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Diapause affects cuticular hydrocarbon composition and mating behaviour of both sexes in *Drosophila montana*

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

Abstract

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

Key words: cuticular hydrocarbon, diapause, *Drosophila*, male reproduction, male 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

under both natural and sexual selection (e.g. Blows 2002; Frentiu & Chenoweth 2010). The first is suggested to favor long-chain and the latter one short-chain hydrocarbons (Gibbs *et al.* 1997; Kwan & Rundle 2010; Chung & Carrol 2014; Ingleby 2015; Otte *et al.* 2018).

Rajpurohit *et al.* (2017) have shown that flies' CHC profiles respond rapidly and adaptively to environmental parameters that covary with latitude and season in *Drosophila melanogaster*. Also, in several insect species, diapausing individuals have been found to differ from the reproducing ones in their CHCs at pupal (Coudron & Nelson 1981; Yoder *et al.* 1995; Kaneko & Katagiri 2004) or adult (Benoit & Denlinger 2007; Jurenka *et al.* 1998) stage. Because diapausing *D. montana* flies encounter different abiotic conditions than the reproducing ones (diapausing flies face up to 7 months of winter and start to reproduce in spring when temperature rises above 10ºC), we anticipate that natural selection has driven the CHC composition of overwintering flies towards longer-chain CHCs. CHC profiles of diapausing and reproducing *D. montana* flies could be further diverged due to hormones like the juvenile hormone, which is involved in diapause regulation in many insect species (Tauber *et al.* 1986). In *D. melanogaster*, topical application of juvenile hormone analogue has been found to decrease the amount of long-chain hydrocarbons on the cuticle (Wicker & Jallon 1995), which mimics the hormonal changes occurring during sexual maturation and termination of diapause. In *D. montana*, CHCs have been found to show quantitative variation among populations, while sex differences are modest or absent (Bartelt *et al.* 1986; Suvanto *et al.* 2000; Veltsos *et al.* 2012; Jennings *et al.* 2014).

In this study our aim was i) to find out whether male diapause exists in *D. montana*, ii) to examine the behavior of males toward diapausing and non-diapausing females, and iii) to compare CHCs of diapausing and non-diapausing flies. We predicted that: 1) males will be in diapause when they are kept under conditions that induce diapause in females in order to save resources and be prepared for harsh environmental conditions, 2) males recover from diapause faster than females to be able to mate as soon as females are receptive, 3) CHCs of diapausing males and females consist of longer chain hydrocarbons than those of reproducing ones, which should increase their survival during overwintering and 4) males discriminate between appropriate and non-appropriate mating partner, i.e. the percentage of courting males increases along with an increase in the percentage of fertile females. Our study showed all these predictions to be true and gives support to the dual role of CHCs in adaptation and mate choice.

2. Materials and methods

2.1 Stocks and maintenance

Experimental flies were descendants of the flies collected from riparian habitats in Oulanka (66°22'N, 29°19'E), Finland, in the summer of 2008. Once in the laboratory, isofemale lines were established from the progenies of fertilized wild-caught females and maintained in half-pint bottles on Lakovaara malt medium. From each isofemale line (N = 20), 20 F_3 males and 20 F_3 females (800 total flies) were transferred into a 25 × 25 × 60 cm wooden population cage with a Plexiglas top and eight food bottles for feeding, oviposition and larval rearing, and bred in overlapping generations under constant light and temperature (19°C). Constant light (or long day length) is necessary to prevent flies from undergoing reproductive diapause (Lumme 1978). Experimental flies were collected on the day of eclosion from the food bottles using CO_2 as an

anaesthetizing agent and moved in malt vials to either diapause or sexual maturation inducing conditions for 21 days (see below). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

