**Continuous activity and no cycling of clock genes in the Antarctic midge
during the polar summer**

**Alena Kobelkova^{a,#}, Shin G. Goto^{b,#}, Justin T. Peyton^a, Tomoko Ikeno^b, Richard E. Lee,
Jr.^c and David L. Denlinger^{a,*}**

^a *Departments of Entomology and Evolution, Ecology and Organismal Biology, Ohio State
University, Columbus, OH 43210, USA*

^b *Graduate School of Science, Osaka City University, Osaka 558-8585, Japan*

^c *Department of Zoology, Miami University, Oxford, OH 45056, USA*

[#] *Equal contributors*

**Corresponding author. Email address: denlinger.1@osu.edu*

Abstract

The extreme seasonal shifts of daylength in polar regions, ranging from constant light in the summer to constant darkness in the winter, pose an intriguing environment for probing activity rhythms and the functioning of circadian clocks. Here, we monitor locomotor activity during the summer on the Antarctic Peninsula and under laboratory conditions, as well as the accompanying patterns of clock gene expression in the Antarctic midge, the only insect endemic to Antarctica. Larvae and adults are most active during the warmest portion of the day, but at a constant

temperature they remain continuously active regardless of the photoregime, and activity also persists in constant darkness. The canonical clock genes *period*, *timeless*, *Clock*, and *vri* are expressed in the head but we detected no cycling of expression in either the field or under diverse photoregimes in the laboratory. The timekeeping function of the clock has possibly been lost, enabling the midge to opportunistically exploit the unpredictable availability of permissive thermal conditions for growth, development, and reproduction during the short summer in Antarctica.

Keywords: polar biology, circadian rhythms, clock genes, natural conditions, continuous light

Abbreviations: *Bmall*, Brain and muscle ARNT-like 1; *Clk*, *Clock*; *cry*, cryptochrome; FDR, false discovery rate; LED, light emitting diode; LD, light-dark; PCR, polymerase chain reaction; *per*, *period*; qPCR, quantitative PCR; *rp 49*, ribosomal protein 49; *tim*, *timeless*; *vri*, *vri*; ZT, Zeitgeber time.

1. Introduction

In spite of our rapidly expanding knowledge of biological clocks in diverse species from tropical and temperate latitudes (Sandrelli et al., 2008; Young and Kay, 2009), it is not at all clear how time-keeping mechanisms function in polar environments where the seasonal change in day length is most dramatic, ranging in the extreme from constant light in summer to constant darkness in winter. Of particular interest is how the circadian clock functions during the polar

summer with protracted periods of light (Bloch et al., 2013), a condition that leads to arrhythmicity in species from lower latitudes.

Activity and hormonal rhythms persist during the summer in some polar animals: bumble bees in the high Arctic continue to display rhythms of foraging (Stelzer and Chittka, 2010), a rhythm of melatonin release is observed in the willow warbler (Silverin et al., 2009) and in the Lapland longspur (Ashley et al., 2013), and a daily rhythm of body temperature is noted in Arctic ground squirrels during the summer (Williams et al., 2012), but in Antarctic penguins, rhythms of melatonin, prolactin (Miché et al., 1991; Cockrem, 1991) and corticosterone (Vleck and van Hook, 2002) appear to stop. Among four Arctic-breeding birds, substantial variation in daily activity rhythms are noted, depending on species, sex and breeding stage (Steiger et al., 2013). Interestingly, Norwegian reindeer (van Oort et al., 2005) and the Svalbard ptarmigan (Reierth and Stokkan, 1998) show rhythmic behavior in the spring and fall, but switch to arrhythmic behavior in the summer. Less is known about the functioning of the clock mechanism in polar environments. Cultured fibroblasts from reindeer display little or no rhythmicity in expression of the clock genes *Bmal1* and *per2* (Lu et al., 2010), while a daily rhythm of *cry2* is evident in Antarctic krill (Teschke et al., 2011). Where rhythms have been documented there is little evidence to suggest whether the cycles are in direct response to daily environmental perturbations or represent a functional circadian clock.

In this study we seek evidence for an activity rhythm in the Antarctic midge, *Belgica antarctica* (Diptera), and monitor expression of the major clock genes that would be expected to drive such a rhythm. This midge is the southernmost free-living insect and is the only insect endemic to Antarctica, where it has a patchy distribution along the western coast of the Peninsula, between 61-68°S (Convey and Block, 1996). Two years are required for completion of larval

development; larvae pupate, emerge as wingless, non-feeding adults in early January, mate, lay eggs and die within 7-10 days after adult eclosion. At this latitude the summer sun briefly drops below the horizon at night, but civil twilight persists; light intensity remains above that of full moonlight (0.2-0.5 lx), the threshold used by temperate insects to distinguish day from night (Beck, 1980). And, during cloudy days, light intensity can sometimes drop below that of the night. Thus, light is available, albeit in varying intensity, throughout the 24-h day. Air temperature during the summer on the Antarctic Peninsula at Palmer Station is usually slightly above freezing but varies, especially in response to sunlight intensity. Adult midges are present in the field only during the extremely long day lengths around the time of the summer solstice; larvae experience the annual range of extreme photoperiods but are active only as long as the substrate remains unfrozen (late-December to mid-March) and are encased in ice most of the year.

