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

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ABSTRACT

The extreme seasonal shifts of day length 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 *vrille* 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.

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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 *Bmal*1 and *per*2 (Lu et al., 2010), while a daily rhythm of *cry*2 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.

Abbreviations: *Bmal*1, *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*, *vrille*; ZT, Zeitgeber time.

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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 and 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. 5 °C. Digital images were captured using a webcam equipped with an infrared LED (DC-NCR13U or DC-NCR20U, Hanwha Japan, Tokyo; λ_{max} = 870 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 (Schmid et al., 2011). The start of the 24-h day in field experiments was designated by 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.

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).

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). 5× 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 s at 94 °C, 10 s at 60 °C (*rp*49, *per*, *tim*) or 65 °C (*Clk*, *vri*), 20 s 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 *rp*49. Genomic DNA was isolated from 1 to 2 larval bodies using DNeasy Blood & Tissue Kit (Qiagen). Intron-overlapping primers for *per*, *tim*, *Clk* and *rp*49 are listed in Table 3, and their positions are shown in Supplementary data 3.

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, *rp*49, 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

Table 1
Environmental conditions prevailing at Palmer Station, Antarctica on the dates we observed locomotory activity of the midge *Belgica antarctica* during the summer of 2012. 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. T_{max}, T_{min} (°C) refer to maximum and minimum air temperatures recorded by HOBO loggers during the experimental periods.

Locomotor activity	Date	LD	Sunrise	Sunset	Civil dawn	Civil dusk	T_{max} (°C)	T_{min} (°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

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. 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. T_{max}, T_{min} (°C) refer to maximum and minimum air temperatures recorded by HOBO loggers during the experimental periods.

Expression profiles	Date	LD	Sunrise	Sunset	Civil dawn	Civil dusk	T_{max} (°C)	T_{min} (°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

(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 ± 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 ± 0.3 °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, Fig. C). 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 2 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 day length (sunrise to sunset) was approximately 21 h, were transferred to the laboratory and subjected to one LD 21:3 cycle, followed by 2 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, Fig. D). 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.

Table 3
Gene-specific primers used in qPCR.

Transcript	Primer	Primer sequence 5′–3′	Product size (bp)
period	Forward	GTATGCACTCAAACACTCACATCAG^CCTG	126
	Reverse	CTGCTGCTGGCGGACGATTCGAG	
timeless	Forward	CGACTGAAGGCGTCTCCATCAA^TGGC	170
	Reverse	CAGTTGCTGGACGACAGCGATTCTGG	
Clock	Forward	AAAACAGTTCACTTAGTCAG^CGGTC	161
	Reverse	GTGAGGCATGAAGTTCGTTGACTG	
vrille	Forward	CGACCAATCCACTCGAAAACTTCG	151
	Reverse	ATGTTCTTGATGGTGTGGATTTCCG	
rp49	Forward	CGGACCGATATGACAAAGTCAAG^GAAGC	149
	Reverse	GAAGCCGTTGGGGAGCATGTGGC	

Intron position is marked with a caret (^).

3.3. Clock genes of B. antarctica

Full length sequences of the circadian clock genes *period* and *timeless* and partial sequences of *Clock* and *vrille* were obtained.

