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

Emma Berdan[1*], Swantje Enge[2], Göran Nylund[1], Maren Wellenreuther[3,4], Gerrit A. Martens[5], and Henrik Pavia[1]

[1]Department of Marine Sciences University of Gothenburg, Göteborg, Sweden

[2]Institute for Chemistry and Biology of the Marine Environment, Carl-von-Ossietzky University Oldenburg, Wilhelmshaven, Germany

[3]The New Zealand Institute for Plant & Food Research Limited, Nelson, New Zealand

[4]School of Biological Sciences, the University of Auckland, New Zealand

[5]Hochschule Bremen, City University of Applied Sciences, Bremen, Germany

* - Corresponding author: Emma Berdan, Department of Marine Sciences, Box 461, 405 30 Göteborg, Sweden. Email: emma.berdan@gmail.com

ABSTRACT

Sexual signals facilitate female choice, reducing mating costs. The seaweed fly, *Coelopa frigida* is a species 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), which act as sex pheromones in most dipterans. We characterized CHCs in *C. frigida* for the first time and find that CHCs likely act as sexual signals and that CHC composition is highly varied. Variation in CHC composition was caused by both genetic (sex and population of origin) and environmental (larval diet) effects. We identified individual compounds that contribute to these differences as well as general shifts in chemistry. Changes in proportion of methylated compounds, mean chain length, proportion of alkenes, and normalized total CHCs were found to differ between these groups. We combined this data with whole genome resequencing data to examine the genetic underpinnings of these changes. We identified 11 genes related to CHC synthesis and find putative F_{ST} outlier SNPs in 5 of them that are concordant with the phenotypic differences we observe. This reveals that 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

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, in some cases 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 commonly suffering injuries (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 a minute, disrupting normal activities like foraging (Magurran & Nowak, 1991).

Females can minimize the cost of unwanted copulations by making it hard to be detected by males (Wellenreuther *et al.*, 2014) or evolving mate rejection responses (Andersson, 1994); however, these responses often carry associated costs (Watson *et al.*, 1998). Consequently, females frequently endure costly matings with males when the costs of rejection outweigh the costs of mating. Females can further modulate mating interactions by evolving defined mate preferences. Mate preferences allow females to obtain high

44 quality or compatible genes from a male, with indirect benefits that include improved
45 offspring fecundity, survival and immune response (Neff & Pitcher, 2005). In many
46 solitary and social insects, cuticular hydrocarbons (CHCs) are used as one of the primary
47 recognition cues to recognize, and possibly discriminate, species, sex, or kin (Bagneres *et*
48 *al.*, 1996; Ferveur, 2005; Blomquist & Bagnères, 2010; Van Oystaeyen *et al.*, 2014).

49

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

64

65 CHC composition is affected by both genetic and environmental factors. Changes in gene
66 expression (Dallerac *et al.*, 2000a; Chertemps *et al.*, 2007; Shirangi *et al.*, 2009;

67 Feldmeyer *et al.*, 2014) and/or coding sequences (Keays *et al.*, 2011; Badouin *et al.*,
68 2013; Kulmuni *et al.*, 2013) can affect CHC composition and length. Multiple
69 environmental factors have also been shown to influence CHC composition. For
70 example, CHCs can vary under different temperature regimes (Toolson & Kupersimbron,
71 1989; Savarit & Ferveur, 2002; Rouault *et al.*, 2004), diets (Stennett & Etges, 1997;
72 Liang & Silverman, 2000; Stojkovic *et al.*, 2014) or climatic variables (Howard *et al.*,
73 1995; Kwan & Rundle, 2010). Moreover, multiple studies have found that CHCs vary
74 between populations (ex: Haverty *et al.*, 1990; Etges & Ahrens, 2001; Frentiu &
75 Chenoweth, 2010; Perdereau *et al.*, 2010) but it is unknown how much of this variation is
76 due to plasticity vs genetic divergence. These can be teased apart using laboratory
77 studies: genetic divergence can be assessed by raising different populations in the same
78 environment and plasticity can be assessed by raising the same population in multiple
79 environments.

80

81 The seaweed fly *Coelopa frigida* is a common inhabitant of "wrackbeds" (accumulations
82 of decomposing seaweed) on shorelines. These wrackbeds act as both habitat and food
83 source for larvae and adults of this species. Fitness trade-offs and local adaptation in
84 response to wrackbed composition have been demonstrated in Swedish *C. frigida*
85 (Wellenreuther *et al.*, 2017). The mating system in *C. frigida* is characterized by sexual
86 conflict. Males will approach and mount females. Females are reluctant to mate and use a
87 variety of responses to dislodge the male, such as downward curling of the abdomen (to
88 prevent contact), kicking of the legs, and shaking from side to side (Day *et al.*, 1990).
89 Although there is no male courtship of any kind it has been consistently reported that

mating attempts by large males are more likely to result in copulation (Butlin *et al.*, 1982; Gilburn *et al.*, 1992; 1993). Two lines of evidence show that females are actively choosing larger males rather than "giving up" due to exhaustion. First, under "giving up" there should be a negative correlation between struggle time and the size differential between the male and female. However, there is absolutely no correlation between these factors (Edward, 2008). Second, if females only allow copulation when they have been exhausted then struggle times should differ between mountings in which copulation occurs and mountings where copulation does not occur. However, there is no significant difference and a slight trend towards longer struggle times for interactions that do not end in copulation (Edward, 2008). Currently, the mechanism by which female choice occurs is unknown in this system.