2. Materials and Methods

2.1 Collection and maintenance of insects

Larvae and adults of *B. antarctica* Jacobs were collected in the vicinity of Palmer Station, Antarctica (64°46' S, 64°04' W) during January-March 2011 and 2012, but adults were available only during the first two weeks of January in both field seasons. Substrate containing larvae was shipped to Ohio State University in temperature controlled containers (ca. 4°C for one week) and subsequently larvae were maintained at 4°C and a LD 18:6 photoperiod, with high humidity. Larvae could be maintained under such conditions for more than 12 months, but we were not able to propagate the midges in the laboratory.

2.2 Activity monitoring

Activity was monitored in January 2012 (Table 1). Larvae (4-5 mm) and adults (2-3 mm) were placed individually in wells (22 mm diameter) of a 12-well tissue culture plate on the day of collection. Moist filter paper was placed at the bottom of each well to prevent desiccation, and no food was provided. Adults do not feed at all, and larvae readily survive more than a week without food, indicating that lack of food is unlikely to be an unnatural condition. Activity levels did not change for most individuals throughout the recording period. Movements of larvae and adults appeared to be natural and did not appear to be restricted by the size of the arena. Some larvae followed the edge of the well, but many others moved throughout the central portion of the well. Adults moved freely throughout the arena.

Although activity monitoring was initiated for more adults, 3-4 day records were obtained for only 5 males. We do not suspect that death of adults was caused by handling, unnatural conditions or sickness, but rather was the consequence of the adult's short life span: males live 8 days on average, and females die within a day after oviposition, resulting in a generally shorter life span of 5 days (Harada et al., 2014). The field-collected adults were of unknown age at the time of collection, and a further 2-day period of laboratory acclimation restricted the number of adults that could be monitored for multiple days with the limited number of cameras available during the narrow time window when adults were present.

Depending on the experiment, plates were placed either outdoors near the station at a site protected from direct sunlight or in an environmental room at ca. 4°C. Digital images were captured using a webcam equipped with an infrared LED (DC-NCR13U or DC-NCR20U, Hanwha Japan, Tokyo; $\lambda_{\text{max}} = 870 \text{ nm}$) every 2 s for adults or every 20 s for larvae, using

camera-controlling software (LiveCapture2; <http://www2.wisnet.ne.jp/~daddy/>). Images were manually reviewed and rhythmicity was determined by the Lomb-Scargle test in ActogramJ (Schmidt et al., 2011). The start of the 24-h day in field experiments was designated as sunrise (see Table 1) or by time of lights-on in laboratory conditions. An infrared LED was used for experiments conducted under photoperiodic conditions in the environmental room. The LED was not needed under outdoor conditions because light intensity in the field was sufficient for recording, even after sunset.

Table 1.

Environmental conditions prevailing at Palmer Station, Antarctica on the dates we observed locomotory activity in field samples of the midge *Belgica antarctica* during the summer of 2012

Locomotor activity	Date	LD	Sunrise	Sunset	Civil Dawn	Civil Dusk	Tmax (°C)	Tmin (°C)
Adults	Jan 8-12th	21:3	3:02 - 3:16	23:41 - 23:30	no	no	15.2	-1.2
Larvae	Jan 13-18th	20:4	3:20 - 3:39	23:27 - 23:11	no	no	18.3	-0.8

Times of sunrise / sunset and civil dawn / dusk were obtained from the online database of the United States Naval Observatory. Civil twilight is defined by a 6° angle of the sun below the horizon, reflecting illumination of approximately 1 lux. At this latitude the center of the sun did not drop below 6° of the horizon between Nov 18 and Jan 24. Tmax, Tmin (°C) refer to maximum and minimum air temperatures recorded by HOBO loggers during the experimental periods.

2.3 RNA extraction and qPCR

For qPCR experiments, field-collected adults and fourth instar larvae were sealed in a Petri dish and maintained under field conditions; 20 adults or larvae were decapitated every 4 h

for 24 h (first time point was sunrise; see Table 2) and heads were transferred to RNA Later ICE (Ambion).

Table 2.

Environmental conditions prevailing at Palmer Station, Antarctica on the dates we monitored clock gene expression in field samples of the midge *Belgica antarctica* during the summer of 2012

Expression profiles	Date	LD	Sunrise	Sunset	Civil Dawn	Civil Dusk	Tmax (°C)	Tmin (°C)
Adults	Jan 9th	21:3	3:05	23:38	no	no	5.6	-0.4
Larvae	Jan 10th	21:3	3:09	23:36	no	no	5.5	0.4
	Jan 25th	19:5	4:11	22:44	0:48	2:10	4.3	0

Times of sunrise / sunset and civil dawn / dusk were obtained from the online database of the United States Naval Observatory. Civil twilight is defined by a 6° angle of the sun below the horizon, reflecting illumination of approximately 1 lux. At this latitude the center of the sun did not drop below 6° of the horizon between Nov 18 and Jan 24. Tmax, Tmin (°C) refer to maximum and minimum air temperatures recorded by HOBO loggers during the experimental periods.

