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Circadian clock involvement in zooplankton diel vertical migration

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Summary

Biological clocks are a ubiquitous ancient and adaptive mechanism enabling organisms to anticipate environmental cycles and to regulate behavioral and physiological processes accordingly [1]. Whilst terrestrial circadian clocks are well understood, knowledge of clocks in marine organisms is still very limited [2–5]. This is particularly true for abundant species displaying large-scale rhythms like diel vertical migration (DVM) that contribute significantly to shaping their respective ecosystems [6]. Here, we describe exogenous cycles and endogenous rhythms associated with DVM of the ecologically important and highly abundant planktic copepod *Calanus finmarchicus*. In the laboratory, *C. finmarchicus* shows circadian rhythms of DVM, metabolism, and most core circadian clock genes (*clock*, *period1*, *period2*, *timeless*, *cryptochrome2*, *clockwork orange*). Most of these genes also cycle in animals assessed in the wild though expression is less rhythmic at depth (50-140 m) relative to shallow caught animals (0-50 m). Further, peak expressions of clock genes generally occurred at either sunset or sunrise, coinciding with peak migration times. Including one of the first field investigations of clock genes in a marine species [5,7] this study couples clock gene measurements with laboratory and field data on DVM. While the mechanistic connection remains elusive, our results imply a high degree of causality between clock gene expression and one of the planet's largest daily migration of biomass. We thus suggest that the circadian clocks increase zooplankton fitness by optimizing the temporal trade-off between feeding and predator avoidance especially when environmental drivers are weak or absent [8].

Key words: *Calanus finmarchicus*, circadian clock, clock genes, diel vertical migration, respiration, zooplankton

Results & Discussion

Diel vertical migration (DVM) in one of the most abundant and ecologically important marine copepods, *Calanus finmarchicus*, is paralleled by endogenous circadian rhythmicity at behavioral, physiological and molecular levels. In the laboratory, copepods collected from an actively migrating field population showed endogenous rhythms of swimming, respiration and core circadian clock gene oscillations under constant darkness. In the field, most clock gene oscillations mimicked laboratory findings with some genes becoming less rhythmic in animals collected from depth. Peaks of gene expression follow sunset/sunrise, the periods of greatest vertical migrations over the solar day. Our data indicates that circadian timekeeping is an important component of DVM and particularly adaptive at maintaining migratory rhythmicity in habitats where the principle exogenous driver of DVM, light, is limiting.

DVM of marine zooplankton is one of the most profound coordinated movements of organisms on the planet. It contributes fundamentally to ecological interactions in both marine and freshwater habitats [9], and to global biogeochemical cycles [10]. DVM also structures predator prey-interactions, since increased predation risk from visually hunting predators drives zooplankton to depths during the day, whilst at night they return to the surface to feed [8]. Current mechanistic knowledge of DVM suggests that diel light changes are the main environmental cue of migration behavior [11]. However, paradoxically DVM still occurs in deepwater habitats or at high latitudes during the winter where light is limiting suggesting alternative control mechanisms [12–15].

In terrestrial organisms, endogenous temporal synchronization is achieved by a circadian clock cellular machinery involving an intricate network of gene/protein feedback loops that create a cycle of ~24 h length [16]. The clock is primarily entrained by light to ensure synchronization with the environment and is a potent tool of rhythm regulation controlling diel activity patterns [17]. However, studies addressing the role of molecular clock mechanisms in marine organisms are scarce [2–4,6], primarily due to the non-model nature of

most marine species and a lack of genetic resources. Furthermore, marine organisms are often difficult to maintain in the laboratory and sampling them in the field is often expensive and labor intensive. However, understanding marine clock mechanisms, especially in key ecological species, is crucial to predicting how the rhythmic life of these organisms may be affected by changes in environmental conditions [18].

Copepods occupy a central position in marine pelagic food webs, providing an important energy source for their predators [19]. *C. finmarchicus* accumulates large lipid reserves [20] and is the main link between phytoplankton and higher trophic levels in the North Atlantic thereby sustaining one of the world's most productive fisheries [21]. It is well recognized that *C. finmarchicus* undergoes DVM [22] and recently published transcriptomic resources [23,24] make it an ideal model to examine the molecular clock machinery.

