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1 **Photosensitive alternative splicing of the circadian clock gene *timeless* is population
2 specific in a cold-adapted fly, *Drosophila montana***

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18

19 **Abstract**

20

21 To function properly, organisms must adjust their physiology, behavior and metabolism in

22 response to a suite of varying environmental conditions. One of the central regulators of

23 these changes is organisms' internal circadian clock, and recent evidence has suggested that

24 the clock genes are also important in the regulation of seasonal adjustments. In particular,

25 thermosensitive splicing of the core clock gene *timeless* in a cosmopolitan fly, *Drosophila*

26 *melanogaster*, has implicated this gene to be involved in thermal adaptation. To further

27 investigate this link we examined the splicing of *timeless* in a northern malt fly species,

28 *Drosophila montana*, which can withstand much colder climatic conditions than its southern

29 relative. We studied northern and southern populations from two different continents

30 (North America and Europe) to find out whether and how the splicing of this gene varies in

31 response to different temperatures and day lengths. Interestingly, we found that the

32 expression of *timeless* splice variants was sensitive to differences in light conditions, and

33 while the flies of all study populations showed a change in the usage of splice variants in

34 constant light compared to LD 22:2, the direction of the shift varied between populations.

35 Overall, our findings suggest that the splicing of *timeless* in northern *Drosophila montana*
36 flies is photosensitive, rather than thermosensitive and highlights the value of studying
37 multiple species and populations in order to gain perspective on the generality of gene
38 function changes in different kinds of environmental conditions.
39

40 **Introduction**

41

42 Adaptation to environmental changes e.g. in temperature and day length, is crucial for the
43 survival of most organisms. While insects' responses to cold temperatures can be highly
44 variable, they usually involve changes in reproduction or diapause strategies and
45 metabolism, as well as in gene expression linked with these traits (e.g. Thieringer *et al.*
46 1998; Hoffmann *et al.* 2003; Vesala *et al.* 2012a, b; Parker *et al.* 2015) However, gene
47 expression studies typically overlook changes at the transcript level, such as differential
48 variant usage resulting from alternative splicing (AS). Because of the overwhelming
49 prevalence of AS forms across the genome of many Eukaryotes and across different
50 functional classes of genes (Irimia *et al.* 2007), it is very likely that the alternatively spliced
51 variants of central genes influence the way in which organisms adjust to environmental
52 change.

53

54 To maximize their fitness, organisms have to adjust their physiology and behavior in
55 response to a suite of varying environmental conditions. Central to the regulation of these
56 changes is an organism's internal circadian clock (Takahashi and Zatz 1982; Boothroyd *et al.*
57 2007; Zheng and Sehgal 2008), and recent evidence has suggested that clock genes are
58 important also in the regulation of seasonal adjustments (Montelli *et al.* 2015; Denlinger *et*
59 *al.* 2017). Regulation of the circadian clock itself has been well described in *Drosophila*
60 *melanogaster*, where both the temperature and the photoperiod have been found to alter
61 the expression of two core clock genes, *timeless* (*tim*) and *period* (*per*) in fly heads
62 (Boothroyd *et al.* 2007). In light-entrainment, the expression of these genes is tightly
63 coupled, but the coupling breaks down in temperature-entrainment, which leads to an

64 advance in the expression of *per* and delay in the expression of *tim*, but induces no
65 differences at protein level (Boothroyd *et al.* 2007). There are several potential explanations
66 on how the equal accumulation of PER and TIM proteins could be maintained regardless of
67 the different expression levels. First, there could be a shift in the timing of the expression of
68 both genes (Boothroyd *et al.* 2007) or the advance in the expression of *per* could be caused
69 by thermosensitive splicing of this gene, which would then effect the expression levels of
70 *tim* (Majercak *et al.* 1999; Collins *et al.* 2004). Finally, splicing occurring in both of these
71 genes could explain the difference in their expression levels (Boothroyd *et al.* 2007). Studies
72 on *D. melanogaster* have shown that *tim* has two different transcript variants that are
73 thermosensitively spliced (Boothroyd *et al.* 2007; Montelli *et al.* 2015): $tim^{spliced}$ and
74 $tim^{unspliced}$. The latter one is a longer transcript, in which the last intron is retained causing a
75 premature stop codon and resulting in the production of a truncated protein (the amino
76 acids encoded by the last exon are missing (Boothroyd *et al.* 2007)). This truncated protein
77 lacks part of the cytoplasmic localization domain, which may contribute to fine-tuning of
78 PER and TIM protein oscillations with the daily thermal cycle (Boothroyd *et al.* 2007). It is
79 also known that the longer variant ($tim^{unspliced}$) in *D. melanogaster* flies has higher expression
80 levels at low temperature (18°) than at high temperature (25°) (Boothroyd *et al.* 2007).
81 Montelli *et al.* (2015) also investigated different splicing variants of *tim* in natural conditions
82 throughout the seasons. They found that $tim^{spliced}$ expression is increasing at higher
83 temperatures and also that the total amount of *tim* mRNA expressed is influenced by
84 seasonal and even daily changes of temperature and day length in the wild (Montelli *et al.*
85 2015).
86

