Title:

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

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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

57 clock, which helps the animals to adjust behavior, physiology and gene expression to the day-
58 night cycle (*17*). Similarly, a study on Norwegian krill, *Meganyctiphanes norvegica*, suggested
59 swimming activity in krill is under clock control, with increased activity during the dark phase
60 (*18*). In Antarctic krill, similar studies have been conducted to test the hypothesis of a clock
61 involvement in DVM behavior (*19, 20*). While these studies gave first hints that daily
62 behavioral activity in krill is partly under endogenous control, a large variability hindered a
63 clear characterization of the circadian behavioral activity of krill.
64 Previous studies on endogenous rhythms in krill identified the molecular components of a krill
65 circadian clock (*21, 22*) . It has further been shown that the krill circadian clock drives daily
66 rhythms in gene expression, metabolism and behavior (*20, 23, 24*). Part of krill's success lies
67 in its strong adaptation to the extreme seasonality of the Southern Ocean environment (*25*),
68 which is reflected in a fundamental, seasonal regulation in body composition, metabolic
69 activity, feeding, growth (*26, 27*), sexual maturity (*28, 29*) and gene expression (*30, 31*). Results
70 from laboratory studies showed that photoperiod is a major driver for seasonal
71 ecophysiological changes in krill (*32-34*), and it has been suggested that an endogenous
72 timekeeping system (i.e. biological clock) is involved, which uses photoperiod as a
73 synchronizing cue (*33, 35*).
74 Circadian clocks are widespread and well-studied endogenous timing mechanisms which allow
75 an organism to fine-tune biological functions to specific times of the day-night cycle. The
76 endogenous oscillation is produced by transcriptional-translational feedback loops (TTFL) of a
77 set of clock genes and their translation products (*36*), which, under the absence of external
78 cues, produce a temporal signal with a period (τ) of about 24 h. Under natural conditions, the
79 rhythm of the central oscillator is synchronized to exactly 24 h by predictable environmental
80 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.

104 # Results

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

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

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

126 As synchronization between individuals and robustness of activity patterns under DD within
127 individuals was strongest in krill from the short day treatment, we used these data for initial
128 assessments of circadian activity rhythms. Under constant darkness conditions in the short
129 day treatment, krill swimming activity continued in several individuals (n = 6; 54%) with
130 significant circadian rhythmicity over the 5 days of DD conditions (Fig. 2a and b, Fig. S4),
131 indicating the involvement of a circadian clock in the daily regulation of swimming activity.
132 Nevertheless, in most individuals, the amplitude of the rhythm decreased over time (Fig. 2a,
133 Fig. 4a, Fig. S4). The patterns of swimming activity observed at the individual level were
134 reflected at the group level, showing a strong synchronization of increased swimming activity
135 with the dark phase under light-dark conditions (Fig 3a, Fig. S2), and persistent significant
136 circadian swimming activity under constant conditions with a continuously decreasing
137 amplitude throughout the experiment (Fig. 2c and d; Fig. 4a: Paired t-test, p-value = 0.005, Fig.
138 4c: Paired t-test, p-value = 0.012).

139

140 **Light affects the amplitude and phase of swimming activity**

141 Comparing the mean swimming activity of rhythmic individuals over an average day from the
142 short day and long day treatments under LD conditions reveals a clear synchronization of
143 swimming activity with the dark phase (Fig. 3), visible in the sharp increase in activity around
144 the times of lights-off and decrease at lights-on. Furthermore, the time of increased activity
145 during the dark phase exhibits a discernible pattern comprising three distinct activity bouts
146 (Fig. 3). First, an increase in activity is observable in the evening (i.e., end of illumination),
147 followed by a decrease in activity towards midnight. A second increase in swimming activity is
148 observable in the latter half of the dark phase, followed by a decline in activity just before

149 morning. A third short activity bout is observed in the morning (i.e., onset of illumination).
150 This distinctive activity pattern during the dark phase is observed regardless of the
151 photoperiod, resulting in a compression of the three activity bouts in short nights, while they
152 spread out in long nights (Fig. 3), demonstrating that krill can entrain to the long and short
153 days provided. Furthermore, comparing the activity pattern during short-day LD conditions
154 with the respective activity pattern under DD conditions reveals the persistence of evening
155 and late-night activity increases under constant darkness conditions. In contrast, the morning
156 activity bout is not visible (Fig. S5). This suggests that the circadian clock drives a distinct
157 bimodal activity pattern with two activity peaks in one day, i.e. the evening and late-night
158 activity bouts. In contrast, the morning activity bout is triggered by the onset of illumination
159 in the experimental set-up. Furthermore, the amplitude of swimming activity is higher under
160 LD than DD conditions (Fig. 4 B, Mann-Whitney-U test: *p-value* < 0.001; Fig. 4 D, *p-value* <
161 0.001).
162

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

165 To gain insights into potential seasonal differences in the regulation of krill swimming activity,
166 we sampled krill from the field in summer (summer treatment), late summer (late summer
167 treatment), autumn (autumn treatment), and winter (winter treatment) and recorded their
168 swimming activity for 4 to 8 days under constant darkness conditions, without previous
169 entrainment in the activity monitor (see Tab. 1 for details).
170 Similar to our previous analysis of individual krill swimming activity under constant darkness
171 conditions, a large proportion of the recorded individuals in each experiment (44% to 100%)
172 exhibited significant rhythmicity in the circadian range (Fig. S7-10), which is also reflected in

173 rhythmic swimming activity at the group level (Fig. 5a-d, Fig. S6). Interestingly, the pattern of
174 circadian swimming activity, with two peaks during the dark phase, appears to become clearer
175 and higher in amplitude with shortening photoperiods towards winter (Fig. 5a-d). This is
176 supported by rhythm analysis of the group behavior, which revealed a higher power and a
177 period estimation closer to 24 h with shortening photoperiod (Fig. S11). While the morning
178 peak is mainly visible in autumn and winter, the late-night activity peak is visible throughout
179 the year, at the same time of the subjective 24-h-day (Fig. 5e-h). This pattern appears
180 irrespective of the natural photoperiod the animals were entrained to in their natural
181 environment before they were sampled for the respective experiment.
182

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

185 To investigate whether krill swarms exhibited daily behavioral patterns in swimming behavior
186 in the field before they were sampled for seasonal experiments, hydroacoustic data were
187 recorded from the fishing vessel, continuously over a three-day period prior to sampling for
188 the seasonal experiments described above (for vessel positions during hydroacoustic
189 recordings see Fig. S1). The data provide information about the vertical distribution of krill
190 swarms in the upper water column (<220 m) below the vessel over time (Fig. 6a-d). The
191 hydroacoustic data in summer, late summer and autumn show that krill swarms performed
192 diel vertical migration, highly synchronized with the local light regime (Fig. 6a-c). The swarms
193 regularly ascended to the surface layers (<50 m) around sunset and returned to deeper layers
194 (>100 m) around sunrise. In winter, little signal is visible in the upper water layer, indicating
195 very low density or the absence of krill swarms (Fig. 6d). Consequently, krill for the winter
196 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.

