and sex for U-PC1 (species: $F(2, 429.29)$, $P < 0.0001$; sex: $F(1, 46.97)$, $P < 0.0001$) and U-PC2 (species: $F(2, 10.54)$, $P = 0.00043$; sex: $F(1, 181.69)$, $P < 0.0001$),

154 but had no sex or species effect for U-PC3 (species: $F(2, 0.31)$, $P = 0.737$; sex: $F(1, 0.57)$, $P = 0.458$)(Figure
155 2). Notably, *D. a. texana* of both sexes differed from the other two species along the U-PC1 axis (Tukey
156 HSD; Nov-Tex: *P-adjust* < 0.0001; Ame-Tex: *P-adjust* < 0.0001; Nov-Ame: *P-adjust* = 0.28), which also
157 explains most of the CHC variation between samples (69.9%). U-PC1 was positively loaded for most of
158 the shorter carbon chain length compounds in both alkene and methyl-branched alkane classes of
159 compounds (Table S2); consequently, negative values were strongly loaded for the compounds with
160 longer carbon chain length, and this axis can be interpreted as a composite of average compound length
161 across the CHC profile as a whole (Figure 2). Accordingly, both sexes of *D. a. texana* had a higher
162 abundance of longer-chain alkenes and methyl-branched alkanes than did either *D. a. americana* or *D.*
163 *novamexicana*.

164 In contrast to U-PC1, U-PC2 appeared to primarily differentiate sexes within species, but also *D.*
165 *a. americana* males from males of the other two species. This axis was most heavily loaded for two
166 compounds: C21:1 and Me-C28, followed by C27:1 and Me-C26, with much smaller loadings for all other
167 compounds (Table S2). C21:1 is a male-specific compound that is not detected in females; Me-C28, Me-
168 26, and C27:1 abundance was also consistently different between sexes, with males always having more
169 of these CHCs than females for each species (Figure 2). Therefore, negative U-PC2 values can be
170 interpreted as primarily representing more male-specific profiles, while positive U-PC2 values
171 correspond to more female-like profiles. With respect to the detected species difference in U-PC2, post-
172 hoc tests reveal that this was driven by *D. a. americana* (Tukey HSD; Nov-Tex: *P-adjust* = 0.99; Ame-Tex:
173 *P-adjust* = 0.0062; Nov-Ame: *P-adjust* = 0.0088). Furthermore, a post-hoc test specifically comparing the
174 abundance of the male C21:1 compound confirms a difference between *D. a. americana* and males of
175 the other two species, but not between *D. novamexicana* and *D. a. texana* (Tukey HSD; Nov-Tex: *P-*
176 *adjust* = 0.98; Ame-Tex: *P-adjust* = 0.016; Nov-Ame: *P-adjust* = 0.022).


A
Males
Females
1.00
0.75
0.50
0.25
0.00
C21:1
C23:1
C25:1
C27:1
C27:3
C29:1
C29:3
C31:2
Me-C24
Me-C26
Me-C28
Me-C30
D. novamexicana
D. a. americana
D. a. texana
Alkenes
Methyl-branched alkanes
B
U−PC2 (19.7%)
U−PC1 (69.9%)
2
0
−2
−6
−4
−2
0
2
D. a. americana
D. novamexicana
D. a. texana
Males
Females

177

178 **Figure 2: Cuticular hydrocarbon composition of unmanipulated males and females of each species**
179 **stock. A) Relative log-scale abundance of compounds for males (upper) and females (lower), for each**
180 **major compound type (line = mean, box = central quartile, whiskers = S.E.). *n* = 5 samples for each sex**
181 **and line. Test statistics for species differences are in Table S3. B) First two principal components (U-**
182 **PC1 and U-PC2) of composite CHC variation among males (circle) and females (triangle). Ellipses**
183 **indicate 90% bivariate normal density for each species-sex group. Species and sex significantly**
184 **influenced both U-PC1 and U-PC2.**
185
186 *Heterospecific perfuming influences* D. a. texana *female acceptance of con- and hetero-specific males*

187 We found that patterns of sexual isolation could be modified by specifically changing the CHC
188 profiles of males of different species. To do this, we used perfuming assays to manipulate the
189 pheromone profile of males by co-housing them with either con- or hetero-specific males, and then
190 evaluated how this manipulation influenced mating rates among species. For these analyses, we focused
191 on two species pairs in two separate, analogous experiments—*D. novamexicana* and *D. a. texana* ("Nov-
192 Tex pair"), and *D. a. americana* and *D. a. texana* ("Ame-Tex pair")— because *D. a. texana* shows the
193 strongest sexual isolation from the two other species, and the largest differences in CHC composition.
194 Within each perfuming experiment, we generated four combinations of target and donor male
195 identities: each species perfumed by conspecific males (control) and each species perfumed by
196 heterospecific males. Each class of perfumed males was evaluated for mating rate with *D. a. texana*
197 females, because these females were the most discriminating against heterospecific males in our
198 previous mating assays (Table 2). The observed mating rate of males perfumed with conspecific males
199 (the control manipulation) was similar to that observed in unperfumed mating trials: *D. novamexicana*
200 and *D. a. americana* males perfumed with their own species performed poorly with *D. a. texana*
201 females, relative to conspecific *D. a. texana* perfumed with their own males, which had a 100% mating
202 rate (Table 2). In strong contrast, heterospecific perfumed males showed altered mating rates relative to
203 control perfumed males. For Nov-Tex pairings, *D. novamexicana* males perfumed with *D. a. texana*
204 males successfully mated with *D. a. texana* females 94% of the time, a significantly higher mating rate
205 than *D. novamexicana* males perfumed with their own males (19%; Mann-Whitney U-test: U = 0, *P* =

206 0.025). Likewise, the reciprocal treatment—male *D. a. texana* perfumed with *D. novamexicana* males—
207 showed significantly reduced copulation success (19%) relative to conspecific-perfumed *D. a. texana*
208 (Mann-Whitney U-test: U = 0, P = 0.018). For Ame-Tex pairings, *D. a. texana* male mating rate was also
209 significantly reduced when perfumed with *D. a. americana* males (Mann-Whitney U-test: U = 0, P =
210 0.018), compared to *D. a. texana* males perfumed with conspecifics. In contrast, *D. a. americana* males
211 perfumed with *D. a. texana* males did not significantly differ from the mating rate of control
212 (conspecific-perfumed) males (Mann-Whitney U-test: U = 5.5, P = 0.54).

