Genetic divergence and phenotypic plasticity contribute to variation in cuticular hydrocarbons in the seaweed fly *Coelopa frigida*

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ABSTRACT

Male sexual signals facilitate female choice and thereby reduce mating costs. The seaweed fly *Coelopa frigida* is characterized by intense sexual conflict, yet it is unclear which traits are under sexual selection. In this study, we investigated phenotypic and genetic variation in cuticular hydrocarbons (CHCs) in *C. frigida* for the first time. CHCs are ubiquitous in insects and they act as sex pheromone in many dipterans. We characterized CHCs and found that composition was affected by both genetic (sex and population origin) and environmental (larval diet) factors. We identified general shifts in CHC chemistry as well as individual compounds and found that the methylated compounds, mean chain length, proportion of alkenes, and normalized total CHCs differed between sexes and populations. We combined this data with whole genome re-sequencing data to examine the genetic underpinnings of these differences. We identified 11 genes related to CHC synthesis and found population level outlier SNPs in 5 that are concordant with phenotypic differences. Together these results reveal that the CHC composition of *C. frigida* is dynamic, strongly affected by the larval environment, and likely under natural and/or sexual selection.

Keywords: cuticular hydrocarbons, *Coelopa frigida*, sexual conflict, sexual selection, population differentiation, diet.

INTRODUCTION

Females and males often differ in optimal reproductive strategies, particularly regarding the mode and frequency of matings. Males can increase their fitness by mating with multiple females, while one or a few matings are generally sufficient for females to maximize their reproductive success (Bonduriansky, 2010). This sexual conflict over mating rates commonly leads to males harassing females to increase the likelihood that their genes get passed on to the next generation (Sánchez‐Guillén *et al.*, 2017). Such male mating harassment can carry considerable costs for females, e.g. by reducing reproductive success and increasing mortality (Gosden & Svensson, 2007). For example, repeated matings are the norm in the blue-tailed damselfly *Ischnura elegans*, particularly when population densities are high (Corbet, 1999), with females showing elevated immune responses, possibly because of mating related injuries or sexually transmitted diseases (Chauhan *et al.*, 2016). Likewise, in the guppy *Poecilia reticulata,* males attempt to copulate with females by thrusting their gonopodium towards the female's genital pore as often as once every minute, disrupting normal activities like foraging (Magurran & Nowak, 1991).

Females can minimize the cost of unwanted copulations by making it difficult for males to detect them (as seen in species with female limited colour polymorphism, Wellenreuther *et al.*, 2014) or evolving mate rejection responses (Andersson, 1994). However, these responses often carry associated costs (Watson *et al.*, 1998) and females therefore frequently endure costly matings with males when the costs of rejection

43 outweigh the costs of mating. Mating interactions can further be modulated by the
44 evolution of divergent inter-and intraspecific mate preferences. Mate preferences in
45 females, for example, can allow them to obtain high quality or compatible genes from a
46 male, with indirect benefits that include improved offspring fecundity, survival and
47 immune response (Neff & Pitcher, 2005). In many solitary and social insects, cuticular
48 hydrocarbons (CHCs) are used as one of the primary cues to recognize, and possibly
49 discriminate between species, sexes, and among kin (Bagneres *et al.*, 1996; Ferveur,
50 2005; Blomquist & Bagnères, 2010; Van Oystaeyen *et al.*, 2014).

51

52 Cuticular hydrocarbons are thought to be a primary adaptation to life on land because
53 they protect insects against desiccation (Wigglesworth, 1945; Blomquist & Bagnères,
54 2010). *De novo* synthesis of CHCs is conserved across insects (Howard & Blomquist,
55 2005; Blomquist & Bagnères, 2010): Synthesis takes place in oenocytes and starts with
56 acetyl-CoA. A fatty acid synthase (FAS) then elongates the acetyl-CoA, forming a long-
57 chained fatty acyl-CoA. FASs may also add methyl groups to these fatty acids (de
58 Renobales *et al.*, 1986; Blomquist *et al.*, 1994; Juarez *et al.*, 1996; Chung *et al.*, 2014).
59 Elongases then further lengthen these fatty acyl-CoAs and double bonds or triple bonds
60 are added by desaturases (Howard & Blomquist, 2005; Blomquist & Bagnères, 2010).
61 These are reduced to aldehydes by reductases and then these aldehydes are converted to
62 hydrocarbons by a decarboxylation reaction, which is mediated by a cytochrome P450
63 (Qiu *et al.*, 2012). Coding or expression variation in any number of these enzymes can
64 lead to significant changes in CHC composition (ex: de Renobales *et al.*, 1986; Reed *et*
65 *al.*, 1995; Qiu *et al.*, 2012; Chung *et al.*, 2014; Chen *et al.*, 2016).

CHC composition is affected by both genetic and environmental factors. Changes in gene expression (Dallerac *et al.*, 2000a; Chertemps *et al.*, 2007; Shirangi *et al.*, 2009; Feldmeyer *et al.*, 2014) and/or coding sequences (Keays *et al.*, 2011; Badouin *et al.*, 2013; Kulmuni *et al.*, 2013) can affect both the overall composition of CHCs and the length of specific compounds. Multiple environmental factors have also been shown to influence CHC composition. For example, CHCs can vary under different temperature regimes (Toolson & Kupersimbron, 1989; Savarit & Ferveur, 2002; Rouault *et al.*, 2004), diets (Stennett & Etges, 1997; Liang & Silverman, 2000; Stojkovic *et al.*, 2014) or climatic variables (Howard *et al.*, 1995; Kwan & Rundle, 2010). Moreover, multiple studies have found that CHCs vary between natural populations (ex: Haverty *et al.*, 1990; Etges & Ahrens, 2001; Frentiu & Chenoweth, 2010; Perdereau *et al.*, 2010), but it is unknown how much of this variation is due to plasticity versus genetic divergence. These can be teased apart using laboratory studies: genetic divergence can be assessed by raising different populations in the same environment and plasticity can be assessed by raising the same population in multiple environments (i.e. common garden and reciprocal transplant experiments).

The seaweed fly *Coelopa frigida* is a common inhabitant of "wrackbeds" (accumulations of decomposing seaweed) on shorelines. These wrackbeds act as both habitat and food source for larvae and adults of this species. Fitness trade-offs and local adaptation in response to wrackbed composition have been demonstrated in Swedish *C. frigida* (Wellenreuther *et al.*, 2017). The mating system in *C. frigida* is characterized by intense

89 sexual conflict. Males will approach and forcefully mount females. Females are reluctant
90 to mate and use a variety of responses to dislodge the male, such as downward curling of
91 the abdomen (to prevent contact), kicking of the legs, and shaking from side to side (Day
92 *et al.*, 1990; Blyth & Gilburn, 2011). Although there is no male courtship of any kind, it
93 has been consistently reported that mating attempts by large males are more likely to
94 result in copulation (Butlin *et al.*, 1982; Gilburn *et al.*, 1992; 1993). Two lines of
95 evidence show that females are actively choosing larger males rather than "giving up"
96 their struggle due to exhaustion. First, under "giving up" there should be a negative
97 correlation between struggle time and the size differential between the male and female.
98 However, there is absolutely no correlation between these factors (Edward, 2008).
99 Second, if females only allow copulation when they have been exhausted then struggle
100 times should differ between mountings in which copulation occurs and mountings where
101 copulation does not occur. However, there is no significant difference and a slight trend
102 towards longer struggle times for interactions that do not end in copulation (Edward,
103 2008). To date, the mechanism by which female choice occurs is currently unknown in
104 this system.

