



Calanus finmarchicus diel and seasonal rhythmicity in relation to endogenous timing under extreme polar photoperiods

N. Sören Häfker^{1,2,*}, Mathias Teschke¹, Lukas Hüppe², Bettina Meyer^{1,2,3,*}

¹Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, 27570 Bremerhaven, Germany

²Institute for Chemistry and Biology of the Marine Environment, University of Oldenburg, 26111 Oldenburg, Germany

³Helmholtz Institute for Functional Marine Biodiversity at the University of Oldenburg, 26129 Oldenburg, Germany

ABSTRACT: Changing environmental conditions cause poleward distribution shifts in many marine organisms including the northern Atlantic key zooplankton species *Calanus finmarchicus*. The copepod has diel cycles of vertical migration and feeding, a seasonal life cycle with diapause in winter and a functioning circadian clock. Endogenous clock mechanisms control various aspects of rhythmic life and are heavily influenced by environmental light conditions. Here we explore how the extreme seasonal change in photoperiod (day length) in a high Arctic fjord affects circadian clock functioning as well as diel and seasonal cycles in *C. finmarchicus*. Expression of clock genes was measured in the active life phase at the end of midnight sun, in early diapause when photoperiod was ~12 h, and in late diapause during the polar night. While the clock maintained diel rhythmicity under extremely long photoperiods, it became arrhythmic during diapause. This was probably not due to a lack of light but was related to the physiological state of diapause. Seasonal expression analyses of 35 genes show distinct patterns for each investigated life phase. *C. finmarchicus* is able to maintain diel clock rhythmicity at photoperiods close to 24 h, and it is discussed how this may be related to the nature of the marine environment. The work also evaluates the potential negative consequences of rigid clock-based seasonal timing in a polar environment exposed to climate change and with high interannual variability.

KEY WORDS: *Calanus finmarchicus* · Circadian clock · Midnight sun · Polar night · Photoperiod · Gene expression · Diel cycle · Seasonal cycle

INTRODUCTION

The copepod *Calanus finmarchicus* is a key zooplankton species in the northern Atlantic food web that provides a crucial trophic link between primary production and commercially important fish stocks (Runge 1988, Prokopchuk & Sentyabov 2006). The species inhabits a large latitudinal range from ~40° up to 80° N and often dominates zooplankton biomass (Kwasniewski et al. 2003, Helaouët & Beaugrand 2007). *C. finmarchicus* performs diel vertical migration (DVM), meaning that the copepods migrate to surface waters at night to feed on phytoplankton and retreat to

deeper water layers during the day to hide from visual predators (Dale & Kaartvedt 2000). *C. finmarchicus* further shows diel cycles of feeding as well as respiration and enzyme activity (Båmstedt 1988). The copepod's seasonal life cycle is characterized by distinct physiological phases starting with spawning and active development in surface waters in spring and summer, when phytoplankton is abundant (Hirche 1996a, Baumgartner & Tarrant 2017). During this time, the animals develop through 5 larval stages as well as 5 juvenile copepodid stages (CI–CV) and accumulate a considerable amount of energy-rich wax esters in a lipid sac (Falk-Petersen et al. 2009, Clark et al. 2013).

*Corresponding authors: soeren.haefker@awi.de, bettina.meyer@awi.de

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The lipid-rich CV stages migrate into deeper water layers where they enter diapause, a phase of overall reduced activity and fasting that lasts until spring (Hirche 1996a, Ingvarsdóttir et al. 1999). While *C. finmarchicus* CV copepodids at lower latitudes may molt directly into adults and produce up to 3 generations per year before entering diapause (Melle et al. 2014), at higher latitudes the species spawns only 1 generation per year that then enters diapause in the CV stage (Kwasniewski et al. 2003, Melle et al. 2014). The emergence from diapause starts in winter at depth, indicated by increasing metabolic activity and the development of reproductive tissues (Hirche 1996b, Ingvarsdóttir et al. 1999). Ultimately, the copepods molt to adults and ascend to surface waters to spawn a new generation that can then benefit from the spring phytoplankton bloom (Hirche 1996a, Baumgartner & Tarrant 2017). Although both the seasonal cycle of *C. finmarchicus* and its diel cycle of activity and migration have been described in great detail, the mechanisms controlling diel and seasonal rhythmicity are still poorly understood (Baumgartner & Tarrant 2017, Häfker et al. 2017).

A potent molecular tool for the regulation of diel and seasonal cycles is the circadian clock, which generates an endogenous rhythm of ~24 h length (Latin: *circa dies*, meaning about a day), thus enabling organisms to anticipate the rhythmic changes of the day–night cycle. The molecular machinery of circadian clocks is based on so-called clock genes and their protein products that interact with each other in feedback loops, with the genes ultimately inhibiting their own transcription (Mackey 2007). The expression patterns of most clock genes show diel rhythms, with peak levels often being associated with sunset or sunrise (Meireles-Filho & Kyriacou 2013). Several clock-associated genes regulate and delay the accumulation of the clock proteins as well as their import into the nucleus, resulting in a cycle period of ~24 h (Mackey 2007). Although this cycle persists under constant darkness, the clock is not perfectly precise and needs to be entrained (synchronized) by external cues on a regular basis to stay in tune with the environment. Light is by far the most common cue used for this entrainment (Aschoff 1954). Circadian clocks affect organisms on various organizational levels, reaching from diel rhythms of gene expression and enzyme activity to the control of the sleep–wake cycle and the regulation of DVM in fish and zooplankton including *C. finmarchicus* (Dunlap & Loros 2016, Häfker et al. 2017).

Another crucial feature of the circadian clock is the ability to measure photoperiod (day length) and thus to provide information about the seasonal cycle (Goto

2013). Various arthropods, including several copepod species, use changes in photoperiod as a cue for the timing of seasonal life cycle events like reproduction or diapause (Marcus & Scheef 2010, Goto 2013). For *C. finmarchicus*, the involvement of clock-based photoperiod measurement in seasonal timing and diapause has been proposed but is controversial (Miller et al. 1991, Baumgartner & Tarrant 2017).

Circadian clocks have been explored in great detail in terrestrial model species such as *Drosophila* and mice, but investigations of marine clock systems are still scarce (Tessmar-Raible et al. 2011). This is particularly true for high-latitude systems with the most poleward study on marine clocks conducted at 65° S (Teschke et al. 2011). However, the polar regions pose a special challenge to circadian clocks due to the extreme seasonal changes in photoperiod, reaching from permanent light in summer (midnight sun) to permanent darkness in winter (polar night). There are several studies reporting diel rhythms in marine organisms during midnight sun or the polar night (e.g. Dale & Kaartvedt 2000, Last et al. 2016), but the mechanistic basis of these rhythms remains completely unknown. Also, the productive period at high latitudes is short, meaning that precise timing of seasonal life cycle events like the emergence from diapause can be crucial for survival and population size (Søreide et al. 2010). In the face of changing environmental conditions, *C. finmarchicus* and many other marine species have experienced a poleward distribution shift (Reygondeau & Beaugrand 2011, Chivers et al. 2017). As a consequence, the circadian clock of the originally boreal *C. finmarchicus* is exposed to the more extreme polar light conditions that were shown to affect circadian clock functioning and rhythmicity in various species (Cottier et al. 2006, Beale et al. 2016). This could affect copepod fitness, population size and distribution (Søreide et al. 2010, Saikkonen et al. 2012). Here we investigate how the strong seasonal changes in photoperiod in an Arctic fjord affect the diel rhythmicity as well as the seasonal life cycle of *C. finmarchicus* with respect to gene expression, physiology and behavior and discuss how endogenous clock systems may increase or reduce fitness under the extreme polar light conditions.

