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Title: Diapause affects cuticular hydrocarbon composition and mating behavior of both sexes in Drosophila montana

Year: 2020

Version: Accepted version (Final draft)

Please cite the original version:

Ala-Honkola, O., Kauranen, H., Tyukmaeva, V., Boetzl, F. A., Hoikkala, A., & Schmitt, T. (2020). Diapause affects cuticular hydrocarbon composition and mating behavior of both sexes in Drosophila montana. Insect Science, 27(2), 304-316. https://doi.org/10.1111/1744-7917.12639

1 **Diapause affects cuticular hydrocarbon composition and mating**

2 **behaviour of both sexes in *Drosophila montana***

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15 Running title: Diapause, mating and CHCs in *D. montana*

16

17 **Abstract**

18

19 Environmental cues, mainly photoperiod and temperature, are known to control female
20 adult reproductive diapause in several insect species. Diapause enhances female
21 survival during adverse conditions and postpones progeny production to the favorable
22 season. Male diapause (a reversible inability to inseminate receptive females) has been
23 studied much less than female diapause. However, if the males maximized their chances
24 to fertilize females while minimizing their energy expenditure, they would be expected
25 to be in diapause at the same time as females. We investigated *Drosophila montana*
26 male mating behavior under short day conditions that induce diapause in females and
27 found the males to be reproductively inactive. We also found that males reared under
28 long day conditions (reproducing individuals) court reproducing post-diapause females,
29 but not diapausing ones. The diapausing flies of both sexes had more long-chain and
30 less short-chain hydrocarbons on their cuticle than the reproducing ones, which
31 presumably increase their survival under stressful conditions, but at the same time
32 decrease their attractiveness. Our study shows that the mating behavior of females and
33 males is well coordinated during and after overwintering and it also gives support to the
34 dual role of insect cuticular hydrocarbons in adaptation and mate choice.

35

36 Key words: cuticular hydrocarbon, diapause, *Drosophila*, male reproduction, male
37 choice

1. Introduction

Insect diapause is a neurohormonally mediated state of low metabolic activity, which involves the cessation of development and/or reproduction (Tauber *et al.* 1986). It is typically induced by environmental cues, such as photoperiod and temperature, and occurs at a certain developmental stage, which varies between species. In several insect species, including many *Drosophila* species, females undergo adult reproductive diapause in order to prepare for unfavorable conditions and postpone their sexual maturation and reproduction to the next growing season (Lumme 1978).

While female diapause has been extensively studied (e.g. Danks 1987; Leather *et al.* 1993; Tauber *et al.* 1986), males have usually not been included into those experiments (Pener 1992). In species that overwinter as diapausing adults and mate in spring, males should not invest resources in courtship or sperm production when females are non-receptive. Spermatogenesis has indeed been found to discontinue or diminish in diapausing males in e.g. male desert beetles *Omorgus freyi* (Friedlander & Scholtz 1993) and the seven-spotted lady-beetle, *Coccinella septempunctata brucki* (Okuda 2000). On the other hand, males should be ready to copulate as soon as females are receptive, and therefore males are expected to recover faster from diapause than females and be coadapted to the timing of female receptivity (Pener 1992). This has been found to be true in several species, such as in the grasshopper, *Oedipoda miniata* (Pener & Orshan 1980), the monarch butterfly, *Danaus plexippus* (Herman 1981), the carabid beetle *Pterostichus nigrita* (Ferenz 1975; Thiele 1977) and the rice bug *Leptocorisa chinensis* (Tachibana & Watanabe 2007). Pener (1992) defines male diapause as "a reversible state of inability to fertilize receptive females", which is due to e.g. underdeveloped testis, cessation of spermatogenesis, or absence of male mating behavior. In this article, we use the definition of Pener (1992) for male diapause.

In several *Drosophila virilis* group species, including our study species *D. montana* (Tyukmaeva *et al.* 2011), females prepare for overwintering by arresting their oocyte development under short day conditions. Aspi *et al.* (1993) have shown that in this species reproductive stage clearly affects fly behavior in the wild, reproducing individuals being actively engaged in seeking feeding and/or breeding sites and the diapausing ones hiding themselves from harsh environmental conditions and showing no interest in each other. As *D. montana* females do not store sperm over the winter but mate in spring/early summer (Aspi *et al.* 1993), there should be no selection on males to use energy for the costly sperm production when females are in diapause (Wedell *et al.* 2002).

Like in all insects, the cuticle of *Drosophila* flies is coated with a thin layer of cuticular hydrocarbons (CHCs), including straight-chain alkanes as well as unsaturated and methyl-branched hydrocarbons (Ferveur 2005). Their presumed ancestral functions have been to increase desiccation tolerance (Gibbs 2002) and to provide an important barrier for bacterial or fungal infections (Gołębiowski *et al.* 2014). CHCs have also been found to play a crucial role in insect communication and act as sex pheromones in *Drosophila* courtship (Howard & Blomquist 2005; Coyne & Oyama 1995; Ferveur *et al.* 1997; Chung & Carrol 2015). Therefore, it is not surprising that CHC profiles have been shown to be