200 ## Discussion:

201 Our study revealed continued rhythmic swimming activity under constant darkness, showing
202 the involvement of a circadian clock in swimming behavior of krill, as has been previously
203 shown for other marine pelagic crustaceans (*17, 18, 44*). To date, only three studies have
204 investigated the involvement of a circadian clock in the regulation of swimming behavior in
205 krill species, including *E. superba* (*19, 20*) and *Meganyctiphanes norvegica* (*18*). Data from
206 Piccolin et al. (*20*) showed a strong damping of the amplitude and indication of a remarkably
207 short (~12 h) free running period (FRP) of vertical swimming behavior of a group of krill under
208 constant darkness (*20*). The short period found in Piccolin et al. (*20*) is in line with our findings
209 of a bimodal activity pattern under DD conditions on the individual level, suggesting that the
210 ~12 h rhythm in group swimming behavior in Piccolin et al. (*20*) could have resulted from a
211 bimodal activity pattern at the individual level, as found in our study. Bimodal locomotor
212 activity rhythms are well known across different species, possibly most prominently in the
213 fruit fly (*Drosophila melanogaster*), where two coupled endogenous oscillators control
214 morning and evening activity bouts (*45*). In our experiments, we observed a strong weakening
215 of the evening activity under constant conditions, especially after entrainment to long days,
216 while the late-night activity peak appeared more robust. The differential variation in the
217 morning and late-night activity suggests that the circadian activity bouts could also be
218 controlled separately at the neuronal level in krill.

219 Comparing the daily pattern of swimming activity under LD conditions with those under DD,
220 it is clear that the amplitude of the rhythm was significantly higher under LD than under DD.
221 In addition, the activity under LD was organized into three activity phases, one in the evening,
222 one late at night and a smaller one in the morning. In contrast, under DD there were a
223 maximum of two activity phases during the subjective night. Light is known to play a dual role

224 in regulating daily behavioral activity. On the one hand, light functions as a *Zeitgeber*, which
225 synchronizes the circadian clock with the day-night cycle, a process called *entrainment* (*46*).
226 On the other hand, it can directly affect clock output functions such as physiology and
227 behavior, known as *masking* (*46, 47*). The masking response depends on the activity type of
228 the organism, and light was shown to promote activity in diurnal animals while inhibiting
229 activity in nocturnal ones (*48, 49*). Krill are regularly observed performing nocturnal DVM (*13,*
230 *14*), including in the present study, with higher activity during the night, including the times of
231 twilight, related to foraging and feeding activity and vertical ascend and descend movements
232 (*50, 51*). The observed effect of light on reducing krill swimming activity under LD conditions
233 in our study is thus in line with previous concepts of the exogenous effects of light on nocturnal
234 organisms (*46, 47*). However, under LD conditions, the observed swimming activity pattern in
235 krill is not solely driven by light. The increase in activity in the evening and the second half of
236 the dark phase under LD conditions flexibly adjust to the length of the night and seem to
237 persist under constant darkness, suggesting these bouts to be under clock control, as
238 discussed above.
239 The reduced amplitude in swimming activity after the switch to DD conditions and further
240 damping of amplitude over time under constant darkness has been found in other studies in
241 krill (*20*) and other marine zooplankton organisms (*17, 44*). A rapidly damping output signal
242 under constant conditions could indicate a weak, low amplitude circadian oscillator, where
243 the damping amplitude of the clock output signal is caused by a rapid desynchronization of
244 clock neurons, when Zeitgeber signals are absent (*52*). Interestingly, weak oscillators have
245 been found in different fruit fly species (*53, 54*), the Svalbard reindeer (*55*), and Svalbard
246 ptarmigan (*56*), native to high latitudes and species that exhibit pronounced seasonal
247 rhythmicity, such as aphids (*57, 58*). It is known that weaker clocks are more flexible and can

248 be entrained more efficiently than those that are more rigid and self-sustained (*52, 59*). This
249 could confer a significant adaptive advantage to species inhabiting environments
250 characterized by extreme photic conditions (*53, 54, 60*), such as phases of polar night or
251 midnight sun as well as rapid changes in daylength, or species that rely on precise
252 photoperiodic time measurement for accurate seasonal adaptation. In the Southern Ocean,
253 photoperiod becomes extreme around the solstices, and its daily change is rapid around the
254 spring and autumn equinoxes. A weak oscillator could, therefore, ensure that krill remains
255 synchronized with the extreme and rapidly changing photic conditions and, thus, its
256 environment.
257 Indeed, our data from activity recordings under DD conditions of krill sampled in different
258 seasons indicate persistent circadian swimming activity, irrespective of the season. Our
259 findings suggest that the krill circadian clock remains functional and drives rhythmic
260 behavioral output in natural conditions throughout the year. This finding is complemented by
261 the hydroacoustic recordings in the days preceding krill sampling for activity experiments. In
262 summer, late summer and autumn, krill swarms exhibited apparent diel vertical migration
263 behavior, synchronized to the natural photoperiod. While we did not observe DVM in the
264 upper water column during sampling in winter, acoustic recordings from other studies in
265 winter indicate that krill may perform DVM also in winter (*61*), potentially in deeper layers.
266 Our data show that light acts in concert with, and is likely modulated by, the circadian clock
267 to achieve the observed daily regulation in krill swimming activity. Nevertheless, further
268 studies at the behavioral, molecular, and neuronal levels are required to elucidate the
269 endogenous and exogenous effects of light and the circadian clock on the different behavioral
270 aspects observed and to gain further insights into the characteristics of the molecular
271 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

