Journal of Crustacean Biology

The Crustacean Society

Journal of Crustacean Biology

Journal of Crustacean Biology (2018) 1–9. doi:10.1093/jcbiol/ruy035

Photoperiodic modulation of circadian functions in Antarctic krill *Euphausia superba* Dana, 1850 (Euphausiacea)

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(Received 29 December 2017; accepted 17 April 2018)

ABSTRACT

An endogenous circadian clock influences metabolic output rhythms in the Antarctic krill (*Euphausia superba* Dana, 1850), a key species in the Southern Ocean ecosystem. Seasonal changes in photoperiod in Antarctica, ranging from midnight sun (24 h light) during mid-summer to very short days (3–4 h light) during mid-winter, represent a challenge for the synchronization of the krill circadian clock. We analyzed clock gene activity and clock output functions in krill exposed to different light conditions during a long-term photoperiodic simulation in the laboratory. In simulated early-autumn (light/dark or LD 16:8) and late-winter (LD 8:16) conditions, the circadian clock of krill was functional and the metabolic output was synchronized to the light/dark cycle, the clock genes Es*per* and Es*clk* peaked in antiphase around simulated dusk/dawn and most metabolic-related genes showed upregulation around simulated dusk. In contrast, in simulated mid-summer (light/light or LL) and mid-winter (LD 3:21) conditions, the synchronization of the circadian clock and the metabolic output appeared to be weaker, with clock gene expression becoming arrhythmic and upregulation of metabolic genes occurring at different times during the day. Early-autumn and late-winter photoperiodic cues in the laboratory thus seem to be sufficient to entrain the krill clock and promote metabolic synchronization, whereas mid-winter and mid-summer photoperiodic cues seem to be insufficient for krill entrainment. Krill in the field may overcome the seasonal lack of overt photoperiodic cycle occurring during mid-summer and mid-winter by using alternative light-related *Zeitgebers* (i.e., varying light intensity rather than the presence or absence of light) to promote basic homeostatic rhythms over 24 h.

Key Words: circadian clock, clock genes, clock output, extreme light conditions, high latitude, midnight sun, Southern Ocean

THIRD INTERNATIONAL SYMPOSIUM ON KRILL

INTRODUCTION

Antarctic krill, *Euphausia superba* Dana, 1850 (hereafter krill), are key players in the Southern Ocean (SO) ecosystem in terms of distribution, abundance, and trophic relevance (Quetin & Ross, 1991). During the last decades, due to warming in the Atlantic sector of the SO and increasing interest in krill by commercial fisheries, concerns have been raised about the well-being of the krill population (Atkinson *et al.*, 2004), and efforts to understand the interaction between krill and their environment have been intensified.

The high-latitude environment of the SO is characterized by strong seasonal fluctuations in day length, sea-ice cover, and primary production, which affect the dynamics of light and food

availability over the year (Knox, 2006). Prolonged day length (up to 24 h) and absence of sea-ice cover in summer favor high irradiance at the sea surface, triggering elevated primary production, whereas in winter shortened day length (3–4 h) and extended sea-ice cover cause significant reduction of irradiance at the sea surface, preventing primary production.

In response, krill display seasonal cycles of metabolic activity and sexual maturity, with lower metabolic rates and sexual regression during winter and higher metabolic rates and sexual maturity during summer (Kawaguchi *et al.*, 2007; Meyer *et al.*, 2010). Moreover, krill display seasonal changes in their vertical distribution and Diel Vertical Migration (DVM) behavior, being generally shallower in summer compared to winter, and performing a more pronounced DVM in spring-autumn compared to summer (Quetin & Ross, 1991; Taki *et al.*, 2005).

The mechanisms underlying these rhythmic functions in krill are still debated. Investigations on the influence of light regime and food availability on the seasonal cycles of metabolic activity and sexual maturity suggested that light might play a prominent role (Atkinson *et al.*, 2002; Teschke *et al.*, 2008). Even though light conditions are generally considered to be the main proximate cue for zooplankton DVM (Cohen & Forward, 2009), other factors like food availability, predation pressure, and social interactions may play a major role in krill (Gaten *et al.*, 2008). Laboratory analyses have indicated that krill rhythmic functions may persist also in the absence of external environmental stimuli, suggesting the interplay with an internal (i.e., endogenous) timing system, possibly a circadian clock (Teschke *et al.*, 2007; Gaten *et al.*, 2008; Brown *et al.*, 2013).

The circadian clock is a molecular oscillator based on the rhythmic expression of clock genes, which free-runs with a period of approximately 24 h. The clock can be synchronized to the day/night cycle by the interaction with light cues (Dunlap, 1999) and can promote output rhythms in metabolism, physiology, and behavior at the daily and seasonal levels (Allada & Chung, 2010; Dardente *et al.*, 2010; Goto, 2013). It has been suggested that the circadian clock in krill might be implicated in the regulation of DVM (Gaten *et al.*, 2008), metabolic activity (Teschke *et al.*, 2011), and transcription (De Pittá *et al.*, 2013), but actual knowledge about krill clock functions is still limited. The recent identification of the most known (from model organisms) set of clock genes in the transcriptome of krill and the molecular characterization of krill circadian feedback loop have opened new opportunities to study the clock at the molecular level (Biscontin *et al.*, 2017; Hunt *et al.*, 2017).

For high latitude organisms like krill, the strong seasonality of the photoperiodic cycle might represent a limitation for the activity of the circadian clock and the entrainment of daily rhythms, especially at those times of the year when photoperiodic cues get weaker (mid-winter) or even disappear (mid-summer). To overcome this problem, high-latitude organisms may either suppress their clock functions and use other mechanisms to entrain daily rhythms when necessary (Reierth *et al.*, 1999; van Oort *et al.*, 2005; Lu *et al.*, 2010), or preserve a functional clock and switch to alternative *Zeitgebers* (entraining cues) when photoperiodic conditions become insufficient (Ashley *et al.*, 2013, 2014).

