Sexual selection affects the evolution of contact pheromones in *Drosophila melanogaster* populations isolated by differential sexual conflict

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Abstract - The ability of 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 the replicates of Drosophila melanogaster populations under differential sexual conflict found that divergent mate preference evolved among replicates under high sexual conflict regime. The precopulatory isolating mechanism underlying such divergent mate preference could be sexual signals such as cuticular lipids since they evolve rapidly and are involved in D. melanogaster mate recognition. Using Drosophila melanogaster replicates used in the previous study, we investigate whether cuticular lipid divergence bears signatures of sexually antagonistic coevolution that led to reproductive isolation among replicates of high sexual conflict regime. We found that their cuticular lipid profiles are sexually dimorphic. Although replicates with male biased sex ratio evolved isolation in reproductive traits due to high sexual conflict, the patterns of cuticular lipid divergence in high and low sexual conflict regimes suggest that sexual selection is the dominant selection pressure rather than sexual conflict affecting the cuticular lipid profile. We also find cuticular lipid divergence patterns to be suggestive of the Buridan's Ass regime which is one of the six possible mechanism to resolve sexual conflict. Although both sexes of male biased replicates have divergent cuticular lipid profiles, isolation of male biased replicates as a result of cuticular lipid divergence cannot be credited to sexually antagonistic coevolution.

Key Words - Sexual conflict, sexual selection, reproductive isolation, Drosophila, cuticular hydrocarbon, random forest analysis.

INTRODUCTION

Mathematical models of sexual conflict based on mating rate envisage six possible evolutionary outcomes out of which two lead to speciation: allopatric speciation as a byproduct of sexually antagonistic coevolution (SAC) and sympatric speciation as a byproduct of divergence in the reproductive traits of females followed by the male sex (Gavrilets and Hayashi 2005). 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 (Arnqvist et al. 2000). 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 (Gage et al. 2002, Morrow et al. 2003). 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 (Gavrilets 2014).

By evolving independent replicates of a dung fly population for 35 generations under monogamous (low) and polygamous (high) sexual conflict) regime, Martin and Hosken (2003) 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* populations 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 (Syed et al. 2017). 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" (Gage et al. 2002, Morrow et al. 2003, Bacigalupe et al. 2007, Gay et al. 2009, Plesnar-Bielak et al. 2013).

Syed et al. (2017) indicate that the differences in mate preference among replicate male biased (3 male : 1 female sex ratio) *Drosophila melanogaster* populations arise due to sexually antagonistic coevolution. Prezygotic isolation by mate preference is often mediated by cuticular lipid profiles that provide information about species identity, sex, mating status, age, etc. to a receiver and play a pivotal role in sexual communication in *Drosophila* sp. (Ferveur et al. 1989, Ferveur 1997, Ferveur and Cobb 2010). It is, therefore, possible that the cuticular lipid profiles form the mechanistic basis for mate preference among replicate male biased populations in *D. melanogaster* studied by Syed et al. (2017). However, this possibility assumes that the cuticular lipid profiles have also differentiated among replicates as a byproduct of sexual conflict within each allopatric replicate. Cuticular lipid profiles of *Drosophila melanogaster* are dimorphic (Ferveur and Cobb 2010) and those cuticular lipids that take part in mate preference are known to have independent genetic controls in the two sexes, often linked to sex determination genes, for their biosynthesis (Ferveur 2005, Chenoweth et al. 2008, Dembeck et al. 2015). This is a possible signature of past sexually antagonistic selection (Cox and Calsbeek 2009) and current interlocus sexual conflict. Many previous studies have implicated differentiated cuticular lipid profiles in the evolution of reproductive isolation in *Drosophila* sp. through sexual selection (Smadja and Butlin 2008, Laturney 2012, Shahandeh et al. 2017) but in this study we look into the possibility of cuticular lipid divergence under differential sexual conflict.

We test the hypothesis that cuticular lipid profiles (that play a role in mate attraction) are differentiated in conjunction with the differentiation in mate preference by sexual conflict.

Using the same *D. melanogaster* populations as Syed et al. (2017), we investigate the patterns of cuticular lipid divergence among the replicates of male and female biased regimes. We expect all *D. melanogaster* populations to show sexual dimorphism in cuticular lipid profiles. We predict that due to high sexual conflict the male cuticular lipid profiles of male biased (3 male : 1 female) replicates will diverge more than the male cuticular lipid profiles of the female biased (1 male : 3 female) populations. Our result suggests that reproductive isolation among M replicates due to high sexual conflict is not explained by the patterns of cuticular lipid divergence. Instead we find other evolutionary mechanisms that are contributing to cuticular lipid divergence.

