# Full title: Cuticular hydrocarbon divergence in *Drosophila melanogaster* populations evolving under differential operational sex ratios

Short title: Evolution of cuticular hydrocarbons under sexual conflict

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## Abstract

The ability of interlocus sexual conflict to facilitate reproductive isolation is widely anticipated. However, very few experimental evolutionary studies have convincingly demonstrated the evolution of reproductive isolation due to sexual conflict. Recently a study on replicate populations of *Drosophila melanogaster* under differential sexual conflict found that divergent mate preference evolved among replicate populations under high sexual conflict regime. The precopulatory isolating mechanism underlying such divergent mate preference could be sexual signals such as cuticular hydrocarbons since they evolve rapidly and are involved in *D. melanogaster* mate recognition. Using *D. melanogaster* replicates used in the previous study, we investigate whether cuticular hydrocarbon divergence bears signatures of sexually antagonistic coevolution that led to reproductive isolation among replicates of high sexual conflict regime. We found that *D. melanogaster* cuticular hydrocarbon profiles are sexually dimorphic. Although replicate populations under high sexual conflict displayed assortative mating, we found no significant differences in the cuticular hydrocarbon profile between the high and low sexual conflict regimes. Instead we find cuticular hydrocarbon divergence patterns to be suggestive of the Buridan's Ass regime which is one of the six possible mechanisms to resolve sexual conflict. Sexual selection that co-vary between populations under high and low sexual conflict regimes may also have contributed to the evolution of cuticular hydrocarbons. This study indicates that population differentiation as a result of cuticular hydrocarbon divergence cannot be credited to sexual conflict despite high sexual conflict regime evolving divergent cuticular hydrocarbon profiles.

## Introduction

Mathematical models of interlocus sexual conflict based on mating rate envisage six possible evolutionary outcomes, out of which two lead to speciation: allopatric speciation as a by-product of sexually antagonistic coevolution (SAC) and sympatric speciation as a result of males responding to female strategy under Buridan's Ass regime (1). SAC occurs when males' mate harming traits and female traits countering such mate harm continually evolve until a stabilising selection restricts further evolution of traits. Buridan's Ass regime refers to genetic differentiation of females in response to harm caused by males such that males lose out on fitness due to genetic mismatch. Males may, however, gain advantage by mirroring female genetic diversification that may lead to isolation of divergent genetic groups within a population (2). A comparative study on groups of insect species has shown that the prevalence of high sexual conflict makes a taxonomic group more speciose than those with low or no sexual conflict (3). However, the support for speciation through sexual conflict is not universal and comparative studies on other groups of species have not found a similar trend (4, 5). Experimental studies that directly investigate speciation linked to sexual conflict too are inconsistent in their support for the hypothesis presumably due to the occurrence of four other possible dynamic outcomes predicted in the mathematical models that do not result in speciation (6).

By evolving independent replicates of a dung fly population for 35 generations under monogamous (low) and polygamous (high) sexual conflict regime, Martin and Hosken (7) showed that mating success between individuals of the same population was significantly higher than that of pairs consisting of males and females from different populations under polygamous regime. In the monogamous regime, there was no

difference in mating success between individuals from hetero-population and same population. Recently, a study on *Drosophila melanogaster* population using male biased (high sexual conflict) and female biased (low sexual conflict) operational sex ratio regimes showed that evolution of both premating and post mating prezygotic isolation was possible within 105 generations in the high sexual conflict regime but not in the low sexual conflict regime (8). Contrary to these two studies, few other studies were unable to find any support for sexual conflict driven reproductive isolation leading us to question the potential of sexual conflict as an "engine of speciation" (4, 5, 9-11).

Manipulating sex ratio in *D. melanogaster* population in favour of either sex leads to differential sexual conflict. Males of male biased regime evolved higher mate harming ability indicated by higher mortality of ancestral females that they mated with while males from female biased regime were found to be relatively benign to ancestral females (12). Concurrently, females from male biased regime show increased fecundity and longevity when continuously exposed to ancestral males (13). These results indicate that the males from male biased regimes evolved more mate harming abilities and females from the same regimes have evolved resistance to mate harm compared to male and females from female biased population. It is important to note that both male biased and female biased regimes had three independent replicate populations each and as such, these replicate populations could be construed as allopatric populations, each potentially following its own evolutionary trajectory. The allopatric male biased replicates have now shown within replicate assortative mating that indicates reproductive isolation due to SAC (8).

86 Premating isolation by mate choice in *Drosophila* sp. is often mediated by cuticular
87 hydrocarbon (henceforth CHC) profiles that provide information about species
88 identity, sex, mating status, age, etc. to a receiver (14-16). Specifically, CHCs such
89 as 7-tricosene, 5-tricosene, 9-pentacosene, 7-pentacosene, 7,11-heptacosadiene and
90 7,11-nonacosadiene play a pivotal role in sexual communication during various
91 heterosexual courtship rituals in *Drosophila melanogaster* (17). Since reproductive
92 isolation in male biased *D. melanogaster* replicates is most likely mediated by mate
93 choice rather than any conspicuous female reluctance to mating (8), it is possible that
94 the CHC profiles form the mechanistic basis for mate preference within male biased
95 replicates. The possibility of mate choice forming the basis for premating reproductive
96 isolation under SAC has cropped up recently (8). Until now, it has been suggested that
97 traits undergoing SAC evolve directly due to natural selection on female resistance to
98 mate harm (18). However, this novel idea assumes that the CHC profiles have also
99 differentiated among male biased replicates as a by-product of SAC within each
100 allopatric replicate.
101
102 CHC profiles of *Drosophila melanogaster* are dimorphic (17) and have the ability to
103 evolve rapidly (19). Those CHCs that take part in mate preference are known to have
104 independent genetic controls in the two sexes, often linked to sex determination genes,
105 for their biosynthesis (14, 19-21). Many previous studies have implicated
106 differentiated CHC profiles in the evolution of reproductive isolation in *Drosophila*
107 sp. through sexual selection (22-25). It is, therefore, impossible to ignore that the
108 different operational sex ratio may also lead to differential sexual selection in male
109 biased and female biased *D. melanogaster* regime. Male biased population may
110 experience high sexual selection as males face higher mate competition and choosy

111 females. Female biased population may experience lowered sexual selection pressures
112 as males experience less competition for mates and females cannot be as choosy (or
113 may even have to compete for mates). In case, SAC does not affect CHC profiles of
114 male biased replicates (mate attraction being multimodal in *Drosophila* sp. (26)), it is
115 equally possible for us to see the evolutionary outcome of sexual selection in CHC
116 profiles of both male biased and female biased regime. It is important to note that
117 sexual selection and sexual conflict are not mutually exclusive mechanisms through
118 which cuticular hydrocarbon profiles of populations under altered sex ratio could
119 evolve (27). In this study, we investigate the possibility of CHC divergence under
120 selection pressure imposed by different operational sex ratio that may lead to
121 differential sexual conflict or differential sexual selection in *D. melanogaster*.
122
123 Our primary objective is to examine the patterns of CHC divergence among the
124 replicates of *D. melanogaster* male biased and female biased regimes, the same
125 population previously used by Syed et al (8). Assuming that CHC profiles (that play
126 a role in mate attraction in *D. melanogaster*) have evolved independently in male
127 biased replicates (isolated by mate choice as indicated by Syed et al (8)) due to SAC,
128 we expect that the CHC profiles of male biased replicates will diverge more than the
129 CHC profiles of the female biased replicates. However, if CHC profiles of male biased
130 regime is not affected by SAC, we may see convergence of CHC profiles of males
131 compared to females due to sexual selection. In male biased regime, males will have
132 to compete for females and can potentially evolve a common CHC profile most
133 preferred by females. The CHC profiles among replicates of female biased regime are
134 not expected to be affected by SAC, unlike male biased regime. We also expect that
135 all *D. melanogaster* populations will show sexual dimorphism in CHC profiles. Our

136 results suggest that reproductive isolation among male biased replicates due to high
137 sexual conflict is not explained by the patterns of CHC divergence. Instead we find
138 sexual selection may predominantly be shaping CHC divergence in *D. melanogaster*
139 populations maintained under different operational sex ratio.

140 # Methods

141 ## *D. melanogaster* population maintenance

142 In this study, we used 168-170 generations old *Drosophila melanogaster* MCF
143 population (henceforth MCF) whose adult flies are maintained at three operational
144 sex-ratio. A 3:1::male:female sex ratio (24 males + 8 females per vial) led to male
145 biased regime (henceforth M). Similarly, a 1:3::male:female sex ratio (8 males + 24
146 females per vial) gave rise to female biased regime (henceforth F). The control regime
147 (henceforth C) had 1:1 sex ratio (16 males + 16 females per vial). Each regime has
148 three replicate populations ($M_{1,2,3}$ in the M regime, $F_{1,2,3}$ in the F regime and $C_{1,2,3}$ in
149 the C regime). All nine populations trace their ancestry to a large, laboratory adapted
150 *D. melanogaster* LHst population (12).

151

152 Nandy et al (12, 28) derived three populations, labelled $C_{1,2,3}$, from LHst (a recessive
153 but benign scarlet eyed *D. melanogaster* population named after Lawrence Harshman).
154 The $C_{1,2,3}$ populations were maintained at a 1:1 adult sex ratio for five generations.
155 Subsequently, from $C_{1,2,3}$ populations, we derived $M_{1,2,3}$ and $F_{1,2,3}$ regime with the
156 subscripted numbers indicating common ancestry. For example, $M_1$, $F_1$ and $C_1$ were
157 derived from $C_1$. Moreover, during population maintenance, MCF populations with
158 the same subscript are handled on the same day and, therefore, can be treated as
159 statistical blocks.

