

“A circadian clock drives behavioral activity in Antarctic krill (*Euphausia superba*) and provides a potential mechanism for seasonal timing”

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This **important** study substantially advances our understanding of the circadian clock in Antarctic krill, a key species in the Southern Ocean ecosystem. Through logistically challenging shipboard experiments conducted across seasons, the authors provide **compelling** evidence for their conclusions. The study will be of broad interest to marine biologists and ecologists.

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Abstract

Antarctic krill is a species with fundamental importance for the Southern Ocean ecosystem. Their large biomass and synchronized movements, like diel vertical migration (DVM), significantly impact ecosystem structure and the biological carbon pump. Despite decades of research, the mechanistic basis of DVM remains unclear. Circadian clocks help organisms anticipate daily environmental changes, optimizing adaptation. In this study, we used a recently developed activity monitor to record swimming activity of individual, wild-caught krill under various light conditions and across different seasons. Our data demonstrate how the krill circadian clock, in combination with light, drives a distinct bimodal pattern of swimming activity, which could facilitate ecologically important behavioral patterns, such as DVM. Rapid damping and flexible synchronization of krill activity indicate that the krill clock is adapted to a life at high latitudes and seasonal activity recordings suggest a clock-based mechanism for the timing of seasonal processes. Our findings advance our understanding of biological timing and high-latitude adaptation in this key species.

Introduction

Antarctic krill (*Euphausia superba*, hereafter referred to as krill) is a pelagic, up to 6 cm long crustacean, which is endemic to the Southern Ocean. With an estimated biomass of 300 to 500 million tons (1), krill is amongst the most abundant wild species worldwide (2) and an important prey for predators such as whales, seals and penguins (3). Its huge abundance and central position in the food web makes krill a key species of fundamental importance for the functioning of the Southern Ocean ecosystem (4, 5).

Krill is mostly found in swarms, the largest of which can be hundreds of meters long and tens of meters in height, likely reflecting a behavioral adaptation to minimize the risk of predation (6, 7). Along with their pronounced swarming behavior, krill display diel vertical migration (DVM), a common behavior among many pelagic zooplankton organisms across different taxa found in all oceans (8). DVM is commonly characterized by an ascent to the surface layers in the dark of the night to feed and a descent to darker parts of the water column during the day to avoid visually hunting predators (8, 9). The regular, synchronized movement of this large biomass significantly shapes the structure of ecosystems (10) and has large impacts on biogeochemical cycles (11, 12). This includes the recycling of nutrients, stimulation of primary production as well as transport and storage of carbon in the deep ocean (4). Although such a “regular” DVM pattern has been commonly observed for krill swarms (13, 14), there is considerable behavioral variation, including reverse DVM, higher frequency migrations or no synchronized migrations at all (13, 15). While the adaptive advantage of balancing increased predation pressure during the day with the need to feed seems obvious, the underlying mechanisms of DVM and its modulations are not understood. Light is thought to be the most reliable and thus important environmental cue for DVM (16). However, a study in the marine copepod *Calanus finmarchicus* indicates that DVM behavior is underpinned by a circadian clock, which helps the animals to adjust behavior, physiology and gene expression to the day-night cycle (17). Similarly, a study on Norwegian krill, *Meganyctiphanes norvegica*, suggested swimming activity in krill is under clock control, with increased activity during the dark phase (18). In Antarctic krill, similar studies have been conducted to test the hypothesis of a clock involvement in DVM behavior (19, 20). While these studies gave first hints that daily behavioral activity in krill is partly under endogenous control, a large variability hindered a clear characterization of the circadian behavioral activity of krill.

Previous studies on endogenous rhythms in krill identified the molecular components of a krill circadian clock (21, 22). It has further been shown that the krill circadian clock drives daily rhythms in gene expression, metabolism and behavior (20, 23, 24). Part of krill’s success lies in its strong adaptation to the extreme seasonality of the Southern Ocean environment (25), which is reflected in a fundamental, seasonal regulation in body composition, metabolic activity, feeding, growth (26, 27), sexual maturity (28, 29) and gene expression (30, 31). Results from laboratory studies showed that photoperiod is a major driver for seasonal ecophysiological changes in krill (32–34), and it has been suggested that an endogenous timekeeping system (i.e. biological clock) is involved, which uses photoperiod as a synchronizing cue (33, 35).

Circadian clocks are widespread and well-studied endogenous timing mechanisms which allow an organism to fine-tune biological functions to specific times of the day-night cycle. The endogenous oscillation is produced by transcriptional-translational feedback loops (TTFL) of a set of clock genes and their translation products (36), which, under the absence of external cues, produce a temporal signal with a period (τ) of about 24 h. Under natural conditions, the rhythm of the central oscillator is synchronized to exactly 24 h by predictable environmental cycles, called *Zeitgeber*, of which light is usually the most important (37). The rhythmic signal of the central oscillator is

superimposed on downstream processes, which results in the temporal regulation of several biological functions, such as gene expression, physiology and behavior. Thus, the circadian clock allows organisms to anticipate daily environmental cycles and adapt their biology, ultimately increasing survival and fitness (38 [↗](#)). Since the circadian clock implicitly measures day length, it is also capable of providing seasonal information. Consequently, the circadian clock has also been shown to be involved in the timing of seasonal functions, such as the induction of reproduction or diapause (39 [↗](#), 40 [↗](#)).

While most knowledge about circadian clocks and their involvement in organism's daily and seasonal processes stems from terrestrial model organisms, little is known about clocks in the marine environment (41 [↗](#)). This is especially true for marine habitats in polar regions, characterized by extreme photic conditions, including polar night and midnight sun phases. An understanding of the mechanistic underpinnings of biological timing is essential to comprehend how organisms adapt to their specific environment. This is particularly important in times of rapid environmental change, which can alter the temporal synchronization of trophic interactions, including DVM, feeding and swimming behaviour, and, thus, the functioning of whole ecosystems (42 [↗](#)).

In this study, we use the novel Activity Monitor for Aquatic Zooplankter (AMAZE) (43 [↗](#)) to investigate the mechanistic basis of swimming activity of wild-caught Antarctic krill under controlled environmental conditions onboard a commercial krill fishing vessel. We provide novel evidence that the circadian clock underlies ecologically important behaviors, such as DVM, and discuss how the krill circadian clock could control the timing of seasonal life cycle functions in krill.

Results

Two sets of experiments were conducted for this study (Fig. 1 [↗](#)). In the first set of experiments (*experiment 1*) we adopt a standard chronobiological experiment design, where we monitor krill behavioral activity under simulated short days (*short day treatment*) or long days (*long day treatment*), before they are transferred into constant darkness for several days to investigate the influence of a circadian clock on krill behavior. In a second set of experiments (*experiment 2*) krill vertical migration behavior in the field was recorded for several days before sampling and transferring krill into constant darkness. This experiment was conducted in four seasons (*summer treatment*, *late summer treatment*, *autumn treatment*, and *winter treatment*).

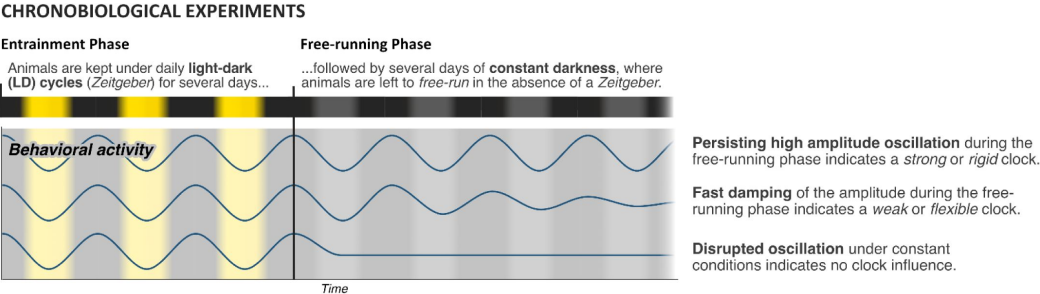
A circadian clock is involved in the daily regulation of swimming activity

To investigate whether a circadian clock is involved in regulating daily rhythms of swimming activity, we sampled krill from the field under short-day conditions in winter (*short day treatment*, sampling date: May 31st 2021, local photoperiod: 5.5 h) and under long-day conditions in late summer (*long day treatment*, sampling date: February 16th 2022, local photoperiod: 15.3 h; see Tab. 1 [↗](#) for details). We exposed 11 and 9 sampled individuals to 3 days of light-dark conditions in the activity monitor simulating short-day conditions and to 5 days of LD simulating long-day conditions, respectively. The photoperiod in the activity monitor approximated the natural photoperiod in the field during sampling. The period of LD was followed by 5 days of constant darkness. The data under LD conditions show that krill swimming activity at both individual and group level increased during the dark phase and showed a strong synchronization with the light-dark cycle provided (Fig. S2 [↗](#) and S3 [↗](#)).

Figure 1

Experimental design of chronobiological studies.

Overview of the basic principle of chronobiological experiment design and how it is adopted in our study to characterize the influence of the circadian clock on swimming activity of wild-caught Antarctic krill.



EXPERIMENTAL DESIGN IN THIS STUDY

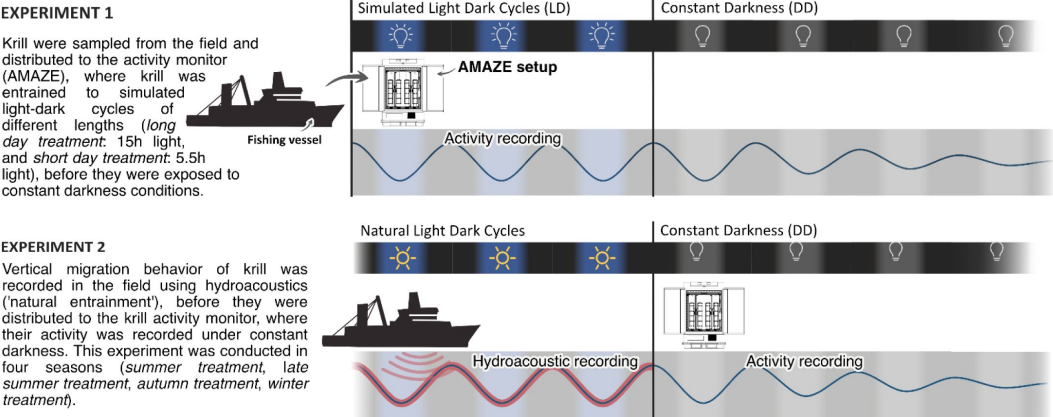


Table 1

Meta data for behavioral experiments.

For each experiment (Experiment ID), the meta data show the light regime during the experiment in the activity monitor (LD: light-dark cycle, DD: constant darkness), the duration of the experiments under the respective light condition, the local date and time, Region (BS: Bransfield Strait, SOI: South Orkney Islands), exact location, and depth for krill sampling, the local natural photoperiod at the time of krill sampling, the number of krill used for each experiment, and their mean length and standard deviation.

Experiment ID	Experimental light regime	Experiment duration [days]	Sampling Date [UTC-2]	Sampling Time [UTC-2]	Sampling Region	Sampling location [Latitude °S/Longitude °W]	Sampling Depth [m]	Natural photoperiod [h]	n Krill [total/male/female]	Mean ±SD length [mm]
Experiment 1 (<i>short day treatment</i>)	LD-DD	3 (LD), 5 (DD)	2021-05-31	13:02	BS	62.95/57.77	170	5.5	11/5/6	45 ±2.6
Experiment 1 (<i>long day treatment</i>)	LD-DD	5 (LD), 5 (DD)	2022-02-16	15:50	SOI	60.04/46.45	94	15.3	9/4/5	44.4 ±3.7
Experiment 2 (<i>summer treatment</i>)	DD	8	2022-01-22	17:03	SOI	60.08/46.46	157	17.5	9/8/1	42.4 ±1.5
Experiment 2 (<i>late summer treatment</i>)	DD	6	2022-03-01	11:00	SOI	60.0/44.73	86.5	14.0	9/4/5	48.9 ±4.4
Experiment 2 (<i>autumn treatment</i>)	DD	7	2022-03-15	08:40	SOI	60.28/46.43	119.2	12.6	10*/4/5	51.3 ±1.6*
Experiment 2 (<i>winter treatment</i>)	DD	4	2021-06-10	12:56	SOI	60.5/46.11	185	5.9	9/2/7	49.2 ±3.9

* Length and sex of one individual undetermined.

As synchronization between individuals and robustness of activity patterns under DD within individuals was strongest in krill from the short day treatment, we used these data for initial assessments of circadian activity rhythms. Under constant darkness conditions in the short day treatment, krill swimming activity continued in several individuals ($n = 6$; 54%) with significant circadian rhythmicity over the 5 days of DD conditions (**Fig. 2a and b**, **Fig. S4**), indicating the involvement of a circadian clock in the daily regulation of swimming activity. Nevertheless, in most individuals, the amplitude of the rhythm decreased over time (**Fig. 2a**, **Fig. 4a**, **Fig. S4**). The patterns of swimming activity observed at the individual level were reflected at the group level, showing a strong synchronization of increased swimming activity with the dark phase under light-dark conditions (**Fig 3a**, **Fig. S2**), and persistent significant circadian swimming activity under constant conditions with a continuously decreasing amplitude throughout the experiment (**Fig. 2c and d**; **Fig. 4a**: Paired t-test, p -value = 0.005, **Fig. 4c**: Paired t-test, p -value = 0.012).