213 Overall, these results indicate that perfuming males with heterospecific CHCs influences the
214 frequency of successful copulation with *D. a. texana* females. *D. a. texana* males perfumed with
215 heterospecific profiles (of either *D. novamexicana* or *D. a. americana*) had significantly reduced mating
216 rates with their own females. Conversely, perfuming *D. novamexicana* males with heterospecific *D. a.*
217 *texana* males significantly increased their mating rate with *D. texana* females, almost completely
218 reversing the pattern of sexual isolation observed for unmanipulated males. This large shift in mating
219 rate for heterospecifically-perfumed *D. novamexicana* indicates that differences in male CHC profile play
220 a critical role in sexual isolation in the Nov-Tex pair. In contrast with the three other classes of
221 heterospecifically-perfumed males, perfuming *D. a. americana* with *D. a. texana* males did not
222 significantly change their mating rate. It possible that this manipulation might have been less effective at
223 changing the CHC profile of *D. a. americana* males (see next section), and/or that mating isolation in the
224 Ame-Tex pair might depend on more complex factors than a simple shift in CHC composition (see
225 Discussion).
226

227 **Table 2: Mean copulation success (number of matings out of 4 per trial) with *D. a. texana* females for**
228 **each perfuming identity in manipulated (perfumed) 4x4 mating assays.**
229

Target male	Donor male	Copulation success (Mean ± SE)	Pairwise Mann-Whitney U test	
	Nov-Tex pair		**U-score**	***P*-value**
D. novamexicana	*D. novamexicana*	0.25 ± 0.15	} 0	0.0247
D. novamexicana	*D. a. texana*	0.94 ± 0.063		
D. a. texana	*D. a. texana*	1.00 ± 0	} 0	0.018
D. a. texana	*D. novamexicana*	0.19 ± 0.063		
	Ame-Tex pair			
D. a. americana	*D. a. americana*	0.44 ± 0.12	} 5.5	0.54
D. a. americana	*D. a. texana*	0.56 ± 0.12		
D. a. texana	*D. a. texana*	1.00 ± 0	} 0	0.018
D. a. texana	*D. a. americana*	0.50 ± 0.063		

Note: *n* = 5 for all crosses

230

231 *Perfuming shifts CHC composition towards the donor male profile*

232 Using CHCs extracted from an additional set of perfumed males (see Methods), we confirmed
233 that our heterospecific perfuming manipulation produced quantitative changes in composite male CHC
234 profiles in both the Nov-Tex and Ame-Tex pairs, by shifting male CHCs closer to the donor male profile.
235 PCAs were performed separately for each species pair (N-PCA for the Nov-Tex pair, and A-PCA for the
236 Ame-Tex pair, hereafter), as were analyses of differences among classes of perfumed males. In both
237 cases, of the first three PCs of CHC composition, PC1 (i.e. N-PC1 or A-PC1) varied by both donor and
238 target male identity (Table S4), indicating that perfuming significantly shifted CHC profiles along the
239 primary axis of variation in both perfuming experiments. Nonetheless, the specific donor-target
240 manipulation that was most successful in this regard differed between the Nov-Tex and Ame-Tex pairs.
241 In the Nov-Tex pair, *D. novamexicana* males perfumed with *D. a. texana* males significantly differed from
242 control (*D. novamexicana*) perfumed samples in CHC composition (N-PC1) ($t(5.9) = -2.8$, $P = 0.031$);
243 however, heterospecific-perfumed *D. a. texana* did not differ from conspecific *D. a. texana*-perfumed
244 samples along the same axis ($t(3.53) = 1.91$, $P = 0.14$). In contrast, in the Am-Tex pair, *D. a. texana*
245 perfumed with *D. a. americana* showed a significant shift in A-PC1 ($t(4.85) = 4.23$, $P = 0.0088$), but there

246 was no significant shift for *D. a. texana*-perfumed *D. a. americana* compared to conspecific-perfumed
247 controls (t(4.68) = 0.055, *P* = 0.96). The difference in pattern between these pairs might be attributed to
248 high variation seen among samples within a given group (in particular conspecific perfumed *D. a.*
249 *texana*). Regardless, it is clear from these results that this perfuming assay can produce significant,
250 detectable differences in CHC profiles after heterospecific perfuming, most notably in *D. novamexicana*
251 males.
252
253 *Patterns of gene expression and sequence variation implicate an elongase gene that contributes to CHC*
254 *variation between species*
255 From among a set of 23 candidate genes whose orthologs have functions related to cuticular
256 hydrocarbon variation, both transcriptome and sequence comparisons implicated one specific elongase
257 locus as potentially causal in CHC composition differences between our species. Using whole-
258 transcriptome RNA-seq data from the same three populations (previously generated in Davis and Moyle
259 2020), we found that 3 of our candidate genes in males and 2 candidates in females were within the top
260 10th percentile of genes most differentially expressed between species across the whole transcriptome
261 (i.e. they showed a stronger species effect than 90% of all 11301 expressed genes) (Table 3). Of these
262 candidate genes, only CG17821 showed elevated gene expression specifically in *D. a. texana* compared
263 to the other two species; this pattern was observed in both sexes but is more significant in females
264 (Figure 3).
265 Closer inspection of our CG17821 sequences revealed that alleles in *D. a. americana* and *D.*
266 *novamexicana* share a thymine insertion mutation that causes a premature stop codon 4 amino acids
267 upstream of the end of the gene, compared to the annotated gene model in outgroup *D. virilis* and the
268 allele in our *D. a. texana* stock (Figure 3); these four terminal amino acids are not present in RNA
269 transcripts for either *D. a. americana* and *D. novamexicana*. Among our three lines, this suggests an

270 association between the truncated protein product of CG17821 and the "short" CHC phenotype we
271 observe. This association is further supported by additional CHC and sequence data from another *D.*
272 *americana* population, originally collected in Anderson, IN (species stock center line 15010-0951.00).
273 Lamb et al. 2020 characterized CHC divergence between females from this *D. a. americana* stock and
274 another *D. novamexicana* line (species stock center 15010-1031.04). The profile for the *D.*
275 *novamexicana* line had absence of long (>C30) compounds, low abundance of C29 compounds, and
276 presence of multiple short alkenes (<C27), consistent with the "short" CHC profile we observe for our *D.*
277 *novamexicana* line. Likewise, the Anderson *D. a. americana* profile they observed is broadly consistent
278 with the "long" profile we observed here in *D. a. texana*, as both show presence of compounds longer
279 than 30 carbons, a high abundance of C29 length alkenes, and absence of compounds shorter than 27
280 carbons. In addition, using a genome assembly from the same Anderson *D. americana* stock (Kim et al.
281 unpubl.), we found that this population has the non-truncated allele of CG17821. These data are
282 consistent with our inference that truncation of this allele could contribute to the difference between
283 "long" and "short" CHC phenotypes in these species.
284
285 *Evolutionary history of elongase CG17821*
286 Several lines of evidence indicate that CG17821 has a complex evolutionary history within the *D.*
287 *americana* subgroup, that likely includes post-speciation introgression. First, the presence of the non-
288 truncated allele in outgroup *D. virilis* (Figure 3) indicate that the truncated allele is derived from a
289 thymine insertion mutation event that took place within the *D. americana* subgroup. Second, the
290 observation that *D. novamexicana* and our western *D. a. americana* line share an identical derived allele
291 at CG17821 indicates an evolutionary history at this locus that disagrees with expected phylogenetic
292 relationships among these three taxa (i.e. where lines from the two *D. americana* subspecies are
293 expected to be most closely related). Gene trees for each of our 23 candidate genes confirmed that

294 CG17821 has a topology that is discordant with the expected species relationships, placing our western
295 *D. a. americana* as sister to *D. novamexicana* rather than grouping it with *D. a. texana* (Figure 3). Four
296 other CHC candidate genes also show this discordant topology, most notably CG18609 which is located
297 immediately downstream of CG17821 (within 1kB) according to the annotated genome of *D. virilis*
298 (Figure 3). For 4 out of 5 of these loci—including both CG17821 and CG18609—using the allele from the
299 eastern (Anderson, IN) *D. a. americana* stock instead produces a genealogy where *D. a. americana*
300 groups with *D. a. texana*, as expected for the species tree (Figure 3).