87 Interestingly, in a northern, highly cold tolerant *D. montana* fly, peak expression levels of
88 *tim* and *per* genes have been found to be similar to each other in several conditions from
89 continuous light in early summer to the shorter day lengths and cooler temperatures in late
90 summer, typical to northern environments (Kauranen *et al.* 2016). Consequently, both the
91 photoperiod and the daily temperature cycles are important cues for seasonal changes in
92 the circadian rhythm of *D. montana* (Kauranen *et al.* 2016). This offers a good possibility to
93 the study fundamentals of expression level differences, including patterns of AS, in *tim* and
94 to get some insights on their role in adaptation to seasonally varying environmental
95 conditions. In the current study, we collected information from the splicing variants of *tim* in
96 *D. montana* flies from two temperature and photoperiodic conditions. We also examined
97 the effects of these conditions on the expression levels of *tim* variants in northern and
98 southern populations from two continents. Alternative splicing is an important genetic
99 mechanism that may also help species to adapt to different environments (e.g. Jakšić and
100 Schlötterer 2016) and information on specific clock genes and their splicing patterns could
101 be of the utmost importance to understand these patterns.
102
103 **Materials and Methods**
104
105 **Fly strains and rearing.** We used isofemale strains of *D. montana* established from the
106 progenies of mated females collected in the wild from two extremes of a latitudinal cline in
107 the USA (Fairbanks, Alaska; 64°55’N and Azalea, Oregon; 42°48’N) and in Finland
108 (Pyhätunturi; 67°06’N and Lahti; 60°59’N) (Figure 1, Supplementary material, Table S1.). The
109 flies were collected using malt traps near small rivers and streams and reared in 250 ml
110 bottles containing malt medium (Lakovaara 1969) in constant light (LL 24:0) and at 19° since

111 their establishment. For the current experiment, ten randomly chosen females and ten
112 males from each of the studied strains were moved into new 250 ml malt medium bottles
113 and transferred into a maintenance chamber with two temperature and two lighting
114 conditions (i.e. we had 4 replicate bottles from each strain). After the flies had mated and
115 laid eggs, the founder flies were removed from the bottles to ensure that the flies (progeny)
116 used in this study had been reared in the correct conditions from egg to adult. Half of the
117 flies were reared in constant light (LL) and the other half in day length of 22 hours of light
118 and two hours of darkness (LD 22:2). Both of these light regimes prevent the flies of all our
119 study populations from entering diapause, which could cause differences in the expression
120 level of many circadian clock genes (Salminen *et al.* 2015). In both lighting conditions, the
121 flies were further divided into two temperature groups: 19°C and 16°C. The higher
122 temperature was the same where the original isofemale strains had been maintained since
123 their establishment and the lower temperature used was 16°. Both of these temperatures
124 resemble those in flies' natural sites during the summer months except the temperatures
125 from southern Azalea which would be too high for northern flies and already cause thermal
126 acclimation (See Supplementary Table S8). We collected two females randomly from each
127 isofemale strain and used three strains for constant light and two strains for LD 22:2 from
128 each population (Supplementary Table S1). Flies were collected at the same time of the day
129 at 1.00 PM (13:00) from both light dark-cycles at the age of 2-3 weeks (i.e. when they were
130 sexually mature) quickly frozen with liquid nitrogen, and stored at -84° prior to RNA
131 extraction. The dark period of 2 hours in LD 22:2 was always at 00.00-02.00 AM.
132

133

134 **Figure 1. *D. montana* fly populations used in the study.** Fly collection sites in Fairbanks (Alaska,
135 northern population) and Azalea (Oregon, southern population) in North America, with geographical
136 distance between the collection sites more than 4000 km. Finnish flies originated from Pyhätunturi
137 (northern population) and Lahti (southern population) with a distance between the sites approximately
138 700 km.

139

140 **RNA extractions, cDNA synthesis and molecular cloning of the different transcripts.** RNA
141 was extracted from whole female flies with Direct-zol™ RNA MiniPrep Kit (Zymo Research)
142 with DNAse treatment, and the purity of the RNA was measured with a NanoDrop® ND-1000
143 (Thermo Scientific) using ND-1000 V3.8.1 software. RNA concentration of each sample was
144 measured with Qubit® 2.0 Fluorometer (Invitrogen, Thermo Fisher Scientific) using Qubit®
145 RNA HS Assay Kit. cDNA synthesis was carried out with SuperScript® III First-Strand Synthesis
146 System for RT-PCR (Invitrogen, Thermo Fisher Scientific) using equal amounts (100 ng) of
147 RNA. The 3′ end of the gene (from exon 13 until beginning of 3'UTR region, Figure 2) was
148 amplified from the cDNA using PCR and tim_ex13_1F and tim_3'UTR_Rb primers
149 (Supplementary Table S2) and following protocol: 95° for 1 min, 95° for 15 s, 55° for 15 s,
150 72° for 20 s, 20° for 2 min. Molecular cloning was used to investigate the presence of
151 different transcripts in different samples. The cloning was carried out using a CloneJET PCR
152 Cloning Kit (Fermentas, Thermo Fisher Scientific) using manufacturer's instructions and

153 LuriaBroth ampicillin plates. PCR products were ligated into the vector (pJET1.2/blunt
154 Cloning vector) and transformed into *E. coli* Zymo 5 alfa (Zymo Research, T3007) cells. Plates
155 were incubated at 37° overnight (14-16 hours) and 70 colonies from each sample were
156 picked from the plates. From 1680 collected colonies, 228 were randomly chosen to be
157 amplified with PCR using pJET1.2 F and R primers (Supplementary Table S2) and run in
158 agarose gels to check which of them had the expected transcript lengths (about 500 and
159 1000 bp). A total of 19 products with expected lengths were then chosen randomly to be
160 sequenced with ABI 3130xl Genetic Analyzer (Thermo Fisher Scientific) using pJET1.2 F and R
161 primers. Sequenced products were analyzed using Sequence Analysis software (Thermo
162 Fisher Scientific) and then aligned using PRANK (v.100802, Löytynoja and Goldman 2005)
163 with default options.