Light affects the amplitude and phase of swimming activity

Comparing the mean swimming activity of rhythmic individuals over an average day from the short day and long day treatments under LD conditions reveals a clear synchronization of swimming activity with the dark phase (**Fig. 3**), visible in the sharp increase in activity around the times of lights-off and decrease at lights-on. Furthermore, the time of increased activity during the dark phase exhibits a discernible pattern comprising three distinct activity bouts (**Fig. 3**). First, an increase in activity is observable in the evening (i.e., end of illumination), followed by a decrease in activity towards midnight. A second increase in swimming activity is observable in the latter half of the dark phase, followed by a decline in activity just before morning. A third short activity bout is observed in the morning (i.e., onset of illumination). This distinctive activity pattern during the dark phase is observed regardless of the photoperiod, resulting in a compression of the three activity bouts in short nights, while they spread out in long nights (**Fig. 3**), demonstrating that krill can entrain to the long and short days provided. Furthermore, comparing the activity pattern during short-day LD conditions with the respective activity pattern under DD conditions reveals the persistence of evening and late-night activity increases under constant darkness conditions. In contrast, the morning activity bout is not visible (**Fig. S5**). This suggests that the circadian clock drives a distinct bimodal activity pattern with two activity peaks in one day, i.e. the evening and late-night activity bouts. In contrast, the morning activity bout is triggered by the onset of illumination in the experimental set-up. Furthermore, the amplitude of swimming activity is higher under LD than DD conditions (**Fig. 4 B**, Mann-Whitney-U test: p -value < 0.001; **Fig. 4 D**, p -value < 0.001).

Circadian swimming activity persists with a stable phase after entrainment to a wide range of natural photoperiods

To gain insights into potential seasonal differences in the regulation of krill swimming activity, we sampled krill from the field in summer (summer treatment), late summer (late summer treatment), autumn (autumn treatment), and winter (winter treatment) and recorded their swimming activity for 4 to 8 days under constant darkness conditions, without previous entrainment in the activity monitor (see **Tab. 1** for details).

Similar to our previous analysis of individual krill swimming activity under constant darkness conditions, a large proportion of the recorded individuals in each experiment (44% to 100%) exhibited significant rhythmicity in the circadian range (**Fig. S7-S10**), which is also reflected in rhythmic swimming activity at the group level (**Fig. 5a-d**, **Fig. S6**). Interestingly, the pattern of circadian swimming activity, with two peaks during the dark phase, appears to become clearer and higher in amplitude with shortening photoperiods towards winter (**Fig. 5a-d**). This is supported by rhythm analysis of the group behavior, which revealed a higher power and a period estimation closer to 24 h with shortening photoperiod (**Fig. S11**). While the morning peak is

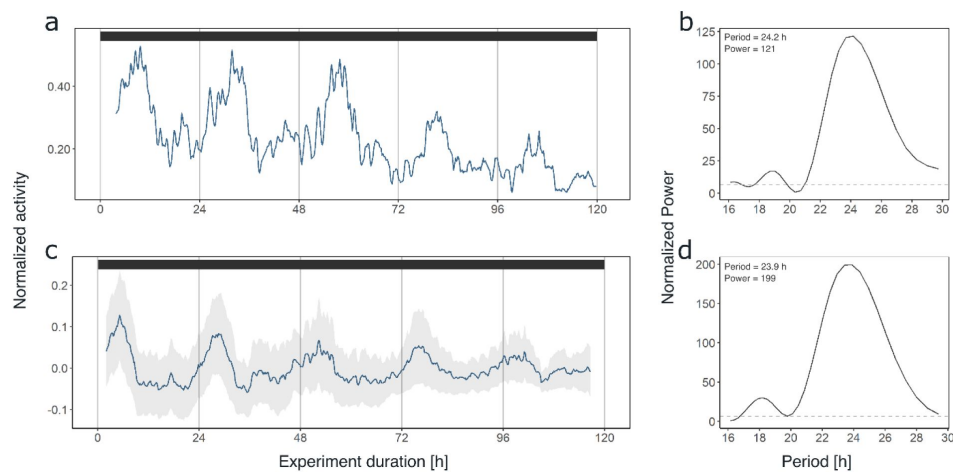


Figure 2

Krill swimming activity persists under constant conditions.

Swimming activity under 5 days of DD conditions in experiment 1 of individual #9 after entrainment to simulated short-day conditions (a) and group mean swimming activity of rhythmic individuals under 5 days of DD conditions from the same experiment ($n = 6$, c). The result from LSP analysis shows significant circadian rhythmicity of swimming activity of individual #9 (period: 24.2 h, b) as well as for group mean swimming activity (period: 23.9 h, d) during DD conditions of experiment 1 (short day treatment). Grey shading represents standard error of the mean (s.e.m.). Color bars at the top indicate light conditions (constant darkness).

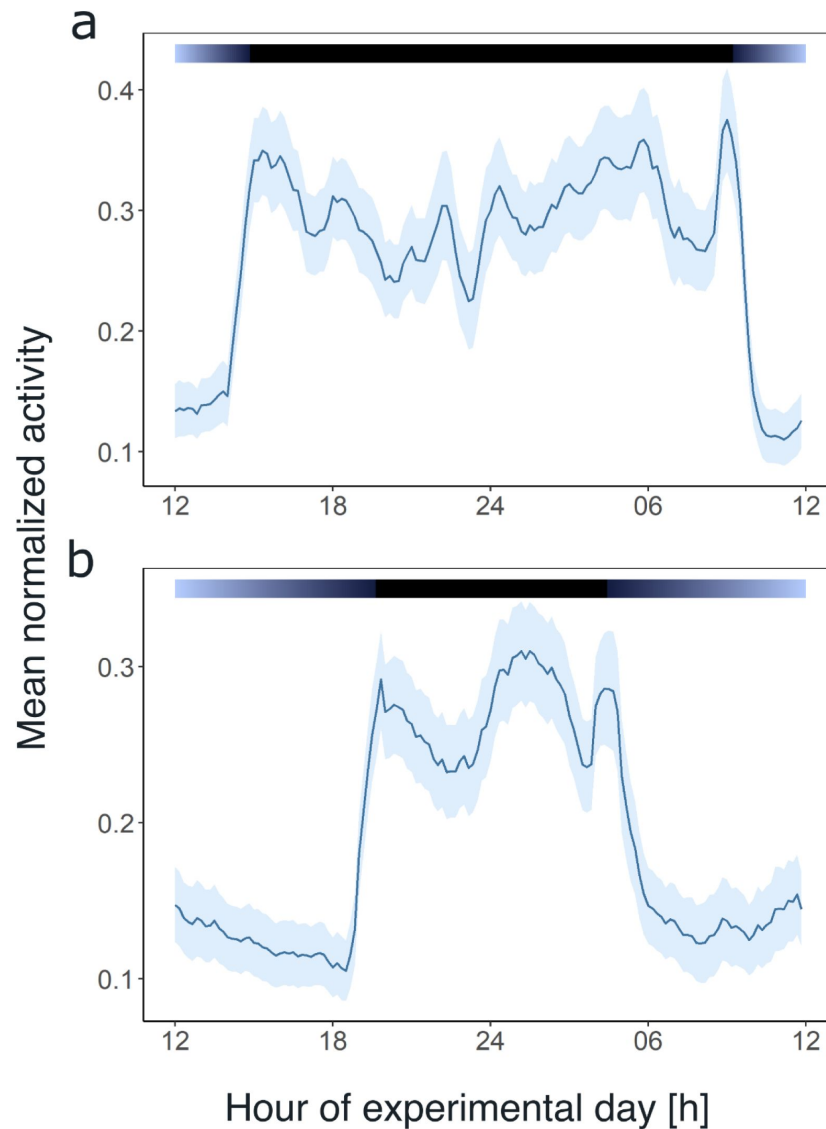


Figure 3

Light and the circadian clock shape the daily activity profile of krill.

Average day analysis of the group mean swimming activity during experiment 1 over 3 days of short-day simulations (short day treatment, $n = 11$, a) and 5 days of long-day simulations (long day treatment, $n = 9$, b). Color bars at the top indicate light regime under day-night simulations. Shading around the line represents the s.e.m.. Data in (b) from Hüppe et al. (43).

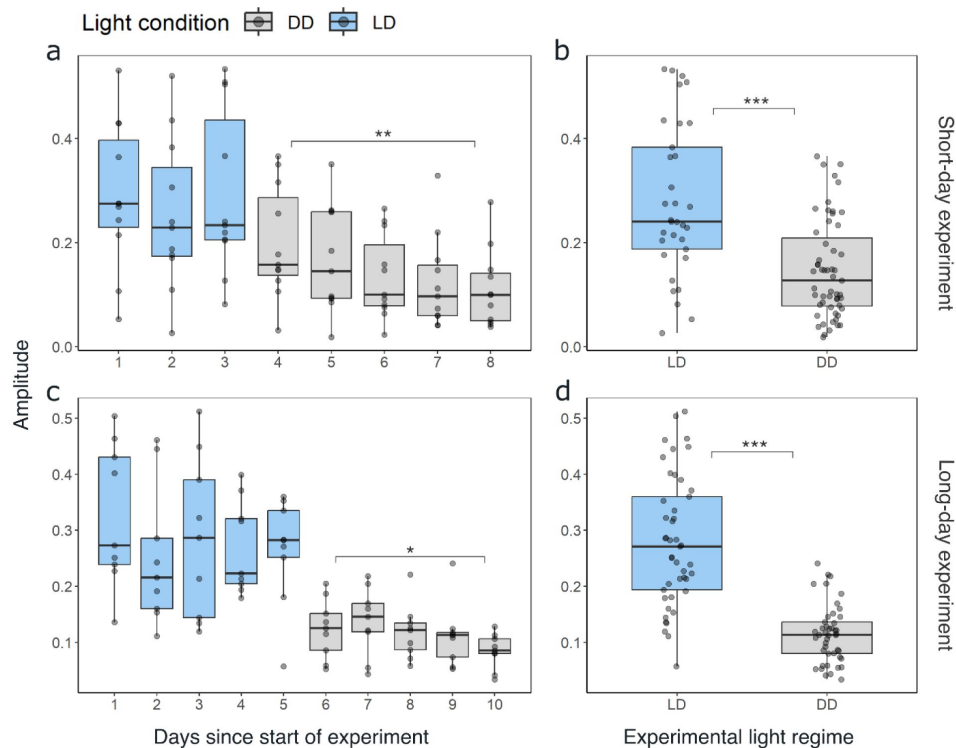


Figure 4

The amplitude of swimming activity decreases in DD conditions.

Distribution of the daily amplitudes of swimming activity visualized with boxplots for every day of experiment 1 for short-day (a), and long-day treatments (b). Differences in the daily amplitude of swimming activity between LD and DD conditions for short-day (b) and long-day (d) treatments. Lower and upper hinges of the boxes correspond to the 25th and 75th percentiles, the upper and lower whiskers extend to the largest and smallest value no further than 1.5 of the interquartile range, respectively, the horizontal line shows the median. Points represent daily amplitudes of individuals for each day and light condition. Differences in amplitude between first and last day under DD tested with paired t-test for short day (a; $n = 11$, $t = 3.197$, $df = 10$, $p\text{-value} = 0.005$) and long day treatments (c; $n = 9$, $t = 2.731$, $df = 8$, $p\text{-value} = 0.012$), and between LD and DD conditions with Mann-Whitney-U-Test for experiment 1 (b; $n = 88$, $p\text{-value} < 0.001$) and experiment 2 (d; $n = 90$, $p\text{-value} < 0.001$). Significance levels: $p < 0.05$: *, $p < 0.01$: **, $p < 0.001$: ***.

mainly visible in autumn and winter, the late-night activity peak is visible throughout the year, at the same time of the subjective 24-h-day ([Fig. 5e-h](#)). This pattern appears irrespective of the natural photoperiod the animals were entrained to in their natural environment before they were sampled for the respective experiment.

Synchronized diel vertical migration in the field is present across a wide range of natural photoperiods

To investigate whether krill swarms exhibited daily behavioral patterns in swimming behavior in the field before they were sampled for seasonal experiments, hydroacoustic data were recorded from the fishing vessel, continuously over a three-day period prior to sampling for the seasonal experiments described above (for vessel positions during hydroacoustic recordings see [Fig. S1](#)). The data provide information about the vertical distribution of krill swarms in the upper water column (<220 m) below the vessel over time ([Fig. 6a-d](#)). The hydroacoustic data in summer, late summer and autumn show that krill swarms performed diel vertical migration, highly synchronized with the local light regime ([Fig. 6a-c](#)). The swarms regularly ascended to the surface layers (<50 m) around sunset and returned to deeper layers (>100 m) around sunrise. In winter, little signal is visible in the upper water layer, indicating very low density or the absence of krill swarms ([Fig. 6d](#)). Consequently, krill for the winter treatment was sampled from deeper waters (185 m). This agrees with observations that krill shift their distribution to deeper waters in winter, where krill have been observed to perform DVM also during this time of year ([15](#)). The available data only covers the upper 220 m of the water column, meaning that the dynamics of swarms below this depth are missed.