Here we investigate cuticular hydrocarbons, a possible male signal that could enable female choice in *C. frigida.* Specifically, we investigate if the CHC signatures vary between sexes, populations, and wrackbed diet. We also explore putative genes involved in CHC synthesis and look for correlations between genetic and phenotypic variation. We discuss out findings in light of sexual selection in this species and others and outline future research areas that deserve attention.

METHODS

Study System

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

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)). Larvae were brought to Tjärnö Marine Biological 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° with a 12h/12h light-dark cycle. After adults eclosed in the lab they were transferred to a new pot filled with standard wrack consisting of 50% *Fucus spp*. and 50% *Saccharina latissima* which had been chopped, frozen, and then defrosted. The exception to this is a subset of Ystad adults that were allowed to mate

129 on the same material they had been collected in (field wrack). Thus we had 5 treatment
130 groups: Skeie on standard wrack, Østhassel on standard wrack, Stavder on standard
131 wrack, Ystad on standard wrack, and Ystad on field wrack. Adult flies were allowed to
132 lay eggs on the provided wrack and this second generation was raised entirely in a
133 temperature controlled room at 25° with a 12h/12h light dark cycle. As larvae pupated
134 they were transferred to individual 2 mL tubes with a small amount of cotton soaked in
135 5% glucose to provide moisture and food for the eclosing adult. This ensured virginity in
136 all eclosing flies. The pots were checked every day for eclosing adults and the date of
137 eclosure was noted. Two days after eclosure adult flies were frozen at -80° and kept there
138 until CHC extraction.

139 CHC Analysis

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

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

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

157 Statistical Analysis

158 The effects of sex and population on CHC profile were assessed using flies raised on
159 standard wrack using a balanced sampling design (N=13 for each combination) and 127
160 peaks identified by R package "GCalignR". The effects of sex and diet (i.e. wrack type)
161 during the larval stage on CHC profiles were analyzed using a balanced design of flies
162 from the Ystad population (N=12 for each combination) and 111 peaks identified by R
163 package "GCalignR". Peak areas were normalized on the peak area of the internal
164 standard and on the weight of the fly before being auto-scaled prior to statistical analysis.
165 Clustering of samples was visualized with a PCA, and group differences were analyzed
166 using a PERMANOVA followed by multiple group comparisons using the PRIMER-E 7
167 software. We also analyzed group differences via (O)PLS-DA using the "ropls"-packages
168 in R (Thevenot, Roux, Xu, Ezan, & Junot, 2015). Candidate compounds for group
169 differentiation were determined from variables of importance for OPLS-DA projection
170 (VIP scores), which were further assessed by univariate analysis using a false discovery
171 adjusted significance level of $\alpha = 0.05$.

172

We examined sex and country effects in more depth to look for 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 used 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.

We also analysed the distribution of chain lengths in our samples. We subset all peaks where we had accurate length information (107 peaks). We calculated weighted mean chain length as

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

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

198 $$D = \Sigma\, p_i(l_i - N)^2$$

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

201 Candidate gene analysis

202 Based on a literature search for genes involved in CHC synthesis, we identified 37
203 reference protein sequences, which were downloaded from Flybase (flybase.org) in
204 October 2017. We used tblastx with the default settings to identify orthologs in the *C.*
205 *frigida* transcriptome (Berdan & Wellenreuther, unpublished). We then used gmap (Wu
206 & Watanabe, 2005) to find areas of the *C. frigida* genome (Wellenreuther, *et al*.
207 unpublished) that might contain these genes. We output all alignments as a gff3 file. We
208 have a previously made a VCF file created from 30x whole genome re-sequencing of 46
209 *C. frigida* across five populations in Scandinavia (Berdan *et. al*, unpublished). We used
210 this VCF file and the gff3 output from gmap in SNPeff (Cingolani *et al.*, 2012) to
211 annotate the SNPs in these genes.

212

213 To find which SNPs are diverged between populations we used vcftools (Danecek *et al.*,
214 2011) to calculate Cockerham and Weir's F_{ST} estimate (Weir & Cockerham, 1984) for all
215 SNPs in our reference genome. We retained the top 5% of this distribution ($F_{ST} \geq 0.142$,
216 mean F_{ST} 0.024) as SNPs that were potentially divergent. We then subset the VCF file to
217 include only outlier SNPs located in our previously identified genomic regions.