MATERIALS AND METHODS

Study site characteristics

Kongsfjorden is an Arctic fjord situated in the western part of the Svalbard archipelago (Fig. 1). Because

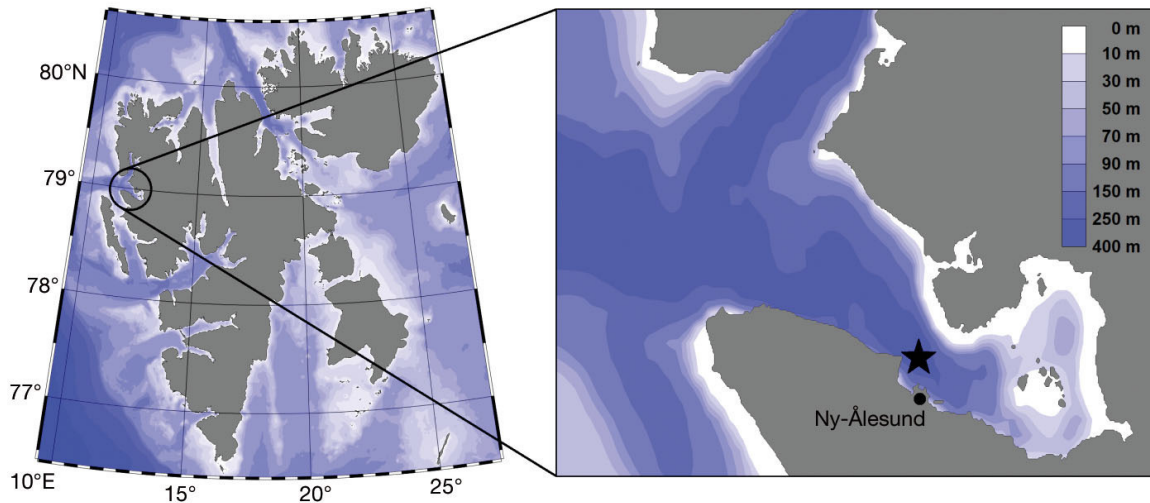


Fig. 1. Kongsfjorden and the Svalbard archipelago. Samples were collected at sampling site KB3 (star). Copepods in early and late diapause (September 2014 and January 2015, respectively) were collected and sorted onboard the R/V 'Helmer Hanssen', while active copepods (August 2016) were collected onboard the R/V 'Teisten' and sorted at the Kings Bay Marine Laboratory in Ny-Ålesund

of its depth of >300 m and the lack of a sill at the fjord entrance, it can be influenced by the influx of Arctic and Atlantic water masses (Cottier et al. 2005). Data on diel changes in surface photosynthetically active radiation (PAR, measured at 385–695 nm in Ny-Ålesund) were obtained from the database of the Baseline Surface Radiation Network. PAR attenuation with depth was calculated based on the mean surface PAR from 10:00 to 14:00 h for a 1 wk period ending with the day of the respective sampling and considering a 10% loss at the air–water interface (J. Cohen pers. comm.). The effect of phytoplankton on attenuation with depth was considered by calculating the attenuation coefficient (K_{PAR}), based on the mean phytoplankton concentration in the water column (data provided by Malin Daase, The Arctic University of Norway [UiT], Tromsø) according to the reports of Schanz (1985) and Lutz et al. (1996).

Table 1. Life phase samplings in Kongsfjorden. Samplings were performed in September 2014 (early diapause), January 2015 (late diapause) and August 2016 (active phase). Samples for gene expression were collected according to the diel time series. Samplings for dry weight, carbon to nitrogen (C:N) ratio and lipid analyses were performed at midday for each life phase

Life phase	Diel time series (no. of diel time points)	Depth
Active phase	Aug 25 00:00–Aug 25 08:00 + Aug 27 12:00–Aug 28 00:00 (3 + 4)	Surface–150 m
Early diapause	Sep 26 00:00–Sep 27 00:00 (7)	200 m–bottom ^a
Late diapause	Jan 13 04:00–Jan 14 00:00 (6)	200 m–bottom

^aNet closing mechanism failed for the 08:00 h sampling, so the entire water column was sampled

Decrease of PAR with depth was then calculated in 1 m intervals based on the Beer-Lambert equation $PAR_x = PAR_0 \times e^{-K_{PAR} \times x}$ with PAR_0 representing the mean surface PAR for the respective season –10% and x representing the depth in meters.

Copepod sampling

Calanus finmarchicus CV stages were collected at Kongsfjorden permanent sampling station KB3 (78° 57' N, 11° 57' E) onboard the R/V 'Helmer Hanssen' (UiT) in September 2014 and January 2015 and onboard the R/V 'Teisten' (Kings Bay AS, Ny-Ålesund) in August 2016 (Table 1). In September 2014 and August 2016, a WP3 net (Ø = 1 m, mesh size = 1000 µm) was used, whereas in January 2015, a WP2 net (Ø = 0.5 m, mesh size = 200 µm) was used. Different net types were not expected to have an effect, as both types should sample the relatively large *C. finmarchicus* CV stage efficiently (Kwasniewski et al. 2003). In September 2014 and January 2015 (early and late diapause, respectively), the net was opened ~10 m above the bottom, towed vertically and closed at 200 m depth. Bottom depth at the sampling site varied because of boat drift but was mostly between 340 and 355 m, with 2 exceptions (285 and 315 m). In August 2016 (active phase), the net

was lowered to 150 m, opened and then towed to the surface. For each life phase, a diel time series was created by collecting samples at 4 h intervals over a period of 20 to 24 h from midnight to midnight. Sampling intervals of 4 h have been used successfully to display diel clock gene cycling before, and as clock genes showed strongest expression changes at sunrise and sunset, starting and ending the sampling at midnight ensures a proper coverage of these times of strongest change (Häfker et al. 2017). While in January 2015 net failure resulted in the loss of the first diel time point, weather conditions in August 2016 forced an interruption of the sampling, resulting in a gap in the diel time series (Table 1).

After net retrieval, copepods for gene expression analysis were fixed as bulk samples immediately in RNeasy[®] Lysis Buffer (Qiagen) and later sorted for *C. finmarchicus* CV stages in RNeasy[®] Lysis Buffer at 4°C using a dissection microscope. Copepods for analyses of lipid content and carbon to nitrogen (C:N) ratio were collected once at midday for each life phase and were sorted alive using dissecting microscopes either on-board (September and January) or at the Kings Bay Marine Laboratory, Ny-Ålesund (August), and were fixed in liquid nitrogen. The exposure to light was minimized (red light used for sorting), and copepods were kept at *in situ* temperature before and during sorting. To avoid contamination of samples with the congener species *C. glacialis*, samples were sorted based on prosome length classes by Kwasniewski et al. (2003). Genetic analyses showed, however, that prosome length alone is not a sufficiently reliable identification feature, and thus individuals with reddish antennae were also excluded, as this is a strong indicator for *C. glacialis* (Gabrielsen et al. 2012, Nielsen et al. 2014). Sorting of copepods for lipid and C:N ratio analyses took a maximum of 1 h.