Discussion

Our study revealed continued rhythmic swimming activity under constant darkness, showing the involvement of a circadian clock in swimming behavior of krill, as has been previously shown for other marine pelagic crustaceans ([17](#), [18](#), [44](#)). To date, only three studies have investigated the involvement of a circadian clock in the regulation of swimming behavior in krill species, including *E. superba* ([19](#), [20](#)) and *Meganyctiphanes norvegica* ([18](#)). Data from Piccolin et al. ([20](#)) showed a strong damping of the amplitude and indication of a remarkably short (~12 h) free running period (FRP) of vertical swimming behavior of a group of krill under constant darkness ([20](#)). The short period found in Piccolin et al. ([20](#)) is in line with our findings of a bimodal activity pattern under DD conditions on the individual level, suggesting that the ~12 h rhythm in group swimming behavior in Piccolin et al. ([20](#)) could have resulted from a bimodal activity pattern at the individual level, as found in our study. Bimodal locomotor activity rhythms are well known across different species, possibly most prominently in the fruit fly (*Drosophila melanogaster*), where two coupled endogenous oscillators control morning and evening activity bouts ([45](#)). In our experiments, we observed a strong weakening of the evening activity under constant conditions, especially after entrainment to long days, while the late-night activity peak appeared more robust. The differential variation in the morning and late-night activity suggests that the circadian activity bouts could also be controlled separately at the neuronal level in krill.

Comparing the daily pattern of swimming activity under LD conditions with those under DD, it is clear that the amplitude of the rhythm was significantly higher under LD than under DD. In addition, the activity under LD was organized into three activity phases, one in the evening, one late at night and a smaller one in the morning. In contrast, under DD there were a maximum of two activity phases during the subjective night. Light is known to play a dual role in regulating daily behavioral activity. On the one hand, light functions as a *Zeitgeber*, which synchronizes the circadian clock with the day-night cycle, a process called *entrainment* ([46](#)). On the other hand, it can directly affect clock output functions such as physiology and behavior, known as *masking*

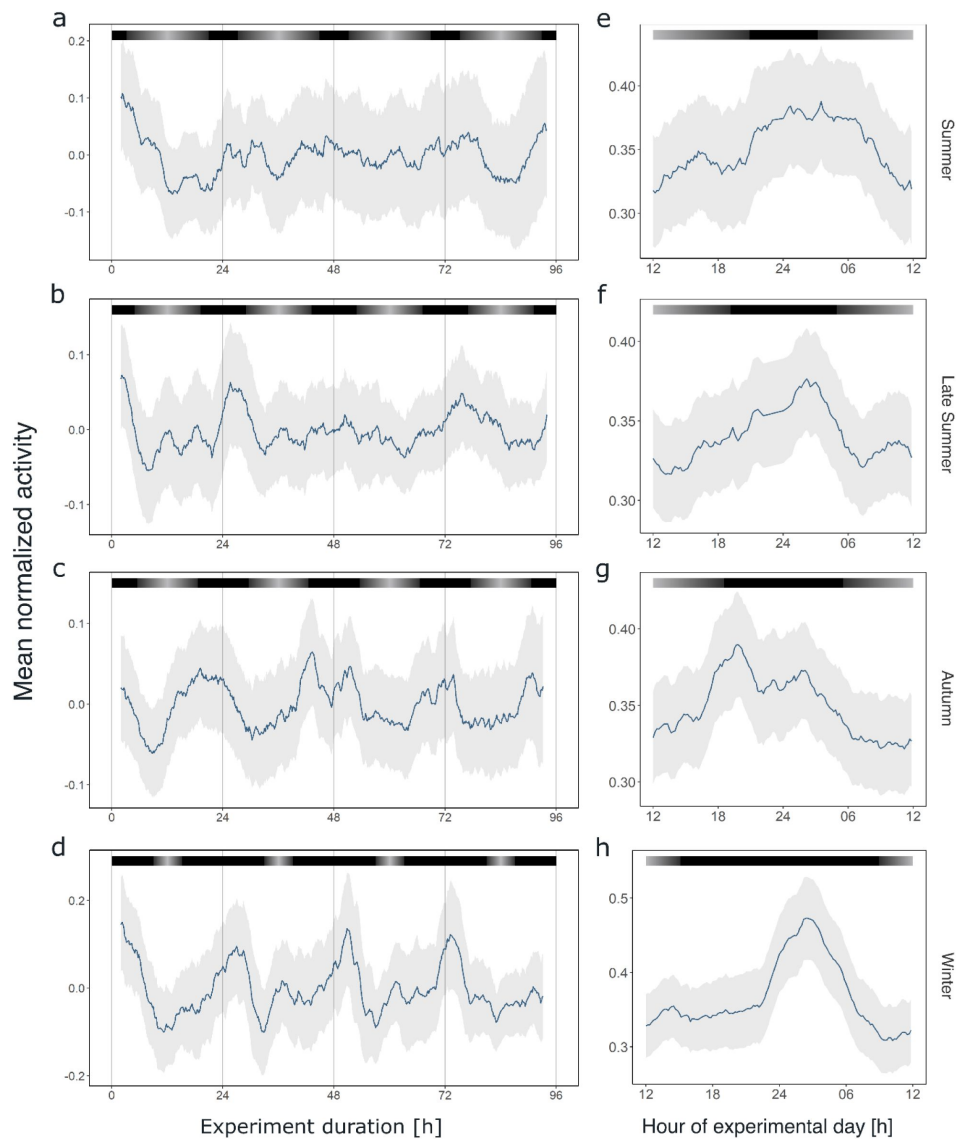


Figure 5

Circadian swimming activity persist across seasons.

Group mean swimming activity of rhythmic individuals during the first four days under DD conditions of experiment 2 for krill sampled for the summer (n individuals = 5, a), late summer (n individuals = 8, b), autumn (n individuals = 10, c), and winter treatments (n individuals = 4, d), as well as corresponding average day analysis of the first four days of experiment 2 in summer (e), late summer (f), autumn (g), and winter (h). Grey shading represents the s.e.m.. Color bars at the top indicate the natural photoperiod at the day of sampling.

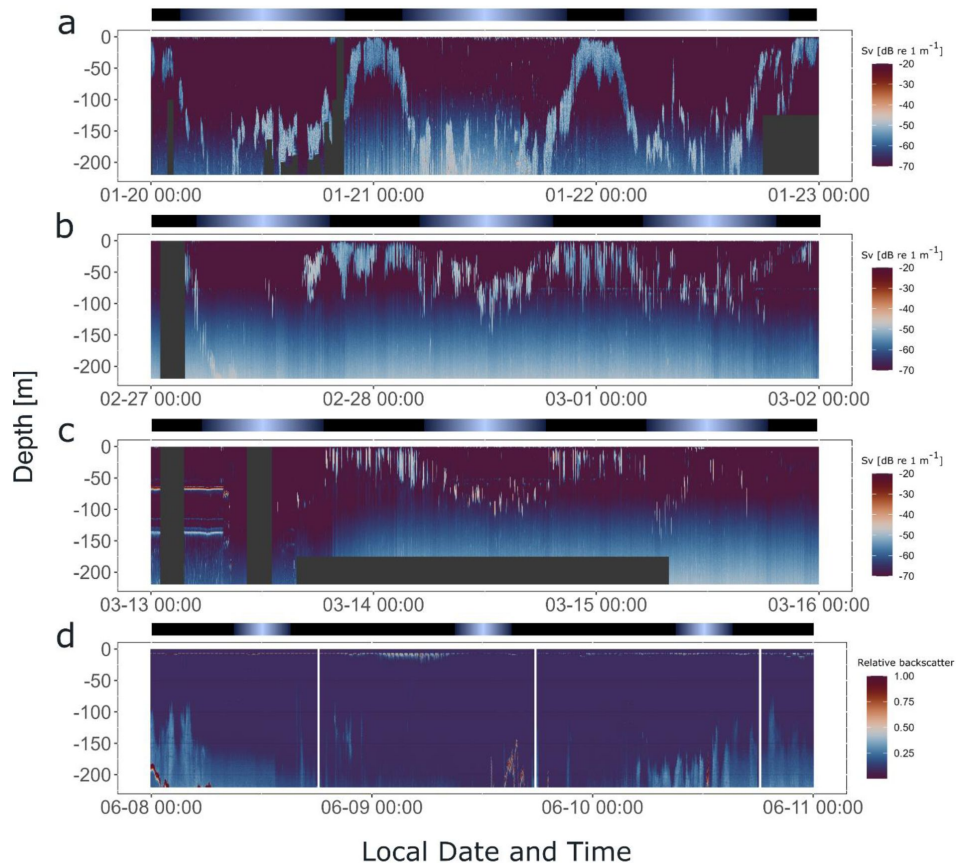


Figure 6

Krill display synchronized DVM in the field across a wide range of photoperiods.

Hydroacoustic recordings showing the vertical distribution of krill swarms in the upper water column (<220 m) below the vessel, visualized by the mean volume backscattering signal (200 kHz), on the three days prior to krill sampling for experiment 2, in summer (a), late summer (b), autumn (c), and winter (d). Diffuse blue shading between ~100–220 m represents instrument noise. Grey areas depict missing data. Color bars at the top indicate the natural light regime.

(46 [↗](#)). On the other hand, it can directly affect clock output functions such as physiology and behavior, known as *masking* (46 [↗](#), 47 [↗](#)). The masking response depends on the activity type of the organism, and light was shown to promote activity in diurnal animals while inhibiting activity in nocturnal ones (48 [↗](#), 49 [↗](#)). Krill are regularly observed performing nocturnal DVM (13 [↗](#), 14 [↗](#)), including in the present study, with higher activity during the night, including the times of twilight, related to foraging and feeding activity and vertical ascend and descend movements (50 [↗](#), 51 [↗](#)). The observed effect of light on reducing krill swimming activity under LD conditions in our study is thus in line with previous concepts of the exogenous effects of light on nocturnal organisms (46 [↗](#), 47 [↗](#)). However, under LD conditions, the observed swimming activity pattern in krill is not solely driven by light. The increase in activity in the evening and the second half of the dark phase under LD conditions flexibly adjust to the length of the night and seem to persist under constant darkness, suggesting these bouts to be under clock control, as discussed above.

The reduced amplitude in swimming activity after the switch to DD conditions and further damping of amplitude over time under constant darkness has been found in other studies in krill (20 [↗](#)) and other marine zooplankton organisms (17 [↗](#), 44 [↗](#)). A rapidly damping output signal under constant conditions could indicate a weak, low amplitude circadian oscillator, where the damping amplitude of the clock output signal is caused by a rapid desynchronization of clock neurons, when Zeitgeber signals are absent (52 [↗](#)). Interestingly, weak oscillators have been found in different fruit fly species (53 [↗](#), 54 [↗](#)), the Svalbard reindeer (55 [↗](#)), and Svalbard ptarmigan (56 [↗](#)), native to high latitudes and species that exhibit pronounced seasonal rhythmicity, such as aphids (57 [↗](#), 58 [↗](#)). It is known that weaker clocks are more flexible and can be entrained more efficiently than those that are more rigid and self-sustained (52 [↗](#), 59 [↗](#)). This could confer a significant adaptive advantage to species inhabiting environments characterized by extreme photic conditions (53 [↗](#), 54 [↗](#), 60 [↗](#)), such as phases of polar night or midnight sun as well as rapid changes in daylength, or species that rely on precise photoperiodic time measurement for accurate seasonal adaptation. In the Southern Ocean, photoperiod becomes extreme around the solstices, and its daily change is rapid around the spring and autumn equinoxes. A weak oscillator could, therefore, ensure that krill remains synchronized with the extreme and rapidly changing photic conditions and, thus, its environment.

Indeed, our data from activity recordings under DD conditions of krill sampled in different seasons indicate persistent circadian swimming activity, irrespective of the season. Our findings suggest that the krill circadian clock remains functional and drives rhythmic behavioral output in natural conditions throughout the year. This finding is complemented by the hydroacoustic recordings in the days preceding krill sampling for activity experiments. In summer, late summer and autumn, krill swarms exhibited apparent diel vertical migration behavior, synchronized to the natural photoperiod. While we did not observe DVM in the upper water column during sampling in winter, acoustic recordings from other studies in winter indicate that krill may perform DVM also in winter (61 [↗](#)), potentially in deeper layers.

Our data show that light acts in concert with, and is likely modulated by, the circadian clock to achieve the observed daily regulation in krill swimming activity. Nevertheless, further studies at the behavioral, molecular, and neuronal levels are required to elucidate the endogenous and exogenous effects of light and the circadian clock on the different behavioral aspects observed and to gain further insights into the characteristics of the molecular mechanism of the krill clock.

In a high-latitude region, such as the Southern Ocean, the timing of crucial life history functions to seasonal fluctuations of light, food availability, and sea ice extent is key to organisms' success. In contrast to early studies, which suggested that only food availability was the main driver of krill's seasonal physiological functions (62 [↗](#)), later studies have revealed that photoperiod, likely in concert with circannual timing, drives the seasonal regulation of key lifecycle functions (26 [↗](#), 32 [↗](#)–35 [↗](#)).

Although the exact molecular mechanism of seasonal timing is still unknown, there is clear evidence that the circadian clock measures day length and provides information about the seasonal progression (39, 40). Several studies in insects (63) and plants (64) support the external coincidence model (65). This model explains how the circadian clock regulates the expression of a light-sensitive substance during the late dark phase. Under long days, this substance is degraded by morning light, suppressing a winter response, while the substance accumulates under short days (and long nights), inducing a winter-like response. In the case of krill, this response could include sexual regression, increased lipid accumulation (28, 33), and a reduction in overall metabolism (26, 35).

Our data on krill circadian swimming activity of animals sampled from the field in different seasons show a remarkably stable peak in late-night activity (Fig. 5e-h). Assuming that the behavioral activity under constant darkness reflects the direct influence of the circadian clock, our findings suggest that the krill circadian clock can measure the length of the day (or night) via external coincidence. The increased rhythmicity and synchronization of animals under shortening photoperiods indicate the potential of precise daylength measurement especially during seasonal physiological changes (26). The day length information could subsequently be used directly to synchronize physiological functions with the environment, and/or indirectly to entrain a circannual clock (66), which subsequently drives rhythmic annual output. While the individual activity recordings provide first insights into potential mechanisms for the control of daily and seasonal processes in krill, it must be noted that behavioral output does not always reflect the detailed characteristics of the circadian oscillator (67). Further investigations into the molecular clock mechanism and its downstream functions across seasons are needed to clarify the involvement of the circadian clock in photoperiodism and circannual rhythms in krill.