85   under both natural and sexual selection (e.g. Blows 2002; Frentiu & Chenoweth 2010).
86   The first is suggested to favor long-chain and the latter one short-chain hydrocarbons
87   (Gibbs *et al.* 1997; Kwan & Rundle 2010; Chung & Carrol 2014; Ingleby 2015; Otte *et*
88   *al.* 2018).
89   Rajpurohit *et al.* (2017) have shown that flies' CHC profiles respond rapidly and
90   adaptively to environmental parameters that covary with latitude and season in
91   *Drosophila melanogaster*. Also, in several insect species, diapausing individuals have
92   been found to differ from the reproducing ones in their CHCs at pupal (Coudron & Nelson
93   1981; Yoder *et al.* 1995; Kaneko & Katagiri 2004) or adult (Benoit & Denlinger 2007;
94   Jurenka *et al.* 1998) stage. Because diapausing *D. montana* flies encounter different
95   abiotic conditions than the reproducing ones (diapausing flies face up to 7 months of
96   winter and start to reproduce in spring when temperature rises above 10°C), we anticipate
97   that natural selection has driven the CHC composition of overwintering flies towards
98   longer-chain CHCs. CHC profiles of diapausing and reproducing *D. montana* flies could
99   be further diverged due to hormones like the juvenile hormone, which is involved in
100 diapause regulation in many insect species (Tauber *et al.* 1986). In *D. melanogaster*,
101 topical application of juvenile hormone analogue has been found to decrease the amount
102 of long-chain hydrocarbons on the cuticle (Wicker & Jallon 1995), which mimics the
103 hormonal changes occurring during sexual maturation and termination of diapause. In *D.*
104 *montana*, CHCs have been found to show quantitative variation among populations, while
105 sex differences are modest or absent (Bartelt *et al.* 1986; Suvanto *et al.* 2000; Veltsos *et*
106 *al.* 2012; Jennings *et al.* 2014).
107
108 In this study our aim was i) to find out whether male diapause exists in *D. montana*, ii) to
109 examine the behavior of males toward diapausing and non-diapausing females, and iii) to
110 compare CHCs of diapausing and non-diapausing flies. We predicted that: 1) males will
111 be in diapause when they are kept under conditions that induce diapause in females in
112 order to save resources and be prepared for harsh environmental conditions, 2) males
113 recover from diapause faster than females to be able to mate as soon as females are
114 receptive, 3) CHCs of diapausing males and females consist of longer chain hydrocarbons
115 than those of reproducing ones, which should increase their survival during overwintering
116 and 4) males discriminate between appropriate and non-appropriate mating partner, i.e.
117 the percentage of courting males increases along with an increase in the percentage of
118 fertile females. Our study showed all these predictions to be true and gives support to the
119 dual role of CHCs in adaptation and mate choice.
120
121   ## 2. Materials and methods
122 ### 2.1 Stocks and maintenance
123 Experimental flies were descendants of the flies collected from riparian habitats in
124 Oulanka (66°22'N, 29°19'E), Finland, in the summer of 2008. Once in the laboratory,
125 isofemale lines were established from the progenies of fertilized wild-caught females
126 and maintained in half-pint bottles on Lakovaara malt medium. From each isofemale
127 line (N = 20), 20 F3 males and 20 F3 females (800 total flies) were transferred into a 25
128 × 25 × 60 cm wooden population cage with a Plexiglas top and eight food bottles for
129 feeding, oviposition and larval rearing, and bred in overlapping generations under
130 constant light and temperature (19°C). Constant light (or long day length) is necessary
131 to prevent flies from undergoing reproductive diapause (Lumme 1978). Experimental
132 flies were collected on the day of eclosion from the food bottles using CO_2 as an

133   anaesthetizing agent and moved in malt vials to either diapause or sexual maturation
134   inducing conditions for 21 days (see below). All applicable international, national,
135   and/or institutional guidelines for the care and use of animals were followed.
136
137   **2.2 Experiment 1: Mating behavior, egg and offspring production and**
138   **CHC composition**
139
140   The day length varies in Oulanka from 21 to 22.6 hours during *D. montana* breeding
141   season in June and decreases to 15 hours as the progeny eclose until August. The
142   critical day length (CDL), at which 50 % of females will enter diapause, is 18.7 hours in
143   Oulanka and when the day length is 16 hours, 100 % of females will enter diapause
144   (Tyukmaeva *et al.* 2011). Since there is daylight for almost 24 hours during the early
145   summer, we used 24 hours light (24L) as conditions inducing sexual maturation and 16
146   hours light (L) and 8 hours dark (D) (16L:8D) as diapause-inducing conditions (16°C).
147   On the day of eclosion, half of the males and half of the females (randomly chosen)
148   were allocated into the 24L treatment and the other half were allocated into the 16L:8D
149   treatment. This treatment is appropriate as *D. montana* females adjust their development
150   (sexual maturation vs. diapause) according to the post eclosion conditions (Salminen *et*
151   *al.* 2012). Flies were kept in single-sex food vials with 10 flies in each vial and changed
152   into new vials once a week during the 21 days maturation period.
153
154   We performed a full factorial experiment in two replicates (Rep) to investigate how
155   diapause inducing conditions affect male and female mating behavior and reproductive
156   output. The sample sizes are reported in Table 1. In the first replicate, we only measured
157   fly mating behavior and in the second one, we measured mating behavior and thereafter
158   egg and offspring production during the 8 days the male and the female were paired. To
159   minimize observer bias, blinded methods were used when all behavioral data were
160   recorded. During the mating experiment, we recorded the time when the male and the
161   female were transferred into the vial as a pair, the time of the first courtship song (the
162   song, produced by wing vibration, is obligatory for successful mating (Liimatainen et al.
163   1992)) and the beginning and the end of copulation. The flies were observed for two
164   hours. However, as courtship and copulation mainly occurred in the treatment where
165   both males and females were at reproductive state (i.e. 24L females with 24L males),
166   the data were coded as categorical: courtship/no courtship and copulation/no copulation.
167   Egg and offspring production was measured in Rep 2 by transferring the fly pairs into
168   new vials daily for 8 days and counting the number of eggs and eclosing offspring from
169   each vial. The flies were kept in 24L at 18°C during egg laying. To make sure that the
170   females entered diapause in 16L:8D and reproductive state in 24L, 23 females from
171   both treatments were dissected under a stereomicroscope at the age of 21 days.
172
173   Finally, we extracted CHCs from 15 males and females from both the 24L and 16L:8D
174   treatment. These flies were frozen at -20°C after 21 days under the respective
175   conditions. CHCs were extracted at the University of Jyväskylä (Finland) by immersing
176   individual flies in 200 µl n-hexane for 10 min (gently vortexing them twice) in 1.5 ml
177   glass vials. Flies were then removed from the solvent and the vials were left in a fume
178   hood at room temperature until the solvent evaporated. Extracts were sealed and stored
179   at -20°C until they were shipped on dry ice to the University of Würzburg, Germany, for
180   gas chromatography/mass spectrometry analysis.