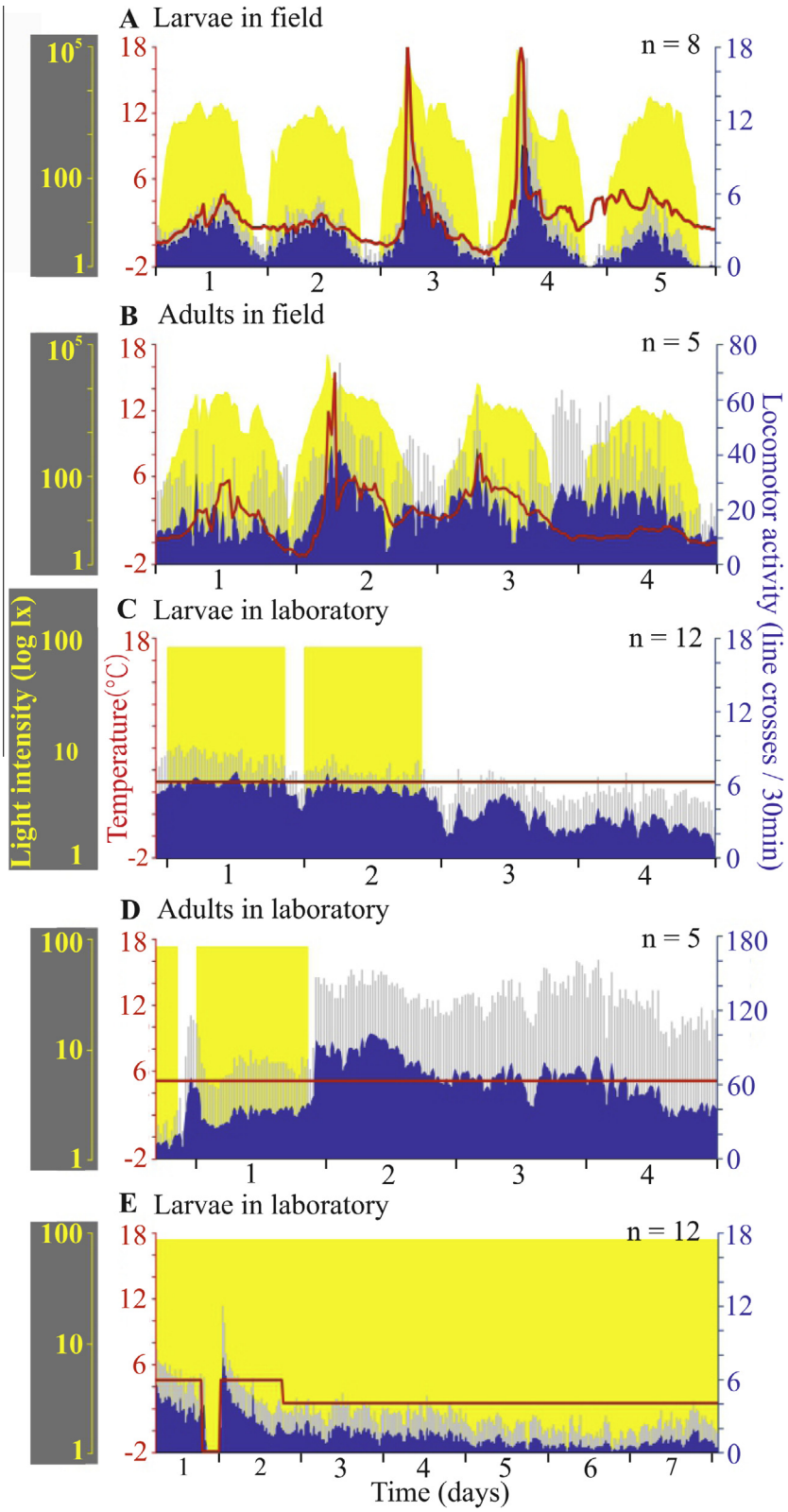

Fig. 1. Locomotory activity of *Belgica antarctica* under field and laboratory conditions. Field monitoring of larvae (A) and adults (B) occurred during the austral summer in Antarctica. Laboratory experiments on larvae (C) and adults (D) monitored activity at 4.9 ± 0.3 °C with a photoperiod of LD 21:3, followed by constant darkness. Larval activity (E) was determined 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. Mean activity is plotted in blue (+SD, gray) together with light intensity (log lx; yellow) and temperature (°C; red). Number of individuals (*n*) is indicated in each diagram. Individual actograms are presented in Supplementary data 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Sequences suggest no conspicuous features predicting anything unusual about these genes. No deletions or insertions that would appear to interfere with translation are present in the sequences.

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

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

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

Protein alignments of the clock genes of *D. 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 (Fig. 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 *D. 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 and Hardin, 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