296 to entrain a circannual clock (*66*), which subsequently drives rhythmic annual output. While
297 the individual activity recordings provide first insights into potential mechanisms for the
298 control of daily and seasonal processes in krill, it must be noted that behavioral output does
299 not always reflect the detailed characteristics of the circadian oscillator (*67*). Further
300 investigations into the molecular clock mechanism and its downstream functions across
301 seasons are needed to clarify the involvement of the circadian clock in photoperiodism and
302 circannual rhythms in krill.
303
304 Circadian clocks have the potential to anticipate the daily light-dark cycle and thereby enable
305 a fine-tuned synchronization of behavioral and physiological functions with environmental
306 cycles, which increases an organism's fitness (*38*). Consequently, synchronizing krill behavioral
307 output with the day-night cycle provides potential adaptive advantages on various levels.
308 DVM of zooplankton seems to be a trade-off between predation risk and feeding (*16*). As it is
309 complex to directly sense predation risk, light is a robust proxy (*68*) and a circadian clock
310 provides an additional mechanism to anticipate and synchronize the times of vertical
311 migration, active feeding and foraging with nighttime. Our data show that light, in addition to
312 the clock, ensures robust, synchronized activity patterns. Differences between entrained and
313 free-running conditions further indicate that evening and late-night activity are under clock
314 control. At the same time, the activity peak in the morning is a response to the onset of light.
315 A differential regulation of certain phases of DVM has previously been suggested for marine
316 zooplankton species (*17, 44*). A clock driven evening ascent would allow for the anticipation
317 of sunset and thus the timely initiation of migration when krill swarms are in deeper water
318 layers, resulting in optimized surface feeding time. The clock-controlled late-night activity
319 could support a second feeding bout and preparation for a timely morning descent before

320 sunrise increases visual predation risk. Interestingly, the decrease in clock-controlled
321 swimming activity during the early night, right after the evening activity bout, may further
322 facilitate a phenomenon called “midnight sinking”, which describes the sinking of animals to
323 intermediate depths after the evening ascent, followed by a second rise to the surface before
324 the morning descend. This behavior has been observed in a number of zooplankton species,
325 including calanoid copepods (see *69, 70* and references therein) and krill (*71*). While previous
326 studies suggested several exogenous factors, such as satiation or predator presence, as drivers
327 of the midnight sink (*69, 70*), our study suggests that this pattern may be partly under
328 endogenous control. In-situ hydroacoustic observations have demonstrated increased
329 swimming speeds during nighttime for both *M. norvegica* and *E. superba* (*50, 51*). This increase
330 is likely related to active foraging and feeding behavior, as higher swimming speeds increase
331 the chance of encountering food particles and thus increase food uptake. Nevertheless,
332 swimming in krill is energetically costly and can make up more than 70% of the total energy
333 expenditure (*72*). Reducing swimming activity to the baseline level during the day thus
334 represents a significant opportunity for energy conservation.
335 At high latitudes, biological clocks are exposed to extreme changes in photoperiod throughout
336 the year, including phases of midnight sun and polar night. Many organisms inhabiting polar
337 regions show arrhythmicity, at least during parts of the year, and potential mechanisms
338 include direct impacts on the central oscillator and uncoupling between output functions and
339 the circadian clock (*60*). Previous investigations of krill clock gene expression in the laboratory
340 indicated a weaker oscillation of the krill clock and metabolic output functions under
341 simulated extreme photic conditions (i.e. LL and LD 3:21), compared to more balanced day-
342 night cycles (*73*). Data on the transcriptomic and organismic level further indicate that krill can
343 flexibly adapt to the photic conditions at different latitudes (*31, 33*). This aligns with our

344 findings, where krill, sampled across a wide range of photoperiods, remain behaviorally
345 synchronized with the local photoperiod under entrained conditions, but show reduced
346 endogenous rhythmicity under DD after entrainment to long day conditions. Krill inhabits a
347 broad range of latitudes, including regions with clear day-night cycles and regions with polar
348 night and midnight sun during the seasonal extremes (*74*). At the same time krill is transported
349 rapidly between regions via ocean currents (*75*), potentially exposing the same individuals to
350 various photic conditions during their life span. A weak circadian clock in close interaction with
351 the ambient light regime could thus allow krill to adopt the best strategy for the environmental
352 conditions prevailing in the current location. For example, at high latitudes, the adaptive
353 advantage of predator evasion with DVM diminishes in summer when the upper water column
354 is illuminated constantly. In this case, arrhythmic behavior would allow flexibly exploiting food
355 patches at any time of day, when the need for food is high to fuel reproductive processes (*76*).
356 Further, a study on the marine annelid *Platynereis dumerilii* recently highlighted a differential
357 regulation of clock-controlled processes, where behaviorally arrhythmic individuals show
358 increased rhythmicity in physiological output (*67*), indicating the possibility of flexibly adapting
359 timed processes to environmental conditions.
360 Adapting to a fluctuating environment involves significant seasonal physiological changes in
361 krill to survive the harsh, food-scarce winter (*26, 77*). Crucial processes, such as accumulating
362 enough lipids, must begin long before winter, requiring an anticipatory mechanism. Recent
363 evidence suggests that photoperiod and circannual timing drive these seasonal changes in krill
364 physiology, using photoperiod as a proxy for seasonal progression (*26, 32-35*). Rapid warming
365 in the Southern Ocean is observed to cause shifts in phytoplankton bloom timing (*78*), which
366 may lead to temporal mismatches between photoperiod synchronized krill physiology and
367 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.

392

393 # Methods:

394 **Animal collection**

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

414 to the tip of the telson, excluding setae), and their sex was determined under a stereo
415 microscope.

416 **Krill swimming activity recording and experimental design**

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

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

437 To investigate seasonal changes in krill circadian activity, four additional experiments were
438 conducted, namely summer treatment (sampling date: January 22nd 2022), late summer
439 treatment (sampling date: March 1st 2022), autumn treatment (sampling date: March 15th
440 2022) and winter treatment (sampling date: June 10th 2022). For these experiments, krill were
441 caught at the dates described above and after acclimation, 10 animals per experiment were
442 distributed to the activity monitor, where their activity was recorded for 4 to 8 days under
443 constant darkness conditions (see Tab. 1 for details).

444 **Behavioral data analysis**

445 *Experiment 1 (LD-DD)*

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

457 To calculate the group activity of rhythmic krill under DD conditions, we first detrended the
458 normalized swimming activity data of each individual to account for individual variability in
459 baseline shifts of activity over time. This was done by subtracting the daily mean activity from
460 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)

484 Group activity and average day activity for seasonal experiments (Experiment 2, summer, late
485 summer, autumn, and winter treatment) was done as for DD conditions described above, but
486 only the first 4 days of activity of each experiment were considered. This was to allow for a
487 better comparison and to account for the effect of damping rhythms after several days under
488 DD.
489 All data handling, analysis and visualization was done with the *R programming language*
490 (version 4.1.2 (*79*)) in *RStudio* (version 2023.12.1.402), using the *tidyverse* package (version
491 2.0.0 (*80*)). Rhythm analysis and period estimation was done with the R package *lomb* (*81*),
492 local sun data (i.e. sunset and sunrise) were retrieved from the *suncalc* package (version 0.5.1).