The aim of this study was to investigate the impact of the seasonal photoperiodic cycle of the Southern Ocean on the activity of the circadian clock and the regulation of circadian functions in Antarctic krill, with particular emphasis on light patterns typical of those times of the year when photoperiodic cues might be strongly reduced (mid-winter, day length below 3–4 h) or even missing (mid-summer, when the sun never goes below the horizon). We exposed krill in aquaria to a long-term photoperiodic simulation in the absence of other *Zeitgebers* and examined clock gene activity and the regulation of daily output rhythms at four simulated seasonal light regimes corresponding to mid-summer (midnight sun, 24 h light), early-autumn (long day, 16 h light), mid-winter (very short day, 3 h light) and late-winter (short day, 8 h light). To monitor the activity of the clock, we analyzed daily patterns of expression of the clock genes Es*clk* and Es*per*, the krill homologues of the canonical *Drosophila* clock genes *clock* and *period* (Dunlap, 1999), and to monitor changes in clock output functions, we analyzed daily patterns of regulation of a selection of genes controlling rate-limiting steps in carbohydrate, lipid, and energy metabolism.

MATERIALS AND METHODS

Sampling and laboratory maintenance of experimental krill

Specimens of *E. superba* were collected in East Antarctica between 65°19'S, 125°37'E (17 September 2007) and 64° 08' S, 119° 16' E (9 October, 2007) in the upper 200 m of the water column using a rectangular midwater trawl (RMT 8) during voyage V1 07/08 of RSV *Aurora Australis*. All krill were immediately transferred to 200 l tanks located in a constant-temperature room at 0 °C and supplied with a continuous flow of chilled seawater. Individuals were kept in conditions of dim light and in the absence of food, and dead animals and molts were removed daily from the tanks. After arrival in Hobart, Tasmania (17 October 2007), krill were transported from the ship to the krill research aquarium at the Australian Antarctic Division (AAD) in Kingston, Tasmania, and transferred to a 1670 l holding tank connected to a 5000 l chilled seawater recirculation system. Temperature of seawater in the tank was maintained constant at 0.5 °C. Water was continuously recirculated through an array of mechanical and biological filters and constantly monitored for quality following King *et al.* (2003). Light in the aquarium was provided by fluorescent tubes covered with a gel filter (Lee Roll 131 Marine Blue; ARRI, Munich, Germany). A PC-controlled timer and dimming system (winDIM v4.0e; EEE, Lisbon, Portugal) provided a light regime similar to that occurring in the Southern Ocean at 66° latitude south (66° S) and 30 m depth. A sinusoidal annual cycle with monthly variations of photoperiod and daily variation of light intensity was calculated by assuming continuous light and a maximum light intensity of 100 lux at the surface of the tank (equal to 1% light penetration to 30 m depth) during summer midday (December at 66° S). The system was adjusted every month to reflect SO conditions. Krill were fed daily with a mixture of living algae at the final concentration of 1.5×10^4 cells ml^{-1} of the pennate diatom *Phaeodactylum tricornutum* Bohlin, 1897, 2.2×10^4 cells ml^{-1} of the chlorophyte *Pyramimonas gelidicola* (McFadden, Moestrup & Wetherbee, 1982), and 2×10^4 cells ml^{-1} of the cryptophyte *Geminigera cryophila* (Taylor & Lee) Hill, 1991. Instant algae were added to yield final concentration of 1×10^4 cells ml^{-1} of *Thalassiosira weissfloggii* (Fryxell & Hasle, 1977) (1200TM; CCMP1051/TWsp.; Reed Mariculture, Campbell, CA, USA), 5.1×10^4 cells ml^{-1} *Isochrysis sp.* (1800TM; Reed Mariculture), and 4.8×10^4 cells ml^{-1} *Pavlova sp.* (1800TM; Reed Mariculture). Krill also received 2 g per tank per day of nutritional supplements (1 g of Frippak #1 CAR; 1 g of Frippak #2 CAR; INVE; Nonthaburi, Thailand). After the phytoplankton mix was added, water flow in the tank was shut off for 2 h to enable krill to feed on the algal mixture. This feeding regime has been used successfully in several experiments trials at the AAD prior to this study, and maintains krill in good conditions, with low mortality and high feeding rates, in long-term laboratory experiments (King *et al.*, 2003; Kawaguchi *et al.*, 2010). Animals were fed *ad libitum*, thus keeping feeding conditions always at their optimum. Dead individuals and molts were removed from the tank daily.

Experimental setup and sampling of krill in the laboratory

The experiment started at the beginning of December 2009. 300 adult krill of mixed sexes were collected at random from the holding tank and transferred to a cylindrical 100 l tank connected to

the aquarium's recirculating seawater facility. The tank was placed into a black lightproof plastic container to create a separate light compartment and enable complete control over light conditions throughout the experiment. Light inside the container was provided by fluorescent tubes (Osram L18W/640 Cool White) covered with a gel filter (Lee Roll 131 Marine Blue; ARRI) simulating light as attenuated to 30 m depth in the ocean. The duration of the light phase and light intensity were regulated using the same PC-controlled system as for the holding tank. The duration of the light phase was adjusted every month to mimic seasonal changes of natural day length at 66° S (Fig. 1). Light intensity at the surface of the tank was 0 lux during the dark phase and 100 lux during the light phase. The transition between light and dark phases was gradual to simulate natural dawn and dusk conditions. Temperature was held constant around 0.5 °C throughout the experiment. The krill were fed with the same phytoplankton mixture and the same procedure described for the holding tank. A fixed volume of food was added to the tank daily to reach a feeding optimum (*ad libitum*) and keep daily food availability constant throughout the experiment.

We sampled four 24-hour time-series (Fig. 1): December 2009 (simulated mid-summer conditions, near-constant light or LL), February 2010 (simulated early-autumn conditions, 16 h light/8 h darkness or LD 16:8), June 2010 (simulated mid-winter conditions, 3 h light/21 h darkness or LD 3:21), and August 2010 (simulated late-winter conditions, 8 h light/ 16 h darkness or LD 8:16). Mid-summer (LL) and mid-winter (LD 3:21) conditions were selected to investigate the effect of light regime towards the extremes of the seasonal cycle, whereas early-autumn (LD 16:8) and late-winter (LD 8:16) were selected to investigate the effect of long and short days, respectively. Moreover, early-autumn and late-winter represent turning points in the seasonal life cycle of krill (processes related to overwintering and sexual regression start in early-autumn, whereas processes related to termination of quiescence and sexual maturity begin in late-winter), and it was therefore deemed worthwhile to investigate the effect of the clock during those times of the year. Sampling was conducted always at the beginning of the third week of the corresponding month. During sampling, food supply was interrupted and six animals were randomly collected every 3 h, starting at 0600 and ending at 0600 on the following day. Sampling during dark phases was conducted under dim red light. All animals were immediately frozen in liquid nitrogen and stored at –80°C.