Vertical migration in the field

To determine DVM of copepods in their natural environment an acoustic mooring was deployed in Loch Etive in the Bonawe deep (~145 m), UK (56°45´N, 5°18´W, Fig. S1). Acoustic Doppler Current Profilers (ADCPs) generated backscatter profiles as sound scattering layers representing the vertical distribution of zooplankton biomass. The ADCP generated data indicated clear DVM behavior of zooplankton with near 24 h periodicity during the field campaign (May 2015) (Fig. 1). The main scattering layer was located in the upper 40 m depth at night, whereas during daytime this was typically between 40 and 80 m depth. The timing of the upward and downward migrations coincided closely with the time of local sunset (20:12) and sunrise (4:24).

C. finmarchicus is the dominant zooplankton species in Loch Etive [25]. As such, the recorded DVM signals were assumed to primarily reflect the vertical migration of *C. finmarchicus*. This assumption was supported by net catches (data not shown) that established high abundance of these animals in the water column during ADCP recordings.

Phenotypic rhythmicity

DVM behavior and respiration were determined in *C. finmarchicus* collected from Loch Etive, to investigate if the cyclic migrations observed in the field also persist under entrained and constant laboratory conditions. The animals were exposed to a simulated light-dark (LD) photoperiod (LD 16 h: 8 h) mimicking field conditions, followed by constant darkness (DD). The copepods exhibited 24 h cycling in DVM under LD and near 24 h rhythms under DD conditions with clear downward movement in the subjective morning (Fig.2A, Tab.S1). These data clearly suggest an endogenous circadian regulation of DVM behavior. The rapid evening ascent and morning descent under LD, with light triggering a direct negative phototactic response, contrasted with the more gradual depth change and lower amplitude rhythm under DD, which dampened over time. Endogenous DVM rhythms have previously been described for zooplankton species and several of these studies also reported lower amplitude DVM rhythms under DD [26,27]. While some of these studies found more robust endogenous rhythms of zooplankton DVM than detailed here, direct comparisons are not appropriate as DVM differ between species and life stages [22]. Nevertheless, the persistence of DVM in copepods under constant darkness strongly suggests circadian clock involvement.

Swimming during vertical migration requires energy and is therefore accompanied by increased metabolic activity [28]. Respiration experiments revealed that oxygen consumption under LD increased in *C. finmarchicus* during the late afternoon/early night, a pattern repeated over the subsequent two days under DD (Fig.2B, Tab.S1). While the peak respiration in the second night between the two DD days was phase delayed by ~8 hours toward the late night, peak respiration was once again in phase by the last night of the experiment suggesting that the endogenous rhythm was still running “on time”. The delay initially observed under DD could be related to the transition from LD to constant darkness constituting “after-effects”

115 suggested to reflect an adaption of the endogenous rhythm to unnatural changes in light
116 regime [29].

117 The evening increase in respiration matches the time when the copepods undertake the
118 energy demanding migration towards the surface [28] whereas the decrease in respiration
119 towards sunrise may reflect passive copepod sinking or reduced energy costs for downward
120 migration facilitated through negative buoyancy [30]. Of relevance here is that respiration
121 increases before the time of upward migration, indicating an endogenously regulated
122 anticipatory process. Rudjakov [12] hypothesized that DVM may actually be a result of an
123 endogenous rhythm of metabolic activity that initiates upward migration around sunset
124 followed by passive sinking around sunrise. Overall, these data reveal that *C. finmarchicus*
125 possesses an endogenous rhythm of metabolic activity that matches to DVM swimming
126 behavior and is in line with previous finding [31].