2.2 Experiment 1: Mating behavior, egg and offspring production and CHC composition

The day length varies in Oulanka from 21 to 22.6 hours during *D. montana* breeding season in June and decreases to 15 hours as the progeny eclose until August. The critical day length (CDL), at which 50 % of females will enter diapause, is 18.7 hours in Oulanka and when the day length is 16 hours, 100 % of females will enter diapause (Tyukmaeva *et al.* 2011). Since there is daylight for almost 24 hours during the early summer, we used 24 hours light (24L) as conditions inducing sexual maturation and 16 hours light (L) and 8 hours dark (D) (16L:8D) as diapause-inducing conditions (16ºC). On the day of eclosion, half of the males and half of the females (randomly chosen) were allocated into the 24L treatment and the other half were allocated into the 16L:8D treatment. This treatment is appropriate as *D. montana* females adjust their development (sexual maturation vs. diapause) according to the post eclosion conditions (Salminen *et al.* 2012). Flies were kept in single-sex food vials with 10 flies in each vial and changed into new vials once a week during the 21 days maturation period.

We performed a full factorial experiment in two replicates (Rep) to investigate how diapause inducing conditions affect male and female mating behavior and reproductive output. The sample sizes are reported in Table 1. In the first replicate, we only measured fly mating behavior and in the second one, we measured mating behavior and thereafter egg and offspring production during the 8 days the male and the female were paired. To minimize observer bias, blinded methods were used when all behavioral data were recorded. During the mating experiment, we recorded the time when the male and the female were transferred into the vial as a pair, the time of the first courtship song (the song, produced by wing vibration, is obligatory for successful mating (Liimatainen et al. 1992)) and the beginning and the end of copulation. The flies were observed for two hours. However, as courtship and copulation mainly occurred in the treatment where both males and females were at reproductive state (i.e. 24L females with 24L males), the data were coded as categorical: courtship/no courtship and copulation/no copulation. Egg and offspring production was measured in Rep 2 by transferring the fly pairs into new vials daily for 8 days and counting the number of eggs and eclosing offspring from each vial. The flies were kept in 24L at 18ºC during egg laying. To make sure that the females entered diapause in 16L:8D and reproductive state in 24L, 23 females from both treatments were dissected under a stereomicroscope at the age of 21 days.

Finally, we extracted CHCs from 15 males and females from both the 24L and 16L:8D treatment. These flies were frozen at -20ºC after 21 days under the respective conditions. CHCs were extracted at the University of Jyväskylä (Finland) by immersing individual flies in 200 µl n-hexane for 10 min (gently vortexing them twice) in 1.5 ml glass vials. Flies were then removed from the solvent and the vials were left in a fume hood at room temperature until the solvent evaporated. Extracts were sealed and stored at -20ºC until they were shipped on dry ice to the University of Würzburg, Germany, for gas chromatography/mass spectrometry analysis.

The CHC extracts were analyzed with a QP2010 Ultra CI gas chromatograph (GC) coupled with a mass spectrometer (MS) (Shimadzu, Duisburg, Germany). The GC (split/splitless-injector in splitless mode for 1 min, injected volume: 1 µl at 250°C) was equipped with a DB-5 Fused Silica capillary column (30 m x 0.25 mm ID, *df* = 0.25 µm, J&W Scientific, Folsom, USA). Helium served as a carrier gas with a linear velocity of 146.8 kPa. The following temperature program was used: start temperature 60°C hold for 1 min, temperature increase by 5°C per minute up to 300°C, isotherm at 300°C for 10 minutes. The transfer line had a temperature of 300°C. The electron ionization mass spectra (EI-MS) were acquired at an ionization voltage of 70 eV (source temperature: 230°C).

Chromatograms and mass spectra were recorded and quantified via integrated peak areas with the software GC solution V2.41 (Shimadzu, Duisburg, Germany). Individual CHC compounds were characterized by considering the MS data base Wiley275 (John Wiley & Sons, New York, USA), retention indices, and the detected diagnostic ions (Carlson *et al.* 1998). Double-bond positions in alkenes and, if possible, in alkadienes were determined by DMDS derivatization as stated in Dunkelblum *et al.* (1980). Retention indices of all compounds were calculated using an alkane standard. Given that some substances could not be accurately separated with the above instrument and settings, we calculated their combined quantity by integrating over all substances within a peak in these cases.