493

494 **Hydroacoustic data recording and visualization**

495 Hydroacoustic data were collected using a hull-mounted SIMRAD ES80 echosounder
496 (Kongsberg Maritime AS) aboard the Antarctic Endurance, covering three days before the
497 sampling for each of the seasonal behavioral experiments of experiment 2. The signal received
498 from the 200 kHz band was used to visualize the vertical distribution of krill swarms beneath
499 the ship. The raw acoustic data from the summer, late summer, and autumn periods were
500 converted to mean volume backscattering strength and binned to a time resolution of 1 s and
501 depth bins of 0.5 m using Echopype (*82*). For the period of winter, raw acoustic data were not
502 available. An alternative approach was employed, utilizing a novel method proposed by
503 Bahlburg et al. (*83*), to reconstruct the backscattering signal from a dataset of screenshots
504 displaying the visualized hydroacoustic signal of the echosounder.
505 We only included data during active fishing periods and the vessel is specifically targeting *E.*
506 *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).

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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). Supplementary figures are available in the Supplementary Material file.

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.

Competing Interests statement

The authors declare no competing interests

Figures

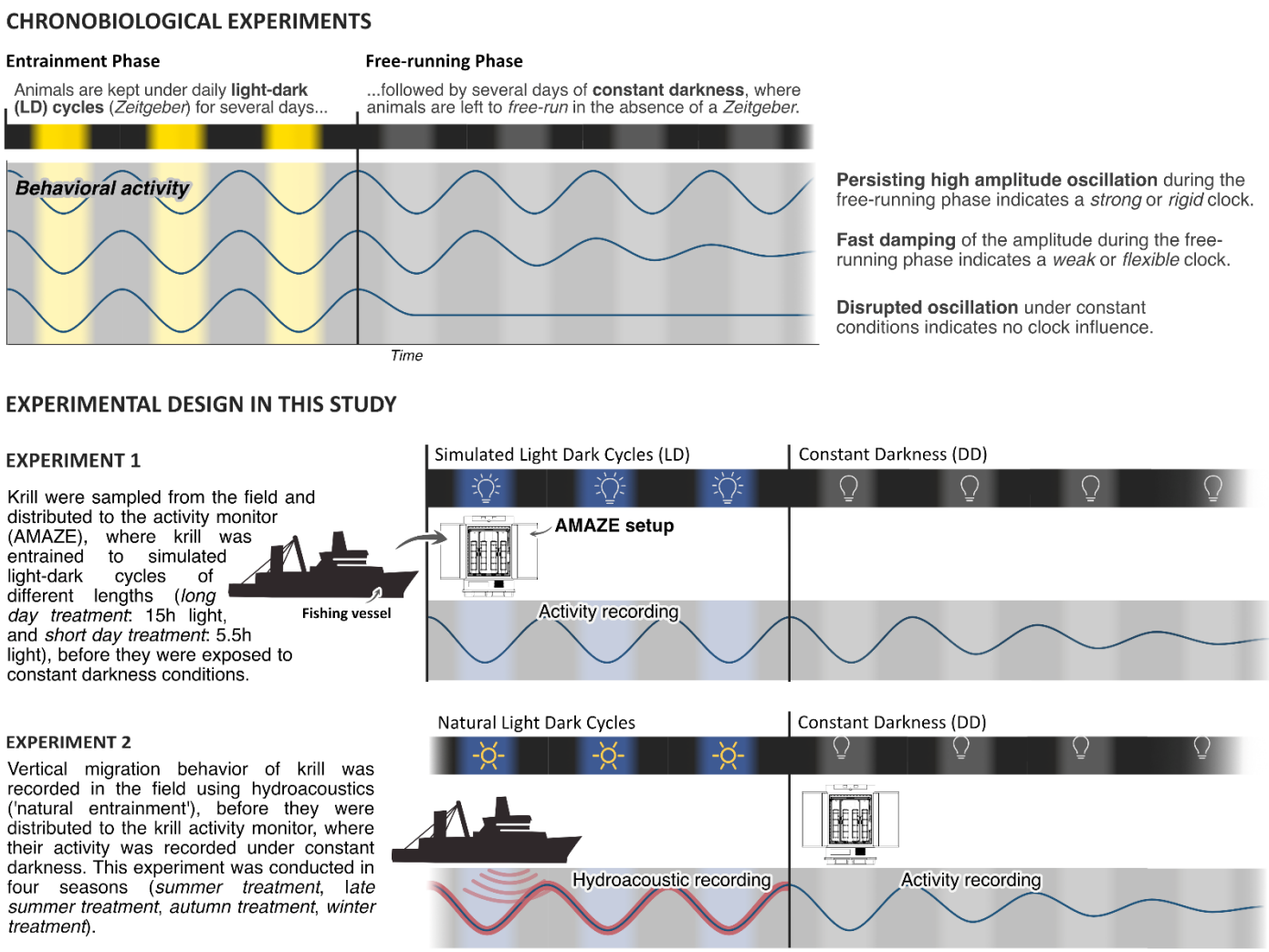
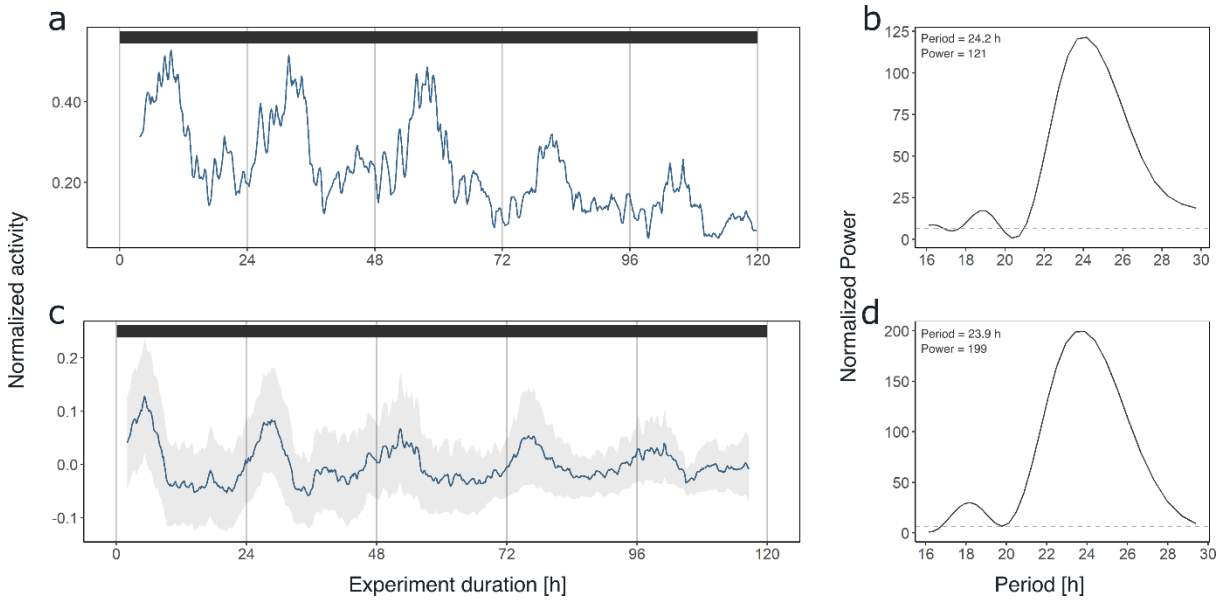