181
182 The CHC extracts were analyzed with a QP2010 Ultra CI gas chromatograph (GC)
183 coupled with a mass spectrometer (MS) (Shimadzu, Duisburg, Germany). The GC
184 (split/splitless-injector in splitless mode for 1 min, injected volume: 1 µl at 250°C) was
185 equipped with a DB-5 Fused Silica capillary column (30 m x 0.25 mm ID, df = 0.25
186 µm, J&W Scientific, Folsom, USA). Helium served as a carrier gas with a linear
187 velocity of 146.8 kPa. The following temperature program was used: start temperature
188 60°C hold for 1 min, temperature increase by 5°C per minute up to 300°C, isotherm at
189 300°C for 10 minutes. The transfer line had a temperature of 300°C. The electron
190 ionization mass spectra (EI-MS) were acquired at an ionization voltage of 70 eV (source
191 temperature: 230°C).
192
193 Chromatograms and mass spectra were recorded and quantified via integrated peak
194 areas with the software GC solution V2.41 (Shimadzu, Duisburg, Germany). Individual
195 CHC compounds were characterized by considering the MS data base Wiley275 (John
196 Wiley & Sons, New York, USA), retention indices, and the detected diagnostic ions
197 (Carlson *et al.* 1998). Double-bond positions in alkenes and, if possible, in alkadienes
198 were determined by DMDS derivatization as stated in Dunkelblum *et al.* (1980).
199 Retention indices of all compounds were calculated using an alkane standard. Given
200 that some substances could not be accurately separated with the above instrument and
201 settings, we calculated their combined quantity by integrating over all substances within
202 a peak in these cases.
203
204 **2.3 Experiment 2: Female recovery time from diapause *vs* male sexual**
205 **interest**
206
207 In the second set of experiments we investigated how well the males are able to track
208 female recovery from diapause, i.e. we asked whether the males become sexually
209 interested in females at the same time as the females become fertile after their recovery
210 from diapause (in experiment 1 this happened 5-7 days after females were taken into
211 24L conditions). Flies for this experiment were collected from a new population cage
212 (the older cage used in experiment 1 was contaminated) that was established from F3
213 descendants of 104 females caught from the wild in Oulanka, Finland, in 2013. The
214 cage was established and maintained as explained above.
215
216 We transferred newly eclosed females into the 16L:8D condition at 16°C every two
217 days for 8 days. When the females had stayed 20 days under those conditions, they were
218 transferred into continuous light (24L at 18°C) and allowed to recover from diapause for
219 either 0, 2, 4, 6 or 8 days before the mating experiment (N = 20 per time treatment).
220 Males for this experiment were collected as virgins, kept at 24L at 18°C and used 20-26
221 days post eclosion. In the mating experiment, we recorded the time when a male and a
222 female were introduced in the vial as a pair, the time of the first courtship song and the
223 beginning and end of copulation. Again, the data was coded as categorical: courtship/no
224 courtship and copulation/no copulation. The pairs were observed for 90 minutes.
225
226 **2.4 Statistical analysis**
227

228   We used R (version 3.0.2) for statistical analysis (R development core team 2013). We
229   analyzed the mating and the courtship data with a generalized linear model (GLM) with
230   logit link function and binomial error structure using sample sizes as weight following
231   Crawley (2007). The full model included the female light treatment, the male light
232   treatment, their interaction and replicate. The full model was simplified until only
233   significant factors remained by removing each term in turn and comparing nested
234   models with and without the given term with an analysis of deviance. The models were
235   not overdispersed. Analyzing the number of egg and offspring data was problematic
236   because of the large number of zeros (some treatments had only zeros in first days),
237   which caused numerical problems in generalized linear models (GLM) and zero-inflated
238   models. Also, variances differed a lot among treatments (see Figure 1). In order to
239   evaluate how long it takes for the females and males to recover from diapause, we
240   therefore analyzed egg and offspring production separately for each day. Egg and
241   offspring production in days 1-4 were analyzed with Kruskal-Wallis test and multiple
242   comparisons were performed with Dunn's test (Dunn 1964; library "dunn.test" in R;
243   Dinno 2015) with Bonferroni correction. Egg and offspring production in days 5-8 were
244   analyzed with GLMs with negative binomial distribution (Poisson models were
245   overdispersed) using the function "glm.nb" in library "MASS" (Venables & Ripley
246   2002). The significance of the factor "treatment" was assessed with likelihood ratio test
247   (L-ratio) by comparing nested models with and without that factor (Zuur *et al.* 2009).
248   We performed model validations by examining the homogeneity and independence of
249   errors. Multiple comparisons (Tukey's test) were performed with library "multcomp"
250   (Hothorn *et al.* 2008).
251
252   For the comparison of CHC profiles of both sexes under diapause and non-diapause
253   conditions, we only considered CHC compounds that had relative quantitative
254   abundance more than 0.1 % of the total quantitative CHC abundance in the respective
255   extracts and which were recognized in more than 50% of the samples within each
256   group. The CHC compositions of all individuals were compared by means of
257   multivariate methods. Therefore, we log-ratio transformed all quantitative CHC values
258   and calculated Bray-Curtis dissimilarities (Bray & Curtis 1957; taking into account
259   compound identities and their relative contributions to the CHC profiles) between all
260   pairs of samples using the vegdist function of the vegan package (version 2.0-10)
261   (Oksanen *et al.* 2013) of the R statistical software (version 3.0.2). The Bray-Curtis
262   dissimilarity values were subsequently displayed in a two-dimensional graph via non-
263   metric multidimensional scaling (NMDS) using the metaMDS function of the vegan
264   package. The spatial distances between points in the NMDS plot indicates the chemical
265   differences between samples and the corresponding stress value indicates the goodness
266   of fit of the two-dimensional representation to the initial multidimensional distances,
267   with a stress value < 15 indicating a good fit. Note that NMDS does not require a priori
268   knowledge of what samples likely represent a group. Any data structures emerging from
269   these visualization methods are purely based on the similarities of the chemical
270   compositions of the analyzed extracts.
271
272   In experiment 2, we compared the number of courting males in each treatment (0, 2, 4, 6
273   and 8 days recovery time from diapause) with the expected number of fertile females at
274   each day, which we counted using data from experiment 1. The expected number is the
275   average of the proportion of fertile females in the18L:8D females/24L males and