2.3 Experiment 2: Female recovery time from diapause *vs* male sexual interest

In the second set of experiments we investigated how well the males are able to track female recovery from diapause, i.e. we asked whether the males become sexually interested in females at the same time as the females become fertile after their recovery from diapause (in experiment 1 this happened 5-7 days after females were taken into 24L conditions). Flies for this experiment were collected from a new population cage (the older cage used in experiment 1 was contaminated) that was established from F3 descendants of 104 females caught from the wild in Oulanka, Finland, in 2013. The cage was established and maintained as explained above.

We transferred newly eclosed females into the 16L:8D condition at 16ºC every two days for 8 days. When the females had stayed 20 days under those conditions, they were transferred into continuous light (24L at 18ºC) and allowed to recover from diapause for either 0, 2, 4, 6 or 8 days before the mating experiment (N = 20 per time treatment). Males for this experiment were collected as virgins, kept at 24L at 18ºC and used 20-26 days post eclosion. In the mating experiment, we recorded the time when a male and a female were introduced in the vial as a pair, the time of the first courtship song and the beginning and end of copulation. Again, the data was coded as categorical: courtship/no courtship and copulation/no copulation. The pairs were observed for 90 minutes.

2.4 Statistical analysis

We used R (version 3.0.2) for statistical analysis (R development core team 2013). We analyzed the mating and the courtship data with a generalized linear model (GLM) with logit link function and binomial error structure using sample sizes as weight following Crawley (2007). The full model included the female light treatment, the male light treatment, their interaction and replicate. The full model was simplified until only significant factors remained by removing each term in turn and comparing nested models with and without the given term with an analysis of deviance. The models were not overdispersed. Analyzing the number of egg and offspring data was problematic because of the large number of zeros (some treatments had only zeros in first days), which caused numerical problems in generalized linear models (GLM) and zero-inflated models. Also, variances differed a lot among treatments (see Figure 1). In order to evaluate how long it takes for the females and males to recover from diapause, we therefore analyzed egg and offspring production separately for each day. Egg and offspring production in days 1-4 were analyzed with Kruskal-Wallis test and multiple comparisons were performed with Dunn's test (Dunn 1964; library "dunn.test" in R; Dinno 2015) with Bonferroni correction. Egg and offspring production in days 5-8 were analyzed with GLMs with negative binomial distribution (Poisson models were overdispersed) using the function "glm.nb" in library "MASS" (Venables & Ripley 2002). The significance of the factor "treatment" was assessed with likelihood ratio test (L-ratio) by comparing nested models with and without that factor (Zuur *et al.* 2009). We performed model validations by examining the homogeneity and independence of errors. Multiple comparisons (Tukey's test) were performed with library "multcomp" (Hothorn *et al.* 2008).

For the comparison of CHC profiles of both sexes under diapause and non-diapause conditions, we only considered CHC compounds that had relative quantitative abundance more than 0.1 % of the total quantitative CHC abundance in the respective extracts and which were recognized in more than 50% of the samples within each group. The CHC compositions of all individuals were compared by means of multivariate methods. Therefore, we log-ratio transformed all quantitative CHC values and calculated Bray-Curtis dissimilarities (Bray & Curtis 1957; taking into account compound identities and their relative contributions to the CHC profiles) between all pairs of samples using the vegdist function of the vegan package (version 2.0-10) (Oksanen *et al.* 2013) of the R statistical software (version 3.0.2). The Bray-Curtis dissimilarity values were subsequently displayed in a two-dimensional graph via non-metric multidimensional scaling (NMDS) using the metaMDS function of the vegan package. The spatial distances between points in the NMDS plot indicates the chemical differences between samples and the corresponding stress value indicates the goodness of fit of the two-dimensional representation to the initial multidimensional distances, with a stress value < 15 indicating a good fit. Note that NMDS does not require a priori knowledge of what samples likely represent a group. Any data structures emerging from these visualization methods are purely based on the similarities of the chemical compositions of the analyzed extracts.