Gene expression analysis

A total of 35 genes were investigated based on their involvement in different metabolic processes including the circadian clock machinery, energy and lipid metabolism, digestion, stress response and light perception. A detailed gene list is given in Table S1 in the Supplement at www.int-res.com/articles/suppl/m603/p079_supp.pdf. Gene sequences were identified from a *C. finmarchicus* transcriptome (Christie et al. 2013, Lenz et al. 2014) using annotated sequences from other crustaceans. All obtained gene sequences were verified and checked for palindromic sequences and repeats as described by Häfker et al. (2017). Gene se-

quences were used to design custom TaqMan[®] low-density array cards (Applied Biosystems, <https://www.thermofisher.com/order/custom-genomic-products/tools/gene-expression/>), and designed primer and probe sequences were checked via BLASTN against NCBI for gene specificity. NCBI accession numbers of genes identified from the transcriptome as well as TaqMan primer and probe sequences can be accessed via PANGAEA (<https://doi.pangaea.de/10.1594/PANGAEA.884073>).

RNA of *C. finmarchicus* CV stages was extracted using the RNeasy[®] Mini Kit (Qiagen) with 0.14 M β -mercaptoethanol added to the extraction buffer. For each sample, 15 copepods were pooled. Genomic DNA residues were removed with the TURBO DNA-free Kit (Life Technologies). Extracted RNA was checked for degradation (2100 Bioanalyzer/RNA 6000 Nano Kit, Agilent Technologies) and investigated for contaminants and RNA concentration (NanoDrop 2000 Spectrophotometer, Thermo Fisher Scientific). Next, 2 μ g RNA per sample were converted to cDNA using RevertAid H Minus Reverse Transcriptase (Invitrogen). Finally, samples were distributed to TaqMan[®] cards, and gene expression was measured via real-time quantitative PCR (ViiA[™] 7, Applied Biosystems).

Gene expression was normalized according to Livak & Schmittgen (2001) using the $2^{-\Delta\Delta CT}$ method. For investigations of diel expression patterns, each of the diel time series from each of the 3 life phases (active phase, early diapause, late diapause) was normalized individually against the geometric mean of the housekeeping genes *elongation factor 1 α* , *RNA polymerase II* and *actin* (6–7 diel time points per life phase, n = 5 replicates per diel time point and gene). For life phase comparisons, each of the 3 diel time series was pooled, resulting in gene expression data for 3 life phases (active, early diapause, late diapause) that were normalized against the geometric mean of *elongation factor 1 α* and *RNA polymerase II* (3 life phases, n = 30–35 replicates per life phase and gene). Housekeeping genes were chosen based on expression stability and the results of previous studies (Hansen et al. 2008, Tarrant et al. 2008, Clark et al. 2013, Fu et al. 2013, Häfker et al. 2017).

Dry weight, C:N ratio and lipid content

For the determination of dry weight and C:N ratio, n = 24 individual copepods per life phase were freeze dried in pre-weighed tin caps. The dry weight was then measured gravimetrically. This was followed by

measurement of the C:N ratio via elemental analyzer (EuroEA, EuroVector).

Lipid samples were measured according to Folch et al. (1957). For each sample, 20 CV stages were pooled with $n = 5$ replicates per life phase. Frozen copepods were homogenized in 4 ml chloroform:methanol (2:1 v/v) (T10 ULTRA-TURRAX® disperser, IKA). The homogenate was then filtered through a pre-washed Whatman® No. 1 filter (GE Healthcare). Next, 1 ml potassium chloride (0.88% w/v) was added, and the homogenate was thoroughly vortexed and centrifuged at $400 \times g$ for 2 min to induce phase separation. The upper aqueous phase was discarded, and the remaining lower phase was dried under a stream of nitrogen gas. Finally, samples were freeze dried for 6 h, and total lipid content ($\mu\text{g ind.}^{-1}$) was determined gravimetrically (XP6U Micro Comparator, Mettler-Toledo).

Statistics

Statistical analyses were done in RStudio (v. 0.99.442, R Development Core Team 2013). Diel time series of clock gene expression were checked for 24 h cycling using the R package RAIN (Thaben & Westermarck 2014). A significance level of $\alpha = 0.001$ was used to account for the number of investigated genes (8 clock genes tested). Life phase differences in gene expression, dry weight, lipid content and C:N ratio were identified via Kruskal-Wallis ANOVAs on ranks followed by Dunn's multiple comparison post hoc tests provided in the FSA R package. For gene expression analyses, $\alpha = 0.0001$ was used (35 genes

tested), whereas for dry weight, lipid content and C:N ratio, the significance level was $\alpha = 0.05$. Graphs were created via SigmaPlot (v. 12.5).

RESULTS

Light conditions in Kongsfjorden

During the active phase in August, photoperiod (from sunrise to sunset) was close to 24 h, but there was still a clear diel rhythm of surface PAR intensity with a noon maximum of $\sim 400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 2A). In September (early diapause), photoperiod was ~ 12 h and PAR intensity peaked at $\sim 190 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. During late diapause in January, there was no clear diel rhythm in PAR intensity and maximum values reached $\sim 1.5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. It is, however, possible that the reflection of artificial light by clouds led to an overestimation of irradiance in January. PAR attenuation with depth was similar in August and September, with the detection limit reported for *Calanus* ($5 \times 10^{-8} \mu\text{mol photons m}^{-2} \text{s}^{-1}$, Båtnes et al. 2015) reached at ~ 120 m depth (Fig. 2B). During the polar night in January, surface PAR was much weaker and the detection limit was reached at ~ 80 m depth or, if artificial light caused an overestimation, at even shallower depth.

Diel clock gene expression

The 24 h cycling of clock gene expression was mostly confined to the active life phase in surface

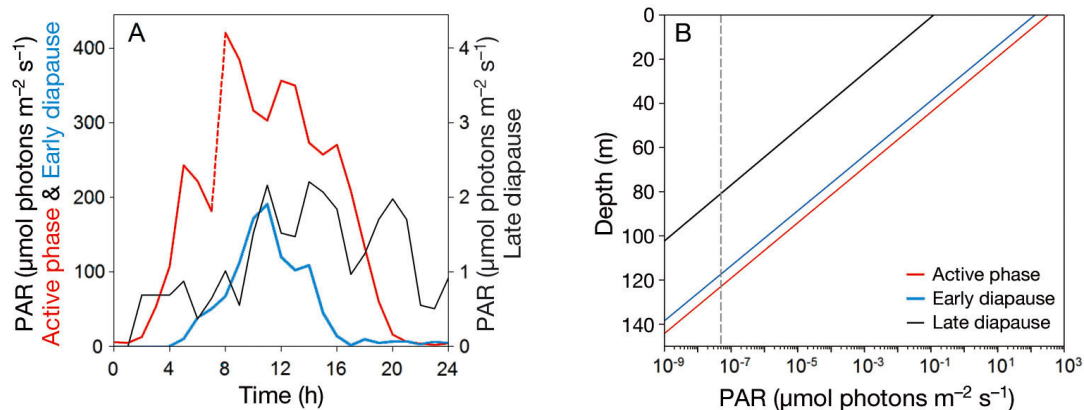


Fig. 2. Light conditions in Kongsfjorden. (A) Diel change in surface photosynthetically active radiation (PAR) during the samplings. The sampling during the active phase had to be interrupted due to bad weather conditions (see also Table 1); hence, light data from August 25 and 27 are shown (gap indicated by dashed line). (B) PAR attenuation with depth. Attenuation was calculated considering phytoplankton concentration (data provided by Malin Daase, The Arctic University of Norway [UIT], Tromsø). The dashed line indicates the light detection limit reported for *Calanus* ($5 \times 10^{-8} \mu\text{mol photons m}^{-2} \text{s}^{-1}$, Båtnes et al. 2015)