METHODS AND MATERIALS

D. melanogaster population maintenance. In this study, we used nine populations of Drosophila melanogaster belonging to 168-170 generation raised under three different selection regimes. These three selection regimes were identical in all respect, except for the adult sex-ratio. The male biased regime (henceforth M) had a 1:3 :: female:male adult sex ratio, while the female biased regime (henceforth F) had a 3:1 :: female:male adult sex ratio. The control regime (henceforth C) had a 1:1 adult sex ratio. Each regime had three replicate populations (M1,2,3 in the M regime, F1,2,3 in the F regime and C1,2,3 in the C regime). All nine populations ultimately trace their ancestry to a large, laboratory adapted population, LHst which has an autosomal, recessive scarlet eye-colour marker. We first derived three populations from the LHst population and labeled them C1,2,3. The C1,2,3 populations were maintained at a 1:1 adult sex ratio for five generations. Subsequently, from each C population, we derived one population for the M regime, one for the F regime and one for the C regime. M, C and F populations with the same subscript were derived from the same C population and therefore are closely related in terms of ancestry. For example, M1, F1 and C1 were derived from C1. Moreover, during subsequent population maintenance, M, C and F populations with the same subscript are handled on the same day and therefore can be treated as statistical blocks. The details of maintenance of M, C and F populations have been described elsewhere(Nandy et al. 2013a). Briefly, these populations are maintained on a 14-day discrete generation cycle, at 25˚C and 60% relative humidity (RH), 12:12 hours light/dark cycle and standard cornmeal– molasses–yeast food in standard vials (90-mm length × 30-mm diameter). In each regime, every generation we collect virgin males and females and combine them in appropriate sex ratios. Males and females interact for around 48 hours, after which they are transferred to fresh vials for oviposition. Eggs laid are trimmed to a

125 density of around 140-160 eggs per vial containing 6-8 ml food. These eggs are used to start
126 the next generation.

127 *Generating flies for cuticular lipid extraction.* Before cuticular lipid extraction
128 from virgin M, C, and F flies, we allowed one generation of "standardization" or common-
129 garden rearing at a 1:1 sex ratio following Nandy et al. (2013a) to account for any potential
130 non-genetic parental effect (Rose 1984). In order to generate flies for cuticular lipid
131 extraction, we collected eggs from "standardised" flies. On the 10th day after egg collection
132 from the M, C, and F populations, we collected newly eclosed flies as virgins within 6 hours
133 of eclosion under light CO2 anesthesia. The flies were isolated by keeping each of them
134 singly in a vial for 2 days. On 12th day (ensuring flies of same age to minimize age-specific
135 variation of cuticular lipids) the cuticular lipids extraction assay was conducted.

136 *Cuticular lipid extraction*. We transferred each virgin individual to a 1.5 ml gas
137 chromatography (GC) vial without anesthetising it. We then poured a 200 µl Hexane solvent
138 containing 10ng/µl Pentadecane (C15H32, internal standard) into the GC vials. The vials
139 were left undisturbed for 4 minutes and then vortexed for 1 minute. We removed the dead
140 individual from each GC vial and subsequently left the hexane to dry out. The extracted
141 samples in the GC vials were then stored at 4˚C until gas chromatography was performed.
142 Cuticular lipids were extracted from the male and female D. melanogaster flies belonging to
143 all blocks of MCF population [n for M = 119 (60 males + 59 females), n for F = 115 (58
144 males + 57 females) and n for C = 118 (59 males + 59 females)].

145 *Gas chromatography.* Before gas chromatography, the dried extract adhering to the
146 glass GC vials was brought to solution by pipetting 20 µl Hexane and vortexed for 30 sec.
147 We injected 2µl of this solution manually into splitless Shimadzu GC-2010 Plus instrument

148 fitted with a Restek Rxi-5Sil MS (30m x 0.25µm df x 0.25mm ID) capillary column. The
149 24.88 minutes' temperature program was set to increase from initial 57˚C held for 1.1
150 minutes to 190˚C held for 1.2 minutes at 100˚C per minute. The temperature increased from
151 190˚C to 270˚C at 5˚C per minute and finally raised to 300˚C at 120˚C per minute with hold
152 time of 5 minutes at 300˚C (Bedhomme et al. 2011). The carrier gas was Nitrogen with
153 column flow rate fixed at 2ml per minute.

154 *Cuticular lipid identification*. Before gas chromatography of *D. melanogaster*
155 samples, a chromatographic run was performed with a mix of known straight chained alkane
156 standards (C_{10} to C_{40}) increasing in even numbers of Carbon. This chromatogram was used to
157 measure Kovats Retention Index (KI) for all relevant unknown sample peaks by referring to
158 retention time for internal standard (Pentadecane: C_{15}) and hydrocarbon compounds in the
159 standard mix. The KI was used to identify cuticular lipid peaks based on already published
160 KIs of cuticular lipids (using similar method followed by Bedhomme et al. (2011)).

161 *Cuticular lipid quantification*. All peaks that consistently appeared in the samples
162 when compared with a blank chromatographic run (using Hexane that was used in the
163 extraction process) were considered relevant to this study. The area under the peak value was
164 calculated by drawing a baseline to each relevant peak using the Shimadzu GCSolutions
165 software. We manually checked the integrated peaks in all the sample chromatograms for any
166 irregularities. The peak areas of the 352 samples was standardized by dividing the peak value
167 with that of internal standard (Pentadecane) from respective chromatograms following
168 Bedhomme et al. (2011). Each standardized value was then multiplied by 105 to enable us to
169 use more significant figures in multivariate analysis. While standardization using internal
170 standard allow us to minimise any handling effect during gas chromatography performed
171 over many days, we further normalized the standardized peak values of all samples to reduce

any handling effect that may have cropped up during extraction process. Extractions were performed on three different days for three different blocks of M, C and F. This entire exercise was repeated at two different times. We use the following min-max feature scaling formula for normalizing cuticular lipid peak values: $Xnew = \frac{X-Xmin}{Xmax-Xmin}$ where Xnew is the normalized peak value, X is the actual peak value, Xmax is the maximum peak value within a group and Xmin is the minimum peak value within a group. However, we also ran Random Forest analysis (see below) with non-normalised data to examine whether any effect of procuring samples at different times affected the cuticular lipid divergence pattern. We find that cuticular lipid divergence pattern is qualitatively similar for both normalized and non-normalised data. We use divergence pattern from normalized data in this study while corresponding cuticular lipid divergence pattern from non-normalised data is presented as supplementary information (see Supplementary Information).