160

161 These populations are maintained on a 14-day discrete generation cycle, at 25˚C and
162 60% relative humidity, 12:12 hours light/dark cycle and standard cornmeal–
163 molasses–yeast food in standard vials (90-mm length × 30-mm diameter). We collect
164 virgin males and females every generation and combine them in appropriate sex ratios

165 for each regime. Males and females interact for around 48 hours, after which they are
166 transferred to fresh vials for oviposition. Eggs laid are trimmed to a density of around
167 140-160 eggs per vial containing 6-8 ml food. These eggs are used to start the next
168 generation (refer (12) for detailed maintenance protocol).

## 169 Generating flies for cuticular hydrocarbon extraction

170 Before CHC extraction from virgin MCF flies, we allowed one generation of
171 "standardization" or common-garden rearing at a 1:1 sex ratio (12) to account for any
172 potential non-genetic parental effect (29). In order to generate flies for CHC
173 extraction, we collected eggs from "standardised" flies. On the 10th day after egg
174 collection from the MCF population, we collected newly eclosed flies as virgins within
175 6 hours of eclosion under light carbon dioxide anesthesia. The flies were isolated by
176 keeping each of them singly in a vial for 2 days. On 12th day (ensuring flies of same
177 age to minimize age-specific variation of CHCs) the CHC extraction assay was
178 conducted.

## 179 Cuticular hydrocarbon extraction

180 We transferred each virgin individual to a 1.5 ml gas chromatography (GC) vial
181 without anesthetising it. We then poured 200 µl HPLC grade 95% *n*-Hexane solvent
182 (AlfaAesar, 39199) containing 10ng/µl (10µl Pentadecane in 1ml *n*-Hexane) 99% pure
183 Pentadecane ($C_{15}H_{32}$, AlfaAesar (A10336), internal standard) into the GC vials. The
184 vials were left undisturbed for 4 minutes and then vortexed for 1 minute (30). We
185 removed the dead individual from each GC vial and subsequently left the *n*-Hexane to
186 dry out. The extracted samples in the GC vials were then stored at 4˚C until gas
187 chromatography was performed. We extracted 119 M ($M_1$ = 20 males + 20 females,
188 $M_2$ = 20 males + 20 females and $M_3$ = 20 males + 19 females) flies, 115 F ($F_1$ = 19
189 males + 18 females, $F_2$ = 20 males + 19 females and $F_3$ = 19 males + 20 females) flies

190 and 118 C ($C_1$ = 20 males + 20 females, $C_2$ = 19 males + 20 females and $C_3$ = 20 males
191 + 19 females) flies in total. The extraction of 352 samples was conducted at two
192 different time periods each having approximately equal sample size. Each extraction
193 time period consisted of three days, a day each for three replicates of MCF population.
194 To be precise, replicates 1, 2 and 3 of MCF was extracted on the days 1, 2 and 3
195 respectively on two different extraction events. We used this information later in
196 linear mixed effects analysis (see below) under the variable termed "time of
197 extraction" that have six levels.

198 ## Gas chromatography

199 Prior to GC-FID (gas chromatography fitted with flame ionization detector) analysis,
200 the dried extract adhering to the walls of glass GC vials was re-dissolved in 20 µl *n*-
201 Hexane and vortexed for 30 sec. We injected 2µl of this solution manually into
202 splitless Shimadzu GC-2010 Plus instrument fitted with a Restek Rxi-5Sil MS (30m x
203 0.25mm x 0.25µm) capillary column. The 24.88 minutes' temperature program began
204 by holding at 57˚C for 1.1 minutes, ramping at 100˚C/min to 190˚C with a hold time
205 of 1.2 minutes. Further, with a ramp rate of 5˚C/min, the temperature was increased
206 to 270˚ and finally raised to 300˚C at 120˚C/min with a hold time of 5 minutes at
207 300˚C (30). The carrier gas was Nitrogen with column flow rate fixed at 2ml per
208 minute.

209 ## Cuticular hydrocarbon identification

210 CHCs were identified with the help of GC-MS (gas chromatography mass
211 spectrometer) using Agilent Technologies 7890B GC system fitted with Agilent J&W
212 GC column (HP-5MS, 30m x 0.25mm x 0.25µm) and mass spectrometer (model no.
213 5977B GCMSD). The resultant chromatograms were analysed using Agilent
214 MassHunter Qualitative Analysis Navigator B.08.00 by comparing mass spectra of

215 each peak and SUPELCO *n*-alkane mixture standard (C8-C40) with characteristic
216 retention indices. Further, the distinctive diagnostic ions were complemented with
217 PUBCHEM compound summaries and probabilistic match of the mass spectras to the
218 NIST Mass Spectral search program (version 2.3).

## 219 Cuticular hydrocarbon quantification

220 All peaks that consistently appeared in the samples when compared with a blank
221 chromatographic run (using only *n*-Hexane solvent) were considered relevant to this
222 study. The area under the peak value was calculated by drawing a baseline to each
223 relevant peak using the Shimadzu GCSolutions software. We manually checked the
224 integrated peaks in all the sample chromatograms for any irregularities. The peak areas
225 of the 352 samples was standardized by dividing area under each peak with that of
226 internal standard (*n*-Pentadecane) from respective chromatograms. Each standardized
227 value was then multiplied by 10000 ng/ml (concentration of *n*-Pentadecane) to derive
228 relative concentration of each cuticular hydrocarbon in all samples for use in
229 subsequent analysis.

## 230 Linear mixed effects analysis

231 Linear mixed effects analysis involved two models that examined how different
232 variables recorded during sampling from the MCF population affect abundance of each
233 CHC peak in all samples. Selection regime (M, C and F), sex (male and female) and
234 their interaction were considered explanatory variables with fixed effects while
235 replicates within each selection regime (blocks 1, 2, and 3) and time of extraction
236 were assigned as variables with random effects (30). We used "lme4" package in R
237 (31) and modelled the fixed and random effects on the response variables according
238 to the following formula:
239

240 Model 1 = Selection regime + Sex + (Selection regime x Sex) + (1|Block) + (1|Time)

241 Model 2 = Selection regime + Sex + (1|Block) + (1|Time)

242

243 For the sake of simplicity, we present only Model 1 that includes all biologically
244 relevant explanatory variables and their interaction. The simpler Model 2 (detailed
245 analysis presented in S1 Supplementary Information) either had a higher Akike
246 Information Criterion (AIC) or did not significantly differ from the Model 1 in the
247 analysis of deviance, especially, where there was no significant effect of selection and
248 sex interaction term on CHC variance. The distribution of each peak that are affected
249 by the selection was further examined in details and their means were compared across
250 sex and populations using Wilcoxon test to observe trends of differences in peak
251 abundance across populations.

## 252 Random Forest analysis

253 Closely related populations such as MCF usually show divergence in combinations of
254 many CHCs rather than in a single peak. Therefore, we used Random Forest based
255 multivariate analysis to study the divergence of CHCs. Multivariate analysis like
256 principal component analysis and discriminant function analysis are generally used in
257 studies that investigates group patterns within samples based on cuticular profiles
258 (32). However, these statistical techniques are adversely impacted by small sample-
259 size(n):variables(p), non-independence of CHCs in a sample (since many CHCs share
260 common biochemical pathways (21)) and disproportionate effects of CHCs found in
261 trace amounts (33). To overcome this drawbacks of traditional techniques, we used
262 Random Forest analysis (R package "Party" (34)) that performs well under "small n
263 large p" conditions, unaffected by highly correlated variables and is unbiased towards
264 the nature of variables used (35, 36).

265

266 By comparing identities of the samples derived from the Random Forest analysis with
267 their already known group identity, we calculate the percentage correct prediction of
268 individual identities. Based on the probabilities of individual identities, proximity
269 analysis (identical to similarity-dissimilarity matrix/ correlation matrix in traditional
270 multivariate analysis) gives us a measure of samples' group affinity. Multidimensional
271 scaling (identical to Principal Component Analysis) of the proximity matrix is then
272 illustrated in a scatterplot to show how experimental individuals group with respect
273 to each other (37).

274

275 We performed Random Forest analysis using relative amount of all relevant CHC
276 peaks. Seed (a random number) was set to 1220. We checked the stability of the
277 classification scheme by increasing the number of subsampling events gradually from
278 5000 to 20000 and decided to set the number of trees (ntree) parameter in the analysis
279 at 10000. The number of variables (mtry = 6) to be used in the analysis was fixed to
280 rounded square root of the number of predictor variables (in the present study, the
281 number of peaks analysed in chromatograms) in the data (Strobl et al. 2009b). Multi-
282 dimensional scaling (MDS) of proximity matrix was used to produce two dimensional
283 scatterplots showing clustering of individuals.

284

285 # Results

286 ## Characterisation of MCF cuticular hydrocarbons

287 We found a total of 38 CHCs peaks that reliably and consistently appeared in MCF
288 chromatograms (see Figure 1 and Table 1). Most of the CHCs that were identified
289 using GCMS had hydrocarbons chain lengths between $C_{20}$ and $C_{30}$. Peak 17 turned out
290 to be a combined peak of the hydrocarbon *n*-Tetracosane, and a non-hydrocarbon
291 Adipate and was left out of further analyses. Only two peaks (Peak 3 and Peak 6)
292 could not be precisely identified into known classes of CHCs. The appearance of
293 different CHCs in *D. melanogaster* MCF flies appeared to follow a fixed pattern (or
294 CHC fingerprint): methyl substituted straight chain *n*-alkanes appears first followed
295 by differently positioned straight chain *n*-alkenes and finally, corresponding straight
296 chain *n*-alkanes (Peak 10-14, Peak 18-23, Peak 26-30 and Peak 32-35).