105

106 Here we investigate cuticular hydrocarbons as a possible male mating signal to test
107 whether this may be involved in female choice in *C. frigida.* Specifically, we examine if
108 CHC signatures vary between sexes, populations, and larval environment/diet
109 (wrackbed). We also explore putative genes involved in CHC synthesis and look for
110 correlations between genetic and phenotypic variation. We discuss our findings in light of

sexual selection in this species and others and outline future research areas that deserve attention.

METHODS

Study Species

Coelopa frigida belongs to the group of acalyptrate flies which exclusively forage on decomposing seaweed (Cullen *et al.*, 1987). This species is found along the seashores of Northern Europe (Mcalpine, 1991), plays a vital role in coastal environmental biodiversity (Griffin *et al.*, 2018) and health by accelerating the decomposition of algae, allowing for faster release of nutrients (Cullen *et al.*, 1987). Additionally, larvae are an important food source for predatory coastal invertebrates and seabirds (Summers *et al.*, 1990; Fuller, 2003).

Sampling

Fly larvae were collected in April and May 2017 from two Norwegian populations, namely Skeie (58.69733, 5.54083) and Østhassel (58.07068, 6.64346), and two Swedish populations, namely Stavder (57.28153, 12.13746) and Ystad (55.425, 13.77254). These populations are situated along an environmental cline in salinity, wrackbed composition, and wrackbed microbiome (Berdan et al., in prep; Day *et al.*, 1983) from the North Sea to the Baltic Sea. Larvae were transported to Tjärnö Marine Laboratory of the University of Gothenburg where they completed development in an aerated plastic pot filled with their own field wrack in a temperature controlled room at 25 °C with a 12h/12h light-dark cycle. After adults eclosed in the lab, they were transferred to a new pot filled with

132 standard wrack consisting of 50 % *Fucus spp*. and 50 % *Saccharina latissima* which had
133 been chopped, frozen, and then defrosted. The exception to this is a subset of Ystad
134 adults that were allowed to mate on the same material they had been collected in (field
135 wrack). Thus we had 5 treatment groups: All four populations (Skeie, Østhassel, Stavder
136 ,Ystad) on standard wrack, and Ystad also on field wrack. Adult flies were allowed to lay
137 eggs on the provided wrack and this second generation was raised entirely in a
138 temperature controlled room at 25 °C with a 12 h/12 h light dark cycle. As larvae pupated
139 they were transferred to individual 2 mL tubes with a small amount of cotton soaked in
140 5 % (w/v) glucose to provide moisture and food for the eclosing adult. This ensured
141 virginity in all eclosing flies. The tubes were checked every day and the date of eclosure
142 of each fly was noted. Two days after eclosure adult flies were frozen at -80 °C and kept
143 there until CHC extraction.

144 CHC Analysis

145 Frozen flies were placed in 12-well porcelain plates to defrost and dry, as moisture can
146 affect lipid dissolution. Each fly was then placed in a 1.5 ml high recovery vial
147 containing 300 µl of *n*-hexane, vortexed at a low speed for 5 second, and extracted for 5
148 minutes. Afterwards flies were removed from the vial and allowed to air dry on the
149 porcelain plates before they were weighed (Sartorius Quintix 124-1S microbalance) to
150 the nearest 0.0001 grams and sexed. Extracts were evaporated until dry under nitrogen
151 gas and then stored at -20°. Before analysis 20 µl of *n*-hexane containing 1 µg/ml *n*-
152 nonane was added as an internal standard to each vial, which was then vortexed at
153 maximum speed for 10 seconds.

154 The extracts from 20 male and 20 female flies from every treatment group were analyzed
155 on a GC (Agilent GC 6890) coupled to a MS (Agilent 5973 MSD) using a HP-5MS
156 capillary column (Agilent) (see Table S1 in supplementary materials for instrument
157 settings).

158 The total ion chromatograms were quality checked, de-noised and Savitzky-Golay
159 filtered before peak detection and peak area integration in OpenChrom®. Peaks were
160 then aligned using the R-package 'GCalignR'(Ottensmann, Stoffel, & Hoffman, 2017)
161 prior to statistical analysis. Peaks were tentatively identified by their mass spectra and
162 retention time index based on a C21-C40 *n*-alkane standard solution (Sigma-Aldrich).

163 Statistical Analysis

164 The effects of sex and population on CHC profile were assessed using flies raised on
165 standard wrack using a balanced sampling design (N=13 for each combination) and 127
166 peaks were identified by the R package "GCalignR". The effects of sex and diet (i.e.
167 wrack type) during the larval stage on CHC profiles were analyzed using a balanced
168 design of flies from the Ystad population (N=12 for each combination) and 111 peaks
169 were identified by R package "GCalignR". Peak areas were normalized on the peak area
170 of the internal standard and based on the weight of the fly before being auto-scaled prior
171 to statistical analysis. Clustering of samples was visualized with a PCA, and group
172 differences were analyzed using a PERMANOVA followed by multiple group
173 comparisons using the PRIMER-E 7 software. We also analyzed group differences via
174 (O)PLS-DA using the "ropls"-packages in R (Thevenot, Roux, Xu, Ezan, & Junot, 2015).
175 Candidate compounds for group differentiation were determined from variables of

importance for OPLS-DA projection (VIP scores) (Galindo‐Prieto *et al.*, 2014), which were further assessed by univariate analysis using a false discovery adjusted significance level of $\alpha = 0.05$ using the Benjamini and Hochberg's FDR-controlling procedure (Benjamini & Hochberg, 1995).

We examined sex and country effects in more depth to examine shifts in chemistry that could be linked to our genetic data (see below). Our results above indicated that the differences in CHC profiles between sexes and populations of *C. frigida* were due to quantitative rather than qualitative differences (i.e. changes in relative amounts rather than presence/absence of certain compounds). Thus, we looked for general shifts in CHC chemistry by examining 1. The total normalized peak area (i.e. the sum of all peaks used in analysis), 2. The proportion of alkenes, and 3. The proportion of methylated compounds. For #1 we used all 127 peaks used in the previous analysis and for #2 and #3 we used the subset of these peaks that could be accurately categorized (116 peaks). For the total peak area we transformed the data with a log transformation and then used the 'dredge' function from the MuMIn package (Barton, 2017) to compare AIC values for nested glm (Gaussian distribution, link=identity) models with the terms sex, country, and country*sex. If there were two models that differed in AIC by less than 1, we chose the simpler model. For alkenes and methylated compounds our data was proportional and did not include 1 or 0 so we used the 'betareg' function from the betareg package (Grun *et al.*, 2012). We again used the 'dredge' function to compare AIC values for nested models with the terms sex, country, and country*sex and took the simpler model in case of an AIC difference of less than 1.