Circadian clocks have the potential to anticipate the daily light-dark cycle and thereby enable a fine-tuned synchronization of behavioral and physiological functions with environmental cycles, which increases an organism's fitness (38). Consequently, synchronizing krill behavioral output with the day-night cycle provides potential adaptive advantages on various levels.

DVM of zooplankton seems to be a trade-off between predation risk and feeding (16). As it is complex to directly sense predation risk, light is a robust proxy (68) and a circadian clock provides an additional mechanism to anticipate and synchronize the times of vertical migration, active feeding and foraging with nighttime. Our data show that light, in addition to the clock, ensures robust, synchronized activity patterns. Differences between entrained and free-running conditions further indicate that evening and late-night activity are under clock control. At the same time, the activity peak in the morning is a response to the onset of light. A differential regulation of certain phases of DVM has previously been suggested for marine zooplankton species (17, 44). A clock driven evening ascent would allow for the anticipation of sunset and thus the timely initiation of migration when krill swarms are in deeper water layers, resulting in optimized surface feeding time. The clock-controlled late-night activity could support a second feeding bout and preparation for a timely morning descent before sunrise increases visual predation risk. Interestingly, the decrease in clock-controlled swimming activity during the early night, right after the evening activity bout, may further facilitate a phenomenon called “midnight sinking”, which describes the sinking of animals to intermediate depths after the evening ascent, followed by a second rise to the surface before the morning descent. This behavior has been observed in a number of zooplankton species, including calanoid copepods (see 69, 70 and references therein) and krill (71). While previous studies suggested several exogenous factors, such as satiation or predator presence, as drivers of the midnight sink (69, 70), our study suggests that this pattern may be partly under endogenous control. In-situ hydroacoustic observations have demonstrated increased swimming speeds during nighttime for both *M. norvegica* and *E. superba* (50, 51). This increase is likely related to active foraging and feeding behavior, as higher swimming speeds increase the chance of encountering food particles and thus

increase food uptake. Nevertheless, swimming in krill is energetically costly and can make up more than 70% of the total energy expenditure (72 [↗](#)). Reducing swimming activity to the baseline level during the day thus represents a significant opportunity for energy conservation.

At high latitudes, biological clocks are exposed to extreme changes in photoperiod throughout the year, including phases of midnight sun and polar night. Many organisms inhabiting polar regions show arrhythmicity, at least during parts of the year, and potential mechanisms include direct impacts on the central oscillator and uncoupling between output functions and the circadian clock (60 [↗](#)). Previous investigations of krill clock gene expression in the laboratory indicated a weaker oscillation of the krill clock and metabolic output functions under simulated extreme photic conditions (i.e. LL and LD 3:21), compared to more balanced day-night cycles (73 [↗](#)). Data on the transcriptomic and organismic level further indicate that krill can flexibly adapt to the photic conditions at different latitudes (31 [↗](#), 33 [↗](#)). This aligns with our findings, where krill, sampled across a wide range of photoperiods, remain behaviorally synchronized with the local photoperiod under entrained conditions, but show reduced endogenous rhythmicity under DD after entrainment to long day conditions. Krill inhabits a broad range of latitudes, including regions with clear day-night cycles and regions with polar night and midnight sun during the seasonal extremes (74 [↗](#)). At the same time krill is transported rapidly between regions via ocean currents (75 [↗](#)), potentially exposing the same individuals to various photic conditions during their life span. A weak circadian clock in close interaction with the ambient light regime could thus allow krill to adopt the best strategy for the environmental conditions prevailing in the current location. For example, at high latitudes, the adaptive advantage of predator evasion with DVM diminishes in summer when the upper water column is illuminated constantly. In this case, arrhythmic behavior would allow flexibly exploiting food patches at any time of day, when the need for food is high to fuel reproductive processes (76 [↗](#)). Further, a study on the marine annelid *Platynereis dumerilii* recently highlighted a differential regulation of clock-controlled processes, where behaviorally arrhythmic individuals show increased rhythmicity in physiological output (67 [↗](#)), indicating the possibility of flexibly adapting timed processes to environmental conditions.

Adapting to a fluctuating environment involves significant seasonal physiological changes in krill to survive the harsh, food-scarce winter (26 [↗](#), 77 [↗](#)). Crucial processes, such as accumulating enough lipids, must begin long before winter, requiring an anticipatory mechanism. Recent evidence suggests that photoperiod and circannual timing drive these seasonal changes in krill physiology, using photoperiod as a proxy for seasonal progression (26 [↗](#), 32 [↗](#)–35 [↗](#)). Rapid warming in the Southern Ocean is observed to cause shifts in phytoplankton bloom timing (78 [↗](#)), which may lead to temporal mismatches between photoperiod synchronized krill physiology and their primary food source, with the potential to reduce reproductive success (42 [↗](#)) and negatively impact the krill-dependent ecosystem. Understanding the mechanistic basis of species adaptation and their flexibility is therefore crucial in times of rapid environmental change (42 [↗](#)).

We used a new krill activity monitor to identify the endogenous underpinnings of swimming activity of wild-caught Antarctic krill. The results of our experiments provide clear evidence that a circadian clock underlies krill behavior, driving a distinct bimodal pattern of clock-controlled swimming activity. The damping of the activity amplitude under constant darkness suggests that krill possesses a “weak”, highly flexible circadian clock adapted to a life under extreme and variable conditions. These findings are further supported by experiments conducted under simulated short-day and long-day conditions, demonstrating a flexible adaptation of the krill swimming activity pattern to a wide range of photoperiods. The results provide novel insights into the combined impacts of the circadian clock and light on the daily regulation of swimming activity. Furthermore, hydroacoustic recordings demonstrate that most krill swarms sampled exhibited synchronized DVM in the field in the days directly before sampling for behavioral experiments, indicating that in this region, krill remain behaviorally synchronized across a wide range of photoperiods. Seasonal recordings of field-entrained circadian swimming activity suggest that the krill circadian clock may be involved in measuring day length and, consequently, the timing of

seasonal events. The findings provide novel insights into the mechanistic underpinnings of daily and seasonal timing in Antarctic krill, a marine pelagic key species, endemic to a high-latitude region. Mechanistic studies are a prerequisite for understanding how krill adapt to their specific environment and their flexibility in responding to environmental changes.

Methods

Animal collection

Sampling of Antarctic krill (*Euphausia superba*) for experimental purposes was conducted at various points throughout the seasonal cycle from the Bransfield Strait and South Orkney Island regions (for detailed locations, please refer to **Tab. 1** and **Fig. S1**) by use of the continuous fishing system onboard the krill fishing vessel Antarctic Endurance. During fishing, the vessel trawled at a speed of 1.5–2 knots using a commercial trawl. The krill was pumped on board by creating a vacuum in a hose connected to the cod-end of the trawl. On board, the krill was separated from the water on a metal grate, from which the krill was sampled. Sampled krill were kept in surface seawater at densities of ~1 Ind. per L, at 1°C under constant darkness for an acclimation period between 4 - 10 h, to reduce the impact of sampling stress and to check for the condition of krill individuals before transfer to the experimental set-up. Prior to the start of each experiment, the experimental columns were filled with seawater sampled from the surface. For experiments conducted during summer, late summer and autumn, when high food concentrations were to be expected, seawater was filtered through a 0.25 µm filter. During winter, field measurements showed a very low surface chlorophyll a level (below 0.5 µg/L; data not shown) and any potential larger particles in the water were excluded by sedimentation. The columns were distributed to the set-up and the temperature was adjusted to 0.8°C. Only krill that were unharmed and lively were selected for behavioral experiments in the set-up. At the end of each experiment, the krill were removed from the set-up, evaluated for overall condition and total length (measured from the front of the eye to the tip of the telson, excluding setae), and their sex was determined under a stereo microscope.

Krill swimming activity recording and experimental design

Krill swimming activity was recorded using the AMAZE setup, described in detail in Hüppe et al. (43). In short, the recording principle is based on krill swimming in vertical acrylic experimental columns (height: 80 cm, diameter: 9 cm). Each column contains five infrared (IR) detector modules, equally spaced over the height of the column. Vertical movements of krill are detected by IR light beam breaks. A computer on each column controls data acquisition and stores beam break data. The experimental columns are placed in a compressor cooled incubator, which allows for a precise temperature control. Programmable LED light bars in the top of the incubator, simulate underwater light spectra and daily light intensity cycles.

To investigate the behavioral activity of individual krill under light-dark conditions, 12 krill were exposed to three days of simulated short-day conditions (short day treatment), and 10 krill to 5 days of simulated long-day conditions (long day treatment). Due to technical problems, only data from 11 krill were analyzed from experiment 1 and data from 9 krill from experiment 2. Light-dark cycles were adjusted to a photoperiod of 5.5 h simulating short days and 15 h simulating long days. Day length was arranged symmetrically around local solar noon, to approximate the natural photoperiodic light regime at the time of sampling. The intensity and spectrum in the activity monitor were adjusted to field measurements, as described in Hüppe et al. (43). In short, light regime increased and decreased in a linear fashion from a maximum light intensity of 8.8 mW·m⁻² during midday. To further investigate the involvement of an endogenous clock in krill activity, the initial phase of day-night simulations was followed by 5 days of constant darkness conditions in both experiments.

To investigate seasonal changes in krill circadian activity, four additional experiments were conducted, namely summer treatment (sampling date: January 22nd 2022), late summer treatment (sampling date: March 1st 2022), autumn treatment (sampling date: March 15th 2022) and winter treatment (sampling date: June 10th 2022). For these experiments, krill were caught at the dates described above and after acclimation, 10 animals per experiment were distributed to the activity monitor, where their activity was recorded for 4 to 8 days under constant darkness conditions (see **Tab. 1** [for details](#)).

Behavioral data analysis

Experiment 1 (LD-DD)

Swimming activity of krill individuals was calculated from raw beam breaks as described in Hüppe et al. (43 [43](#)). In short, we only considered upward swimming movements of individuals to separate baseline activity from increased activity, as krill are negatively buoyant. To accomplish this, we organized the raw beam break data from all five detector modules in each experimental column in chronological order. We selected only those beam break detections that occurred after a detection in the detector module positioned lower on the column. Like this, we consider upward swimming movements throughout the full height of the column. Beam breaks caused by upward swimming were summed over 10-minute intervals and normalized between 0 and 1 for each individual. Data were smoothed by a centered moving average, with a smoothing window of 6 data points under light-dark simulations and 24 data points under constant darkness conditions.

To calculate the group activity of rhythmic krill under DD conditions, we first detrended the normalized swimming activity data of each individual to account for individual variability in baseline shifts of activity over time. This was done by subtracting the daily mean activity from each activity value of the same day. As the individual free running period (FRP) varies between individuals, in the next step we corrected the data for each individual's FRP and assigned them to a 24 h day. To achieve this, we tested the detrended and smoothed activity data of each krill for rhythmic activity and determined the period of the rhythm using the Lomb-Scargle-Periodogram analysis (LSP). We considered individuals with a period τ between 20 and 28 hours, a power of ≥ 50 , and a p-value of less than 0.01 to be significantly rhythmic. The activity data of each rhythmic krill were subsequently assigned a new modified timestamp to cover the individual FRP within a 24-hour experimental day. This maintains the original resolution of the time series data while allowing for comparative analysis of individuals with differing FRPs. Group behavior was determined by calculating the mean and standard error of the mean (s.e.m.) per 10-minute time interval and subsequent smoothing, as described above. Average day analysis for a group of krill under LD cycles was done by determining the mean and s.e.m. per 10-minute time interval of a 24 h day over all experimental days and all individuals in one experiment and subsequent smoothing. Average day analysis for a group of krill under DD conditions were done in the same way, but based on the detrended and FRP-corrected data. The amplitude of swimming activity under LD and DD conditions was based on individual, normalized, detrended, and smoothed activity data, and was calculated for every experimental day as the difference between the daily maximal and minimal swimming activity. Differences in the activity amplitude between light conditions (i.e. LD vs. DD) were tested with the Mann-Whitney-U-Test and amplitude differences between the first and last day under DD conditions were tested with a paired t-test (R *stats* package version 4.1.2). All statistical analysis assumed a significance level of $p > 0.05$.

Experiment 2 (DD)

Group activity and average day activity for seasonal experiments (Experiment 2, summer, late summer, autumn, and winter treatment) was done as for DD conditions described above, but only the first 4 days of activity of each experiment were considered. This was to allow for a better comparison and to account for the effect of damping rhythms after several days under DD.

All data handling, analysis and visualization was done with the *R programming language* (version 4.1.2 (79 [link](#))) in *RStudio* (version 2023.12.1.402), using the *tidyverse* package (version 2.0.0 (80 [link](#))). Rhythm analysis and period estimation was done with the R package *lomb* (81 [link](#)), local sun data (i.e. sunset and sunrise) were retrieved from the *suncalc* package (version 0.5.1).