297

298 Fig. 1 Representative chromatogram showing 38 cuticular hydrocarbon peaks of
299 *Drosophila melanogaster* MCF population.

300

301 The abundance of CHC peaks was measured using area under the peak relative to that
302 of internal standard, *n*-Pentadecane that had a known concentration of 10 ng/ul in *n*-
303 Hexane. Male and female chromatographic profiles significantly differed in
304 abundance of most peaks. Peak 19 (6, 10/4, 10-pentacosadiene) apart from being the
305 only alkadiene in male was totally absent in females indicating a likely sex specific
306 role (alkadienes usually have pheromonal functions in female *D. melanogaster* (38))
307 in MCF population. Peak 29 (5-heptacosene) was generally absent from male
308 chromatograms (% zero presence = 88.9) but were found in most females (% zero
309 presence = 14.7). Peak 33 (7-nonacosene) that appeared consistently in female

310 chromatograms were found to be missing in a few males (% zero presence = 7.6). Only
311 eight CHCs occurred in similar quantities in both males and female.
312
313 *D. melanogaster* MCF males predominantly produce monoenes (e.g. Tricosene,
314 Pentacosene, Heptacosene, Nonacosene) while the females predominantly produce
315 dienes (e.g. Tricosadiene, Pentacosadiene, Heptacosadiene) (17). Most major and
316 minor CHCs that are known to act as contact pheromones in *Drosophila melanogaster*
317 during reproduction such as 7-tricosene (major), 5-tricosene (minor), 9-pentacosene
318 (minor), 7-pentacosene (minor) and 5,9/7,11-heptacosadiene (major) were found in
319 MCF population (17). However, a major female contact pheromone, 7,11-
320 nonacosadiene was absent from the MCF chromatograms. It is interesting to note that
321 7-monoalkenes (Peaks 12, 21 and 28) occur in males only but corresponding peaks in
322 females are a mixture of 5,9- and 7,11- alkadienes. The 5,9- alkadiene isomer is
323 predominant over 7,11- alkadiene isomer in MCF females. 5,9-alkadiene was found to
324 be present in 17 *D. melanogaster* Genetic Reference Panel recently due to presence of
325 *Desat2* allele that was initially thought to occur only in African and Caribbean *D.*
326 *melanogaster* (21).

| **Peak** | **Rt** | **Chemical ID** | **Diagnostic Ion** | **Relative Concentration (in ng/ml)** | | **Proportional Amount (in %)** | | **% Zero-Presence** | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Male (m ± sd) | Female (m ± sd) | Male | Female | Male | Female |
| 1 | 5.262 | 4-methylheptadecane | 71, 253 | 1.013 ± 0.880 | 0.974 ± 0.888 | 0.80 | 0.73 | 0 | 0 |
| 2 | 5.612 | *n*-octadecane | 57, 254 | 0.090 ± 0.050 | 0.085 ± 0.047 | 0.07 | 0.06 | 0 | 0 |
| 3 | 7.219 | unidentified hydrocarbon | 57, 71, 85 | 0.322 ± 0.159 | 0.260 ± 0.158 | 0.26 | 0.20 | 0 | 0 |
| 4 | 8.747 | *n*-henicosane | 57, 296 | 1.832 ± 0.660 | 0.324 ± 0.091 | 1.45 | 0.24 | 0 | 0 |
| 5 | 9.181 | 4-methyleicosane | 71, 296 | 0.426 ± 0.257 | 0.433 ± 0.301 | 0.34 | 0.33 | 0 | 0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 6 | 9.621 | unidentified hydrocarbon | 57, 71, 85 | 0.816 ± 0.649 | 0.799 ± 0.664 | 0.65 | 0.60 | 0 | 0 |
| 7 | 9.748 | 3,7-dimethylnonadecane | 71, 127 | 0.564 ± 0.199 | 0.156 ± 0.145 | 0.45 | 0.12 | 0 | 0 |
| 8 | 9.858 | x-docosene | 55, 308 | 7.786 ± 5.472 | 0.058 ± 0.063 | 6.17 | 0.04 | 0.85 | 0 |
| 9 | 9.972 | *n*-docosane | 57, 310 | 1.826 ± 0.486 | 0.648 ± 0.309 | 1.45 | 0.49 | 0 | 0 |
| 10 | 10.803 | 2-methyldocosane | 281, 309, 324 | 0.529 ± 0.253 | 0.483 ± 0.181 | 0.42 | 0.36 | 0 | 0 |
| 11 | 10.957 | 9-tricosene | 125, 322 | 5.259 ± 2.006 | 0.410 ± 0.190 | 4.17 | 0.31 | 0 | 0 |
| 12 | 11.064 | 7-tricosene (in male) \| 5,9/7,11-tricosadiene (in female) | 97, 322 \| 69, 125, 320 | 46.489 ± 13.095 | 5.639 ± 2.668 | 36.83 | 4.24 | 0 | 0 |
| 13 | 11.174 | 5-tricosene | 69, 322 | 2.149 ± 0.599 | 0.384 ± 0.182 | 1.70 | 0.29 | 0 | 0.86 |
| 14 | 11.306 | *n*-tricosane | 57, 324 | 11.486 ± 2.858 | 5.727 ± 1.562 | 9.10 | 4.30 | 0 | 0 |
| 15 | 12.571 | 7-tetracosene | 97, 336 | 3.014 ± 0.929 | 1.467 ± 0.963 | 2.39 | 1.10 | 0 | 0 |
| 16 | 12.674 | x,x-dimethyldocosane | 71, 99, 113, 127, 338 | 2.299 ± 0.686 | 1.580 ± 0.654 | 1.82 | 1.19 | 0 | 0 |
| 17 | 12.747 | Adipate (non-hydrocarbon) + *n*-tetracosane | 57, "A-129", 338 | NA | NA | NA | NA | 0 | 0 |
| 18 | 13.669 | 2-methyltricosane | 295, 323, 338 | 3.660 ± 1.529 | 1.783 ± 0.657 | 2.90 | 1.34 | 0 | 0 |
| 19 | 13.704 | 6,10/4,10-pentacosadiene | 83, 141, 338 | 1.732 ± 0.852 | 0 | 1.37 | 0.00 | 0 | 100 |
| 20 | 13.814 | 9-pentacosene | 125, 350 | 3.661 ± 1.425 | 8.333 ± 2.575 | 2.90 | 6.26 | 0 | 0 |
| 21 | 13.902 | 7-pentacosene (in male) 5,9/7,11-pentacosadiene (in female) | 97, 350 \| 69, 125, 348 | 7.472 ± 4.469 | 19.826 ± 10.715 | 5.92 | 14.90 | 0 | 0 |
| 22 | 14.061 | 5-pentacosene | 69, 350 | 0.135 ± 0.098 | 0.789 ± 0.369 | 0.11 | 0.59 | 0 | 0.86 |
| 23 | 14.149 | *n*-pentacosane | 57, 352 | 2.390 ± 0.750 | 8.404 ± 2.201 | 1.89 | 6.32 | 0 | 0 |
| 24 | 14.534 | 6,x-hexacosadiene | 85, 362 | 0.223 ± 0.098 | 0.230 ± 0.143 | 0.18 | 0.17 | 0 | 0 |
| 25 | 15.549 | *n*-hexacosane | 57, 366 | 0.328 ± 0.208 | 0.821 ± 0.338 | 0.26 | 0.62 | 0 | 0 |
| 26 | 16.387 | 2-methylhexacosane | 337, 365, 380 | 6.427 ± 1.761 | 15.113 ± 11.917 | 5.09 | 11.36 | 0 | 0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 27 | 16.626 | 9-heptacosene | 125, 378 | 0.173 ± 0.096 | 7.482 ± 2.812 | 0.14 | 5.62 | 0 | 0 |
| 28 | 16.792 | 7-heptacosene (in male) 5,9/7,11-heptacosadiene (in female) | 97, 378 \| 69, 125, 376 | 0.402 ± 0.194 | 25.603 ± 12.821 | 0.32 | 19.25 | 0 | 0 |
| 29 | 16.844 | 5-heptacosene | 69, 378 | 0.004 ± 0.013 | 0.774 ± 0.487 | 0.00 | 0.58 | 88.9 | 14.7 |
| 30 | 16.936 | *n*-heptacosane | 57, 380 | 1.082 ± 0.384 | 5.979 ± 1.794 | 0.86 | 4.49 | 0 | 0 |
| 31 | 18.278 | *n*-octacosane | 57, 394 | 0.351 ± 0.417 | 0.416 ± 0.424 | 0.28 | 0.31 | 0 | 0 |
| 32 | 19.118 | 2-methyloctacosane | 365, 393, 408 | 4.977 ± 1.427 | 8.595 ± 6.943 | 3.94 | 6.46 | 0 | 0 |
| 33 | 19.308 | 7-nonacosene | 69, 407 | 0.138 ± 0.105 | 0.774 ± 2.039 | 0.11 | 0.58 | 7.6 | 0.86 |
| 34 | 19.406 | 5-nonacosene | 69, 406 | 0.225 ± 0.216 | 1.556 ± 0.917 | 0.18 | 1.17 | 0 | 0.86 |
| 35 | 19.599 | *n*-nonacosane | 57, 408 | 0.369 ± 0.113 | 0.927 ± 0.327 | 0.29 | 0.70 | 0 | 0 |
| 36 | 19.996 | 2-methylnonacosane | 379, 407, 422 | 4.947 ± 6.139 | 4.327 ± 5.443 | 3.92 | 3.25 | 0 | 0 |
| 37 | 20.135 | *x*-triacontene | 55, 69, 420 | 0.748 ± 0.186 | 0.626 ± 0.141 | 0.59 | 0.47 | 0 | 0 |
| 38 | 20.790 | 2-methyltriacontane | 393, 421, 436 | 0.868 ± 0.263 | 1.246 ± 0.500 | 0.69 | 0.94 | 0 | 0 |

Table 1: List of 38 cuticular hydrocarbon peaks along with their retention time (Rt), chemical identities (Chemical ID), diagnostic ions from mass spectrum found in *D. melanogaster* MCF population. Relative concentration (mean (m) and standard deviation (sd)), proportional amount of each cuticular hydrocarbon peak (in %) and % zero presence of 38 cuticular hydrocarbon peaks in both sexes of 352 *D. melanogaster* MCF population is also furnished here. % zero presence is the percentage of individuals that have absence (or zero presence) of a particular CHC.