Figure 1: Experimental design of chronobiological studies.

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.



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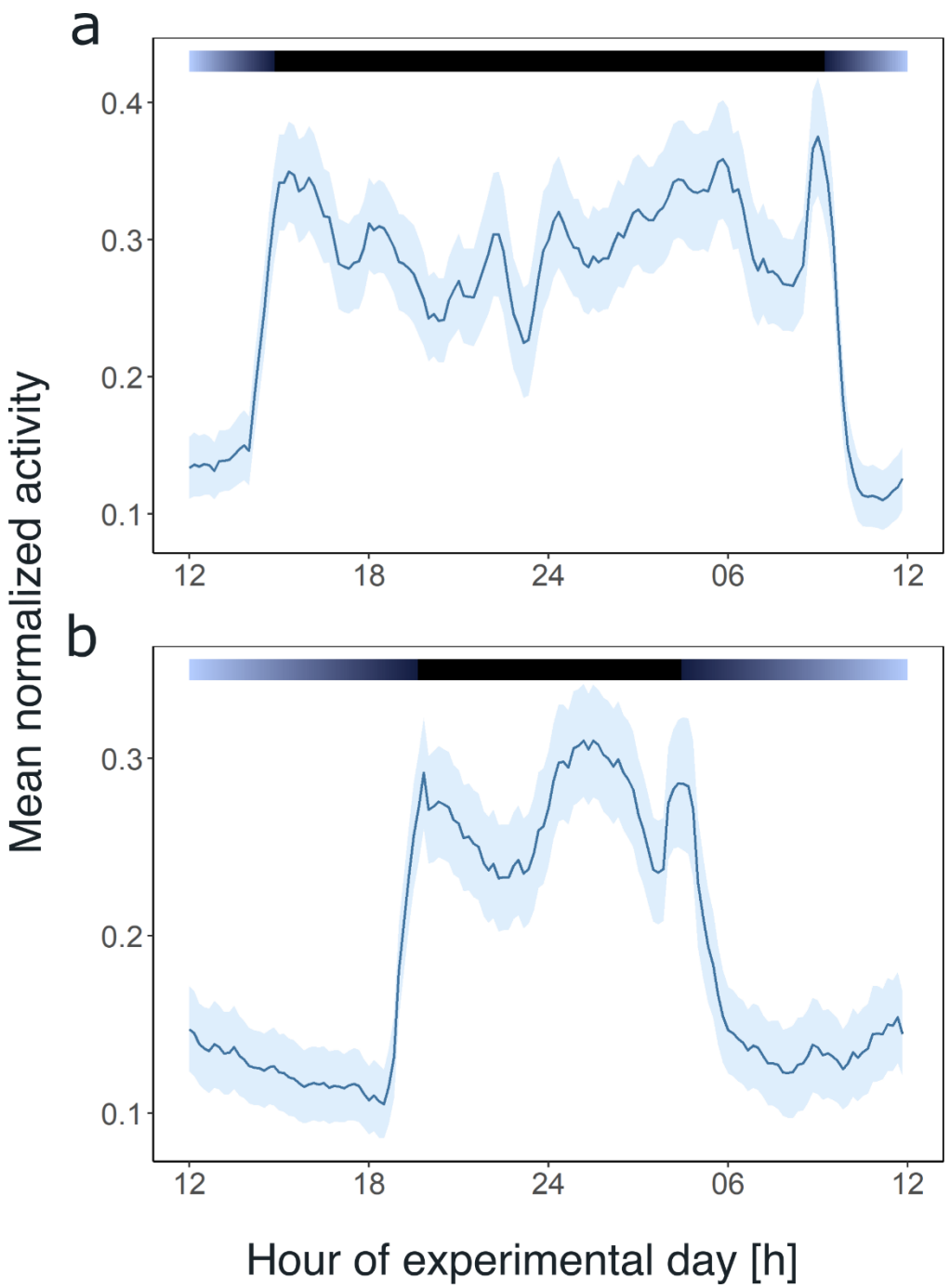
742