127

128 Clock gene expression

129 To investigate the expression of clock genes under controlled conditions, copepods were
130 collected in Loch Etive and, as for DVM and respiration experiments, transferred to the
131 laboratory where they were exposed to LD and DD conditions. Only “core” clock genes that
132 interact via gene/protein feedback loops to create endogenous circadian rhythms were
133 investigated [16]. The results indicated strong 24 h rhythmicity in six of eight core clock
134 genes: *clock* (*clk*); *period1* (*per1*); *period2* (*per2*); *timeless* (*tim*); *cryptochrome2* (*cry2*) and;
135 *clockwork orange* (*cwo*). The two remaining core genes *cycle* (cyc) and *vrille* (*vri*) showed
136 weak rhythmicity (Fig.3A-H, Tab.S2). Times of peak gene expression were closely associated
137 with the time of sunset or sunrise and generally matched expression patterns of terrestrial
138 model species [32,33]. Rhythmic gene expression persisted under DD, confirming the
139 endogenous nature of the clock in *C. finmarchicus*.

The presence and rhythmic expression of a mammalian type *cry2* gene which peaks in the evening, indicates a clock mechanism similar to the ancestral clock model known from the monarch butterfly *Danaus plexippus*, where *cry2* acts as a transcriptional repressor [33]. Laboratory studies in this insect found rhythmic *cry2* expression to peak in the early day, as with the Antarctic krill *Euphausia superba*, the water flea *Daphnia pulex* and the marine annelid *Platynereis dumerilii* [3,6,33,34]. In contrast, *C. finmarchicus cry2* expression in the laboratory peaked at sunset (Fig.3F).

In addition to the core clock genes, expression was also measured in a suite of genes associated with the modification and localization of core clock proteins (*doubletime2*, *widerborst1*, *twins*, *casein kinase II α*, *shaggy*) or light entrainment (*cryptochrome1*) [35]. In accordance with previous findings, none of these clock-associated genes showed consistent circadian expression (Tab.S2) [35].

To investigate the functioning of a circadian clock in the field we conducted a 28 h sampling campaign at Bonawe deep. Clock gene expression of *C. finmarchicus* was measured in two depth layers (5-50 m, 50-140 m). Generally, the expression patterns of the clock genes resembled those recorded in the laboratory (Fig.3I-O). However, gene rhythms were less overt in the field and the number of rhythmic genes was reduced, especially in copepods from the deeper layer (Tab.S2). Temperature changes and food availability can entrain clock activity [36,37] and it is possible that the vertical migration through layers of different temperature and phytoplankton concentration (Fig.S2) may have affected clock gene expression and resulted in more labile rhythms when compared with laboratory experiments. Further the overall reduced rhythmicity at 50-140 m could reflect the physiological state of the copepods. At the time of the sampling animals in the deep layer may already have started transitioning to seasonal diapause, a phase of inactivity in deep waters characterized by metabolic downregulation and without any known diel activity cycle [25,38]. Data collected later in the year (not shown) suggests that cyclic clock gene expression ceases during

diapause. It is also noteworthy that the more labile gene rhythms at 50-140 m depth were mirrored by the weaker DVM signal acoustically recorded in this layer in Loch Etive (Fig.1) further suggesting a coupling between clock and DVM. Nevertheless, the existence of clock gene cycles in animals in the deeper layer shows that circadian clocks can operate under very low light intensities providing an explanation for the observations of diel migrations in meso-/bathypelagic habitats [13] and at high latitudes during winter months [14,15].

In summary, circadian clock gene expression in *C. finmarchicus* demonstrates pronounced rhythms well-suited for evoking the observed rhythms in DVM and respiration. Expression patterns mostly persist in the field, strongly suggesting that the copepod possesses an endogenous clock that is also functioning under natural conditions.

Ecological implications

The adaptive significance of a circadian clock underpinning DVM in *C. finmarchicus* and other vertically migrating organisms is clear. Primarily the clock would provide a mechanism for the copepods to anticipate the day/night cycle, thereby temporally adjusting behavioral functions, physiology, and gene expression accordingly. However, circadian clocks have also been implicated in the sensitivity to predator cues and avoidance behavior [39]. Copepods and many other planktic organisms are prey to visual predators during the day [40]. The circadian clock would provide a mechanism for anticipating sunrise to return to deep, dark waters before sufficient light enables visual predation. For example, the sea urchin *Centrostephanus coronatus* shows an endogenous cycle in nocturnal foraging which is closely tuned to the resting times of its predator, a diurnally active fish [41], increasing the urchins chance of survival and also maximizing the time it can spend foraging. Circadian clock involvement in vertical swimming may also explain “midnight sinking” behavior which is characterized by a descent to intermediate depth in the middle of the night followed by a second upward migration closely before sunrise [12,27]. This behavior has been suggested to be an avoidance