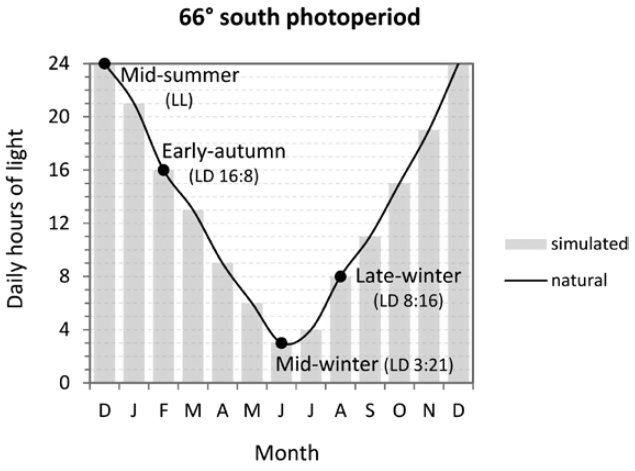

Figure 1. Laboratory simulation of the natural photoperiodic cycle occurring at 66° latitude south in the Southern Ocean. Black solid line indicates the natural course of the photoperiodic cycle at 66° S. Gray-shaded bars indicate monthly changes in day length in the simulation. Black dots indicate time-points of collection of 24 h samples.

Molecular analyses

Total RNA for gene expression analyses was extracted from krill heads. For each time-point, six frozen heads were dissected on dry ice and individually transferred in separated Precellys® tubes (Bertin Instruments; Montigny-le-Bretonneux, France) containing the TRIzol® reagent (ThermoScientific, Waltham, MA, USA). Tissue homogenization was performed using a Precellys®24 tissue homogenizer (Bertin Instruments) connected to a Cryolys cooling element; the process was carried out at 4°C. The homogenates were removed from the tubes and treated with chloroform/isopropanol for phase separation and precipitation. The RNA pellets were washed twice with 75% ethyl alcohol and resuspended in RNase-free water. We checked RNA concentration and purity using a NanoDrop™2000 UV-Vis spectrophotometer (ThermoScientific), and RNA integrity using an Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA). To prevent genomic contamination, all samples were treated with the TURBO DNA-Free kit from Ambion (ThermoScientific). After DNA removal, for each sample 2 µg of total RNA were retro-transcribed to cDNA using the RevertAid H Minus Reverse Transcriptase kit from Invitrogen (ThermoScientific) to a final volume of 50 µl per sample (40 ng/µl). Gene expression was measured using custom-designed TaqMan® Low-Density Array Cards (ThermoScientific). Primers for qPCR analysis were designed around sequences of interest using the Custom TaqMan® Assay Design Tool (ThermoScientific). The primer sets for the clock genes Es*clk* and Es*per* and the ones for the metabolic genes *glycogen synthase* (*gys*), *phosphofructokinase*-6 (*pfk*6), *citrate synthase* (*cs*), *hydroxyacyl-coenzyme* A *dehydrogenase* (*hadh*), *acetyl-coenzyme* A *carboxylase* (*acc*), and ATP *synthase* (*atp*) were designed using the sequences available online at http://krilldb.bio.unipd.it (Sales *et al.*, 2017) (Supplementary material Table S1). For each sample we used 20 µl of cDNA (40 ng/µl), 30 µl of RNase-free water and 50 µl of TaqMan® Gene Expression Master Mix (ThermoScientific), for a final volume of 100 µl. The cards were analyzed using a ViiA™ 7 Real-Time PCR System (ThermoScientific). Standard curves were conducted to verify the efficiency of each primer pair on the card.

Normalization and relative quantification

The levels of transcription of all genes were normalized and quantified following the $2^{-\Delta\Delta Ct}$ method implemented by the ddCt-Expression-method function of the ddCT R-package (Zhang *et al.*, 2015). To select the most stable genes to use in the normalization procedure, we measured 24 h patterns of gene expression of five different candidate reference genes in the head of adult krill in the four different photoperiodic conditions considered and used Genorm (Vandesompele *et al.*, 2002), Bestkeeper (Pfaffl *et al.*, 2004), and Normfinder (Andersen *et al.*, 2004) to identify reference genes that did not show a sinusoidal expression during the 24 h period. Our candidate reference genes were *ubiquitin carboxyl-terminal hydrolase* 46 (*usp*46), *ryanodine receptor* (*ryr*), *elongation factor* 1-*alpha* (*ef*1a), *ribosomal protein* S13 (*rps*13), and *ribosomal protein* L32 (*rpl*32). *Usp*46 and *ryr* had showed constant mRNA levels during the daily 24 h cycle in a previous experiment conducted in LD/DD conditions (data not shown) and in previous experiments on daily patterns of expression of opsin-related mRNAs in the head of adult krill (Biscontin *et al.*, 2016). *Ef*1a, *rps*13 and *rpl*32 had showed constant mRNA levels in previous experiments involving manipulation of photoperiod in the lepidopteran *Plutella xylostella* (Linnaeus, 1758) (Fu *et al.*, 2013). Here, *usp*46 and *rps*13 showed constant mRNA expression levels in all simulated photoperiodic conditions (Supplementary material Fig. S2). We therefore used a combination of *usp*46 and *rps*13 as a reference for normalization in the ddCT package. Daily changes in mRNA levels were then calculated relative to the daily average for each target gene.

Statistical analysis

We used the R-package Rain (Thaben & Westermark, 2014) to test for the presence of 24 h rhythmicity in the daily patterns of gene expression. The package fits the data to sinusoidal or sawtooth curves having the required period (24 h or 12 h in our case) and returns a probability value (*P*) indicating the likelihood of the fit and the phase of the fitted curve (corresponding to the time-point where the amplitude of the oscillation is maximal). *P* values were corrected for multiple testing using the Bonferroni method implemented by the package.

Since the Rain software is designed specifically to detect sinusoidal and sawtooth waves, more complex or less structured rhythmic expression profiles might sometimes be overlooked. To overcome this limitation, when we failed to detect a significant fit but the expression profile showed clear changes over the 24 h, we used the Wilcoxon-Mann-Whitney non-parametric test implemented by R to put in evidence significant differences between peaks and troughs of gene expression.

