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Current Biology

Circadian Clock Involvement in Zooplankton Diel Vertical Migration

Highlights

- The copepod *Calanus finmarchicus* shows circadian vertical migration and metabolism
- *C. finmarchicus* shows strong circadian rhythmicity in clock gene expression
- Clock gene rhythmicity was also found in a vertically migrating field population
- The results strongly suggest that a circadian clock controls diel vertical migrations

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In Brief

Häfker et al. describe endogenous rhythms in diel vertical migration in the ecologically important copepod *Calanus finmarchicus*. These behavioral rhythms correspond with rhythms in metabolic activity and clock gene expression. Together, the results strongly suggest that diel vertical migration is affected by an endogenous circadian clock.

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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]. Although 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*, and *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 migrations of biomass. We thus suggest that 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].

RESULTS AND 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 indicate 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 limited.

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, while 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 and at high latitudes during the winter where light is limited, 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-hr length [16]. The clock is primarily entrained by light to ensure synchronization with the environment, and it 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

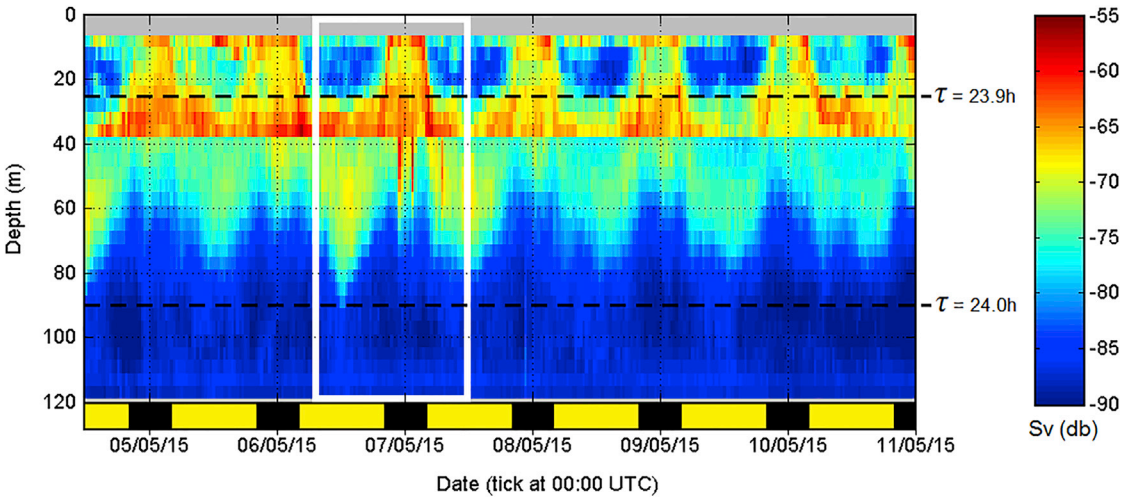

Figure 1. Backscatter Profile at Bonawe Deep, Loch Etive, in May 2015

DVM rhythms had periods (τ) of 23.9 and 24.0 hr at 25 and 90 m, respectively (TSA Cosinor analysis, May 4–11). Color bars indicate local sunrise/sunset; 28-hr field sampling is indicated by white box. The sharp backscatter change at ~38 m is a measuring artifact caused by the two acoustic profilers. Sampling site and water column characteristics are detailed in Figures S1 and S2, respectively.

[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; Figure 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-hr periodicity during the field campaign (May 2015) (Figure 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 (8:12 p.m.) and sunrise (4:24 a.m.).

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 a 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 16:8 hr) mimicking field conditions, followed by constant darkness (DD). The copepods exhibited 24-hr cycling in DVM under LD and near 24-hr rhythms under DD conditions, with clear downward movement in the subjective morning (Figure 2A; Table 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 DVMs 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 2 days under DD (Figure 2B; Table S1). While the peak respiration in the second night between the 2 DD days was phase delayed by ~8 hr 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 aftereffects suggested to reflect an adaption of the endogenous rhythm to unnatural changes in light regime [29].