response to larger vertically migrating predators, which ascend later and descend earlier [42]. While predation risk can usually not be sensed until the predator is present, circadian clocks are highly suitable for controlling crepuscular activity patterns [12] and could thus explain the two upward migrations at sunset and sunrise characteristic for “midnight sinking”.

Circadian clocks would also be adaptive for maintaining DVM rhythms in photoperiodically extreme environments such as high latitudes during the polar night and the meso-/bathypelagic zone. In both these habitats light as an entrainment cue is only temporarily available and/or extremely weak and food levels are relatively constant over the course of the day [43,44]. Indeed, DVM occurrence in polar night habitats and the synchronized evening ascent of animals from the aphotic depths beyond 1000 m support the hypothesis that DVM is underpinned by a circadian clock [13–15]. Interestingly, a recent study found that vertical migration shifted from diel (24 h) to lunar-day (24.8 h) cycles under the influence of the full moon during the darkest part of the Arctic polar night [15]. This may indicate that during the polar night strong lunar light can either override endogenous rhythmicity or can act as an entrainment cue lengthening the period of a circadian clock underlying the vertical migration pattern.

Furthermore, *C. finmarchicus* digestive enzymes are probably produced before feeding to speed up digestion thereby increasing the overall amount of food that can be consumed and digested while being at the surface for a limited time [31]. A similar preparatory mechanism could be involved in the endogenous and light-entrained feeding rhythms in the copepod *Acartia tonsa* [45] as too the clock-controlled anticipatory enzyme production in the shrimp *Palaemon squilla* [46].

Circadian clocks have the capacity to regulate seasonal rhythmicity by measuring photoperiod [47]. This can be achieved by a light sensitive phase at the transition between day and night, which is associated with clock gene peak activity (external coincidence model, [48]). The presence or absence of light during this critical phase of the day/night cycle

provides information about the photoperiod and hence season. Alternatively, peaks in clock gene activity might shift over the season following either sunset or sunrise and the phase difference between these peaks would provide another measure of photoperiodic time measurement (internal coincidence model, [29]). The seasonal life cycle of many insects is affected by photoperiod [47] as too are various aspects of copepod biology, including diapause, reproduction, activity, and feeding [49]. As with many of its congeners, *C. finmarchicus* undergoes seasonal diapause fueled by its large lipid reserves [20] where lipid content, food availability and temperature are considered important regulators of this resting phase [50]. However, a clear understanding of the mechanisms initiating and terminating *Calanus* diapause is still missing leading to the tantalizing suggestion that this critical life-history transition maybe underpinned by a circadian clock as an integral part in the timing of *C. finmarchicus*' annual cycle.

Conclusions

Our results provide a detailed description of clock gene expression in an ecologically important marine species combined with measurements of DVM and metabolic activity. *C. finmarchicus* shows robust clock gene cycling in the wild and endogenous 24 h oscillations in the laboratory. The persistence of circadian rhythms in DVM and respiration under constant conditions suggests circadian clock involvement in the regulation of these processes. So far, the mechanistic link between clock rhythmicity and phenology remains elusive where functional analyses of the clock machinery and its output pathways is now required. DVM has previously been shown to occur in the high Arctic during the polar night, in the aphotic depths beyond 1000 m and spontaneously as midnight sinking, all of which contradict the assumption of DVM being driven by purely exogenous cues. Given the ecological benefits offered by endogenous timekeeping it seems likely that circadian clocks are extant in the regulation of vertical migration patterns. Furthermore, investigations of clock systems and

DVM in marine phytoplankton and cyanobacteria [5,51] have led to the suggestion that circadian DVM could exist even in these primordial organisms [52]. Our study provides a basis for better understanding the mechanisms of DVM and also for exploring the adaptive advantages of ancestral clock systems, which are hypothesized to have originated in the aquatic environment [53].