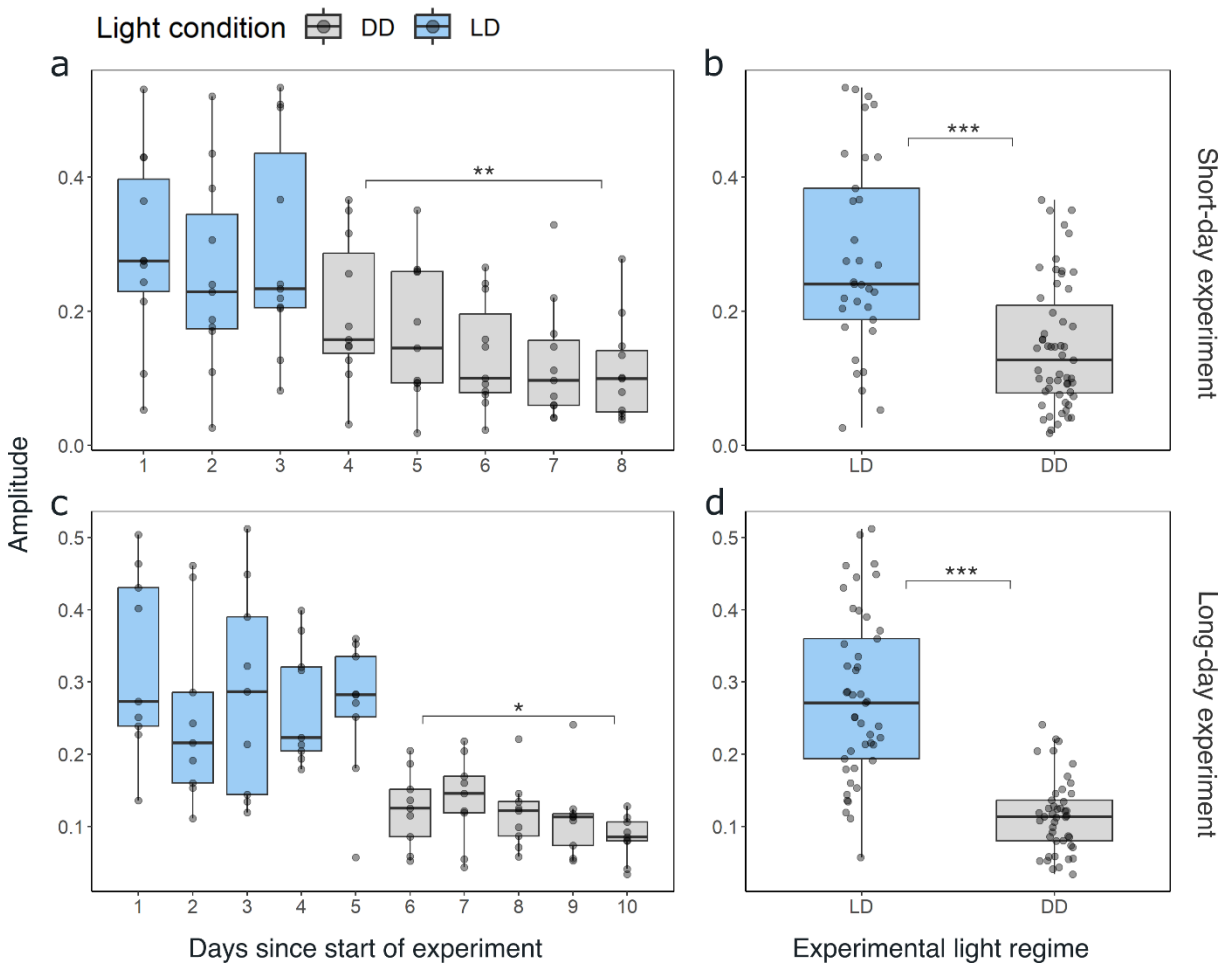
743 **Figure 2: Krill swimming activity persists under constant conditions.**
744 Swimming activity under 5 days of DD conditions in experiment 1 of individual #9 after
745 entrainment to simulated short-day conditions (a) and group mean swimming activity of
746 rhythmic individuals under 5 days of DD conditions from the same experiment (n = 6, c). The
747 result from LSP analysis shows significant circadian rhythmicity of swimming activity of
748 individual #9 (period: 24.2 h, b) as well as for group mean swimming activity (period: 23.9 h,
749 d) during DD conditions of experiment 1 (short day treatment). Grey shading represents
750 standard error of the mean (s.e.m.). Color bars at the top indicate light conditions (constant
751 darkness).



752

753 **Figure 3: Light and the circadian clock shape the daily activity profile of krill.**
754 Average day analysis of the group mean swimming activity during experiment 1 over 3 days of
755 short-day simulations (short day treatment, n = 11, a) and 5 days of long-day simulations (long
756 day treatment, n = 9, b). Color bars at the top indicate light regime under day-night
757 simulations. Shading around the line represents the s.e.m.. Data in (b) from Hüppe et al. (*43*).