In experiment 2, we compared the number of courting males in each treatment (0, 2, 4, 6 and 8 days recovery time from diapause) with the expected number of fertile females at each day, which we counted using data from experiment 1. The expected number is the average of the proportion of fertile females in the18L:8D females/24L males and

18L:8D females/18L:8D males treatments (i.e. diapausing females with fertile or diapausing males) on the precise day and the preceding and the following days multiplied by 20 (20 = N per treatment), except for day 8, for which we used the average of days 7 and 8 and for day 0, for which we used the data from day 1. We compared the distributions of these observed and expected numbers using Fisher's exact test.

3. Results

3.1 Experiment 1: Mating behavior, egg and offspring production and CHC composition

3.1.1 Female ovary development

We dissected 23 females from 24 L treatment and 22 (96 %) of these females had fully developed ovaries, whereas one female (4 %) had undeveloped ovaries. Of the 23 females we dissected from the 16L:8D treatment, all (100 %) had undeveloped ovaries. We therefore conclude that our treatment conditions worked as expected and 24 L treatment produced reproductive females and 16L:8D treatment produced diapausing females.

3.1.2 Courtship and mating success

Both the male and the female light treatment influenced the occurrence of courtship (male treatment: deviance = 38.2, p (χ^2 df = 1) < 0.001; female treatment: deviance = 87.7, p (χ^2 df = 1) < 0.001) and mating (male treatment: deviance = 51.2, p (χ^2 df = 1) < 0.001; female treatment: deviance = 87.9, p (χ^2 df = 1) < 0.001), but there was no interaction between the male and the female light treatment (courtship: deviance = 2.0, p (χ^2 df = 1) = 0.16; mating: deviance = 0.1, p (χ^2 df = 1) = 0.78). Final models are presented in Table 2. Courtship and mating occurred in 80 % of pairs when the flies of both sexes were reproductively active, while hardly any occurred when the females were in diapause (Table 1). Most males were reproductively inactive when kept in diapause inducing conditions as only 22 % of diapausing males courted reproductively active females and only 13 % mated with them. When females were in diapause, only three males out of over a hundred courted them, suggesting that diapausing females were not at all attractive.

3.1.3 Egg and offspring production

It took 7 days until the egg and offspring production of the females that had been maintained in diapause inducing conditions had recovered to the same level as that of females that were in continuous light (no significant treatment effect in egg (L-ratio 2.36, p (χ^2) = 0.50) or offspring (L-ratio 1.72, p (χ^2) = 0.63) production in day 7 or day 8 (eggs: L-ratio 1.49, p (χ^2) = 0.68; offspring: L-ratio 1.06, p (χ^2) = 0.79) (Figure 1). In days 1-6 treatments differed in egg and offspring production (p (treatment) = 0.003 for day 6 offspring production, all other p < 0.001).

The egg and offspring production of the females that had mated with the males reared in diapause inducing conditions reached the same level as that of the females mated with reproducing males on day 4 (Figure 1; eggs: comparison of 24L female/24L male *vs* 24L female/16L:8D male Dunn's test p = 0.71; offspring: 24L female/24L male *vs* 24L female/16L:8D male Dunn's test p = 1.0). Curiously, on day 2, egg production of these groups did not differ (24L female/24L male *vs* 24L female/16L:8D male Dunn's test p = 0.94), while offspring production did (24L female/24L male *vs* 24L female/16L:8D male Dunn's test p = 0.028). This suggests that it takes about 4 days for the males to fully recover from reproductive inability induced by short day length.

3.1.4 CHC profiles of diapausing and reproductive flies

The CHC profiles showed distinct differences between reproductively active and diapausing flies. However, there was no difference between the CHC composition of males and females, neither in the reproductively active flies nor in the diapausing ones (Figure 2). The qualitative composition of CHC profiles of the reproductively active individuals were congruent with already published data on *D. montana* populations (Jennings *et al.* 2014). A detailed chemical analysis revealed that the differences of the profiles could be attributed to a shift in chain-length of the entire profile. Reproductive individuals exhibited CHCs from C23 to C31 whereas diapausing individuals started with CHCs of the chain-length C27 to C35. The composition varies from mainly alkenes and 2-methylbranched alkanes of the chain-length C23 to mainly alkadienes and 2-methylbranched alkanes of the chain-length of C31 in reproductively active individuals. We detected a similar pattern in the CHC profile of diapausing individuals, but it was shifted adding 4 C-atoms (Table 3).