301 The observation of phylogenetically discordant sites can be due to several factors, notably
302 incomplete lineage sorting (ILS) or post-speciation introgression. However, evidence from SNP variants
303 across the whole transcriptome and specifically at CG17821 is more consistent with introgression
304 between *D. novamexicana* and our western *D. a. americana* population. Genome-wide, of 34441 total
305 SNPs detected in 11301 loci within the transcriptome dataset (Davis and Moyle 2020), 14902 supported
306 *D. americana* subspecies as sister (in accordance with the species tree), 13372 SNPs grouped *D.*
307 *novamexicana* and *D. a. americana*, and 6167 grouped *D. novamexicana* with *D. a. texana*. These data
308 indicate a significant excess of shared variants between *D. novamexicana* and our western *D. a.*
309 *americana* line ($D = 0.369$, $P < 0.0001$), consistent with a history of introgression between the
310 progenitors of these two populations. Moreover, gene tree topologies at and around CG17821 also
311 indicate this specific genomic region shares recent ancestry between *D. novamexicana* and western *D. a.*
312 *americana* due to introgression. CG17821, the adjacent downstream CHC candidate CG18609, and the
313 next nearest gene (List, a neurotransmitter located ~14 kB downstream) all display shared derived SNPs
314 between *D. novamexicana* and our western *D. a. americana* line, and discordant gene tree topologies
315 that group these two lines as sister. In contrast, topologies for the next two nearest up/downstream
316 genes in this region reflect the species tree, indicating that recent shared ancestry between *D.*
317 *novamexicana* and our western *D. a. americana* extends across a genomic window between 18 and 68

318 kB long around the specific region containing CG17821 (Figure 3C). Estimates of linkage disequilibrium
319 (LD) from *D. melanogaster* autosomes indicate that most LD decays within 200 bp, and r^2 decreases to
320 around 0.1 within a 1 kB window (Franssen et al. 2015); therefore the size of the window observed here
321 is well outside the range expected to show discordant ancestry due to sorting from ancestral variation
322 (i.e. ILS). Coupled with genetic and phenotypic evidence above, these results indicate introgression of
323 these genes between *D. novamexicana* and western *D. a. americana*—and not ILS—is most likely
324 responsible for their shared allele at CG17821 and therefore potentially for their similarity in "short"
325 CHC phenotypes.
326
327

328 **Table 3: Species differences in quantitative gene expression for 23 candidate genes associated with**
329 **CHC function, analyzed separately by sex, with *P*-value and rank compared to all genes in dataset.**

		Males		Females	
Candidate gene	**motif**	***P*-value**	**Rank (%)**	***P*-value**	**Rank (%)**
Cyp4g1	Cyp4g	0.87	10780 (95%)	0.86	9834 (87%)
Cyp4g15	Cyp4g	0.22	3702 (33%)	0.41	4286 (38%)
Cyt-b5-r	desat	0.19	3244 (29%)	0.51	5946 (53%)
ifc	desat	0.89	10862 (96%)	0.52	5906 (52%)
CG17928	desat	0.40	6375 (56%)	0.98	10883 (96%)
CG8630	desat	0.11	2110 (19%)	0.30	3086 (27%)
CG9743	desat	0.15	2660 (24%)	0.25	2443 (22%)
desat2	desat	0.81	10465 (93%)	0.45	5138 (45%)
Baldspot	elongase	0.49	7711 (68%)	0.45	5078 (45%)
bond	elongase	0.38	5988 (53%)	0.76	8901 (79%)
ELOVL	**elongase**	**0.019**	**646 (6%)**	0.15	1535 (14%)
CG5326	elongase	0.087	1763 (16%)	0.87	10008 (89%)
CG17821	**elongase**	0.11	2099 (19%)	**0.0033**	**99 (0.8%)**
CG18609	**elongase**	**0.026**	**781 (7%)**	0.24	2325 (21%)
CG30008	**elongase**	0.21	3460 (31%)	**0.069**	**807 (7%)**
CG31522	elongase	0.52	8052 (71%)	0.70	8052 (73%)
CG31523	**elongase**	**0.040**	**1024 (9%)**	0.95	10710 (95%)
CG33110	elongase	0.65	9332 (83%)	0.67	7863 (70%)
CG6660	**elongase**	0.39	6150 (54%)	0.36	3752 (33%)
FASN1	fatty-acid-synthase	0.32	5197 (46%)	0.62	7194 (64%)
FASN2	fatty-acid-synthase	0.25	4136 (37%)	0.41	4385 (39%)
ebony	pigment	0.22	3686 (33%)	0.38	3948 (35%)
tan	pigment	0.31	5029 (45%)	0.72	8460 (75%)

Note: **Bold** genes indicate gene expression difference in the top 10th percentile