164

165 **Quantitative real time PCR (qPCR).** Differences between the expression levels of the two
166 splice variants of *timeless* were measured with qPCR using the above-mentioned cDNA
167 samples and tim_intron_13_2FR, tim_exon_14F and tim_3'UTR_Rb primers (Figure 2,
168 Supplementary Table S2) with Bio-Rad's CFX96™ Real-Time System C1000 Touch™ thermal
169 cycler. qPCR protocol used was: 95° for 3 min, 95° for 10 s, 55° for 10 s, 72° for 30 s, 95° for
170 10 s and finally melting curve analysis. The gene expression levels were calculated with
171 normalized expression method (ΔΔ(Ct)) (Livak and Schmittgen 2001) using real efficiency
172 values for the primers (Supplementary Table S3) and CFX Manager v. 3.1 (BioRad).
173 *Ribosomal protein L23* (*RpL32*) and *18S Ribosomal RNA* (*18S*), which showed equal
174 expression levels in all samples, were used as control genes. Each run included 3 technical
175 replicates for each sample, and the final threshold value (Cq) was defined as a mean of the
176 technical replicates that produced good quality data. The primers used in qPCR bind either

177 to intron 13 or to exon 14 and the 3'UTR region (Figure 2, Supplementary Figure S1). The
178 primer pair that binds only to intron 13 tracks the amount of expression of the longer
179 variant (intron 13 included, *Dmon-tim*^{unspliced} see Figure 2), whereas the primer pair that
180 binds to exon 14 and in 3'UTR tracks the expression levels of both variants, as both of them
181 include exon 14 at the transcriptional level (*Dmon-tim*^{unspliced} and *Dmon-tim*^{spliced}, Figure 2).
182
183 **Statistical analyses.** The expression values collected from the qPCR runs were used to
184 calculate the ratio of expression levels between the intron-containing long transcript
185 (*Dmon-tim*^{unspliced}) and both transcripts containing the exon region (hereby referred to as the
186 long/both variant(s) ratio), for each sample. A type III 3-way ANOVA implemented in R
187 (version 3.1.2) (R Core Team 2016) using the car package (Fox and Weisberg 2011) was used
188 to trace the effects of temperature and lighting treatments and population, as well as
189 possible interactions between them, on the long/both variant(s) ratio in our samples. The
190 assumptions of ANOVA were met, as the data were normally distributed (normality of the
191 dependent variable (long/both variant(s) ratio) was checked with Shapiro–Wilk test
192 (Royston 1995) and the samples were independent. After ANOVA, Tukey's HSD test (Tukey's
193 honest significant difference) (Miller 1981) was used to examine pairwise differences
194 between the groups.
195
196 **Data availability.** All generated sequences have been deposited into GenBank under the
197 following accession numbers: MG279509 - MG279527.
198 Supplementary Material includes Tables S1-S8 and Figures S1 and S2.
199 Supplementary Data S1 contains the full alignment of the sequenced areas of *timeless* gene.
200

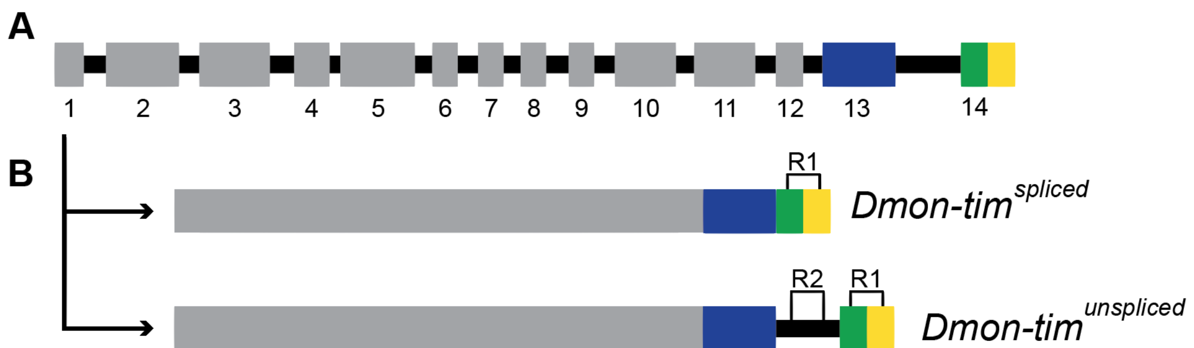
201 **Results**

202

203 **Different *tim* splicing variants in *D. montana*.** Sequencing of the cDNA collected from two
204 different temperature and light-dark (LD) treatments identified two different splicing
205 variants, *Dmon-tim*^{spliced} and *Dmon-tim*^{unspliced}, with the product lengths of 284 and 800
206 nucleotides (Figure 2, Supplementary Figure S2). The length difference was caused by the
207 retention of the intron between exons 13 and 14 in the longer transcript. Before
208 sequencing, the variants were cloned to separate the long and short transcripts from each
209 other and run on agarose gel electrophoresis to identify their length in each picked colony.
210 The difference between the product lengths was 516 bp, which caused both the PCR and
211 cloning to be biased towards the shorter transcript (less than 1/10 of the colonies had the
212 longer product).