335 Effects of selection regime, sex, replicate and the time of extraction on the
336 MCF cuticular hydrocarbons

337 We considered 37 MCF CHCs (Peak 17 was not considered since it is a combined peak
338 of Adipate and *n*-tetracosane) for the linear mixed effects analysis (see S1
339 Supplementary Information). Our model considered time of extraction and replicates
340 of MCF population as variables with random effects and selection line, sex and their
341 interaction as variables with fixed effect on the CHCs. Time of extraction (see
342 Methods section under subsection Cuticular hydrocarbon extraction for details) refers
343 to six different time points when CHC extractions of *D. melanogaster* samples were
344 done. Among variables with random effect, we compared the variance in replicates
345 and time of extraction with that of the individual (residual) variance (see S1
346 Supplementary Information). We found no peaks where MCF replicates had more
347 variance than the residuals. In fact, most of the CHC peaks had negligible variance
348 for blocks indicating no effect of replicate populations on peak variability. However,
349 six peaks (Peaks 1, 3, 5, 6, 36 and 37) were affected by time of extractions (variance
350 due to time of extraction was more than the residual). These peaks were not considered
351 for Random Forest analysis. The sex of the sampled *D. melanogaster* MCF individuals
352 is the most important variable affecting 30 CHC peaks (see Table 2) as seen previously
353 in Table 1. The variables with fixed effects such as selection regime affected 19 CHCs
354 while its interaction with sex of the MCF individuals affected 10 CHCs (see Table 2).
355 Only three CHCs (Peak 24, 31 and 36) are not affected by any of the explanatory
356 variables.

| **Peak** | **Selection** | **Sex** | **Selection x Sex** | **Time** | **Block** |
|---|---|---|---|---|---|
| 1 | NS | NS | p = 0.005413 ** | Effect present | NS |
| 2 | p = 0.02986 * | NS | NS | NS | NS |
| 3 | p = 0.01683 * | p < 2e-16 *** | NS | Effect present | NS |
| 4 | NS | p < 2e-16 *** | NS | NS | NS |
| 5 | P = 0.02936 * | NS | NS | Effect present | NS |

| | | | | | |
|---|---|---|---|---|---|
| 6 | NS | NS | p = 0.01266 * | Effect present | NS |
| 7 | NS | p < 2e-16 *** | NS | NS | NS |
| 8 | NS | p < 2e-16 *** | NS | NS | NS |
| 9 | p = 0.03019 * | p < 2e-16 *** | NS | NS | NS |
| 10 | NS | p = 0.02483 * | NS | NS | NS |
| 11 | NS | p < 2e-16 *** | NS | NS | NS |
| 12† | p = 0.04202 * | p < 2e-16 *** | NS | NS | NS |
| 13† | NS | p < 2e-16 *** | NS | NS | NS |
| 14 | p = 0.004357 ** | p < 2e-16 *** | NS | NS | NS |
| 15 | p = 7.744e-05 *** | p < 2e-16 *** | NS | NS | NS |
| 16 | NS | p < 2e-16 *** | NS | NS | NS |
| 18 | NS | p < 2e-16 *** | NS | NS | NS |
| 19 | p = 0.016364 * | p < 2e-16 *** | p = 0.009102 ** | NS | NS |
| 20† | p = 0.005125 ** | p < 2e-16 *** | NS | NS | NS |
| 21† | p = 0.01224 * | p < 2e-16 *** | NS | NS | NS |
| 22 | p = 0.001317 ** | p < 2e-16 *** | p = 0.009922 ** | NS | NS |
| 23 | p = 0.0001536 *** | p < 2e-16 *** | p = 0.0285458 * | NS | NS |
| 24 | NS | NS | NS | NS | NS |
| 25 | p = 0.002671 ** | p < 2e-16 *** | NS | NS | NS |
| 26 | NS | p < 2e-16 *** | NS | NS | NS |
| 27 | p = 0.001985 ** | p < 2e-16 *** | p = 0.001615 ** | NS | NS |
| 28† | p = 0.0009216 *** | p < 2e-16 *** | p = 0.0007424 *** | NS | NS |
| 29 | p = 0.04211 * | p < 2e-16 *** | p = 0.03855 * | NS | NS |
| 30 | p = 0.003904 ** | p < 2e-16 *** | p = 0.008833 ** | NS | NS |
| 31 | NS | NS | NS | NS | NS |
| 32 | NS | p = 7.81e-13 *** | NS | NS | NS |
| 33 | NS | p = 2.547e-05 *** | NS | NS | NS |
| 34 | p = 0.04416 * | p < 2e-16 *** | NS | NS | NS |
| 35 | NS | p < 2e-16 *** | p = 0.01684 * | NS | NS |
| 36 | NS | NS | NS | Effect present | NS |
| 37 | p = 0.03032 * | p < 2e-16 *** | NS | Effect present | NS |
| 38 | NS | p < 2e-16 *** | NS | NS | NS |

Table 2: Influence of variables with fixed effects (selection, sex and their interactions) and random effects (block and time of extraction) on 37 cuticular hydrocarbons of *D. melanogaster* MCF population assessed by linear mixed effect model analysis. Those fixed effect variables with statistically significant effect have been represented by a p-value based on *Type II Wald chi-square* test. Those variables with no significant effect on cuticular peak abundance has been denoted NS. Effect of replicates of selection lines (Block) and time of extraction (Time) was assessed by comparing their variance with that of the residual (see S1 Supplementary Information). † represents known sex pheromones in *D. melanogaster*.

367 The 19 peaks affected by differential operational sex ratio i.e. selection regime as
368 shown in linear mixed effects analysis are an obvious point of interest in our study.
369 However, 3 (Peak 3, 5 and 37) of those 19 peaks were affected by differences in time
370 of extractions arising out of logistical reasons. Rest of the 16 peaks includes four
371 known sex pheromones (Peak 12, 20, 21 and 28). To examine the trend of these 16
372 CHC peak distributions that were not affected by the time of extraction but affected
373 by imposed selection, we plotted grouped Box and Whisker graphs (see S2
374 Supplementary Information). The differences in mean CHC amounts between sexes
375 mirrored linear mixed effects results (see Table 3). We found that, unlike the rest of
376 15 CHC peaks, sexual dimorphism in Peak 2 did not exist in F and C populations.
377 Peak 2 was sexually dimorphic in M population only. By comparing means of peak
378 distribution of M and F populations in each sex with respect to C population, we found
379 that M and F males and females are not different from C in most of the cases. Both M
380 and F males was different from C males for Peak 19 while only M males was different
381 from C males for Peak 9. In case of females, we found differences between M
382 population and C population in four peaks (Peak 20, 23, 27 and 30) but there was no
383 difference between F population and C in any peak distribution. Qualitatively, in
384 comparison to C population, it appears that the amount of some CHCs have changed
385 only in M population than F population. Comparison of means of CHC peak
386 distribution between M and F population indicates that only Peak 15 is significantly
387 different in males while most of the differences between M and F populations are due
388 to differences in 11 CHC peaks in females (Peak 2, 20, 21, 22, 23, 25, 27, 28, 29, 30
389 and 34). The analysis indicates that male M and F CHC profiles have not evolved to
390 be different from that of C males and the differences between M and F males
391 themselves are based on only one peak. Any divergence in M and F CHC profiles may

392 have been because of the M females that have significantly diverged from C females
393 (see S2 Supplementary Information). Although F females have not diverged from C
394 females in most peaks, M females were found to be significantly different from F
395 females in majority of the CHC peaks. It is interesting to note that 7 CHCs found on
396 both M and F females that were not significantly different from C turned out to be
397 significantly different between M and F populations themselves.