3.2 Experiment 2: Female recovery time from diapause *vs* male sexual interest

The proportion of females that produced offspring after the given recovery period from diapause in experiment 1 is given in Table 4. These numbers were used to calculate the expected number of fertile females in experiment 2 (see statistical analysis and Table 4). The number of males that courted the females that had been recovering from diapause for 0, 2, 4, 6, or 8 days is also presented in Table 4. The observed number of courtship does not differ from the expected number of fertile females (Fisher's exact test: p = 0.22), which suggests that males are able to track female fertility state accurately and start to court only after females have matured.

4. Discussion

Diapause is an essential survival strategy for many insect species in temperate zone during harsh winter conditions. While in some Diptera species, such as *Culex pipiens*, males die in autumn shortly after mating and sperm is stored in female's spermatheca over winter (Denlinger & Armbruster, 2016), in *D. montana* diapause is equally important for both sexes as mating occurs in northern populations mainly in spring (Aspi *et al.* 1993). In this species both the females and the males prepare for winter by reducing their CO_2 production and increasing their total body lipid content (Tyukmaeva, unpublished), the usual characteristics of "diapause syndrome", which increases their chances for survival.

In the present study we found *D. montana* males to become reproductively inactive, i.e. enter diapause, when kept under conditions that induce adult reproductive diapause in females. According to our results males recover from diapause faster than females (4 days *vs* 7 days) and are therefore ready to fertilize females as soon as they are receptive. Both findings are in accordance of Pener's (1992) predictions about male diapause and make evolutionary sense as males should not invest resources in sperm and courtship when females are non-receptive. Interestingly, a recent study by Kubrak *et al.* (2016) found an opposite result where *D. melanogaster* males needed more time to recover from dormancy than females. This might be explained by different energy requirements during a "weaker" type of dormancy in this species compared to *D. montana.* Alternatively, Kimura (1988) suggests that for species with generations overlapping within one growing season, such as *D. melanogaster*, earlier development of mating activity might be disadvantageous due to possible competition with older males later in the growing season. This, however, would not be the case with *D. montana* flies in Oulanka as they have only one generation per year (Tyukmaeva *et al.* 2011).

Another interesting finding was that males were not at all interested in diapausing females, i.e. they did not court or try to mate with them. Possibly CHCs of diapausing females are unattractive to males. Diapausing flies of both sexes had longer-chained CHCs than the reproducing ones but there were no sex differences in CHC composition, which is in accordance with earlier studies (Bartelt *et al.* 1986; Suvanto *et al.* 2000; Veltsos *et al.* 2011; Jennings *et al.* 2014) and may explain the relatively high frequency of homosexual courtships in this species (Hoikkala & Liimatainen 1992; Hoikkala & Aspi 1993). Despite CHCs being qualitatively sexually monomorphic in *D. montana*, Veltsos *et al.* (2011) did find that CHCs clearly predicted *D. montana* male and female mating success, even though their impact is smaller than that of the male courtship song. However, Jennings *et al.* (2014) did not find a correlation between courtship latency and female CHC profile in Oulanka population but CHCs played a role in two North American populations of this species.

It has been shown that CHC profiles of several insect species serve as indicators of female fertility status (e.g. Smith & Liebig, 2017; Bilen *et al*. 2013). In our study CHC chain-length in diapausing *D. montana* flies varied between 27 and 35 carbon atoms while that of reproductive flies was between 23 and 29 carbons. In accordance with our behavioral assays we hypothesize that males have evolved an ability to detect female fertility based on their CHC profile, as only females with shorter chain-length CHCs (C23 to C25) evoked male interest. Whether short-chain CHCs are used by males as a cue for female fertility needs to be tested in future studies. The ability to identify female reproductive status efficiently and avoid energy loss from costly courtship should be especially advantageous in spring when the females are recovering from diapause. The mating season of northern *D. montana* populations is short (Aspi *et al.* 1993), which may lead to situations where the number of receptive females exceeds male mating ability, one key factor in the evolution of male mate choice (Edward & Chapman 2011). Previously, males have been shown to respond adaptively to differences e.g. in female mating status, age, size and fecundity in *D. melanogaster* (Byrne & Rice 2006; Friberg 2006; Lüpold *et al.* 2011) and in female genetic quality in *Drosophila littoralis* (Ala-Honkola *et al.* 2015). Adaptive male responses have also been detected against sperm competition risk e.g. in ground squirrels, *Spermophilus tridecemlineatus*, (Schwagmeyer & Parker 1990) and mosquito fish, *Gambusia holbrooki* (Wong & McCarthy 2009). Our study demonstrates