The evening increase in respiration matches the time when the copepods undertake the energy demanding migration toward the surface [28], whereas the decrease in respiration toward sunrise may reflect passive copepod sinking or reduced energy costs for downward migration facilitated through negative buoyancy [30]. Of relevance here is that respiration increases before

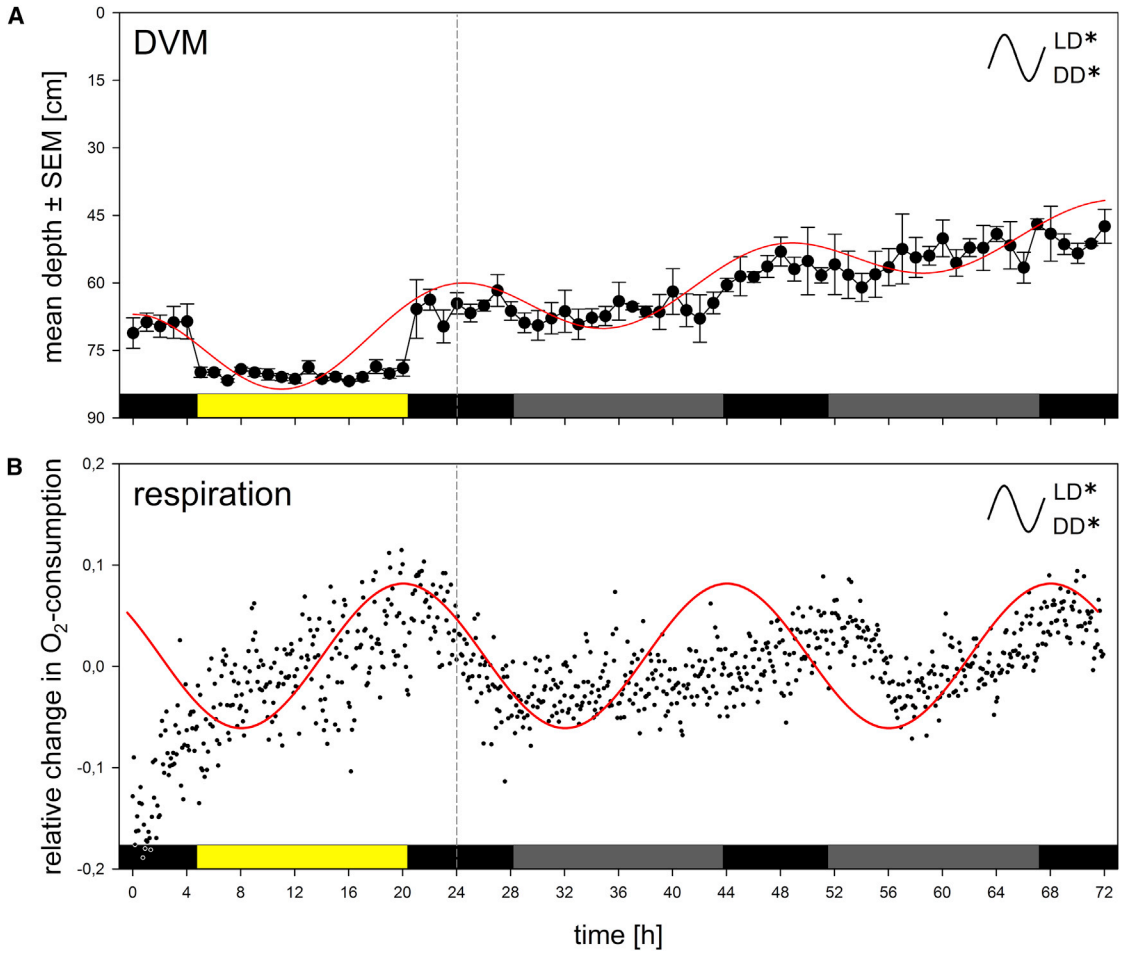

Figure 2. DVM and Respiration Rhythms in the Laboratory

(A) DVM. Depth of *C. finmarchicus* copepodid 5 (CV) stages in 90-cm DVM columns is shown. Data are derived from video recordings. Mean values (n = 4) ± 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 hr) and the two following days in constant darkness (DD) were analyzed separately, as indicated by the dashed gray line. Asterisks (*) indicate significant 24-hr rhythmicity. Sinusoidal curves (red) were fitted to illustrate the partially damped but still highly significant rhythms. For exact p values, see Table S1.

the time of upward migration, indicating an endogenously regulated anticipatory process. Rudjakov [12] hypothesized that DVM may actually be a result of an endogenous rhythm of metabolic activity that initiates upward migration around sunset, followed by passive sinking around sunrise. Overall, these data reveal that *C. finmarchicus* possesses an endogenous rhythm of metabolic activity that matches DVM swimming behavior and is in line with previous findings [31].

