Author(s): Kauranen, Hannele; Ala-Honkola, Outi; Kankare, Maaria; Hoikkala, Anneli

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Circadian clock of D*rosophila montana* is adapted to high variation in summer day lengths and temperatures prevailing at high latitudes

Hannele Kauranen[a], Outi Ala-Honkola[a], Maaria Kankare[a] and Anneli Hoikkala[a]

[a]University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, Jyväskylä, Finland

Corresponding author:
Hannele Kauranen
Department of Biological and Environmental Science
P.O. Box 35, University of Jyväskylä
40014 Jyväskylä, Finland
Phone: +35840 8053885; fax: +35814 617239
E-mail: hannele.kauranen@jyu.fi

ABSTRACT

Photoperiodic regulation of the circadian rhythms in insect locomotor activity has been studied in several species, but seasonal entrainment of these rhythms is still poorly understood. We have traced the entrainment of activity rhythm of northern *Drosophila montana* flies in a climate chamber mimicking the photoperiods and day and night temperatures that the flies encounter in northern Finland during the summer. The experiment was started by transferring freshly emerged females into the chamber in early and late summer conditions to obtain both non-diapausing and diapausing females for the studies. The locomotor activity of the females and daily changes in the expression levels of two core circadian clock genes, *timeless* and *period*, in their heads were measured at different times of summer. The study revealed several features in fly rhythmicity that are likely to help the flies to cope with high variation in the day length and temperature typical to northern summers. First, both the non-diapausing and the diapausing females showed evening activity, which decreased towards the short day length as observed in the autumn in nature. Second, *timeless* and *period* genes showed concordant daily oscillations and seasonal shifts in their expression level in both types of females. Contrary to *D. melanogaster*, oscillation profiles of these genes were similar to each other in all conditions, including the extremely long days in early summer and the cool temperatures in late summer, and their peak expression levels were not locked to lights-off transition in any photoperiod. Third, the diapausing females were less active than the non-diapausing ones, in spite of their younger age. Overall, the study showed that *D. montana* clock functions well under long day conditions, and that both the photoperiod and the daily temperature cycles are important zeitgebers for seasonal changes in the circadian rhythm of this species.

Keywords: Seasonal adaptation, photoperiod, temperature, circadian clock, *timeless, period*.

ABBREVIATIONS

D	Diapausing female
DD	Constant darkness

E peak	Evening locomotor activity peak
LD	Light dark cycle
LL	Continuous light
M peak	Morning locomotor activity peak
ND	Non-diapausing female
ZT	Zeitgeber time; environmental signal that adjust the circadian clock of the individual

1. INTRODUCTION

Organization of biological activities into daily and seasonal cycles is universal in organisms from cyanobacteria to humans, and in many species endogenous rhythms in physiological and behavioral traits are controlled by a circadian clock. Even though the clock-driven rhythms can persist with a period of about 24 h in the absence of environmental cues (free-running rhythms), they have to be reset every day by environmental signals (entrainment) to keep up proper phase relationship with the day-to-night cycle (Dubruille and Emery, 2008). Seasonal phase shifts in the circadian clock have also been found to lead to corresponding changes in various kinds of metabolic, physiological and behavioral traits, including insect locomotor activity (Saunders, 2002). Furthermore, the circadian clock has been suggested to function in cooperation with the photoperiodic timer, which can induce shifts e.g. in insects' dormancy and diapause (Koštál, 2011).

Insects' circadian rhythms have usually been studied by monitoring the oviposition, eclosion and/or locomotor activity rhythms of groups of individuals (Hamblencoyle et al., 1992; Sheeba et al., 2001). For example *Drosophila melanogaster* flies start to move actively before the lights-on and lights-off transition, which leads to a bimodal locomotor activity pattern (Hamblencoyle et al., 1992; Wheeler et al., 1993). In this species, flies' morning (M) and evening (E) activity peaks have been suggested to be induced by two separate circadian oscillators, the morning and the evening oscillators (Aschoff, 1966; Pittendrigh and Daan, 1976), so that seasonal changes in the phase angle between these activity peaks help the flies to adjust their behavior to match with forthcoming environmental changes (Majercak et al., 1999; Rieger et al., 2003). The morning peak of the flies has also been found to synchronize with the temperature increase in the morning and the evening peak with the temperature decrease in the afternoon in natural-like temperature cycles (Bywalez et al., 2012). Several other insect species have been found to show unimodal activity pattern, and thus their seasonal time measuring cannot be based on above-mentioned system. For example housefly *Musca domestica* (Helfrich et al., 1985) and some *D. virilis* group species (Bahn et al., 2009; Kauranen et al., 2012) show only evening activity peak, while *D. ananassae* shows only morning activity peak (Joshi, 1999).

Differences between the species with uni- and bimodal activity rhythms can be detected also in fly brains. Bahn et al. (2009), Hermann et al. (2012) and Kauranen et al. (2012) have detected differences in the number and location of PDF-neuropeptide and CRY-protein expressing neurons between in *D. virilis* group species and *D. melanogaster.* According to the authors, these differences account, at least partly, for the lack of flies' morning activity, their reduced circadian rhythmicity in constant darkness and their ability to maintain rhythmicity in continuous light. Interestingly, unimodal activity patterns seem to be common among northern *Drosophila* species (Simunovic and Jaenike, 2006), which raises a question on whether this kind of rhythmicity / clock mechanism is adaptive to high latitudes. Ability to retain the rhythmic circadian behavior in constant light is not unique for *D. montana*, as e.g. bumblebees *Bombus terrestris* and *B. pascuorum* have been found to retain their foraging rhythm in constant light during summer in northern hemisphere (Stelzer and Chittka, 2010). However, in some other northern insect species the circadian clock stops working under constant light; e.g. Antarctic midges, *Belgica antarctica,* lose their activity rhythm, as well as rhythmic clock gene expression (Kobelkova et al. 2015).