that males target their courtship effort towards fertile females that have recovered from diapause. Our data also show that males are able to accurately track changes in female fertility, as the proportion of courting males raised along with an increase in the expected proportion of fertile females after a given recovery time from diapause.

In several *Drosophila* species CHCs show minor quantitative differences under different light regimes and at different times of the day (e.g. Kent *et al.* 2007; Kent *et al.* 2008; Krupp *et al.* 2008; Gershman *et al.* 2014). One might therefore argue that if flies perceived the time of the day differently in the 16L:8D treatment than in the 24L treatment, the difference in perceived time of the day could explain the differences in CHC composition between our light treatments. However, contrary to *D. melanogaster* (Konopka *et al.* 1989), *D. montana* flies' clock functions well under long day conditions (Kauranen *et al*. 2012; Kauranen *et al*. 2016) suggesting that the flies in the two light treatments perceived the time of the day quite similarly. In addition, there are several long-chained hydrocarbons that are missing on sexually mature (24L) flies and several short-chained hydrocarbons that are missing on diapausing (16L:8D) flies. In the context of our knowledge about CHCs it is very unlikely that differences in the circadian rhythmicity of flies' CHC profiles can explain the large qualitative differences between the sexually mature and diapausing individuals.

Why do diapausing flies produce longer-chained CHCs than reproductive flies? For recognition and communication insects are likely to more utilize short-chain CHCs (e.g. Blomquist & Bagnères 2010; Menzel *et al*. 2017). Higher amounts of long-chained alkanes or mono methyl-branched CHCs in the profile generate a waxier texture and thus, create a stable, protective barrier against desiccation e.g. in *D. melanogaster* (Gibbs *et al*. 1997; Ferveur 2005), which may be beneficial in decreasing temperature where insects can be under serious drought stress (Chown *et al.* 2011). Long-chained CHCs may be of special importance for adjusting water balance in diapausing insects, as many other potential mechanisms for this require energy, which is not available during dormancy (Danks 2000). Alterations in the desaturation levels of the membrane phospholipids have also been suggested to affect insects' cold tolerance by helping to maintain membrane fluidity at low temperatures (Overgaard *et al.* 2005), and thus adaptation to cold could also involve changes in fatty acid synthesis leading to changes in CHC profiles (Chung & Carrol 2015). In *D. melanogaster*, flies with relatively long chain-length CHCs have been found to be overrepresented in the late season collections, while the ones with relatively short chain CHCs are more common in early season (Rajpurohit *et al.* 2017). The same phenomenon has been found in the grasshopper *Melanoplus sanguinipes*, where CHC profile compositions differ between populations under different climatic conditions (Rourke 2000). In adult face flies, *Musca autumnalis*, CHC profiles of both sexes change dramatically during diapause, reproducing flies having more alkenes and less methyl-branched alkanes than the diapausing ones (Jurenka *et al.*1998). In some other species like flesh flies, *Sarcophaga crassipalpis* (Yoder *et al.* 1995), and mosquitoes, *Culex pipiens* (Benoit & Denlinger 2007), CHC profiles of diapausing and nondiapausing puparia reflect quantitative rather than qualitative differences. The extreme differences between CHCs of diapausing and nondiapausing *D. montana* might have been driven by the extreme cold and drought stress these flies face in their environment at the arctic circle. Generally, natural selection is thought to favor the production of longer-chained non-volatile CHCs over

the shorter-chained more volatile compounds favored by sexual selection (Gibbs *et al.*, 1997; Kwan & Rundle 2010; Ingleby 2015; Otte *et al.* 2018).