For laboratory entrainment, larvae were entrained two weeks under light or temperature regimes and then rapidly frozen at -80°C at 4 h intervals (Zeitgeber time ZT0 = lights-on), using 20 heads/sample. RNA was isolated using a RiboPure Kit (Ambion) and cDNA was synthesized from 1 µg total RNA using SuperScript VILO Mix (Invitrogen). 5x diluted cDNA was used as a template for qPCR (iQ SYBR Green Supermix, Bio-Rad). qPCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 20 sec at 94°C, 10 sec at 60°C (*rp49*, *per*, *tim*) or 65°C (*Clk*, *vri*), 20 sec at 72°C, 35-40 cycles. We also cloned partial genomic sequences of the four circadian clock genes (*per*, *tim*, *Clk*, *vri*) and the reference gene *rp49*. Genomic DNA was isolated from 1-2 larval bodies using DNeasy Blood & Tissue Kit (Qiagen). Intron-overlapping

primers for *per*, *tim*, *Clk* and *rp49* are listed in Table 3, and their positions are shown in Supplementary data 3.

Table 3.

Gene-specific primers used in qPCR.

Transcript	Primer	Primer sequence 5` - 3`	Product size (bp)
<i>period</i>	forward	GTATGCACTCAAACACTCACATCAG^CCTG	126
	reverse	CTGCTGCTGGCGGACGATTCGAG	
<i>timeless</i>	forward	CGACTGAAGGCGTCTCCATCAA^TGGC	170
	reverse	CAGTTGCTGGACGACAGCGATTCTGG	
<i>Clock</i>	forward	AAAACAGTTCACTTAGTCAG^CGGTC	161
	reverse	GTGAGGCATGAAGTTCGTTGACTG	
<i>vrille</i>	forward	CGACCAATCCACTCGAAAACCTTCG	151
	reverse	ATGTTCTTGATGGTGTGGATTTCCG	
<i>rp49</i>	forward	CGGACCGATATGACAAAGTCAAG^GAAGC	149
	reverse	GAAGCCGTTGGGGAGCATGTGGC	

Intron position is marked with a caret (^).

Background-subtracted fluorescence data was exported from Bio-Rad iQ5 software. Relative Log Concentration (RLC) was computed similar to Larionov (Larionov et al., 2005), utilizing standard curves that were performed for each primer set. A crossing threshold of 0.2 was used throughout. Parameters estimated from multiple standard curves from the same primer set were averaged. The RLC for technical replicates were averaged. Normalized RLCs (NRLC) were computed by subtracting the RLC of the control gene, *rp49*, from the RLC of the gene of interest. Normalized mRNA levels were computed by $10^{(NRLC)}$. Normalized mRNA levels were centered about 1 by dividing the average of normalized mRNA level for each combination of gene of interest and environmental exposure, producing relative mRNA levels

2.4 Statistical analysis of qPCR results

An analysis of variance (ANOVA) with all pair wise comparisons was performed in SigmaPlot on the relative mRNA levels for each combination of gene of interest and environmental exposure. The family wise error rate was controlled via the Holm-Sidak method for each combination that passed the F-test. Because of the potential of increased sensitivity, circadian activity was modeled for each combination using nlinfit in MATLAB. The circadian model $\{y = p1 + p2 * \cos(2 * \pi * (ZT - p3) / 24)\}$ was fit to relative mRNA levels and a model utility test was performed against the reduced model $\{y = p1\}$ (F (2, N - 3)). Additionally a randomization test (100,000 simulations) was performed on R^2 . False Discovery Rate (*FDR*) was controlled according to Benjamini and Hochberg (1995).

3. Results and Discussion

3.1 Rhythmic activity in the field during the Antarctic summer

Activity patterns of larvae and adult males were monitored for 5 and 4 days, respectively, under field conditions during the polar summer. All larvae showed significant rhythmicity in locomotor activity, with a periodicity of 23.9 ± 0.4 h, mean $\pm$ SD (Fig. 1A). All but one adult displayed a rhythmic activity pattern with a period 24.5 ± 1.1 h (Fig. 1B). Under these natural

conditions both larvae and adults displayed rhythmic patterns, with peak activity coinciding with the highest daily temperatures and light intensities.

3.2 Absence of activity rhythms under photoperiodic conditions at constant temperature

To test whether activity patterns observed in the field were driven by the daily light cycle, larval activity was also monitored in newly-field collected larvae at a constant temperature of $4.9 \pm 0.3^{\circ}\text{C}$ (near the prevailing mean January air temperature at Palmer Station) under a daily photoregime of 21 h light and 3 h dark (LD 21:3, a regime that corresponds to the natural light regime the larvae received in the field in January) for 2 days, and then after transfer to constant darkness (Fig. 1C). Four of 10 larvae showed significant rhythmicity at LD 21:3, however rhythmicity persisted in only one larva in constant darkness (larva no. 4 in Supplementary data 1). Following transfer to constant darkness, 3 of 10 larvae maintained high activity throughout, while the other 6 showed diverse patterns with discontinuous bursts of activity. When compared with the conspicuously rhythmic activity observed under field conditions, profound activity peaks were abolished under laboratory conditions when two light-dark cycles were followed by two days of constant darkness. We cannot discount the possibility that additional days of recording may have revealed low amplitude rhythms that we did not detect under our experimental regime.