330

331

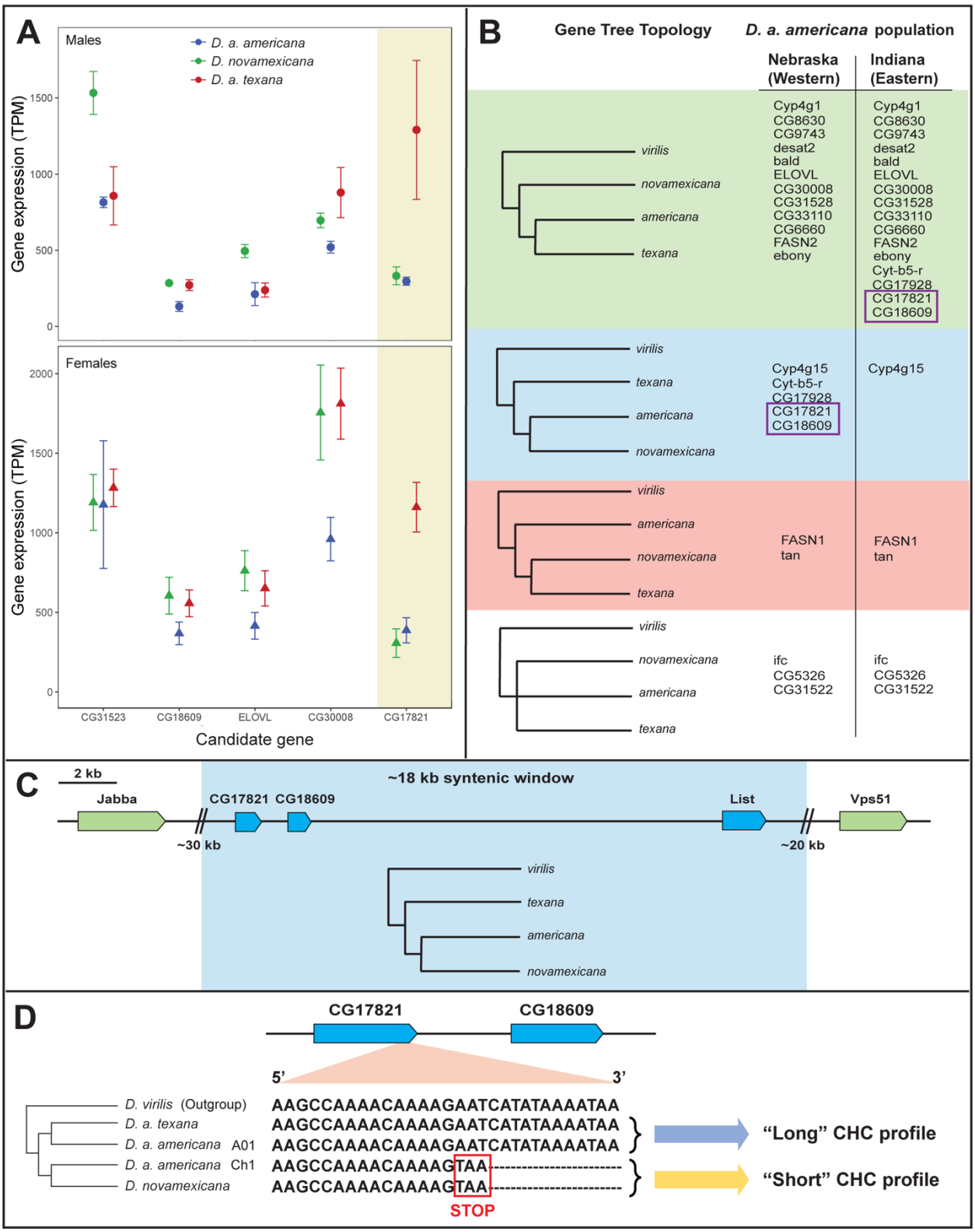


Figure 3: CHC candidate gene expression, genealogical relationships, and sequence variation among *D. a. americana* group lines. A) Gene expression variation showing mean and standard error between focal species lines for candidate genes with the largest differences in either males (upper, circles) or

females (lower, triangles). Gene expression is measured in transcripts per million (TPM, n = 3 for each sample). B) Gene tree topologies for all 23 candidate genes using either western (Nebraska) or eastern (Indiana) populations for *D. a. americana*. C) Schematic of loci (green/blue boxes) in the genomic window surrounding candidates CG17821 and CG18609. Blue boxes indicate a genealogy that groups the western *D. a. americana* population with *D. novamexicana*; gene topology of shaded blue region displayed below line. Green boxes indicate loci with genealogies matching the expected species tree. D) 3' terminal nucleotides and occurrence of inferred ancestral and derived (truncated) allele in CG17821 among lineages with sequence data.

344 **Discussion**

345 Identifying the genetic and evolutionary mechanisms underlying the first steps of reproductive
346 isolation between species is essential for understanding the factors that drive speciation. Evolutionary
347 divergence in sexual signals may be an especially potent contributor to this process (Schemske 2000,
348 Coyne and Orr 2004, Ritchie 2007, Schluter et al. 2009, van Doorn et al. 2009). However, demonstrating
349 the connection between sensory signal divergence and emerging reproductive isolation can be
350 challenging, as it requires identification and demonstration of the direct role of specific signals
351 mediating sexual isolation between species and knowledge of the specific mechanistic changes that
352 have given rise to lineage differences in this signal. Here we have demonstrated that sexual isolation
353 between species in the *D. americana* group is based on female choice of male chemical signals,
354 identified both the specific phenotypic shift between species in pheromone chemistry and a genetic
355 variant likely contributing to this phenotypic change, and uncovered evidence of a complex evolutionary
356 history for this hypothesized causal locus. Together these data support a clear role for sensory signal
357 divergence in the evolution of premating species barriers in this diverse wild group and provide data on
358 the likely genetic and evolutionary mechanisms involved in its evolutionary emergence.

359

360 *Female choice of male CHC variation is responsible for isolation between* D. novamexicana *and* D. a.
361 texana

362 Differences in copulation success between species are the product of variation in male choice
363 (via differences in courtship intensity and copulation attempts, depending upon female identity), female
364 choice (via differences in acceptance rates of males, depending upon their identity), or a combination of
365 these factors. Disentangling these alternatives is critical for identifying the specific trait and
366 corresponding preference variation responsible for the emergence of prezygotic species barriers. Here
367 we showed that female choice of a male sensory signal results in strong sexual isolation within the *D.*

368 *americana* subgroup, specifically *D. a texana* female preference for CHC profiles of their own males and
369 rejection of male with heterospecific profiles. The role of CHCs is exceptionally clear in the case of
370 premating isolation between male *D. novamexicana* and female *D. a. texana*: otherwise complete sexual
371 isolation in this cross can be almost entirely reversed by perfuming *D. novamexicana* males with *D. a.*
372 *texana* male CHCs. In contrast, we find little evidence for differential male preference of females, even
373 though female CHC profiles are also divergent between species. Instead, our behavioral data indicates
374 that males did not alter their courtship behavior in response to female species identity. Interestingly,
375 these observations also suggest evidence for female choice of male courtship behaviors, whereby *D. a.*
376 *texana* courtship consistently induced females to copulate sooner than courtship behaviors of other
377 species males.

378 These findings fit within a body of studies that have identified either male or female choice of
379 sensory signals as critical for sexual isolation among *Drosophila* species (patterns reviewed Yukilevich
380 and Patterson 2019). In *Drosophila melanogaster,* many studies examining CHC divergence have focused
381 on male choice based on female compounds, and found that specific female compounds play a role in
382 isolation between closely related heterospecifics (Coyne et al 1995, Billeter et al. 2009) as well as
383 between intraspecific populations (Wu et al. 1995, Hollocher et al. 1997, Yukilevich and True 2008).
384 Female choice of male CHC variation has received comparably less mechanistic attention, however has
385 been demonstrated as a prezygotic barrier in several diverse *Drosophila* groups, most notably between
386 *D. santomea* and *D. yakuba* (Coyne et al. 2002, Mas and Jallon 2005), between the mycophagous *D.*
387 *subquinaria* and *D. recens* (Curtis et al. 2013, Dyer et al. 2013), and between *D. mojavensis* males reared
388 on different cactus substrates (Havens and Etges 2013). Our results therefore complement a growing
389 body of evidence that shows female choice can be an important factor dictating patterns of sexual
390 isolation among diverse wild *Drosophila* species.
391

392 *CHC divergence and the role of Elongases*

393 Dissecting the finer details of phenotypic divergence in sensory signals can help pinpoint
394 underlying mechanisms and associated genes responsible, and more clearly demonstrate how these
395 signal changes contribute to species isolation. Here we found that CHC divergence between our lines
396 occurs primarily on the basis of compound length, with *D. novamexicana* and *D. a. americana* profiles
397 both having similar enrichment of shorter compounds ("short" phenotype) compared to an enrichment
398 of longer compounds for *D. a. texana* ("long" phenotype). These differences in abundance of shorter-
399 versus longer-chain compounds were observed across both sexes, and across both alkenes and methyl-
400 branched alkanes (Figure 2, Table S3). In contrast, we find no evidence for variation in other features
401 such as double bond or methyl branch location or number. These features themselves suggest that the
402 striking difference between 'shorter' and 'longer' profile phenotypes could be due to variation in fatty-
403 acid elongase activity, which globally influences the carbon chain length of CHC precursors, and
404 therefore can have consistent downstream effects on both alkenes and methyl-branched alkanes,
405 because both are modified after the elongation step (Pardy et al. 2018).

