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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

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

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16 **ABSTRACT**

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

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

39 **ABBREVIATIONS**

40 D Diapausing female
41 DD Constant darkness

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

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49 **1. INTRODUCTION**

50 Organization of biological activities into daily and seasonal cycles is universal in organisms from
51 cyanobacteria to humans, and in many species endogenous rhythms in physiological and
52 behavioral traits are controlled by a circadian clock. Even though the clock-driven rhythms can
53 persist with a period of about 24 h in the absence of environmental cues (free-running rhythms),
54 they have to be reset every day by environmental signals (entrainment) to keep up proper phase
55 relationship with the day-to-night cycle (Dubruille and Emery, 2008). Seasonal phase shifts in the
56 circadian clock have also been found to lead to corresponding changes in various kinds of
57 metabolic, physiological and behavioral traits, including insect locomotor activity (Saunders,
58 2002). Furthermore, the circadian clock has been suggested to function in cooperation with the
59 photoperiodic timer, which can induce shifts e.g. in insects' dormancy and diapause (Košťál,
60 2011).

61 Insects' circadian rhythms have usually been studied by monitoring the oviposition,
62 eclosion and/or locomotor activity rhythms of groups of individuals (Hamblencoyle et al., 1992;
63 Sheeba et al., 2001). For example *Drosophila melanogaster* flies start to move actively before the
64 lights-on and lights-off transition, which leads to a bimodal locomotor activity pattern
65 (Hamblencoyle et al., 1992; Wheeler et al., 1993). In this species, flies' morning (M) and evening
66 (E) activity peaks have been suggested to be induced by two separate circadian oscillators, the
67 morning and the evening oscillators (Aschoff, 1966; Pittendrigh and Daan, 1976), so that
68 seasonal changes in the phase angle between these activity peaks help the flies to adjust their
69 behavior to match with forthcoming environmental changes (Majercak et al., 1999; Rieger et al.,
70 2003). The morning peak of the flies has also been found to synchronize with the temperature
71 increase in the morning and the evening peak with the temperature decrease in the afternoon in
72 natural-like temperature cycles (Bywalez et al., 2012). Several other insect species have been
73 found to show unimodal activity pattern, and thus their seasonal time measuring cannot be based
74 on above-mentioned system. For example housefly *Musca domestica* (Helfrich et al., 1985) and
75 some *D. virilis* group species (Bahn et al., 2009; Kauranen et al., 2012) show only evening
76 activity peak, while *D. ananassae* shows only morning activity peak (Joshi, 1999).

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

92 Molecular models of the circadian clock underlying behavioral rhythms are based on the
93 oscillations in the transcription and translation of the central circadian clock genes, which is
94 largely regulated by the proteins coded by other clock genes (reviewed in Hardin, 2004). This
95 system is best understood in *D. melanogaster*, where the circadian genes *Clock* (*Clk*) and *cycle*
96 (*cyc*) activate the transcription of genes like *timeless* (*tim*) and *period* (*per*), whose transcript
97 levels show highest expression during the early night (Hardin et al., 1990; Sehgal et al., 1995).
98 Price et al. (1995) have suggested that *D. melanogaster* clock will stop in continuous light (LL),
99 since PER protein level does not show any rhythmic changes in this condition. According to Lee
100 et al. (1996) and Myers et al. (1996) this is likely to be due to that the level of TIMELESS protein
101 is reduced in LL, which prohibits the cycling of *per* expression. Rhythmicity of *D. melanogaster*
102 flies can, however, be rescued in continuous light through temperature cycling (Yoshii et al,
103 2005), and in low temperatures also through temperature-dependent splicing of *tim* and *per*
104 (Dubruille and Emery, 2008).