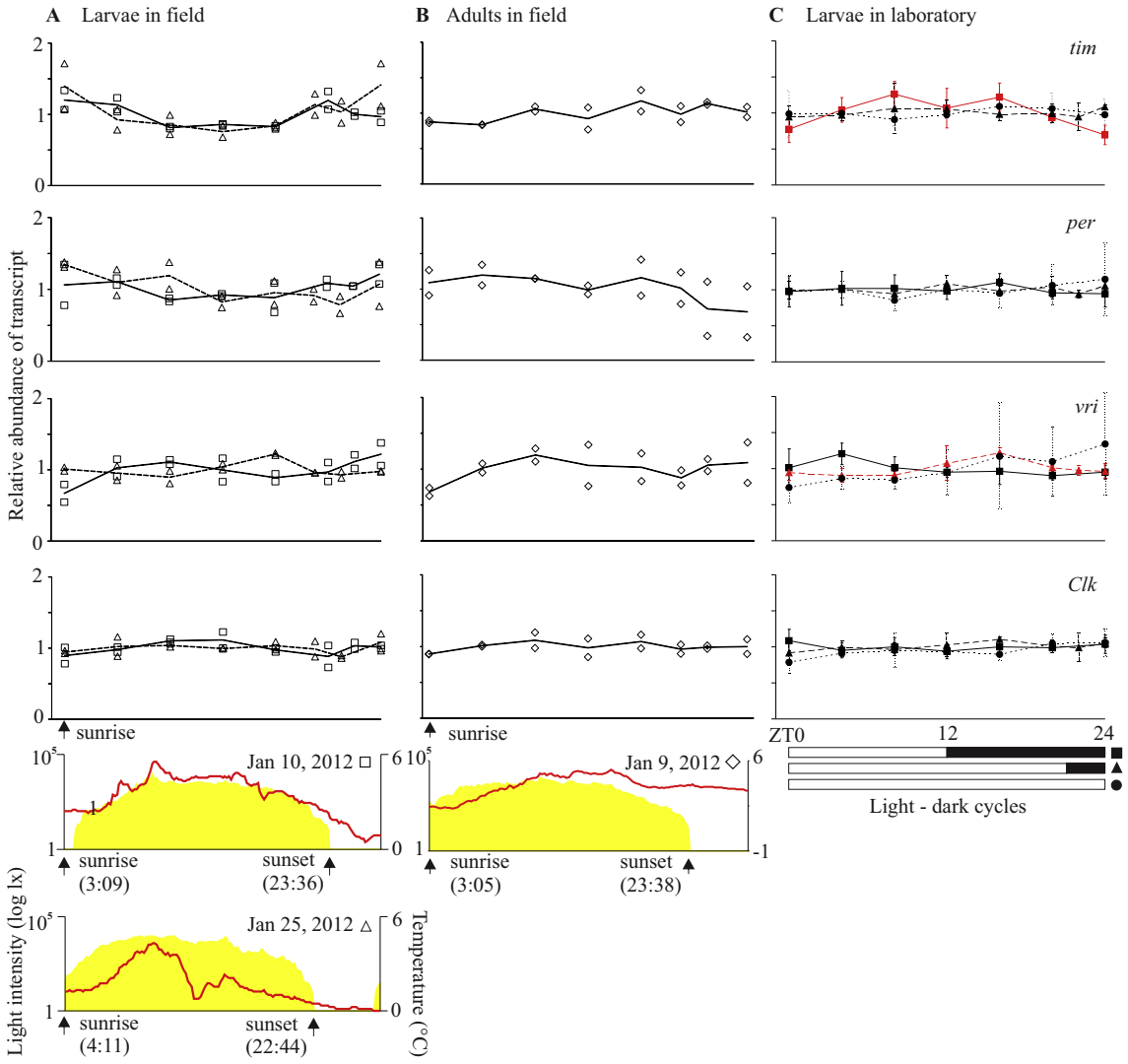

Fig. 2. Daily expression profiles of circadian clock genes in larval and adult heads of *Belgica antarctica* from the field or in the laboratory under different photoregimes. Transcript levels of *period*, *timeless*, *vrille* and *Clock* were measured by quantitative PCR. (A) Larvae used for the field data were collected under the natural conditions that prevailed at two different dates in January, during the austral summer at Palmer Station, Antarctica (Jan. 10, solid line and open rectangle; Jan. 25, broken line and open triangle). Each time point represents an independent biological repeat. Light intensity (log lux, yellow) and temperature (°C, red) were measured during the corresponding days; sunrise and sunset are designated with arrows (A; lower panels). (B) Adults were collected under the natural conditions on Jan, 9 (solid line and open diamonds) during the austral summer at Palmer Station, Antarctica. Each time point represents an independent biological repeat. Light intensity (log lux, yellow) and temperature (°C, red) were measured during the corresponding day; sunrise and sunset are designated with arrows (B; lower panel). (C) Three different photoperiods were used to entrain larvae in the laboratory experiments: LD 12:12 (solid line), LD 21:3 (broken line), LL (dotted line). Open and black bars below graphs refer to light (L) and dark (D) periods. Significant oscillations for *tim* and *vri* mRNAs (both *FDR* < 0.05) are highlighted in red. Each time point represents an average of independent biological repeats: $n = 5$ for LD 12:12; $n = 3$ for LD 21:3 and LL. Error bars indicate ± SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 indispensible, 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2015.07.008.

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