waters in August (Fig. 3). During the active phase when photoperiod was close to 24 h, the clock genes *period1* (*per1*), *timeless* (*tim*), *cryptochrome2* (*cry2*) and *clockwork orange* (*cwo*) showed significant 24 h cycling. During early diapause in September, the photoperiod was close to 12 h and diel expression cycling at depth below 200 m was confined to the genes *clock* (*clk*) and *period2* (*per2*). In late diapause during the polar night (January), 24 h rhythmicity was completely absent. The clock genes *cycle* (*cyc*) and *vri* (*vri*) did not show significant cycling in any of the life phases.

In the active phase, cycling clock genes showed maximum expression in the afternoon, with peak times reaching from close to noon (*cwo*) to around 16:00 h (*per1*, *tim*) to sunset (*cry2*) (Fig. 3). In early diapause, *clk* and *per2* showed minimum expression before sunrise, but while *clk* reached maximum expression around noon, expression of *per2* gradually increased and peaked at 20:00 h, i.e. after sunset.

Life phase differences in gene expression

Most of the investigated genes showed clear expression differences between life phases (Table S1 in the Supplement). The majority of genes peaked in expression during the active phase, including most of the clock genes (e.g. *clk*, *per2*, *cwo*) and several clock-associated genes (e.g. *cry1*, *double-time2* [*dbt2*]) (Fig. 4). Other genes elevated during the active phase were involved in food digestion and metabolization (*chymotrypsin* [*chtrp*], *phosphofructokinase*

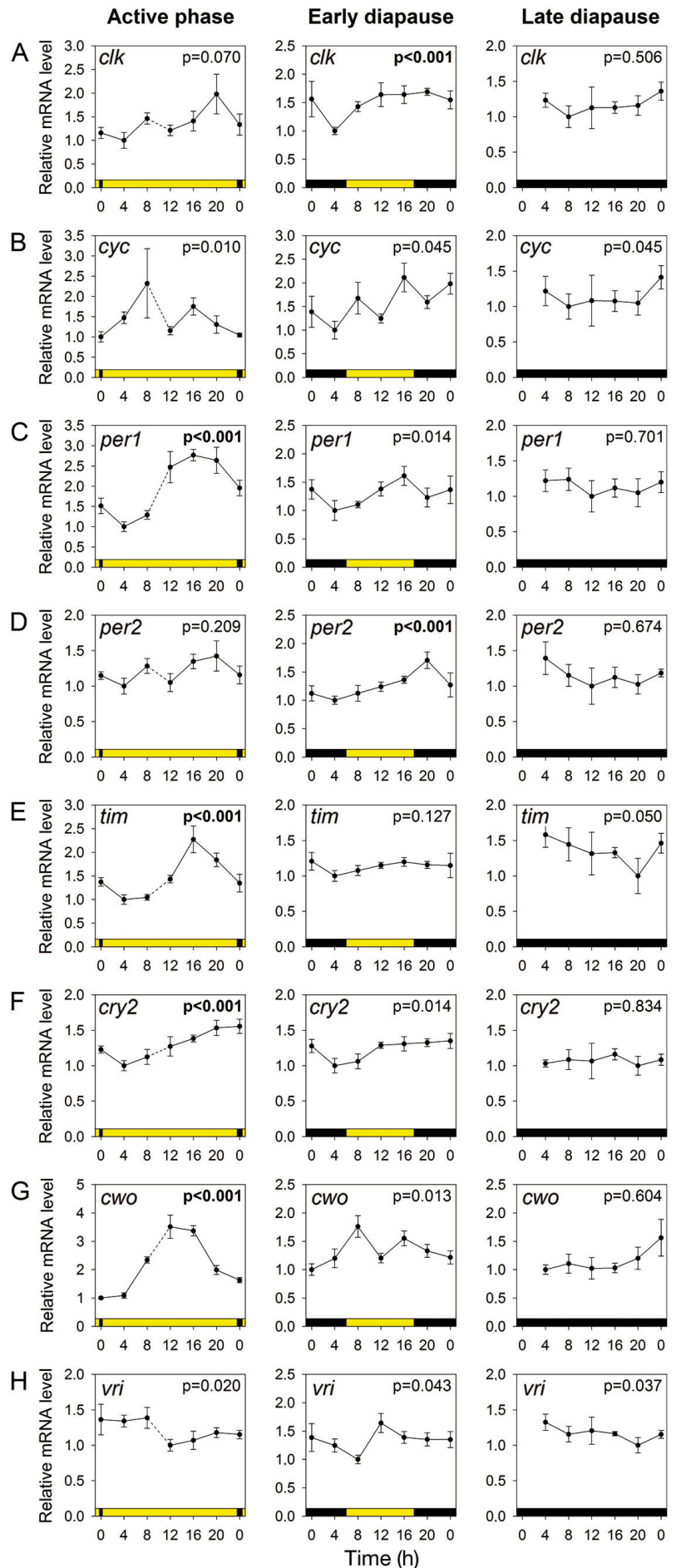


Fig. 3. Diel clock gene expression. Diel expression changes of the clock genes (A) *clock* (*clk*), (B) *cycle* (*cyc*), (C) *period1* (*per1*), (D) *period2* (*per2*), (E) *timeless* (*tim*), (F) *cryptochrome2* (*cry2*), (G) *clockwork orange* (*cwo*) and (H) *vri* (*vri*) are shown. Color bars indicate day (yellow) and night (black). The sampling during the active phase had to be interrupted because of bad weather conditions (see also Table 1); hence, data from August 25 and 27 are shown (gap indicated by dashed lines). Genes were investigated for 24 h rhythmicity via RAIN analysis, and p-values are shown. Significant 24 h cycling ($p < 0.001$) is indicated in **bold**. Mean values \pm SEM are shown

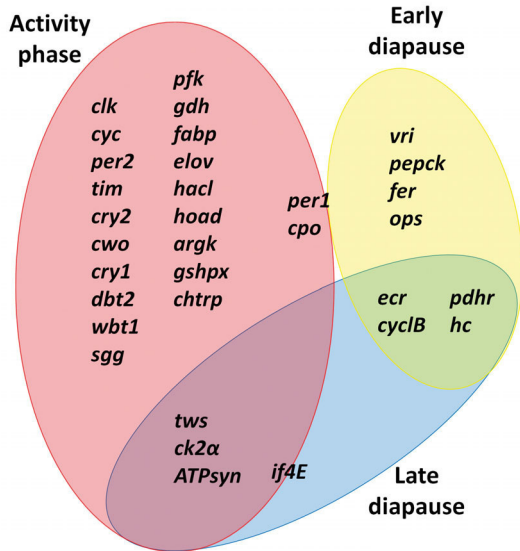


Fig. 4. Life phase-dependent changes in gene expression. Life phases of *Calanus finmarchicus* CV copepodid stages were compared via Kruskal-Wallis ANOVA on ranks and Dunn's post hoc test. Circles indicate the life phases during which the respective genes showed elevated expression. For a detailed characterization of individual gene patterns (including abbreviation names), see text and Table S1 in the Supplement

[*pfk*], *glutamate dehydrogenase* [*gdh*], *2-hydroxyacyl-CoA lyase* [*hacl*]) and lipid accumulation (*fatty acid-binding protein* [*fabp*], *elongation of very long fatty acids protein* [*elov*]).