| **Peaks** | **Sexual Dimorphism** | | | **Male** | | | **Female** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **M** | **C** | **F** | **M vs C** | **F vs C** | **M vs F** | **M vs C** | **F vs C** | **M vs F** |
| 2 | * | NS | NS | NS | NS | NS | NS | NS | **, F>M |
| 9 | *** | *** | *** | *, C>M | NS | NS | NS | NS | NS |
| 12† | *** | *** | *** | NS | NS | NS | NS | NS | NS |
| 14 | *** | *** | *** | NS | NS | NS | NS | NS | NS |
| 15 | *** | *** | *** | NS | NS | *, F>M | NS | NS | NS |
| 19 | *** | *** | *** | *, C>M | **, C>F | NS | Peak absent in females | | |
| 20† | *** | *** | *** | NS | NS | NS | *, C>M | NS | *, F>M |
| 21† | *** | *** | *** | NS | NS | NS | NS | NS | *, F>M |
| 22 | *** | *** | *** | NS | NS | NS | NS | NS | **, F>M |
| 23 | *** | *** | *** | NS | NS | NS | *, C>M | NS | **, F>M |
| 25 | *** | *** | *** | NS | NS | NS | NS | NS | *, F>M |
| 27 | *** | *** | *** | NS | NS | NS | *, C>M | NS | ***, F>M |
| 28† | *** | *** | *** | NS | NS | NS | NS | NS | **, F>M |
| 29 | *** | *** | *** | NS | NS | NS | NS | NS | *, F>M |
| 30 | *** | *** | *** | NS | NS | NS | *, C>M | NS | ***, F>M |
| 34 | *** | *** | *** | NS | NS | NS | NS | NS | **, F>M |

398 Table 3: Sexual dimorphism in sixteen cuticular hydrocarbons (that was affected by
399 differential operational sex ratios but not time of extraction as shown in linear mixed
400 effects analysis) found on *D. melanogaster* MCF population. M represents male biased
401 population, F represents female biased population and C represents control population
402 of unit sex ratio. Sex-wise comparison using Wilcoxon test of the means of peak
403 distributions between M and F populations when compared to C as well as between
404 themselves are presented. * represents statistically significant difference (* means p
405 $\leq 0.05$, ** means $p \leq 0.01$ and *** means $p \leq 0.001$) and † represents known sex
406 pheromones in *D. melanogaster* (see S2 Supplementary Information). Stars coloured
407 red indicate males have higher amount of the particular CHC whereas golden stars
408 indicate females have higher amount of the particular CHC than males.

409 Divergence patterns of MCF cuticular hydrocarbons

410 We used 31 out of 37 MCF CHCs, whose variance were not affected by time of
411 extraction as shown in linear mixed effects analysis, for multivariate analysis using
412 Random Forest. Multidimensional scaling of proximity matrix showed that sexes
413 formed distinct groups in *D. melanogaster* MCF population with 100 % correct sex
414 identification in random forest analysis. In C population, Random Forest analysis
415 produced 94.1% correct prediction of individuals belonging to 3 different replicates.
416 It is important to note that the triplicates of C are maintained at unit sex ratio and are
417 derived from $LH_{st}$ while M and F populations are derived from the C replicates (see
418 Methods section for details). Therefore, the divergence pattern of C in Fig. 2 can be
419 considered most close to ancestral pattern and is subsequently used as reference
420 pattern to M and F populations. The CHC divergence pattern in Fig. 3 suggests that
421 the M females are more dispersed than the M males that are tightly grouped (91 %
422 correct identification of M individuals from 3 replicate populations). In comparison

423 to C population, M males appear to have evolved CHC profiles which are more similar
424 to each other while M females have evolved a more variable CHC profile than C
425 females. In F population (Fig. 4), we see an opposite trend where the F males are
426 dispersed more than the F females that form tight group (87 % correct identification
427 of F individuals belonging to 3 replicate populations). Comparison with C females
428 suggests that F females have evolved CHC profiles that are similar to each other while
429 F males have increased the variability of their CHC profile.
430
431 Fig. 2 Multi-dimensional scaling (MDS) of the proximity matrix of C individuals
432 ($C_{1/2/3}$ are C blocks, m= males and f= females)
433 Fig. 3 Multi-dimensional scaling (MDS) of the proximity matrix of M individuals
434 ($M_{1/2/3}$ are M blocks, m= males and f= females)
435 Fig. 4 Multi-dimensional scaling (MDS) of the proximity matrix of F individuals
436 ($F_{1/2/3}$ are F blocks, m= males and f= females)
437
438 When we compare male CHC profile of M and F replicates, Random Forest analysis
439 predicted 86.4% correct identification of the $M_{1,2,3}$ and $F_{1,2,3}$ males (see Fig. 5). In
440 Fig. 5, males of M replicates formed separate groups with few overlaps among
441 themselves. However, F replicates did not group separately from the M replicates as
442 predicted but overlapped with the corresponding M replicates. In Random Forest
443 analysis involving females of M and F replicates, we could identify $M_{1,2,3}$ and $F_{1,2,3}$
444 females with 85.3% correct prediction (see Fig. 6). Fig. 6 revealed that M females
445 from different replicates formed separate groups with few overlaps. We again find a
446 considerable overlap of F replicates on corresponding M replicates except for females
447 from second F replicate.

448

449 Fig. 5 MDS of proximity matrix derived from Random Forest analysis of males from
450 three M and F replicates
451 Fig. 6 MDS of proximity matrix derived from Random Forest analysis of females from
452 three M and F replicates
453

454 We also performed Random Forest analysis with the 16 CHC peaks that were
455 significantly affected by differential operational sex ratios but not time of extraction
456 (see linear mixed effects analysis, Table 2). The MDS plots based on these peaks are
457 presented in S3 Supplementary Information. While there are minor differences
458 between MDS based on 31 and 16 peaks, the overall pattern of scatter (i.e. tightly
459 packed M males and F females in M and F populations respectively; divergence of M
460 and F males and females) still held strong.

461 # Discussion

462 Reproductive isolation evolved as a by-product of SAC within each M replicates (8).
463 The allopatric M replicates of *D. melanogaster* MCF population diverged from each
464 other in terms of mate preference, copulation duration and sperm defense ability over
465 105 generations. However, mating latency that is considered to be a measure of female
466 reluctance to mate did not differ among M replicates. Therefore, it was concluded that
467 the divergent mate preference in the high sexual conflict regime of the MCF
468 population may most likely be due to underlying female choice (8). Since mate
469 preference did not diverge in F replicates under low sexual conflict, we predicted that
470 male and female CHC profiles of the three replicates within the M regime should be
471 more divergent from each other compared to the CHC profiles of the three replicates
472 within the F regime. Here we assume that the putative chemical cues such as the CHCs
473 form the basis of divergent mate choice in M and have also diverged as a by-product
474 of SAC.
475
476 Abundances of different CHC peaks that are affected by altered operational sex ratio
477 indicate that the predicted divergence of M replicates may not hold true at least for
478 males (see Table 3 and S2 Supplementary Information). The results indicate that most
479 male CHCs of M population have diverged little from either C and F males. Among
480 the CHCs found in *D. melanogaster* MCF population, male CHCs (such as 7-tricosene,
481 9-pentacosene and 7-pentacosene) that have known pheromonal roles show no
482 difference among selection lines. On the other hand, M females evolved CHC
483 differences from C females in four peaks but F females did not differ in CHCs from
484 C females at all. However, M females showed differences in peak abundance with F
485 females in many CHCs including the known contact pheromone Heptacosadiene, in

spite of F females not significantly differing from C females. Any major differences between M and F populations, therefore, are due to CHC abundance on female cuticle. The distribution of CHC abundance in both males and females shows quite a consistent trend whereby M population has decreased peak abundance while F population has increased peak abundance (see S2 Supplementary Information). The CHC peaks of M males also have a near consistent tighter distribution apart from being less abundant than F males. It appears as though M males are compelled to display a typical CHC profile, possibly due to the highly competitive scenario in accessing mates, indicating an intense sexual selection. Less abundant CHCs in M females, however, could be because of various reasons including (a) to reduce attractiveness (viz. decreasing amount of peak abundances) to harassing males, (b) increased energy/resource investment in resistance to male induced harm, etc. However, at this point, we have no evidence supporting any of the possibilities.

Random Forest analysis (see Fig. 5) shows that the three replicates of M males form 3 distinct groups but contrary to our prediction, F males do not form a single group. Instead, the three F replicates overlap with the three corresponding M replicates. A similar divergence pattern is also reflected in two of the three female replicates of M and F (see Fig. 6). Such CHC divergence pattern indicates that SAC did not result in CHC differentiation in M population in a similar way it had led to divergence in mating behaviour in M population. The within-replicate assortative mating in the high sexual conflict regime may, therefore, be mediated by mate choice on mate attracting cues other than CHCs. It is difficult to ascertain how M and F replicates could have an overlapping CHC divergence pattern though. Since M and F populations do not differ from C population in the abundance and distribution of many CHCs, it is

511 possible that the divergence pattern of M and F reflect that of the C population whose
512 sexes show quite a differentiated profile of CHCs among its three replicates (see S4
513 Supplementary Information). The pattern of CHC divergence in MCF sexes could
514 otherwise be linked to ancestry. However, since MCF population was established
515 using a large population size ($N_e$ ($LH_{st}$) ~ 2250, $N_e$ (MCF) ~ 450; (28)), a founder
516 effect during initial establishment is unlikely to have affected the MCF population.
517 This is also confirmed in the linear mixed modelling analysis which shows effect of
518 replicates on the CHC profile is negligible (see Table 2). In conclusion, reproductive
519 isolation (in M regime but not in F regime (8)) as a by-product of SAC cannot be
520 explained by the CHC divergence among males and females of *Drosophila*
521 *melanogaster* M and F regimes. However, CHC divergence in M and F populations at
522 regime level indicate other possible evolutionary mechanisms at play that are
523 discussed below.
524
525 In this study, sexual selection appears to have particularly strong effect on the CHC
526 profiles at the population level (see Fig. 2, Fig. 3 and Fig. 4). It is important to note,
527 here, that CHC profile refers to the combination of different CHCs (CHC bouquet)
528 that acts as a composite pheromonal signal rather than individual CHCs. If we compare
529 the CHC profiles of C and M populations, we see that M females have diverged with
530 respect to C females while M males have converged with respect to C males
531 considerably. Increased sexual selection in M population appears to have resulted in
532 CHC profiles of M males being pushed toward a common optimum point such that M
533 males present a more preferable CHC profile that is under stabilising selection to M
534 females. Similar CHC profiles of the M males may also be explained by suppression
535 of sexually selected CHCs such that variability of male CHCs is reduced over time.