Clock Gene Expression

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

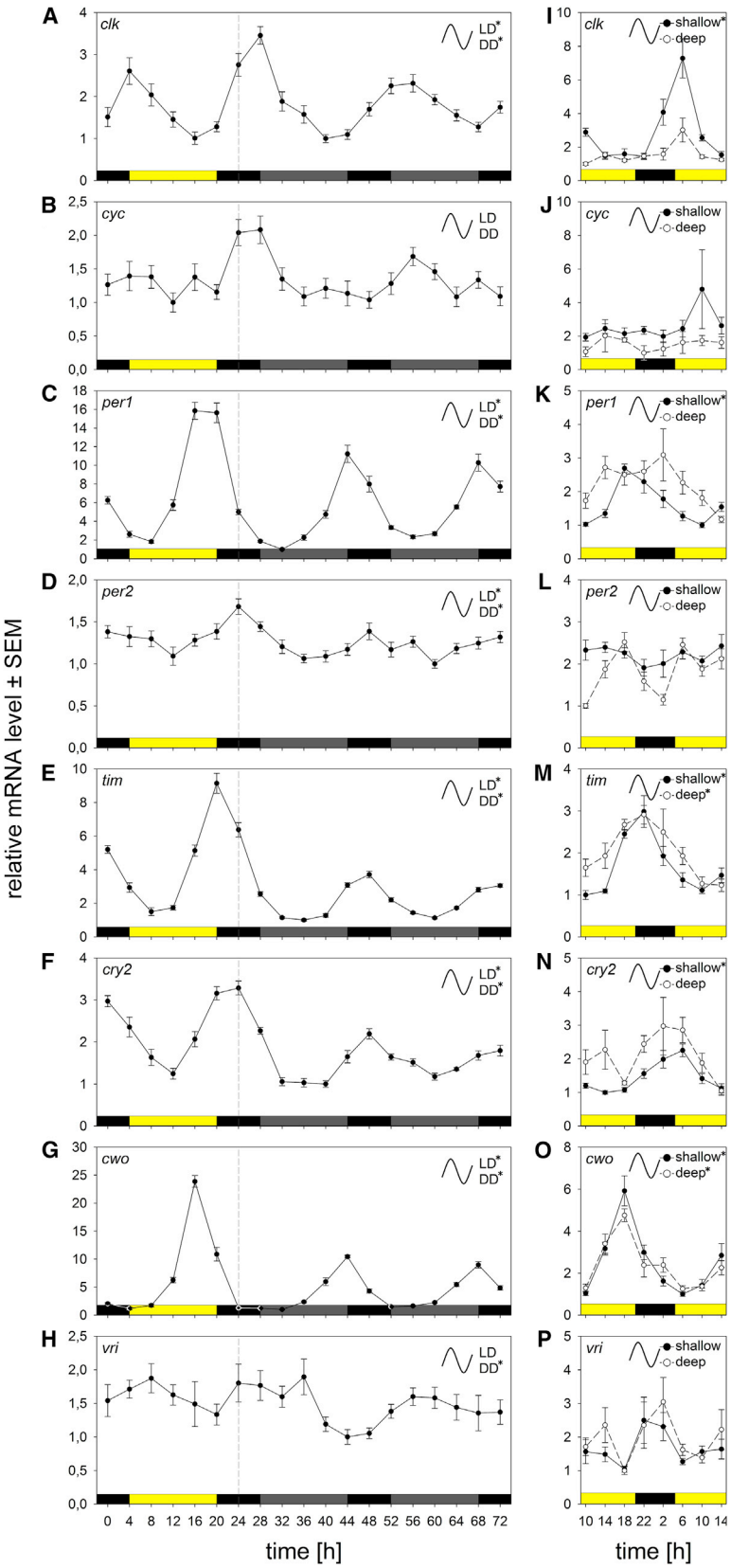

Figure 3. Diel Expression Patterns of Core Clock Genes in the Laboratory and in the Field

Expression patterns were recorded in *C. finmarchicus* CV stages and the investigated genes were as follows: *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.

(A–H) In the laboratory experiments, rhythm analysis of the clock genes *clock* (*clk*, A), *cycle* (*cyc*, B), *period1* (*per1*, C), *period2* (*per2*, D), *timeless* (*tim*, E), *cyrptochrome2* (*cry2*, F), *clockwork orange* (*cwo*, G), and *vrille* (*vri*, H) was done separately for LD (photoperiod = 16 hr) and DD intervals, as described in Figure 2. Per time point, n = 10 replicates were pooled from two identical experimental runs.