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

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

120 **2. MATERIAL AND METHODS**

121 *2.1. Study material*

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

129 *2.2. Study design*

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

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

138 *D. montana* females originating from Oulanka develop ovaries, if the day length during
139 the first days after their emergence is more than 18-19 hours, while the females emerging under
140 shorter day length in late summer will enter reproductive diapause (Tyukmaeva et al., 2011).
141 Accordingly, the first set of freshly emerged female vials was transferred into the chamber in
142 Mid-June conditions (photoperiod 24 LL and the day and night temperatures of 19°C; see Fig. 1).
143 This set of females represented the non-diapausing (ND) generation, which is very small
144 compared to the diapausing (D) and overwintering one in wild *D. montana* populations in
145 northern Finland (Aspi et al., 1993). The second set of females was placed into the chamber in a
146 photoperiod of 16:8 LD and the day and night temperatures of 16°C and 12°C, corresponding to
147 19th ~ 20th of August. These females entered diapause. The reproductive stage of the females used
148 in the experiments was determined on the basis of their ovarian development stage (see
149 Tyukmaeva et al., 2011). It should be noted that once the females have developed ovaries, they
150 cannot enter diapause even if the environmental conditions would change substantially (Salminen
151 and Hoikkala, 2013).

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

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164 LD and 136 days in 14:10 LD experiment. The ages of the D females were 14 and 35 days in
165 16:8 LD and 14:10 LD experiments, respectively.

166 *2.3. Analysis of female locomotor activity data*

167 The raw locomotor activity data for the females were displayed as double-plotted actograms (48
168 hour plots) for 14 days under free-running and/or entraining conditions to determine the daily
169 activity rhythms of the females. The primary analysis was done with the ActogramJ program
170 (Schmid et al., 2011; available at http://actogramj.neurofly.de. The presence of daily rhythms in
171 actograms was traced using the Lomb-Scargle periodogram method with a significance level of
172 0.05; if the periodogram analysis detected significant periodicity in fly's activity rhythm across
173 consecutive days, the fly was determined to be rhythmic.

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

177 *2.4. Gene expression samples*

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

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

194 *2.5. Quantitative real-time PCR (qPCR) on tim and per genes*

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

209 Primers for *tim* and *per* and two reference genes *Actin42A* and *E1alpha48D* were
210 designed using NetPrimer (http://www.premierbiosoft.com/netprimer/index.html) program
211 (primer sequences are available Table A1). Amplification efficiency values of all primer pairs
212 were defined using 2-fold serial dilutions of pooled cDNA (from all treatments) with three
213 technical replicates and 7-9 dilution points (Table A1). Expression patterns of experimental genes
214 were traced with qPCR using 5-6 biological replicates and 3 technical replicates from all
215 treatments and ZTs. qPCR reactions contained the following mixture: 10 µl 2x Power SYBR
216 Green PCR Master Mix (Bio-Rad Laboratories, Hercules, CA, USA), 0.3 µl of each gene-specific
217 primer and 1 µl of cDNA solution; the total volume of reaction was 20 µl. qPCR reactions were
218 run with Bio-Rad CFX96 instrument (Bio-Rad Laboratories, Hercules, CA, USA) with following
219 cycling conditions: initiation at 95°C for 3 min, denaturation at 95°C for 10 seconds, annealing at
220 55°C for 10 seconds and extension at 72°C for 30 seconds. Denaturation, annealing and extension

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221 phases of the PCR were repeated 40 times and they were followed by a melting curve analysis to
222 check the purity of the qPCR reaction.
223 Relative gene expression values for all samples in each treatment were calculated using mean
224 Cq values (3 technical replicates) for all genes and biological replicates and applying real
225 efficiency values. As the expression levels of the reference genes did not remain constant in ND
226 and D females in different LD cycles, the expression levels of *tim* and *per* were normalized using
227 a data driven normalization algorithm in NORMA-Gene program. This method has been shown
228 to be very efficient at reducing variance due to experimental bias even when using only four
229 study genes (Heckmann et al., 2011) and has been used also e.g. in Colinet et al. 2013 and
230 Waagner et al. 2013. In our case we used *Actin42A* and *E1alpha48D* genes in addition to our
231 experimental genes *tim* and *per*.