199 We also analysed the distribution of chain lengths in our samples. To do this, we
200 subsetted all peaks where we had accurate length information (107 peaks) and then
201 calculated the weighted mean chain length as

202 $$N = \Sigma\, l_i p_i$$

203 where l_i is the length of a carbon chain and p_i is the proportional concentration of all
204 chains of that length. We also calculated dispersion around this mean as

205 $$D = \Sigma\, p_i (l_i - N)^2$$

206 We analyzed these in the same manner as described above, using AIC values to conduct
207 model choice from glm models (Gaussian distribution, link=identity).

208 Candidate gene analysis

209 Based on a literature search for genes involved in CHC synthesis, we identified 37
210 reference protein sequences, which were downloaded from Flybase (flybase.org) in
211 October 2017. We used tblastx with the default settings to identify orthologs in the *C.*
212 *frigida* transcriptome (Berdan & Wellenreuther, unpublished). We then used gmap (Wu
213 & Watanabe, 2005) to find areas of the *C. frigida* genome (Wellenreuther, *et al*.,
214 unpublished) that might contain these genes. We output all alignments as a gff3 file. We
215 have a previously made a VCF file created from 30x whole genome re-sequencing of 46
216 *C. frigida* across five populations in Scandinavia (Berdan *et. al*, unpublished). This VCF
217 file was made by aligning raw reads to the *C. frigida* genome using bwa-mem (Li &
218 Durbin, 2010), duplicate reads were marked using 'picard' (http://picard.sourceforge.net).
219 SNPs were called with the Genome Analysis Toolkit (GATK; DePristo *et al.* 2011; Van

220 der Auwera *et al.* 2013) specifically the GATK-module 'UnifiedGenotyper' (Van der
221 Auwera *et al.* 2013). VCF filtering was done as described in Berdan *et al.* 2015. We used
222 this VCF file and the gff3 output from gmap in SNPeff (Cingolani *et al.*, 2012) to
223 annotate the SNPs in these genes.

224

225 To find which SNPs are diverged between populations we used vcftools (Danecek *et al.*,
226 2011) to calculate Cockerham and Weir's F_{ST} estimate (Weir & Cockerham, 1984) for all
227 SNPs in our reference genome. We retained the top 5% of this distribution ($F_{ST} \geq 0.142$,
228 mean F_{ST} 0.024) as SNPs that were potentially divergent. We then subset the VCF file to
229 include only outlier SNPs located in our previously identified genomic regions.

230 RESULTS

231 CHC analysis

232 A diverse blend of hydrocarbons with more than 100 different compounds were found in
233 the cuticular extract of *C. frigida*. The majority of CHCs ranged in chain length from 23
234 to 33 carbon atoms. They contained odd numbered *n*-alkanes, methyl-, dimethyl- and
235 trimethyl-alkanes, as well as odd numbered alkenes. However, some even numbered
236 alkanes were also present but in lower quantities.

237 Sex and population effects

238 Results of the PERMANOVA indicated significant differences between females and
239 males and between populations but also a significant interaction between sex and
240 population (Table 1A). Pairwise tests on the interaction showed a significant difference

241 between males and females for all populations except Ystad (Table S2). Multiple
242 comparisons of populations showed that the Norwegian populations (Skeie and
243 Østhassel) were similar while the Swedish populations (Ystad and Stavder) differed for
244 males but not for females. In general the Norwegian populations tended to differ from the
245 Swedish and a similar pattern was also shown in the results of the PCA and PLS-DA
246 (Figure 2A, C, Table S3). After combining the Swedish and Norwegian populations, the
247 PERMANOVA indicated highly significant differences between females and males as
248 well as between Sweden and Norway (Table 1B). OPLS-DA models for both sex and
249 country were significant and lead to separate clusters of females and males, and Sweden
250 and Norway, respectively (Figure 2B, D, Table S3). The OPLS-DA model revealed 41
251 peaks that contributed with a VIP score >1 to the differentiation between females and
252 males. Of these, 31 were also significant in t-tests with a FDR adjusted p-values (Table
253 2). Generally compounds were more abundant in males with the exception of only 3
254 peaks that were more abundant in females. Looking at country differentiation between
255 Swedish and Norwegian populations we found 43 compounds with a VIP score >1, of
256 which 29 were significant in t-tests with FDR adjusted p-values and consisted of an equal
257 mix of compounds more abundant in either of the countries (Table 2). Differences in
258 CHC profiles between sexes and populations were due to quantitative differences,
259 affecting a wide range of compounds instead of qualitative differences, i.e. the presence/
260 absence of a few distinct compounds

261

262 <u>Sex and diet effects</u>

263 PERMANOVA demonstrated a significant difference between females and males in the
264 Ystad population and also a significant effect of the larval diet (wrack type) (Table 1C).
265 Both OPLS-DA models for either sex or wrack type were significant and lead to separate
266 clusters of females and males and of flies raised on standard or field wrack (Figure 3B,C,
267 Table S3). The OPLS-DA model revealed 38 peaks that contributed to the differentiation
268 between females and males with a VIP score >1. Of these, 24 compounds were also
269 significant in a t-test with FDR adjusted p-values (Table 2). All of these, except for 3
270 peaks, were more abundant in males. Overall 65% (26 compounds) were shared between
271 this analysis and the previous analysis investigating sex and population effects. Looking
272 at differentiation between flies raised on a diet consisting of standard or field wrack we
273 found 33 compounds with a VIP score >1. Of these, 27 compounds were significant
274 using a t-test with FDR adjusted p-values, all with higher amounts in flies reared on field
275 wrack (Table 2). Flies reared on field wrack had more CHCs per gram body weight than
276 flies reared on standard wrack (normalized totals: Females field wrack 7.96 ± 0.94,
277 females standard wrack 4.09 ± 0.47, males field wrack 11.24 ± 1.03, males standard
278 wrack 7.09 ± 1.21). Differences in CHC profiles between sexes and diets were
279 exclusively due to quantitative differences, affecting a wide range of compounds instead
280 of qualitative differences, i.e. the presence/ absence of a few distinct compounds

281

282 <u>Shifts in Chemistry</u>

283 As our results above indicated an overall effect of country rather than population we used
284 country and sex for our terms in the glm models. For total normalized peak area, the best
285 model contained only sex as a main effect (for a full AIC comparison for all models see

286 Table S4A-E). In general, males had more CHCs per gram body weight than females
287 (Figure 4A). For the proportion of alkenes, the best model contained country and sex as
288 main effects (for type II analysis of deviance tables for all best models see Table S5A-E).
289 Females tended to have more alkenes than males and individuals from Norway had more
290 alkenes than individuals from Sweden (Figure 4B). The best model for the proportion of
291 methylated compounds contained sex and country as the main effects. As with alkenes,
292 females tended to have more methylated compounds than males and individuals from
293 Norway tended to have more methylated compounds than individuals from Sweden
294 (Figure 4C).