Molecular models of the circadian clock underlying behavioral rhythms are based on the oscillations in the transcription and translation of the central circadian clock genes, which is largely regulated by the proteins coded by other clock genes (reviewed in Hardin, 2004). This system is best understood in *D. melanogaster*, where the circadian genes *Clock* (*Clk*) and *cycle* (*cyc*) activate the transcription of genes like *timeless* (*tim*) and *period* (*per*), whose transcript levels show highest expression during the early night (Hardin et al., 1990; Sehgal et al., 1995). Price et al. (1995) have suggested that *D. melanogaster* clock will stop in continuous light (LL), since PER protein level does not show any rhythmic changes in this condition. According to Lee et al. (1996) and Myers et al. (1996) this is likely to be due to that the level of TIMELESS protein is reduced in LL, which prohibits the cycling of *per* expression. Rhythmicity of *D. melanogaster* flies can, however, be rescued in continuous light through temperature cycling (Yoshii et al, 2005), and in low temperatures also through temperature-dependent splicing of *tim* and *per* (Dubruille and Emery, 2008).

During the summer, northern *Drosophila* species have to cope with quite different combinations of day lengths and temperatures than the southern species, and studies on the daily

and seasonal rhythms of these species may give new insight on the clock mechanisms behind the rhythms. Our study species, *D. montana*, is a good representative of the northern *Drosophila* species with its unimodal daily activity rhythm and an ability to maintain free-running locomotor activity rhythm in continuous light, but not in constant darkness (Kauranen et al., 2012). *D. montana* females enter photoperiodic adult reproductive diapause under short day conditions (Tyukmaeva et al., 2011), which offers good possibilities for simultaneous studies on daily and seasonal changes in fly behavior. The main questions of this study were: (1) Do *D. montana* females show unimodal locomotor activity also in LD cycles with temperature fluctuations, and does their activity decrease? (2) Does *D. montana*'s circadian clock involve daily and seasonal oscillations in the expression levels of *tim* and *per*, and does the mutual phase relationship of these genes break down under long day conditions and/or in cool temperatures? (3) Do the non-diapausing and diapausing females show differences in their activity level under late summer conditions?

2. MATERIAL AND METHODS

2.1. Study material

The study flies came from a mass-bred *D. montana* population established from the F3 progenies of 20 females collected in Oulanka (northern Finland, 66° 40'N) in 2008. The flies were maintained in a wooden population cage attached to eight malt bottles (Lakovaara, 1969) and kept in continuous light, 19°C and 60% humidity for about 16 generations prior to the experiment. Females were collected from cage bottles within 1 day after eclosion and transferred in malt vials (15-20 females per vial) into an experimental chamber (Sanyo MLR-351H, Sanyo, San Diego, CA, USA) in early and late summer conditions (see below).

2.2. Study design

The experimental conditions in the climate chamber were set to mimic the photoperiod and the day and night temperature conditions typical to northern Finland from June to the beginning of September. Photoperiod was decreased during this period stepwise from 24 LL (continuous light) to LD 14:10 (14 hours light and 10 hours dark), the day temperature from 19°C to14°C and the

night temperature from 13°C to 11°C (see Fig. 1). Light intensity was kept at ~950 lux and humidity at 60 ± 10% throughout the experiment. Conditions were changed at about two week intervals, which enabled us to perform 14 days locomotor activity recordings at different times of summer in given photoperiods and temperatures.

D. montana females originating from Oulanka develop ovaries, if the day length during the first days after their emergence is more than 18-19 hours, while the females emerging under shorter day length in late summer will enter reproductive diapause (Tyukmaeva et al., 2011). Accordingly, the first set of freshly emerged female vials was transferred into the chamber in Mid-June conditions (photoperiod 24 LL and the day and night temperatures of 19°C; see Fig. 1). This set of females represented the non-diapausing (ND) generation, which is very small compared to the diapausing (D) and overwintering one in wild *D. montana* populations in northern Finland (Aspi et al., 1993). The second set of females was placed into the chamber in a photoperiod of 16:8 LD and the day and night temperatures of 16°C and 12°C, corresponding to 19th ~ 20th of August. These females entered diapause. The reproductive stage of the females used in the experiments was determined on the basis of their ovarian development stage (see Tyukmaeva et al., 2011). It should be noted that once the females have developed ovaries, they cannot enter diapause even if the environmental conditions would change substantially (Salminen and Hoikkala, 2013).

The activity rhythms of the females were studied in five different photoperiods (free-running rhythm in continuous light and entrained rhythms in four LD cycles) and temperatures prevailing in the chamber at different times of summer. Females (64-96 females/LD) were transferred individually into glass tubes inserted in Trikinetics *Drosophila* Activity Monitors (Waltham, MA, USA) and placed back into the chamber in these monitors. The locomotor activity of these females was registered in each of the five photoperiods/temperature conditions for 14 days. After this, the females were stored at -20°C until their reproductive stage was determined on the basis of the developmental stage of their ovaries (see Tyukmaeva et al., 2011). The data for the early summer conditions (24 LL, 22:2 LD and 18:6 LD) consisted of only ND females, while the data for the late summer conditions (16:8 LD and 14:10 LD) involved both ND and D females (see Fig 1). The age of the ND females was 24 days at the beginning of the first locomotor experiments (24 LL) and increased to 51 days in 22:2 LD, 86 days in 18:6 LD, 115 days in 16:8

LD and 136 days in 14:10 LD experiment. The ages of the D females were 14 and 35 days in 16:8 LD and 14:10 LD experiments, respectively.