276 18L:8D females/18L:8D males treatments (i.e. diapausing females with fertile or
277 diapausing males) on the precise day and the preceding and the following days
278 multiplied by 20 (20 = N per treatment), except for day 8, for which we used the
279 average of days 7 and 8 and for day 0, for which we used the data from day 1. We
280 compared the distributions of these observed and expected numbers using Fisher's exact
281 test.
282
283 ## 3. Results
284
285 ### 3.1 Experiment 1: Mating behavior, egg and offspring production and
286 CHC composition
287
288 ### 3.1.1 Female ovary development
289
290 We dissected 23 females from 24 L treatment and 22 (96 %) of these females had fully
291 developed ovaries, whereas one female (4 %) had undeveloped ovaries. Of the 23
292 females we dissected from the 16L:8D treatment, all (100 %) had undeveloped ovaries.
293 We therefore conclude that our treatment conditions worked as expected and 24 L
294 treatment produced reproductive females and 16L:8D treatment produced diapausing
295 females.
296
297 ### 3.1.2 Courtship and mating success
298
299 Both the male and the female light treatment influenced the occurrence of courtship
300 (male treatment: deviance = 38.2, p (χ^2 df = 1) < 0.001; female treatment: deviance =
301 87.7, p (χ^2 df = 1) < 0.001) and mating (male treatment: deviance = 51.2, p (χ^2 df = 1) <
302 0.001; female treatment: deviance = 87.9, p (χ^2 df = 1) < 0.001), but there was no
303 interaction between the male and the female light treatment (courtship: deviance = 2.0,
304 p (χ^2 df = 1) = 0.16; mating: deviance = 0.1, p (χ^2 df = 1) = 0.78). Final models are
305 presented in Table 2. Courtship and mating occurred in 80 % of pairs when the flies of
306 both sexes were reproductively active, while hardly any occurred when the females
307 were in diapause (Table 1). Most males were reproductively inactive when kept in
308 diapause inducing conditions as only 22 % of diapausing males courted reproductively
309 active females and only 13 % mated with them. When females were in diapause, only
310 three males out of over a hundred courted them, suggesting that diapausing females
311 were not at all attractive.
312
313 ### 3.1.3 Egg and offspring production
314
315 It took 7 days until the egg and offspring production of the females that had been
316 maintained in diapause inducing conditions had recovered to the same level as that of
317 females that were in continuous light (no significant treatment effect in egg (L-ratio
318 2.36, p (χ^2) = 0.50) or offspring (L-ratio 1.72, p (χ^2) = 0.63) production in day 7 or day
319 8 (eggs: L-ratio 1.49, p (χ^2) = 0.68; offspring: L-ratio 1.06, p (χ^2) = 0.79) (Figure 1). In
320 days 1-6 treatments differed in egg and offspring production (p (treatment) = 0.003 for
321 day 6 offspring production, all other p < 0.001).
322

323 The egg and offspring production of the females that had mated with the males reared in
324 diapause inducing conditions reached the same level as that of the females mated with
325 reproducing males on day 4 (Figure 1; eggs: comparison of 24L female/24L male *vs*
326 24L female/16L:8D male Dunn's test p = 0.71; offspring: 24L female/24L male *vs* 24L
327 female/16L:8D male Dunn's test p = 1.0). Curiously, on day 2, egg production of these
328 groups did not differ (24L female/24L male *vs* 24L female/16L:8D male Dunn's test p =
329 0.94), while offspring production did (24L female/24L male *vs* 24L female/16L:8D
330 male Dunn's test p = 0.028). This suggests that it takes about 4 days for the males to
331 fully recover from reproductive inability induced by short day length.
332
333 **3.1.4 CHC profiles of diapausing and reproductive flies**
334
335 The CHC profiles showed distinct differences between reproductively active and
336 diapausing flies. However, there was no difference between the CHC composition of
337 males and females, neither in the reproductively active flies nor in the diapausing ones
338 (Figure 2). The qualitative composition of CHC profiles of the reproductively active
339 individuals were congruent with already published data on *D. montana* populations
340 (Jennings *et al.* 2014). A detailed chemical analysis revealed that the differences of the
341 profiles could be attributed to a shift in chain-length of the entire profile. Reproductive
342 individuals exhibited CHCs from C23 to C31 whereas diapausing individuals started
343 with CHCs of the chain-length C27 to C35. The composition varies from mainly
344 alkenes and 2-methylbranched alkanes of the chain-length C23 to mainly alkadienes and
345 2-methylbranched alkanes of the chain-length of C31 in reproductively active
346 individuals. We detected a similar pattern in the CHC profile of diapausing individuals,
347 but it was shifted adding 4 C-atoms (Table 3).
348
349 **3.2 Experiment 2: Female recovery time from diapause *vs* male sexual**
350 **interest**
351
352 The proportion of females that produced offspring after the given recovery period from
353 diapause in experiment 1 is given in Table 4. These numbers were used to calculate the
354 expected number of fertile females in experiment 2 (see statistical analysis and Table 4).
355 The number of males that courted the females that had been recovering from diapause
356 for 0, 2, 4, 6, or 8 days is also presented in Table 4. The observed number of courtship
357 does not differ from the expected number of fertile females (Fisher's exact test: p =
358 0.22), which suggests that males are able to track female fertility state accurately and
359 start to court only after females have matured.
360
361 ## 4. Discussion
362
363 Diapause is an essential survival strategy for many insect species in temperate zone during
364 harsh winter conditions. While in some Diptera species, such as *Culex pipiens*, males die
365 in autumn shortly after mating and sperm is stored in female's spermatheca over winter
366 (Denlinger & Armbruster, 2016), in *D. montana* diapause is equally important for both
367 sexes as mating occurs in northern populations mainly in spring (Aspi *et al.* 1993). In this
368 species both the females and the males prepare for winter by reducing their CO_2
369 production and increasing their total body lipid content (Tyukmaeva, unpublished), the
370 usual characteristics of "diapause syndrome", which increases their chances for survival.