Linear mixed effects analysis. To quantify whether variables such as selection regimes, sex, replicates and their interactions explain the distribution of the peak values of each cuticular lipid, we used linear models for mixed effects analysis. Following Bedhomme et al. (2011), selection regime (M, C and F), sex (male and female) and their interaction were considered explanatory variables with fixed effects while replicates within each selection regimes (block 1, 2, and 3) and its interactions with explanatory variables were assigned as variables with random effects. Although each individual within blocks were unique, block themselves were nested within each selection regime. Additionally, the same subscript in block identity from different selection regime denoted common ancestry. A common ancestry has the possibility of confounding the independence of cuticular lipid profiles of the studied Drosophila individuals stochastically since these populations have evolved for around 170 generations, making it necessary to consider block identity as random effect. Therefore, using

196 normalized peak values as response variables in this analysis, we modelled the fixed and
197 random effects on the response variables according to the following two formula:

198 Model 1 = Selection regime + Sex + (Selection regime x Sex) + Block

199 Model 2 = Selection regime + Sex + (Selection regime x Sex) + Block + (Selection
200 regime x Block) + (Block x Sex) + (Selection regime x Block x Sex)

201 While Model 1 represents a simpler model out of the two models containing all variables
202 with fixed effects and only block identity having random effect, Model 2 incorporates all
203 biologically relevant explanatory variables with fixed and random effects. We used "lme4"
204 package in R and selected a model out of the two aforementioned models for each cuticular
205 lipid that had a lower Akike Information Criterion (AIC). Linear mixed effects analysis
206 explains how different variables recorded during sampling from the *D. melanogaster*
207 populations affect each cuticular lipid individually. However, to further understand how the
208 combinations of all cuticular lipids determine grouping of *Drosophila* M, C and F
209 populations, we used Random Forest based multivariate analysis.

210 *Random Forest analysis*. Multivariate analysis like principal component analysis and
211 discriminant function analysis are generally used in studies that investigates group patterns
212 within samples based on cuticular profiles (Kather and Martin 2012). However, these
213 statistical techniques are adversely impacted by small sample-size(n):variables(p), non-
214 independence of cuticular lipids in a sample (since many cuticular lipids share common
215 biochemical pathways (Dembeck et al. 2015)) and disproportionate effects of cuticular lipids
216 found in trace amounts (Martin and Drijfhout 2009). To overcome this drawbacks of
217 traditional techniques, we used Random Forest analysis (R package "Party") that performs
218 well under "small n large p" conditions, unaffected by highly correlated variables and is

unbiased towards the nature of variables used (Strobl et al. 2009b, 2009a). Random Forest Analysis is a machine learning algorithm that forms numerous classification schemes without prior expectation of the data structure by repeated subsampling of the data and random selection of a few predictor variables (Strobl et al. 2009b). Using these classification schemes (also called base learners), individuals are reassigned their identity based on their probability to appear as a particular group in the base learners. When these new identities of the samples are compared to their already known group identity, we calculate the percentage correct prediction of individual identities from Random Forest analysis. Based on the probabilities of individual identities, proximity analysis (identical to similarity-dissimilarity matrix/ correlation matrix in traditional multivariate analysis) gives us a measure of samples' group affinity. Multidimensional scaling (identical to Principal Component Analysis) of the proximity matrix is then illustrated in a scatterplot to show how experimental individuals group with respect to each other.

We performed Random Forest analysis using normalized and standardised area under the peak values of all relevant peaks. Seed (a random number) was set to 1220. We checked the stability of the classification scheme by increasing the number of subsampling events gradually from 5000 to 20000 and decided to set the number of trees (ntree) parameter in the analysis at 15000. The number of variables (mtry = 6) to be used in the analysis was fixed to rounded square root of the number of predictor variables (in the present study, the number of peaks analysed in chromatograms) in the data (Strobl et al. 2009b). Multi-dimensional scaling (MDS) of proximity matrix was used to produce two dimensional scatterplots showing clustering of individuals.

RESULTS

Characterisation of cuticular lipids in D. melanogaster populations under differential sexual conflict. We found a total of 44 peaks that consistently appeared in MCF chromatograms. Out of the 44 peaks, 37 were cuticular hydrocarbons (CHCs), 3 belonged to other chemical groups found on insect cuticle and 4 could not be identified. We also found 14 new peaks that have not been reported for *Drosophila melanogaster* in literature before. Most of the CHCs had hydrocarbons chain lengths between C_{20} and C_{30} (see Supplementary Information). Male and female chromatographic profiles significantly differed in relative abundance of 35 peaks out of the 44 identified peaks based on Welch two sample *T test* ($p<0.05$). The 9 cuticular lipids which occur in similar quantities in both males and females are most likely involved in primary anti-dessication function of cuticular lipids. *Drosophila melanogaster* males predominantly produce monoenes (e.g. Tricosene, Pentacosene, etc) while their females predominantly produce dienes (e.g. Heptacosadiene, Nonacosadiene, etc.) (Ferveur and Cobb 2010). Among the dimorphic cuticular lipids (see Supplementary Information), we found that the *D. melanogaster* males produced few monoenes (peak 7, 8, 15, 16, 19 and 43) significantly more than the females. Peak 19 (a Pentacosene isomer) that is always present in males was totally absent in females indicating a likely sex specific role. However, we also found certain monoenes (peak 20, 21, 25, 26, 27, 28 and 30) to be significantly more in the females than males. The *D. melanogaster* females almost always produced significantly more dienes (peak 22, 23, 31, 32, 33, 34 and 41) than the males in our study (only exception being peak 14/ (Z,Z)-7,11-Tricosadiene). In our *D. melanogaster* populations, Peak 33 (9,13-Heptacosadiene) was generally absent from male chromatograms but were found in most females. Apart from Peak 19 and Peak 33, all other peaks appeared consistently in all female samples while except Peak 33 and Peak 39 (14-

Methyloctacosane), all peaks appeared consistently in all male chromatograms (see Supplementary Information).