295

296 The best model for weighted mean chain length was country and sex. Overall, males had
297 longer mean chain lengths than females and individuals from Sweden had longer mean
298 chain lengths than individuals from Norway (Figure 5A). The best model for dispersion
299 around mean chain length was also country and sex. Males had higher dispersion than
300 females and individuals from Norway had higher dispersion than individuals from
301 Sweden (Figure 5B).

302

303 Genetic analysis

304 We identified 15 isoforms from 13 transcripts in our transcriptome with matches to our
305 query proteins. These transcripts mapped to 16 unique areas of the genome assembly. We
306 blasted these areas against the NCBI nr database (accessed in April 2018) to confirm our
307 annotation. Five of these areas had either no match or matched to an unrelated protein

308 and were thus removed. The remaining genes include 2 putative fatty acid synthases, 2
309 putative desaturases, 5 putative elongases, 1 putative cytochrome P450 reductase, and 1
310 putative cytochrome P450-4G1 (Table 3). We found 1042 SNPs within these genes
311 (including 5K upstream and 5K downstream), of these 11 had an F_{ST} value > 0.142 (i.e.
312 in the top 5% of the F_{ST} distribution, Table 4).

313

314 DISCUSSION

315 In species with sexual conflict females may minimize unwanted copulations via mate
316 rejection responses; however, these responses typically carry associated costs (Watson *et*
317 *al.*, 1998). The ability to modulate mating outcomes and thus the level of male
318 harassment experienced by the female based on male traits is especially beneficial in
319 systems where no "courtship" exists. Here we explored a potential male trait, cuticular
320 hydrocarbons (CHCs), and detected considerable phenotypic CHC variation attributable
321 to sex, population, and diet and genetic variation in candidate genes involved in CHC
322 synthesis that was attributable to population. We discuss these results and their
323 consequences below.

324

325 Males and females differed in their CHC composition. Not only did the composition
326 between males and females differ (Table 2, Figure 2-5) but, in addition to that, males
327 consistently had more CHC per gram body weight than females (Figure 4A). This is a
328 strong indication that CHCs play a role in sexual communication in *C. frigida* under a
329 female choice scenario, as it is unlikely that desiccation risk would differ between males

330 and females. A role for CHCs in sexual selection in *C. frigida* would be in line with the
331 general finding that CHCs play a major role in communication in insects, specifically in
332 Diptera (Howard & Blomquist, 2005; Blomquist & Bagnères, 2010; Ferveur & Cobb,
333 2010). Differences in CHCs have been shown to be involved in a wide variety of
334 behaviours in diptera such as courtship, aggregation, and dominance (Ferveur & Cobb,
335 2010). For instance, 11-*cis*-vaccenyl acetate, a male specific hydrocarbon in *Drosophila*
336 *melanogaster*, increases male-male aggression while suppressing male mating (Wang &
337 Anderson, 2010). The same compound is also involved in aggregation behavior in both
338 *D. simulans* and *D. melanogaster* (Bartelt *et al.*, 1985; Schaner *et al.*, 1987). Other
339 aggregation hydrocarbons, such as (Z)-10-heneicosene, in *D. virilis* have been shown to
340 attract males and females of certain ages (Bartelt & Jackson, 1984). Behavioural tests
341 will be necessary to confirm that CHCs are used for mate selection in *C. frigida* but
342 attention should also be paid to their possible role in behaviours such as aggression and
343 aggregation. This may be particularly relevant to *Coelopa frigida* as this species has been
344 described as forming large aggregations (Mather, 2011).
345
346 We found a shift in the composition between sexes, populations, and diets rather than
347 differences in the presence/absence of specific compounds. This type of pattern differs
348 from some Dipterans where sex and population differences are mostly qualitative
349 (Carlson & Schlein, 1991; Ferveur & Sureau, 1996; Dallerac *et al.*, 2000b; Gomes *et al.*,
350 2008; Everaerts *et al.*, 2010) although many dipterans show quantitative differences (ex:
351 Jallon & David, 1987; Byrne *et al.*, 1995). This discrepancy is likely to be partially
352 explained by the distribution of compounds that make up the CHCs in *C. frigida*. While

353 *C. frigida* chain lengths and compound classes are comparable with other dipteran
354 species (Ferveur & Cobb, 2010) the distribution of these compounds is somewhat
355 different. Many dipterans have principal CHC components that make up a large
356 proportion of the CHC composition (Ferveur & Sureau, 1996; Etges & Jackson, 2001;
357 Gomes *et al.*, 2008). Cuticular extracts from *C. frigida* have a large number of
358 compounds, but do not appear to have specific compounds that are dominant in either sex
359 or any of the populations. When averaging across all samples, the most abundant
360 compound was the C25 alkane, which made up on average 15% of the CHCs.
361
362 We examined coding variation to determine if we could tie population level phenotypic
363 differences to genetic differences. We observed country level variation with strong
364 differences between Norway and Sweden (Figure 2D, Figure 4, Figure 5). This pattern
365 mirrors a strong genetic split between the two countries (representing approximately 14%
366 of genetic variation, Berdan *et al*, unpublished). We found potential outlier SNPs in
367 desaturases, elongases, a cytochrome P450-4G1, and a cytochrome P450 reductase.
368 Desaturases add double bonds or triple bonds to alkanes (Howard & Blomquist, 2005;
369 Blomquist & Bagnères, 2010). We found an outlier missense variant in a putative
370 Desaturase 1 as well as a missense and two downstream outlier variants in a putative
371 Desaturase (Table 4). This aligns with our phenotypic data showing differences in the
372 proportion of alkanes vs. alkenes between Norway and Sweden. Elongases lengthen fatty
373 acyl-CoAs and are necessary for chains longer than 16 carbon atoms (Blomquist &
374 Bagnères, 2010). We found both synonymous and downstream outlier variants in putative
375 *C. frigida* elongases (Table 4) and corresponding differences in both the mean chain