2.3. Analysis of female locomotor activity data

The raw locomotor activity data for the females were displayed as double-plotted actograms (48 hour plots) for 14 days under free-running and/or entraining conditions to determine the daily activity rhythms of the females. The primary analysis was done with the ActogramJ program (Schmid et al., 2011; available at http://actogramj.neurofly.de. The presence of daily rhythms in actograms was traced using the Lomb-Scargle periodogram method with a significance level of 0.05; if the periodogram analysis detected significant periodicity in fly's activity rhythm across consecutive days, the fly was determined to be rhythmic.

The mean activity level of the females was calculated over 14 days in 5 min bins (how many times a fly moved during each bin) in given environmental conditions. Flies that did not survive throughout the whole experiment were excluded from the analysis.

2.4. Gene expression samples

Daily and seasonal variation in the expression level of *tim* and *per* genes was traced in the same experimental conditions, where the females' activity rhythms were measured. Fresh sets of females (5-6 females/ZT; ZT = Zeitgeber Time = daily sampling time) for this study were collected from the chamber every 6 h over a 24 h period immediately after the locomotor activity experiments had been completed, i.e. the females used for the activity experiments and for the RNA extractions at the same photoperiod were of the same age. As above, the samples of ND females were collected from all five photoperiods and the ones of D females at photoperiods 16:8 LD and 14:10 LD. In each photoperiod, the first sample was collected immediately before the lights-on transition (ZT = 0 in darkness) and the 2nd, 3rd and 4th sample 6, 12 and 18 h after the lights-on transition (ZT 6, ZT 12 and ZT 18). ZT 0 samples were collected at 10.00 AM both in LD cycles and in 24 LL. The females of all samples were flash-frozen in liquid nitrogen immediately after their removal from the chamber and stored at –84°C. Prior to RNA extractions, they were put in pre-cooled (2 h in –84°C) RNAlaterICE solution (Applied Biosystems, Waltham, MA, USA) and maintained there in –20°C for at least 16 h, after which their heads

were used individually for the RNA extractions. Females' abdomens (with ovaries) were stored in 70% ethanol to determine females' reproductive stage.

2.5. Quantitative real-time PCR (qPCR) on tim and per genes

RNA samples collected for ND and D females in different environmental conditions were used to trace daily changes in the expression profiles of *tim* and *per* genes. Total RNA was extracted individually from the heads of the females using ZR RNA Microprep kit with DNase treatment (ZymoResearch, Irvine, CA, USA) according to the manufacturer's protocol. After extraction, the purity and concentration of each sample was measured with NanoDrop (NanoDrop Technologies, Wilmington, DE, USA) and the integrity of RNA for part of the samples (one to two from each extraction set) was checked with Bioanalyzer (Agilent, Santa Clara, CA, USA). Before cDNA synthesis, RNA samples were diluted to equal concentrations (15 ng/µl) and 2 µl of total RNA of each sample was used as a template for cDNA synthesis using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's protocol. In addition to RNA, the cDNA reaction mixture (20 µl) consisted of 4 µl of 5 x iScript reaction mixture, 1 µl of reverse transcriptase enzyme and dH_2O. The PCR runs were run with Bio-Rad C1000 instrument (Bio-Rad Laboratories, Hercules, CA, USA) and the cycling conditions were 5 minutes at 25°C, 30 min at 42°C and 5 min at 85°C.

Primers for *tim* and *per* and two reference genes *Actin42A* and *E1alpha48D* were designed using NetPrimer (http://www.premierbiosoft.com/netprimer/index.html) program (primer sequences are available Table A1). Amplification efficiency values of all primer pairs were defined using 2-fold serial dilutions of pooled cDNA (from all treatments) with three technical replicates and 7-9 dilution points (Table A1). Expression patterns of experimental genes were traced with qPCR using 5-6 biological replicates and 3 technical replicates from all treatments and ZTs. qPCR reactions contained the following mixture: 10 µl 2x Power SYBR Green PCR Master Mix (Bio-Rad Laboratories, Hercules, CA, USA), 0.3 µl of each gene-specific primer and 1 µl of cDNA solution; the total volume of reaction was 20 µl. qPCR reactions were run with Bio-Rad CFX96 instrument (Bio-Rad Laboratories, Hercules, CA, USA) with following cycling conditions: initiation at 95°C for 3 min, denaturation at 95°C for 10 seconds, annealing at 55°C for 10 seconds and extension at 72°C for 30 seconds. Denaturation, annealing and extension

phases of the PCR were repeated 40 times and they were followed by a melting curve analysis to check the purity of the qPCR reaction.

Relative gene expression values for all samples in each treatment were calculated using mean Cq values (3 technical replicates) for all genes and biological replicates and applying real efficiency values. As the expression levels of the reference genes did not remain constant in ND and D females in different LD cycles, the expression levels of *tim* and *per* were normalized using a data driven normalization algorithm in NORMA-Gene program. This method has been shown to be very efficient at reducing variance due to experimental bias even when using only four study genes (Heckmann et al., 2011) and has been used also e.g. in Colinet et al. 2013 and Waagner et al. 2013. In our case we used *Actin42A* and *E1alpha48D* genes in addition to our experimental genes *tim* and *per*.

2.6. Statistical analysis

All statistical analyses were performed with R 3.10.1 for (R Development Core Team 2013).

The effect of seasonal sampling time on the activity level of ND females with five data points was analysed using general linear model (lm) with a photoperiod as a factor. In the two shortest photoperiods (16:8 LD and 14:10 LD), where both types of females were present, also the effect of females' reproductive state (ND vs. D) on their activity level was tested. These data were analysed using lm with the photoperiod and female reproductive state and their interaction as factors. Because of heteroskedasticity in residuals, female activity levels were $\log_{10}$-transformed. All models were simplified by removing non-significant interactions, and multiple comparisons were performed with Tukey's test.