Effects of selection regime, sex and replicate on the cuticular lipids of D. melanogaster populations under differential sexual conflict. We considered 37 peaks out of 44 cuticular lipids for the linear mixed effects analysis. Peak 15, 25-29 and 42 were ambiguous since they often appeared as more than one peak within their retention time in many samples. We assigned Model 1 for most cuticular lipids while 6 cuticular lipids were assigned Model 2 as the best fit model based on the lower AIC value. Even in case of these 6 cuticular lipids, Model 2 outperformed Model 1 on a thin difference of AIC varying between 0.008-4.38 (exception being Peak 36: difference in AIC between Model 1 and Model 2 was 28.52). This indicates that variables with random effects played an insignificant role in influencing the cuticular lipid profiles of *D. melanogaster* M, C and F populations. When we compared the standard deviation of variable(s) with random effects to that of the residual for each peak (that were in itself close to 0 in all cases), we found that the standard deviation of residual was always more (in many cases, order of magnitude more) than the standard deviation of variable(s) with random effects for all peaks under consideration (see Supplementary Information for detailed results). This means block or its interactions are not substantial than other unaccounted random effects and confirms that there is no effect of common ancestry of the replicates of the selection lines on the cuticular lipid profiles of the M, C and F populations. The sex of the sampled *D. melanogaster* individuals is the most important variable affecting the cuticular lipid profiles of the M, C and F populations (see Table 1) as is seen in the *T test* (see Supplementary Information). The variables with fixed effects such as selection regime and its interaction with sex of the individuals also affect a few cuticular lipids (see Table 1). Only two cuticular lipids (Peak 2 and Peak 10) are not affected by any of the explanatory variables. Since this test failed to give us any indication

290 whether replicates of M were more diverged in terms of cuticular lipids than that of the F
291 populations, we subsequent performed multivariate analysis to investigate whether cuticular
292 lipid bouquet resulted in grouping among M populations compared to that of the F
293 populations.

Peak ID	Selected model	Selection regime	Sex	Selection regime x Sex	Block
Peak 1	Model 1	No effect	No effect	p = 0.04237 *	No effect
Peak 2	Model 1	No effect	No effect	No effect	No effect
Peak 3	Model 1	p = 0.03458 *	p < 2e-16 ***	No effect	No effect
Peak 4	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak 5	Model 1	No effect	No effect	p = 0.01148 *	No effect
Peak 6	Model 1	No effect	No effect	p = 0.004846 **	No effect
Peak 7	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak 8	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak 9	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak 10	Model 2	No effect	No effect	No effect	No effect
Peak 11	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak 12	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak 13	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak 14	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak 16	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak 17	Model 2	No effect	p = 2.285e-08 ***	No effect	No effect
Peak 18	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak 19	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak 20	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak 21	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak 22	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak 23	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak 24	Model 1	p = 0.004373 **	No effect	p = 0.007562 **	No effect
Peak 30	Model 2	No effect	p < 2e-16 ***	p = 0.008085 **	No effect
Peak 31	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak 32	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak 33	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak 34	Model 1	p = 0.01104 *	p < 2e-16 ***	p = 0.02210 *	No effect
Peak 35	Model 1	No effect	p = 0.03364 *	No effect	No effect
Peak 36	Model 2	No effect	p = 2.745e-06 ***	No effect	No effect
Peak 37	Model 1	No effect	No effect	p = 0.003142 **	No effect
Peak 38	Model 2	p = 0.0007705 ***	p = 0.0093053 **	No effect	No effect
Peak 39	Model 2	No effect	p < 2e-16 ***	No effect	No effect
Peak 40	Model 1	No effect	p < 2e-16 ***	No effect	No effect
Peak_41	Model 1	p = 0.043603 *	p < 2e-16 ***	p = 0.009622 **	No effect

Peak_43	Model 1	p = 0.01067 *	p < 2e-16 ***	No effect	No effect
Peak_44	Model 1	No effect	p < 2e-16 ***	p = 0.00130 **	No effect

Table 1**:** Influence of variables with fixed effects (selection line, sex and their interactions) and random effects (block and its interaction with other variables) on 37 cuticular lipids of *Drosophila* M-C-F populations under linear mixed effect modelling framework. Model with lower AIC was selected for each cuticular lipid. Those variables with statistically significant effect have been assigned a p-value (p) based on *Type II Wald chi-square* tests on variables with fixed effects. Effect of replicates from selection regime (block) was assessed by comparing the standard deviation of block with that of the residual random effects (see Supplementary Information). We also performed *MANOVA* with *Pillai's test* on the 37 cuticular lipid peaks that showed all peaks individually as well as together contributes significantly (Pillai statistic = 5.67, F (646, 5321) = 4.12, p < 0.0001) to differences between the sexes and among the blocks of all 3 *Drosophila* selection regime (M, C and F) (see Supplementary Information).