406 Strikingly, analyses of gene expression and/or sequence variation revealed two putative fatty-
407 acid elongases (Szafer-Glusman et al. 2008, Gaudet et al. 2011)—CG17821 and CG18609—with patterns
408 of variation associated with species differences. Both could functionally contribute to our observed
409 variation between longer and shorter CHC profiles, as alleles at both loci are shared between our
410 western *D. a. americana* and *D. novamexicana*, differ from our *D. a. texana* line and the eastern *D. a.*
411 *americana* Anderson stock, and are therefore associated with the "short" versus "long" CHC
412 phenotypes. The variation we observed at CG17821 is particularly interesting, because we observe a
413 thymine insertion mutation that results in premature truncation of CG17821 specifically in *D.*
414 *novamexicana* and our western *D. a. americana* (Figure 3) and because our gene expression data also
415 indicates reduced expression of the CG17821 allele specifically in these lines. In the latter case, while we

416 might expect gene expression differences to be tissue-specific (that is, observed specifically in the
417 oenocytes: the CHC-producing organs), our data indicate that the species-specific expression signal at
418 this locus is strong enough to detect from whole-body transcriptome data. Both observed sequence and
419 expression changes at CG17821 could individually produce a greater abundance of short CHC products,
420 by reducing elongase functionality: gene expression changes could result in a lower abundance of
421 elongase proteins, while the truncation insertion mutation could reduce the efficiency or activity of the
422 resulting elongase enzymes. Therefore, although our current data cannot differentiate which change
423 may have occurred first (or whether they are pleiotropic), either change could produce the pattern of
424 phenotypic difference observed between species. Overall, these data suggest allelic variation in
425 CG17821 could be responsible for the major axis of CHC divergence observed between *D. a. texana* and
426 the other two species, consistent with a hypothesis of a simple underlying basis for chain length
427 variation. Moreover, because this axis of CHC divergence appears primarily responsible for sexual
428 isolation between *D. novamexicana* and *D. a. texana*, this points to a large role for this simple allelic
429 change in CG17821 in the emergence of a strong isolating barrier between these two species.
430
431 *Complex patterns of isolation and multimodality of sexual signals*
432 While our analysis indicates that sexual isolation between *D. novamexicana* males and *D. a*
433 *texana* females could be due to a single trait change of large effect, it also reveals that isolation in other
434 species combinations is likely influenced by more complex factors. This is particularly evident for cross
435 combinations involving our *D. a. americana* and *D. a. texana* lines. Despite having a similar "short" CHC
436 profile to *D. novamexicana*, western *D. a. americana* has more modest and bi-directional isolation from
437 *D. a. texana*, including more frequent acceptance of *D. a. americana* males by *D. a. texana* females
438 (compared to the complete rejection of *D. novamexicana* males). Furthermore, perfuming of *D. a.*
439 *americana* males did not alter mating rate with *D. a. texana* females. It is possible that these more

complex patterns are due to the additional contribution of other sensory signals to sexual interactions between *D. a. texana* and *D. a. americana*, including via the two other main sensory modalities used in insect courtship—visual and auditory signals (reviewed Bontonou and Wicker-Thomas 2014, Jezovit et al. 2017). Nonetheless, it is unlikely that auditory signals make a major contribution here, as song differences between the *D. americana* group species are much smaller than those between other subgroups in the virilis clade, and these differences have been shown not to influence female choice (Hoikkala and Lumme 1987). Similarly, unlike other *Drosophila* groups (e.g., Fuyama 1979, Massey et al. 2020), *D. americana* group males spend relatively little time during courtship on visual cues such as wing displays (Spieth 1951, personal observation), and visual display intensity did not appear to influence mating success in our trials (Table S5). Overall, these data suggest that chemical communication is the primary means of sexual signaling in this subgroup. This would explain why a shift in CHCs has such a large effect on mating in the *D. novamexicana-D. a. texana* pair, but also suggests that more subtle variations in chemical signals might also be responsible for more complex isolation patterns involving *D. a. americana,* especially between *D. a. americana* and *D. a. texana*. This possibility is supported by our findings that, in addition to the primary axis of CHC variation between species, *D. a. americana* also shows male specific divergence on the U-PC2 axis (Figure 2). This axis is loaded heavily for the male-specific compound C21:1—the only major compound with species-specificity that differentiates *D. a. americana* from both *D. novamexicana* and *D. a. texana*—and *D. a. americana* has significantly less C21:1 than the other two species (Results). Lower C21:1 abundance might be critical for the persistence of sexual isolation even after *D. a. americana* males are perfumed by *D. a. texana*, if *D. a. texana* females require high abundance of C21:1 in males as a stimulus for mating, but transfer of C21:1 during perfuming is insufficient. Indeed, CHC quantification indicates that heterospecific-perfumed *D. a. americana* males do not differ from conspecific-perfumed males, suggesting incomplete transfer of compounds may play a role. This preference would not impact mating patterns with *D. novamexicana*

464 males—who have a high abundance of C21:1 regardless of perfuming—but could explain continued
465 isolation from *D. a. americana* males even after perfuming manipulation. Therefore, in addition to *D. a.*
466 *texana* females discriminating against "short" CHC phenotypes, our data also indicate they might use
467 C21:1 as a threshold trait for assessment of male quality or identity.

468 Lastly, the expression of sexual isolation in some of our other cross combinations might also be
469 modulated by additional complex effects on the order and timing of courtship behaviors (Ruedi and
470 Hughes 2008, Gaertner et al. 2015). For instance, we observed that female *D. a. americana* accept fewer
471 *D. novamexicana* males in 1x1 trials (20%) compared to 4x4 (100%), suggesting the density of flies
472 during these mating opportunities might influence behaviors of males or females, with consequences
473 for mating rates. Male-male interactions may subtly alter courtship patterns in *Drosophila* (Baxter et al.
474 2018), for example, so that courtship intensity differences between trials in which single or multiple
475 males were present might explain these more complex *D. a. americana* mating patterns. Regardless, it is
476 clear that the primary signal modality—and major cause of sexual isolation—between *D. a. texana*
477 females and *D. novamexicana* males is female choice on the basis of male pheromone chemistry.
478

479 *The evolutionary history of CHC divergence and consequences for past and future sexual isolation in this*
480 *group*

481 Together, our data demonstrate sexual isolation between *D. a. texana* and *D. novamexicana* is
482 due to CHC divergence in compound length, and suggest that variation between truncated and non-
483 truncated alleles of the elongase CG17821 contribute to this phenotypic variation. Moreover, both
484 genome-wide SNP variation, as well as localized variation specifically around this this putative elongase
485 gene (Figure 3C), indicate that this phenotypic variation involves a history of introgression. These data
486 point to a model for the evolutionary history of transitions involved in the change in CHC profiles

487 between species and, potentially, in the emergence and expression of sexual isolation that depends
488 upon this phenotype.