376 length and variance of the chain length (dispersion around the mean) due to country of
377 origin. P450 reductases are responsible for reducing fatty acids to aldehydes (Blomquist
378 & Bagnères, 2010). Knockdown of P450 reductases in *D. melanogaster* leads to a
379 striking reduction of cuticular hydrocarbons (Qiu *et al.*, 2012). P450-4G genes, which are
380 unique to insects, encode an oxidative decarboxylase that catalyzes the aldehyde to
381 hydrocarbon reaction. Work in other insect species has found that a knockdown or
382 knockout of this gene leads to a decrease in overall hydrocarbon levels (Reed *et al.*, 1995;
383 Qiu *et al.*, 2012; Chen *et al.*, 2016). We found a downstream outlier variant in our
384 cytochrome P450-4G1 and multiple kinds of outlier variants in a cytochrome P450
385 reductase. However, the total amount of CHCs did not differ between populations, only
386 sexes. As males and females share a genome (with the exception of the sex chromosome)
387 it is unlikely that these SNPs affect this difference. Finally, we also found variation
388 between Sweden and Norway in the proportion of methylated compounds. Other studies
389 suggest that differences in the proportion of methylated compounds are often caused by
390 either variation in Fatty Acid Synthase (FAS, de Renobales *et al.*, 1986; Blomquist *et al.*,
391 1994; Juarez *et al.*, 1996) or multiple copies of FAS with different functions (Chung *et*
392 *al.*, 2014). We found two different FAS' in our genome (Table 3) but neither of them
393 contained divergent SNPs. Future studies are needed to examine if these genetic and
394 phenotypic changes are related. Specifically, investigations into both RNA expression
395 and tests of function of these loci will be necessary to provide a direct link. However, the
396 association between the known function of several of these genes (elongases, desaturases)
397 and the corresponding differences in populations are marked. Given that we see shifts in

398 multiple chemical aspects (length, methylation, and alkene/alkane ratio) it is likely that
399 multiple loci are responsible for these patterns.
400
401 The wrackbed itself also had a strong influence on the CHC composition (Figure 3C).
402 Wrackbed composition and microbiome (i.e. the food source for *C. frigida* larvae) varies
403 across *C. frigida* populations in Europe (Berdan, et al., in prep, Day *et al.*, 1983; Butlin &
404 Day, 1989; Wellenreuther *et al.*, 2017), indicating that natural populations may be have
405 different CHC composition. This is concordant with other research showing that larval
406 diet impacts CHC composition (Liang & Silverman, 2000; Rundle *et al.*, 2005; Etges &
407 de Oliveira, 2014; Stojkovic *et al.*, 2014). Adding to this, we found strong population
408 (country) level differences in CHC composition even when larvae were reared on the
409 same substrate. We also found several outlier SNPs in a variety of genes involved in
410 CHC synthesis. Together these results indicate several possibilities: 1. Natural selection
411 may select for a different CHC composition in different environments and natural
412 polymorphism in CHC genes may be under direct selection due to this. For example,
413 *Drosophila serrata* and *D. birchii* differ in the environment they inhabit (habitat
414 generalist vs. habitat specialist for humid rainforests). These two species also differ in the
415 concentration of methyl branched CHCs (especially important in desiccation resistance)
416 caused, in part, by changes in the cis-regulatory sequence likely under natural selection
417 (Chung *et al.*, 2014). 2. Natural populations differ strongly in their CHC composition. If
418 assortative mating or selection on female preferences is present that could lead to locally
419 adapted male traits and female preferences (Chung & Carroll, 2015). This would reduce
420 effective migration between populations, as foreign males would be at a disadvantage.

Differences in CHC composition and corresponding preferences have been shown to cause behavioral isolation between many different *Drosophila* species or populations (Coyne *et al.*, 1994; Rundle *et al.*, 2005; Etges & de Oliveira, 2014).

In conclusion, the analysis of cuticular hydrocarbon extracts from male and female *C. frigida* from multiple populations revealed a complex and variable mix of more than 100 different compounds. We found extensive phenotypic variation attributable to diet, sex, and country as well as associated genetic variation attributable to the latter. This reveals that CHC composition is dynamic, strongly affected by the larval environment, and most likely under natural and/or sexual selection. Further work is needed to confirm that CHCs are used as sexual signals in this system and to explore the evolutionary consequences of these differences.

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Tables

Table 1– PERMANOVA and PERMDISP results for A. Sex and Population, B. Sex and Country, and C. Sex and Wrack type. * < 0.05 ** < 0.01 *** < 0.001

A.

	PERMANOVA: Sex and Population						PERMDISP
	df	SS	MS	F	p-value		
Sex	1	658.1	658.1	5.86	<0.001	***	see S2
Population	3	1150.3	383.4	3.41	<0.001	***	
Sex:Population	3	486.9	162.3	1.44	0.015	*	
Residuals	96	10786.0	112.4				
Total	103	13081					

B.

	PERMANOVA: Sex and Country						PERMDISP		
	df	SS	MS	F	p-value		F	p-value	
Sex	1	658.0	658.1	5.60	<0.001	***	8.94	0.003	**
Country	1	517.8	517.8	4.41	<0.001	***	1.19	0.279	
Sex:Country	1	154.1	154.1	1.31	0.128				
Residuals	100	11751.0	117.5						
Total	103	13081							

C.

	PERMANOVA: Sex and Wrack type						PERMDISP	
	df	SS	MS	F	p-value		F	p-value
Sex	1	360.9	360.9	3.73	<0.001	***	2.36	0.129
Wrack	1	481.3	481.3	4.98	<0.001	***	2.29	0.139
Sex:Wrack	1	122.3	122.3	1.27	0.1629			
Residuals	44	4252.5	96.7					
Total	47	5217						

Table 2 – List of compounds that are significantly important for differentiation of at least factor (sex, diet, or country). If compounds are significant for differentiation of any category in any comparison a * appears under that factor. Color indicates the direction of the differentiation (red- higher in females, blue – higher in males, grey – higher in Norwegian populations, green – higher in Swedish populations, yellow – higher in flies raised on standard wrack, purple – higher in flies raised on field wrack).

				Importantance in differentiation for factor			
Compound number	RI	Identified as	Methyl group position	Sex in Sex & Country	Country in Sex & Country	Sex in Sex & Diet	Wrack type in Sex & Diet
1	2096	unknown alkane					*
2	2101	C21			*		
3	2176	unknown alkane			*		
5	2202	C22		*			
7	2300	C23		*	*	*	
8	2344	unknown alkane		*		*	
9	2365	2-ME-C23			*		
11	2400	C24		*		*	*
14b	2478	25:x-C25			*		
16	2500	C25					*
17	2524	unknown alkane					*
20	2564	2-ME-C25					*
21	2576	3-ME-C25					*
22	2584	5,X-Me-C25	X=9,11,13,15	*		*	
23	2600	C26					*
24	2638	unknown alkane		*			
26	2665	2-Me-C26					*
27	2680	27:x-C27		*		*	
29	2701	C27			*		*
30	2712	unknown alkane					*
31	2735	11+13-Me-C27			*		*
33	2765	2-Me-C27 + 11,15-Me-C27			*		*
34	2775	3-ME-C27					*
37a	2807	3,X-Me-C27	X=15,13,11,7				*
40	2850	29:x,x-C29					*
41	2857	29:x,x-C29			*		
42	2865	2-ME-C28 or x:C29			*		*
44	2880	29:x-C29				*	*
45	2882	29:x-C29			*		
46	2891	4,12-Me-C28					*
47	2902	C29			*		*
48	2933	15+13+11-Me-C29			*		*
50	2961	11,X-ME-C29	X=15,17	*	*		
51	2972	7,X-ME-C29	X=11,13,15	*	*	*	
52	2982	5,13-Me-C29		*	*		
53	3007	3,13-ME-C29			*		*
54	3033	3,X,15-Me-C29	X= 11,13	*		*	
55	3042	3,7,15-Me-C29			*		
56	3054	31:x,x-C31		*			
57	3058	12,16-Me-C30			*		
60	3074	31:x-C31 or 6,16-Me-C30			*		
61	3082	31:x-C31			*	*	
62	3090	31:x-C31 or 4,16-Me-C30		*			
63	3101	C31		*	*	*	*
64	3118	unknown alkane		*	*		*
65	3131	15+13+11+9-Me-C31		*		*	*
66	3157	15,19-Me-C31		*		*	
67	3163	9,X-Me-C31	X=13,15	*	*	*	
68	3170	7,X-Me-C31	X=15,17	*		*	
69	3180	5,15-Me-C31		*		*	
71	3194	unknown alkane		*		*	
72	3205	3,X-Me-C31	X=9,11,13	*		*	
73a	3231	3,X,Y-Me-C31	X=9,11 ; Y=13,15	*		*	
74	3246	33:x,x,x,x-C33		*			
75	3256	33:x,x-C33			*		
76	3272	33:x-C33			*		
77	3284	33:x-C33			*		
78	3300	unknown alkane		*	*	*	*
79	3315	unknown alkene					*
80	3330	17+15+13+11-Me-C33		*		*	*
81	3353	11,X-Me-C33	X=13,15,17	*			
82	3359	9,X-Me-C33	X=11,13,15		*		
83a	3365	7,X-Me-C33	X=11,13,15,17			*	
83b	3368	7,X-Me-C33	X=11,13,15,17	*		*	
84	3377	5,X-Me-C33	X=15,17	*		*	
85a	3391	7,13,17-Me-C33		*		*	
85b	3393	7,13,17-Me-C33		*			