Divergence patterns of cuticular lipids in D. melanogaster populations under differential sexual conflict. We used the same 37 cuticular lipids from linear mixed effects analysis for multivariate analysis using Random Forest. As indicated in the table from Supplementary Information, MDS of proximity matrix showed sexes formed distinct groups in *D. melanogaster* M, C and F populations with 100 % correct sex identification in random forest analysis. In C population, Random Forest analysis produced 98.3% correct prediction of individuals belonging to 3 different replicates. It is important to note that the triplicates of C are maintained at unit sex ratio and are derived from LH_{st} while M and F populations are derived from the C replicates (see Methods section for details). Therefore, the divergence pattern of C in Fig. 1 (see Supplementary Information for 3D representation) can be considered as the ancestral pattern and is subsequently used as

reference pattern to M and F populations. The cuticular lipid divergence pattern in Fig. 2 (see Supplementary Information for 3D representation) suggests that the M females are more dispersed than the M males that are tightly grouped (95 % correct identification of M individuals from 3 replicate populations). In comparison to C population, M males appear to have evolved cuticular lipid profiles which are more similar to each other while M females have either evolved a more variable cuticular lipid profile or maintained their cuticular lipid profile variability as seen in C females. In F population (Fig. 3 (see Supplementary Information for 3D representation)), we see an opposite trend where the F males are dispersed more than the F females that form tight group (93 % correct identification of F individuals belonging to 3 replicate populations). Comparison with C females suggests that F females have evolved identical cuticular lipid profiles while F males have either increased or maintained their variable cuticular lipid profile as seen in C males. Corresponding 3-dimensional scatterplot representations for Fig. 1, Fig. 2 and Fig. 3 are provided in the supplementary information.

Fig. 1 About here.

Fig. 2 About here.

Fig. 3 About here.

When we compare male cuticular lipid profile of M and F replicates, Random Forest analysis predicted 89% correct identification of the $M_{1,2,3}$ and $F_{1,2,3}$ males (see Fig. 4). In Fig. 4, males of M replicates formed separate groups with few overlaps among themselves. However, F replicates did not group separately from the M replicates as expected but overlapped with corresponding M replicates. In Random Forest analysis involving females of M and F replicates, we could identify $M_{1,2,3}$ and $F_{1,2,3}$ females with 87.1% correct prediction (see Fig.

340 5). Fig. 5 revealed that M females from different replicates formed separate groups with few
341 overlaps. We again find a considerable overlap of F replicates on corresponding M replicates.

342 Fig. 4 About here.

343 Fig. 5 About here.

344

DISCUSSION

Divergent reproductive behavior in high sexual conflict regimes of allopatric MCF populations evolved over 105 generations as a byproduct of sexually antagonistic coevolution within each M replicates (Syed et al. 2017). Since reproductive behavior did not diverge in F replicates under low sexual conflict, we predicted that male and female cuticular lipid profiles of the three replicates within the M regime should be more divergent from each other compared to the differences in the cuticular lipid profiles of the three replicates within the F regime (assuming the putative chemical cues such as the cuticular lipids form the basis of divergent mate choice in M). Random Forest analysis (see Fig. 4) 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. The same divergence pattern is also reflected in the females of M and F replicates (see Fig. 5). Such cuticular lipid divergence pattern indicates that sexual conflict has little or no role in reproductive isolation via male cuticular lipid differentiation within M and F regimes that have been under high and low sexual conflict, respectively, for around 170 generations at the time of this study. Rather the cuticular lipid divergence in M and F regime appears to follow an inexplicable pattern. There are two possible reasons for the block-wise cuticular lipid divergence pattern that we see in the replicates of M and F sexes. The cuticular lipid divergence pattern of M and F males and females (Fig. 4 and Fig. 5) may be construed as a result of handling effect since extraction process spanned over three days (for three blocks) and repeated at two different times (see Methods section for details). Using Random Forest analysis on normalized and non-normalised data, we tried to address this issue. However, congruence in cuticular lipid divergence patterns from these two treatments of cuticular lipid peak values (see Supplementary Information for cuticular lipid divergence pattern using non-normalised dataset) indicate that there was no significant effect of handling during the extraction process done over three days at two different

370 times. Ferveur and Cobb (2010) suggest that the variability in the cuticular lipid profiles of
371 different populations under different selection pressure (as is observed in this study) is possibly
372 due to different initial and undergoing evolutionary mechanism. Alternatively, the divergence
373 pattern of M and F males and females (Fig. 4 and Fig. 5) could be a result of ancestry. However,
374 since M, C and F populations were established using a large population size (N_e (LH_{st}) ~ 2250,
375 N_e (MCF) ~ 450; (Nandy et al. 2013b)), a founder effect during initial establishment is unlikely
376 to affect the MCF populations. This is also confirmed in the linear mixed modelling analysis
377 which shows effect of replicates on the cuticular lipid profile is negligible, if any in many cases
378 (see Table 1). In conclusion, reproductive isolation (in M regime but not in F regime (Syed et
379 al. 2017)) as a byproduct of sexually antagonistic coevolution cannot be explained by the
380 cuticular lipid divergence among males and females of *Drosophila melanogaster* M and F
381 regimes when analysed separately. However, cuticular lipid divergence in M and F at regime
382 level indicate other possible evolutionary mechanisms at play that are discussed below.