489 First, the distribution of both “long” versus “short” CHC phenotypes (shown here and in Lamb et
490 al. 2020), and allelic variation in CG17821, indicate that the “short” phenotype and the CG17821
491 truncated allele are derived states that arose within the *D. americana* group. This shift most likely
492 occurred in western lineages that gave rise to contemporary *D. novamexicana*. The evolutionary forces
493 responsible for the persistence and spread of this phenotype are not yet known. The relationship
494 between variation in environmental factors, insect stress physiology, and features of CHC length and
495 branching is known to be complex. Prior work has associated aspects of CHC divergence with abiotic
496 variation such as latitude (Frentiu and Chenoweth 2010, Rajpurohit et al. 2017, but see Gibbs et al.
497 2003), and physiological traits such as desiccation resistance (reviewed Chung and Carroll 2015), that
498 suggest a role for natural selection in shaping CHC composition, but the specific sources(s) of selection
499 can be challenging to pinpoint. In the *D. americana* group, species habitats are differentiated primarily
500 on the basis of water availability, and species differ in key physiological traits such as desiccation
501 resistance (Davis and Moyle 2019). These ecological and physiological differences, combined with
502 evidence of elevated nonsynonymous molecular evolution at CG17821 across the whole *D. virilis*
503 subclade (Table S6), suggest there could be strong selection acting on CHC composition in this group;
504 this selection may have contributed to the emergence and persistence of the novel “short” phenotype in
505 the western distribution of the *D. americana* species group.

506 Based on our data associating phenotypic divergence with sexual isolation, the appearance of
507 this new CHC phenotype would have reduced sexual compatibility between derived “short” males and
508 females with strong preferences for the “long” ancestral CHC profile. Persistence of this phenotype
509 would therefore have required a broadening of female preference to accommodate males with “short”
510 CHC phenotypes, perhaps via selection of rare females that are already responsive to the new

511 pheromone blend (e.g., as observed in male *Ostrinia* moths; Roelofs et al. 2002). Our data support this
512 expectation, as *D. novamexicana* females are more accepting of both (putatively derived) *D.*
513 *novamexicana* and (ancestral) *D. a. texana* male CHC phenotypes, while *D. a. texana* females
514 discriminate against derived "short" CHC phenotypes. Interestingly, this model for the evolution of
515 asymmetric sexual isolation is broadly consistent with Kaneshiro's (1976, 1980) model for peripatric
516 speciation. Kaneshiro observed that females from derived populations frequently have broad
517 preferences for both derived and ancestral male phenotypes; he proposed that this was due to relaxed
518 selection on narrow female preferences in genetically bottlenecked island populations, where founder
519 effects have led to the loss of elements of male courtship. Although Kaneshiro's model explicitly invokes
520 genetic drift in male trait evolution, our observations indicate his explanation of sexual isolation
521 asymmetry could extend more generally to all cases where evolutionary change in male sexual traits
522 precipitates broadening of female preferences for them in derived populations. Regardless of the
523 evolutionary force that might have driven the initial trait change, this process results in the emergence
524 of strong premating asymmetry specifically between females with ancestral trait preferences and males
525 with derived trait values.

526 Intriguingly, our data also support the subsequent movement of this CHC phenotype from *D.*
527 *novamexicana* into western *D. a. americana* lineages. Although shared variation in CG17821 and CHC
528 phenotypes could have been due to segregating ancestral variation present in both *D. a. americana* and
529 *D. novamexicana* (that is, ILS), our data strongly support the alternative hypothesis of more recent
530 introgression of this trait from *D. novamexicana* into western *D. a. americana* populations. Both their
531 significant excess of shared genome-wide variation and a genomic region of at least 18 kB of shared
532 recent ancestry surrounding CG17821, is consistent with introgression between *D. novamexicana* and
533 western *D. a. americana*, that was accompanied by a similar shift to the "short" CHC phenotype in
534 western *D. a. americana*.

535 Given the effect of the "short" CHC phenotype on sexual isolation between *D. novamexicana*
536 and *D. a texana*, this introgression is expected to have consequences for reproductive isolation involving
537 recipient *D. a. americana* lineages. Interestingly, our data indicate that current patterns of sexual
538 isolation differ from those seen in *D. novamexicana*, even though western *D. a. americana* shows the
539 general shift to shorter chain CHC length associated with the introgressed region. One explanation might
540 be that this introgression-mediated shift occurred on a novel *D. a. americana* genomic background that
541 has additional variation in other aspects of CHC composition (such as the abundance of C21:1, see
542 above) and/or different patterns and strengths of female preference. A concrete example of these
543 background effects can be seen for (putatively) introgressed locus CG18609, where our western *D. a.*
544 *americana* shares the *D. novamexicana* allele at this locus but not its patterns of gene expression.
545 Instead, this locus has significantly reduced expression in *D. a. americana* compared to the other two
546 lineages (Figure 3A). These and similar effects of genomic background could be responsible for more
547 complex mating relationships seen in the *D. a. americana* line examined here, that might also extend to
548 patterns more broadly among *D. a. americana* populations depending on their individual histories of
549 divergence and introgression. Recent work (Sramkowski et al. 2020) has described rare *D.*
550 *novamexicana*-like pigmentation alleles in geographically disparate *D. a. americana* populations,
551 similarly suggesting past gene exchange into diverse genomic backgrounds. If alleles for specific sexual
552 signals, behaviors, and/or preferences are being differentially exchanged throughout the range of *D.*
553 *americana*, we could expect to observe variable levels of mating success between disparate populations.
554 Indeed, 4x4 mating trials performed by Spieth (1951) support this expectation, by demonstrating up to
555 10-fold variation in mating success among seven populations of *D. a. americana* from disparate
556 geographic origins. These lines of evidence suggest that trait variation arising from introgression of a
557 locus with major effects on a signaling phenotype—such as CG17821—could initiate the further
558 emergence of reproductive isolating barriers between populations within the broad and diverse *D. a.*

559 *americana* subspecies. This expectation is testable with work examining mating success between
560 geographically diverse *D. americana* populations—particularly between eastern and western
561 populations—and its covariation with CHC phenotypic and genotypic variation.

562 Regardless of the collateral consequences for isolation within *D. a. americana*, our data clearly
563 support the role of divergent cuticular hydrocarbon profiles—specifically a general shift in carbon chain
564 length—in sexual isolation between *D. novamexicana* and *D. a. texana*. They also implicate a causal role
565 for the gene CG17821 in determining CHC phenotype via a global change in CHC elongation activity early
566 in the generation of these compounds. These data provide a strong example of how a recently derived
567 allele in a single gene with large phenotypic effects on a sexual signal could underpin complete
568 asymmetric sexual isolation between closely related allopatric species. Moreover, they suggest that
569 subsequent introgression of this allele into genomic backgrounds of other closely related populations
570 could lead to additional emerging yet complex barriers to reproduction.

571 **Methods**

572 *Experimental Fly stocks*

573 One stock from each of three species was obtained from the University of California San Diego
574 Drosophila Species Stock Center (DSSC): a *Drosophila novamexicana* stock from San Antonio, NM
575 (15010-1031.08); a *D. americana americana* stock from Chadron, NE (15010-0951.06); and, a *D.*
576 *americana texana* stock from Jamestown, SC, (15010-1041.29). All stocks were originally collected
577 between 1946 and 1950. *D. americana* is divided into subspecies according to presence of a
578 chromosomal fusion of the X- and 4-chromosomes in *D. a. americana* that shows a distinct latitudinal
579 cline (McAllister 2002). For simplicity we refer to them by their subspecies names. All fly stocks were
580 reared on standard cornmeal media prepared by the Bloomington Drosophila Stock Center (BDSC) at
581 Indiana University, and were kept at room temperature (~22 °C). Every assay in this study used virgins
582 isolated within 8 hours of eclosion and aged for 7 days prior to the start of experiments.