536 This may be true since *D. serrata* males have been found to lower their CHC
537 expression when female:male ratio is decreased (39). We cannot, also, rule out the
538 possibility of the male CHC profile of M population coevolving with other sexually
539 selected trait(s). On the other hand, divergence of CHC profiles of M females could
540 be a result of lack of selection pressure due to abundant mating options or as predicted
541 in populations following Buridan's ass regime (see below).
542
543 The F males and females show a diametrically opposite pattern to that of the M regime.
544 Under low sexual selection, the selection pressure is possibly weaker and hence,
545 absence of directional selective pressures allowed diversification of CHC profiles in
546 F males compared to the C males. In F regime where female:male is high, males may
547 evolve to display their most attractive CHCs to potentially improve mating success
548 (as seen in *D. serrata* (39)) leading to varied CHC profiles in the process. The F
549 females appear to be more tightly grouped in terms of their CHC profile than C females
550 (and also M females). This indicates that F females are probably under selection
551 pressure to maintain similarity of their CHC profiles. The reason for this is not obvious
552 and may be a result of correlated evolution in some other trait affected by sexual
553 selection. Although not experimentally tested, *D. melanogaster* females do possess
554 specific mate attracting contact pheromones that suggest some degree of female
555 control over mating outcome and fitness derived from such mating (17). Another
556 possibility, therefore, exist where F females may also be affected by increased
557 competition due to altered sex ratio where they have to maintain identical profile to
558 be acceptable to males that are fewer in number. Overall, the CHC divergence pattern
559 of M and F regime appears to be strongly influenced by sexual selection although

560 other possible sexual conflict resolution mechanism such as Buridan's ass paradigm
561 (see below) cannot be ruled out.
562
563 Apart from speciation, a population under sexual conflict may follow other
564 evolutionary stable strategy to resolve the conflict between sexes (6). For example, in
565 a large population and/or population with significant rates of mutations, there might
566 be diversification of female traits with respect to male traits (Buridan's ass regime)
567 such that males, in an attempt to adapt to the variability in female traits, are caught in
568 limbo unable to increase their own fitness in the population as a whole (1). This is
569 relevant to the results from the current study where multivariate analysis show M
570 females of each replicate are more dispersed and M males form a tight group with
571 respect to their CHC profile (see Fig. 3). It is known that the male mating behavior of
572 *Drosophila melanogaster* is partially mediated by female CHCs (17). The M
573 population also meet the criterion for Buridan's ass regime since the population size
574 is large ($N_e$ ~ 450) and the block-wise differences among M replicates (see Fig. 5)
575 suggest that possibility of random mutation accumulation over several generations in
576 allopatry. It is, therefore, possible that sexual conflict on CHC profiles in M regime
577 has followed Buridan's ass phenomenon where M males have been caught in an
578 uncertain intermediate stage failing to pursue either ends of CHC divergence in M
579 females. The fact that this pattern is repeated in case of females of F regime is a
580 revelation since effect of sexual conflict has been traditionally focused on males rather
581 than females (17).
582

583 # Conclusion

584 Experimental studies and comparative analysis of taxonomic groups suggest that
585 speciation is possible under sexual conflict. The mathematical models support this
586 possibility but also suggest that dynamics of evolution under sexual conflict is quite
587 varied and stochastic to a point that evolution of reproductive isolation in allopatry is
588 only one of the several other outcomes. This study is an example of different
589 reproductive traits being affected differently by sexual conflict such that isolation
590 based on mate preference, copulation duration and sperm defense ability is not
591 reflected in the mate attraction cues such as CHCs. Rather sexual conflict, if any, is
592 probably reconciled in form of Buridan's ass regime. In fact, *D. melanogaster*
593 individuals from high and low sexual conflict regime appear to be significantly
594 affected by sexual selection on CHC profiles. We conclude that since CHCs form a
595 basis for mate attraction, M males under high sexual selection may be likely to keep
596 their CHC profiles similar to be acceptable by the limited number of females in the
597 male biased regime. The same may also be true for the females of female biased regime
598 or their CHC divergence pattern may be by-product of sexual selection pressure on
599 any other hitherto unknown trait.