383 Isolation among M replicates in form of diverged mate preference could possibly be maintained
384 by sexual selection acting on mating signals (Syed et al. 2017). Since cuticular lipid profiles of
385 *D. melanogaster* MCF populations are dimorphic, one possible mechanism for this
386 reproductive isolation among M replicates could be mate choice over the divergent cuticular
387 lipid profiles. In this study, sexual selection appears to have particularly strong effect on the
388 cuticular lipid profiles in a different way at the regime level (see Fig. 1, Fig. 2 and Fig. 3). If
389 we compare the cuticular lipid profiles of males and females of C regime and M regime, we
390 see that M females have diverged with respect to C females while M males have converged
391 with respect to C males considerably. This pattern indicates that under increased sexual
392 selection, M males are pushed towards a common optimum point in terms of cuticular lipid
393 profile in order to present a more preferable cuticular lipid profile to M females. Similar
394 cuticular lipid profiles of the M males may also be explained by suppression of sexually

selected cuticular lipids such that variability of male cuticular lipid is reduced over time. This may be true since *D. serrata* males have been found to lower their cuticular lipid expression when female : male ratio is decreased (Gershman and Rundle 2017). The female cuticular lipid divergence of M could either be a result of maintaining ancestral pattern as seen in C or have diverged as predicted in populations following Buridan's ass regime (see below). On the contrary, F males and F females show a diametrically opposite pattern to that of the M regime. Under low sexual selection, the selection pressure is possibly weaker and hence, F males diversified and/or maintain their cuticular lipid variation as seen in the C regime. *Drosophila serrata* males exhibit cuticular lipid profile associated with increased mating success when female : male ratio is increased (Gershman and Rundle 2017). In F regime where female : male is high, males may evolve to display their most attractive cuticular lipids to potentially improve mating success leading to varied cuticular lipid profiles in the process. The F females appear to be more tightly grouped in terms of their cuticular lipid profile (in comparison to M females) than C population. This indicates that F females are probably under selection pressure to maintain similarity of their cuticular lipid profiles. The reason for this is not obvious and may be a result of correlated evolution in some other trait affected by sexual selection. Alternatively, F females may also be affected by high sexual selection (increased competition) due to altered sex ratio where they have to maintain identical profile to be acceptable to males that are fewer in number. Overall the cuticular lipid divergence pattern of M and F regime appears to be strongly influenced by sexual selection although other possible sexual conflict resolution mechanism such as Buridan's ass paradigm (see below) cannot be ruled out.

Apart from speciation, a population under sexual conflict may follow other evolutionary stable strategy to resolve the conflict between sexes (Gavrilets 2014). For example, in a large population and/or population with significant rates of mutations, there might be diversification of female traits with respect to male traits (Buridan's ass regime) such that males, in an attempt

to adapt to the variability in such traits, are caught in limbo unable to increase its own fitness in the population as a whole (Gavrilets and Hayashi 2005). This is relevant to the results from the current study where multivariate analysis show M females are more dispersed and M males form a tight group with respect to their cuticular lipid profile (see Fig. 2). It is known that the male mating behavior of *Drosophila melanogaster* is partially mediated by female cuticular lipids (Ferveur and Cobb 2010). The M population also meet the criterion for Buridan's ass regime since the population size is large ($N_e \sim 450$) and the block-wise differences among M replicates (see Fig. 4) suggest that possibility of random mutation accumulation over several generations due to allopatry. It is therefore possible that sexual conflict on cuticular lipid trait in M regime has followed Buridan's ass phenomenon where M males have been caught in an uncertain intermediate stage failing to pursue either ends of cuticular lipid divergence in M females. The fact that this pattern is repeated in case of females of F regime is a revelation since effect of sexual conflict has been traditionally focused on males rather than females (Ferveur and Cobb 2010).

CONCLUSION

Experimental studies and comparative analysis of taxonomic groups suggest that speciation is possible under sexual conflict. The mathematical models support this possibility but also suggest that dynamics of evolution under sexual conflict is quite varied and stochastic to a point that evolution of reproductive isolation in allopatry is only one of the several other outcomes. This study is an example of different reproductive traits being affected differently by sexual conflict such that isolation based on mate preference, copulation duration and sperm defense ability is not reflected in the mate attraction cues such as cuticular lipids. Rather sexual conflict, if any, is probably reconciled in form of Buridan's ass regime. In fact, *D. melanogaster* individuals from high and low sexual conflict regime appear to be significantly affected by

444 sexual selection on cuticular lipid profiles. We conclude that since cuticular lipids form a basis
445 for mate recognition, M males under high sexual selection tend to keep their cuticular lipid
446 profiles similar to be acceptable by the limited number of females in the male biased regime.
447 The same may also be true for the females of female biased regimes or their cuticular lipid
448 divergence pattern may be by-product of sexual selection pressure on any other hitherto
449 unknown trait.