Ethics Statement

All animal work was conducted according to relevant national and international guidelines. Krill catches, welfare, and experimentation were based on permission of the Department of Environment and Heritage (DEH) of the Australian Government, and were conducted in accordance with the Antarctic Marine Living Resources Conservation Act 1981 (permit 06_09_2220) and the Environment Protection and Biodiversity Conservation Act 1999 (permit: WT2007-1480).

RESULTS

Clock gene expression

To monitor changes in the activity of the circadian clock of krill in the different photoperiodic conditions, we analyzed the daily patterns of expression of the clock genes Es*clk* and Es*per*, which are the krill homologs of the *Drosophila* core clock genes *clock* and *period*. Due to the opposite roles played by *clock* and *period* within the circadian molecular feedback loop (*clock* is a promoter, *period* a suppressor), the daily patterns of expression of these two clock genes usually show an antiphase relationship, with *period* peaking around dusk and *clock* peaking around dawn (Dunlap, 1999; Hardin, 2005).

In krill exposed to near-constant light (mid-summer conditions, LL), the expression patterns of Es*clk* and Es*per* did not show a clear antiphase relationship (Fig. 2A, B). Both genes showed a peak during the first half of the 24 h cycle, at CT 6 (CT = Circadian Time, indicating time intervals in the absence of Zeitgeber starting at 0000), and a trough during the second half of the 24 h cycle, at CT 15. The daily profiles of expression looked irregular and the variability between biological replicates (standard error mean) was high. This indicates that in LL the circadian molecular feedback loop did not work properly and that no synchronization was present among the clocks of different individual krill.

In krill exposed to long-day conditions (early-autumn, LD 16:8) the expression pattern of Es*clk* and Es*per* showed a clear antiphase relationship (Fig. 2C, D), with Es*per* showing 24 h rhythmicity with the phase of the oscillation set towards the end of the light phase (ZT 14; ZT = Zeitgeber Time, indicating the time intervals from the beginning of the light phase), and Es*clk* showing a well-defined daily pattern with a peak of expression at the beginning of the light phase (ZT 2). The daily profiles of expression looked regular, and the variability between biological replicates was low, especially for Es*per*. This indicates that in LD 16:8 the circadian molecular feedback loop was working properly and the clocks of different individual krill were synchronized. The occurrence of peaks and troughs of expression at the beginning and end of the light phase indicates that photoperiod was acting as an entraining cue.

In krill exposed to very short-day conditions (mid-winter, LD 3:21) the expression patterns of Es*clk* and Es*per* did not show a clear antiphase relationship (Fig. 2E, F). The mRNA levels of Es*clk* were high around ZT 7–8, whereas Es*per* showed a peak at ZT 4–5 at the beginning of the dark phase. The daily profile of expression of Es*clk* was irregular, and the variability between replicates was high, whereas the profile of Es*per* was more regular and the variability was lower. Even though the expression pattern of Es*per* showed some synchronization with the LD cycle, the absence of a clear antiphase relationship between Es*clk* and Es*per* and the irregular expression profile of Es*clk* indicated that in LD 3:21 the circadian molecular feedback loop was not working properly, and that the synchronization among clocks of different individual krill was not tight.

In krill exposed to short-day conditions (late-winter, LD 8:16), the expression patterns of Es*clk* and Es*per* (Fig. 2G, H) were similar to those observed in long-day conditions (early-autumn or LD 16:8, Fig. 2C, D). In particular, Es*clk* shows almost the same pattern with mRNA expression levels rising during the dark phase, peaking at the end of the dark phase (ZT 22), dropping during the light phase and reaching a trough at the beginning of the dark phase (ZT 13). Es*per* showed low mRNA levels at the beginning of the light phase with a trough at ZT 1, followed by the mRNA levels rising towards the end of the light phase and during the first half of the dark phase, reaching a peak at ZT 16. The profiles of expression of both genes looked regular, and the variability between biological replicates was low, indicating that in LD 8:16 the clock in krill was active and synchronized to the photoperiodic cycle.

Metabolic gene expression

To investigate potential changes in the daily patterns of regulation of the metabolic output in the different photoperiodic conditions, we paralleled our clock gene expression analysis with that of a selection of key metabolic genes including *glycogen synthase* (*gys*), *phosphofructokinase*-6 (*pfk*6) and *citrate synthase* (*cs*), which play prominent roles in carbohydrate metabolism, *hydroxyacyl*-CoA *dehydrogenase* (*hadh*) and *acetyl*-CoA *carboxylase* (*acc*), which play a fundamental role in the metabolism of fatty acids, and ATP *synthase gamma subunit* (*atp*), which regulates the synthesis of ATP.

Daily rhythms of metabolic regulation were present in simulated mid-summer conditions (LL), but the different pathways showed upregulation at different times of the day and the oscillations in the mRNA expression levels were characterized by different periods and different phases (Fig. 3A, B, C). Genes involved in the citric acid cycle (*cs*), glycogen synthesis (*gys*), and beta-oxidation of fatty acids (*hadh*) showed upregulation mostly during the second half of the 24 h cycle, at CT 15–18, whereas genes involved in glycolysis (*pfk*6), ATP production (*atp*), and fatty acids synthesis (*acc*) showed upregulation mostly at CT 6 during the first half of the 24 h cycle. Among the genes showing significant daily rhythmicity, *gys* was characterized by a 12 h period of oscillation with phase set around CT 18 (Fig. 3B), whereas *acc* showed a 24 h period with phase set around CT 6 (Fig. 3C).

Metabolic regulation seemed to be synchronized to the light/dark (LD) cycle in simulated early-autumn conditions (LD 16:8). All pathways showed upregulation at the end of the light phase (ZT 11–14–17; Fig. 3D). This was particularly evident for the genes involved in the synthesis of ATP (*atp*; Fig. 3F) and in the beta-oxidation of fatty acids *(hadh*; Fig. 3G), which showed 24 h rhythmic expression with low mRNA levels at the beginning of the LD cycle (ZT 2–5), increasing during the light phase, peaking at the end of the light phase (ZT 14), and then dropping again during the dark phase towards the end of the LD cycle. A very similar pattern was also present for *gys*, even if it is not statistically significant (Fig. 3E).