The effects of photoperiod and ZT (daily sampling time) on the expression levels of *tim* and *per* genes were analysed separately for ND and D females with lm or generalized least squares model (GLS; in cases where variance covariates were needed, see below) with photoperiod and ZT and their interaction as factors. As the interaction between photoperiod and ZT was significant in both genes in both female types (tim_{ND}: $F_{12, 98} = 26.1$, $p < 0.001$; tim_D: $F_{3, 38} = 131.0$, $p < 0.001$; per_{ND}: $F_{12, 99} = 22.6$, $p < 0.001$; per_D: $F_{3, 38} = 89.0$, $p < 0.001$), the effect of ZT on gene expression was analyzed separately in each photoperiod.

Model validations were performed by examining the homogeneity and independence of errors. Heteroskedasticity, which was detected in several cases in lm, was solved by using ZT as a variance covariate (function varIdent in R) in GLS models, as this improves the models based on likelihood ratio tests (Zuur et al., 2009). Variance covariate was added into the models in 16:8 LD for *tim* in D females and in 14:10 LD for *per* in D females.

3. RESULTS

3.1. Locomotor activity of the females

About half of the studied *D. montana* females showed a free-running locomotor activity rhythm in continuous light (24 LL) and 19°C temperature (Table 1; all females were non-diapausing in this condition), and nearly all females showed a clear entrained activity rhythm in photoperiods involving a dark period and different day and night temperatures (Table 1). Rhythmicity of ND females was lowest (54.8%) in 24 LL, followed by 84.1% in 22:2 LD, 91.1% in 18:6 LD, 80.0% in 16:8 LD and 92.7% in 14:10 LD (Table 1). D females showed about the same level of rhythmicity in the two entrained photoperiods where they were studied (87.5% in 16:8 LD and 82.8% in 14:10 LD; Table 1).

In 24 LL with constant temperature, the rhythmic females free-run with a period of τ = 22.76 ± 0.41 (Table 1); this rhythm cannot be detected in Fig. 2 A as the free-running rhythms of different females were not running in the same phase. In all entrained conditions, i.e. the ones involving light and dark phase, females showed a 24 h rhythm. In these conditions females had a clear evening activity peak, but no morning activity peak (Fig. 2 B-G; in 16:8 LD the activity profile of ND females was slightly bimodal). The females showed highest activity at the end of the light period, and their activity level decreased rapidly before the lights off transition (Fig. 2 B-G).

The mean activity level of both ND and D females decreased clearly towards the autumn (Table 2). Activity levels of the flies during the light period differed significantly between different photoperiods both among ND ($F_{4, 392}$ = 45.6, $p<0.001$) and D females ($F_{1, 291}$ = 15.0, $p<0.001$). Subsequent Tukey tests showed that the activity level of the ND females differed between all LD comparisons, except between 24 LL and 22 LD, 16:8 LD and 18:6 LD and 14:10

LD and 16:8 LD (Table 3). D females moved less than ND females in the two shortest photoperiods ($F_{1, 291} = 6.53$, $p=0.01$) involving both female types.

3.2. Changes in the expression level of tim and per genes

tim and *per* genes showed significant daily oscillation in all photoperiods used in this study (Fig. 2), and their expression peaks differed significantly from the expression levels measured at other time points in all photoperiods in both female types (Table A3). The only exceptions were 22:2 LD and 14:10 LD for ND females, where the highest expression level of *per* differed significantly from only two out of three other samples (Fig. 2; Table A 3). Oscillations in the expression levels of these genes also coincided with each other in most sampling points.

In continuous light, the highest expression levels of *tim* and *per* occurred in ND females at ZT 18 (Fig. 2 A). In photoperiods 22:2 LD and 18:6 LD, both genes showed highest expression at an earlier time of day compared to continuous light, their expression peaking at ZT 6 and ZT 0, respectively (Fig. 2 B,C). In photoperiod 16:8 LD, where the expression levels of these genes were studied for both ND and D females, the highest peaks of both genes were detected in ZT 12 in both types of females (Fig. 2 D, E). In 14:10 LD *tim* expression peaked at ZT 0 and *per* expression at ZT 0 and ZT 12 (difference between ZT 0 and ZT 12 was not significant) in ND females (Fig. 2 F). In the same photoperiod, the expression levels of both genes peaked at ZT 0 in D females (Fig. 2 G). It is worth to note that generally *tim* and *per* cycling was quite similar in ND and D females in the same photoperiods and temperatures.

4. DISCUSSION

Bahn et al. (2009) have suggested that neural and molecular bases of the biological clock system have evolved uniquely among insect species, perhaps to maximize adaptive fitness to their natural environment. Our study revealed several interesting features in *D. montana* flies' rhythmicity and clock mechanism that are likely to be adaptive to high latitudes.

The role of the circadian clock in controlling insects' behavioral rhythms has traditionally been studied under continuous light and temperature conditions, but during recent years several studies have been performed in more natural environments, especially in *D. melanogaster* (e.g. Yoshii et al., 2009; Vanin et al., 2012; Menegazzi et al., 2013). For example, Yoshii et al. (2009)

found the flies' locomotor activity rhythms to be most robust under the combination of LD and temperature cycles. They suggested that these rhythms are entrained synergistically by two zeitgebers (photoperiod and temperature) and that although the photoperiod is the most important zeitgeber for the circadian clock, flies' activity pattern is more strongly affected by the temperature. In the present study, we used light and temperature conditions that mimicked the photoperiods and the mean day and night temperatures in northern Finland at different times of summer. This kind of strategy provided us information on the joint effects of photoperiod and temperature cycles on fly rhythmicity in a range of environmental conditions that the flies encounter at their home site during the breeding season. Comparing the results with those of our earlier study on *D. montana* females' locomotor activity rhythms in LDs 16:8, 20:4 and 22:2 in constant temperatures of 16°C and 20°C (Kauranen et al., 2012) also enabled us to distinguish the effects of photoperiod and temperature fluctuations from each other. However, the used conditions cannot be called completely natural, as the switches in light and temperature occurred abruptly, without dawns and dusks.