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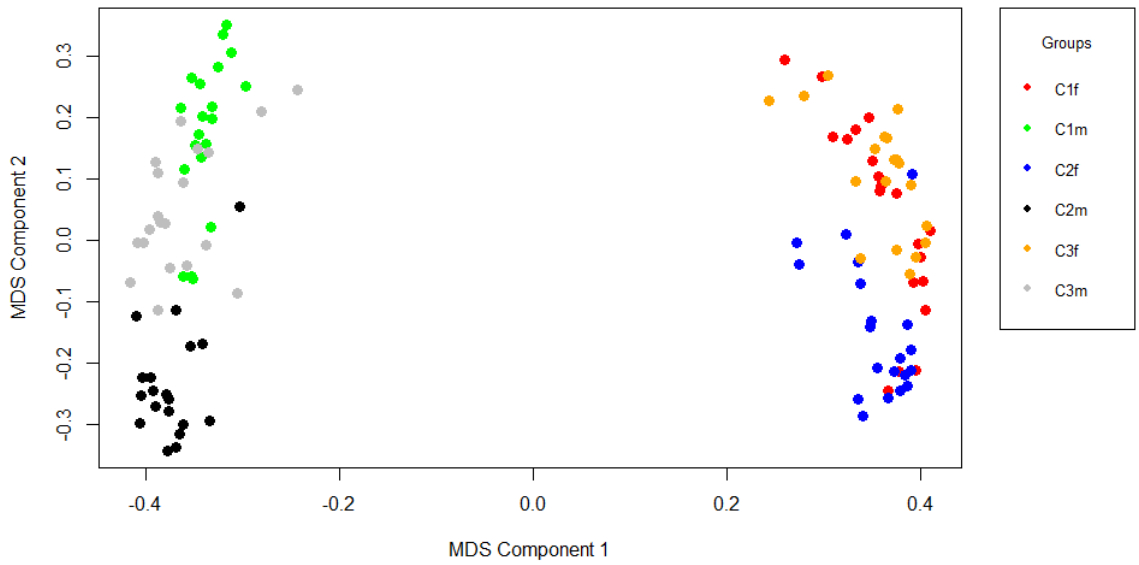
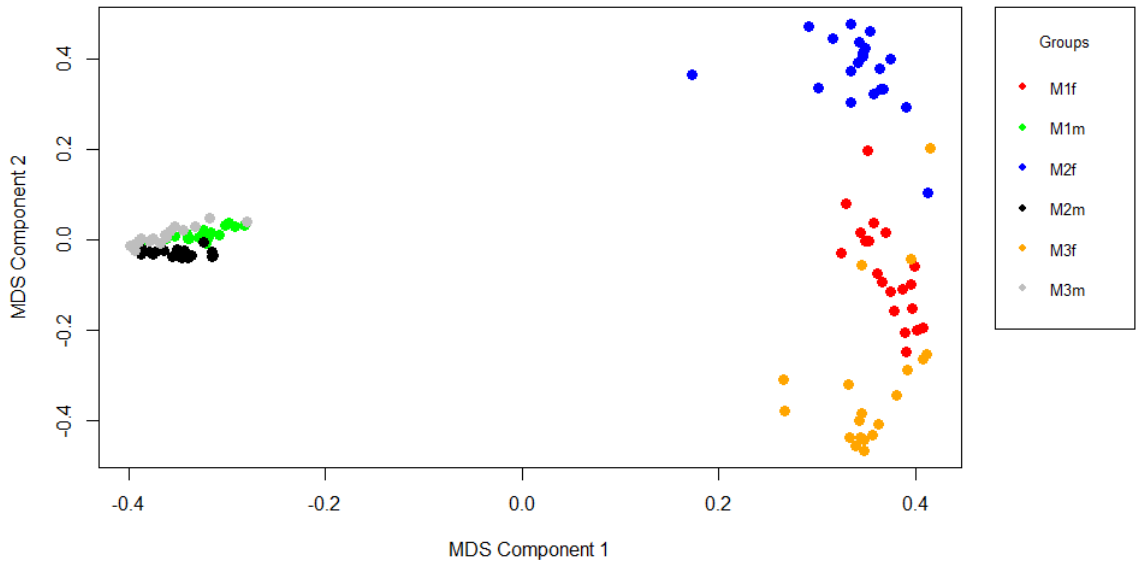


528

529 Fig. 1 Multi-dimensional scaling (MDS) of the proximity matrix of C individuals ($C_{1/2/3}$ are C

530 blocks, m= males and f= females)



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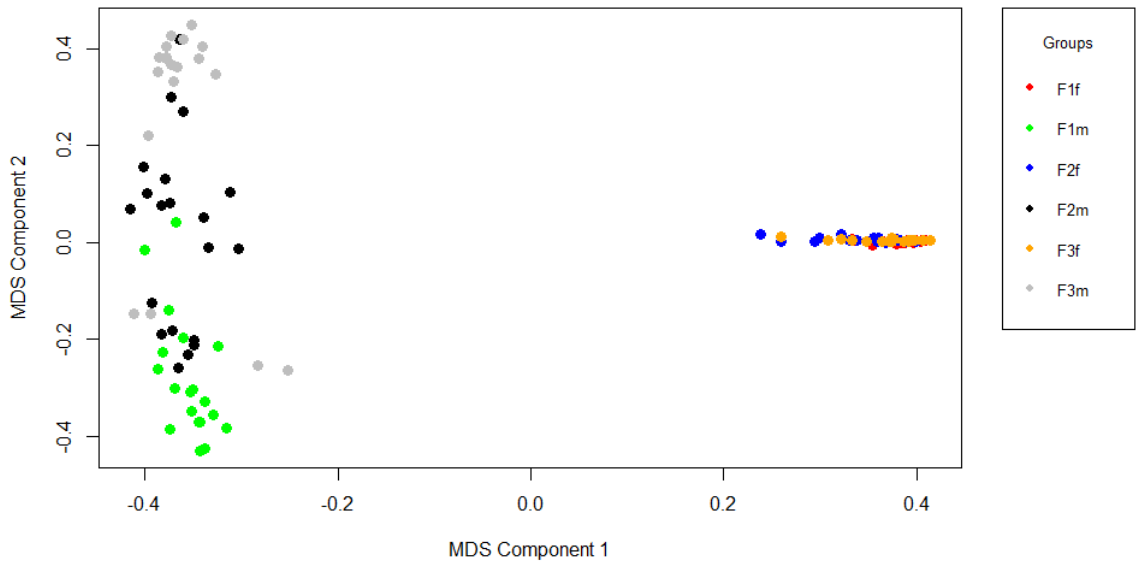
532 Fig. 2 Multi-dimensional scaling (MDS) of the proximity matrix of M individuals ($M_{1/2/3}$ are