Adults, pretrained in the field during a time when daylength (sunrise to sunset) was approximately 21 h, were transferred to the laboratory and subjected to one LD 21:3 cycle, followed by two days of constant darkness and constant temperature (Fig. 1D). Adults displayed behavioral patterns that differed from those observed in the field. Unlike the observations with

field adults, there was no peak of activity during the photophase, and no significant rhythmicity was observed under constant darkness, except for one specimen with a long and rather obscure period (adult no. 5 in Supplementary data 1). Unlike the field adults, adults were more active during the scotophase under laboratory conditions when temperature was held constant. This suggests that locomotor activity in the field is predominantly temperature dependent, as temperature drops at night and activity ceases as well. Under laboratory conditions with a continuously favorable constant temperature, the scotophase may be a slightly preferred time for activity.

Monitoring activity patterns in adults of *B. antarctica* is especially challenging due to their short life span, their limited availability restricted to a 1-2 week window in early January, and the inability to generate adults in culture. Thus, meeting the standards of chronobiology research done on *Drosophila* is not realistic, yet we argue that the results we have obtained strongly support our research interpretation. To the best of our knowledge these actograms are the first recorded for a polar insect in its natural habitat.

Larvae exposed to constant light and a thermocycle with a 19 h thermophase of 4.5°C and 5 h cryophase of -1.9°C, followed by constant 2.5°C were active during the 4.5 °C thermophase and at a constant temperature of 2.5°C, but ceased moving immediately when temperature dropped to -1.9°C (Fig. 1E). What is clear from this set of experiments is that larvae quickly responded to changes in temperature, and at the cryophase temperature employed here, activity stopped and then quickly restarted when the larvae were again exposed to a higher temperature.

3.3 Clock genes of *B. antarctica*

239

240 Full length sequences of the circadian clock genes *period* and *timeless* and partial
241 sequences of *Clock* and *vrille* were obtained. Sequences suggest no conspicuous features
242 predicting anything unusual about these genes. No deletions or insertions that would appear to
243 interfere with translation are present in the sequences.

244 *Baper* encodes a 1083 amino acid (aa) protein; the genomic sequence contains 9 introns
245 within the coding sequence and one long intron (1.6 kb) at the 5'UTR; the aa sequence has
246 39.9% identity with DmPER (aligned by Clustal W, DNASTAR Inc.). The relatively low
247 similarity is consistent with the fact that *per* is a rapidly evolving gene showing high variability
248 outside the PAS domains. Identity is 63% within the PAS domains of these two species,
249 including 80% and 57% for PAS-A and PAS-B, respectively. The nuclear localization signal
250 (RKKKK) is present at the 5' end of the gene. Like many other species, *Baper* lacks the TG-
251 repeats present in *D. melanogaster*.

252 *Batim* encodes a 1148 aa protein, and the genomic sequence contains 9 introns.
253 Alignment of BaTIM and DmTIM shows 47.2% overall aa identity with 61.3% and 58.3%
254 identity in PER1 and PER2 domains, respectively.

255 The partial genomic sequence of *BaClk* contains 5 introns and encodes a 515 aa protein
256 that includes a PAS domain. Alignments of BaCLK and DmCLK show 46% overall identity and
257 60.6% identity of their PAS domains. The *Bavri* sequence encodes a 588 aa protein, lacks
258 introns, and shows 32% overall aa identity to DmVRI and 90.6% aa identity within the bZIP
259 domains.

Protein alignments of the clock genes of *Drosophila melanogaster* and *B. antarctica* are presented in Supplementary data 3. Sequences were deposited in GenBank: KF437828, KF437829, KF437830, KF437831.

Within larval heads, expression of *per*, *tim* and *Clk* was restricted to the brain, whereas *vri* was also expressed in non-brain tissues (Supplementary data 2, Fig. 2A).

3.4 No cycling of clock genes in field populations during the Antarctic summer

Daily expression profiles of clock genes were monitored in both larvae and adults to determine whether they show the cyclic patterns of expression well documented in temperate species. Expression profiles of the circadian clock genes were determined in heads of larvae (Fig. 2A) and adults (Fig. 2B) collected under field conditions. The circadian clock genes *per*, *vri* and *Clk* do not cycle under field conditions (modeled by nlinfit in MATLAB, $FDR > 0.05$; tested by ANOVA, *per* df = 9, $F = 1.24$, $P = 0.31$; *vri* df = 9, $F = 0.68$, $P = 0.73$; *Clk* df = 9, $F = 1.3$, $P = 0.28$). The ANOVA analysis shows a significant differential expression for *timeless* (df = 9, $F = 2.47$, $P = 0.04$), however there is no evidence for cycling (MATLAB, $FDR > 0.05$). The results presented for the adults must be considered preliminary due to the limited number of adults available for sampling, yet the data are consistent in supporting noncyclical expression of clock genes in the field.