Hydroacoustic data recording and visualization

Hydroacoustic data were collected using a hull-mounted SIMRAD ES80 echosounder (Kongsberg Maritime AS) aboard the Antarctic Endurance, covering three days before the sampling for each of the seasonal behavioral experiments of experiment 2. The signal received from the 200 kHz band was used to visualize the vertical distribution of krill swarms beneath the ship. The raw acoustic data from the summer, late summer, and autumn periods were converted to mean volume backscattering strength and binned to a time resolution of 1 s and depth bins of 0.5 m using Echopype (82 [link](#)). For the period of winter, raw acoustic data were not available. An alternative approach was employed, utilizing a novel method proposed by Bahlburg et al. (83 [link](#)), to reconstruct the backscattering signal from a dataset of screenshots displaying the visualized hydroacoustic signal of the echosounder. We only included data during active fishing periods and the vessel is specifically targeting *E. superba*, which occurs in large monospecific aggregations. Further, krill fishery bycatch rates are very low (0.1-0.3%, 84), which makes it highly probable that the recorded signal represents krill swarms. Data handling and visualization were done with R in RStudio, using the packages *tidyverse* and *scico* (version 1.3.1).

Data availability

The datasets and analysis scripts used for the analysis of krill swimming activity, as well as of hydroacoustic recordings are available on the Zenodo repository (<https://zenodo.org/records/14766951> [link](#)). Supplementary figures are available in the Supplementary Material file.

Supplementary figures

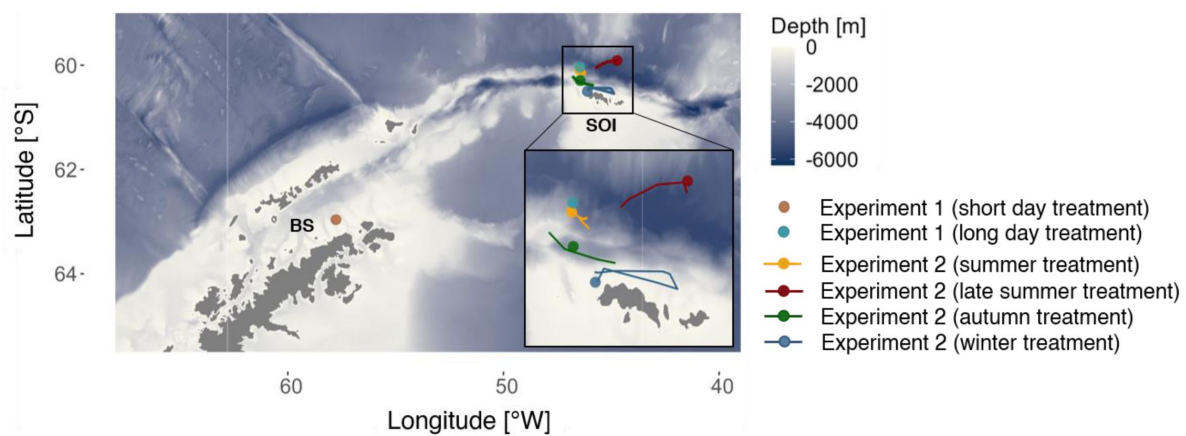


Figure S1

Locations of krill sampling and hydroacoustic recordings for behavioral experiments.

Colored dots indicate sampling locations for the respective behavioral experiments. Colored lines indicate the cruise track of the fishing vessel during recording of hydroacoustic data prior to sampling for behavioral experiment 2. BS: Bransfield Strait. SOI: South Orkney Islands. Bathymetric data were retrieved from the International Bathymetric Chart of the Southern Ocean (500m× 500 m resolution, <https://ibcsa.org/>).

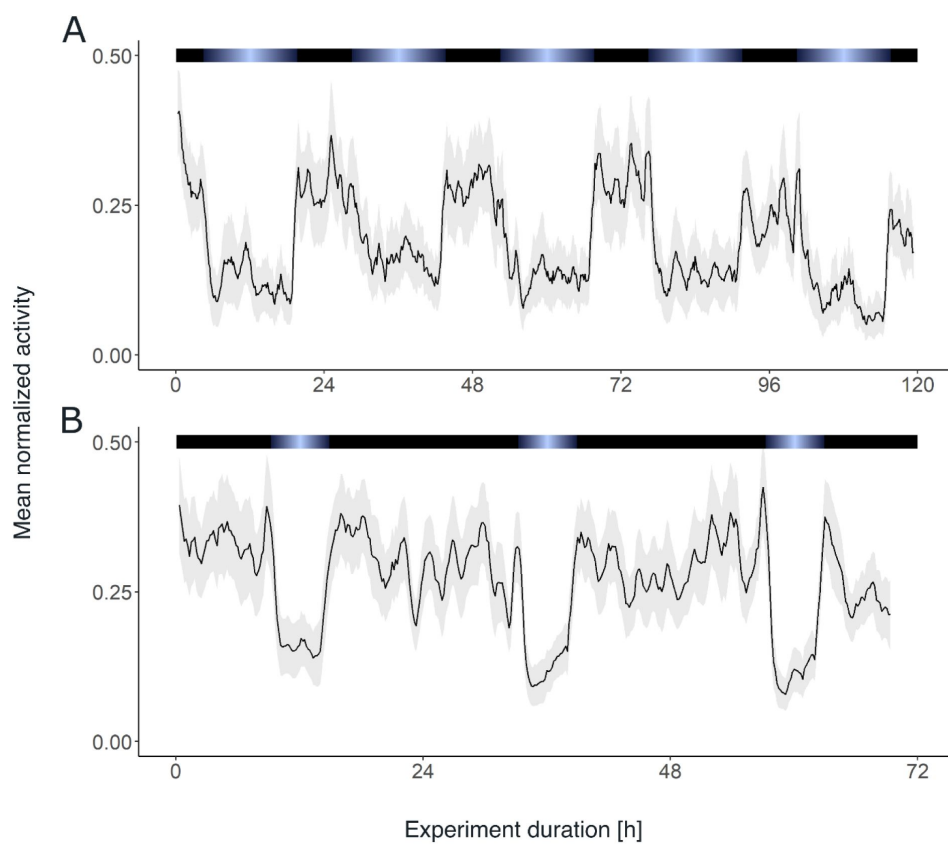


Figure S2

Krill group activity under LD conditions.

Group mean swimming activity during experiment 1 of krill under long-day (n=9, A) and short-day (n=11, B) simulations. Shading around the line depicts the standard error of the mean (s.e.m.). Color bars at the top indicate the light regime provided. Data for (A) from Hüppe et al. (43 [link](#)).

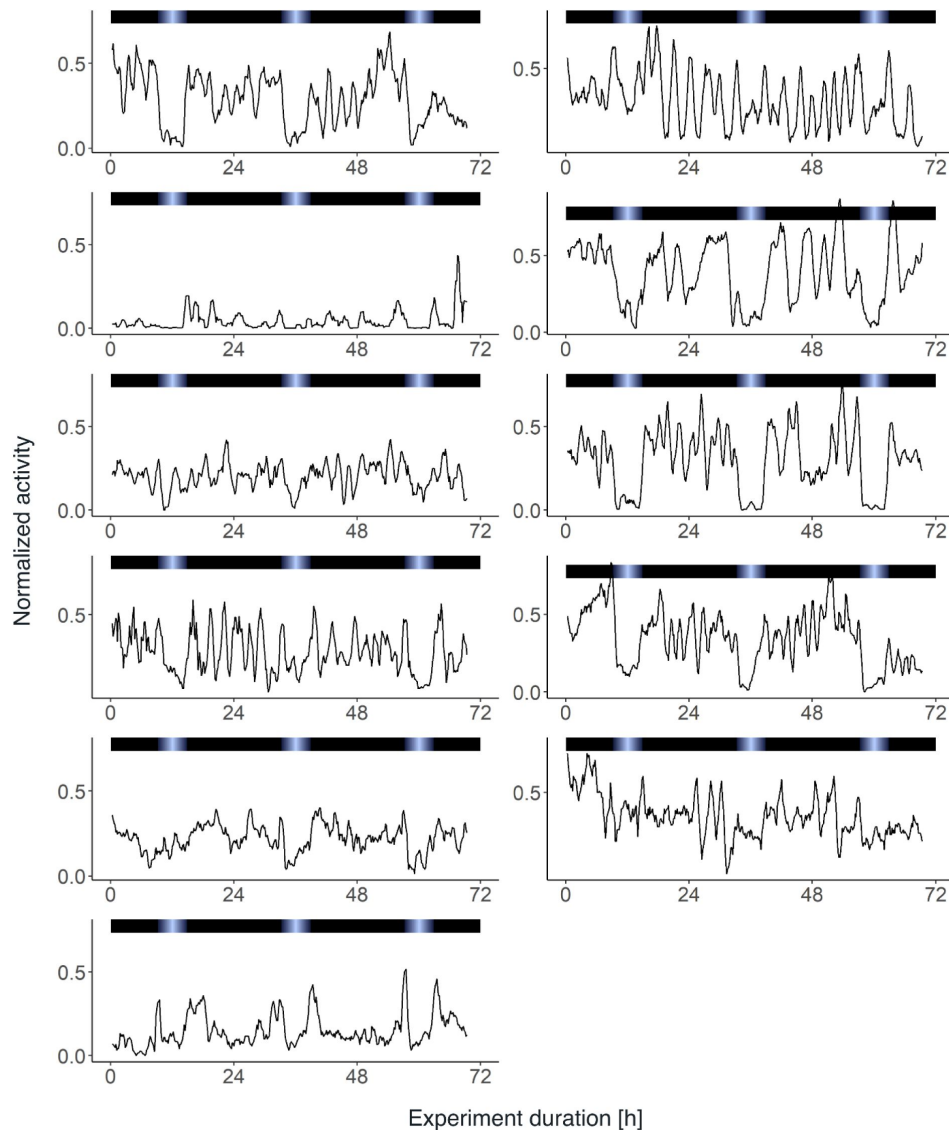


Figure S3

Swimming activity of individual krill under LD conditions.

Normalized swimming activity during experiment 1 under 5 days of simulated short-day conditions for each individual. Color bars at the top indicate the light regime provided.

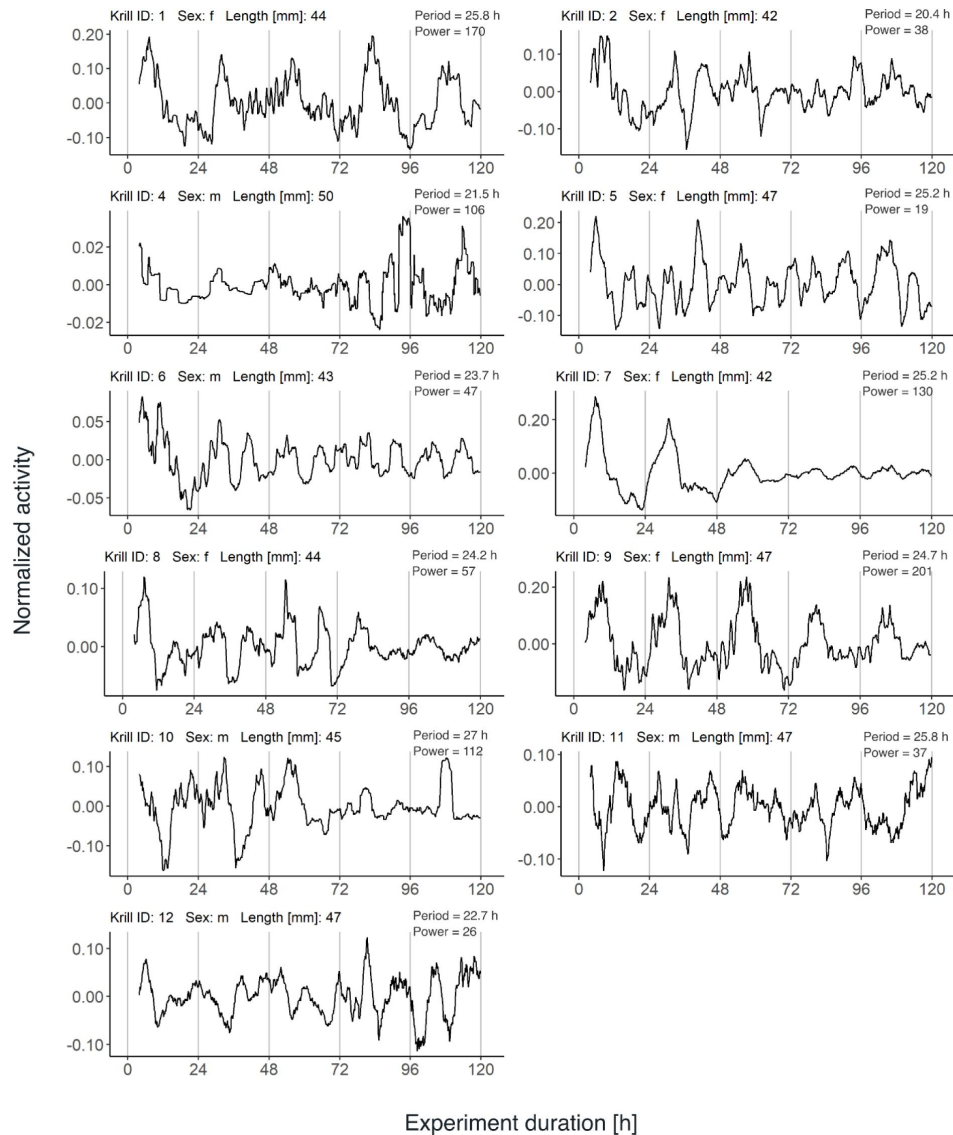


Figure S4

Swimming activity of individual krill under DD conditions.

Normalized and detrended swimming activity under 5 days of constant darkness (DD) after entrainment to simulated short-day conditions (experiment 1) for each individual. Period and power estimates from Lomb-Scargle Periodogram (LSP) analysis are detailed for each individual.