218 # RESULTS

219 CHC analysis

220 A rich blend of hydrocarbons with more than 100 different compounds were found in the
221 cuticular extract of *Coelopa frigida*. The majority of CHCs ranged in chain length from
222 23 to 33 carbon atoms. They contained odd numbered *n*-alkanes, methyl-, dimethyl- and
223 trimethyl-alkanes as well as odd numbered alkenes. However, some even numbered
224 alkanes were present in lower amounts.

225 Sex and population effects

226 Results of the PERMANOVA indicated significant differences between females and
227 males and between populations but also a significant interaction between the two factors
228 (Table 1A). Pair-wise tests on the interaction showed a significant difference between
229 males and females for all populations except Ystad (Table S2). Multiple comparisons of
230 populations showed that most of our populations differed from one another and a PCA
231 and PLS-DA indicated that our Swedish (Ystad and Stavder) and Norwegian (Skeie and
232 Østhassel) populations seemed to group together by country (Figure 2A, C, Table S3).
233 After combining the Swedish and Norwegian populations, the PERMANOVA indicated
234 highly significant differences between females and males as well as between the Sweden
235 and Norway (Table 1B). OPLS-DA models for either sex or country were significant and
236 lead to separate clusters of females and males and Sweden and Norway, respectively
237 (Figure 2B, D, Table S3). According to the OPLS-DA model there were 41 peaks that
238 contributed with a VIP score >1 to the differentiation between females and males. Of
239 these 31 turned out to be also significant in t-tests with a FDR adjusted p-values (Table

240 2). These were highly male biased with all except 3 peaks more abundant in males.
241 Looking at differentiation between Swedish and Norwegian populations we found 43
242 compounds with a VIP score >1, 29 of which were significant in t-tests with FDR
243 adjusted p-values and consisted of an equal mix of compounds more abundant in either of
244 the countries (Table 2).

245 Sex and diet effects

246 PERMANOVA demonstrated a significant difference between females and males in the
247 Ystad population and also a significant effect of the larval diet (wrack type) (Table 1C).
248 Both OPLS-DA models for either sex or wrack type were significant and lead to separate
249 clusters of females and males and of flies raised on standard or field wrack (Figure 3B,C,
250 Table S3). According to the OPLS-DA model there were 38 peaks that contributed to the
251 differentiation between females and males with a VIP score >1. Of these, 24 compounds
252 were also significant in a t-test with FDR adjusted p-values (Table 2). These were highly
253 male biased with all except 3 peaks more abundant in males. Overall 65% (26
254 compounds) were shared between this analysis and the previous analysis of sex and
255 population effects. Looking at differentiation between flies raised on diet of standard or
256 field wrack we found 33 compounds with a VIP score >1. Of these, 27 compounds were
257 significant using a t-test with FDR adjusted p-values, all with higher amounts in flies
258 reared on field wrack (Table 2). Flies reared on field wrack had more CHCs per gram
259 body weight than flies reared on standard wrack (normalized totals: Females field wrack
260 7.96 ± 0.94, females standard wrack 4.09 ± 0.47, males field wrack 11.24 ± 1.03, males
261 standard wrack 7.09 ± 1.21).

262

263 Overall, the results indicated that the differences in CHC profiles between sexes,
264 populations and diet type of *C. frigida* were due to quantitative differences, affecting a
265 wide range of compounds instead of qualitative differences, i.e. the presence/ absence of
266 a few distinct compounds.

267

268 Shifts in Chemistry

269 As our results above indicated an overall effect of country rather than population we used
270 country and sex for our terms in these models. For total normalized peak area, the best
271 model contained only sex as a main effect (for full AIC comparison for all models see
272 Table S4A-E). In general, males had more CHCs per gram body weight than females
273 (Figure 4A). For proportion of alkenes, the best model contained country and sex as main
274 effects (for type II analysis of deviance tables for all best models see Table S5A-E).
275 Females tended to have more alkenes than males and individuals from Norway tended to
276 have more alkenes than individuals from Sweden (Figure 4B). The best model for the
277 proportion of methylated compounds contained sex and country as the main effects. As
278 with alkenes, females tended to have more methylated compounds than males and
279 individuals from Norway tended to have more methylated compounds than individuals
280 from Sweden (Figure 4C).

281

282 The best model for weighted mean chain length was country + sex. Overall, males had
283 longer mean chain lengths than females and individuals from Sweden had longer mean
284 chain lengths than individuals from Norway (Figure 5A). The best model for dispersion

285 around mean chain length was also country + sex. Males had higher dispersion than
286 females and individuals from Norway had higher dispersion than individuals from
287 Sweden (Figure 5B).