3.5 Expression of clock genes under controlled temperatures and photoperiods

As a next step, we assessed expression of clock genes in the laboratory at constant temperatures and photoperiods, including constant light at 10°C, LD 21:3 at 5°C, and LD 12:12 at 10°C (Figure 2C). With constant light at 10°C, none of the four clock genes showed rhythmicity (MATLAB, $FDR > 0.05$) or differential expression (ANOVA, *tim* df = 6, $F = 0.32$, $P = 0.91$; *per* df = 6, $F = 0.38$, $P = 0.88$; *vri* df = 6, $F = 0.64$, $P = 0.69$; *Clk* df = 6, $F = 1.19$, $P = 0.37$). At long day length (LD 21:3) and 5°C, only *vri* displayed evidence for cycling when fitted to a circadian model ($FDR < 0.05$), but the data showed no significant temporal pattern by ANOVA (df = 7, $F = 2.40$, $P = 0.07$). Under LD 12:12 and 10°C, *per*, *vri*, and *Clk* did not cycle (MATLAB, $FDR > 0.05$; ANOVA, *per* df = 6, $F = 0.49$, $P = 0.8$; *Clk* df = 6, $F = 1.01$, $P = 0.43$; *vri* df = 6, $F = 1.98$, $P = 0.1$); *tim* showed significant temporal patterns (ANOVA, *tim* df = 7, $F = 6.81$, $P < 0.001$) supporting oscillation ($FDR < 0.05$), but phase and amplitude do not correspond to *tim* expression patterns noted in *Drosophila melanogaster* (Sehgal et al., 1995) or other insect species (Gentile et al., 2009; Codd et al., 2007; Kobelkova et al., 2010).

To validate our methodology we also monitored *tim* expression in heads of *D. melanogaster* (Supplementary data 2, Fig. 2B) and showed that the amplitude of change in *tim* from *B. antarctica* (*Batim*) is far lower (1.8 fold change) than we observed at the same photoperiod for the *tim* transcript from *D. melanogaster* (*Dmtim*) (22 fold change), and the phases of maximum and minimum expression differ (max. ZT8, min. ZT24 for *Batim*; max. ZT16, min. ZT4 for *Dmtim*). Although slight oscillations were noted for *Batim* in LD 12:12 and *Bavri* in LD 21:3, oscillations were of extremely low amplitude, peaked at the “wrong” time, were not consistent among different photoregimes tested, and did not oscillate in concert with expected partner clock genes. In the field, larvae of *Belgica* would experience LD 12:12 in mid-March and mid-September, at which times they are frozen solid within an encasement of ice,

thus the LD 12:12 experiments were not ecologically relevant but provided a photoregime that could easily be compared to data from temperate species.

The lack of cyclic transcription of clock genes in the heads of adults and larvae under field and laboratory conditions offers several hypotheses about the circadian clock mechanism in this species. Cycling of *per* and *tim* also does not occur in *D. melanogaster* and other insect species under constant light or under extremely long days, with photophases longer than 16 h (Qiu et al., 1996, Codd et al., 2007; Kobelkova et al., 2015). But, lack of *per*, *vri* and *Clk* rhythmic expression and only mild and atypical *tim* cycling in the midge under experimental conditions of LD 12:12 suggests that these clock genes fail to cycle under a range of photophases, not only extremely long photophases. Our observations suggest that the Antarctic midge lost the capacity for rhythmic expression of the clock genes. We recognize, of course, that a photoregime of LD 12:12 is not ecologically relevant for this species because adults are not present during that time of year, and larvae are already frozen solid in an ice matrix.

In addition to long photophases, low temperature is another factor known to be capable of halting clock function. Fruit flies are completely inactive in winter when temperatures are lower than 10°C; at such temperatures, PER and TIM are not detectable (Menegazzi et al., 2013). Low temperature is clearly a limiting factor for activity, reproduction and survival of *Drosophila*, a species that is of tropical origin. But, the situation of the Antarctic midge is quite different. The temperatures we used for our experiments were low by temperate zone standards but were within the normal temperature range for this species. This midge is most active and reproduces during the polar summer, with prevailing air temperatures between -2°C and +10°C. It is thus unlikely that the temperatures used in these experiments would be interpreted as being sufficiently cold to compromise rhythmic transcription in the midge.

Although we argue strongly that the transcription of the clock genes is not cyclic in the Antarctic midge, we cannot discount the possibility that the clock proteins cycle in the absence of RNA cycling, as reported for certain situations in *D. melanogaster* (Yang and Sehgal, 2001). Clock protein cycling in the absence of transcript cycling is an intriguing possibility worthy of future attention. Attempts to measure levels of PER and TIM in the midge brain using antibodies from *Drosophila* thus far have failed (data not shown), thus we anticipate that it will be necessary to generate antibodies specific to *B. antarctica* to adequately address this question.

It is quite possible that the circadian clock mechanism in *B. antarctica* is completely turned-off. Clearly, the clock genes are still present and transcribed in *B. antarctica*, but in addition to their well-defined role as mediators of circadian events, clock genes are involved in regulating diverse non-clock activities, including oogenesis (Beaver et al., 2003), long-term memory formation (Sakai et al., 2004), development (George and Terracol, 1997) and gut function (Bajgar et al., 2013). Within the brain of *D. melanogaster*, clock genes are expressed in both neural and non-neural cells, and only a subset of neurons carry out circadian clock functions (Kaneko and Hall, 2000; Kaneko et al., 1997; Ewer et al., 1992). Thus, the clock genes are indispensable, displaying a wide array of functions, and expression persists even in the absence of a clock function.