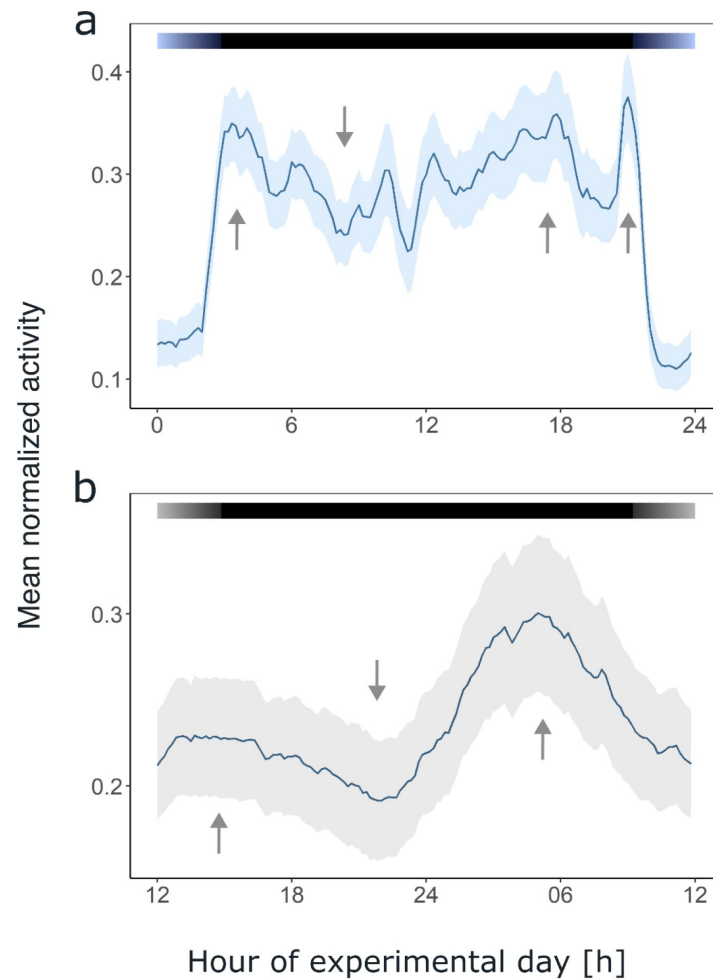


Figure S5

Comparison of daily swimming activity between LD and DD conditions.

Group mean swimming activity of rhythmic individuals over an average day under 3 days of simulated short-day conditions (A) and 5 days of constant darkness (B) during the short day treatment of experiment 1. Color bars at the top indicate the light regime provided, as well as subjective day and night. Arrows indicate times of increased or decreased activity which lead to the characteristic activity pattern during the dark phase. Shading indicates the s.e.m..

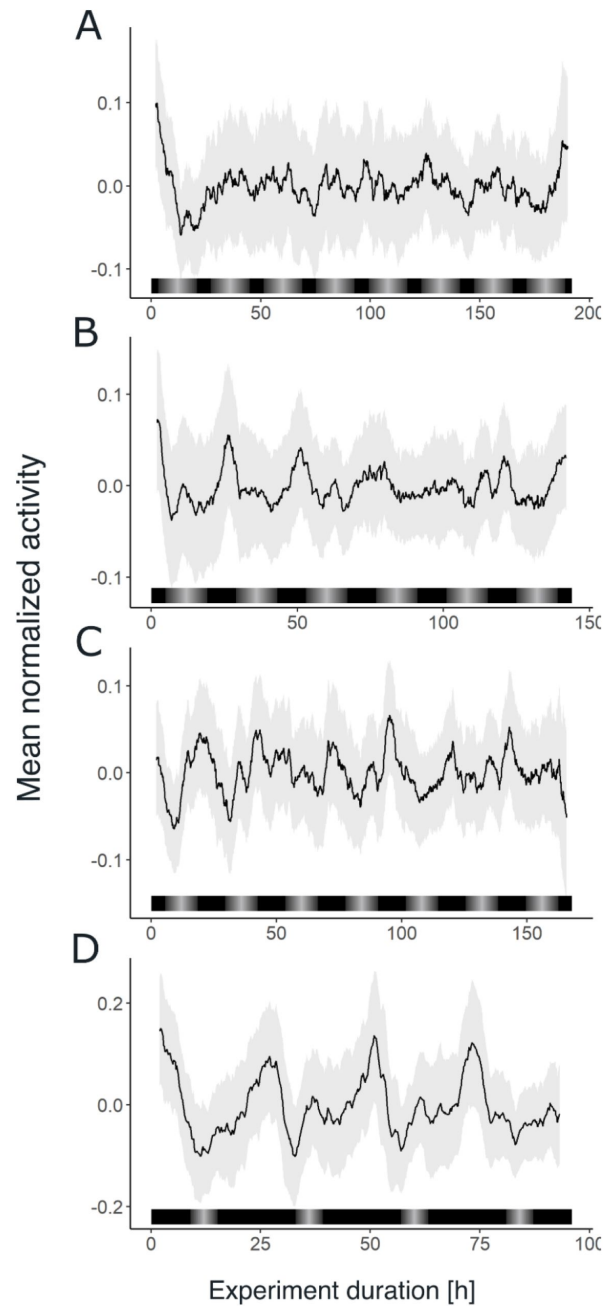


Figure S6

Group swimming activity under DD conditions across seasons.

Group mean swimming activity of rhythmic individuals under DD conditions after individuals have been sampled from their natural environment during summer (A), late summer (B), autumn (C), and winter (D) for experiment 2. Grey shading indicates the s.e.m..

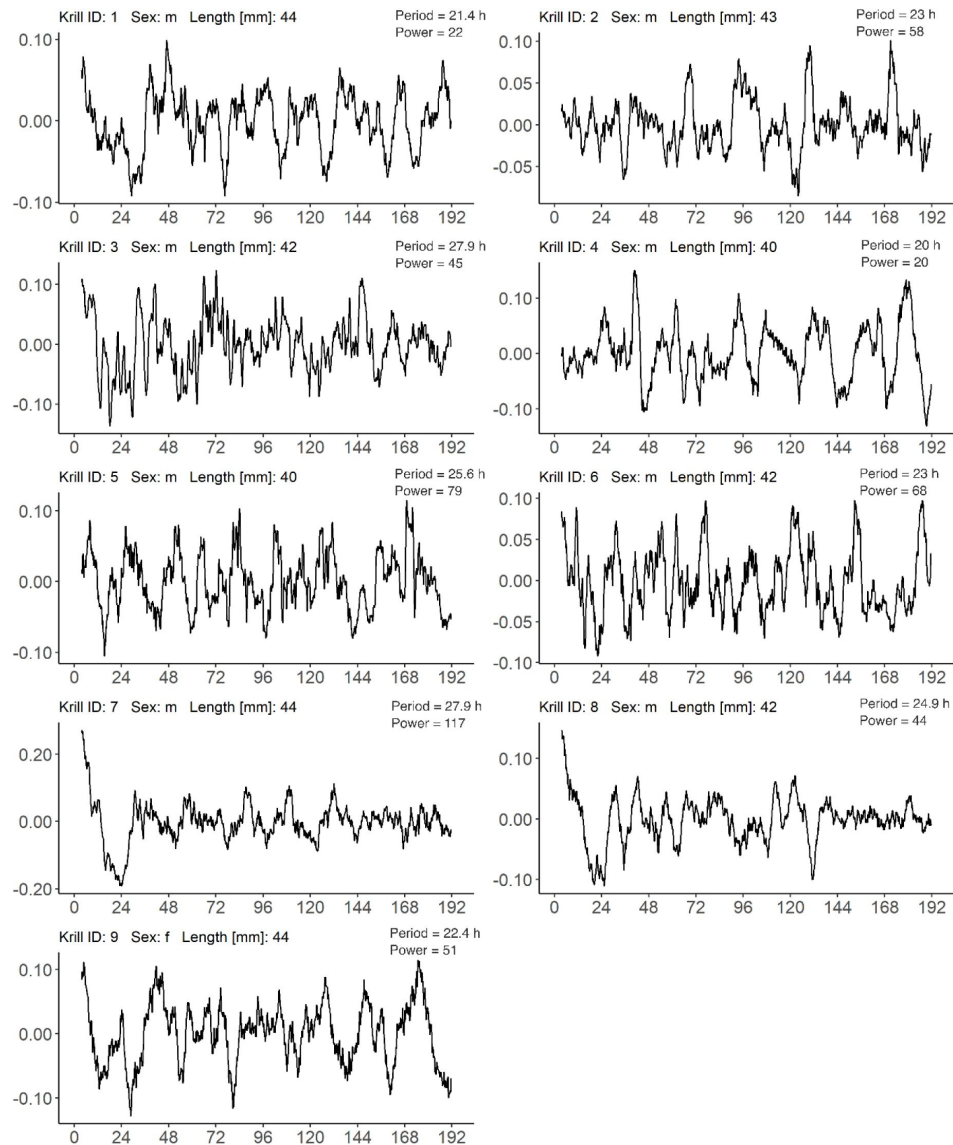


Figure S7

Individual swimming activity under DD conditions of krill sampled in Summer.

Normalized and detrended swimming activity under 8 days of constant darkness (DD) after individuals have been sampled from their natural environment during the summer treatment of experiment 2 (photoperiod: 17.5 h). Period and power estimates from LSP analysis of the first four days under DD are detailed for each individual.

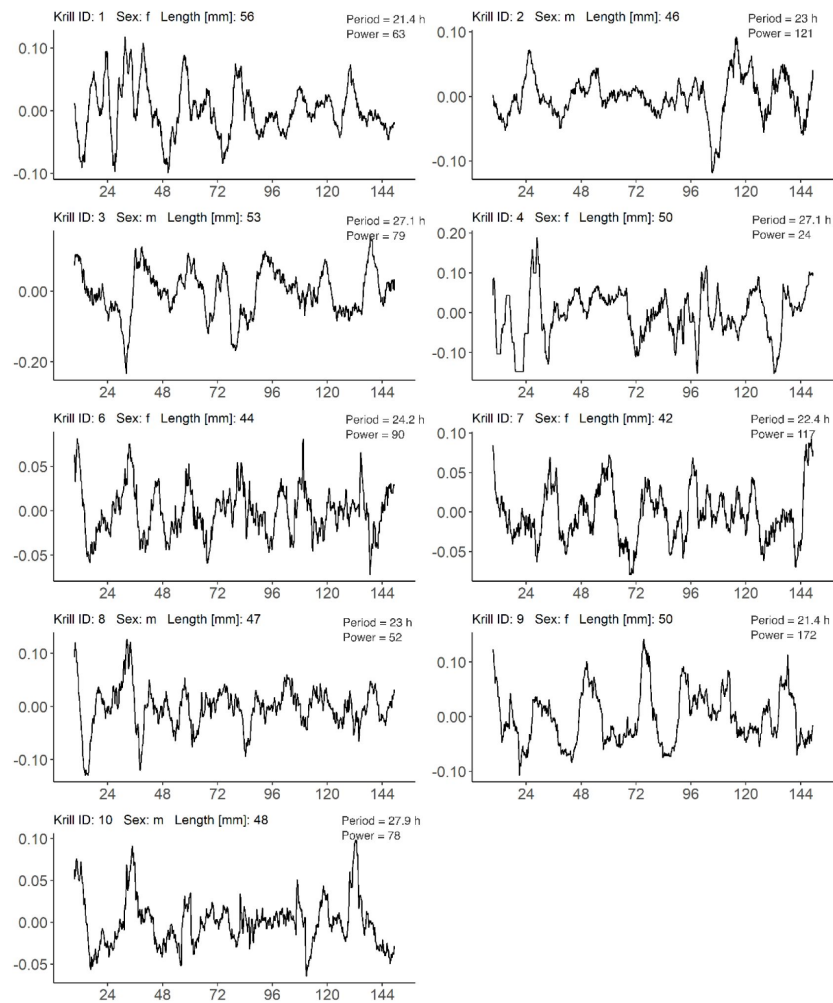


Figure S8

Individual swimming activity under DD conditions of krill sampled in Late Summer.

Normalized and detrended swimming activity under 6 days of constant darkness (DD) after individuals have been sampled from their natural environment during the late summer treatment of experiment 2 (photoperiod: 14 h). Period and power estimates from LSP analysis of the first four days under DD are detailed for each individual.