288

289 Genetic analysis

290 We identified 15 isoforms from 13 transcripts in our transcriptome with matches to our
291 query proteins. These transcripts mapped to 16 unique areas of the genome assembly. We
292 blasted these areas against the NCBI nr database (accessed in April 2018) to confirm our
293 annotation. Five of these areas had either no match or matched an unrelated protein and
294 were removed. The remaining genes include 2 putative fatty acid synthases, 2 putative
295 desaturases, 5 putative elongases, 1 putative cytochrome P450 reductase, and 1 putative
296 cytochrome P450-4G1 (Table 3). We found 1042 SNPs within these genes (including 5K
297 upstream and 5K downstream), of these 11 had an F_{ST} value > 0.142 (i.e. in the top 5% of
298 the F_{ST} distribution, Table 4).

299

300 DISCUSSION

301 In a sexual conflict system females may minimize the cost of unwanted copulations via
302 mate rejection responses; however, these responses typically carry associated costs
303 (Watson *et al.*, 1998). The ability to modulate mating responses and the level of
304 harassment based on male traits is especially beneficial in systems where no traditional
305 "courtship" exists. Here we explored a potential male trait, cuticular hydrocarbons
306 (CHCs) and assessed variation between sexes, populations, and different diets. We

307 supplemented this with a scan of the *C. frigida* genome to search for candidate genes for
308 CHC synthesis and to determine if genetic variation could be tied to the phenotypic
309 patterns we saw. We found phenotypic variation attributable to sex, population, and diet
310 and genetic variation attributable to population. We discuss these results and their
311 consequences below.
312
313 Males and females differed in their CHC composition. Not only did the composition
314 between males and females differ (Table 2, Figure 2-5) but, in addition, males
315 consistently had more CHC per gram body weight than females (Figure 4A). This is a
316 strong indication that CHCs play a role in sexual communication in *C. frigida* under a
317 female choice scenario, as it is unlikely that desiccation risk would differ between males
318 and females. A role for CHCs in sexual selection in *C. frigida* would be in line with the
319 general finding that CHCs play a major role in communication in insects, specifically in
320 Diptera (Howard & Blomquist, 2005; Blomquist & Bagnères, 2010; Ferveur & Cobb,
321 2010). Hydrocarbons may be involved in a wide variety of behaviours in Diptera such as
322 courtship, aggregation, and dominance (Ferveur & Cobb, 2010). For instance, 11-*cis*-
323 vaccenyl acetate, a male specific hydrocarbon in *Drosophila melanogaster*, increases
324 male-male aggression as well as influencing male mating behavior (Wang & Anderson,
325 2010). Future behavioural tests will be necessary to confirm that CHCs are used for mate
326 selection but attention should also be paid to behaviours such as aggression and
327 aggregation. Aggregation hydrocarbons, such as (Z)-10-heneicosene in *D. virilis* can
328 attract males and females of certain ages (Bartelt & Jackson, 1984). *Coelopa frigida* is
329 famous for forming large aggregations that often disrupt human activity (Mather, 2011).

330

331 The majority and most abundant CHCs found in *C. frigida* had chain lengths from 23 to
332 33 carbon atoms. The majority consisted of odd numbered *n*-alkanes, methyl-, dimethyl-
333 and trimethyl-alkanes as well as odd numbered alkenes, which is consistent with the
334 general biosynthetic pathway of long-chain hydrocarbons derived from fatty acids
335 (Blomquist, 2010). However, some even numbered alkanes (mainly C30 and C26) were
336 present in lower amounts.

337

338 We found shifts in CHC composition between sexes, populations, and diet. As we only
339 examined coding variation, only one of these factors, population, could be tied to genetic
340 differences. Rather than population specific variation we observed country level variation
341 with strong differences between Norway and Sweden (Figure 2D, Figure 4, Figure 5).
342 This pattern mirrors a strong genetic split between the two countries (representing
343 approximately 14% of genetic variation, Berdan *et al*, unpublished). We found potential
344 outlier SNPs in desaturases, elongases, a cytochrome P450-4G1, and a cytochrome P450
345 reductase. Desaturases add double bonds or triple bonds to alkanes (Howard &
346 Blomquist, 2005; Blomquist & Bagnères, 2010). We found an outlier missense variant in
347 a putative Desaturase 1 as well as a missense and two downstream outlier variants in a
348 putative Desaturase (Table 4). This aligns with our phenotypic data showing differences
349 in proportion of alkanes vs. alkenes between Norway and Sweden. Elongases lengthen
350 fatty acyl-CoAs and are necessary for chains longer than 16 or so (Blomquist &
351 Bagnères, 2010). We found both synonymous and downstream outlier variants in putative
352 *C. frigida* elongases (Table 4) and corresponding differences in both mean chain length