758



759

760 **Figure 4: The amplitude of swimming activity decreases in DD conditions.**

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

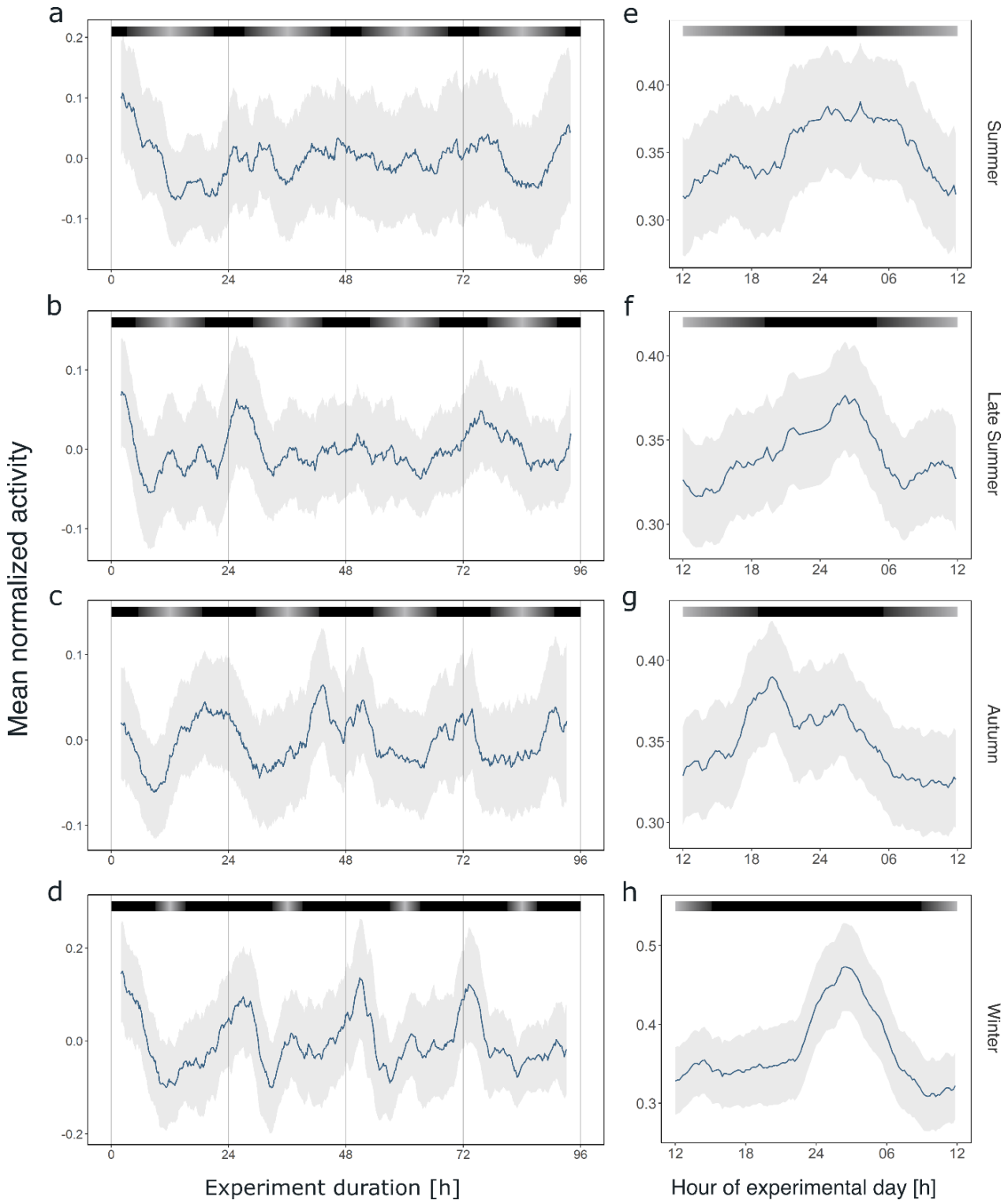


Figure 5: Circadian swimming activity persist across seasons.

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.

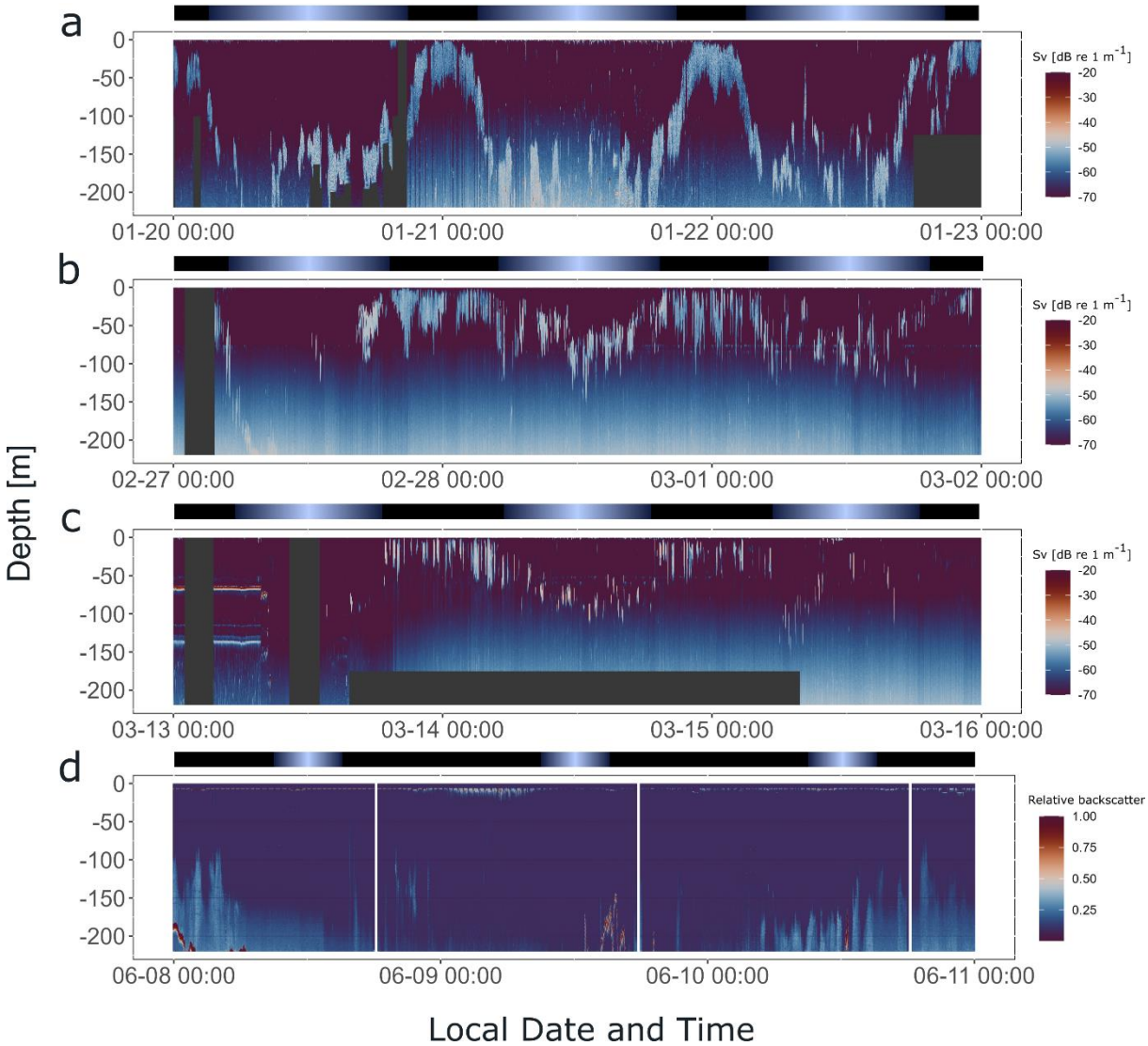


Figure 6: Krill display synchronized DVM in the field across a wide range of photoperiods. Hydroacoustic recordings showing the vertical distribution of krill swarms in the upper water column (<220 m) below the vessel, visualized by the mean volume backscattering signal (200 kHz), on the three days prior 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.

Tables

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 (short day treatment)	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 (long day treatment)	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 (summer treatment)	DD	8	2022-01-22	17:03	SOI	60.08/46.46	157	17.5	9/8/1	42.4 ±1.5
Experiment 2 (late summer treatment)	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 (autumn treatment)	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 (winter treatment)	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.