213

214



215

216 **Figure 2. *timeless* gene in *D. montana*.** A) Schematic presentation of *timeless* gene in *D. montana*
217 showing exons 1-12 (grey), exon 13 (blue), exon 14 (green), UTR (yellow), and introns (black). Note
218 that introns are not in scale. B) Schematic presentation of the two splice variants, *Dmon-tim*^{spliced} and
219 *Dmon-tim*^{unspliced}. In the first variant the intron between exons 13 and 14 has been spliced out,
220 whereas in the second variant it has been retained. R1 and R2 show the regions of the transcripts
221 amplified in the qPCR (see methods for details).

222 In addition to the two expected splice variants, we also found a few variants with
223 unexpected sizes. Up to 16 nucleotide differences in the lengths of these variant were
224 caused by insertions/deletions of the “CCCGATCG” repeat region in the middle of the intron
225 13 (Figure 3A). Moreover, at the 3’UTR, a dinucleotide region of GA repeated 6-9 times
226 (Figure 3B) was found from all four fly populations from Finland and USA (for the full
227 alignment see Supplementary Data S1). We did not find any correlation between the
228 number of repeats and particular population, i.e. none of the repeat types were restricted
229 to a single population.

```
A
        440        450        460
TGGTCGCCCGATCGCCCGATCGCCCGATCGCCCTGAA
TGGTCGCCCGATCGCCCGATCG--------CCCTGAA
TGGTCGCCCGATCG----------------CCCTGAA

B
 900       910        920
GAAAGAGAGAGAGAGAGAGAGACACTAA
GAAAGAGAGAGAGAGAGAGA--CACTAA
GAAAGAGAGAGAGAGAGA----CACTAA
GAAAGAGAGAGAGAGA------CACTAA
```
230

231

232 **Figure 3. Repeat variation in *timeless* intron 13 and 3’UTR.** Sequence alignment produced by
233 PRANK showing the length differences in the *timeless* gene, caused by repeat regions. Numbers
234 indicate base position in full alignment provided in Supplementary Data S1. A) Repeat region of
235 CCCGATCG in the middle of intron 13 and B) dinucleotide repeat of GA in the 3’UTR region.

236

237 **Gene expression levels of different *tim* variants.** Expression level analysis involved two
238 primer pairs, the first of which binds to intron 13 and the second one to exon 14 and 3’UTR
239 region (Supplementary Figure S1). The first pair of primers gave information on the
240 expression level of the longer splice variant (*Dmon-tim*^{unspliced}), including the intron
241 sequence, and the second pair on the expression levels of both products (as both variants

242 include the exon 14). These expression levels were then used to calculate the long/both
243 variant(s) ratio in different fly samples. An ANOVA comparing these ratios between all
244 populations and treatments detected three statistically significant factors, including the
245 effect of population ($P < 0.001$), light treatment ($P < 0.05$) and the interaction between them
246 ($P < 0.001$), while temperature did not have a significant effect ($P > 0.90$) (Table 1).
247 Interestingly, significant interaction between population and light treatment seems to be
248 driven by the fact that in the USA populations (Fairbanks and Azalea) the long/both
249 variant(s) ratio is higher in constant light (LL) than in LD 22:2, while in the Finnish
250 populations (Lahti and Pyhätunturi) the situation is the opposite (Figure 4).

251

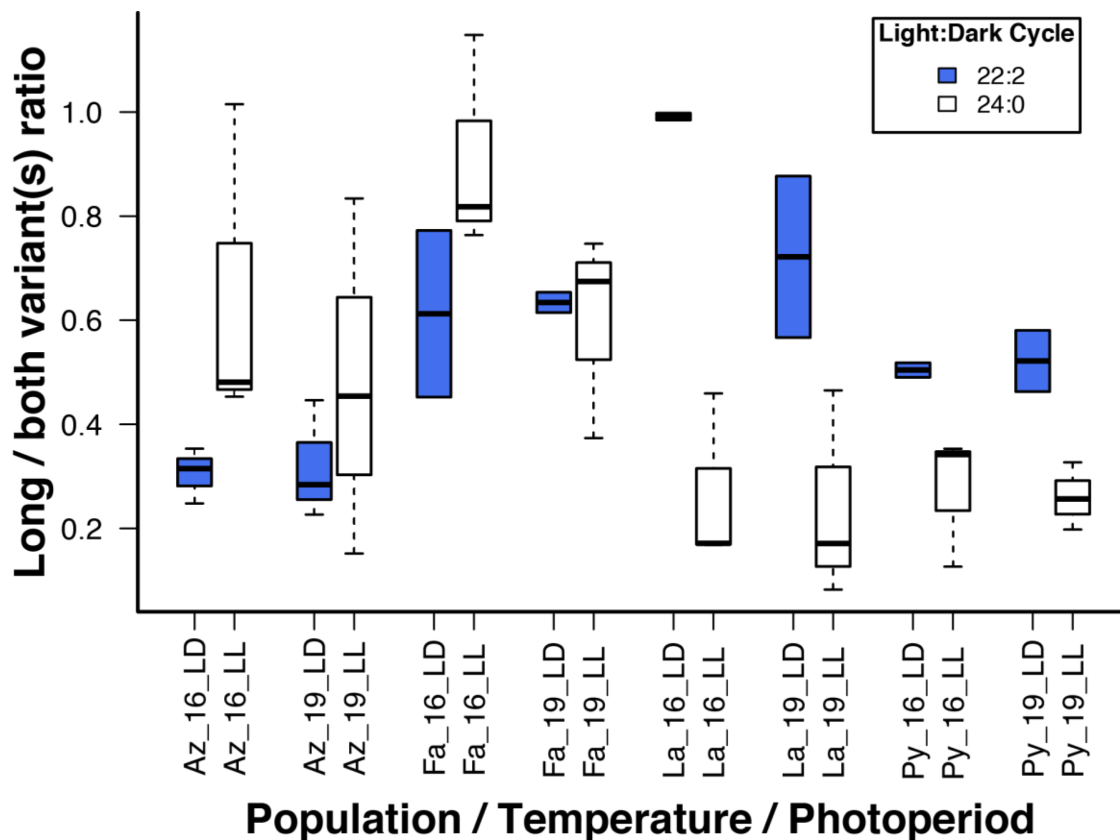
252 **Table 1. The ANOVA analysis comparing long/both variant(s) ratios of *timeless* among all the**
253 **populations and treatments.** The effect of temperature, population and light treatment assessed by
254 ANOVA (full model). Abbreviations: Temp = temperature, Pop = population and Light = light
255 treatment. Significant p-values are bolded. Total number of replicates = 42.