583

584 *4x4 unperfumed mating assay*

585 We performed trials in which four virgin males and four virgin females were paired and
586 observed for mating behavior, following the design used by Speith (1951) which allows for behavioral
587 interactions that might not otherwise be observed in single-pair no-choice assays. Within each trial, all
588 males are from a single subspecies, as are all females; crosses are varied by pairing males and females of
589 alternative lines. For each trial, 4 males and 4 females were transferred to a single vial without
590 anesthetization and observed for 3 hours. The number and duration of each copulation event was
591 recorded for each trial. This assay was repeated for a total of 5 replicates for each possible species
592 combination, in reciprocal (i.e., 9 cross types, each of N = 5). Each trial used 7-day old virgins and testing
593 was started within 30 minutes of lights on in the morning.

594

595 *Courtship behavior assay*

596 To quantify and evaluate differences in courtship behaviors between cross types, flies were
597 observed in single pair (no-choice) mating assays. We used a modified FlyPi setup—that combines a
598 Raspberry Pi (Raspberry Pi Foundation, Cambridge, UK), pi camera, and 3D printed parts (Chagas et al.
599 2017)—to record courtship behaviors. Assays were performed in a modified cell culture plate consisting
600 of six 3 cm-diameter culture wells, each with a small amount of cornmeal media in the bottom, that
601 allowed six total crosses to be recorded simultaneously. For each assay, individual virgin male and
602 female flies of a given cross were aspirated without anesthetization to a cell culture well; after 6 total
603 crosses were set up, the plate was videotaped for a contiguous 3-hour period. The six cross
604 combinations assessed in any particular video trial were randomized to account for variance that might
605 otherwise be explained by date. As in the 4x4 mating assay, we performed 5 replicates of all possible
606 species combinations in reciprocal.

607 Behavioral features were analyzed and scored manually by the same individual to avoid
608 subjective variation among researchers. Three courtship behaviors—male display events, male tapping
609 events, and male licking events—in addition to copulation were scored in each 1x1 trial using the
610 following criteria. A male "display" was counted when a male performed a combination of back and
611 forth movements and occasional wing flicks while maintaining sustained orientation in front of and
612 facing the female. "Tapping" events were defined when a male used his tarsus to touch the abdomen of
613 the female when oriented behind her. "Licking" events were defined when the male's mouthparts
614 contacted the female genital arch. For male display, tapping, and licking events, individual events were
615 scored separately only if a different behavior (including sitting still/walking away) was observed
616 between instances of the defined behavior. This criterion minimized overcounting of discrete behavioral
617 events, especially those with difficult to view aspects such as number of times a male extruded
618 mouthparts during a contiguous licking event. In addition to these behaviors, copulation success and

619 latency to copulation were also recorded for each trial. Note that females also engage in tapping and
620 other behaviors, but because these appear to be less consistent and are more difficult to observe and
621 score, they were not addressed in this study.
622
623 *Extraction and quantification of cuticular hydrocarbons*
624 Cuticular hydrocarbons were extracted from pooled samples by placing five 7-day old virgin flies
625 of a single sex and species identity in a 1.8 mL glass vial (Wheaton 224740 E-C Clear Glass Sample Vials)
626 with 120 µL of hexane (Sigma Aldrich, St Louis, MO, USA) spiked with 10 µg/mL of hexacosane (Sigma
627 Aldrich). After 20 minutes, 100 µL of the solution was removed to a sterilized 1.8 mL glass vial (Wheaton
628 224740 E-C Clear Glass Sample Vials) and allowed to evaporate overnight under a fume hood. Extracts
629 were stored at -20 °C until analysis. Five replicate samples consisting of 5 flies per sample (25 flies total)
630 were prepared for each sample type, and all replicates were extracted on the same day.
631 Gas chromatography mass spectrometry (GC/MS) analysis was performed on a 7820A GC
632 system equipped with a 5975 Mass Selective Detector (Agilent Technologies, Inc., Santa Clara, CA, USA)
633 and a HP-5ms column ((5%-Phenyl)-methylpolysiloxane, 30 m length, 250 µm ID, 0.25 µm film thickness;
634 Agilent Technologies, Inc.). Electron ionization (EI) energy was set at 70 eV. One microliter of the sample
635 was injected in splitless mode and analyzed with helium flow at 1 mL/ min. Two different temperature
636 gradients were used depending on the sample type. For CHC analysis of unmanipulated males and
637 females of each species, the following parameters were used: column was set at 50 °C for 0 min,
638 increased to 210 °C at a rate of 35 °C/min, then increased to 280 °C at a rate of 3 °C/min. The MS was set
639 to detect from m/z 33 to 500. For analysis of samples from perfuming trials, the parameters were
640 modified to increase resolution and sensitivity for less abundant compounds: the column was set at 40
641 °C and held for 3 min, increased to 200 °C at a rate of 35 °C/min, then increased to 280 °C at a rate of 3
642 °C/min and held for 15 minutes. Chromatograms and spectra were analyzed using MSD ChemStation

643 (Agilent Technologies, Inc.). CHCs were identified on the basis of retention time and electron ionization
644 fragmentation pattern. Compounds are identified in this study as: CXX:Y for alkenes or Me-CXX for
645 methyl-branched alkanes, where XX indicates the length of the carbon chain, and Y indicates number of
646 double bonds, e.g. C21:1 is a 21-carbon alkene with a single double bond.

647 The abundance of each compound was quantified by normalizing the area under each CHC peak
648 to the area of the hexacosane signal using homebuilt peak selection software (personal correspondence,
649 Dr. Scott Pletcher, Univ. of Michigan).

650

651 *Perfuming manipulation and mating assay*

652 'Perfuming' involves co-housing target flies in vials filled predominantly with flies of a desired
653 donor identity, so that the CHC profile of the target flies is altered via physical transfer of CHCs from
654 donor flies (Coyne et al. 1994, Dyer et al. 2014, Serrato-Capuchina et al. 2020). Perfuming was
655 performed by placing 2 'target' males with 15 'donor' males within a single vial. All males were 1-day old
656 virgins when perfuming vials were established, and the wings of donor males were removed (under
657 anesthetic) to distinguish them from target males. For the heterospecific perfuming treatments, target
658 males were co-housed with donor males of a different species; conspecific (control) treatments paired
659 donor and target males of the same species. Following 7 days of perfuming, 4 male flies from the same
660 perfuming conditions were transferred (without anesthetization) to vials containing 4 female *D. a.*
661 *texana*. Within each experiment (Nov-Tex, or Ame-Tex), we ran one trial of each perfuming condition (2
662 heterospecific-perfumed types, and 2 conspecific-perfumed types) in parallel on the same day (four 4x4
663 trials in total).