232 *2.6. Statistical analysis*

233 All statistical analyses were performed with R 3.10.1 for (R Development Core Team 2013).
234 The effect of seasonal sampling time on the activity level of ND females with five data
235 points was analysed using general linear model (lm) with a photoperiod as a factor. In the two
236 shortest photoperiods (16:8 LD and 14:10 LD), where both types of females were present, also
237 the effect of females' reproductive state (ND vs. D) on their activity level was tested. These data
238 were analysed using lm with the photoperiod and female reproductive state and their interaction
239 as factors. Because of heteroskedasticity in residuals, female activity levels were $\log_{10}$-
240 transformed. All models were simplified by removing non-significant interactions, and multiple
241 comparisons were performed with Tukey's test.
242 The effects of photoperiod and ZT (daily sampling time) on the expression levels of *tim*
243 and *per* genes were analysed separately for ND and D females with lm or generalized least
244 squares model (GLS; in cases where variance covariates were needed, see below) with
245 photoperiod and ZT and their interaction as factors. As the interaction between photoperiod and
246 ZT was significant in both genes in both female types (tim_{ND}: $F_{12, 98} = 26.1$, $p < 0.001$; tim_D: $F_{3, 38}$
247 $= 131.0$, $p < 0.001$; per_{ND}: $F_{12, 99} = 22.6$, $p < 0.001$; per_D: $F_{3, 38} = 89.0$, $p < 0.001$), the effect of ZT
248 on gene expression was analyzed separately in each photoperiod.

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

254 **3. RESULTS**

255 *3.1. Locomotor activity of the females*

256 About half of the studied *D. montana* females showed a free-running locomotor activity rhythm
257 in continuous light (24 LL) and 19°C temperature (Table 1; all females were non-diapausing in
258 this condition), and nearly all females showed a clear entrained activity rhythm in photoperiods
259 involving a dark period and different day and night temperatures (Table 1). Rhythmicity of ND
260 females was lowest (54.8%) in 24 LL, followed by 84.1% in 22:2 LD, 91.1% in 18:6 LD, 80.0%
261 in 16:8 LD and 92.7% in 14:10 LD (Table 1). D females showed about the same level of
262 rhythmicity in the two entrained photoperiods where they were studied (87.5% in 16:8 LD and
263 82.8% in 14:10 LD; Table 1).

264 In 24 LL with constant temperature, the rhythmic females free-run with a period of τ =
265 22.76 ± 0.41 (Table 1); this rhythm cannot be detected in Fig. 2 A as the free-running rhythms of
266 different females were not running in the same phase. In all entrained conditions, i.e. the ones
267 involving light and dark phase, females showed a 24 h rhythm. In these conditions females had a
268 clear evening activity peak, but no morning activity peak (Fig. 2 B-G; in 16:8 LD the activity
269 profile of ND females was slightly bimodal). The females showed highest activity at the end of
270 the light period, and their activity level decreased rapidly before the lights off transition (Fig. 2
271 B-G).

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

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

279 *3.2. Changes in the expression level of tim and per genes*

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

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

296 **4. DISCUSSION**

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

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

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

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

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335 shorter day lengths flies activity peak overlapped partly with the dark period (Kauranen et al.,
336 2012). The finding that the activity peak of *D. montana* females decreases and shifts to an earlier
337 time of the day in decreasing day lengths and day and night temperatures shows that the
338 temperature acts as an important zeitgeber for the fly rhythmicity.

339 Older flies have been found to have weaker activity rhythm and lower morning activity
340 peak than younger flies in *D. melanogaster*, even though the coupling of the photoperiodic cycles
341 with temperature cycles improves their rhythmicity (Luo et al., 2012). In the present study the
342 age of ND *D. montana* females increased from 24 days at the beginning of the first locomotor
343 experiments (24 LL) to 136 days in the last experiment (14:10 LD), and thus a decrease in their
344 activity towards the autumn could be partly due to aging. However, the D females were only 14
345 (16:8 LD) and 35 (14:10 LD) days old in respective experiments, and they showed in both LDs
346 lower locomotor activity than the ND ones. Also, the activity of D females decreased
347 significantly between these photoperiods. These findings suggest that the diapause state affects
348 the locomotor activity of *D. montana* females more than their age. The reproductive state of *D.*
349 *montana* (and other northern *D. virilis* group species) females has been found to affect their
350 activity also in the wild: while ND flies are actively engaged in seeking feeding or breeding sites
351 e.g. on the malt baits, the D flies are found in excess e.g. under bridges, where they show very
352 low locomotor activity (Aspi et al., 1993). Our finding is likely due to the fact that the diapausing
353 females are already preparing for the winter (the females overwinter as adults in diapause stage;
354 Aspi et al., 1993).