600

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607

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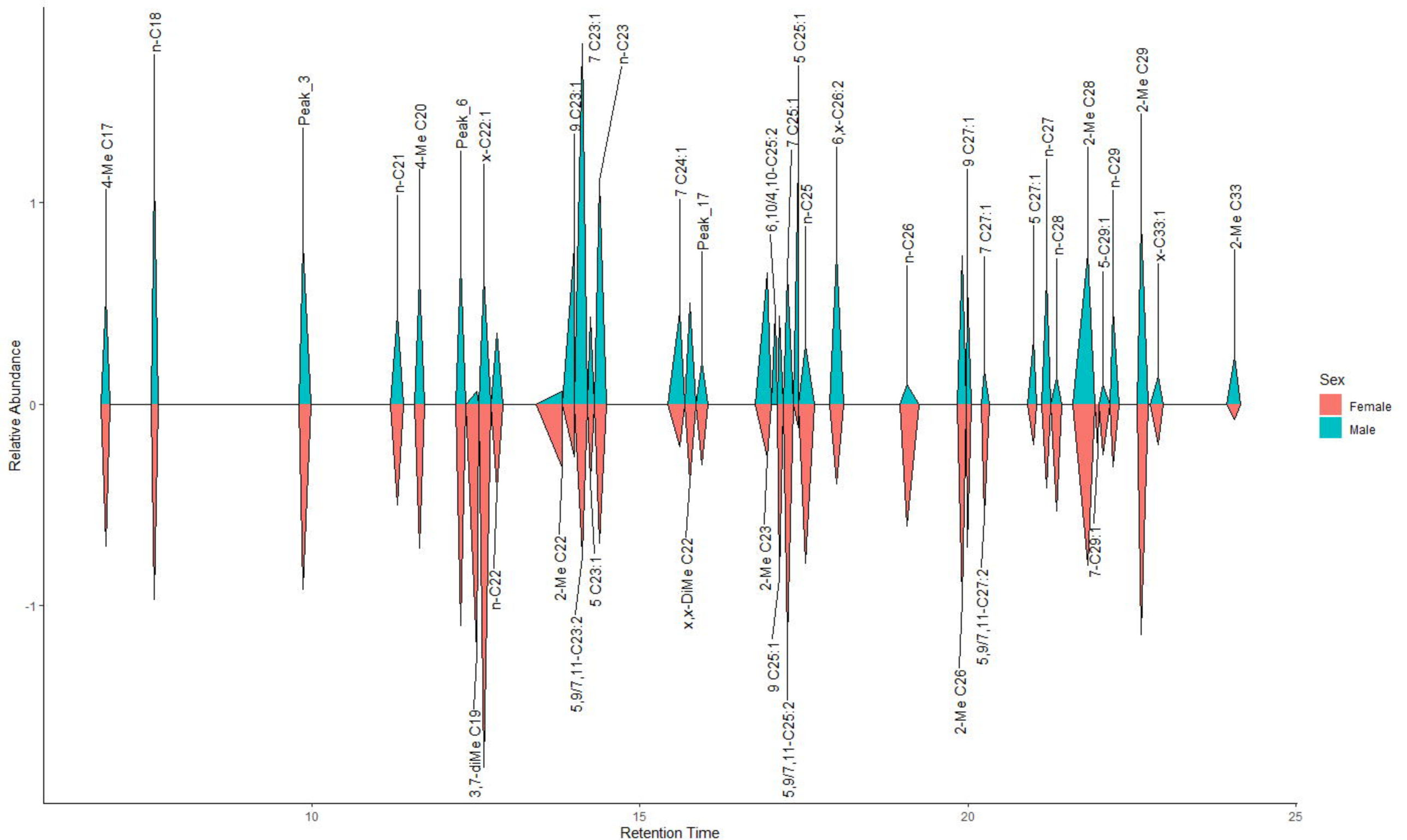
716 S1 Supplementary Information. Results from linear mixed effect model analysis of *D.*
717 *melanogaster* MCF cuticular hydrocarbons.
718 S2 Supplementary Information. Boxplots showing sex-wise differences in *D.*
719 *melanogaster* MCF cuticular hydrocarbon abundance.
720 S3 Supplementary Information. Multi-dimensional scaling of the proximity matrix
721 based on 16 *D. melanogaster* cuticular hydrocarbons of MCF population.
722 S4 Supplementary Information. Multi-dimensional scaling of proximity matrix of the
723 three *D. melanogaster* C replicates.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7 C24:1
Peak_17
6,10/4,10-C25:2
7 C25:1
5 C25:1
n-C25
6,x-C26:2
n-C26