In early diapause, peak expression was shown by the genes *vri* (clock gene), *phosphoenolpyruvate carboxykinase* (*pepck*, gluconeogenesis) and *ferritin* (*fer*, antioxidative defense), and by an *opsin* gene (*ops*, light perception) (Fig. 4). None of the investigated genes was exclusively highly expressed in late diapause. However, the clock-associated genes *twins* (*tws*) and *casein kinase II α* (*ck2α*) as well as the *eukaryotic translation initiation factor 4E* (*if4E*, translation) and the *ATP synthase γ subunit* (*ATPsyn*, mitochondrial energy production) showed high expression during both late diapause and the active phase. In contrast, the genes *ecdysteroid receptor* (*ecr*, molting), *cyclin B* (*cyclB*, cell cycle), *pigment-dispersing hormone receptor* (*pdhr*, light perception) and *hemocyanin* (*hc*, blood oxygen transport) were upregulated in early and late diapause (Fig. 4).

Dry weight, C:N ratio and lipid content

Dry weight, C:N ratio and total lipid content of CV stages differed significantly between life phases ($p <$

0.05, $df = 2$, for each parameter). Dry weight was highest during early diapause (mean \pm SEM: $504 \pm 6 \mu\text{g}$, $n = 24$) compared to the active phase ($183 \pm 3 \mu\text{g}$, $n = 22$) and late diapause ($155 \pm 5 \mu\text{g}$, $n = 22$) when dry weights were similarly low (Fig. 5A). The C:N ratio was lowest in the active phase (4.73 ± 0.05 , $n = 22$) and showed a maximum during early diapause (8.94 ± 0.05 , $n = 24$) before decreasing to intermediate levels in late diapause (6.86 ± 0.05 , $n = 22$) (Fig. 5B). Lowest lipid levels were measured in active

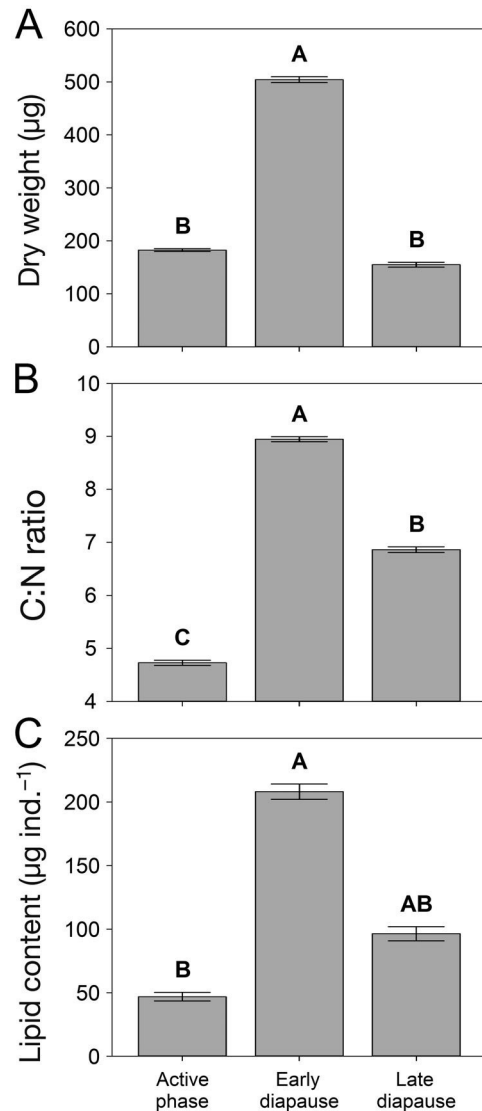


Fig. 5. Life phase-dependent changes in physiology. (A) Dry weight, (B) carbon to nitrogen (C:N) ratio and (C) lipid content of *Calanus finmarchicus* copepod V stages, analyzed via Kruskal-Wallis ANOVA on ranks and Dunn's post hoc test. p-values of Kruskal-Wallis ANOVAs are shown, and significant differences between life phases are indicated by different letters. Lipid content in late diapause was not significantly different from the other life phases. Mean values \pm SEM are shown

CV copepodids ($47 \pm 3 \mu\text{g ind.}^{-1}$, $n = 5$). Lipid content was highest during early diapause ($208 \pm 6 \mu\text{g ind.}^{-1}$, $n = 5$) and at intermediate levels in late diapause ($96 \pm 6 \mu\text{g ind.}^{-1}$, $n = 5$) (Fig. 5C).

DISCUSSION

Diel rhythmicity

Our study provides new insights into the diel and seasonal rhythmicity of the boreal zooplankton key species *Calanus finmarchicus* in a polar environment and under extreme light conditions. During the active life phase in August, when copepods were developing and feeding in surface waters, the diel cycling in clock gene expression was strongest. The clock genes *per1*, *tim* and *cwo* showed the most pronounced diel expression cycles with peak activity in the afternoon, closely resembling laboratory and field patterns of an active *C. finmarchicus* population in Loch Etive, UK (56°N , photoperiod = 16 h) (Häfker et al. 2017). The observation that peak times coincided despite strongly differing photoperiods and latitudes suggests that *C. finmarchicus*' circadian clock can maintain a robust rhythm under long polar photoperiods. Classical terrestrial chronobiology shows that while clock rhythmicity usually persists under constant darkness, the constant presence of light does often disrupt circadian rhythms (e.g. Kobelkova et al. 2015). Accordingly, there are several reports of a lack of diel rhythmicity including DVM during midnight sun (e.g. Cottier et al. 2006). However, surface PAR nevertheless showed a clear diel cycle of light intensity (Fig. 2A) that was also present during the midnight sun period (Cottier et al. 2006). Furthermore, light intensity attenuates with water depth, and active-phase copepods were collected down to 150 m depth. This means that the subjective photoperiod perceived by the copepods in Kongsfjorden was possibly shorter than the actual time between sunrise and sunset (Miller et al. 1991). This would explain why *per1*, *tim* and *cwo*, which typically peak close to sunset (Meireles-Filho & Kyriacou 2013, Häfker et al. 2017), showed highest expression in the afternoon (Fig. 3C,E,G). The shorter subjective photoperiod at depth could support clock rhythmicity during midnight sun, enabling *C. finmarchicus* as well as other polar marine species to maintain a 24 h cycle even under extremely long photoperiods. It can be further speculated that the presence of sea ice would shorten the subjective photoperiod in the marine environment even more.