higher in females
higher in males
higher in Swedish populations
higher in Norwegian populations
higher in flies raised on standard wrack
higher in flies raised on field wrack
* significant t-test with FDA α=0.05

Table 3 – Putative genes involved in CHC synthesis in *C. frigida*

Scaffold[1]	Gene Position[1]	Best Blast Hit[2]
scaffold_129	185034-197861	Desaturase 1
scaffold_155	109640-112144	Cytochrome P450-4G1
scaffold_206	225893-231312	Cytochrome P450 reductase
scaffold_480	98147-106166	CG5326 (elongase family)
scaffold_480	2453-12223	CG31522 (elongase family)
scaffold_545	1553-3217	CG5326 (elongase family)
scaffold_340	1141-16368	Baldspot (elongase family)
scaffold_149	7122-16098	Fatty Acid Synthase 3
scaffold_51	250672-259608	Fatty Acid Synthase 1
scaffold_716	36957-55451	CG2781 (elongase family)
scaffold_994	14376-17314	CG9743 (contains Fatty acid desaturase domain and Acyl-CoA desaturase)

1. From unpublished genome assembly (Wellenreuther *et. al*)
2. From a BLAST against *D. melanogaster* annotated nucleotides on flybase.org

Table 4 – Outlier SNPs putatively involved in geographic differences

Scaffold[1]	Position[1]	Variant type[2]	Best Blast Hit[3]	F_{ST}[4]
scaffold_129	195074	missense variant	Desaturase 1	0.16
scaffold_155	116876	downstream gene variant	Cytochrome P450-4G1	0.22
scaffold_206	225645	downstream gene variant	Cytochrome P450 reductase	0.17
scaffold_206	226804	missense variant	Cytochrome P450 reductase	0.17
scaffold_206	229444	missense variant	Cytochrome P450 reductase	0.20
scaffold_206	230032	intron variant	Cytochrome P450 reductase	0.30
scaffold_480	105896	synonymous variant	CG5326 (elongase family)	0.22
scaffold_480	110918	downstream gene variant	CG5326 (elongase family)	0.16
scaffold_994	16976	missense variant	CG9743 (contains Fatty acid desaturase domain and Acyl-CoA desaturase)	0.16
scaffold_994	18844	downstream gene variant	CG9743 (contains Fatty acid desaturase domain and Acyl-CoA desaturase)	0.20
scaffold_994	19574	downstream gene variant	CG9743 (contains Fatty acid desaturase domain and Acyl-CoA desaturase)	0.20

1. From unpublished genome assembly (Wellenreuther *et. al*)
2. Annotated in SnpEff (see text for details)
3. From a BLAST against *D. melanogaster* annotated nucleotides on flybase.org
4. Calculated from a separate unpublished data set of WGS from 46 *C. frigida*

Figure Legends

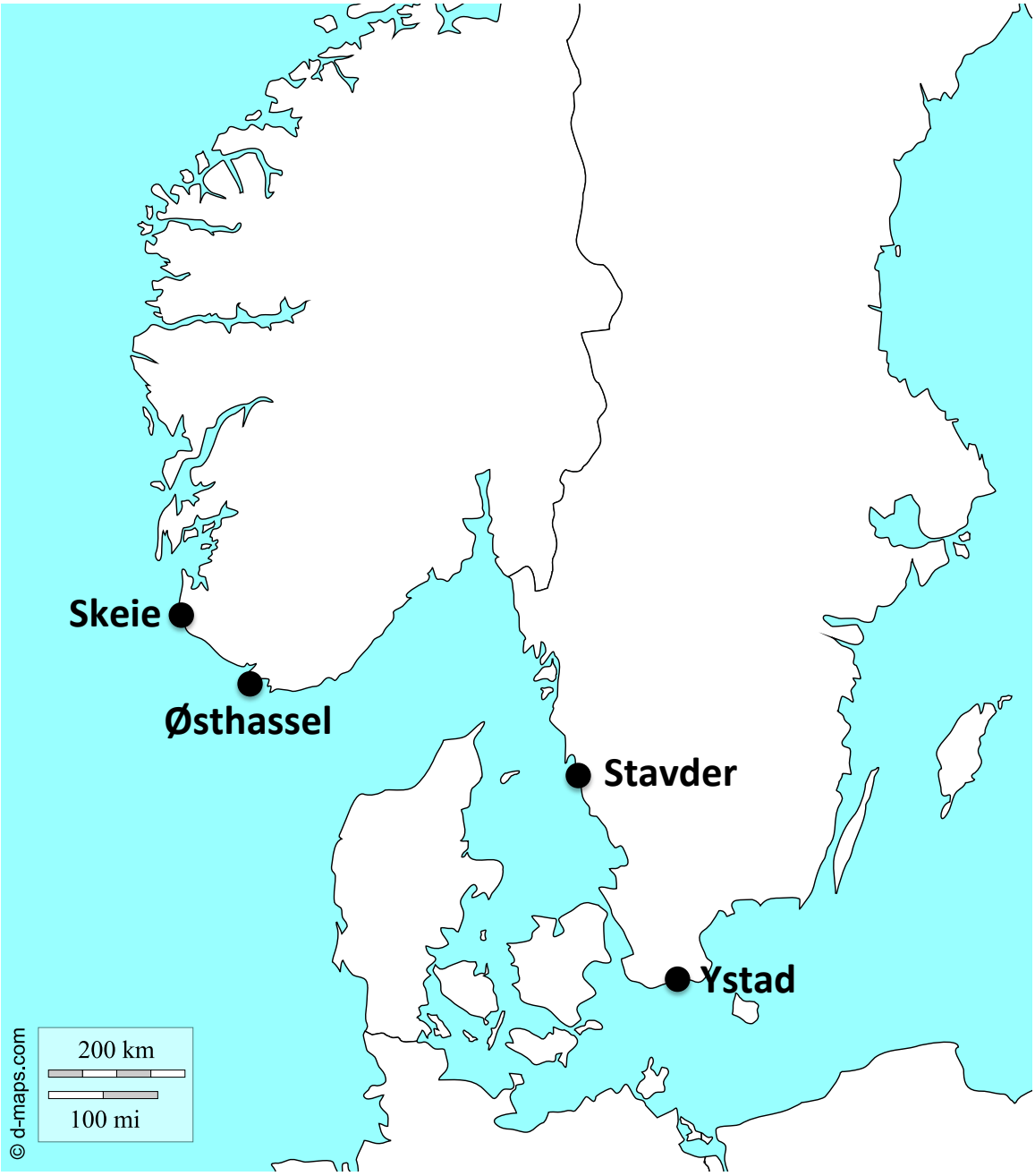
Figure 1 - Map of *C. frigida* populations used in this study. Base map from d-maps (http://d-maps.com/carte.php?num_car=5972)