(I–P) In the field, samples from 5–50 m (shallow) and 50–140 m (deep) were investigated for the same clock genes (photoperiod = 16 hr). n = 5 replicates per time point.

Both laboratory and field data were analyzed for rhythmic expression using the R-package RAIN. Asterisks (*) indicate significant 24-hr rhythmicity. Mean values ± SEM are shown. Color bars indicate (subjective) day and night. For exact p values, see Table S2.

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, and *shaggy*) or light entrainment (*cryptochrome1*) [35]. In accordance with previous findings, none of these clock-associated genes showed consistent circadian expression (Table S2) [35].

To investigate the functioning of a circadian clock in the field, we conducted a 28-hr sampling campaign at Bonawe deep. Clock gene expression of *C. finmarchicus* was measured in two depth layers (5–50 m and 50–140 m). Generally, the expression patterns of the clock genes resembled those recorded in the laboratory (Figures 3I–3O). However, gene rhythms were less overt in the field and the number of rhythmic genes was reduced, especially in copepods from the deeper layer (Table 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 (Figure 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) suggest that cyclic clock gene expression ceases during diapause. It is also noteworthy that the more labile gene rhythms at 50- to 140-m depth were mirrored by the weaker DVM signal acoustically recorded in this layer in Loch Etive (Figure 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 that are 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 urchin's 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 they could thus explain the two upward migrations at sunset and sunrise characteristic of 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 1,000 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-hr) to lunar day (24.8-hr) 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 may be 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-hr 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 are now required. DVM has previously been shown to occur in the high Arctic during the polar night, in the aphotic depths beyond 1,000 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].

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Study site characteristics
  - Vertical migration in the field
  - Field time series
  - DVM experiment
  - Respiration experiment
  - Gene expression experiment
  - Gene expression analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2017.06.025.

AUTHOR CONTRIBUTIONS

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

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
RNA*later*®	Ambion	Cat#AM7021
RNeasy® Mini kit	QIAGEN	Cat#74104
TURBO DNA-*free*™ Kit	Ambion	Cat#AM1907
RevertAid H Minus Reverse Transcriptase	Invitrogen	Cat#EP0452
Taqman® low-density array card (custom designed)	Applied Biosystems	N/A
Deposited Data		
Raw and analyzed data	this paper	https://doi.pangaea.de/10.1594/PANGAEA.875739
Oligonucleotides		
Taqman® primers/probes	this paper	N/A
Software and Algorithms		
TSA Cosinor 6.3 package	Expert Soft Tech	N/A
R package "RAIN"	[54]	http://journals.sagepub.com/doi/10.1177/0748730414553029

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 animal work was conducted in accordance with local legislation. 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 (Figure 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 hr) 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 hr 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).

METHOD DETAILS

Study site characteristics

Loch Etive is a sea loch at the western coast of Scotland, UK (56°45′N, 5°18′W). It is connected to the open ocean by a sill with a width of 200 m and ~7 m water depth and has another sill with ~13 m depth further up the loch [55]. Beyond the second sill there is the upper main basin with the deepest point of the loch (Bonawe deep, ~145 m) where all samplings were done (Figure S1). The sills limit the water exchange leading hypoxic conditions in the deeper layers of the upper basin. Turnover events occur during the strongest spring tides in spring/autumn, but are irregular and only happen every few years [55].

During the sampling of the 28 hr field time series at Bonawe deep (6^{th}/7^{th} May 2015) the water column parameters salinity, temperature, oxygen concentration and Chlorophyll a (Chl a) fluorescence were recorded by a conductivity-temperature-depth (CTD) profiler (SBE 19plus V2 SeaCAT Profiler, Sea-Bird Electronics, USA). The water column was characterized by an approx. 5 m thick surface layer with a low salinity $\leq$ 20 psu (Figure S2). From 5 m on salinity gradually increased to 27 at ~50 m and showed only a minor increase below this depth. Temperature from the surface to 26 m depth ranged between 8.3°C and 8.9°C. Below 26 m temperature sharply rose to a maximum of 12.2°C at 50 m depth before gradually decreasing to 10.4°C at 90 m and below (Figure S2).