4. Conclusions

We have shown that rhythmic behavior observed under natural condition during the polar summer does not correlate with cyclic expression of the canonical clock genes. In a similar fashion, certain *Drosophila* clock mutants also show high levels of rhythmicity under natural

(Vanin et al., 2012) and pseudo-natural conditions (Menegazzi et al., 2012), suggesting that temperature affects the fly activity pattern more strongly than photoperiod under natural conditions (Menegazzi et al., 2013). Our observations with the Antarctic midge are consistent with the idea that the activity cycles observed in the field are primarily a direct response to changes in prevailing temperatures rather than the light-dark cycle.

Warmth is at a premium in the challenging polar environment, and we suspect that our observations reflect an adaptation by the midge to maximize and fully exploit permissive, but unpredictable, thermal conditions for feeding, development and reproduction during the brief austral summer. As long as temperatures are sufficiently high, the midge remains active, disregarding daily light-dark cycles that are so pervasive in dictating activity rhythms in temperate and tropical animals. And, correlated with an activity pattern that is directly responsive to thermal conditions, the midge lacks the rhythmic pattern of clock gene expression that drives many activity rhythms at lower latitudes.

Acknowledgements

We thank Palmer Station staff for support provided during our field seasons and Martina Hajduskova for assistance in preparing figures of the activity patterns. This work was supported by National Science Foundation OPP-ANT-0837613 and OPP-ANT-0837559.

References

371 Ashley, N.T., Schwabl, I, Goymann, W, Buck, C.L., 2013. Keeping time under the midnight sun:
 372 Behavioral and plasma melatonin profiles of free-living Lapland longspurs (*Calcarius*
 373 *lapponicus*) during the arctic summer. J. Exp. Zool. 319A, 10–22. (doi: 10.1002/jez.1768)
 374 Bajgar, A., Jindra, M., Dolezel, D., 2013. Autonomous regulation of the insect gut by circadian
 375 genes acting downstream of juvenile hormone signaling. Proc. Natl. Acad. Sci. USA 110,
 376 4416–4421. . (doi: 10.1073/pnas.1217060110)
 377 Beaver, L.M., Rush, B.L., Gvakharia, B.O., Giebultowicz, J.M., 2003. Noncircadian regulation
 378 and function of clock genes *period* and *timeless* in oogenesis of *Drosophila melanogaster*. J.
 379 Biol. Rhythms 18, 463-472. (doi: 10.1177/0748730403259108)
 380 Beck, S. D., 1980. Insect Photoperiodism. New York: Academic Press.
 381 Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: A practical and powerful
 382 approach to multiple testing. J R Statist Soc B 57: 289–300. (doi: 10.2307/2346101)
 383 Bloch, G., Barnes, B.M., Gerkema, M.P., Helm, B., 2013. Animal activity around the clock with
 384 no overt circadian rhythms: Patterns, mechanisms, and adaptive value. Proc. R. Soc. B 280,
 385 20130019. (doi: 10.1098/rspb.2013.0019)
 386 Cockrem, J.F., 1991. Plasma melatonin in the Adelie penguin (*Pygoscelis adeliae*) under
 387 continuous daylight in Antarctica. J. Pineal. Res. 10, 2–8. (doi: 10.1111/j.1600-
 388 079X.1991.tb00457.x)
 389 Codd, V., Dolezel, D., Stehlik, J., Piccin, A., Garner, K.J., Racey, S.N., Straatman, K.R., Louis,
 390 E.J., Costa, R., Sauman, I., Kyriacou, C.P., Rosato, E., 2007. Circadian rhythm gene
 391 regulation in the housefly *Musca domestica*. Genetics 177, 1539-1551. (doi:
 392 10.1534/genetics.107.079160)

393 Convey, P., Block, W., 1996. Antarctic Diptera: Ecology, physiology and distribution. Eur. J.
394 Entomol. 93, 1-13.

395 Ewer, J., Frisch, B., Hamblen-Coyle, M.J., Rosbash, M., Hall, J.C., 1992. Expression of the
396 *period* clock gene within different cell types in the brain of *Drosophila* adults and mosaic
397 analysis of these cells' influence on circadian behavioral rhythms. J. Neurosci. 12, 3321–
398 3349.

399 Gentile, C., Rivas, G.B.S., Meireles-Filho, A.C.A., Lima, J.B.P., Peixoto, A.A., 2009. Circadian
400 expression of clock genes in two mosquito disease vectors: *cry2* is different. J. Biol. Rhythms
401 24, 444-451. (doi: 10.1177/0748730409349169)

402 George, H., Terracol, R., 1997. The *vrille* gene of *Drosophila* is a maternal enhancer of
403 *decapentaplegic* and encodes a new member of the bZIP family of transcription factors.
404 Genetics 146, 1345-1363.