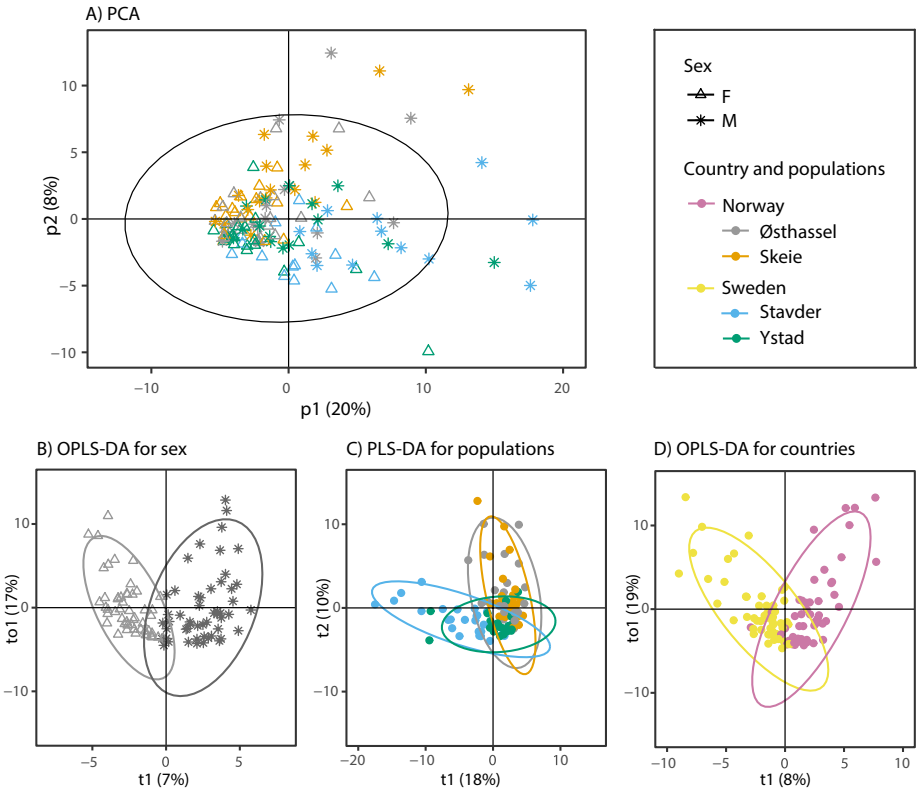
Figure 2 – A. PCA of all samples from the balanced data set indicating population (grey – Østhassel, orange – Skeie, blue – Stavder, green – Ystad) and sex (triangle – female, star – male). B. OPLS-DA for sex using all samples from the balanced data set, C. PLS-DA for population using all samples from the balanced data set, and D. OPLS-DA for country using all samples from the balanced data set (yellow – Sweden, purple – Norway). Circles indicate 95% confidence regions.

Figure 3 – A. PCA of samples from Ystad indicating diet (blue – field, yellow – standard) and sex (triangle – female, star – male). B. OPLS-DA for sex using only Ystad samples. C. OPLS-DA for diet using only Ystad samples. Circles indicate 95% confidence regions.

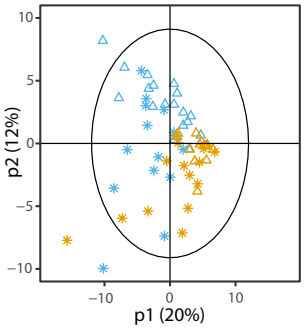
Figure 4 – Boxplot of differences in A. Log transformed total peak area, B. proportion of compounds that are alkenes, C. proportion of methylated compounds. Females are in red, males are in blue.

Figure 5 – Boxplot of differences in A. Weighted mean chain length and B. Dispersion around the mean. Females are in red, males are in blue.

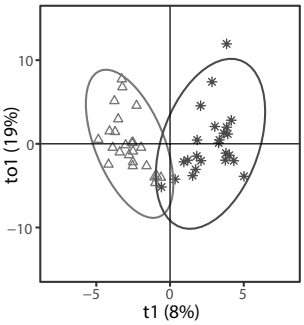

Skeie
Østhassel
Stavder
Ystad
200 km
100 mi
© d-maps.com


A) PCA
p2 (8%)
p1 (20%)
Sex
F
M
Country and populations
Norway
Østhassel
Skeie
Sweden
Stavder
Ystad
B) OPLS-DA for sex
to1 (17%)
t1 (7%)
C) PLS-DA for populations
t2 (10%)
t1 (18%)
D) OPLS-DA for countries
to1 (19%)
t1 (8%)