256

Treatment	Sum of Sq	Df	F value	ω^2	P
(Intercept)	0.2798	1	7.9440	0.078	**0.0091****
Temp	0.0003	1	0.0079	-0.011	0.9300
Pop	0.5772	3	5.4623	0.150	**0.0048****
Light	0.1777	1	5.0458	0.045	**0.0334***
Temp*pop	0.0639	3	0.6044	-0.013	0.6181
Temp*light	0.0252	1	0.7150	-0.003	0.4055
Pop*light	0.9642	3	9.1245	0.273	**0.0003*****
Temp*pop*light	0.1105	3	1.0460	0.002	0.3888
Residuals	0.9158	26			

257 Significance levels: * p<0.05, ** p<0.01, *** p<0.001.

258



259

260

261 **Figure 4. Comparison of long/both variant(s) normalized expression ratio of *timeless* in**
262 **different populations from different light-dark cycles and temperatures.** Conditions are given in
263 the form: population, temperature (°C), and light dark cycle separated by an underscore. Populations
264 are abbreviated as: Az = Azalea, Fa = Fairbanks, La = Lahti and Py = Pyhätunturi. Blue boxes
265 indicates the LD of 22:2 and white boxes constant light (LL).

266

267 Since a number of the higher-order terms in the full ANOVA model were non-significant, a
268 reduced model was produced by sequentially dropping the non-significant highest-order
269 term with the highest p-value. Following this procedure the reduced model consisted of the
270 following terms: temperature, light treatment, population, and the interaction between
271 population and light treatment (Supplementary Table S4). Results from the reduced model
272 were similar to the results obtained from the full model, with population ($P < 0.001$), light
273 treatment ($P < 0.05$) and interaction between of population and light treatment ($P < 0.001$)

274 showing significant effects. Moreover, examination of pairwise differences between the
275 treatment groups for temperature, light and population revealed several significant
276 differences, most of which were between the two Finnish populations (Supplementary Table
277 S5, for statistically significant results of pairwise comparisons see Supplementary Table S6
278 and for all pairwise comparisons see Supplementary Table S7).

279 **Discussion**

280 Alternative splicing of genes is an important genetic mechanism, as it provides an additional
281 source of variation for selection at the genetic level, which may be crucial for the species'
282 adaptation into seasonally changing environments. In many insect species the circadian
283 clock, which regulates many changes in physiological and behavioral traits, is entrained by
284 rhythmic changes in light and temperature cues (Montelli *et al.* 2015; Denlinger *et al.* 2017).
285 Here, we investigated the splicing patterns of a core circadian clock gene, *timeless* (*tim*), in
286 northern and southern *D. montana* populations from two different continents (Europe and
287 North America). Overall, our findings suggest that the splicing of *tim* in northern *D. montana*
288 flies is photosensitive, rather than thermosensitive contrary to the situation in the
289 widespread cosmopolitan species, *D. melanogaster* (Boothroyd *et al.* 2007; Montelli *et al.*
290 2015). Interestingly, we also found that a change in the photoperiod induced a shift in the
291 relative abundances of *tim* variants on both continents, but in different directions.
292 Moreover, in Finnish populations the level of splicing was similar in constant light but not in
293 LD 22:2, while in North America it showed different patterns in both photoperiods. This
294 highlights how idiosyncratic genetic changes can be even within a single species and the
295 value of using multiple species and populations in these type of studies.