405 Harada, E., Lee, R.E., Denlinger, D.L., Goto, S.G., 2014. Life history traits of adults and
406 embryos of the Antarctic midge *Belgica antarctica*. Polar Biol. 37, 1213-1217. (doi:
407 10.1007/s00300-014-1511-0)

408 Kaneko, M., Helfrich-Förster, C., Hall, J.C., 1997. Spatial and temporal expression of the *period*
409 and *timeless* genes in the developing nervous system of *Drosophila*: Newly identified
410 pacemaker candidates and novel features of clock gene product cycling. J. Neurosci. 17,
411 6745–6760.

412 Kaneko, M., Hall, J.C., 2000. Neuroanatomy of cells expressing clock genes in *Drosophila*:
413 Transgenic manipulation of the *period* and *timeless* genes to mark the perikarya of circadian

pacemaker neurons and their projections. J. Comp. Neurol. 422, 66–94. (doi:
10.1002/(SICI)1096-9861(20000619)422:1<66::AID-CNE5>3.0.CO;2-2)

Kobelkova, A., Bajgar, A., Dolezel, D., 2010. Functional molecular analysis of a circadian clock
gene *timeless* promoter from the drosophilid fly *Chymomyza costata*. J. Biol. Rhythms 25,
399-409. (doi: 10.1177/0748730410385283)

Kobelkova, A., Zavodska, R., Sauman, I., Bazalova, O., Dolezel, D., 2015. Expression of clock
genes *period* and *timeless* in the central nervous system of the Mediterranean flour moth,
Ephestia kuehniella. J. Biol. Rhythms 30,104-16. (doi: 10.1177/0748730414568430)

Larionov, A., Krause, A., Miller, W., 2005. A standard curve based method for relative real time
PCR data processing. BMC Bioinformatics 6: 62. (doi:10.1186/1471-2105-6-62)

Lu, W., Meng, Q-J., Tyler, N.J.C., Stokkan, K-A., Loudon, A.S.I., 2010. A circadian clock is not
required in an arctic mammal. Curr. Biol. 20, 533–537. (doi: 10.1016/j.cub.2010.01.042)

Menegazzi, P., Yoshii, T. & Helfrich–Förster, C., 2012. Laboratory versus nature: The two sides
of the *Drosophila* circadian clock. J. Biol. Rhythms 27, 433-442. (doi:
10.1177/0748730412463181)

Menegazzi, P., Vanin, S., Yoshii, T., Rieger, D., Hermann, C., Dusik, V., Kyriacou, C. P.,
Helfrich-Förster, C., Costa, R., 2013. *Drosophila* clock neurons under natural conditions. J.
Biol. Rhythms 28, 3-14. (doi: 10.1177/0748730412471303)

Miché, F., Vivien-Roels, B., Pévet, P., Spehner, C., Robin, J.P., Le Maho, Y., 1991. Daily
pattern of melatonin secretion in an antarctic bird, the emperor penguin, *Aptenodytes forsteri*:
Seasonal variations, effect of constant illumination and of administration of isoproterenol or
propranolol. Gen. Comp. Endocrinol. 84, 249–263. (doi: 10.1016/0016-6480(91)90048-B)

436 Qiu, J., Hardin, P.E., 1996. *per* mRNA cycling is locked to lights-off under photoperiodic
437 conditions that support circadian feedback loop function. *Mol. Cell Biol.* 16, 4182–4188.

438 Reierth, E., Stokkan, K-A., 1998. Activity rhythm in High Arctic Svalbard ptarmigan (*Lagopus*
439 *mutus hyperboreus*). *Can. J. Zool.* 76, 2031–2039. (doi: 10.1139/cjz-76-11-2031)

440 Sakai, T., Tamura, T., Kitamoto, T., Kidokoro, Y., 2004. A clock gene, *period*, plays a key role in
441 long-term memory formation in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 101, 16058–16063.
442 (doi: 10.1073/pnas.0401472101)

443 Sandrelli, F., Costa, R., Kyriacou, C.P., Rosato, E., 2008. Comparative analysis of circadian
444 clock genes in insects. *Insect Mol. Biol.* 17, 447–463. (doi: 10.1111/j.1365-
445 2583.2008.00832.x)

446 Schmid, B., Helfrich-Förster, C., Yoshii, T., 2011. A new ImageJ plugin "ActogramJ" for
447 chronobiological analyses. *J. Biol. Rhythms* 26, 464–467. (doi: 10.1177/0748730411414264)

448 Sehgal, A., Rothenfluh-Hilfiker, A., Hunter-Ensor, M., Chen, Y., Myers, M.P., Young, M.W.,
449 1995. Rhythmic expression of *timeless*: A basis for promoting circadian cycles in *period*
450 gene autoregulation. *Science* 270, 808–810. (doi:10.1126/science.270.5237.808)

451 Silverin, B., Gwinner, E., Van't Hof, T.J., Schwabl, I., Fusani, L., Hau, M., Helm, B., 2009.
452 Persistent diel melatonin rhythmicity during the Arctic summer in free-living willow
453 warblers. *Horm. Behav.* 56, 163–168. (doi: 10.1016/j.yhbeh.2009.04.002)

454 Steiger, S. S., Valcu, M., Spoelstra, K., Helm, B., Wikelski, M., Kempenaers, B., 2013. When
455 the sun never sets: Diverse activity rhythms under continuous daylight in free-living arctic-
456 breeding birds. *Proc. R. Soc. B* 280, 20131016. (doi: 10.1098/rspb.2013.1016)