353 and variance in chain length (dispersion around the mean) due to country of origin. P450
354 reductases are responsible for reducing fatty acids to aldehydes (Blomquist & Bagnères,
355 2010). Knockdown of this in *D. melanogaster* leads to a striking reduction of cuticular
356 hydrocarbons (Qiu *et al.*, 2012). P450-4G genes, which are unique to insects, encode an
357 oxidative decarboxylase that catalyzes the aldehyde to hydrocarbon reaction. Work in
358 other insect species has found that a knockdown or knockout of this gene leads to a
359 decrease in overall hydrocarbon levels (Reed *et al.*, 1995; Qiu *et al.*, 2012; Chen *et al.*,
360 2016). We found a downstream outlier variant in our cytochrome P450-4G1 and multiple
361 kinds of outlier variants in a cytochrome P450 reductase. However, total amount of
362 CHCs did not differ as a result of anything except for sex. As males and females share a
363 genome it is unlikely that these SNPs affect this difference unless they are located on the
364 sex chromosome. Finally, we also found variation between Sweden and Norway in the
365 proportion of methylated compounds. However, research suggests that these differences
366 are often caused by either variation in Fatty Acid Synthase (FAS, de Renobales *et al.*,
367 1986; Blomquist *et al.*, 1994; Juarez *et al.*, 1996) or multiple copies of FAS with different
368 functions (Chung *et al.*, 2014). We found two different FAS' in our genome (Table 3) but
369 neither had divergent SNPs. Future studies are needed to examine if these genetic and
370 phenotypic changes are related. Specifically, investigation into both RNA expression and
371 tests of function of these loci will be necessary to provide a direct link. However, the
372 association between the known function of several of these genes (elongases, desaturases)
373 and the corresponding differences in populations are marked. Given that we see shifts in
374 multiple chemical aspects (length, methylation, and alkene/alkane ratio) it is likely that
375 multiple loci are responsible for these patterns.

We found a shift in the composition rather than differences in the presence absence of specific compounds. This type of pattern differs from some Dipterans where sex and population differences may be mostly qualitative (Carlson & Schlein, 1991; Ferveur & Sureau, 1996; Dallerac *et al.*, 2000b; Gomes *et al.*, 2008; Everaerts *et al.*, 2010) although plenty of Dipterans show quantitative differences as well (ex: Jallon & David, 1987; Byrne *et al.*, 1995). At least part of this discrepancy is likely explained by the number of compounds that make up the CHCs in *C. frigida* versus other Dipterans. Many Dipterans have principal CHC components that make up a large proportion of the CHC composition (Ferveur & Sureau, 1996; Etges & Jackson, 2001; Gomes *et al.*, 2008). Cuticular extracts from *C. frigida*, have a large number of compounds but do not appear to have compounds that are dominant in any one sex or population. When averaging across all samples, the most abundant compound was the C25 alkane, which made up on average 15% of the composition.

The wrackbed itself also had a strong influence on the CHC composition (Figure 3C). Wrackbed composition and microbiome (i.e. the food source for *C. frigida* larvae) varies across *C. frigida* populations in Europe (Berdan, et al., in prep, Day *et al.*, 1983; Butlin & Day, 1989; Wellenreuther *et al.*, 2017), indicating that natural populations may be composed of different CHC composition. Adding to this, we found strong population (country) level differences in CHC composition even when larvae were reared on the same substrate. We also found several outlier SNPs in a variety of genes involved in CHC synthesis. Together these results indicate several possibilities: 1. Natural selection

399 may select for different CHC composition in different environments and natural
400 polymorphism in CHC genes may be under direct selection due to this. 2. Natural
401 populations likely differ strongly in their CHC composition. If assortative mating or
402 selection on female preferences is present that could lead to locally adapted male traits
403 and female preferences. This would reduce effective migration between populations, as
404 foreign males would be at a disadvantage.
405
406 In conclusion, the analysis of cuticular hydrocarbon extracts from male and female *C.*
407 *frigida* from multiple populations revealed a complex and variable mix of more than 100
408 different compounds. We found extensive phenotypic variation attributable to diet, sex,
409 and population and potentially underlying genetic variation attributable to population.
410 This reveals that CHC composition is dynamic, strongly affected by the larval
411 environment, and most likely under natural and/or sexual selection. Further work is
412 needed to test the exact role of CHCs in this system.
413

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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