Author contributions

N.S.H.: principle investigator, study design, fieldwork, laboratory experiments, gene expression analysis, video analysis, rhythm analysis, interpretation, manuscript preparation and review. B.M.: study design, interpretation, and manuscript review. K.S.L: fieldwork, acoustic data analysis, interpretation, and manuscript review, D.W.P.: fieldwork and manuscript review. L.H.: fieldwork, laboratory experiments, and video analysis. M.T.: study design, interpretation, and manuscript review.

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Figure Legends

Figure 1. Backscatter (Sv) profile at Bonawe deep, Loch Etive in May 2015. DVM rhythms had periods (τ) of 23.9 h and 24.0 h at 25 m and 90 m, respectively (TSA Cosinor analysis, 4^{th}-11^{th} May). Color bars indicate local sunrise/sunset. 28 h field sampling is indicated by white box. The sharp backscatter change at ~38 m is a measuring artefact caused by the two acoustic profilers. Sampling site and water column characteristics are detailed in Figures S1 and S2, respectively.

Figure 2. DVM and respiration rhythms in the laboratory. (A): DVM. Depth of *C. finmarchicus* CV stages in 90 cm "DVM-columns" is shown. Data derived from video recordings. Mean values (n = 4) ± standard error of means (SEM) are shown. (B): Respiration. Mean values (n = 6) for each time point are shown. Due to the high sampling rate (5 min), error bars were removed for the sake of clarity. Color bars indicate (subjective) day and night. For both phenotypes, the first day with natural light/dark cycle (LD, photoperiod = 16 h) and the two following days in constant darkness (DD) were analyzed separately, as indicated by the dashed grey line. Asterisks (*) indicate significant 24 h rhythmicity. Sinusoidal curves (red) were fitted to illustrate the partially damped but still highly significant rhythms. For exact p-values see Table S1.

Figure 3. Diel expression patterns of core clock genes in the laboratory (A-H) and in the field (I-P). Expression patterns were recorded in *C. finmarchicus* CV stages and the investigated genes were: *clock* (*clk*), *cycle* (*cyc*), *period1* (*per1*), *period2* (*per2*), *timeless* (*tim*), *cryptochrome2* (*cry2*), *clockwork orange* (*cwo*), and *vrille* (*vri*). Color bars indicate (subjective) day and night. In the laboratory experiments (A-H), rhythm analysis was done separately for LD (photoperiod = 16 h) and DD intervals as described in Fig.2. Per time point

n = 10 replicates were pooled from two identical experimental runs. In the field (I-P), samples from 5-50 m (shallow) and 50-140 m (deep) were investigated (photoperiod = 16 h). n = 5 replicates per time point. Both, laboratory and field data was analyzed for rhythmic expression using the R-package “RAIN”. Asterisks (*****) indicate significant 24 h rhythmicity. Mean values ± standard error of means (SEM) are shown. For exact p-values see Table S2.

STAR★METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents, including the video material of the DVM experiment, the sequences of custom Taqman® probes/primers and the RAIN rhythm analysis script, should be directed to and will be fulfilled by the lead author, Sören Häfker (shaefker@awi.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All investigations were performed on CV life stages of the copepod *Calanus finmarchicus* (Gunnerus, 1770). Copepods were collected at the sampling site Bonawe deep in Loch Etive, Scotland (Fig.S1) and laboratory experiments were performed at the Scottish Association for Marine Science (SAMS) at *in situ* temperature (10°C). During the transfer to the laboratory (max. 1.5 h) the copepods were kept dark and at in situ temperature. For the laboratory experiments filtered and UV-treated seawater was used that was pumped in from below a beach next to the institute. The water was adjusted to a salinity of 27.5 by adding Milli-Q water to match the conditions at the sampling site in ~50 m depth.