533 M blocks, m= males and f= females)



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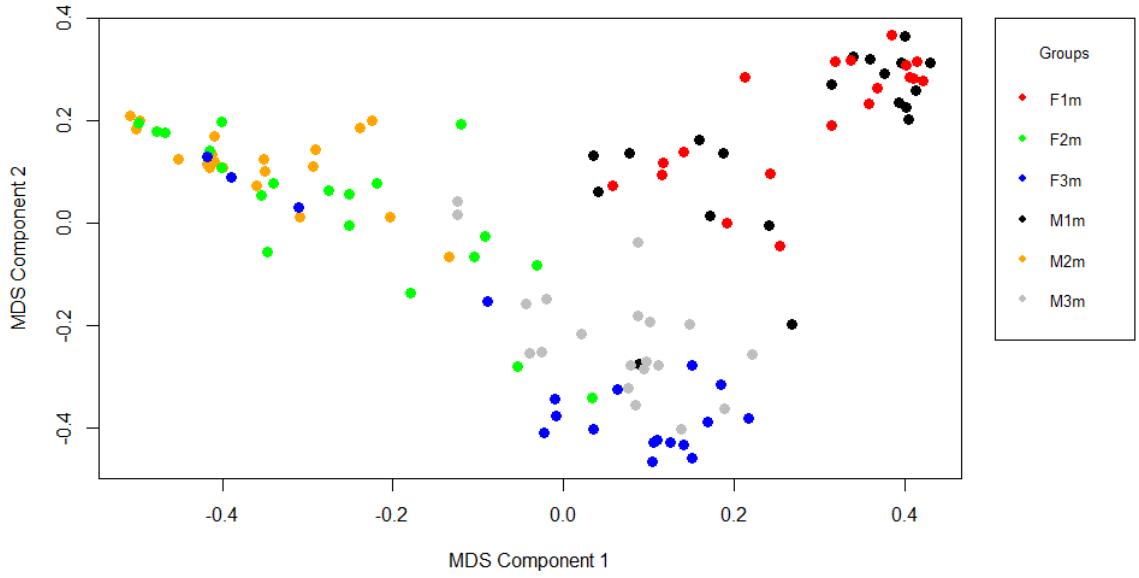
535 Fig. 3 Multi-dimensional scaling (MDS) of the proximity matrix of F individuals ($F_{1/2/3}$ are F

536 blocks, m= males and f= females)



537

538 Fig. 4 MDS of proximity matrix derived from Random Forest analysis of males from three M

539 and F replicates

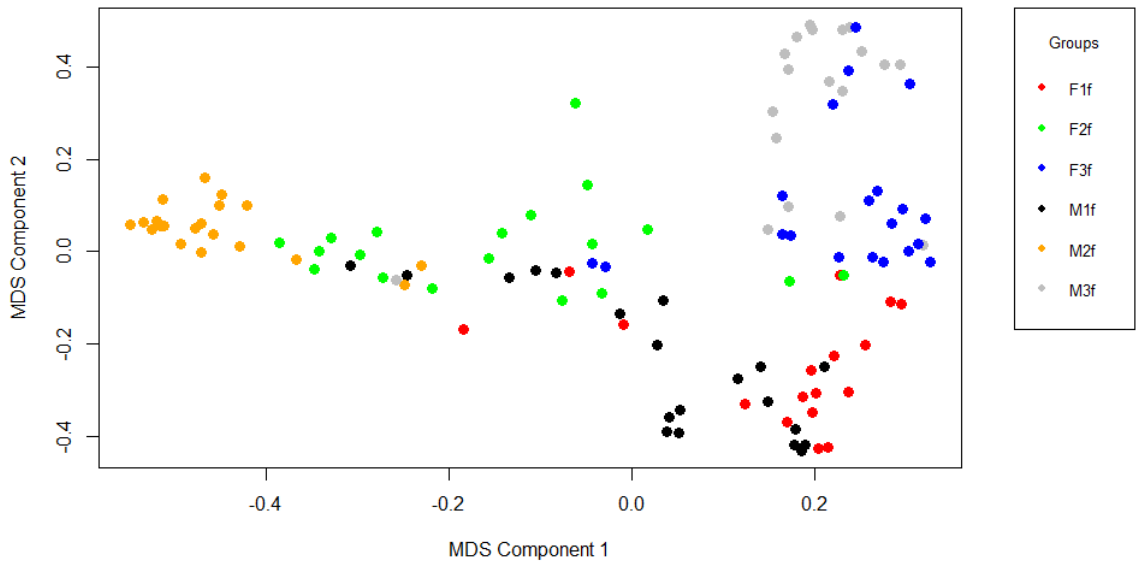


Fig. 5 MDS of proximity matrix derived from Random Forest analysis of females from three M and F replicates