371 In the present study we found *D. montana* males to become reproductively inactive, i.e.
372 enter diapause, when kept under conditions that induce adult reproductive diapause in
373 females. According to our results males recover from diapause faster than females (4 days
374 *vs* 7 days) and are therefore ready to fertilize females as soon as they are receptive. Both
375 findings are in accordance of Pener's (1992) predictions about male diapause and make
376 evolutionary sense as males should not invest resources in sperm and courtship when
377 females are non-receptive. Interestingly, a recent study by Kubrak *et al.* (2016) found an
378 opposite result where *D. melanogaster* males needed more time to recover from dormancy
379 than females. This might be explained by different energy requirements during a
380 "weaker" type of dormancy in this species compared to *D. montana.* Alternatively,
381 Kimura (1988) suggests that for species with generations overlapping within one growing
382 season, such as *D. melanogaster*, earlier development of mating activity might be
383 disadvantageous due to possible competition with older males later in the growing season.
384 This, however, would not be the case with *D. montana* flies in Oulanka as they have only
385 one generation per year (Tyukmaeva *et al.* 2011).
386 Another interesting finding was that males were not at all interested in diapausing
387 females, i.e. they did not court or try to mate with them. Possibly CHCs of diapausing
388 females are unattractive to males. Diapausing flies of both sexes had longer-chained
389 CHCs than the reproducing ones but there were no sex differences in CHC composition,
390 which is in accordance with earlier studies (Bartelt *et al.* 1986; Suvanto *et al.* 2000;
391 Veltsos *et al.* 2011; Jennings *et al.* 2014) and may explain the relatively high frequency
392 of homosexual courtships in this species (Hoikkala & Liimatainen 1992; Hoikkala &
393 Aspi 1993). Despite CHCs being qualitatively sexually monomorphic in *D. montana*,
394 Veltsos *et al.* (2011) did find that CHCs clearly predicted *D. montana* male and female
395 mating success, even though their impact is smaller than that of the male courtship
396 song. However, Jennings *et al.* (2014) did not find a correlation between courtship
397 latency and female CHC profile in Oulanka population but CHCs played a role in two
398 North American populations of this species.
399
400 It has been shown that CHC profiles of several insect species serve as indicators of female
401 fertility status (e.g. Smith & Liebig, 2017; Bilen *et al*. 2013). In our study CHC chain-
402 length in diapausing *D. montana* flies varied between 27 and 35 carbon atoms while that
403 of reproductive flies was between 23 and 29 carbons. In accordance with our behavioral
404 assays we hypothesize that males have evolved an ability to detect female fertility based
405 on their CHC profile, as only females with shorter chain-length CHCs (C23 to C25)
406 evoked male interest. Whether short-chain CHCs are used by males as a cue for female
407 fertility needs to be tested in future studies. The ability to identify female reproductive
408 status efficiently and avoid energy loss from costly courtship should be especially
409 advantageous in spring when the females are recovering from diapause. The mating
410 season of northern *D. montana* populations is short (Aspi *et al.* 1993), which may lead to
411 situations where the number of receptive females exceeds male mating ability, one key
412 factor in the evolution of male mate choice (Edward & Chapman 2011). Previously, males
413 have been shown to respond adaptively to differences e.g. in female mating status, age,
414 size and fecundity in *D. melanogaster* (Byrne & Rice 2006; Friberg 2006; Lüpold *et al.*
415 2011) and in female genetic quality in *Drosophila littoralis* (Ala-Honkola *et al.* 2015).
416 Adaptive male responses have also been detected against sperm competition risk e.g. in
417 ground squirrels, *Spermophilus tridecemlineatus*, (Schwagmeyer & Parker 1990) and
418 mosquito fish, *Gambusia holbrooki* (Wong & McCarthy 2009). Our study demonstrates