It has been reported earlier in *D. montana* (Kankare *et al.* 2010) and its close relative *D. americana* (Reis *et al.* 2015) that flies reared under diapause-inducing conditions show phenotypes more similar to younger flies than one would expect by their age. This is likely due to reduced levels of juvenile hormone (Tatar & Yin 2001; Yamamoto *et al.* 2013), which acts as a switch in CHC chain-length in e.g. *D. melanogaster* (Wicker & Jallon 1995). In addition, Bilen *et al.* (2013) found genetic ablation of corpora allata (the gland secreting juvenile hormone) in *D. melanogaster* to lead to a delay in mating behavior and a decrease in male courtship towards females, along with the significant changes in CHC profiles. Subsequently long-chained CHCs have often been found to be typical to both immature and diapausing insects. In *D. melanogaster,* the CHC profiles of immature flies of both sexes include 29–35 carbon atoms, while in mature flies the chains with 23-29 carbon atoms become predominant (Antony & Jallon 1981; 1982; Pechiné *et al.* 1988). Also, in *D. montana*'s close relative, *D. virilis*, the average chain-length has been found to decrease and the sex differences to enhance when the flies get older and sexually mature (Jackson & Bartelt 1986). In *D. montana*, the courtship directed towards immature females usually includes only orienting and touching, but no licking and singing, which suggests that immature females are not as attractive as the fertile ones (Liimatainen & Hoikkala 1998). However, mature and immature flies do not differ in CHC chain length in all species, e.g. in *D. mojavensis* (Etges & de Oliveira 2014) which might indicate different selection pressures acting on this species. CHC profiles have been found to correlate with ovarian activity also in several eusocial insects such as ants, wasps, bumble-bees and termites (e.g. Ayasse *et al.* 1995; Peeters *et al.* 1999; Liebig *et al.* 2000; Sledge *et al.* 2001; Liebig *et al.* 2009) and they seem to give honest information about an individual's fertility to the nest mates.

To conclude, our results show that *D. montana* males, as well as females, enter reproductive diapause. Males are able to accurately track changes in female fertility, most likely based on CHC differences of diapausing and fertile females. Males are thus able to direct courtship towards fertile females that have recovered from diapause.

5. Disclosure

The authors have no conflicts of interest to declare.

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8. Tables

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

Table 2. Final generalized linear models explaining variance in courtship and mating data. LL = 24 h light treatment that produces reproductive flies; DL = treatment with 16 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