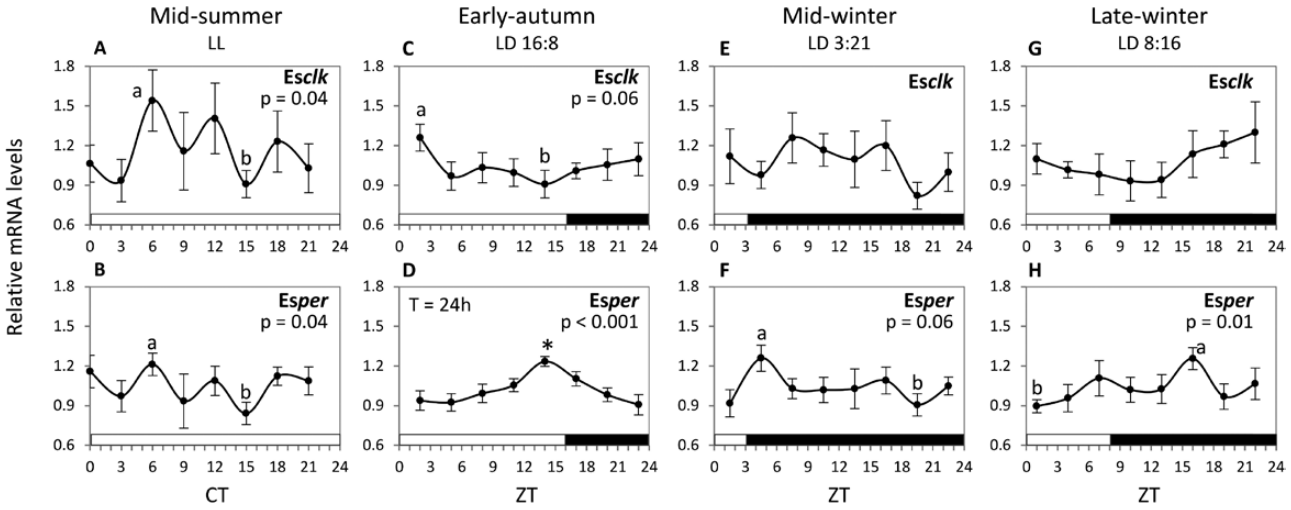

Figure 2. Daily patterns of expression of E*sclk* and E*sper* in mid-summer (**A**, **B**), early-autumn (**C**, **D**), mid-winter (**E**, **F**), and late-winter (**G**, **H**). Data points represent means ± SEM ($N = 6$). Significant differences in probabilities between peaks (a) and troughs (b), and for significant 24 h rhythmicity (T = 24 h) are reported; asterisk indicates phase of oscillation. White/black bars at the bottom of the graphs indicate photoperiod (white, light phase; black, dark phase). ZT, Zeitgeber Time; ZT 0 corresponds to 0400 h in early-autumn, 1030 h in mid-winter, and 0800 h in late-winter; CT, Circadian Time; CT 0 corresponds to 0000 h in mid-summer.

Daily rhythms of metabolic regulation were present in simulated mid-winter conditions (LD 3:21), but the different pathways showed upregulation at different times of the day and the oscillations in the mRNA expression levels were characterized by different periods and different phases (Fig. 3H, I, J, K). Genes involved in carbohydrate metabolism (*gys*, *cs*, and *pfk*6) and ATP production (*atp*) showed upregulation mostly during the second half of the LD cycle, around ZT 13.5, whereas genes involved in fatty acids metabolism (*acc* and *hadh*) showed upregulation mostly during the first half of the LD cycle, around ZT 4.5. Among the genes showing significant daily rhythms of regulation, *gys* was characterized by a 24 h period of oscillation with the phase set at ZT 13.5 (Fig. 3I), whereas *acc* showed a significant 24 h period with the phase set at ZT 4.5 (Fig. 3J). An intermediate pattern was present for *pfk*6, which showed significant 12 h rhythmicity with the phase set at ZT 13.5 and a second peak at ZT 4.5 (Fig. 3K).

Metabolic regulation seemed to be synchronized to the light/dark (LD) cycle in simulated late-winter conditions (LD 8:16), with the upregulation of the metabolic genes taking place mostly during the dark phase, from ZT 7–10 until ZT 16–19 (Fig. 3L). This was particularly evident for the genes involved in the synthesis of glycogen (*gys*, Fig. 3M) and glycolysis (*pfk*6, Fig. 3N), which showed 24 h rhythmic expression with low mRNA levels during the light phase, increasing during the dark phase and peaking in the middle of the dark phase, at ZT 16.

DISCUSSION

While photoperiod during our long-term simulation was adjusted every month to mimic natural conditions, food availability and temperature were held constant throughout the experiment. We observed different levels of modulation of krill circadian clock, depending on the time of the year when the samples had been taken. Krill sampled in February and August (simulated early-autumn and late-winter conditions), when photoperiodic conditions were not extreme (LD 16:8 and LD 8:16, respectively), displayed a functional circadian clock. Positive (E*sclk*) and negative (E*sper*) clock components showed rhythmic daily patterns peaking in antiphase with respect to each other, as in the *Drosophila* circadian feedback loop (Dunlap, 1999; Hardin, 2005). The occurrence of peaks and troughs of expression at the transition between light and dark phases (simulated dawn and dusk) indicated that the photoperiodic cycle was playing a major role in the entrainment of the clock. Alternatively, in krill sampled in December and June (simulated mid-summer and mid-winter conditions) when photoperiodic conditions were extreme (LL and LD 3:21 respectively), the circadian clock seemed to be disrupted, clock gene expression was irregular and the antiphase relationship between positive and negative clock components was not present anymore. Moreover, the clocks seemed not to be synchronized among individual krill, as if a proper entraining cue was missing. Similar disruptive effects of extreme light conditions on the activity of the circadian clock have been observed before in insects (Sauman & Reppert, 1996) and mammals (Ohta *et al.*, 2005), and even if recent findings suggested that high-latitude *Drosophila* species might have evolved specific clock features to mitigate such problem (Kyriacou, 2017; Menegazzi *et al.*, 2017), our results indicated that in krill exposure to extreme photoperiodic conditions in the laboratory can lead to the de-synchronization of the clock.