Laboratory copepods were exposed to an *in situ* photoperiod of 16 h with a gradual change in light intensity and spectral compositions to simulate the natural conditions at Bonawe deep in a depth of ~50 m. From 4:00 (sunrise) on light intensity increased to ~5.5 Lux at noon measured right above the water surface. During this time color temperature shifted from initial 15460 K to 13780 K at noon. The decrease in the afternoon mirrored the morning increase resulting in complete darkness at 20:00 (sunset). To create these light conditions, a programmable LED-system was used (Mitras Lightbar oceanic blue / ProfiLux 3.1T control unit, both GHL Advanced Technology GmbH, Germany).

485

486 METHOD DETAILS

487

488 Study site characteristics

489 Loch Etive is a sea loch at the western coast of Scotland, UK (56°45´N, 5°18´W). It is
490 connected to the open ocean by a sill with a width of 200 m and ~7 m water depth and has
491 another sill with ~13 m depth further up the loch [54]. Beyond the second sill there is the
492 upper main basin with the deepest point of the loch (Bonawe deep, ~145 m) where all
493 samplings were done (Fig.S1). The sills limit the water exchange leading hypoxic conditions
494 in the deeper layers of the upper basin. Turnover events occur during the strongest spring
495 tides in spring/autumn, but are irregular and only happen every few years [54].

496 During the sampling of the 28 h field time series at Bonawe deep (6^{th}/7^{th} May 2015) the
497 water column parameters salinity, temperature, oxygen concentration and Chlorophyll a (Chl
498 a) fluorescence were recorded by a conductivity-temperature-depth (CTD) profiler (SBE
499 19plus V2 SeaCAT Profiler, Sea-Bird Electronics, USA). The water column was
500 characterized by an approx. 5 m thick surface layer with a low salinity ≤20 psu (Fig.S2). From
501 5 m on salinity gradually increased to 27 at ~50 m and showed only a minor increase below
502 this depth. Temperature from the surface to 26 m depth ranged between 8.3°C and 8.9°C.
503 Below 26 m temperature sharply rose to a maximum of 12.2°C at 50 m depth before gradually
504 decreasing to 10.4°C at 90 m and below (Fig.S2). The deeper layers of Bonawe deep were
505 hypoxic during the sampling. From the surface to 26 m depth oxygen concentrations was ≥8.5
506 mg O_2*L^{-1} before sharply decreasing to 3.6 mg O_2*L^{-1} at 40-43 m depth (Fig.S2). Oxygen
507 concentration then continued to gradually decreased to values ≤1.6 mg O_2*L^{-1} in ~80 m depth
508 and below. Chl a fluorescence was high in the upper 10 m (4-16 mg*m^{-3}), showed a second,
509 much smaller maximum at ~25 m and then quickly diminished with depth (Fig.S2). The

conditions were similar in spring 2016 when animals for laboratory experiments on DVM and respiration were collected (data not shown).

Vertical migration in the field

A mooring was deployed close to Bonawe deep (depth: ~135 m) in March 2015 (Fig. S1). The mooring was equipped with two acoustic Doppler current profilers (ADCPs) pointing upwards at 120 m and 45 m depth. The RDI 300 kHz ADCPs have been employed successfully in making biological observation of zooplankton migrations [14,15]. ADCP data were checked for quality using the RDI correlation index (a measure of signal to noise ratio) and absolute volume backscatter (Sv, measured in decibels, dB) was derived from echo intensity following the method described in Deines [55] with derived acoustic mean volume backscattering strength (MVBS). Acoustic data was analyzed via population mean TSA Cosinor analysis for backscatter rhythmicity in 25 m an 90 m depth (time series analysis [TSA] Cosinor 6.3 package). For the period 4^{th} to 11^{th} May 2015 significant backscatter rhythmicity could be detected in both, the shallow (45 m, τ = 23.9 h, % model fit = 49.6) and the deep layer (125 m, τ = 24.0 h, % model fit = 33.3). Tests on tidal (~12 h) and lunar (24.8 h) rhythms did not produce any significant rhythmicity.