419   that males target their courtship effort towards fertile females that have recovered from
420   diapause. Our data also show that males are able to accurately track changes in female
421   fertility, as the proportion of courting males raised along with an increase in the expected
422   proportion of fertile females after a given recovery time from diapause.
423   In several *Drosophila* species CHCs show minor quantitative differences under
424   different light regimes and at different times of the day (e.g. Kent *et al.* 2007; Kent *et al.*
425   2008; Krupp *et al.* 2008; Gershman *et al.* 2014). One might therefore argue that if flies
426   perceived the time of the day differently in the 16L:8D treatment than in the 24L
427   treatment, the difference in perceived time of the day could explain the differences in
428   CHC composition between our light treatments. However, contrary to *D. melanogaster*
429   (Konopka *et al.* 1989), *D. montana* flies' clock functions well under long day conditions
430   (Kauranen *et al.* 2012; Kauranen *et al.* 2016) suggesting that the flies in the two light
431   treatments perceived the time of the day quite similarly. In addition, there are several
432   long-chained hydrocarbons that are missing on sexually mature (24L) flies and several
433   short-chained hydrocarbons that are missing on diapausing (16L:8D) flies. In the
434   context of our knowledge about CHCs it is very unlikely that differences in the
435   circadian rhythmicity of flies' CHC profiles can explain the large qualitative differences
436   between the sexually mature and diapausing individuals.
437
438   Why do diapausing flies produce longer-chained CHCs than reproductive flies? For
439   recognition and communication insects are likely to more utilize short-chain CHCs (e.g.
440   Blomquist & Bagnères 2010; Menzel *et al.* 2017). Higher amounts of long-chained
441   alkanes or mono methyl-branched CHCs in the profile generate a waxier texture and
442   thus, create a stable, protective barrier against desiccation e.g. in *D. melanogaster*
443   (Gibbs *et al.* 1997; Ferveur 2005), which may be beneficial in decreasing temperature
444   where insects can be under serious drought stress (Chown *et al.* 2011). Long-chained
445   CHCs may be of special importance for adjusting water balance in diapausing insects,
446   as many other potential mechanisms for this require energy, which is not available
447   during dormancy (Danks 2000). Alterations in the desaturation levels of the membrane
448   phospholipids have also been suggested to affect insects' cold tolerance by helping to
449   maintain membrane fluidity at low temperatures (Overgaard *et al.* 2005), and thus
450   adaptation to cold could also involve changes in fatty acid synthesis leading to changes
451   in CHC profiles (Chung & Carrol 2015). In *D. melanogaster*, flies with relatively long
452   chain-length CHCs have been found to be overrepresented in the late season collections,
453   while the ones with relatively short chain CHCs are more common in early season
454   (Rajpurohit *et al.* 2017). The same phenomenon has been found in the grasshopper
455   *Melanoplus sanguinipes*, where CHC profile compositions differ between populations
456   under different climatic conditions (Rourke 2000). In adult face flies, *Musca*
457   *autumnalis*, CHC profiles of both sexes change dramatically during diapause,
458   reproducing flies having more alkenes and less methyl-branched alkanes than the
459   diapausing ones (Jurenka *et al.*1998). In some other species like flesh flies, *Sarcophaga*
460   *crassipalpis* (Yoder *et al.* 1995), and mosquitoes, *Culex pipiens* (Benoit & Denlinger
461   2007), CHC profiles of diapausing and nondiapausing puparia reflect quantitative rather
462   than qualitative differences. The extreme differences between CHCs of diapausing and
463   nondiapausing *D. montana* might have been driven by the extreme cold and drought
464   stress these flies face in their environment at the arctic circle. Generally, natural
465   selection is thought to favor the production of longer-chained non-volatile CHCs over

466 the shorter-chained more volatile compounds favored by sexual selection (Gibbs *et al.*,
467 1997; Kwan & Rundle 2010; Ingleby 2015; Otte *et al.* 2018).
468
469 It has been reported earlier in *D. montana* (Kankare *et al.* 2010) and its close relative *D.*
470 *americana* (Reis *et al.* 2015) that flies reared under diapause-inducing conditions show
471 phenotypes more similar to younger flies than one would expect by their age. This is
472 likely due to reduced levels of juvenile hormone (Tatar & Yin 2001; Yamamoto *et al.*
473 2013), which acts as a switch in CHC chain-length in e.g. *D. melanogaster* (Wicker &
474 Jallon 1995). In addition, Bilen *et al.* (2013) found genetic ablation of corpora allata
475 (the gland secreting juvenile hormone) in *D. melanogaster* to lead to a delay in mating
476 behavior and a decrease in male courtship towards females, along with the significant
477 changes in CHC profiles. Subsequently long-chained CHCs have often been found to be
478 typical to both immature and diapausing insects. In *D. melanogaster,* the CHC profiles
479 of immature flies of both sexes include 29–35 carbon atoms, while in mature flies the
480 chains with 23-29 carbon atoms become predominant (Antony & Jallon 1981; 1982;
481 Pechiné *et al.* 1988). Also, in *D. montana*'s close relative, *D. virilis*, the average chain-
482 length has been found to decrease and the sex differences to enhance when the flies get
483 older and sexually mature (Jackson & Bartelt 1986). In *D. montana*, the courtship
484 directed towards immature females usually includes only orienting and touching, but no
485 licking and singing, which suggests that immature females are not as attractive as the
486 fertile ones (Liimatainen & Hoikkala 1998). However, mature and immature flies do not
487 differ in CHC chain length in all species, e.g. in *D. mojavensis* (Etges & de Oliveira
488 2014) which might indicate different selection pressures acting on this species. CHC
489 profiles have been found to correlate with ovarian activity also in several eusocial
490 insects such as ants, wasps, bumble-bees and termites (e.g. Ayasse *et al.* 1995; Peeters
491 *et al.* 1999; Liebig *et al.* 2000; Sledge *et al.* 2001; Liebig *et al.* 2009) and they seem to
492 give honest information about an individual's fertility to the nest mates.
493
494 To conclude, our results show that *D. montana* males, as well as females, enter
495 reproductive diapause. Males are able to accurately track changes in female fertility,
496 most likely based on CHC differences of diapausing and fertile females. Males are thus
497 able to direct courtship towards fertile females that have recovered from diapause.
498
499 ## 5. Disclosure
500
501 The authors have no conflicts of interest to declare.
502
503 ## 6. Acknowledgements
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507

7. References

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773 **8. Tables**
774

775 Table 1. Percentage (and proportion) of courting and copulating males in each treatment
776 combination in the two replicates. LL = 24 h light treatment that produces reproductive
777 flies; DL = treatment with 16 h light/8 h dark that induces diapause; F = female; M =
778 male.