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

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

377 The clock genes *tim* and *per* have been suggested to play a role also in controlling seasonal
378 rhythms in insect behavior and development, including photoperiodic diapause (e.g. Emerson et
379 al., 2009). For example, mutations in *per* gene have been found to disrupt *D. melanogaster*
380 females' ability to discriminate short day lengths and enter diapause (Saunders et al., 1989), and
381 the Drosophilid fly *Chymomyza costata npd*-mutants, which do not transcribe *tim,* lack an ability
382 to enter diapause (Pavelka et al., 2003). Some of the most convincing evidence on the role the
383 circadian clock in insect diapause initiation comes from recent molecular studies by Ikeno et al.
384 (2010, 2011) and Meuti (2015). Ikeno et al. (2010, 2011) have shown that the circadian clock
385 regulates diapause initiation in bean bug *Riptortus pedestris* as a functional unit and not just
386 through individual genes. Meuti et al. (2015), on the other hand, have presented new evidence on
387 the function of circadian clock genes in the overwintering diapause of the northern house
388 mosquito, *Culex pipiens*. Their studies show that the major circadian clock genes, including *per*
389 and *tim*, continue to cycle throughout the diapause, and that RNAi directed against these two
390 genes causes females to avert diapause even when reared under diapause-inducing conditions
391 (Meuti et al., 2015). We detected no differences in *tim* or *per* cycling between non-diapausing
392 and diapausing females in the present study, which indicates that the clock functions the same
393 way in both types of females. This is in slight contrast with our earlier microarray study, where
394 *per* showed differential expression in the initiation, maintenance and overwintering stages of

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

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

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413 also all the persons involved in maintaining the fly strains and performing the behavioral
414 experiments.

415 **COMPETING INTERESTS**

416 The authors declare no competing financial interests.

417 **AUTHOR CONTRIBUTIONS**

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

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567 **FIGURE LEGENDS**

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

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

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586 **Table 1** Percentage of *D. montana* females females showing rhythmicity in different
587 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

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

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593 **Table 2** The mean activity levels of *D. montana* females during day and night (movements
594 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

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

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597 **Table 3** Comparisons for the mean activity levels of the females in different photoperiods
598 (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 ***

599 LD = light-dark cycle used in entrained conditions; ND = non-diapausing; D = diapausing; Difference =
600 Degree and direction of the difference in the mean activity level of the females in particular L:D
601 comparison.
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607 TABLE A1. Primer sequences, amplification efficiency values (E %) and correlation
608 coefficients (R^2) for the genes used in the qPCR analysis.

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

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613 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 ***

614 LD = light-dark cycle used in entrained conditions; ND = non-diapausing, D = diapausing; df_{factor} =
615 degrees of freedom of factor;
616 $df_{residual}$ = degrees of freedom of residual.
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620 TABLE A3. Comparisons for the highest expression peak of *tim* and *per* to the three other ZTs in each photoperiod (Tukey's
621 tests). The time point for the highest expression of *tim* and *per* in each LD is shown in parentheses after the gene's
622 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			

623
624

625 LD = light-dark cycle used in entrained conditions; ND = non-diapausing; D = diapausing; ZT = zeitgeber time; vcov model = variance
626 covariant added to the model.
627

628

ACCEPTED MANUSCRIPT

Figure 1.