457 Stelzer, R.J., Chittka, L., 2010. Bumblebee foraging rhythms under the midnight sun measured
 458 with radiofrequency identification. *BMC Biol.* 8, 93. (doi:10.1186/1741-7007-8-93)

459 Teschke, M., Wendt, S., Kawaguchi, S., Kramer, A., Meyer, B., 2011. A circadian clock in
 460 Antarctic krill: An endogenous timing system governs metabolic output rhythms in the
 461 euphausiid species *Euphausia superba*. *PLoS ONE* 6, e26090. (doi:
 462 10.1371/journal.pone.0026090)

463 van Oort, B. E. H., Tyler, N. J. C., Gerkema, M. P., Folkow, L., Blix, A. S., Stokkan, K-A., 2005.
 464 Circadian organization in reindeer. *Nature* 438, 1095–1096. (doi: 10.1038/4381095a)

465 Vanin, S., Bhutani, S., Montelli, S., Menegazzi, P., Green, E. W., Pegoraro, M., Sandrelli, F.,
 466 Costa, R., Kyriacou, C. P., 2012. Unexpected features of *Drosophila* circadian behavioural
 467 rhythms under natural conditions. *Nature* 484, 371-375. (doi: 10.1038/nature10991)

468 Vleck, C.M., van Hook, J. A., 2002. Absence of daily rhythms of prolactin and corticosterone in
 469 Adelie penguins under continuous daylight. *The Condor* 104, 667–671. (doi: 10.1650/0010-
 470 5422(2002)104[0667:AODROP]2.0.CO;2)

471 Williams, C.T., Barnes, B.M., Richter, M., Buck, C.L., 2012. Hibernation and circadian rhythms
 472 of body temperature in free-living arctic ground squirrels. *Physiol. Biochem. Zool.* 85, 397–
 473 404. (doi: 10.1086/666509)

474 Yang, Z., Sehgal, A., 2001. Role of molecular oscillations in generating behavioral rhythms in
 475 *Drosophila*. *Neuron* 29, 453-467. (doi: 10.1016/S0896-6273(01)00218-5)

476 Young, M.W., Kay, S.A., 2009. Time zones: A comparative genetics of circadian clocks. *Nat.*
 477 *Rev. Genet.* 2, 702–715. (doi:10.1038/35088576)

478

479 **Figure legends**

480 **Fig. 1.** Locomotory activity of *Belgica antarctica* under field and laboratory conditions. Field
481 monitoring of larvae (A) and adults (B) occurred during the austral summer in Antarctica.
482 Laboratory experiments on larvae (C) and adults (D) monitored activity at $4.9 \pm 0.3^{\circ}\text{C}$ with a
483 photoperiod of LD 21:3, followed by constant darkness. Larval activity (E) was determined
484 using a thermoperiod of 4.5°C for 19 h and (-1.9°C) for 5 h, followed by 2.5°C in constant light.
485 Mean activity is plotted in blue (+ SD, gray) together with light intensity (log lx; yellow) and
486 temperature ($^{\circ}\text{C}$; red). Number of individuals (n) is indicated in each diagram. Individual
487 actograms presented in Supplementary data 1, Fig. 1A-E.

488 **Fig. 2.** Daily expression profiles of circadian clock genes in larval and adult heads of *Belgica*
489 *antarctica* from the field or in the laboratory under different photoregimes. Transcript levels of
490 *period*, *timeless*, *vriille* and *Clock* were measured by quantitative PCR. (A) Larvae used for the
491 field data were collected under the natural conditions that prevailed at two different dates in
492 January, during the austral summer at Palmer Station, Antarctica (Jan. 10, solid line and open
493 rectangle; Jan. 25, broken line and open triangle). Each time point represents an independent
494 biological repeat. Light intensity (log lux, yellow) and temperature ($^{\circ}\text{C}$, red) were measured
495 during the corresponding days; sunrise and sunset are designated with arrows (A; lower panels).
496 (B) Adults were collected under the natural conditions on Jan, 9 (solid line and open diamonds)
497 during the austral summer at Palmer Station, Antarctica. Each time point represents an
498 independent biological repeat. Light intensity (log lux, yellow) and temperature ($^{\circ}\text{C}$, red) were
499 measured during the corresponding day; sunrise and sunset are designated with arrows (B; lower
500 panel). (C) Three different photoperiods were used to entrain larvae in the laboratory

501 experiments: LD 12:12 (solid line), LD 21:3 (broken line), LL (dotted line). Open and black bars
502 below graphs refer to light (L) and dark (D) periods. Significant oscillations for *tim* and *vri*
503 mRNAs (both $FDR < 0.05$) are highlighted in red. Each time point represents an average of
504 independent biological repeats: $n = 5$ for LD 12:12; $n = 3$ for LD 21:3 and LL. Error bars
505 indicate $\pm$ SD.

Figureg

Figure 1

Figure 2

Polar summer

with extremely
long days

Antarctic midge

the only insect
endemic to Antarctica

Biological rhythms

Temperature dependent
locomotor activity

No cycling of circadian
clock genes