	Treatment combination			
Female	**FLL**	**FLL**	**FDL**	**FDL**

Male	MLL	MDL	MDL	MLL
Courtship				
Replicate 1	(17/19)	(7/19)	(1/17)	(1/19)
Replicate 2	(26/35)	(5/35)	(0/35)	(1/35)
% courting	80 % (43/54)	22 % (12/54)	2 % (1/52)	4 % (2/54)
Mating				
Replicate 1	(17/19)	(3/19)	(0/17)	(0/19)
Replicate 2	(25/35)	(4/35)	(0/35)	(1/35)
% mating	78 % (42/54)	13 % (7/54)	0 % (0/52)	2 % (1/54)

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781 Table 2. Final generalized linear models explaining variance in courtship and mating
782 data. LL = 24 h light treatment that produces reproductive flies; DL = treatment with 16
783 h light/8 h dark that induces diapause; F = female; M = male).

	Effect	Parameter estimate	SE	z-value	p
Courtship	Intercept (FDL, MDL, Replicate 1)	-4.91	0.74	-6.66	< 0.001
	FLL	4.41	0.70	6.35	< 0.001
	MLL	2.61	0.48	5.45	< 0.001
	Replicate 2	-1.16	0.48	-2.45	0.015
Mating	Intercept (FDL, MDL)	-7.18	1.13	-6.38	< 0.001
	FLL	5.27	1.06	4.99	< 0.001
	MLL	3.17	0.52	6.10	< 0.001

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787 Table 3. List of peaks of cuticular hydrocarbon extracts from diapausing and
788 reproductive *Drosophila montana* males and females with calculated mean proportions
789 and standard deviations. RT = Retention time; LL = 24 h light treatment that produces
790 reproductive flies; DL = treatment with 16 h light/8 h dark that induces diapause; F =
791 female; M = male; ? = M^+ (molecular ion) not found.

792

Peak #	RT	Compound name	DL		LL	
			MDL	FDL	MLL	FLL
1	2291	x-C23en	-	-	-	0.005 ± 0.007
2	2463	2-MeC24	-	-	0.024 ± 0.005	0.027 ± 0.007
3	2474	9-C25en	-	-	0.013 ± 0.006	0.009 ± 0.003
4	2481	7-C25en	-	-	0.026 ± 0.007	0.023 ± 0.005
5	2492	5-C25en	-	-	0.063 ± 0.008	0.057 ± 0.006
6	2660	5,x-C27dien	-	0.001 ± 0.001	0.017 ± 0.007	0.013 ± 0.007
7	2662	2-MeC26	0.124 ± 0.021	0.119 ± 0.025	0.313 ± 0.078	0.305 ± 0.057
8	2668	13-; 11-C27en	0.030 ± 0.009	0.026 ± 0.006	0.072 ± 0.092	0.174 ± 0.110
9	2673	9-C27en	0.066 ± 0.021	0.049 ± 0.013	0.140 ± 0.024	0.098 ± 0.028
10	2680	7-C27en	-	-	0.003 ± 0.002	0.002 ± 0.002
11	2700	C27	-	-	-	0.002 ± 0.003
12	2849	x,x-C29dien	0.019 ± 0.005	0.015 ± 0.005	0.015 ± 0.008	0.014 ± 0.005
13	2851	7,x-C29dien	-	-	0.059 ± 0.012	0.039 ± 0.008
14	2857	7,x-C29dien	0.029 ± 0.010	0.022 ± 0.009	-	-
15	2863	2-MeC28; 13-C29en	0.367 ± 0.044	0.359 ± 0.070	0.243 ± 0.045	0.217 ± 0.049
16	2876	9-C29en	0.035 ± 0.009	0.025 ± 0.006	-	-
17	3044	x,x-C31dien	0.035 ± 0.007	0.032 ± 0.007	0.002 ± 0.001	0.002 ± 0.002
18	3053	x,x-C31dien	0.042 ± 0.008	0.033 ± 0.007	-	-
19	3063	2-MeC30	-	-	0.009 ± 0.004	0.013 ± 0.005
20	3068	8-C31en	0.121 ± 0.020	0.099 ± 0.031	-	-
21	3076	6-C31en	-	0.044 ± 0.037	-	-
22	3236	x,x-C33dien?	0.018 ± 0.024	0.007 ± 0.004	-	-
23	3244	x,x-C33dien	0.023 ± 0.011	0.027 ± 0.005	-	-
24	3252	x,x-C33dien	0.053 ± 0.015	0.069 ± 0.020	-	-
25	3260	x,x-C33dien	0.008 ± 0.009	0.019 ± 0.014	-	-
26	3435	x,x-C35dien?	0.002 ± 0.001	0.004 ± 0.002	-	-
27	3443	x,x-C35dien	0.013 ± 0.004	0.021 ± 0.006	-	-
28	3450	x,x-C35dien	0.016 ± 0.009	0.029 ± 0.012	-	-

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794

795 Table 4. The proportion of females producing offspring after the given recovery period
796 from diapause in experiment 1, the number of males that courted females after the given
797 recovery period from diapause in experiment 2, and the expected number of fertile
798 females after the given recovery period based the data from experiment 1.