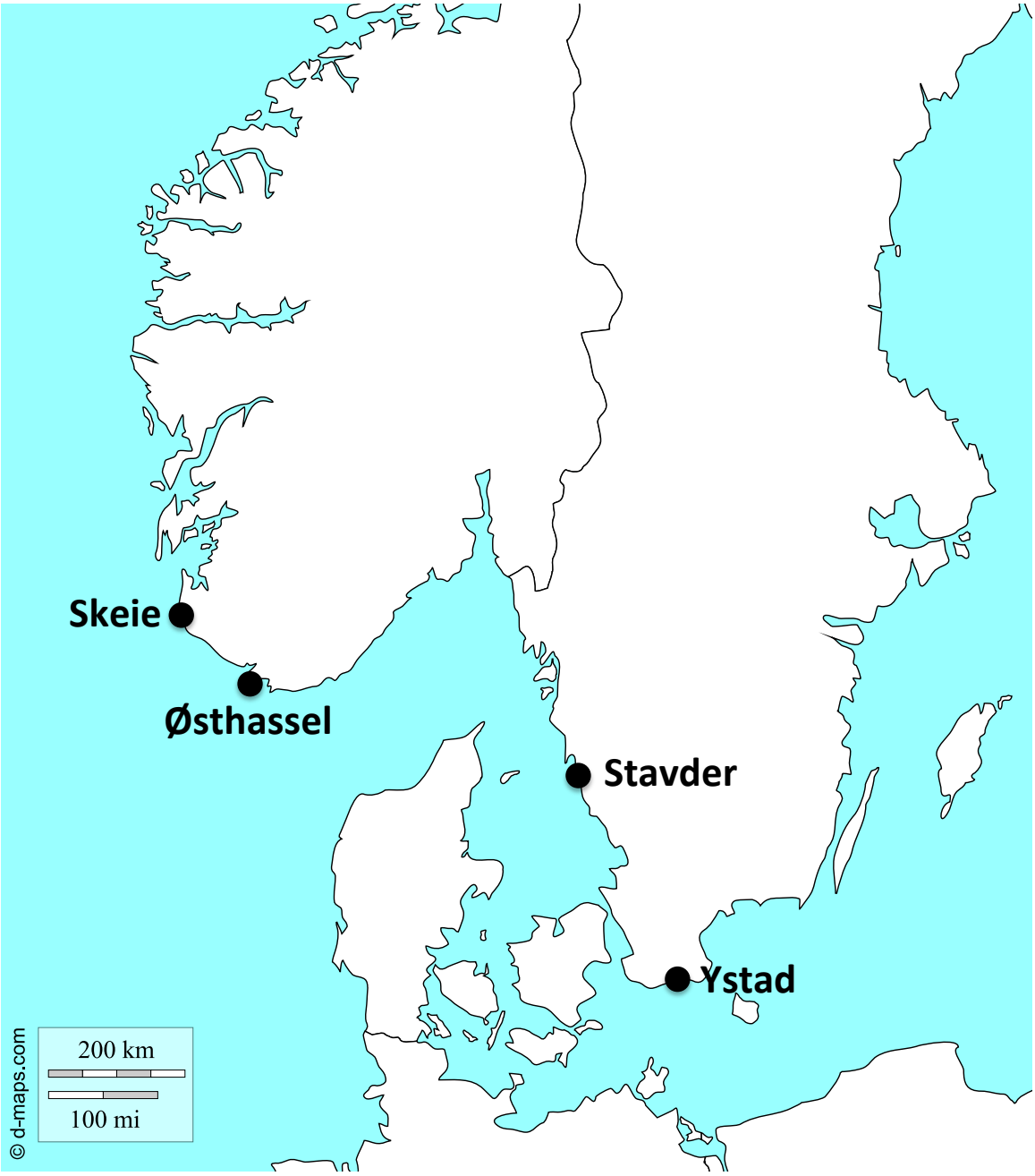
Figure 1 - Map of *C. frigida* populations used in this study. Base map from d-maps (http://d-maps.com/carte.php?num_car=5972)

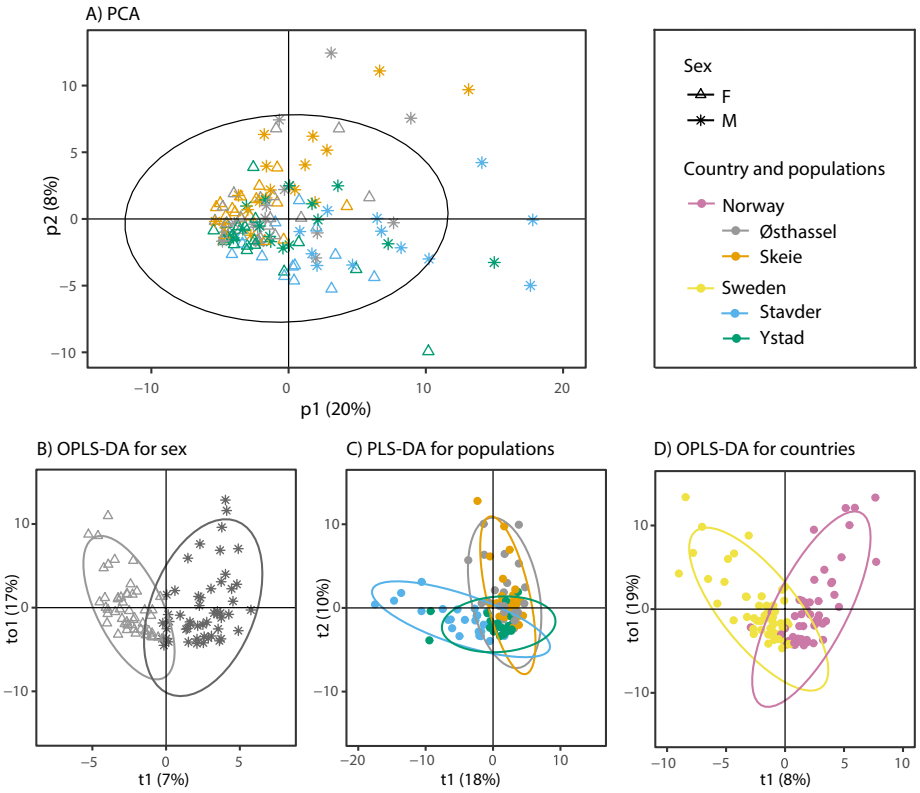
Figure 2 – A. PCA of all samples from the balanced data set indicating population (grey – Østhassel, orange – Skeie, blue – Stavder, green – Ystad) and sex (triangle – female, star – male). B. OPLS-DA for sex using all samples from the balanced data set, C. PLS-DA for population using all samples from 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.