664 For CHC analysis, males were perfumed as described for the perfumed mating assay, for both
665 Nov-Tex and Ame-Tex experimental pairs, with the exception that 2-3 target males (rather than just 2)

666 were co-housed with 15-18 donor males, enabling 2 parallel perfuming vials to generate 5 target males
667 flies per identity.
668
669 *Candidate gene selection*
670 Our candidate list was generated by searching Flybase (Flybase.org) for annotated *Drosophila*
671 *melanogaster* genes with protein-coding domains (as identified by InterProt) that have known functions
672 in CHC synthesis (Pardy et al. 2018), resulting in an initial list of 34 genes. To this we added the
673 pigmentation genes *ebony* and *tan* as they have been shown to alter CHC variation among *D. americana*
674 group species (Lamb et al. 2020, Massey et al. 2019). With these 36 genes, we identified orthologs in
675 *Drosophila virilis* (as annotated in Flybase) and evaluated whether each of these loci had transcript
676 expression in our three species, using previously published whole-body transcriptome data generated
677 from the same three populations using RNA-seq (Davis and Moyle 2020). Our analyses used only gene
678 expression data from the control (ambient) conditions for both males and females from this study, and
679 excluded any desiccation stress treatment data. Of the 36 initial genes, 30 were found to have
680 unambiguous 1-to-1 orthologs in *D. virilis*, and of these, 23 had transcripts present within the Davis and
681 Moyle (2020) dataset. This final set of 23 genes was used to evaluate gene expression and sequence
682 variation among our three lines. For downstream analyses, we also identified alleles of these 23 loci in a
683 genome assembly of *D. a. americana* Anderson (15010-0951.00, also referred to as A01) generated by
684 Kim et al. (unpubl.), using the BLASTn function of BLAST+ version 2.6.0 (Camacho et al. 2009) and
685 retaining the top BLAST hits for each locus.
686
687 *Statistical Analyses*
688 All statistical analyses and figure construction in this study was performed with R version 3.4.3.
689 For the unperfumed 4x4 mating dataset, we used Kruskal-Wallis tests to compare copulation success

690 within 3 hours of observation with male or female species identity (one test for each sex). In addition,
691 we used Wilcoxon rank sum tests to perform post-hoc pairwise comparisons for each sex to evaluate
692 which species most differed from one another in female acceptance of males or in male courtship
693 success of females.

694 In 1x1 mating trials, mating rate was evaluated using a logistic regression with a chi-square test.
695 For copulation latency, the effects of male identity, female identity, and their interaction were evaluated
696 with an ANOVA, followed by a post-hoc Tukey HSD in which we assessed which male identities (species)
697 were specifically different for this trait. For each of the three courtship behaviors (displays, tappings and
698 lickings), we converted the count data for each trial to a rate per unit time within a trial, by dividing the
699 recorded counts for each trait by the latency to copulation (in minutes) or, if no copulation occurred, the
700 maximum amount of observed time (180 minutes). Describing male behaviors in terms of rates accounts
701 for differences among males in their courtship efficiency; for example, it allows us to differentiate males
702 that performed fewer courtship behaviors because they were rapidly accepted by a female, from males
703 that displayed lower courtship intensity across the total 3 hour monitored period. Differences in male
704 courtship behaviors were evaluated with ANOVA. Correlation of individual courtship behaviors with
705 copulation success was analyzed using a logistic regression (with z-test).

706 The perfumed 4x4 mating experiment was analyzed using planned contrasts that compared the
707 mating success of males that had the same target identity but different (con- versus hetero-specific)
708 donor perfumes, when paired with *D. a. texana* females. This enabled us to specifically assess the effects
709 of donor perfume variation on mating success of a given target male species. Each pairwise test was
710 performed using a non-parametric Mann-Whitney U test.

711 Our primary analyses of differences in CHC composition were done after summarizing major
712 axes of variation in CHC phenotypes using a set of Principal Component Analyses (PCA). Separate PCAs
713 were performed on each CHC experiment within this study: one for the initial (unperfumed) parental

714 populations (denoted U-PCA) that included both male and female samples, and one each for the
715 perfumed experimental pairs—Nov-Tex (N-PCA) and Ame-Tex (A-PCA)—with male samples only. Within
716 each dataset, a one-way ANOVA was used on each of the first 3 PCs to examine effects of sex and
717 species (for the unperfumed dataset) or male perfume identity (in each perfuming study) on CHC
718 composition. Additionally, for the perfumed datasets, T-tests were used to compare differences in PC
719 values between target males that were paired with different (con- and hetero-specific) donor males to
720 assess the effects of our perfuming manipulation on major axes of CHC variation. Factor loadings for N-
721 PC an A-PC analyses, as well as individual compound differences in perfuming pairs can be found in in
722 the supplement (Tables S7, S8, S9 and Figures S1, S2).

723 For all gene expression analyses, expression is quantified in transcripts per million (TPM), and
724 therefore is normalized within each sample. Here we analyzed datasets separated by sex, as Davis and
725 Moyle (2020) showed that the majority of genes have differential expression based on sex, whereas our
726 primary interest here is in differences between species that might be implicated in female mating
727 choices and male CHC profile variation. With the dataset for each sex, we ran one-way ANOVAs on every
728 expressed locus (11301 genes total)—including our 23 target candidate genes—in order to determine if
729 gene expression varied by species and to rank order each gene according to their resulting *P*-value. This
730 allowed us to interpret patterns of species variation specifically among candidate genes in the context of
731 species differences across all genes in the transcriptome.

732 Using the same dataset for our 23 candidate genes, we also evaluated patterns of molecular
733 evolution by calculating clade-wide d_N/d_S values for each gene. For each candidate gene, sequences
734 from each species were aligned to the *D. virilis* reference genome (Flybase version 1.7, www.flybase.org)
735 and aligned sequences analyzed in PAML v4.9 (Yang 2007) using the base model in CodeML. CodeML is a
736 maximum likelihood model for codon substitution that estimates the rates of nonsynonymous to
737 synonymous substitutions (d_N/d_S) to test for positive selection on a given gene between species. Gene

trees for each candidate gene were constructed in ClustalW (Thompson et al. 1994), as PAML uses a tree-based model for computing d_N/d_S that is sensitive to use of the correct gene tree for a given gene. Results of these analyses are reported in the supplement (Table S6).

We constructed additional gene trees for each candidate gene using orthologs from the Anderson IN *D. a. americana* genome (from Kim et al. unpublished) in place of our (western) *D. a. americana* sequences, using alignments of this sample with the other species generated from ClustalW (Thompson et al. 1994). Additional gene trees were generated in the same manner for neighboring genes in the region around CG17821/CG18609, to estimate size of a window of shared ancestry.

To assess evidence for genome-wide introgression, we used our transcriptome data in conjunction with the *D. virilis* genome to generate a genome-wide set of SNPs for our three focal populations and the *D. virilis* outgroup. To do so, we used bcftools (Li 2011) to generate a VCF (variant calling format) file and call SNP sites between the 4 taxa after filtering for low depth and masking heterozygous sites from individual taxa. With this, we calculated Patterson's *D*-statistic (Durand et al. 2011) using the Dtrios program from Dsuite (Malinsky et al. 2020) to calculate the 3 taxa *D*-statistic as well as an overall *P*-value using standard errors generated from the default 20 jackknife blocks.

Acknowledgements

We would like to thank Matthew Gibson for help with implementing molecular evolution analyses, Zinan Wang for advice about candidate gene selection, Bernard Kim for sequencing the *Drosophila americana* genome and providing assemblies, Scott Pletcher for sharing hydrocarbon analysis software, and Jonathan Massey for connecting JSD and LCM with JYY.

Funding

761 This work was supported by the IU Department of Biology (LCM, JSD), the Department of Defense
762 United States Army Research Office (Grant No. W911NF1610216) and the National Institutes of Health
763 (Grant No. 1P20GM125508) awarded to JYY. The GC/ MS analysis was performed in the UHM Microbial
764 Genetics and Analytical Laboratory (supported by NIH Grant No. 1P20GM125508).

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