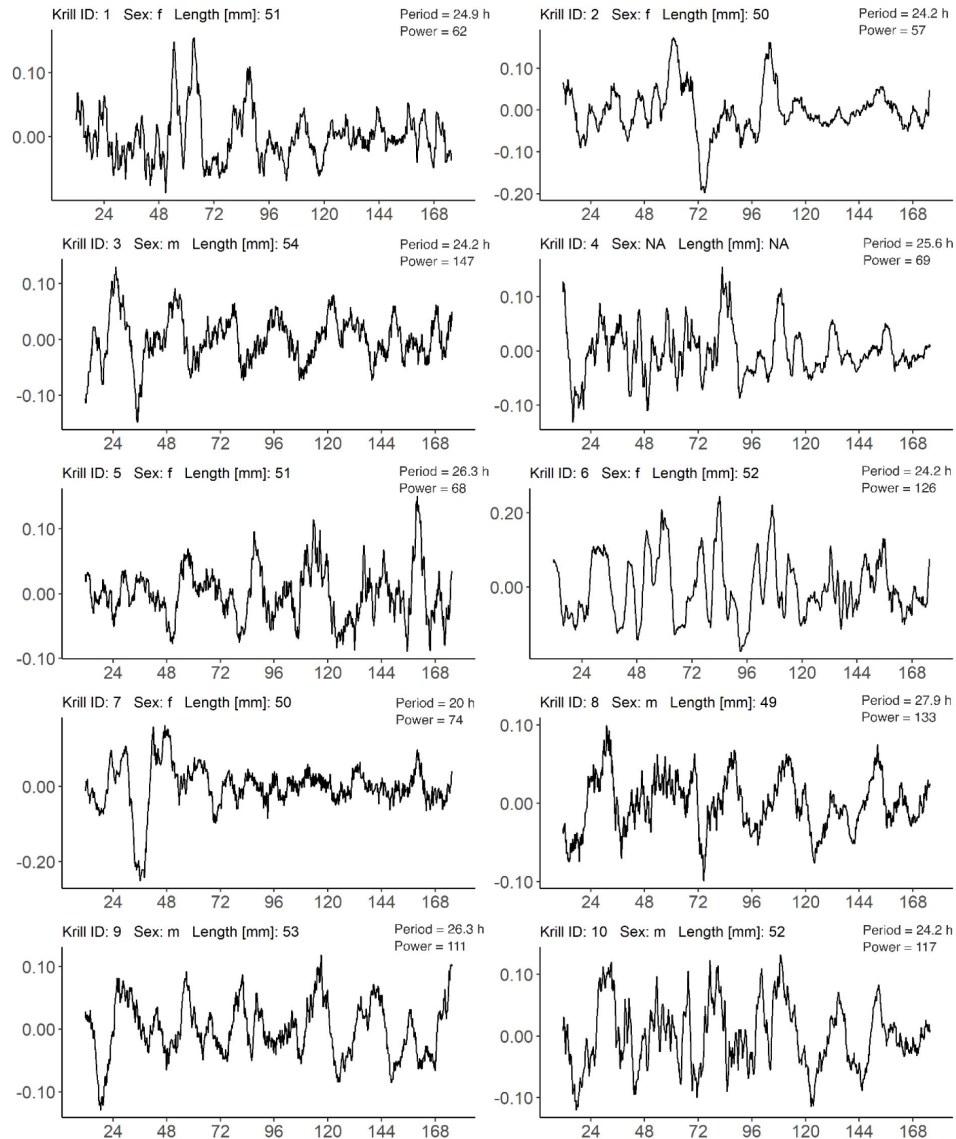


Figure S9

Individual swimming activity under DD conditions of krill sampled in Autumn.

Normalized and detrended swimming activity under 7 days of constant darkness (DD) after individuals have been sampled from their natural environment during the autumn treatment of experiment 2 (photoperiod: 12.6 h). Period and power estimates from LSP analysis of the first four days under DD are detailed for each individual.

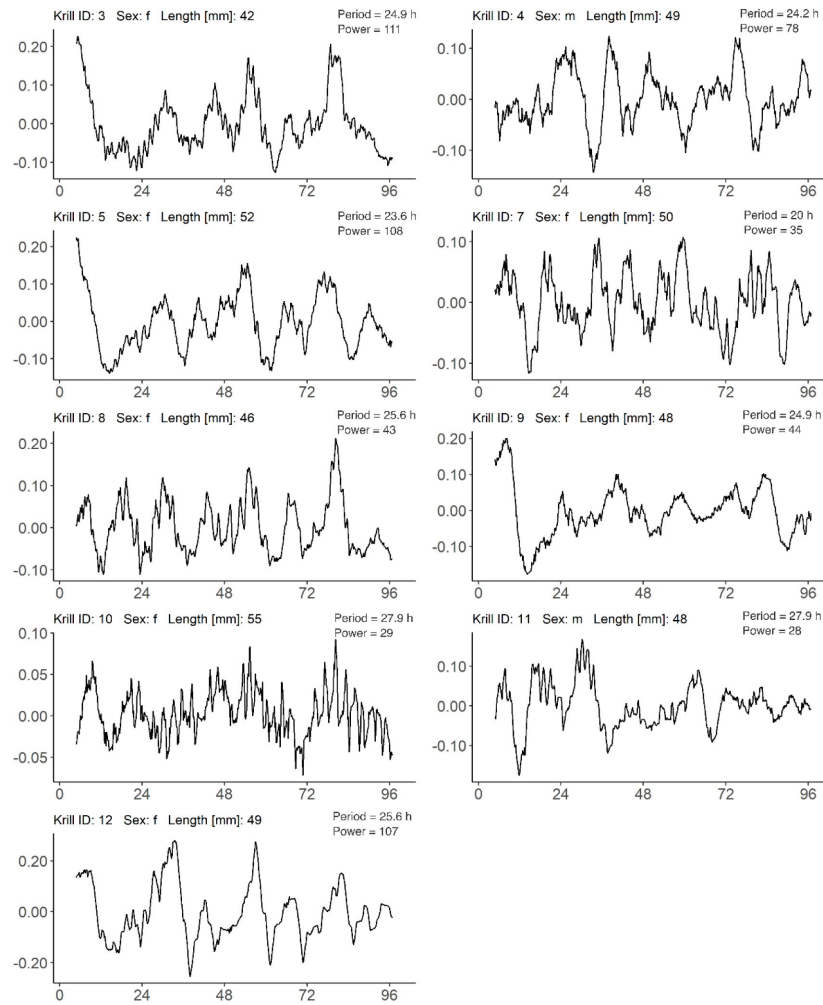


Figure S10

Individual swimming activity under DD conditions of krill sampled in Winter.

Normalized and detrended swimming activity under 4 days of constant darkness (DD) after individuals have been sampled from their natural environment during the winter treatment of experiment 2 (photoperiod: 5.9 h). Period and power estimates from LSP analysis of the first four days under DD are detailed for each individual.

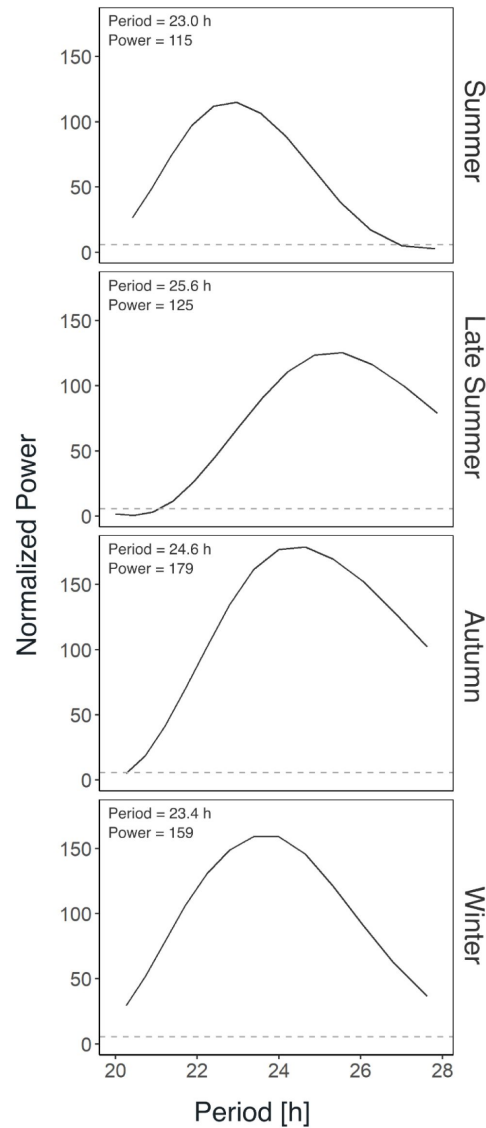


Figure S11

Rhythm analysis of group swimming activity under DD conditions across seasons.

Periodogram derived from Lomb-Scargle Periodogram analysis (LSP) of group mean swimming activity under DD conditions after individuals have been sampled from their natural environment in different seasons for experiment 2 (see [fig. S6](#)).

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Additional information

Author Contributions

LH, BM and CHF designed the study. LH, RD and DB conducted the behavioral experiments. DB processed and analyzed the hydroacoustic data. LH analyzed the behavioral data and wrote the initial draft of the manuscript. All authors revised the initial draft and approved the submitted version.

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Reviewer #1 (Public review):

Hüppe and colleagues had already developed an apparatus and an analytical approach to capture swimming activity rhythms in krill. In a previous manuscript they explained the system, and here they employ it to show a circadian clock, supplemented by exogenous light, produces an activity pattern consistent with "twilight" diel vertical migration (DVM; a peak at sunset, a midnight sink, and a peak in the latter half of the night).

They used light:dark (LD) followed by dark:dark (DD) photoperiods at two times of the year to confirm the circadian clock, coupled with DD experiments at four times a year to show rhythmicity occurs throughout the year along with DVM in the wild population. The individual activity data show variability in the rhythmic response, which is expected. However, their results showed rhythmicity was sustained in DD throughout the year, although the amplitude decayed quickly. The interpretation of a weak clock is reasonable, and they provide a convincing justification for the adaptive nature of such a clock in a species that has a wide distributional range and experiences various photic environments. These data also show that exogenous light increases the activity response and can explain the morning activity bouts, with the circadian clock explaining the evening and late-night bouts. This acknowledgement that vertical migration can be driven by multiple proximate mechanisms is important.

The work is rigorously done, and the interpretations are sound. I see no major weaknesses in the manuscript. Because a considerable amount of processing is required to extract and interpret the rhythmic signals (see Methods and previous AMAZE paper), it is informative to have the individual activity plots of krill as a gut check on the group data.

The manuscript will be useful to the field as it provides an elegant example of looking for biological rhythms in a marine planktonic organism and disentangling the exogenous response from the endogenous one. Furthermore, as high-latitude environments change, understanding how important organisms like krill have the potential to respond will become increasingly important. This work provides a solid behavioral dataset to complement the earlier molecular data suggestive of a circadian clock in this species.

<https://doi.org/10.7554/eLife.103096.2.sa2>

Reviewer #2 (Public review):**Summary:**

This manuscript provides experimental evidence on circadian behavioural cycles in Antarctic krill. The krill were obtained directly from krill fishing vessels and the experiments were carried out on board using an advanced incubation device capable of recording activity levels over a number of days. A number of different experiments were carried out where krill were first exposed to simulated light:dark (L:D) regimes for some days followed by continuous darkness (DD). These were carried out on krill collected during late autumn and late summer. A further set of experiments was performed on krill across three different seasons (summer, autumn, winter), where incubations were all DD conditions. Activity was measured as the frequency by which an infrared beam close to the top of the incubation tube was broken over unit time. Results showed that patterns of increased and decreased activity that appeared synchronised to the LD cycle persisted during the DD period. This was interpreted as evidence of the operation of an internal (endogenous) clock. The amplitude of the behavioural cycles decreased with time in DD, which further suggests that this clock is relatively weak. The authors argued that the existence of a weak endogenous clock is an adaptation to life at high latitudes since allowing the clock to be modulated by external

(exogenous) factors is an advantage when there is a high degree of seasonality. This hypothesis is further supported by seasonal DD experiments which showed that the periodicity of high and low activity levels differed between seasons.

Strengths:

Although there has been a lot of field observations of various circadian type behaviour in Antarctic krill, relatively few experimental studies have been published considering this behaviour in terms of circadian patterns of activity. Krill are not a model organism and obtaining them and incubating them in suitable conditions are both difficult undertakings. Furthermore, there is a need to consider what their natural circadian rhythms are without the overinfluence of laboratory-induced artefacts. For this reason alone, the setup of the present study is ideal to consider this aspect of krill biology. Furthermore, the equipment developed for measuring levels of activity is well-designed and likely to minimise artefacts.

<https://doi.org/10.7554/eLife.103096.2.sa1>

Author response:

The following is the authors' response to the original reviews

Reviewer #1 (Public review):

Hüppe and colleagues had already developed an apparatus and an analytical approach to capture swimming activity rhythms in krill. In a previous manuscript they explained the system, and here they employ it to show a circadian clock, supplemented by exogenous light, produces an activity pattern consistent with "twilight" diel vertical migration (DVM; a peak at sunset, a midnight sink, and a peak in the latter half of the night).

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We appreciate the positive evaluation of our work by Reviewer 1, acknowledging our approach to record locomotor activity in krill and the importance of the findings in assessing krill's potential to respond to environmental change in their habitat.

Reviewer #2 (Public review):

Summary:

This manuscript provides experimental evidence on circadian behavioural cycles in Antarctic krill. The krill were obtained directly from krill fishing vessels and the experiments were carried out on board using an advanced incubation device capable of recording activity levels over a number of days. A number of different experiments were carried out where krill were first exposed to simulated light:dark (L:D) regimes for some days followed by continuous darkness (DD). These were carried out on krill collected during late autumn and late summer. A further set of experiments was performed on krill across three different seasons (summer, autumn, winter), where incubations were all DD conditions. Activity was measured as the frequency by which an infrared beam close to the top of the incubation tube was broken over unit time. Results showed that patterns of increased and decreased activity that appeared synchronised to the LD cycle persisted during the DD period. This was interpreted as evidence of the operation of an internal (endogenous) clock. The amplitude of the behavioural cycles decreased with time in DD, which further suggests that this clock is relatively weak. The authors argued that the existence of a weak endogenous clock is an adaptation to life at high latitudes since allowing the clock to be modulated by external (exogenous) factors is an advantage when there is a high degree of seasonality. This hypothesis is further supported by seasonal DD experiments which showed that the periodicity of high and low activity levels differed between seasons.

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Although there has been a lot of field observations of various circadian type behaviour in Antarctic krill, relatively few experimental studies have been published considering this behaviour in terms of circadian patterns of activity. Krill are not a model organism and obtaining them and incubating them in suitable conditions are both difficult undertakings. Furthermore, there is a need to consider what their natural circadian rhythms are without the overinfluence of laboratory-induced artefacts. For this reason alone, the setup of the present study is ideal to consider this aspect of krill biology. Furthermore, the equipment developed for measuring levels of activity is well-designed and likely to minimise artefacts.