The deeper layers of Bonawe deep were hypoxic during the sampling. From the surface to 26 m depth oxygen concentrations was $\geq$ 8.5 mg O_2*L^{-1} before sharply decreasing to 3.6 mg O_2*L^{-1} at 40-43 m depth (Figure S2). Oxygen concentration then continued to gradually decreased to values $\leq$ 1.6 mg O_2*L^{-1} in ~80 m depth and below. Chl a fluorescence was high in the upper 10 m (4-16 mg*m^{-3}), showed a second, much smaller maximum at ~25 m and then quickly diminished with depth (Figure 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 (Figure S1). The mooring was equipped with two acoustic Doppler current profilers (ADCPs) pointing upward 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 [56] with derived acoustic mean volume backscattering strength (MVBS). Acoustic data were 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 4th to 11th May 2015 significant backscatter rhythmicity could be detected in both, the shallow (45 m, τ = 23.9 hr, % model fit = 49.6) and the deep layer (125 m, τ = 24.0 hr, % model fit = 33.3). Tests on tidal (~12 hr) and lunar (24.8 hr) rhythms did not produce any significant rhythmicity.

Field time series

Samples were collected at Bonawe deep on the 6th/7th May 2015 starting at 11:00 and continuing in 4 hr intervals until 15:00 of the next day, resulting in a total of eight time points over a period of 28 hr. 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 m 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 solution (Ambion, UK) for later gene expression analysis (see below). A possible sample contamination by the congener species *C. helgolandicus* is unlikely due to its limited tolerance to low salinities and the brackish conditions in the loch [25].

DVM experiment

To investigate the diel vertical migration (DVM) behavior, copepods were incubated in four so-called DVM-columns made out of acrylic glass (10*8*90 cm lxwxh, 7.2 L). Animals were collected on the 3rd June 2016, sorted, and per column 50 *C. finmarchicus* CV stages were incubated for a total of three days (LD-DD-DD, photoperiod = 16 hr). The columns were vertically divided into six 15 cm increments and each layer was filmed with a surveillance cameras equipped with filters excluding visible light (SK-B140XP/SO, Sunkwang Electronics, South Korea). Infrared lights were used to illuminate the columns without disturbing the animals. Copepod abundance per layer was then counted by three different persons from the recorded video material at 1 hr intervals. For every column, there was a certain fraction of copepods which was inactive and never left the bottom layer of the column. These animals were excluded from statistical analysis by determining the lowest number of copepods in the bottom layer over the course of the experiment for each column, respectively. This number was then defined as zero for the respective column.

Copepods were not fed during the DVM experiments to avoid particle accumulation at the bottom, which could have affected vertical distribution. At the end of the experiment a vertical oxygen profile was recorded using an oxygen tipping probe (PreSens GmbH, Germany). There was a weak (< 6%), gradual decrease in oxygen from 9.27 mg O_2*L^{-1} near the surface to 8.75 mg O_2*L^{-1} close to the bottom.

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 hr (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 hr). Oxygen content was measured using oxygen-sensitive sensor spots and monitoring equipment (OXY-4, PreSens GmbH, Germany). A moving average over 12 hr 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 were binned to 1 hr 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 hr another bucket was sampled accordingly resulting in a total of 19 time points over a period of three days (72 hr). On the first experimental day (0-24 hr) 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 hr). Copepods were fed with phytoplankton (Shellfish Diet 1800, Reed Mariculture, USA) in 4 hr 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. [57]. Housekeeping genes were newly annotated from the respective transcriptome. All gene annotations were verified via blastn against NCBI database (see Table 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 Table 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 and Schmittgen [58]. Housekeeping genes were chosen based on expression stability over the 24 hr cycle, expression level relative to other investigated genes and the findings of previous studies [59]. 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 hr 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 hr rhythmicity in RStudio (version 0.99.442 [60],) using the RAIN-package. RAIN was specifically designed to detect (circadian) rhythms in biological datasets independent of waveform by using a non-parametric approach [54]. For the 28 hr 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 hr interval (LD) was analyzed separately from the following 48 hr 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 hr 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 Table S1).

For the analyses of DVM and respiration data, an α of 0.05 was used (Table S1). For the gene expression analyses, a p value < 0.001 was considered significant to account for the testing of multiple genes (Table 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 Table 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), and 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).