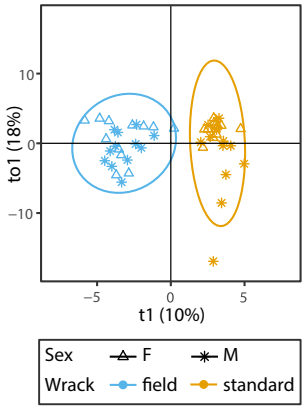
A) PCA

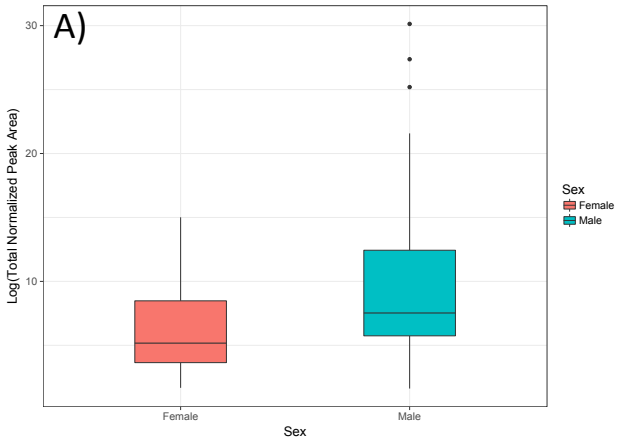


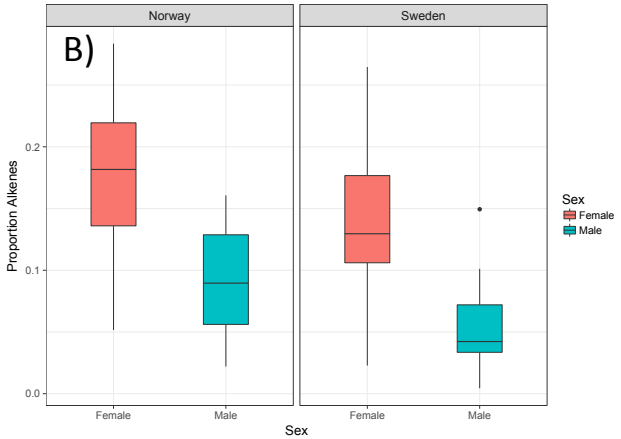
B) OPLS-DA for sex

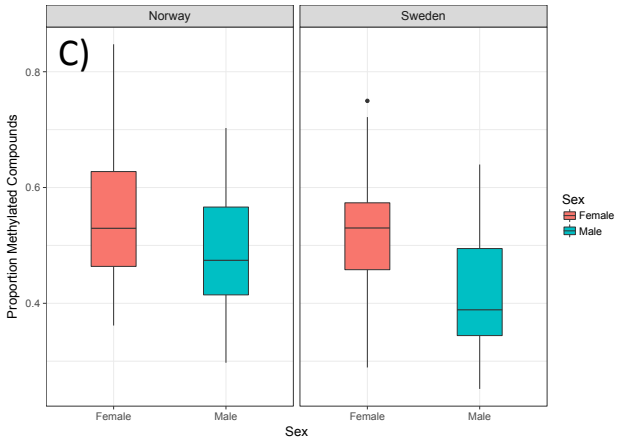


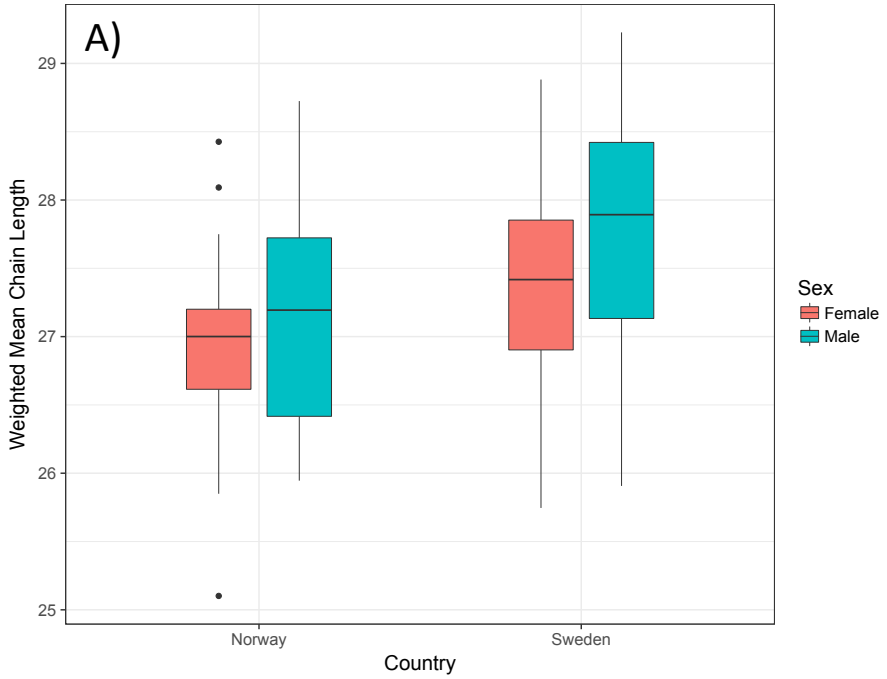
C) OPLS-DA for wrack type

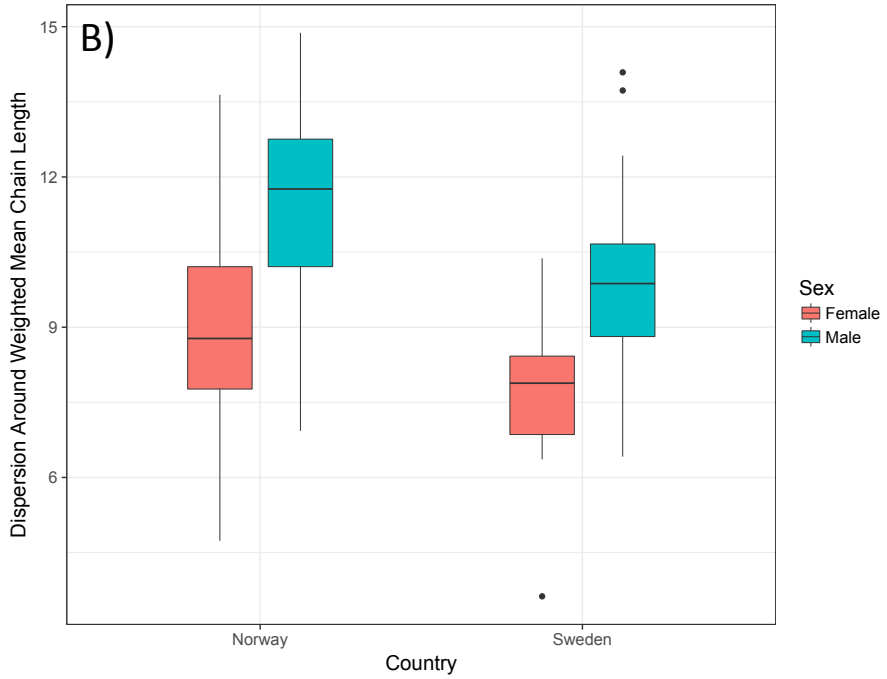



A)
Log(Total Normalized Peak Area)
30
20
10
Female
Male
Sex
Sex
Female
Male


B)
Norway
Sweden
Proportion Alkenes
0.2
0.1
0.0
Female
Male
Female
Male
Sex
Sex
Female
Male


C)
Norway
Sweden
Proportion Methylated Compounds
0.8
0.6
0.4
Female
Male
Female
Male
Sex
Sex
Female
Male


A)
Weighted Mean Chain Length
29
28
27
26
25
Norway
Sweden
Country
Sex
Female
Male


B)
Dispersion Around Weighted Mean Chain Length
15
12
9
6
Norway
Sweden
Country
Sex
Female
Male