Samples of active copepods were collected in August, at the end of the midnight sun period, and the question remains whether *C. finmarchicus* maintains clock rhythmicity over the entire summer period of permanent (although oscillating) light. The study was conducted at a time where DVM becomes rhythmic while it is arrhythmic beforehand, and the same could apply to the clock (Cottier et al. 2006). In this context, Cottier et al. (2006) argued that rhythmic vertical migration during midnight sun may persist on the individual level but is desynchronized within the population, resulting in arrhythmic overall migration patterns, and possibly driven by hunger and/or predator avoidance. However, net catch data of *C. finmarchicus* show rhythmic DVM during midnight sun (Dale & Kaartvedt 2000). For our analysis, we pooled 15 CV copepodids per replicate and still found clear diel clock gene cycling. While this showed that *C. finmarchicus* can maintain clock rhythmicity under extremely long photoperiods, the possibility that individuals were desynchronized on a behavioral level cannot be excluded. If decoupling of migration behavior from the rhythmic clock is present, this could be an adaptation to the permanent risk of visual predation during midnight sun (Cottier et al. 2006). Maintaining clock rhythmicity would, however, still be important for the temporal orchestration of different physiological processes that can interfere with each other (Panda et al. 2002). While further studies are needed for definite answers, it seems possible that *C. finmarchicus*' circadian clock can maintain rhythmicity throughout the midnight sun period.

Cycling clock gene expression during early diapause in September (photoperiod = ~12 h) was limited to the clock genes *clk* and *per2*, and in late diapause during the polar night, 24 h rhythmicity was completely absent (Fig. 3). Diapause samples were collected below 200 m, while based on our calculations, the copepod's light detection limit ($5 \times 10^{-8} \mu\text{mol photons m}^{-2} \text{s}^{-1}$, Båtnes et al. 2015) was reached at ~120 and ~80 m depth during early and late diapause, respectively (Fig. 2B). In spite of a lack of light at depth, cycling of clock genes was reduced but still present during early diapause (Fig. 3), indicating that the clock was running endogenously. Data on *C. finmarchicus* clock gene cycling in a Scottish sea loch at ~ 56°N show that the clock not only was arrhythmic in copepods diapausing at depth in autumn and winter but also stayed arrhythmic in diapausing animals advected to surface waters where they experienced a clear day–night cycle (Häfker et al. 2018). This strongly indicates that the reduction in clock gene cycling in early diapause in Kongsfjorden

as well as the complete lack of clock rhythmicity in late diapause was related to the physiological transition to diapause rather than the lack of a diel light cue for clock entrainment. It is therefore likely that the extreme light conditions during the polar night will have little effect on *C. finmarchicus*.

Seasonal cycle

C. finmarchicus showed strong differences in gene expression between life phases. The majority of genes were upregulated during the active life phase in August (Fig. 4). High expressions of *ATPsyn*, a key component of the mitochondrial respiration chain, as well as *if4E*, which is involved in protein synthesis, indicate high metabolic activity matching expression patterns from lower latitudes (Häfker et al. 2018) and the high respiration rates reported for this life phase (Ingvarsdóttir et al. 1999). Also, the upregulation of several genes involved in the digestion and metabolism of food (*pfk*, *gdh*, *hacl*, *chtrp*) and the accumulation of lipid storages (*fabp*, *elov*) during the active phase is in tune with our findings from lower latitudes in Loch Etive (Häfker et al. 2018), reflecting that this is the time when the copepods are feeding at the surface and are building up large lipid storages for the time of diapause (Falk-Petersen et al. 2009, Clark et al. 2013). This was also visible in the strong difference in dry weight, C:N ratio and lipid content of active copepods that were still feeding in surface waters compared to animals in early diapause that had just descended to deep waters (Fig. 5). The upregulation of *glutathione peroxidase (gshpx)* and *arginine kinase (argk)* suggest an increased protection from oxidative stress (*gshpx*) and an increased capacity for anaerobic energy production (*argk*). This is surprising, as in *C. finmarchicus*, protection from oxidative stress is typically associated with diapause (Tarrant et al. 2008, Häfker et al. 2018), and anaerobic metabolism would rather be expected in deeper water layers that tend to be more hypoxic. A possible explanation may be related to the short polar productive period of phytoplankton. A limited time window for growth and lipid accumulation means that the copepods have to feed and grow as much and as fast as possible, resulting in a high energy turnover that could support oxidative stress and increase the need for additional anaerobic energy sources. Additionally, the high light levels at the end of midnight sun increase predation risk (Fortier et al. 2001), and escape responses depend on short but intense bursts of energy most likely fueled

by anaerobic energy storages. Other genes that are typically associated with diapause but showed upregulation during the active phase were *couch potato (cpo)* and *3-hydroxyacyl-Coa dehydrogenase (hoad)*. While *cpo* is involved in the regulation of diapause in insects (Salminen et al. 2015), *hoad* is a key enzyme for the mobilization of lipid storages and was found to be upregulated during *Calanus* diapause (Freese et al. 2017, Häfker et al. 2018). Nevertheless, the decrease of dry weight, lipid content and C:N ratio from early to late diapause shows that a significant fraction of storage wax esters was metabolized during diapause (Fig. 5). The low expression of *hoad* in diapause thus may reflect that energy demands during this phase are reduced and that lipid storages are depleted rather slowly. The definitive reasons for the expression patterns of *cpo* and *hoad* remain unknown, and more detailed latitudinal comparisons of *C. finmarchicus* populations will be needed to evaluate whether the observed patterns are specifically related to the polar environment.

Most clock genes and clock-associated genes showed highest expression during the active phase, matching the time of strongest clock rhythmicity (Figs. 3 & 4), and it seems reasonable that clock genes show highest expression at the time when the clock is actually ticking. However, at lower latitudes, several clock genes of *C. finmarchicus* showed highest seasonal expression during diapause when the clock was arrhythmic (Häfker et al. 2018). To understand these latitudinal differences, a detailed understanding of the molecular interactions within the *C. finmarchicus* circadian clock machinery will be needed.

Genes upregulated in early diapause in September represent a variety of metabolic processes (Fig. 4). *fer* is part of the copepod's antioxidative defense machinery, and its high expression in diapause matches previous reports (Tarrant et al. 2008, Häfker et al. 2018). Upregulation of *pepck*, a key enzyme of gluconeogenesis, could reflect the mobilization of wax ester lipids from the lipid sac and their conversion to glucose for the consecutive distribution within the body (Falk-Petersen et al. 2009, Poelchau et al. 2013). *pdhr* and a gene encoding an opsin (*ops*) were also upregulated in early diapause, and both genes are involved in light perception. *pdhr* is a receptor for pigment-dispersing hormone, which reduces the eye's sensitivity to light (Strauss & Dirksen 2010). Light can cause arousal in *Calanus* (Miller et al. 1991), and the upregulation of *pdhr* may ease the transition to diapause. The nature of the opsin is unknown, meaning that its increased expression does not necessarily

reflect increased light sensitivity but could also indicate a change in responsiveness to specific wavelengths related to the change in light composition during the descent to diapause depth. Although the exact reasons remain elusive, the upregulation of *pepck*, *pdhr* and *ops* in diapause was also observed in Loch Etive, supporting the correctness of our data (Häfker et al. 2018).

Notably, the clock gene *vri*, which did not show any diel cycling during any life phase both in this study (Fig. 3) or in previous studies (Häfker et al. 2017, 2018), peaked in expression during early diapause. In *Drosophila*'s circadian clock, *vri* is part of a separate negative feedback loop (Mackey 2007), but its mechanistic role within the clock of *C. finmarchicus* is unknown. As peak *vri* expression in Loch Etive also occurred in early diapause (Häfker et al. 2018), it is possible to speculate on a potential role of the gene in the transition to diapause and/or the cessation of clock rhythmicity.