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Supplemental Information

Circadian Clock Involvement in Zooplankton Diel Vertical Migration

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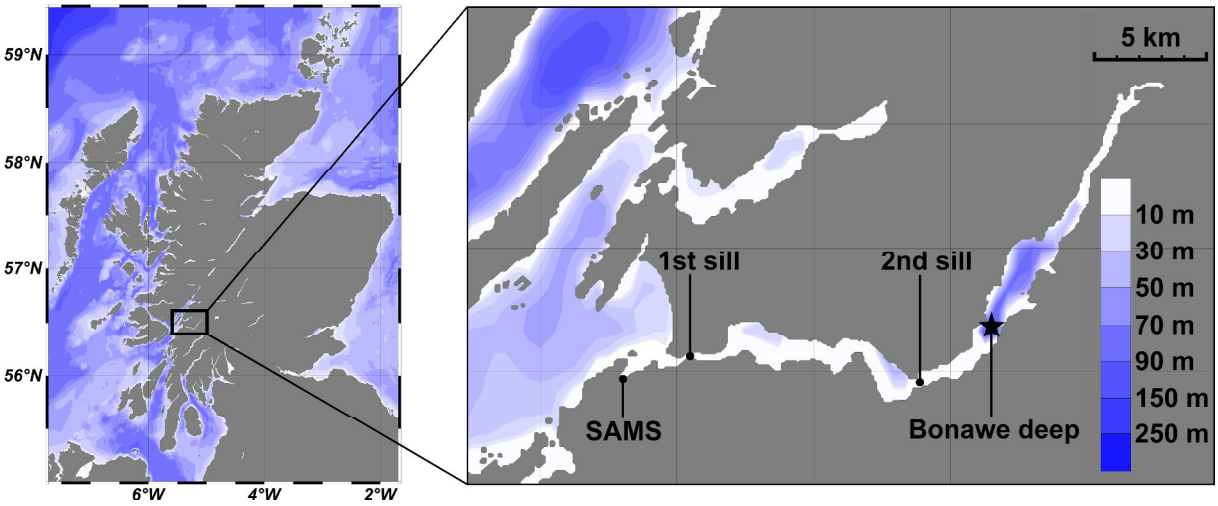

Figure S1. Geographical characteristics of the study area. Related to Figure 1. Loch Etive is a sea loch at the western coast of Scotland, UK (56°45´N, 5°18´W). Water exchange with the ocean is limited by two sills. All samplings as well as the mooring deployment were done at the deepest point of the loch, Bonawe deep (~145 m), at the Scottish Association for Marine Science (SAMS) permanent station RE5. Laboratory studies were conducted at SAMS, in close proximity to Loch Etive. Maps were created with Ocean Data View (v. 4.7.4, [S1]).