Field time series

Samples were collected at Bonawe deep on the 6^{th}/7^{th} May 2015 starting at 11:00 and continuing in 4 h intervals until 15:00 of the next day, resulting in a total of eight time points over a period of 28 hours. At each time point a WP2-net (200 µm mesh size, Hydro-Bios GmbH, Germany) was towed vertically through the water column to collect animals from 5-50 m depth and 50-140 m depth, respectively. Generally, the upper 5 meters of the water column were excluded to avoid hypoosmotic stress for the copepods. Upon retrieval of the net, the sample was immediately (within 1 min) transferred into RNAlater® stabilizing

536 solution (Ambion, UK) for later gene expression analysis (see below). A possible sample
537 contamination by the congener species *C. helgolandicus* is unlikely due to its limited
538 tolerance to low salinities and the brackish conditions in the loch [25].
539
540 DVM experiment
541 To investigate the diel vertical migration (DVM) behavior, copepods were incubated in four
542 so-called DVM-columns made out of acrylic glass (10*8*90 cm lxwxh, 7.2 L). Animals were
543 collected on the 3rd June 2016, sorted, and per column 50 *C. finmarchicus* CV stages were
544 incubated for a total of three days (LD-DD-DD, photoperiod = 16 h). The columns were
545 vertically divided into six 15 cm increments and each layer was filmed with a surveillance
546 cameras equipped with filters excluding visible light (SK-B140XP/SO, Sunkwang
547 Electronics, South Korea). Infrared lights were used to illuminate the columns without
548 disturbing the animals. Copepod abundance per layer was then counted by three different
549 persons from the recorded video material at 1 h intervals. For every column, there was a
550 certain fraction of copepods which was inactive and never left the bottom layer of the column.
551 These animals were excluded from statistical analysis by determining the lowest number of
552 copepods in the bottom layer over the course of the experiment for each column, respectively.
553 This number was then defined as zero for the respective column.
554 Copepods were not fed during the DVM experiments to avoid particle accumulation at the
555 bottom, which could have affected vertical distribution. At the end of the experiment a
556 vertical oxygen profile was recorded using an oxygen tipping probe (PreSens GmbH,
557 Germany). There was a weak (<6%), gradual decrease in oxygen from 9.27 mg O_2*L^{-1} near
558 the surface to 8.75 mg O_2*L^{-1} close to the bottom.
559
560 Respiration experiment

Copepods collected on the 23rd June 2016 and sorted for C. finmarchicus CV stages were distributed to six glass bottles (305 mL) with filtered (0.2 µm) and UV-treated seawater which had been air-equilibrated for >1 h (10 animals per bottles). Two additional bottles without animals served as controls. Bottles were closed tightly without any air bubbles inside and incubated for three days (LD-DD-DD, photoperiod = 16 h). Oxygen content was measured using oxygen-sensitive sensor spots and monitoring equipment (OXY-4, PreSens GmbH, Germany). A moving average over 12 h was calculated to remove the trend of gradually decreasing oxygen within the bottles and to reveal underlying rhythmic oscillations. A simple inverse correlation between oxygen content and animal oxygen consumption was assumed. As the moving average is based on comparing O_2-levels between time points, the resulting relative change in oxygen consumption is dimensionless. Data was binned to 1 h intervals for rhythm analysis (see below).

Gene expression experiment

Copepods were collected on the 22nd May 2015 in 10-60 m depth. In the laboratory the animals were evenly distributed to 19 buckets filled with 20 L seawater. At midnight the sampling started by pouring the animals from the first, randomly chosen bucket through a sieve and fixing them in RNAlater®. Every 4 h another bucket was sampled accordingly resulting in a total of 19 time points over a period of three days (72 h). On the first experimental day (0-24 h) the animals were exposed to a natural light/dark regime (LD, see above) while they were kept in constant darkness (DD) on the second and third day (24-72 h). Copepods were fed with phytoplankton (Shellfish Diet 1800, Reed Mariculture Inc., USA) in 4 h intervals. A constant food concentration of ~200 µg $C*L^{-1}$ was maintained to avoid starvation effects while not introducing a new *Zeitgeber*. The experiment was repeated in the same way (LD-DD-DD) with copepods collected on the 29th May 2015 and the data of both runs was pooled.