Different levels of modulation were observed at the different times of the year in the regulation of the metabolic output, in agreement with the corresponding clock activity. In simulated early-autumn and late-winter conditions, the metabolic output showed synchronization to the LD cycle, with upregulation at the transition between light and dark phase (in simulated early-autumn; Fig. 4B) and during the dark phase (in simulated late-winter, Fig. 4D), suggesting the presence of higher levels of activity during the simulated night. In contrast, in simulated mid-summer and mid-winter conditions the daily patterns of regulation of the main metabolic pathways seemed to lack a clear temporal synchronization, with some pathways showing upregulation during the first half of the 24 h cycle, and some other showing upregulation during the second half (Fig. 4A, C). Moreover, the period of the oscillations was heterogeneous, being in some cases 24 h and 12 h in others. The emergence of 12 h rhythmicity might be interpreted as circasemidian output related to the clock (Teschke *et al.*, 2011; De Pittá *et al.*, 2013), but also as ultradian output, with no direct connection with the clock (Lloyd & Stupfel, 1991), suggesting a tendency towards the occurrence of "around the clock" patterns of activity (Bloch *et al.*, 2013).

The synchronizations of krill circadian clock and metabolic output with the LD cycle observed in simulated early-autumn and late-winter photoperiodic conditions were in agreement with previous observations of rhythmic clock gene activity and oxygen

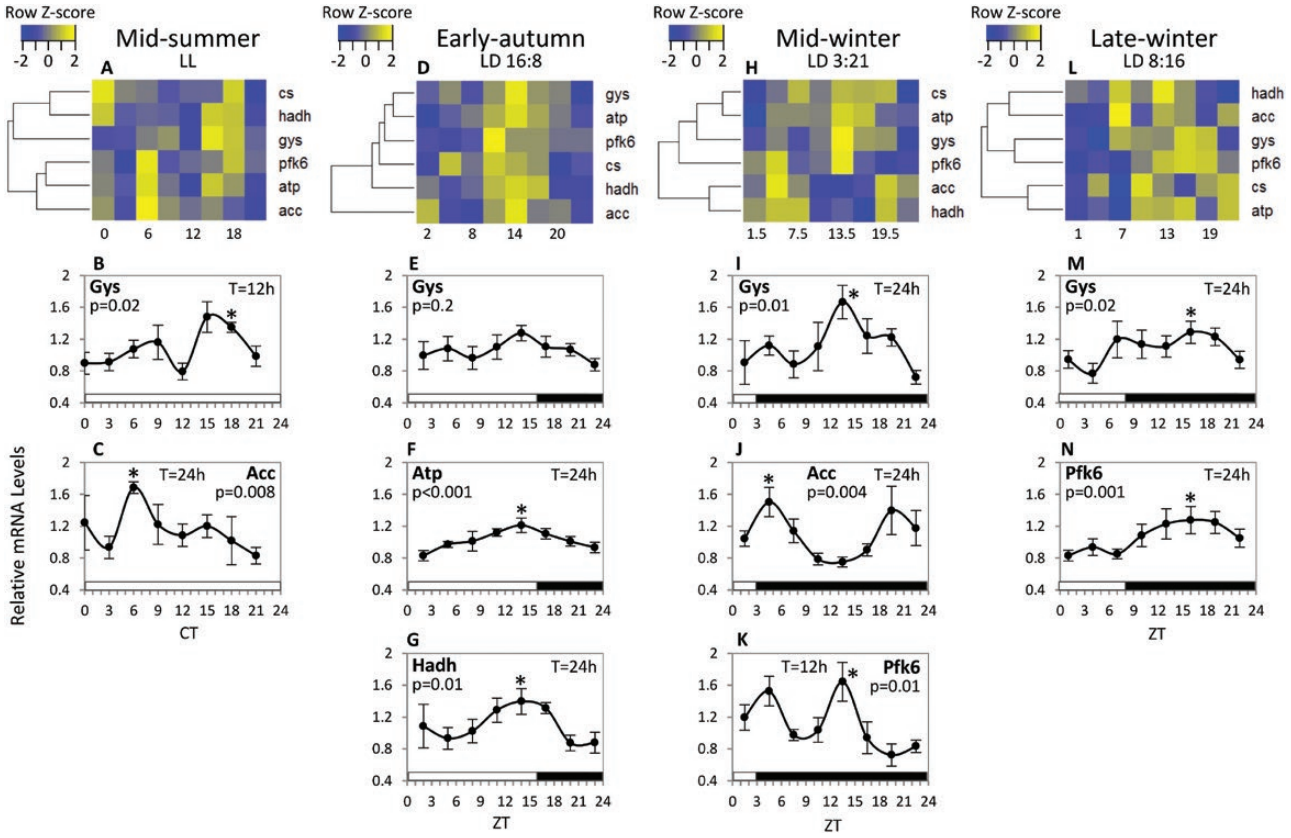

Figure 3. Daily expression signatures of metabolic genes in mid-summer (**A** – **C**), early-autumn (**D** – **G**), mid-winter (**H** – **K**) and late-winter (**L** – **N**). Heatmaps and dendrograms, based on Euclidean distances, show the expression levels of metabolic genes during the 24 h cycle and are represented with a color-coded scale; yellow and blue represent high and low expression levels, respectively. Graphs show daily expression profiles with significant 12 h (T = 12 h) or 24 h (T = 24 h) rhythmicity. Data points represent means ± SEM ($N = 6$). Asterisks indicate phase of oscillation. White/black bars at the bottom of the graphs indicate photoperiod (white, light phase; black, dark phase). ZT, Zeitgeber Time; ZT 0 corresponds to 0400 h in early-autumn, 1030 h in mid-winter, and 0800 h in late-winter; CT, Circadian Time, CT 0 corresponds to 0000 h in mid-summer. The names of genes are provided in Supplementary material Table S1.

consumption in krill exposed to similar LD conditions in a short-term, laboratory photoperiodic simulation (Teschke *et al.*, 2011), which also indicated the presence of higher metabolic activity during the dark phase (simulated night). This suggested that during autumn and spring photoperiodic cues alone could play a major role as *Zeitgeber* for the entrainment of krill circadian clock, possibly promoting the daily rhythms of activity generally observed in the field at those times of the year (i.e., DVM and higher activity during the night; Ross *et al.*, 1996). Conversely, the loss of synchronization observed in simulated mid-summer photoperiodic conditions was in apparent contrast with previous reports of rhythmic clock gene expression and rhythmic transcriptional output in krill collected during the midnight sun in Antarctica, when the sun was never below the horizon (Mazzotta *et al.*, 2010; De Pittá *et al.*, 2013; Biscontin *et al.*, 2017). Because a clear 24 h cycle of light intensity was present in the field at that time of the year (De Pittá *et al.*, 2013), we hypothesize that krill circadian functions were being entrained by alternative rhythmic light cues related to light-intensity. Entrainment of the clock during the midnight sun by means of alternative *Zeitgebers* has been reported before for other high-latitude organisms (Williams *et al.*, 2015), and it might represent an adaptation to preserve homeostatic regulation of basic physiological processes at times of the year when overt photoperiodic cues are missing (Sharma, 2003; Vaze & Sharma, 2013). In that case, due to the major loss of rhythmicity in the environment, even if basic clock functions might be preserved, the daily rhythms of activity might get uncoupled from the clock and lose their synchronization (Bloch *et al.*, 2013). A similar process might be related to the de-synchronization of krill DVM observed during mid-summer.