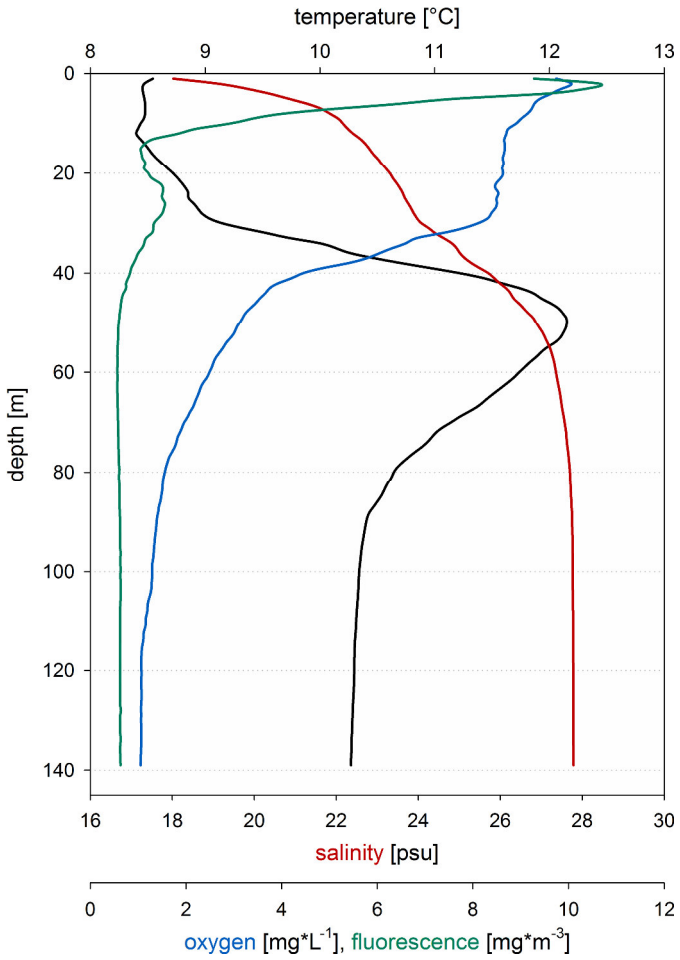

Figure S2. Water column characteristics at the study site. Related to Figure 1. Data was pooled from three CTD hauls conducted during the 28-hr field sampling campaign on the 6th/7th May 2015 at Bonawe deep. Mean values of the pooled hauls are shown.

	Rhythm analysis (24-hr, $\boldsymbol{\alpha = 0.05}$)			
Phenotype	LD	DD	DD - day1	DD - day2
DVM	**< 0.001**	**0.039_{m}**	**< 0.001**	**0.002**
respiration	**< 0.001**	**$< 0.001_{m}$**	**< 0.001**	**< 0.001**

Table S1. Statistical rhythm analysis of DVM and respiration. Related to Figure 2. Analysis was performed with the R-package "RAIN" [S2]. Bold values indicate significant 24-hr rhythmicity ($p < 0.05$). A subscript m ($_{m}$) indicates results derived from mean value analysis.

			Rhythm analysis (24-hr, **α = 0.001**)			
Gene	Function	NCBI Accession No°	Lab LD	Lab DD	Field shallow	Field deep
clock	core clock	GAXK01092177.1	**< 0.001**	**<0.001**	**< 0.001**	–
cycle		GAXK01131751.1	0.024	0.006	–	–
period1		GAXK01127710.1	**< 0.001**	**<0.001**	**< 0.001**	0.048
period2		GAXK01015947.1	**< 0.001**	**<0.001**	–	0.046
timeless		GAXK01195225.1	**< 0.001**	**<0.001**	**< 0.001**	**< 0.001**
cryptochrome2		GAXK01199676.1	**< 0.001**	**<0.001**	**< 0.001**	0.002
clockwork orange		GAXK01116566.1	**< 0.001**	**<0.001**	**< 0.001**	**< 0.001**
vrille		GAXK01130166.1	0.048	**<0.001**	–	–
doubletime2	clock-associated	GAXK01058829.1	–	–	0.026	–
widerborst1		GAXK01125267.1	–	–	–	**< 0.001**
twins		GAXK01019902.1	–	–	–	–
casein kinase II α		GAXK01065631.1	–	–	–	–
shaggy		GAXK01013351.1	–	–	–	–
cryptochrome1	clock light input	GAXK01107177.1	–	–	–	0.020
elongation factor 1 α	housekeeping	GAXK01169633.1	not tested			
RNA polymerase		GAXK01026612.1	not tested			
actin		GAXK01166051.1	not tested			

Table S2. List of investigated genes. Related to Figure 3. Gene sequences were obtained from Lenz et al. [S3] and Christie et al [S4]. Genes were normalized against the geometric mean of the housekeeping genes *elongation factor 1 α*, *RNA polymerase* and *actin*. Rhythm analysis was performed with the R-package "RAIN" [S2]. Bold values indicate significant 24-hr rhythmicity ($p < 0.001$). For the sake of clarity, p-values > 0.05 are not shown (–).

Supplemental References

S1. Schlitzer, R. (2015). Ocean data view. Available on https://odv.awi.de/.

S2. Thaben, P.F., and Westermark, P.O. (2014). Detecting Rhythms in Time Series with RAIN. J. Biol. Rhythms *29*, 391–400.

S3. Lenz, P.H., Roncalli, V., Hassett, R.P., Wu, L.-S., Cieslak, M.C., Hartline, D.K., and Christie, A.E. (2014). *De novo* assembly of a transcriptome for *Calanus finmarchicus* (Crustacea, Copepoda) – the dominant zooplankter of the North Atlantic Ocean. PloS One *9*, e88589.

S4. Christie, A.E., Fontanilla, T.M., Nesbit, K.T., and Lenz, P.H. (2013). Prediction of the protein components of a putative *Calanus finmarchicus* (Crustacea, Copepoda) circadian signaling system using a de novo assembled transcriptome. Comp. Biochem. Physiol. Part D Genomics Proteomics *8*, 165–193.