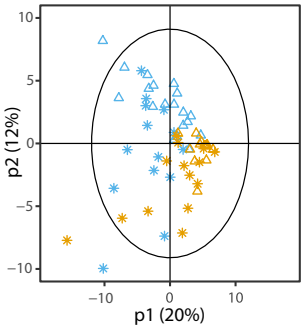
Figure 4 – Boxplot of differences in A. Log transformed total peak area, B. proportion of compounds that are alkenes, C. proportion of methylated compounds. Females are in red, males are in blue.

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.

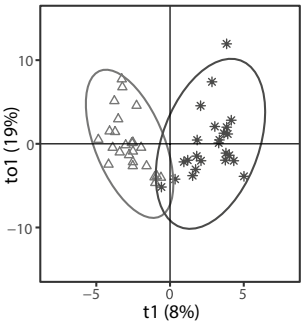

Skeie
Østhassel
Stavder
Ystad
200 km
100 mi
© d-maps.com


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%)

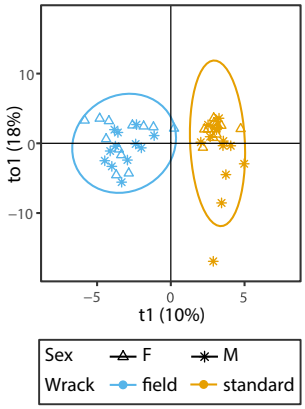
A) PCA

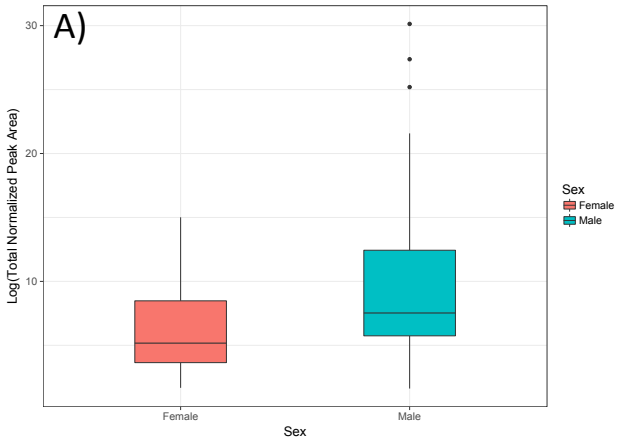


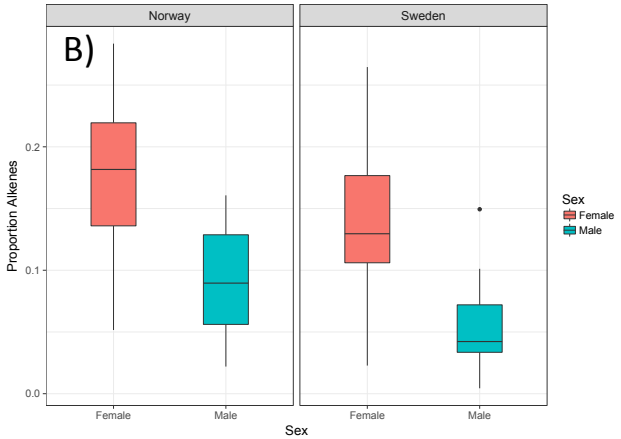
B) OPLS-DA for sex

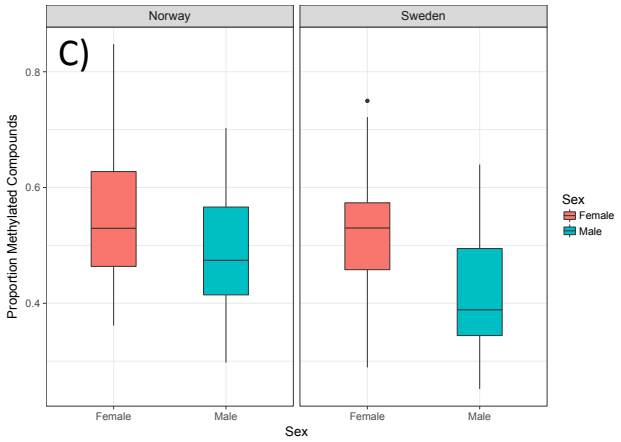


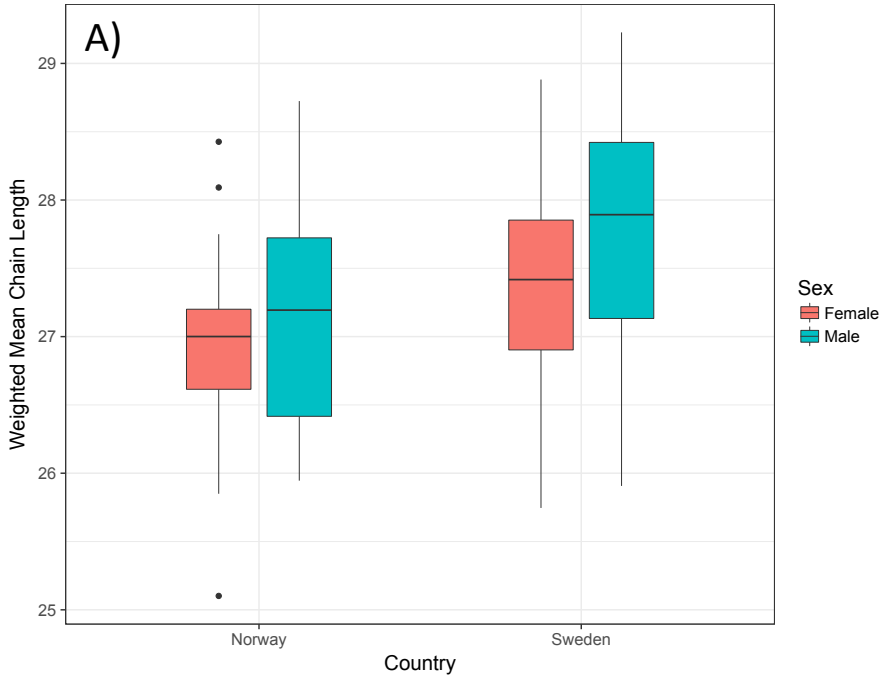
C) OPLS-DA for wrack type

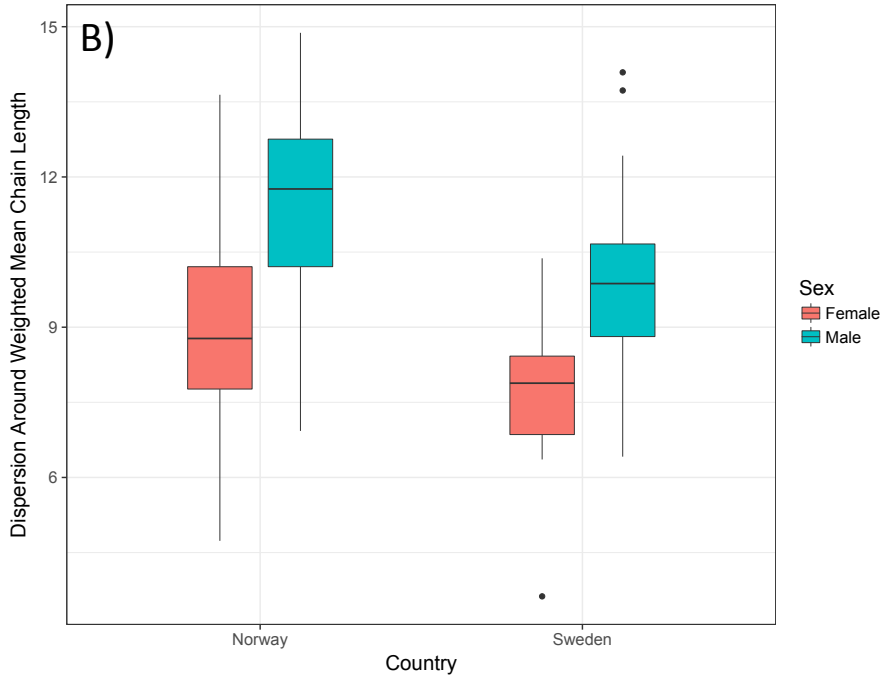



A)
Log(Total Normalized Peak Area)
30
20
10
Female
Male
Sex
Sex
Female
Male


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