296

297 The circadian clock is the main time-keeping mechanism in a wide range of organisms.
298 When investigating the splicing of the core circadian clock genes, like *tim*, it is important to
299 take into account that the role and the function of the clock can vary between the species.
300 Importantly, the northern *D. montana* flies are known to be able to maintain their free-
301 running locomotor activity rhythm in constant light but not in constant darkness, opposite
302 to the behavior of more southern species like *D. melanogaster* (Kauranen *et al.* 2012) and
303 the same pattern was also found from two other northern *D. virilis* group fly species *D.*
304 *ezoana* and *D. littoralis* (Menegazzi *et al.* 2017). This is suggested to be an adaptation to the
305 long (even continuous) days during the flies' mating season in early summer in the northern
306 latitudes (Kauranen *et al*. 2012). Kauranen *et al*. (2016) also investigated locomotor activity
307 of *D. montana* flies in different light-dark cycles for two weeks and found out that in LL (19°)
308 the flies free run with period of 23 hours and in LD 22:2 (19/13°) the flies entrained rhythms
309 were very close to 24 hours. The authors also resolved that the mean locomotor activity did
310 not change between the groups of flies collected from the above mentioned light dark
311 cycles (Kauranen *et al*. 2016). Moreover, *D. montana* lacks the morning activity peak typical
312 to *D. melanogaster* (Kauranen *et al.* 2012) as do also *D. ezoana* and *D. littoralis* (Menegazzi
313 *et al.* 2017). All of these differences could induce the differences detected in the splicing
314 patterns of *tim* in above-mentioned species, but the splicing frequency of the *tim* can also
315 be influenced by the expression of the other core circadian clock genes, including *per*. In *D.*
316 *montana*, the expression levels of *tim* and *per* coincide in the same light-dark cycles and
317 temperatures (Kauranen *et al.* 2016), while in *D. melanogaster* in light-entrainment, the
318 expression of these genes is tightly coupled, but the coupling breaks down in temperature-
319 entrainment leading to an advance in the expression of *per* and delay in the expression of
320 *tim* (Boothroyd *et al.* 2007).

321 In natural conditions, flies need to respond to variation in light and temperature at both
322 daily and seasonal scales. Among the photoperiods used in this study, constant light (24:0)
323 represents situation in early summer at high latitudes (Supplementary Table S8) and in this
324 lighting condition the circadian rhythms of the flies are free-running but the flies still retain
325 their rhythmicity (Kauranen *et al.* 2016). The second photoperiod, light-dark cycle of LD
326 22:2, represents mid-summer at high latitudes (Supplementary Table S8), and here the
327 circadian rhythms are entrained by the two hours dark period. These photoperiods prevent
328 both the northern and the southern flies from entering diapause, which is important for the
329 scope of the present study (Salminen *et al.* 2015). Although a difference of two hours
330 between the light cycles may appear small, it in fact may represent a large difference in the
331 environmental conditions for different populations of northern-distributed fly such as *D.*
332 *montana*. For example, day length increases by 4.8 hours in northern Pyhätunturi (Finland)
333 and by 2.5 hours in Fairbanks (USA) populations from May to June, while in the southern
334 Lahti (Finland) and Azalea (USA) populations the change is only 1.7 and 1.0 hours,
335 respectively (Supplementary Table S8). Hence, for the more northern populations a 2 hour
336 difference in LD equates to a short time period (i.e. only 2 weeks) during midsummer while
337 for more southern species it means a longer time period (up to 2 months) when other
338 environmental conditions such as temperature may have changed greatly. Unexpectedly,
339 the direction of a change induced by the photoperiod (constant light vs. LD 22:2) on *tim*
340 splicing differed between the USA and Finnish populations. In the USA populations the
341 expression of the longer variant (*Dmon*-tim^{unspliced}) was higher in constant light (LL) than in
342 LD of 22:2, while in the Finnish populations the pattern was the opposite. One thing that
343 may have affected our results is that, although Finnish flies from both of the used
344 populations and USA flies from northern Fairbanks experience very long day lengths during

345 the summer in the wild, constant light is natural only for the Pyhätunturi population in June
346 (see Supplementary Table S8). The effect of very long and unnatural light conditions in the
347 splicing of *tim* is not known but it might explain the observed differences between North
348 American and Finnish populations, as 15 hours of light is the longest photoperiod that the
349 southern Azalea flies from USA ever experience in natural conditions while in Lahti (Finland)
350 it is almost 19 hours of light (Supplementary Table S8).
351
352 In addition to the thermosensitive variants at the 3'end of the *timeless* discussed above, *D.*
353 *melanogaster* flies have two other *tim* variants called *ls-tim* and *s-tim*. Among these variants
354 *ls-tim* has two translation starting points, which create long and short variants differing by
355 23 amino acids in the first exon, whereas *s-tim* only codes for the short product (Sandrelli *et*
356 *al.* 2007; Tauber *et al.* 2007). *s-tim* appears to be the ancestral variant present in northern
357 Europe, while *ls-tim* is a newer variant that has spread through Europe (Tauber *et al.* 2007).
358 *ls-tim* makes the circadian clock in *D. melanogaster* less light-sensitive, which is thought to
359 be favorable in colder climates and in environments with more variable photoperiods
360 (Pittendrigh *et al.* 1991; Pegoraro *et al.* 2017). Montelli *et al.* (2015) investigated the affinity
361 of 4 different TIM protein isoforms (combination of S-TIM/L-TIM and $\text{TIM}^{\text{spliced}}$/$\text{TIM}^{\text{unspliced}}$)
362 and found that the light response may be stronger for *s-tim* flies under colder conditions.
363 Interestingly, the earlier translation start site and *ls-tim* variant has not been found in *D.*
364 *montana*, so these northern flies appear to only have *s-tim* variants and with different 3'end
365 splicing variants they seem to be strongly photosensitive and well adapted to northern
366 environmental conditions. We also want to point out that in this study we investigated the
367 alternative splicing patterns of *tim* using whole flies, while studies with *D. melanogaster*
368 have been mainly using the heads of the flies (Sandrelli *et al.* 2007; Montelli *et al.* 2015),