None of the investigated genes was exclusively upregulated in late diapause (Fig. 4). High expression levels of *ATPsyn* and *if4E* suggest an increase in metabolic activity indicative of the emergence from diapause. Resumption of activity in January and preparation for the ascent to surface waters was further supported by the upregulation of *cyclB*, which is involved in the cell cycle and tissue development, most likely representing the formation of reproductive tissues that starts in the late phase of diapause (Hirche 1996b, Jónasdóttir 1999). Also, the copepods were preparing for the final molt as indicated by the high expression of *ecr*, a molting hormone receptor. Hence, the emergence process which ends with the molt to the adult stage and the ascent to the surface is initiated well before the start of the phytoplankton bloom, which in Kongsfjorden happens around April/May (Leu et al. 2006). Furthermore, these patterns also mimic the data from the low-latitude Loch Etive (Häfker et al. 2018).

Ecological implications

C. finmarchicus shows a persistent northward shift in its latitudinal distribution range (Reygondeau & Beaugrand 2011). Similar shifts have also been observed in numerous other marine species, and these distribution shifts have been linked to increasing temperatures as well as changes in food availability (Feng et al. 2016, Chivers et al. 2017). However, for terrestrial species, there is indication that the extreme photoperiods experienced at higher latitudes

could limit poleward range expansions, and the same has been proposed for marine organisms (Kaartvedt 2008, Saikkonen et al. 2012). Studies on drosophilid flies from different latitudes show that high-latitude species can maintain circadian rhythmicity under extremely long photoperiods, whereas lower-latitude species become arrhythmic (Menegazzi et al. 2017). The most likely reason for this limitation is that the strong seasonal changes in photoperiod could exceed the range to which the circadian clocks of species from lower latitudes can adapt (Menegazzi et al. 2017). Latitudinal range limitation would force species to live under suboptimal environmental conditions, reducing overall fitness. Alternatively, if species shift to latitudes with light regimes beyond the adaptive range of their circadian clock, this could cause an impairment of diel cycles in physiology and behavior, also resulting in a loss of fitness. In consequence, species can show reduced growth or population size. This would be particularly severe in ecological key species like *C. finmarchicus* that are a food source for a variety of higher trophic levels. However, our data indicate that *C. finmarchicus*' circadian clock is robust under the extremely long photoperiods during the active phase at the end of midnight sun. As the copepods in diapause do not show any signs of diel rhythmicity, the lack of light during the polar night probably has little effect on *C. finmarchicus* fitness (Hirche 1996a, Häfker et al. 2018).

Circadian clocks are not only central in regulating diel rhythmicity but also important for the control and timing of seasonal life cycles because of their ability to measure photoperiod (Goto 2013). In polar habitats, seasonal timing can be crucial, as low light levels and sea ice cover can limit the time of phytoplankton primary production to a very short period (Niehoff et al. 2002, Leu et al. 2006, Søreide et al. 2010). The influences of copepod lipid content and food availability on the timing of diapause in *C. finmarchicus* have been addressed most frequently (e.g. Hind et al. 2000, Maps et al. 2014, Baumgartner & Tarrant 2017). In contrast, the potential effects of photoperiod on diapause have received relatively little attention (Miller et al. 1991). Nevertheless, there are several reports of photoperiod controlling copepod diapause timing (Marcus & Scheef 2010). The timing of diapause initiation in *C. finmarchicus* as well as the number of generations per year vary strongly between populations and latitudes (Kwasniewski et al. 2003, Melle et al. 2014). In *Drosophila*, clock gene alleles were identified that differ between populations and cause flies to initiate diapause under

different photoperiods (Sandrelli et al. 2007). A similar genetic diversity in clock gene alleles in *C. finmarchicus* could explain the differences in diapause timing between populations. The timing of emergence is of particular importance as the species relies heavily on the phytoplankton bloom to fuel reproduction and development of the new generation (Niehoff et al. 2002). Emergence starts well before phytoplankton is available and is usually well synchronized within a population (Marshall & Orr 1955, Hirche 1996a,b, Ingvarsdóttir et al. 1999, Baumgartner & Tarrant 2017). This led to the hypothesis that emergence timing is based on photoperiod measurement (Miller et al. 1991). Alternatively, emergence by hourglass timers based on decreasing lipid content or slow continuous development have been proposed (Hind et al. 2000, Saumweber & Durbin 2006), but these models struggle to explain synchronized emergence in habitats where *C. finmarchicus* produces several generations per year that all initiate diapause at different times. The lack of clock gene cycling in late diapause during the polar night as well as the low light levels make clock-based photoperiod measurement and emergence at this time highly unlikely. The synchronized emergence could, however, be based on a circannual clock that had been entrained by the circadian clock and photoperiod during the active phase in surface waters (tom Dieck 1991, Goldman et al. 2004). Circannual life cycle timing has been described in *C. finmarchicus*' high Arctic congener *C. hyperboreus* and other calanoid copepods (Conover 1965, Fulton 1973). Such long-range timing mechanisms can run with impressive precision and could explain the synchronized emergence of copepods at depth without any known seasonal cue (Goldman et al. 2004). In general, if diapause timing in *C. finmarchicus* involves endogenous clocks and possibly habitat-specific clock gene alleles, this could hinder adaptation to the seasonal environmental cycle of newly occupied polar habitats. Furthermore, it would increase the vulnerability to changing environmental conditions as exemplified in a study by Søreide et al. (2010). The authors investigated the influence of the timing of sea ice breakup and phytoplankton bloom on the population dynamics of *C. finmarchicus* and its polar congener *C. glacialis* in an Arctic fjord. They found that while a sea ice breakup 2 mo earlier than usual caused a similar shift in the phytoplankton bloom, the timing of copepod emergence and reproduction stayed the same, once again suggesting an endogenous timer independent of acute external stimuli. This caused a mismatch, as food levels were already decreasing as

the newly hatched copepods developed. As a consequence, the biomass of both *C. finmarchicus* and *C. glacialis* was much lower than in the previous year, also affecting higher trophic levels (Søreide et al. 2010). Thus, while endogenous clocks can help to maximize fitness under stable, predictable conditions, they may become detrimental in polar habitats that can show strong interannual variability in environmental parameters (Kahru et al. 2011).

Summary

Calanus finmarchicus in the high Arctic Kongsfjorden maintained diel clock gene cycling under extremely long photoperiods at the end of midnight sun, but clock rhythmicity was reduced in early diapause and absent in late diapause. Gene expression differences between life phases reflect clock rhythmicity as well as metabolic activity and feeding during the active phase and the emergence process in late diapause. The cessation of clock rhythmicity in diapause was most likely due to physiological changes rather than a lack of light. Hence, the circadian clock of *C. finmarchicus* seems to be able to cope with the extreme polar photoperiods. However, in the variable polar environment, seasonal life cycle timing based on rigid clock mechanisms and photoperiod measurement may cause mismatch situations that could lead to reduced fitness and stock size. Thus, while polar marine species such as *C. glacialis* and *C. hyperboreus* may be pushed further poleward by changing environmental conditions, boreal species like *C. finmarchicus* could struggle to take their place because of the inability to adjust their clock-based seasonal timing (Kaartvedt 2008, Saikkonen et al. 2012).