Recovery time from diapause (days)	0	1	2	3	4	5	6	7	8
Experiment 1									
Proportion of females producing offspring		0/67	0/67	0/67	1/67	8/67	26/67	48/67	48/67
Experiment 2									
Number of courting males (out of N = 20)	2		3		6		14		14
Expected number of fertile females (out of N = 20)	0		0		1		8		14

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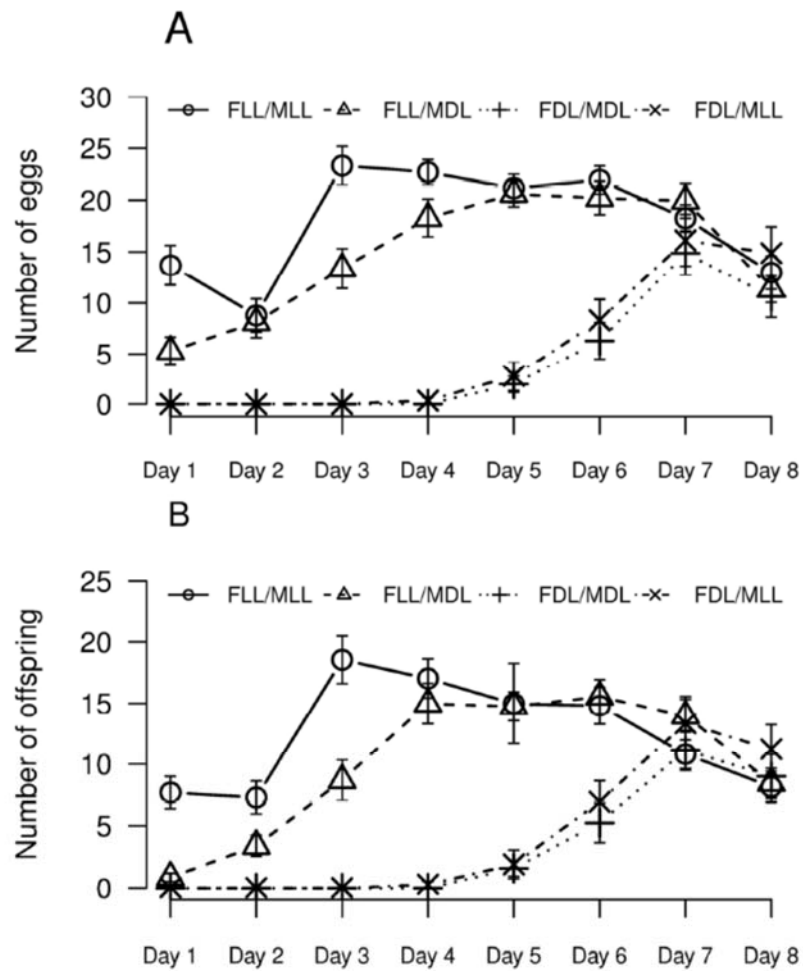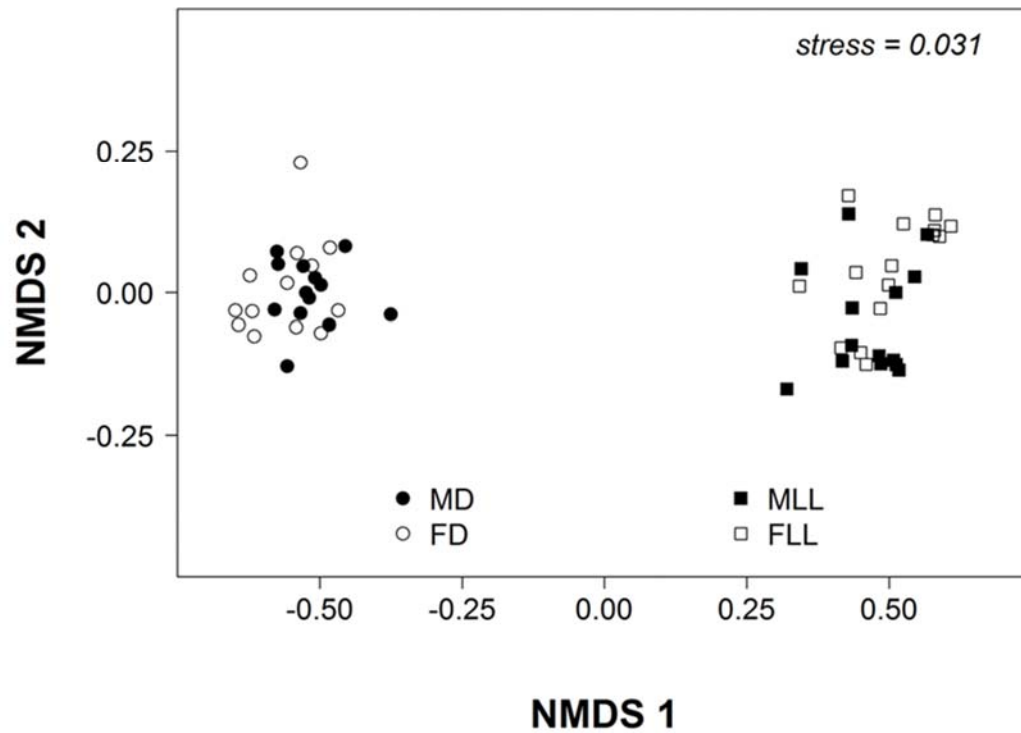802 **9. Figures**
803

A

B

Figure 1

142x165mm (300 x 300 DPI)

804

805 Figure 1. Number of eggs (A) and offspring (B) produced in each female/male treatment
806 combination over 8 days. LL = 24 h light treatment that produces reproductive flies; DL
807 = treatment with 16 h light/8 h dark that induces diapause; F = female; M = male.

NMDS 1

808

809 Figure 2. Similarity of *Drosophila montana* male and female cuticular hydrocarbon
810 (CHC) profiles kept under either 24 h light conditions (LL) that produces reproductive
811 flies (MLL = males; FLL = females) or 16 h light/8 h dark (DL) that induces diapause
812 (MDL = males; FDL = females) displayed in a two-dimensional graph by non-metric
813 multidimensional scaling (NMDS) of Bray–Curtis CHC profile dissimilarities (stress =
814 0.031).