369 which could indeed cause some differences in *tim* expression patterns between the species.
370 However, although the expression of *tim* is known to primarily occur in the head of the flies
371 (Myers *et al.* 1996), it is also expressed in other tissues like digestive system, fat body and
372 ovaries of the flies (Graveley *et al.* 2011) and currently very little is known about the
373 expression or splicing patterns of *tim* or other circadian clock genes in *D. montana* in specific
374 tissues like head (but see Kauranen *et al.* 2016). Consequently, to get an overall idea of the
375 splicing patterns occurring in different populations of *D. montana* from several different
376 environmental conditions, we used whole flies and aim to move to more specific tissues in
377 future studies.

378

379 Interestingly, we also found length differences in the longer variant from all four fly
380 populations caused by insertions/deletions of the "CCCGATCG" repeat region in the middle
381 of the intron 13 as well as from a dinucleotide region of GA repeated 6-9 times 3'UTR
382 region. There was no correlation between the number of repeats and particular population,
383 i.e. none of the repeat types were restricted to a single population. The function of these
384 repeat regions and the reason for the variability of the repeat length is currently unknown,
385 though they might have some regulatory functions and affect e.g. the expression levels of
386 the *tim*. This could be investigated in detailed in future studies as the other core circadian
387 clock gene, *per,* is known to have thermosensitive splicing specifically in the 3'UTR region in
388 *D. melanogaster* (Majercak *et al.* 1999).

389

390 **Conclusions**

391 In this study we investigated the alternative splicing of an important core circadian clock
392 gene *timeless* in several *Drosophila montana* populations in response to different
393 temperatures and day lengths. We found that the expression of different splice variants of
394 *tim* were not thermosensitive, but photosensitive, in contrast to earlier studies in *D.*
395 *melanogaster*. We also found significant differences between the populations on two
396 continents, as the flies from the North America (especially the southern population)
397 expressed the longer, unspliced transcript more in constant light than in specific light-dark
398 cycle, while flies from the Finnish populations showed the opposite expression pattern.
399 Consequently, our results suggest that the splicing patterns of *tim* in different *D. montana*
400 populations from different geographical origins are different, highlighting the importance of
401 using multiple populations in order to gain perspective on the generality of gene function
402 changes in different kinds of environmental conditions. Obviously, more studies with bigger
403 temperature differences, a wide range of photoperiods and even more widely distributed
404 populations are needed to better understand the presence of different variants of *tim* and
405 their possible effects on the circadian clock rhythms of these flies in tough northern
406 environments. Moreover, functional genetic studies with e.g. RNAi and CRISPR/Cas9 gene
407 editing systems enable to experientially manipulate the expression of different *tim* variants
408 in multiple genetic backgrounds to determine if different splicing patterns are able to
409 produce similar phenotypic effects in different photoperiods.

410

411 **References**

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413 Boothroyd, C. E., H. Wijnen, F. Naef, L. Saez, and M. W. Young, 2007 Integration of light and
414 temperature in the regulation of circadian gene expression in Drosophila. PLoS
415 genetics **3**: e54.

416 Collins, B. H., E. Rosato, and C. P. Kyriacou, 2004 Seasonal behavior in *Drosophila*
417 *melanogaster* requires the photoreceptors, the circadian clock, and phospholipase C.
418 Proc. Natl. Acad. Sci. USA **101**: 1945-1950.

419 Denlinger, D. L., D. A. Hahn, C. Merlin, C. M. Holzapfel, and W. E. Bradshaw, 2017 Keeping
420 time without a spine: what can the insect clock teach us about seasonal adaptation?
421 Philos. Trans. R. Soc. Lond., B, Biol. Sci. **372**: 1734.

422 Fox, J., and S. Weisberg, 2011 An R Companion to Applied Regression, Second Edition.
423 Thousand Oaks CA: Sage.
424 URL: http://socserv.socsci.mcmaster.ca/jfox/Books/Companion

425 Graveley, B. R., A. N. Brooks, J. W. Carlson, M. O. Duff, J. M. Landolin*, et al.*, 2011 The
426 developmental transcriptome of *Drosophila melanogaster*. Nature **471**: 473-479.

427 Hoffmann, A. A., J. G. Sørensen, and V. Loeschcke, 2003 Adaptation of Drosophila to
428 temperature extremes: bringing together quantitative and molecular approaches. J
429 Therm Biol. **28**: 175-216.

430 Irimia, M., J. L. Rukov, D. Penny, and S. W. Roy, 2007 Functional and evolutionary analysis of
431 alternatively spliced genes is consistent with an early eukaryotic origin of alternative
432 splicing. BMC Evol. Biol. **7**: 188.

433 Jakšić, A. M. and C. Schlötterer, 2016 The Interplay of Temperature and Genotype on
434 Patterns of Alternative Splicing in Drosophila melanogaster. Genetics **204**: 315-325.