According to Simunovic and Jaenike (2006), daily unimodal activity patterns, like that of *D. montana*, are typical to northern *Drosophila* species. These authors studied the locomotor activity rhythms of 11 *Drosophila* species and found the species from high latitudes to show one activity peak (like *D. montana*) and the ones from lower latitudes two activity peaks during the day. An independent contrast test, correcting for phylogeny, confirmed the latitude to be the main factor separating the species, which suggests that the unimodal activity rhythms have evolved several times in genus *Drosophila*. It also means that the species with unimodal daily activity must be able to anticipate seasonal changes some other way than from the phase angle between morning and evening activity peaks like *D. melanogaster* flies have been suggested to do (Allada and Chung, 2010). In the present study, *D. montana* flies were found to have only evening activity peak, and a rise in the temperature at the beginning of light period did arouse fly activity in the morning. However, simultaneous changes in the day length and the day and night temperature in this study shifted the females' activity to an earlier time of the day under shortening day lengths so that their peak activity did not overlap with the lights-off transition. This differs from the situation in a constant temperature, where *D. montana* flies' activity was found to be highest about 16 h after lights-on transition both in 16°C and 20°C, so that under

shorter day lengths flies activity peak overlapped partly with the dark period (Kauranen et al., 2012). The finding that the activity peak of *D. montana* females decreases and shifts to an earlier time of the day in decreasing day lengths and day and night temperatures shows that the temperature acts as an important zeitgeber for the fly rhythmicity.

Older flies have been found to have weaker activity rhythm and lower morning activity peak than younger flies in *D. melanogaster*, even though the coupling of the photoperiodic cycles with temperature cycles improves their rhythmicity (Luo et al., 2012). In the present study the age of ND *D. montana* females increased from 24 days at the beginning of the first locomotor experiments (24 LL) to 136 days in the last experiment (14:10 LD), and thus a decrease in their activity towards the autumn could be partly due to aging. However, the D females were only 14 (16:8 LD) and 35 (14:10 LD) days old in respective experiments, and they showed in both LDs lower locomotor activity than the ND ones. Also, the activity of D females decreased significantly between these photoperiods. These findings suggest that the diapause state affects the locomotor activity of *D. montana* females more than their age. The reproductive state of *D. montana* (and other northern *D. virilis* group species) females has been found to affect their activity also in the wild: while ND flies are actively engaged in seeking feeding or breeding sites e.g. on the malt baits, the D flies are found in excess e.g. under bridges, where they show very low locomotor activity (Aspi et al., 1993). Our finding is likely due to the fact that the diapausing females are already preparing for the winter (the females overwinter as adults in diapause stage; Aspi et al., 1993).

Expression levels of *tim* and *per* have been found to show clear daily rhythms in several insect species. For example in flesh fly *Sarcopahaga crassipalpis* and blow fly *Protophormia terraenovae* expression levels of both of these genes show phase-shift in concert with the onset of darkness under short photoperiods (Koštál et al., 2009; Muguruma et al., 2010). The same is true for *D. melanogaster*, where the transcription of *tim* and *per* begins before the midday and reaches peak mRNA levels during the early night under 12:12 LD (Nitabach and Taghert, 2008). Qiu and Hardin (1996) have shown that in this species *per* mRNA is locked to the lights-off transition, being at highest level 4 hours after lights-off in the day lengths that are shorter than 16 hours, and that in extremely long photoperiods (>20 hours of light) the flies become arrhythmic due to a progressive break-down of the *tim/per* feedback loop. According to Boothroyd et al. (2007), the

tight coupling between *tim* and *per* expression breaks down also in 25°C/18°C temperature cycle due to a temperature-induced advance in *per* expression and a delay in the expression of the predominant *tim* transcript. Our results show that *D. montana* differs from *D. melanogaster* in all above-mentioned characters: in this species the mRNA levels of *tim* and *per* were not locked to lights-off transition and they showed diel rhythms and mutual phase relationship in practically all studied photoperiods including continuous light and cold temperatures (14°C/11°C) under 14:10 LD. Under the shortest day lengths both genes showed highest expression level during the late scotophase / early photophase, a phenomenon which is not easy to understand on the basis of present knowledge. Furthermore, according to Price et al. (1995) *D. melanogaster* clock will stop in continuous light (LL), since PER protein level does not show any rhythmic changes in this condition. In *D. montana* the expression levels of *per* and *tim* continued to cycle in LL and constant temperature, and about 50 % of females also showed rhythmic activity in this condition.

The clock genes *tim* and *per* have been suggested to play a role also in controlling seasonal rhythms in insect behavior and development, including photoperiodic diapause (e.g. Emerson et al., 2009). For example, mutations in *per* gene have been found to disrupt *D. melanogaster* females' ability to discriminate short day lengths and enter diapause (Saunders et al., 1989), and the Drosophilid fly *Chymomyza costata npd*-mutants, which do not transcribe *tim,* lack an ability to enter diapause (Pavelka et al., 2003). Some of the most convincing evidence on the role the circadian clock in insect diapause initiation comes from recent molecular studies by Ikeno et al. (2010, 2011) and Meuti (2015). Ikeno et al. (2010, 2011) have shown that the circadian clock regulates diapause initiation in bean bug *Riptortus pedestris* as a functional unit and not just through individual genes. Meuti et al. (2015), on the other hand, have presented new evidence on the function of circadian clock genes in the overwintering diapause of the northern house mosquito, *Culex pipiens*. Their studies show that the major circadian clock genes, including *per* and *tim*, continue to cycle throughout the diapause, and that RNAi directed against these two genes causes females to avert diapause even when reared under diapause-inducing conditions (Meuti et al., 2015). We detected no differences in *tim* or *per* cycling between non-diapausing and diapausing females in the present study, which indicates that the clock functions the same way in both types of females. This is in slight contrast with our earlier microarray study, where *per* showed differential expression in the initiation, maintenance and overwintering stages of

diapausing *D. montana* females, when compared to non-diapausing ones (Salminen et al., 2015). The biggest difference between the two studies was that Salminen et al. (2015) extracted RNA from the whole flies with both central and peripheral circadian oscillators, while we used only female heads.