Gene expression analysis

Gene sequences were taken from an Illumina transcriptome of *C. finmarchicus* [23]. Core clock and associated genes had been previously annotated by Christie et al. [56]. Housekeeping genes were newly annotated from the respective transcriptome. All gene annotations were verified via blastn against NCBI database (see Tab.S2 for accession numbers). They were then investigated for common protein domains via blastx and were checked for palindromic sequences and repeats via Oligoanalyzer 3.1 (http://eu.idtdna.com/calc/analyzer) and RepeatMasker 3.0 (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker). Binding regions for probes and primers were placed in sequence intersects that were specific for the respective genes (checked via blastn).

To measured gene expression, copepods were sorted in cooled RNAlater® (4°C) using dissecting microscopes. *C. finmarchicus* CV stages were pooled in groups of 15 copepods and RNA was extracted using the RNeasy® Mini kit (Quiagen, Netherlands). β-mercaptoethanol was added to the lysis buffer (0.14 M) as recommended for lipid-rich samples. DNA residues were removed with the TURBO DNA-free kit (Life Technologies, USA) and RNA was checked for concentration and purity (Nanodrop 2000 Spectrophotometer, Thermo Fisher Scientific, USA) as well as possible degradation (2100 Bioanalyzer / RNA 6000 Nano Kit, Agilent Technologies, USA). RNA was then converted to cDNA using RevertAid H Minus Reverse Transcriptase (Invitrogen GmbH, Germany). Gene expression was analyzed by real-time quantitative PCR (ViiA™ 7, Applied Biosystems, USA) using custom-designed Taqman® low-density array-cards (Applied Biosystems, USA). The list of investigated genes included eight core clock genes, five clock-associated genes, one gene involved in clock entrainment via light, and 3 housekeeping genes (see Tab.S2). Gene expression levels were normalized against the geometric mean of the housekeeping genes *elongation factor 1 α*, *RNA polymerase* and *actin* using the $2^{-\Delta\Delta CT}$-method developed by Livak & Schmittgen [57].

Housekeeping genes were chosen based on expression stability over the 24 h cycle, expression level relative to other investigated genes and the findings of previous studies [58]. For both experimental runs, five replicates were analyzed per time point. As there were no visible differences between the first and the second run, the datasets were pooled and treated as one resulting in n = 10 replicates per time point. For the 28 h field time series, n = 5 replicates were analyzed per time point and depth. Shallow and deep samples were normalized against housekeeping genes together to ensure comparability of expression levels between depths.

QUANTIFICATION AND STATISTICAL ANALYSIS

Datasets of DVM, respiration and gene expression were investigated for 24 h rhythmicity in RStudio (version 0.99.442, [59]) using the RAIN-package. RAIN was specifically designed to detect (circadian) rhythms in biological datasets independent of waveform by using a non-parametric approach [60]. For the 28 h field time series from May 2015, each depth (shallow/deep, n = 5, respectively) was analyzed separately as one dataset. In the laboratory experiments (n = 10), the first 24 h interval (LD) was analyzed separately from the following 48 h interval (DD). The time point at midnight between the two intervals (LD/DD) was used in both analyses. Due to the limited computing capacity of RAIN and the large amount of data from the DVM (n = 4) and respiration experiments (n = 6), the mean values were used to analyses rhythmicity for the 48 h DD interval of these experiments. Thus, to increase the confidence in the obtained results, each DD day in the DVM and respiration experiment was also analyzed individually using the respective replicates (see Tab.S1).

For the analyses of DVM and respiration data, an α of 0.05 was used (Tab.S1). For the gene expression analyses, a p-value <0.001 was considered significant to account for the testing of multiple genes (Tab.S2). Graphs were created with SigmaPlot (v. 12.5).

DATA AND SOFTWARE AVAILABILITY

The mRNA sequences of the investigated genes can be found via the accession numbers summarized in Tab.S2. For the video material of the DVM experiment, the sequences of custom Taqman® probes/primers and the RAIN rhythm analysis script, please contact the lead author (shaefker@awi.de). Data of the DVM experiment (abundance counts), the respiration experiment (moving averages) as well as the gene expression data of the laboratory experiment and the field time series (raw CT-values) are accessible via PANGAEA (https://doi.org/10.1594/PANGAEA.875739).