Field data describing krill clock activity and circadian regulation during mid-winter are missing, but a similar concept of clock entrainment by alternative light cues might apply also during that time of the year. Rhythmic zooplankton DVM has been reported during winter in the Arctic at 80°N (Berge *et al.*, 2009) in association with changes in the phase and height of the moon (Last *et al.*, 2016), whereas daily rhythms of activity have been observed in the scallop *Chlamys islandica* (Müller, 1776), during three consecutive winters at 79°N, possibly entrained by low-intensity light cues (Tran *et al.*, 2016). Field observations conducted during the Polar night at 79°N revealed the presence of low-intensity rhythmic light cues (Cohen *et al.*, 2015) that might have the potential to entrain circadian rhythms during mid-winter (Batnes *et al.*, 2015). Because corresponding information on clock gene activity is missing, it is still unclear whether these wintertime rhythmic activities would be promoted by alternative light cues in a direct way (masking effect), or if an involvement of the clock should be considered instead (Tran *et al.*, 2016). Our results suggest that severely reduced photoperiodic cues in krill might be insufficient for the entrainment of the circadian clock during mid-winter. It will be of particular interest investigating the influence of other light-related cues (e.g., light intensity and spectral composition).

We developed a conceptual model (Fig. 5) that links the seasonal modulation of krill circadian functions with the seasonal changes in photoperiod, sea-ice cover, phytoplankton abundance, and predator pressure in the environment. Predation pressure from visual predators is mainly concentrated during the day during autumn and spring, when a regular day/night cycle is present. At the same seasons, phytoplankton blooms are present in the surface layers, which

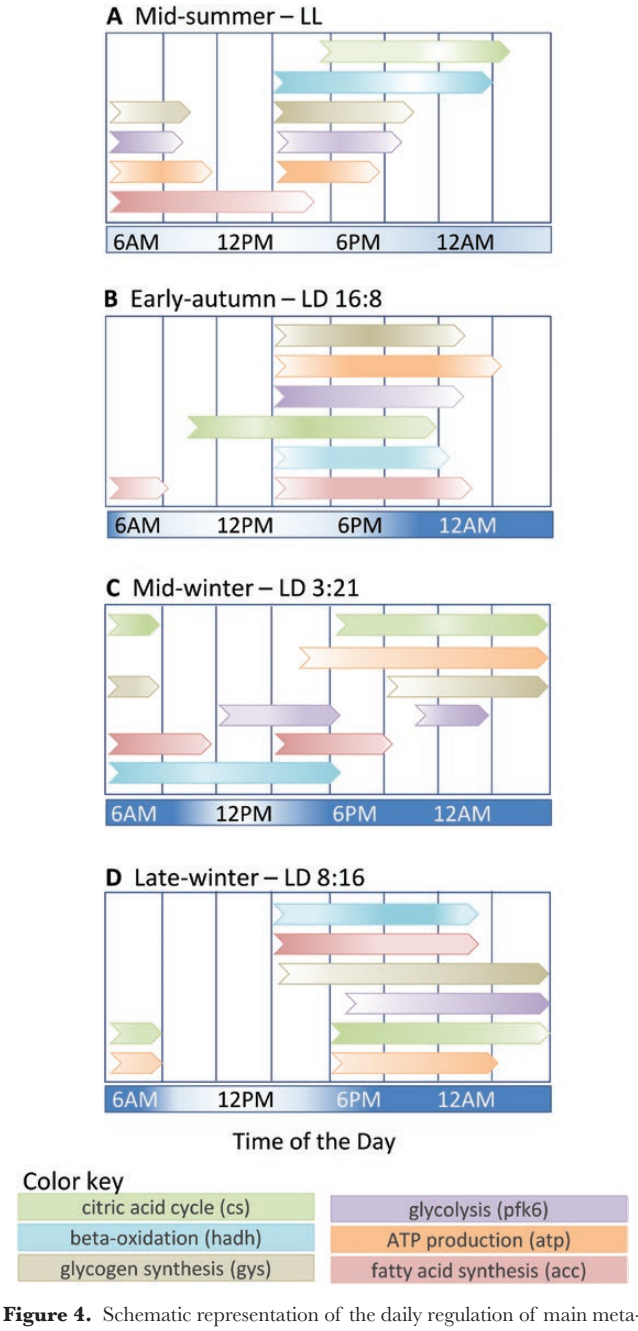

Figure 4. Schematic representation of the daily regulation of main metabolic pathways in mid-summer (**A**), early-autumn (**B**), mid-winter (**C**), and late-winter (**D**) based on gene expression data of key-metabolic genes. The different metabolic processes are indicated by gradient-colored arrows showing the time of the day corresponding to upregulation of key metabolic genes. The lengths of the arrows and darkness of the colors indicate intervals and intensity of upregulation. Time of the day is indicated at the bottom of each figure, where an indicative representation of light intensity is also shown. Each pathway is associated to one color in the color key, with the corresponding gene indicated in parenthesis.

are not covered by sea-ice at all times. Robust rhythmic regulation of krill metabolic activity promoted by the clock might be of great advantage, allowing krill to anticipate the light/dark cycle by implementing synchronized adaptive behaviors (i.e., DVM) at the population level. In mid-summer, when the sun never goes below the horizon, visual predators may occur at all times. At the same time, the surface layers are rich in food and free from sea-ice cover, and krill are in their seasonal phase of high metabolic activity with high energy demand to fuel reproduction. We therefore hypothesize that the costs related to predation when krill are in the surface layers are compensated by the benefits related to increased energy uptake and reproductive output. Less robust rhythmic regulation of the daily schedules might be more advantageous, allowing krill to switch to an "around the clock" activity mode and stay closer to the surface at all times to maximize food intake. The reduction of day length in mid-winter, together with the increase of sea-ice cover would significantly limit the amount of light reaching the surface layers, preventing primary production. By that time, krill have entered their seasonal phase of quiescence, characterized by reduced metabolic rates and feeding activity, and sexual regression. Even if visual predation might still be concentrated in the surface layers during a restricted time of the day, there would be no reason for krill to expose themselves in the food-depleted surface layers at any time. Less robust rhythmic regulation might therefore be advantageous, allowing krill to remain quiescent in the deeper layers.