The present results raised several questions that would be interesting to study in future. The first thing would be to study the molecular background of the circadian clock of *D. montana* in more details to find out how it works in seasonal time measurement. Another interesting task would be find out whether low temperatures induce alternative splicing in *D. montana tim* and *per* genes, as they do in *D. melanogaster* (Boothroyd et al. 2007; Dubruille and Emery 2008), and whether the splicing forms show different rhythms in their expression level in different environmental conditions. It would also be interesting to measure the abundancies of the clock proteins TIM and PER in fly brains (see Menegazzi et al., 2013), as most *tim* and *per* mRNA comes from retinal photoreceptors of the flies and their levels in the eye / head may not reflect the abundancies of respective proteins in the pacemaker neurons. All these studies could be performed on *D. montana* flies from high and low latitudes to find out how important zeitgebers light and temperature are in northern and southern populations of the species.

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COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

H.K., M.K. and A.H. contributed to designing the research. The research was performed by H.K. The locomotor activity data of the flies was analyzed by H.K., gene expression data by H.K. and M.K. and the statistical analysis was done by H.K. and O. A-H. All the authors participated in writing the paper.

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FIGURE LEGENDS

Figure 1. Environmental conditions during the experiment. Day length (grey area) and day (dash line) and night (solid line) temperatures in environmental chamber from the beginning of June to the end of September. The first set of freshly emerged females was placed into the chamber in 24LL representing conditions at the beginning of June and the second set in 16:8 LD representing the condition in mid-August. Thick black lines (ACTIVITY) indicate the timing of the two-week locomotor activity recordings and the arrows on the upper side of the figure show the time points when the females were collected from the chamber for RNA extractions. The first three samples consisted of non-diapausing (ND) females and the two last ones of both non-diapausing and diapausing (D) females.

Figure 2. The locomotor activity patterns of *D. montana* females and the expression levels of *tim* and *per* genes. Mean activity scores (left column) and the normalized expression profiles of *tim* and *per* genes (right column) for non-diapausing (ND) and diapausing (D) *D. montana* females under continuous light and different entraining conditions (shown in the figure). The heights of the bars in activity scores indicate the mean activity levels of the females during 30 minute bin over 8 days. The heights of the bars in gene expression profiles show the normalized expression level of *tim* (grey bars) and *per* (white bars) at ZT0, ZT6, ZT12 and ZT18 (ZT = Zeitgeber Time). Light and dark periods are indicated with white and black horizontal bars under the figures.

Table 1 Percentage of *D. montana* females females showing rhythmicity in different LDs/temperatures, the period of daily rhythms and the power of test.

LD	Day/night temperature	Reproductive stage	N	% of rhythmic females	Period (h) (mean ± SEM)	Power (mean ± SEM)
24:0	19 °C	ND	83	54.8	22.76 ± 0.41	18.15 ± 0.88
22:2	19 °C/ 13 °C	ND	69	84.1	24.05 ± 0.03	54.82 ± 6.13
18:6	17 °C/ 13 °C	ND	79	91.1	23.98 ± 0.06	54.38 ± 3.82
16:8	16 °C/ 12 °C	ND	70	80	24.12 ± 0.08	37.97 ± 2.62
		D	64	87.5	23.98 ± 0.03	61.28 ± 6.79
14:10	14 °C/ 11 °C	ND	96	92.7	23.97 ± 0.03	65. 38 ± 4.65
		D	64	82.8	24.03 ± 0.12	42.58 ± 4.68

LD = light-dark cycle used in entrained conditions; ND = non-diapausing; D = diapausing; N = number of individuals tested; Period (hours) = the length of the free-running rhythm of the flies in 24 LL (i.e. the length of the intrinsic day) and the length of the entrained rhythm in LD cycles; Power = power of periodogram test was defined as the amplitude of the peak in the rhythmic flies from Lomb-Scargle periodogram with significance level of $p < 0.05$.

Table 2 The mean activity levels of *D. montana* females during day and night (movements per 5 min bins) in different photoperiods.

LD	Reproductive stage	N	Mean day activity level	Mean night activity level
24	ND	83	0.58	–
22:2	ND	69	0.58	0.14
18:6	ND	79	0.44	0.04
16:8	ND	70	0.46	0.06
	D	64	0.37	0.04
14:10	ND	96	0.30	0.02
	D	64	0.20	0.03

LD = light-dark cycle used in entrained conditions; ND = non-diapausing; D = diapausing; N = number of females tested

Table 3 Comparisons for the mean activity levels of the females in different photoperiods (Tukey test).

LD comparison	Reproductive stage	Difference	P value
22:2 vs 24:0	ND	-0.03	0.96
18:6 vs 22:2	ND	-0.22	< 0.001 ***
18:6 vs 24:0	ND	-0.26	< 0.001 ***
16:8 vs 24:0	ND	-0.41	< 0.001 ***
16:8 vs 22:2	ND	-0.37	< 0.001 ***
16:8 vs 18:6	ND	-0.15	0.03
14:10 vs 24:0	ND	-0.52	< 0.001 ***
14:10 vs 22:2	ND	-0.48	< 0.001 ***
14:10 vs 18:6	ND	-0.26	< 0.001 ***
14:10 vs 16:8	ND	-0.11	0.12
14:10 vs 16:8	D	-0.11	< 0.001 ***

LD = light-dark cycle used in entrained conditions; ND = non-diapausing; D = diapausing; Difference = Degree and direction of the difference in the mean activity level of the females in particular L:D comparison.