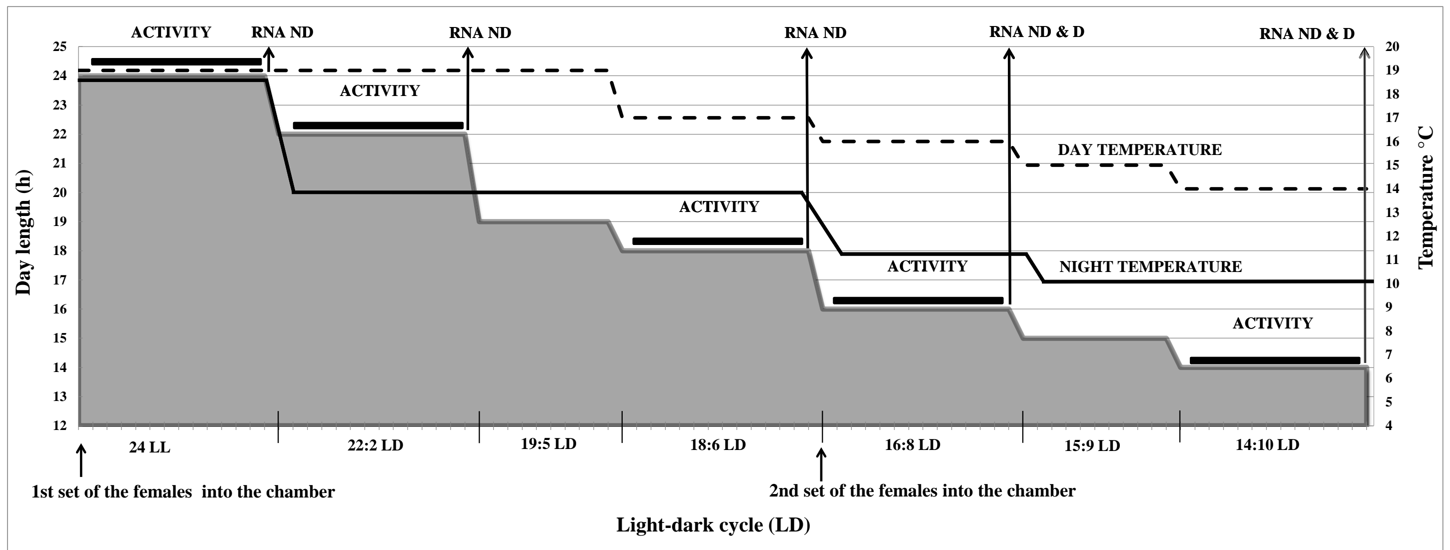

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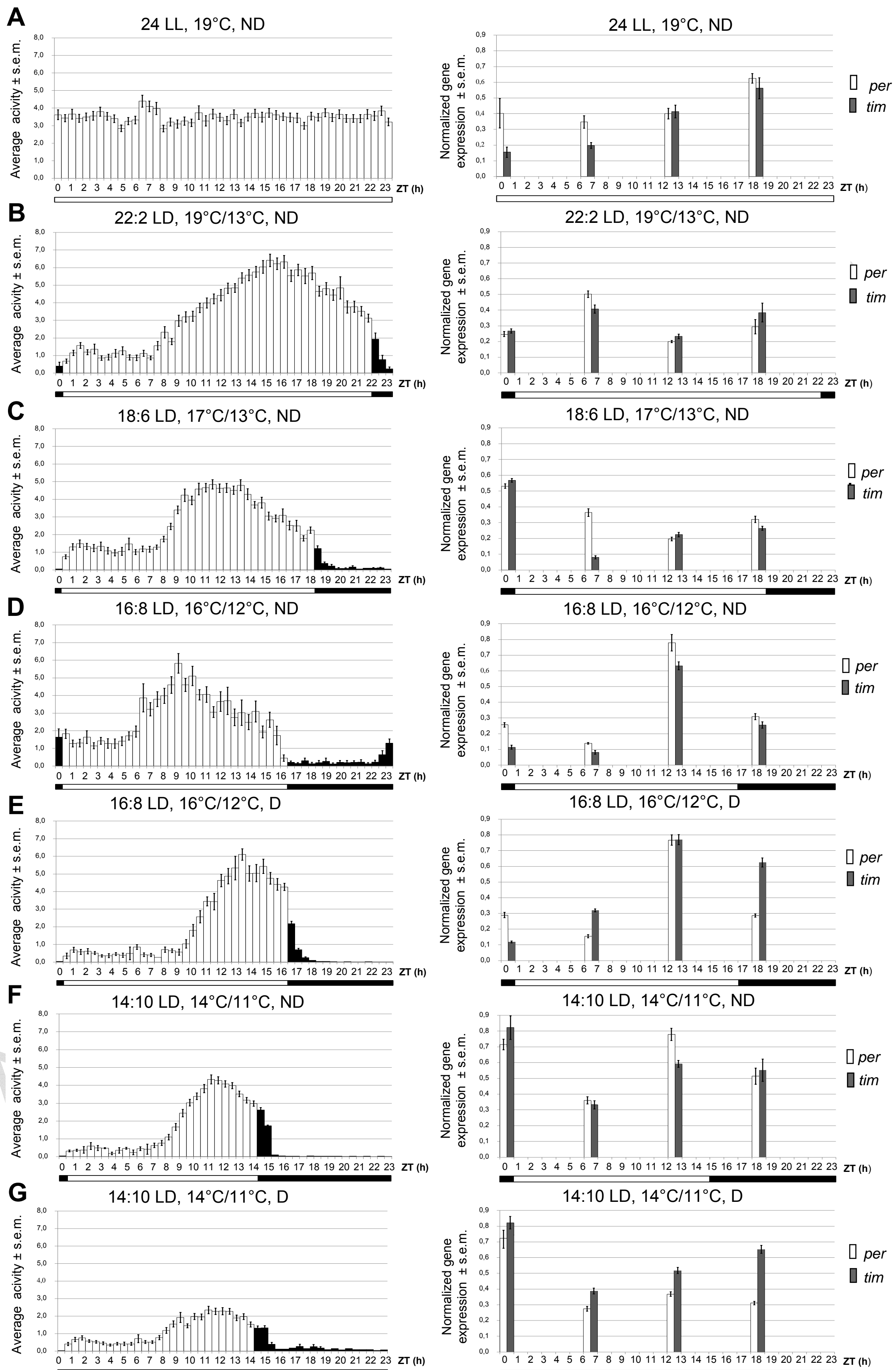
Figure 2