Photoperiodic cues may thus play a major role for the entrainment of krill circadian functions during spring and autumn, when a regular day/night cycle is present. This might promote synchronous DVM and upregulation of metabolic activity during the night, allowing krill to evade visual predation by resting in the deeper layers during the day. During mid-summer and mid-winter, when the day/night cycle is strongly biased towards full light or full dark, photoperiodic cues might not be sufficient to entrain krill circadian functions. Basic clock functions necessary for homeostatic regulation might nevertheless still be promoted by alternative *Zeitgebers* related to the daily cycle of light intensity in mid-summer and to low-intensity light cues in mid-winter. The absence of overt photoperiodic cues might at the same time promote the uncoupling of krill DVM and the emergence of "around the clock" activity patterns, which could be more suitable in a less rhythmic environment.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Crustacean Biology* online.

S1 Table. Sequences of primers and probes used to amplify target genes.

S2 Figure. Changes in raw CT (Cycle Threshold) values of candidate reference genes *usp*46 (*ubiquitin carboxyl-terminal hydrolase* 46) and *rps*13 (*ribosomal protein* S13) along the 24 h cycle in the different photoperiodic conditions.

ACKNOWLEDGEMENTS

This work was funded by the Helmholtz Virtual Institute PolarTime (VH-VI-500: Biological timing in a changing marine environment – clocks and rhythms in polar pelagic organisms) and by the Italian Programma Nazionale di Ricerche in Antartide (grant 2013/C1.05). This work contributes to the PACES (Polar Regions and Coasts in a Changing Earth System) program (Topic 1, WP 5) of the Alfred-Wegener-Institut Helmholtz-Zentrum für Polar- und Meeresforschung, as well as to the Australian Antarctic Science Program Project #4037 (Experimental krill biology: Response of krill to environmental change), and Project R2.2 (Biological responses to environmental change) of the Antarctic Climate and Ecosystems Research Cooperative Centre. We would like to thank the captain and crew of RV *Aurora Australis* for their support on sampling of krill during cruise V1 07/08. We thank Rob King and Tasha Waller at the Australian Antarctic Division for their support with krill maintenance during the experiment. We are grateful to the anonymous reviewers, who provided useful comments that helped us to improve the manuscript.

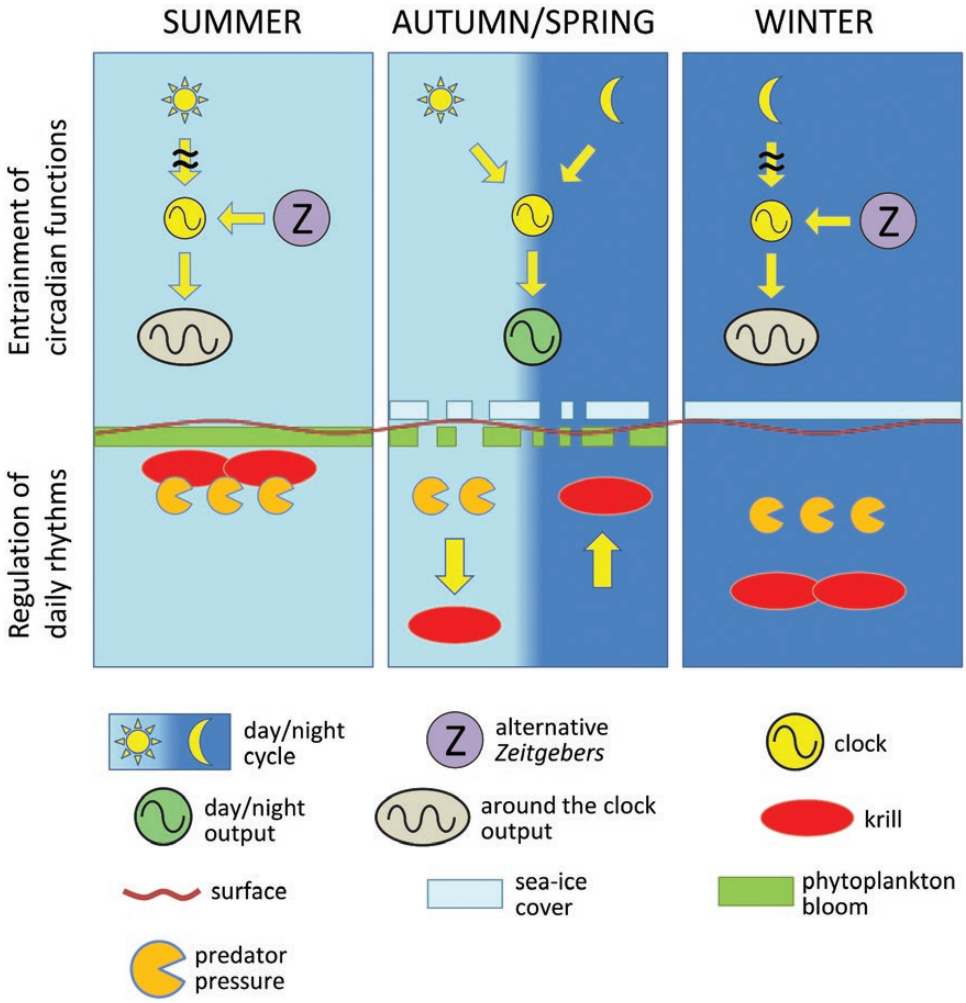

Figure 5. Conceptual model of photoperiodic-dependent modulation of circadian functions in Antarctic krill in different seasons. Photoperiod is the main *Zeitgeber* for the clock in autumn/spring and the daily output rhythms are synchronized to the LD cycle. Daily rhythms of predation are present and krill show DVM being active at the surface during the night. Photoperiod in summer/winter is insufficient to entrain the clock and alternative *Zeitgebers* come into play, promoting "around the clock" output rhythms. Daily rhythms of predation are less predictable and krill modify their DVM behavior (shallower in summer, deeper in winter).

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