We would like to thank Reviewer 2 for their positive assessment of our approach to study the influence of the circadian clock on krill behavior. We are delighted, that Reviewer 2 found our mechanistic approach in understanding daily behavioral patterns of Antarctic krill using the AMAZE set-up convincing, and that the challenging circumstances of working with a polar, non-model species are acknowledged.

Weaknesses

I have little criticism of the rationale for carrying out this work, nor of the experimental design. Nevertheless, the manuscript would benefit from a clearer explanation of the experimental design, particularly aimed at readers not familiar with research into circadian rhythms. Furthermore, I have a more fundamental question about the relationship between levels of activity and DVM on which I will expand below. Finally, it was unclear how the observational results made here related to the molecular aspects considered in the Discussion.

(1) Explanation of experimental design - I acknowledge that the format of this particular journal insists that the Results are the first section that follows the Introduction. This nevertheless presents a problem for the reader since many of the concepts and terms that would generally be in the Methods are yet to be explained to the reader. Hence, right from the start of the Results section, the reader is thrown into the detail of what happened during the LD-DD experiments without being fully aware of why this type of experiment was carried out in the first place. Even after reading the Methods, further explanation would have been helpful. Circadian cycle type research of this sort often entrains organisms to certain light cycles and then takes the light away to see if the cycle continues in complete darkness, but this critical piece of knowledge does not come until much later (e.g. lines 369-372) leaving the reader guessing until this point why the authors took the approach they did. I would suggest the following (1) that more effort is made in the Introduction to explain the exact LD/DD protocols adopted (2) that a schematic figure is placed early on in the manuscript where the protocol is explained including some logical flow charts of e.g. if behavioural cycle continues in DD then internal clock exists versus if cycle does not continue in DD, the exogenous cues dominate - followed by - major decrease in cyclic amplitude = weak clock versus minor decrease = strong clock and so on

We want to thank Reviewer 2 for pointing out that the experimental design and its rationale are not becoming clear early in the manuscript, especially for people outside the field of chronobiology. We added a new figure (now Fig. 1), illustrating the basic principle of chronobiological study design and how we adopted it. We also extended the description at the beginning of the Results section to clarify the rationale behind the experimental design.

(2) Activity vs kinesis - in this study, we are shown data that (i) krill have a circadian cycle - incubation experiments; (ii) that krill swarms display DVM in this region - echosounder data (although see my later point). My question here is regarding the relationship between what is being measured by the incubation experiments and the in situ swarm behaviour observations. The incubation experiments are essentially measuring the propensity of krill to swim upwards since it logs the number of times an individual (or group) break a beam towards the top of the incubation tube. I argue that krill may be still highly active in the rest of the tube but just do not swim close to the surface, so this approach may not be a good measure of "activity". Otherwise, I suggest a more correct term of what is being measured is the level of "upward kinesis". As the authors themselves note, krill are negatively buoyant and must always be active to remain pelagic. What changes over the day-night cycle is whether they decide to expend that activity on swimming upwards, downwards or remaining at the same depth. Explaining the pattern as upward kinesis then also explains by swarms move upwards during the night. Just being more active at night may not necessarily result in them swimming upwards.

We believe there is a slight misunderstanding in how what we call “activity” is measured. The experimental columns are equipped with five detector modules, evenly distributed over the height of the column. In our analysis we count all beam breaks caused by upward movement, i.e. every time a detector module is triggered after a detector module at a lower position has been triggered, and not only when the top detector module is triggered. In this way, we record upward swimming movements throughout the column, and not only when the krill swims all the way to the top of the column. This still means that what we are measuring is swimming activity, caused by upward swimming. We use this measure, to deliberately separate increased swimming activity, from baseline activity (i.e. swimming, which solely compensates for negative buoyancy) and inactivity (i.e. passive sinking).

Higher activity is thus at first interpreted as an increase in swimming activity, which in the field may result in upwards-directed swimming but also could mean a horizontal increase in activity, for example, representing increased foraging and feeding activity. This would explain the daily activity pattern observed under LD cycles (now Fig. 3), which shows a general increase in activity during the dark phase. This nighttime increase could be used for both upward directed migration during sunset and horizontal directed swimming for feeding and foraging throughout the night.

We added the following sentence to the description of the activity metric in the Methods section to clarify this point (lines 465-469):

“To accomplish this, we organized the raw beam break data from all five detector modules in each experimental column in chronological order. We selected only those beam break detections that occurred after a detection in the detector module positioned lower on the column. Like this, we consider upward swimming movements throughout the full height of the column.”

(3) Molecular relevance - Although I am interested in molecular clock aspects behind these circadian rhythms, it was not made clear how the results of the present study allow any further insight into this. In lines 282 to 284, the findings of the study by Biscontin et al (2017) are discussed with regard to how TIM protein is degraded by light via the clock photoreceptor CRYPTOCHROME 1. This element of the Discussion would be a lot more relevant if the results of the present study were considered in terms of whether they supported or refuted this or any other molecular clock model. As it stands, this paragraph is purely background knowledge and a candidate for deletion in the interest of shortening the Discussion.

We agree that this part is not directly related to the data presented in the manuscript. We, therefore, omitted this part in the revised version of the manuscript to keep the discussion concise and focused on the results.

Other aspects

(i) 'Bimodal swimming' was used in the Abstract and later in the text without the term being fully explained. I could interpret it to mean a number of things so some explanation is required before the term is introduced.

We thank the Reviewer for pointing this out. We provided an explanation for the term “bimodal” in the Results section, where the two clock driven activity bouts are described first, by extending the sentence in lines 161-164, which now reads:

“This suggests that the circadian clock drives a distinct bimodal activity pattern with two activity peaks in one day, i.e. the evening and late-night activity bouts, while. In contrast, the morning activity bout is triggered by the onset of illumination in the experimental set-up.”.

(ii) Midnight sinking - I was struck by Figure 2b with regards to the dip in activity after the initial ascent, as well as the rise in activity predawn. Cushing (1951) Biol Rev 26: 158-192 describes the different phases of a DVM common to a number of marine organisms observed in situ where there is a period of midnight sinking following the initial dusk ascent and a dawn rise prior to dawn descent. Tarling et al (2002) observe midnight sinking pattern in Calanus finmarchicus and consider whether it is a response to feeding satiation or predation avoidance (i.e. exogenous factors). Evidence from the present study indicates that midnight sinking (and potential dawn rise) behaviour could alternatively be under endogenous control to a greater or lesser degree. This is something that should certainly be mentioned in the Discussion, possibly in place of the

molecular discussion element mentioned above - possibly adding to the paragraph Lines 303-319.

We would like to thank the Reviewer for pointing this out and agree that adding the idea of an endogenous control of midnight sinking would be interesting to the discussion. We added the following section to the Discussion (lines 335-343):

“Interestingly, the decrease in clock-controlled swimming activity during the early night, right after the evening activity bout, may further facilitate a phenomenon called “midnight sinking”, which describes the sinking of animals to intermediate depths after the evening ascent, followed by a second rise to the surface before the morning descend. This behavior has been observed in a number of zooplankton species, including calanoid copepods (see 69, 70 and references therein) and krill (71). While previous studies suggested several exogenous factors, such as satiation or predator presence, as drivers of the midnight sink (69, 70), our study suggests that this pattern may be partly under endogenous control.”

(iii) Lines 200-207 - I struggled to follow this argument regarding Piccolin et al identifying a 12 h rhythm whereas the present study indicates a ~24 h rhythm. Is one contradicting the other - please make this clear.

In our study, we found that the circadian clock drives a bimodal pattern of swimming activity in krill, meaning it controls two bouts of activity in a 24-hour cycle. Piccolin et al. (2020) identified a swimming activity pattern of ~12 h (i.e. two peaks in 24 h) at the group level, which aligns with our findings at the individual level. We revised the Section in the discussion for more clarity, which now reads:

“Data from Piccolin et al. (20) showed a strong damping of the amplitude and indication of a remarkably short (~12 h) free running period (FRP) of vertical swimming behavior of a group of krill under constant darkness (20). The short period found in Piccolin et al. (20) complements is in line with our findings of a bimodal activity pattern the pattern of swimming activity under DD conditions on the individual level found in the present study, suggesting that the ~12 h rhythm in group swimming behavior in Piccolin et al. (20) could have resulted from a bimodal activity pattern at the individual level, as found in our study.” (lines 212-219).

(iv) Although I agree that the hydroacoustic data should be included and is generally supportive of the results, I think that two further aspects should be made clear for context (a) whether there was any groundtruthing that the acoustic marks were indeed krill and not potentially some other group known to perform DVM such as myctophids (b) how representative were these patterns - I have a sense that they were heavily selected to show only ones with prominent DVM as opposed to other parts of the dataset where such a pattern was less clear - I am aware of a lot of krill research where DVM is not such a clear pattern and it is disingenuous to provide these patterns as the definitive way in which krill behaves. I ask this be made clear to the reader (note also that there is a suggestion of midnight sinking in Fig 5b on 28/2).

To clarify the mentioned points concerning the hydroacoustic data:

a) As mentioned in the Methods section, only hydroacoustic data during active fishing was included in the analysis. *E. superba* occurs in large monospecific aggregations, and the fishery actively targets *E. superba* and monitors their catch and the proportion of non-target species continuously with cameras. Krill fishery bycatch rates are very low (0.1–0.3%, Krafft et al. 2022), and fishing operations would stop if non-target species were caught in significant proportions at any time. Therefore, and supported by our own observations when we

conducted the experiments, we argue that it is a valid assumption that *E. superba* predominantly causes the backscattering signal shown in Figure 5 (now Fig. 6).

b) We are aware of the fact that DVM patterns of Antarctic krill are highly variable and that normal DVM patterns do not need to be the rule (e.g. see our cited study on the plasticity of krill DVM by Bahlburg et al. 2023). The visualized data were not selected for their DVM pattern but represent the period directly preceding the sampling for behavioral experiments in four seasons (experiment 2), including the day of sampling. These periods were chosen to assess the DVM behavior of krill swarms in the field in the days before and during the sampling for behavioral experiments.

To improve understanding, we modified the description in the Results, Discussion, and Methods sections, as well as the caption of Figure 5 (now Fig. 6), which now read:

“To investigate whether krill swarms exhibited daily behavioral patterns in swimming behavior in the field before they were sampled for seasonal experiments, hydroacoustic data were recorded from the fishing vessel, continuously over a three-day period prior to sampling for the seasonal experiments described above...” (lines 191-194).

“Furthermore, hydroacoustic recordings demonstrate that most krill swarms sampled exhibited synchronized DVM in the field in the days directly before sampling for behavioral experiments, indicating that in this region, krill remain behaviorally synchronized across a wide range of photoperiods.” (lines 397-400).

“Hydroacoustic data were collected using a hull-mounted SIMRAD ES80 echosounder (Kongsberg Maritime AS) aboard the Antarctic Endurance, covering three days before the sampling for each of the seasonal behavioral experiments of experiment 2” (lines 512-515).

“We only included data during active fishing periods and the vessel is specifically targeting *E. superba*, which occurs in large monospecific aggregations. Further, krill fishery bycatch rates are very low (0.1-0.3%, 84), which makes it highly probable that the recorded signal represents krill swarms.” (lines 523-526).

“Hydroacoustic recordings showing the vertical distribution of krill swarms in the upper water column (<220 m) below the vessel, visualized by the mean volume backscattering signal (200 kHz), on the three days prior to krill sampling for experiments...” (lines 802-804).

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

As noted in the public review, this is a logical and well-written manuscript. I have very few comments to consider addressing.

The Results lead with a paragraph outlining the experimental approach. This is good, but you use the term "experiments" to refer to both the two sets, and the two or four subsets of experiments. Perhaps consider the subset experiments as "treatments"? I understood what you meant, but it took a few read-throughs to be sure I got it.

We thank the reviewer for pointing this out and changed the nomenclature of the experiments throughout the manuscript. We now refer to the two sets of experiments as experiment 1 and 2, to the subsets of experiment 1 as “short day treatment” and “long day treatment”, and to the subsets of experiment 2 as summer treatment, late summer treatment, autumn treatment, and winter treatment. We also believe that the new Figure 1 is now helping to follow the experimental design more efficiently.

Ln 140: "...off and decrease at lights-on."

We adjusted the sentence accordingly.

Ln 244: Can you define "extreme photic conditions"? I get what you mean, but to be clear to the reader this would help.

We adjusted the sentence, which now reads:

“This could confer a significant adaptive advantage to species inhabiting environments characterized by extreme photic conditions (53, 54, 60), such as phases of polar night or midnight sun as well as rapid changes in daylength, or species that rely on precise photoperiodic time measurement for accurate seasonal adaptation.” (lines 258-261).

Figures: Consider adding an LSP for groups in Fig 1. Also, it would be useful to have LSP period estimates for each individual tested. This could be a separate table, or it could be added to the individual activity plots. Should S3 and S4 be reversed?

We thank the reviewer for their suggestion and added an LSP as figure 1d (now Fig. 2d) to statistically support the group activity shown in Figure 1c (now Fig. 2c) as suggested. We added the individual animals' LSP period estimates to supplementary figures S2, S7, S8, S9, and S10. We also reversed Figures S3 and S4 to match the appearance in the main text.

Fig 5: are the light regime bars for b and c correct? They look similar, but there are only 15 days apart, so perhaps they are correct as is.

We double checked the light regime bars in Fig. 5b and c (now 6b and c) and they are correct as is.

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