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

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	-	-

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

799

800

801

802 **9. Figures**
803

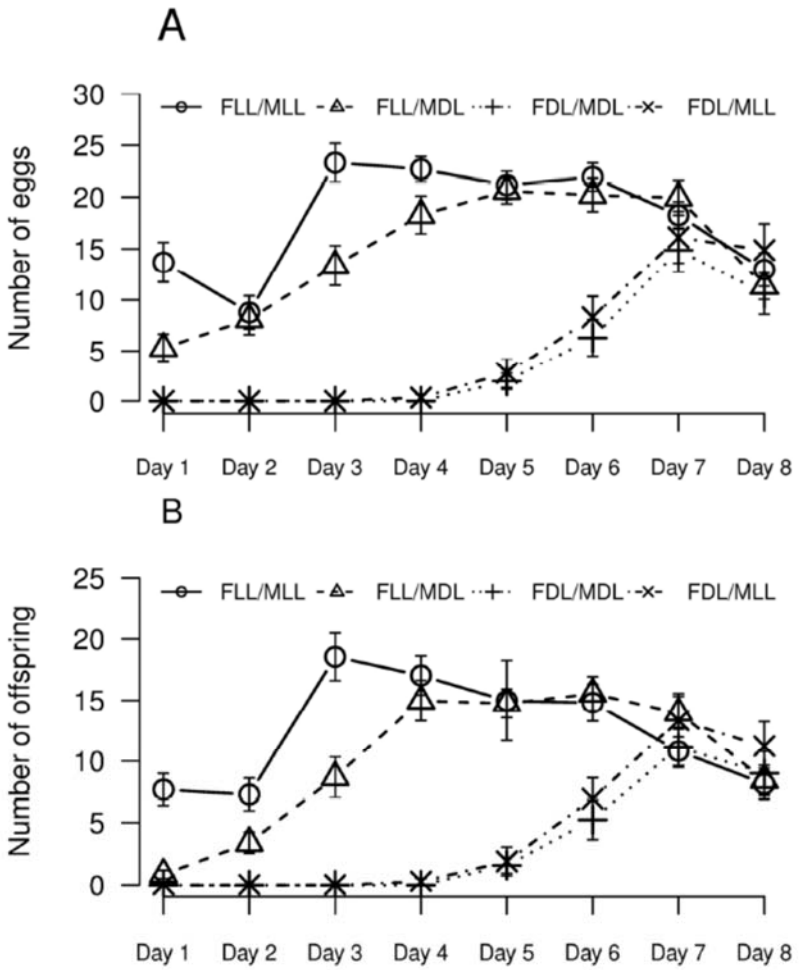

Figure 1

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.

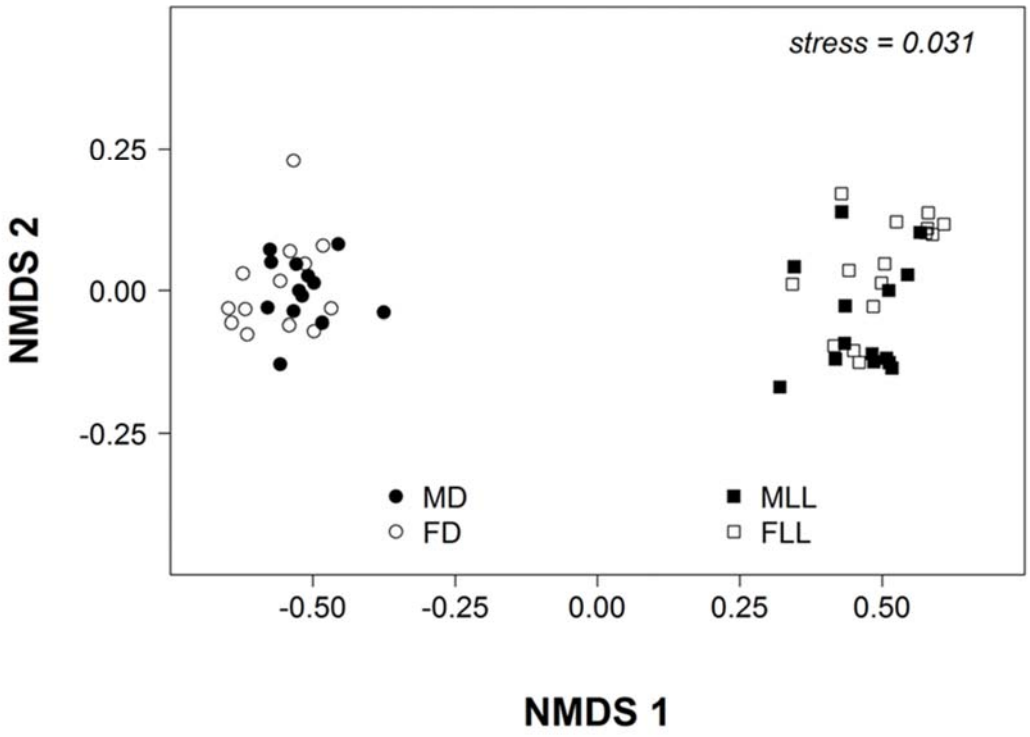

Figure 2. Similarity of *Drosophila montana* male and female cuticular hydrocarbon (CHC) profiles kept under either 24 h light conditions (LL) that produces reproductive flies (MLL = males; FLL = females) or 16 h light/8 h dark (DL) that induces diapause (MDL = males; FDL = females) displayed in a two-dimensional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