The presence of robust clock gene cycling at the end of midnight sun and the cessation of clock rhythmicity with the physiological switch to diapause suggest that *C. finmarchicus* is well equipped to cope with the extreme polar photoperiods in terms of diel rhythmicity. However, to make a similar statement for the copepod's seasonal rhythmicity, the mechanisms controlling diapause timing are still too poorly understood. A better understanding of marine clock systems in boreal key species such as *C. finmarchicus* and related high Arctic species such as *C. glacialis* and *C. hyperboreus* will be essential to determine how clocks affect seasonal timing, how they function under extreme photoperiods, and how they will respond to changing environmental conditions.

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***Calanus finmarchicus* diel and seasonal rhythmicity in relation to endogenous timing under extreme polar photoperiods**

N. Sören Häfker*, Mathias Teschke, Lukas Hüppe, Bettina Meyer

*Corresponding author: soeren.haefker@awi.de, bettina.meyer@awi.de

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Tab.S1: Gene expression in different life phases of *C. finmarchicus* (stage CV). For each gene, the abbreviation (Abbr.), the physiological function, as well as the mean expression \pm SD in the three life phases are shown. Genes were analyzed individually and life phases were compared via Kruskal-Wallis ANOVA on ranks and Dunn's post-hoc test. Significant differences are indicated by different numbers of asterisks (*). In cases where two life phases differ significantly while the third is equal to both of them (e.g. *per1*), the asterisk is shown in brackets.

Gene	Abbr.	Function	Active phase	Early diapause	Late diapause
<i>Clock</i>	<i>clk</i>	Clock gene	* 3.46 \pm 1.37	1.23 \pm 0.34	1.00 \pm 0.35
<i>Cycle</i>	<i>cyc</i>		* 3.22 \pm 1.96	1.00 \pm 0.40	1.31 \pm 0.57
<i>Period1</i>	<i>per1</i>		* 2.22 \pm 0.91	(*) 1.49 \pm 0.46	1.00 \pm 0.37
<i>Period2</i>	<i>per2</i>		** 5.54 \pm 1.44	* 2.51 \pm 0.68	1.00 \pm 0.37
<i>Timeless</i>	<i>tim</i>		* 2.69 \pm 0.90	1.49 \pm 0.29	1.00 \pm 0.40
<i>Cryptochrome2</i>	<i>cry2</i>		* 2.02 \pm 0.42	1.38 \pm 0.27	1.00 \pm 0.32
<i>Clockwork orange</i>	<i>cwo</i>		* 3.76 \pm 1.79	1.00 \pm 0.27	1.29 \pm 0.54
<i>Vrille</i>	<i>vri</i>		1.21 \pm 0.32	* 1.71 \pm 0.46	1.00 \pm 0.26
<i>Cryptochrome1</i>	<i>cry1</i>	Clock light input	* 2.58 \pm 1.18	1.00 \pm 0.36	1.22 \pm 0.53
<i>Doubletime2</i>	<i>dbt2</i>	Clock-associated	* 2.94 \pm 0.78	1.23 \pm 0.32	1.00 \pm 0.34
<i>Casein kinase II α</i>	<i>ck2a</i>		* 1.52 \pm 0.23	1.00 \pm 0.19	* 1.49 \pm 0.35
<i>PP2A subunit twins</i>	<i>tws</i>		* 1.69 \pm 0.27	1.00 \pm 0.25	* 2.21 \pm 0.84
<i>PP2A subunit widerborst</i>	<i>wbt1</i>		* 1.61 \pm 0.21	1.10 \pm 0.14	1.00 \pm 0.14
<i>Shaggy</i>	<i>sgg</i>		* 2.54 \pm 0.50	1.05 \pm 0.25	1.00 \pm 0.26

Tab.S1 (continued)

Gene	Abbr.	Function	Active phase	Early diapause	Late diapause
<i>Phosphofructokinase</i>	<i>pfk</i>	Glycolysis	* 6.71 ± 2.23	1.00 ± 0.25	1.21 ± 0.46
<i>Phosphoenolpyruvate carboxykinase</i>	<i>pepck</i>	Gluconeogenesis	1.28 ± 0.50	* 1.91 ± 0.54	1.00 ± 0.26
<i>Citrate synthase</i>	<i>cs</i>	Citric acid cycle	1.25 ± 0.24	1.15 ± 0.22	1.00 ± 0.35
<i>Glutamate dehydrogenase</i>	<i>gdh</i>	Nitrogen metabolism	** 3.53 ± 0.58	* 1.50 ± 0.18	1.00 ± 0.28
<i>Fatty-acid binding protein</i>	<i>fabp</i>	Lipid anabolism	** 11.37 ± 3.88	* 2.02 ± 0.49	1.00 ± 0.29
<i>Elongation of very long fatty acids protein</i>	<i>elov</i>		* 39.82 ± 12.73	1.25 ± 1.40	1.00 ± 0.40
<i>2-hydroxyacyl-CoA lyase</i>	<i>hacl</i>	Lipid catabolism	* 4.26 ± 1.58	1.28 ± 0.69	1.00 ± 0.45
<i>3-hydroxyacyl-Coa dehydrogenase</i>	<i>hoad</i>		* 2.58 ± 0.53	1.47 ± 0.34	1.00 ± 0.31
<i>ATP synthase γ subunit</i>	<i>ATPsyn</i>	Respiration chain	* 1.96 ± 0.34	1.00 ± 0.17	* 1.48 ± 0.21
<i>Arginine kinase</i>	<i>argk</i>	Anaerobic metabolism	** 4.43 ± 0.90	* 2.80 ± 0.58	1.00 ± 0.38
<i>Eukaryotic translation initiation factor 4E</i>	<i>if4E</i>	Translation	(*) 1.24 ± 0.33	1.00 ± 0.34	* 1.53 ± 0.51
<i>Glutathione peroxidase</i>	<i>gshpx</i>	Stress response	** 9.79 ± 3.20	* 2.22 ± 0.66	1.00 ± 0.38
<i>Ferritin</i>	<i>fer</i>		1.59 ± 0.44	* 4.17 ± 0.93	1.00 ± 0.31
<i>Ecdysteroid receptor</i>	<i>ecr</i>	Molting	1.00 ± 0.85	* 4.22 ± 1.57	* 10.89 ± 7.83
<i>Cyclin B</i>	<i>cyclB</i>	Cell cycle	1.00 ± 2.44	* 1.19 ± 0.41	* 7.43 ± 6.41
<i>Couch potato</i>	<i>cpo</i>	Diapause-associated	* 1.49 ± 0.28	(*) 1.34 ± 0.19	1.00 ± 0.33
<i>Chymotrypsin</i>	<i>chtrp</i>	Digestion	* 64.34 ± 17.51	1.47 ± 3.35	1.00 ± 0.32
<i>Opsin</i>	<i>ops</i>	Light perception	1.05 ± 0.21	* 1.44 ± 0.27	1.00 ± 0.46
<i>Pigment-dispersing hormone receptor</i>	<i>pdhr</i>		1.00 ± 0.25	* 2.36 ± 0.70	* 2.71 ± 1.22
<i>Hemocyanin subunit</i>	<i>hc</i>	Blood O ₂ transport	1.00 ± 0.68	* 2.31 ± 1.09	* 2.00 ± 0.99