TABLE A1. Primer sequences, amplification efficiency values (E %) and correlation coefficients (R^2) for the genes used in the qPCR analysis.

Gene	F/R primer sequence (5' – 3')	E %	R^2
tim	TGTCAGCGATGAGGATGAGA CTTGGGTCGGTTCATTGTCT	95.4	0.995
per	ACGGCTCTGAGAGTCAGCTC CTCCGGATGCTCAACGAT	102.2	0.994
Actin42A	TGCCAGATCTTCTCCATGTC ATGTGTGACGAAGAGGTTGC	99.1	0.997
E1alpha48D	TCTACAAGTGCGGTGGTATC GAGGTACCAGTGATCATGTTC	98.2	0.998

TABLE A2. The effect of ZTs in the expression of *tim* and *per* genes within each photoperiod.

		per				***tim***			
LD	**Reproductive stage**	**df_{factor}**	**$df_{residual}$**	**F**	**p value**	**df_{factor}**	**$df_{residual}$**	**F**	**p value**
24:0	ND	3	20	4.926	0.01*	3	20	19.609	< 0.001 ***
22:2	ND	3	20	17.569	< 0.001 ***	3	20	16.555	< 0.001 ***
18:6	ND	3	19	83.437	< 0.001 ***	3	19	103.09	< 0.001 ***
16:8	ND	3	20	68.028	< 0.001 ***	3	19	180.65	< 0.001 ***
16:8	D	3	19	176.09	< 0.001 ***	3	19	262.413	< 0.001 ***
14:10	ND	3	20	25.225	< 0.001 ***	3	20	13.612	< 0.001 ***
14:10	D	3	19	20.599	< 0.001 ***	3	19	46.815	< 0.001 ***

LD = light-dark cycle used in entrained conditions; ND = non-diapausing, D = diapausing; df_{factor} = degrees of freedom of factor;
$df_{residual}$ = degrees of freedom of residual.

TABLE A3. Comparisons for the highest expression peak of *tim* and *per* to the three other ZTs in each photoperiod (Tukey's tests). The time point for the highest expression of *tim* and *per* in each LD is shown in parentheses after the gene's name.

ZT comparison	24 LL ND				22:2 LD ND				18:6 LD ND			
	tim (ZT 18)		*per* (ZT 18)		*tim* (ZT6)		*per* (ZT6)		*tim* (ZT0)		*per* (ZT0)	
	t value	p value	t value	p value	t value	p value	t value	p value	t value	p value	t value	p value
0 vs 6					-6.457	< 0.001 ***	-7.180	< 0.001***	-10.103	< 0.001 ***	-8.507	< 0.001 ***
0 vs 12									-16.293	< 0.001 ***	-14.159	< 0.001 ***
0 vs 18	6.692	< 0.001 ***	2.823	0.048*					-14.381	< 0.001 ***	-13.487	< 0.001 ***
6 vs 12					4.012	0.003**	3.294	0.0175*				
6 vs 18	5.982	< 0.001 ***	3.529	0.011*	1.307	0.570	2.662	0.066				
12 vs 18	2.44	0.102	2.861	0.044*								

ZT comparison	16:8 LD ND				16:8 LD D				14:10 LD ND				14:10 LD D			
	tim (ZT 12)		*per* (ZT 12)		*tim* (ZT 12)		*per* (ZT 12)		*tim* (ZT0)		*per* (ZT 12)		*tim* (ZT0)		*per* (ZT0)	
	t value	p value	t value	p value	z value	p value	t value	p value	t value	p value	t value	p value	t value	p value	z value	p value
0 vs 6									-6.368	<0.001 ***			-11.276	< 0.001 ***	-6.869	< 0.001 ***
0 vs 12	19.815	< 0.001 ***	10.869	< 0.001*	18.28	< 0.001 ***	16.945	<0.001 ***	-2.988	0.034*	1.195	0.637	-7.888	< 0.001 ***	-5.435	< 0.001 ***
0 vs 18									-3.518	0.011*			-4.412	0.002 **	-6.377	< 0.001 ***
6 vs 12	21.050	< 0.001 ***	13.646	< 0.001 ***	12.37	< 0.001 ***	21.732	<0.001 ***			7.774	< 0.001 ***				
6 vs 18																
12 vs 18	-14.400	< 0.001 ***	-10.009	< 0.001 ***	-3.25	0.005**	-17.076	<0.001 ***			-4.919	< 0.001 ***				
					vcov model										vcov model	

LD = light-dark cycle used in entrained conditions; ND = non-diapausing; D = diapausing; ZT = zeitgeber time; vcov model = variance covariant added to the model.

Figure 1.