ACCEPTED MANUSCRIPT

629
630 - The locomotor activity of Drosophila montana females decreased towards late summer
631 - The diapausing females were less active than the non-diapausing ones
632 - Expression of *timeless* and *period* showed both daily and seasonal oscillations

633

ACCEPTED MANUSCRIPT

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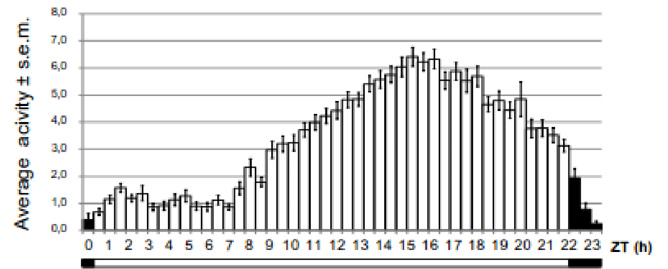

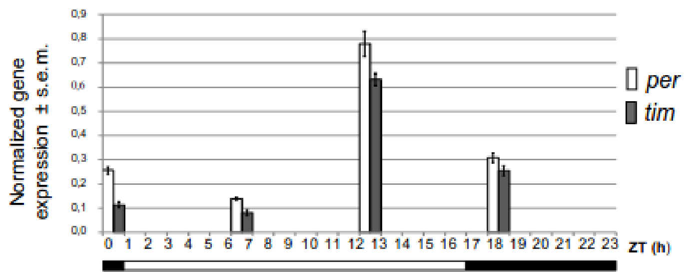

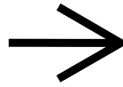

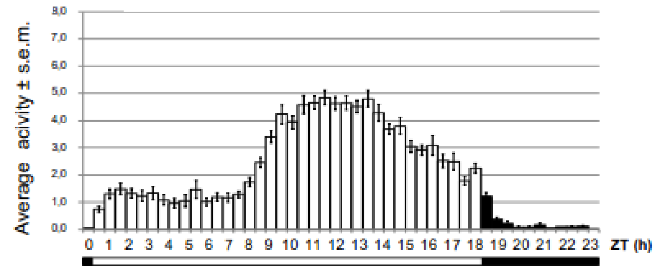

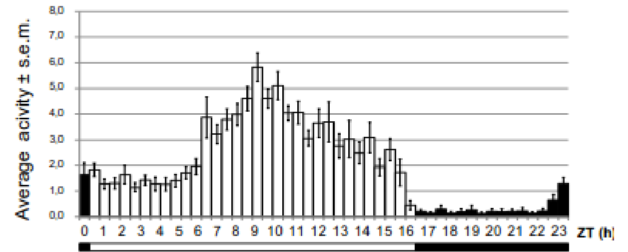

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