435 Kauranen, H., O. Ala-Honkola, M. Kankare, and A. Hoikkala, 2016 Circadian clock of
436 *Drosophila montana* is adapted to high variation in summer day lengths and
437 temperatures prevailing at high latitudes. J Insect Physiol. **89**: 9-18.
438 Kauranen, H., P. Menegazzi, R. Costa, C. Helfrich-Förster, A. Kankainen*, et al.*, 2012 Flies in
439 the north: Locomotor behavior and clock neuron organization of *Drosophila montana*.
440 J. Biol. Rhythms **27**: 377-387.
441 Lakovaara, S., 1969 Malt as a culture medium for Drosophila species. Drosophila Inf.Serv.
442 **44**: 128.
443 Livak, K. J., and T. D. Schmittgen, 2001 Analysis of relative gene expression data using real-
444 time quantitative PCR and the 2− ΔΔCT method. Methods **25**: 402-408.
445 Löytynoja, A., and N. Goldman, 2005 An algorithm for progressive multiple alignment of
446 sequences with insertions. Proc. Natl. Acad. Sci. USA **102**: 10557-10562.
447 Majercak, J., D. Sidote, P. E. Hardin, and I. Edery, 1999 How a circadian clock adapts to
448 seasonal decreases in temperature and day length. Neuron **24**: 219-230.
449 Menegazzi, P., E. Dalla Benetta, M. Beauchamp, M. Schlichting, I. Steffan-Dewenter*, et al.*,
450 2017 Adaptation of circadian neuronal network to photoperiod in high-latitude
451 European drosophilids. Curr. Biol. **27**: 833-839.
452 Miller, R. G., 1981 Normal univariate techniques, 37-108 in *Simultaneous statistical*
453 *inference*. Springer, New York
454 Montelli, S., G. Mazzotta, S. Vanin, L. Caccin, S. Corrà*, et al.*, 2015 *period* and *timeless* mRNA
455 splicing profiles under natural conditions in *Drosophila melanogaster*. J. Biol. Rhythms
456 **30**: 217-227.

457 Myers, M. P., K. Wager-Smith, A. Rothenfluh-Hilfiker, and M. W. Young, 1996 Light-induced
458 degradation of TIMELESS and entrainment of the Drosophila circadian clock. Science
459 **271**: 1736.
460 Parker, D., L. Vesala, M. Ritchie, A. Laiho, A. Hoikkala, et al., 2015 How consistent are the
461 transcriptome changes associated with cold acclimation in two species of the
462 *Drosophila virilis* group? Heredity **115**: 13-21.
463 Pegoraro, M., V. Zonato, E. R. Tyler, G. Fedele, C. P. Kyriacou*, et al.*, 2017 Geographical
464 analysis of diapause inducibility in European *Drosophila melanogaster* populations. J
465 Insect Physiol. **98**: 238-244.
466 Pittendrigh, C. S., W. T. Kyner, and T. Takamura, 1991 The amplitude of circadian
467 oscillations: temperature dependence, latitudinal clines, and the photoperiodic time
468 measurement. J. Biol. Rhythms **6**: 299-313.
469 R Core Team, 2016. R: A language and environment for statistical computing. R Foundation
470 for Statistical Computing, Vienna, Austria. URL: https://www.R-project.org/
471 Royston, P., 1995 Remark AS R94: A remark on algorithm AS 181: The W-test for normality. J
472 R Stat Soc Ser C Appl Stat **44**: 547-551.
473 Salminen, T. S., L. Vesala, A. Laiho, M. Merisalo, A. Hoikkala*, et al.*, 2015 Seasonal gene
474 expression kinetics between diapause phases in *Drosophila virilis* group species and
475 overwintering differences between diapausing and non-diapausing females. Sci. Rep.
476 **5**: 11197.
477 Sandrelli, F., E. Tauber, M. Pegoraro, G. Mazzotta, P. Cisotto*, et al.*, 2007 A molecular basis
478 for natural selection at the *timeless* locus in *Drosophila melanogaster*. Science **316**:
479 1898-1900.

480 Takahashi, J. S., and M. Zatz, 1982 Regulation of circadian rhythmicity. Science **217**: 1104-
481 1111.
482 Tauber, E., M. Zordan, F. Sandrelli, M. Pegoraro, N. Osterwalder, *et al.*, 2007 Natural
483 selection favors a newly derived *timeless* allele in *Drosophila melanogaster*. Science
484 **316**: 1895-1898.
485 Thieringer, H. A., P. G. Jones, and M. Inouye, 1998 Cold shock and adaptation. Bioessays **20**:
486 49-57.
487 Vesala, L., T. Salminen, M. Kankare, and A. Hoikkala, 2012a Photoperiodic regulation of cold
488 tolerance and expression levels of *regucalcin* gene in *Drosophila montana*. J Insect
489 Physiol. **58**: 704-709.
490 Vesala, L., T. Salminen, A. Laiho, A. Hoikkala, and M. Kankare, 2012b Cold tolerance and
491 cold-induced modulation of gene expression in two *Drosophila virilis* group species
492 with different distributions. Insect Mol. Biol. **21**: 107-118.
493 Zheng, X., and A. Sehgal, 2008 Probing the relative importance of molecular oscillations in
494 the circadian clock. Genetics **178**: 1147-1155.
495

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497

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503

504 **Author contributions**

505 M.K. designed the study, R.T. conducted the experiments and collected the data, R.T, M.K.

506 and D.J.P. analyzed the data and wrote the manuscript.