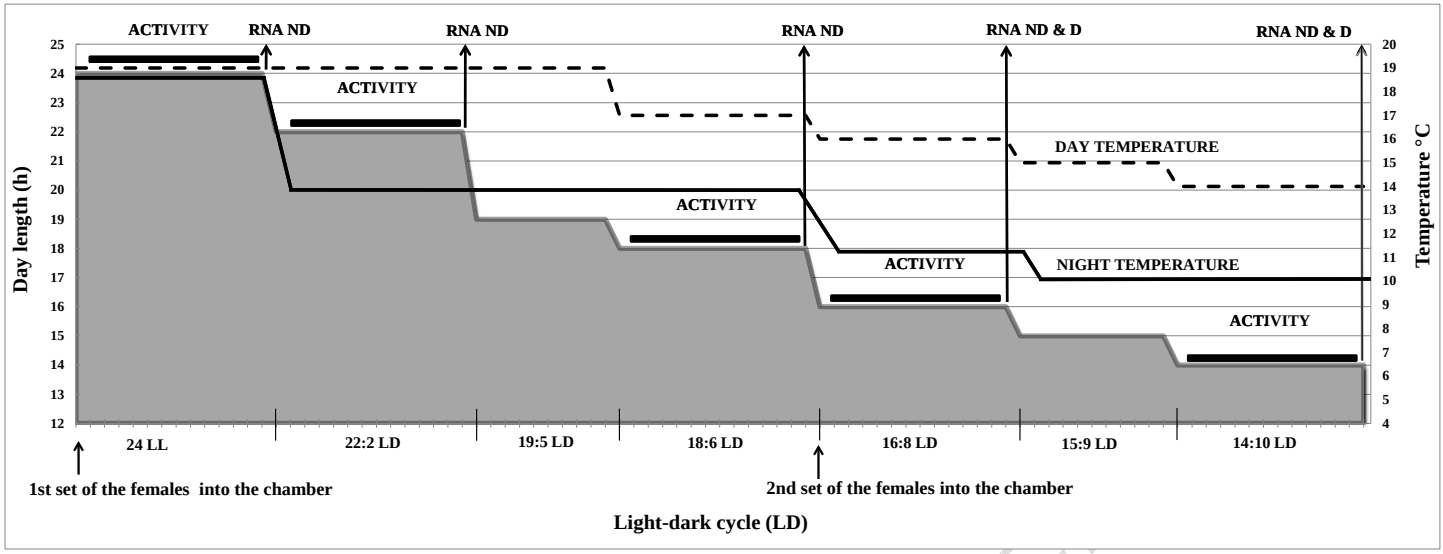
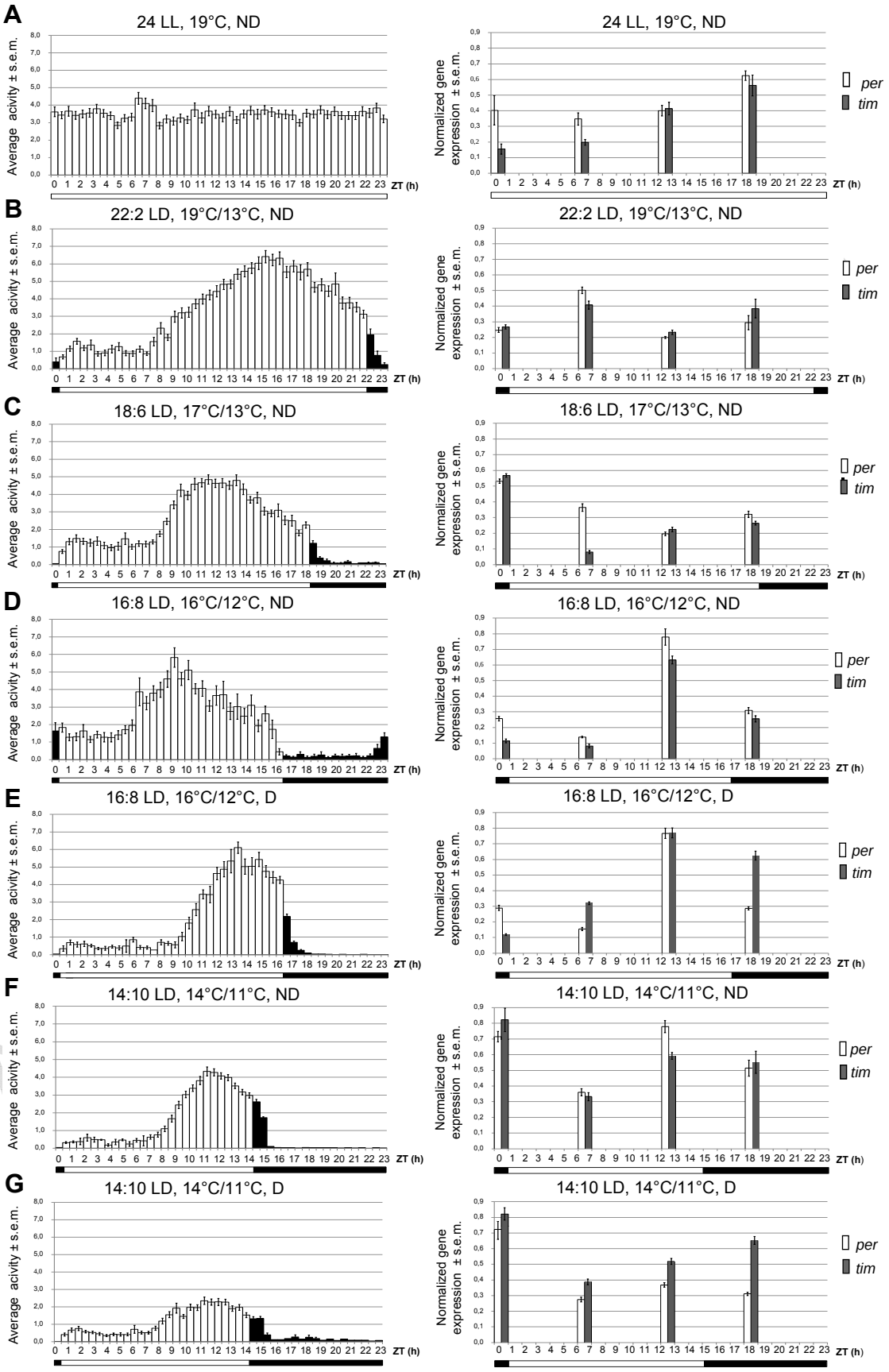


Figure 2



- The locomotor activity of Drosophila montana females decreased towards late summer
- The diapausing females were less active than the non-diapausing ones
- Expression of *timeless* and *period* showed both daily and seasonal oscillations