9 C27:1
7 C27:1
5 C27:1
n-C27
n-C28
2-Me C28
5-C29:1
n-C29
2-Me C29
x-C33:1
2-Me C33
Sex
Female
Male
Relative Abundance
1
0
-1
3,7-diMe C19
n-C22
2-Me C22
5,9/7,11-C23:2
5 C23:1
x,x-DiMe C22
9 C25:1
2-Me C23
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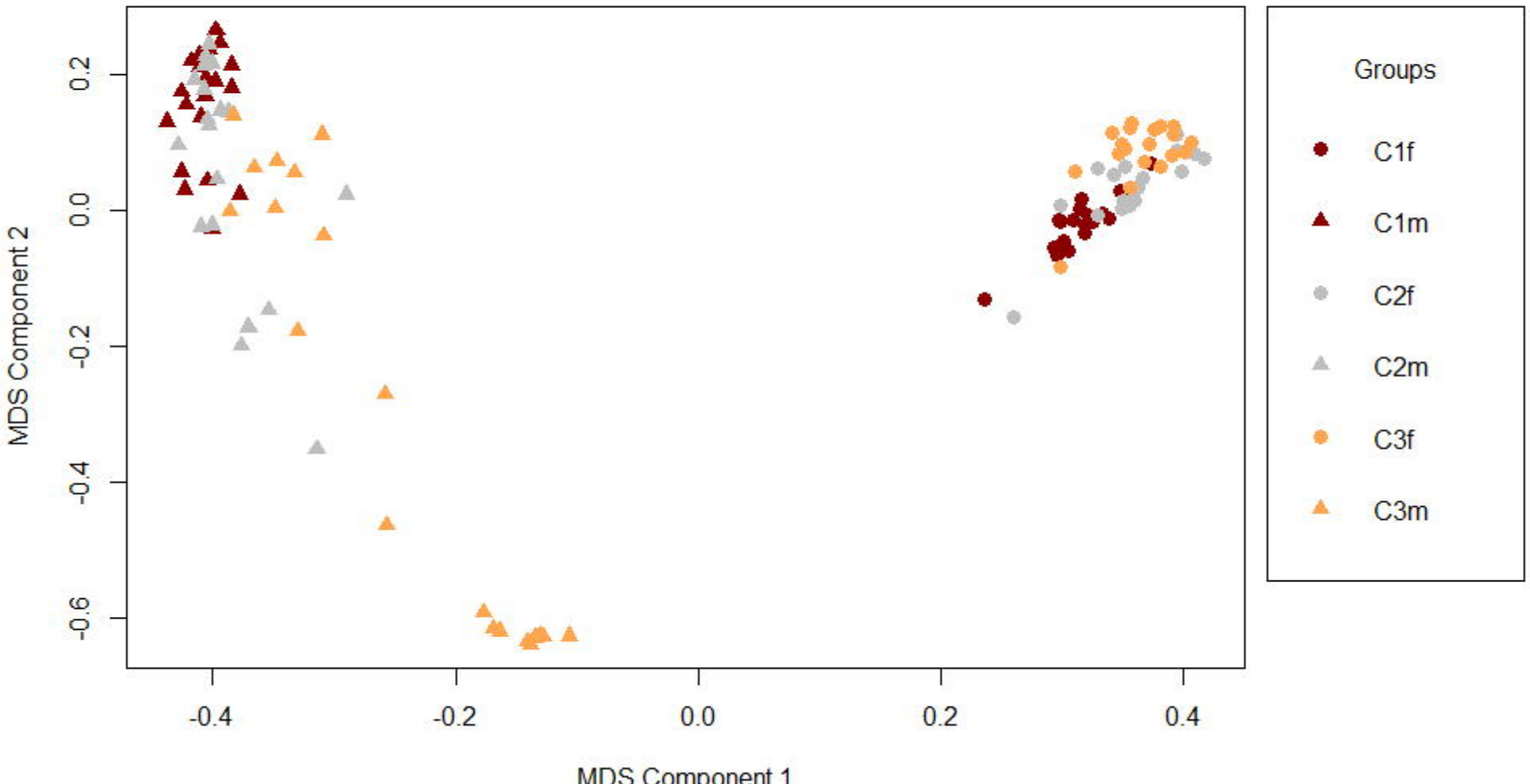
10
15
20
25
Retention Time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C2f
C2m
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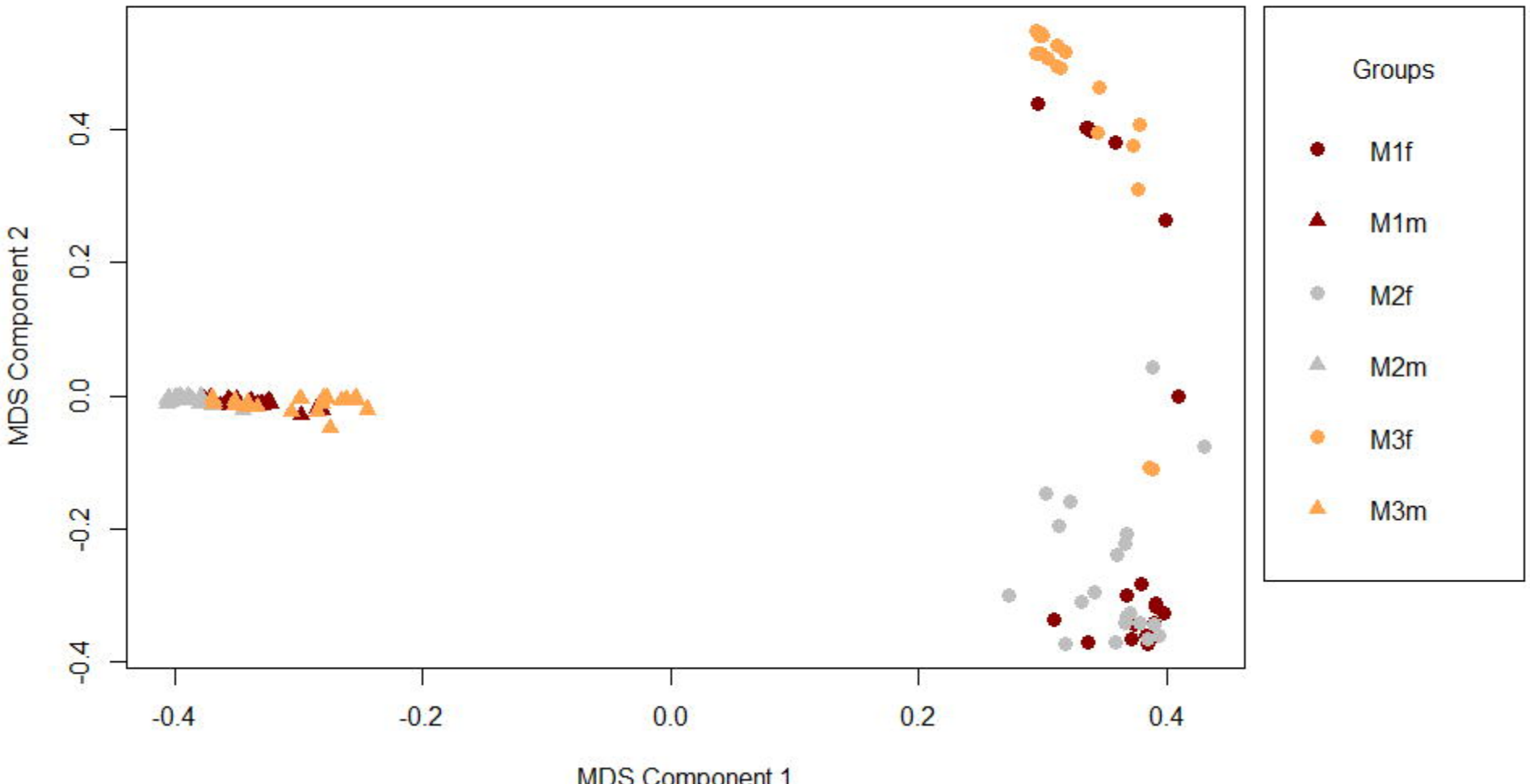
MDS Component 1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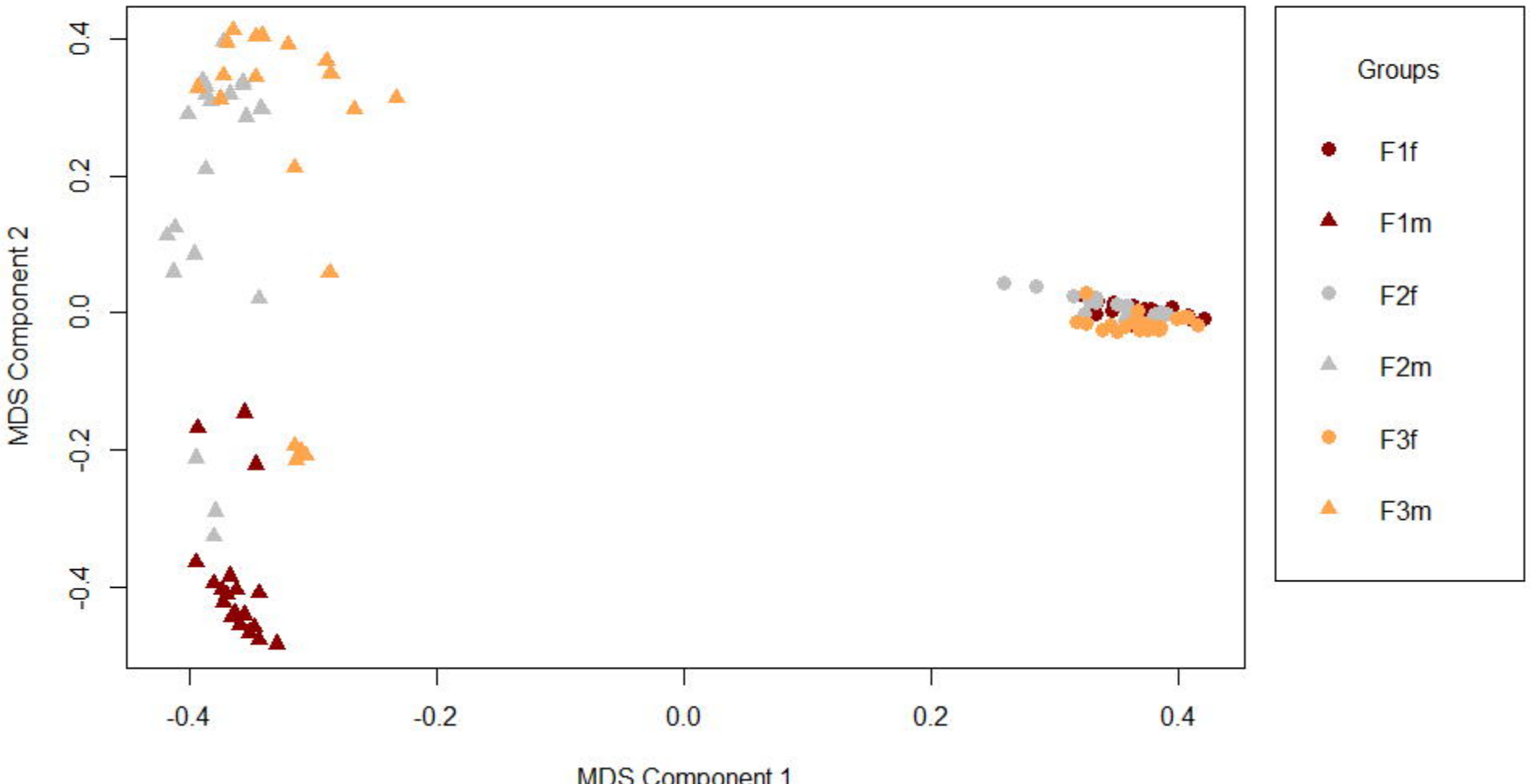
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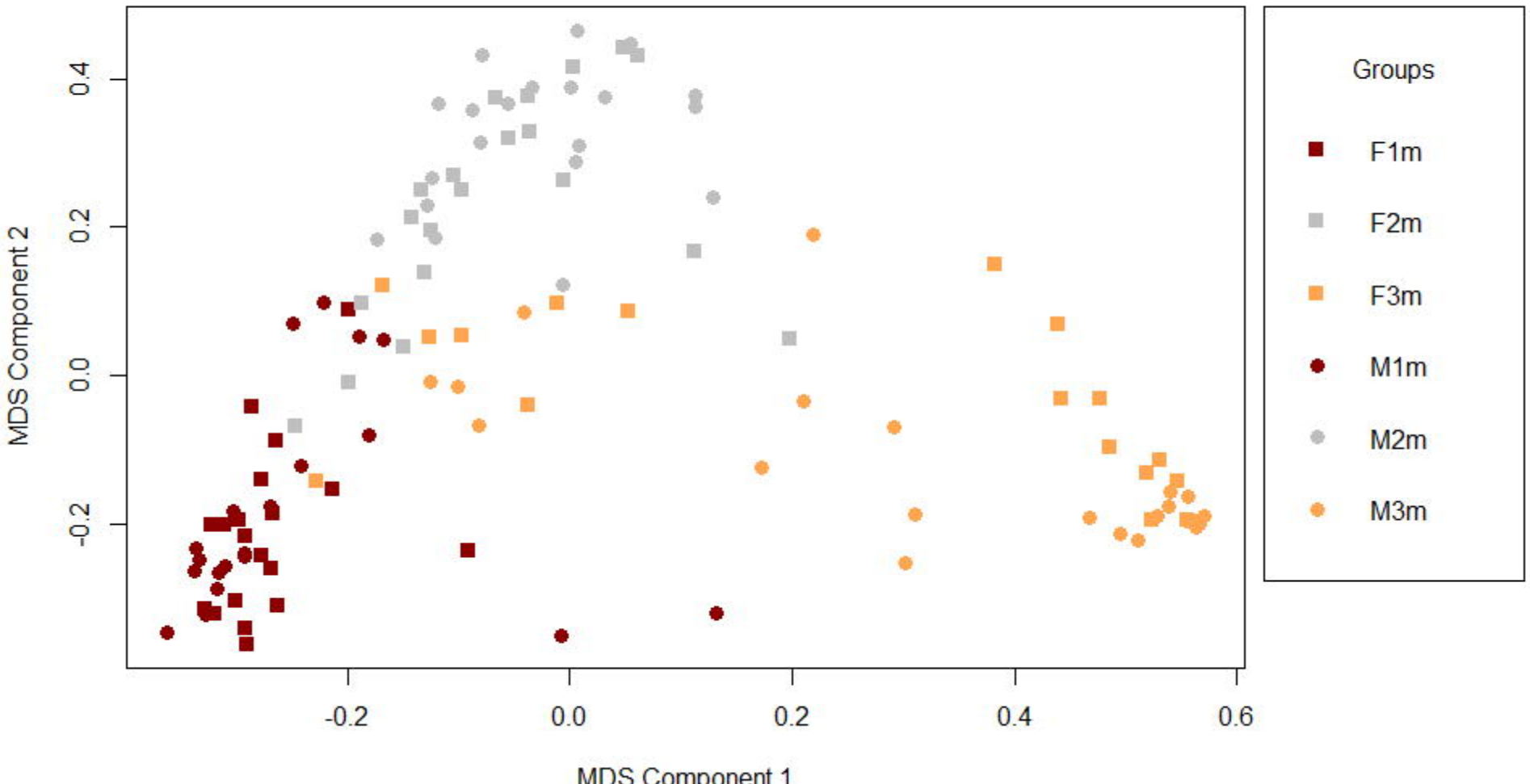
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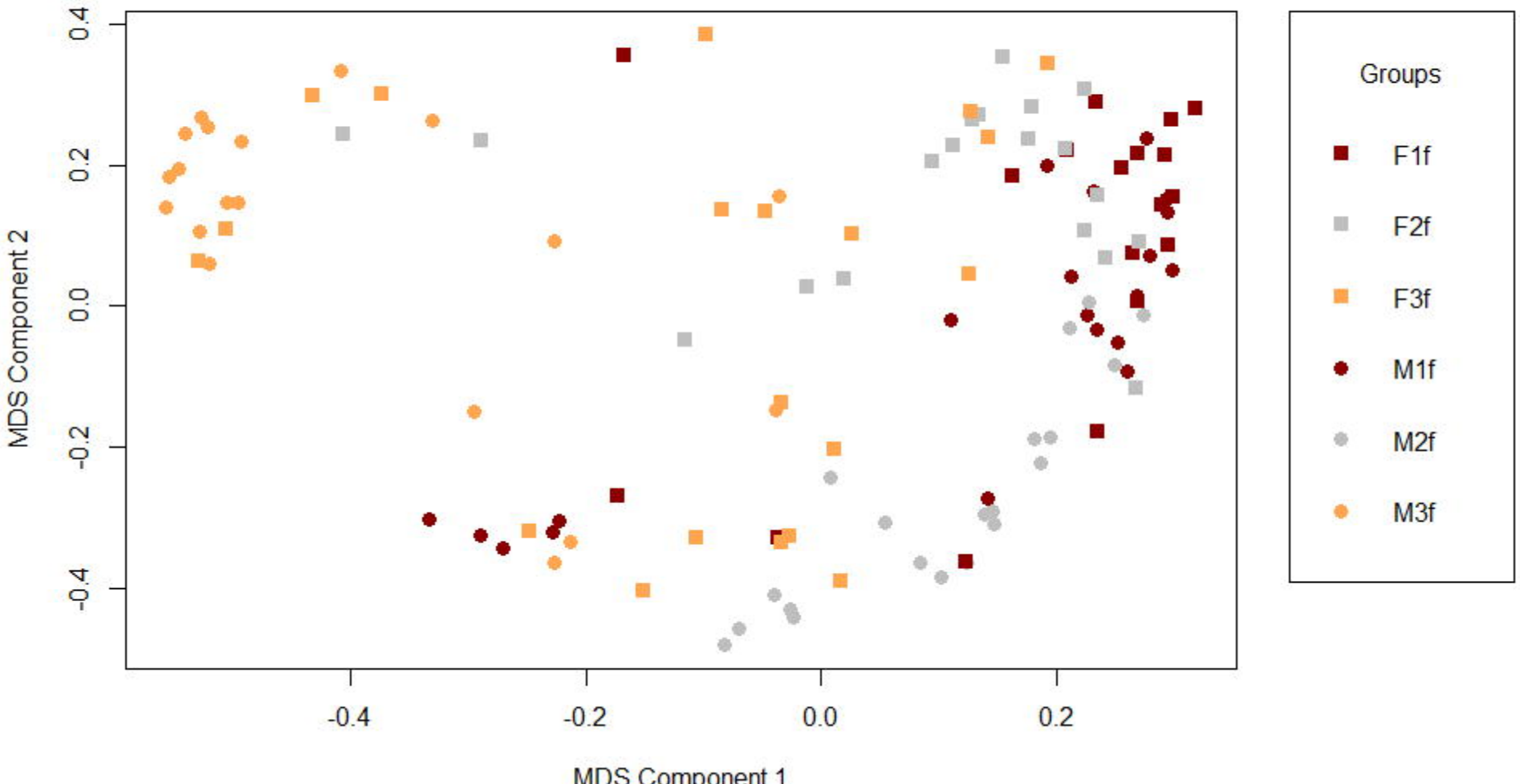
0.4
0.6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