Supplementary Figures

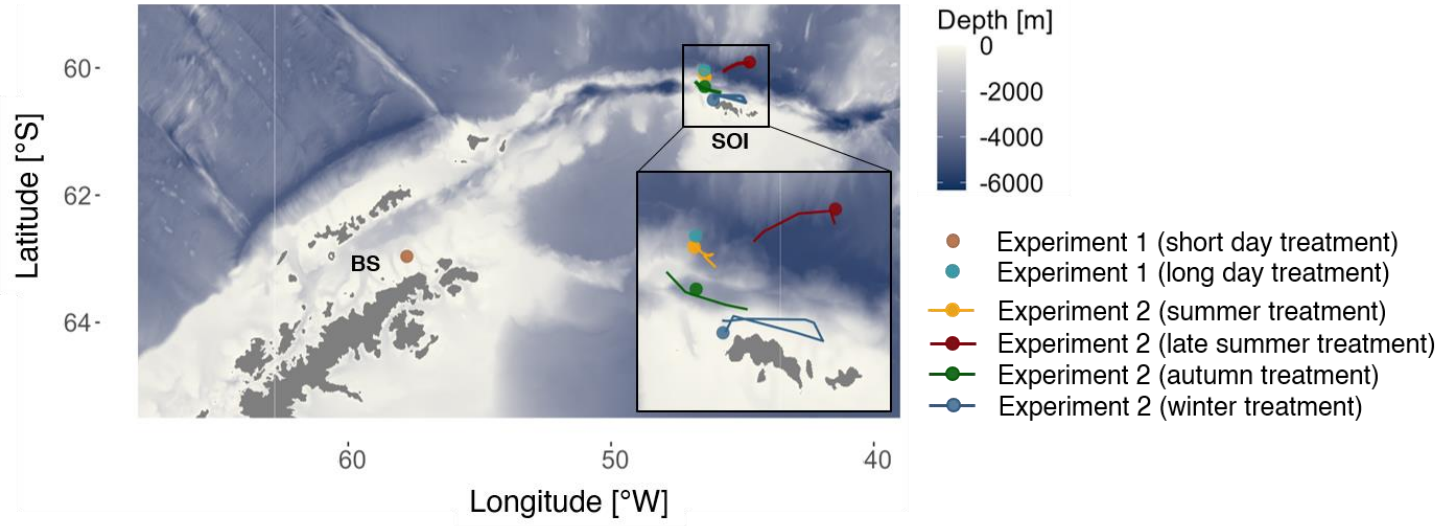


Figure S1: Locations of krill sampling and hydroacoustic recordings for behavioral experiments. Colored dots indicate sampling locations for the respective behavioral experiments. Colored lines indicate the cruise track of the fishing vessel during recording of hydroacoustic data prior to sampling for 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://ibcso.org/).

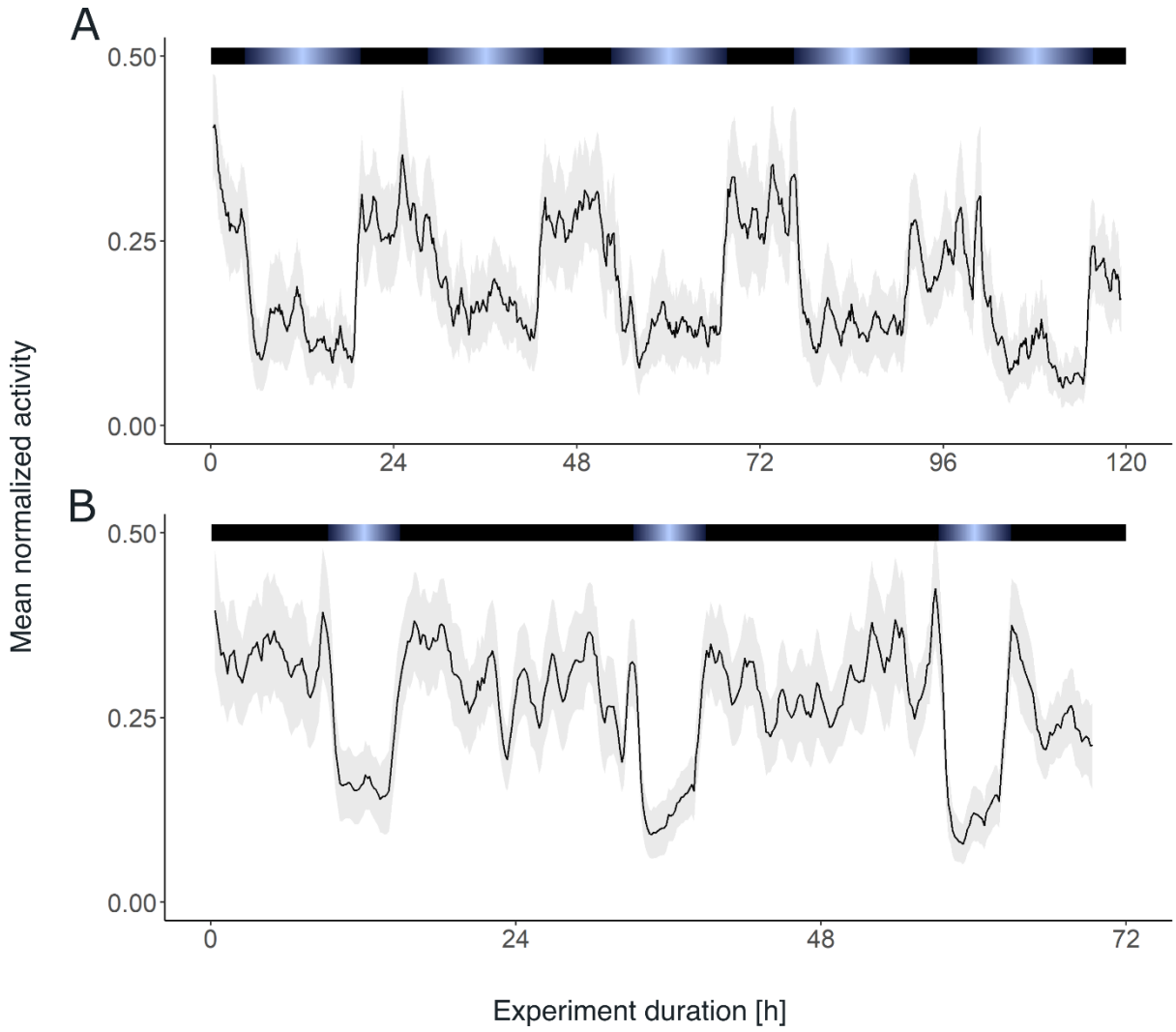


Figure S2: Krill group activity under LD conditions.
Group mean swimming activity during experiment 1 of krill under long-day (n=9, A) and short-day (n=11, B) simulations. Shading around the line depicts the standard error of the mean (s.e.m.). Color bars at the top indicate the light regime provided. Data for (A) from Hüppe et al. (*43*).

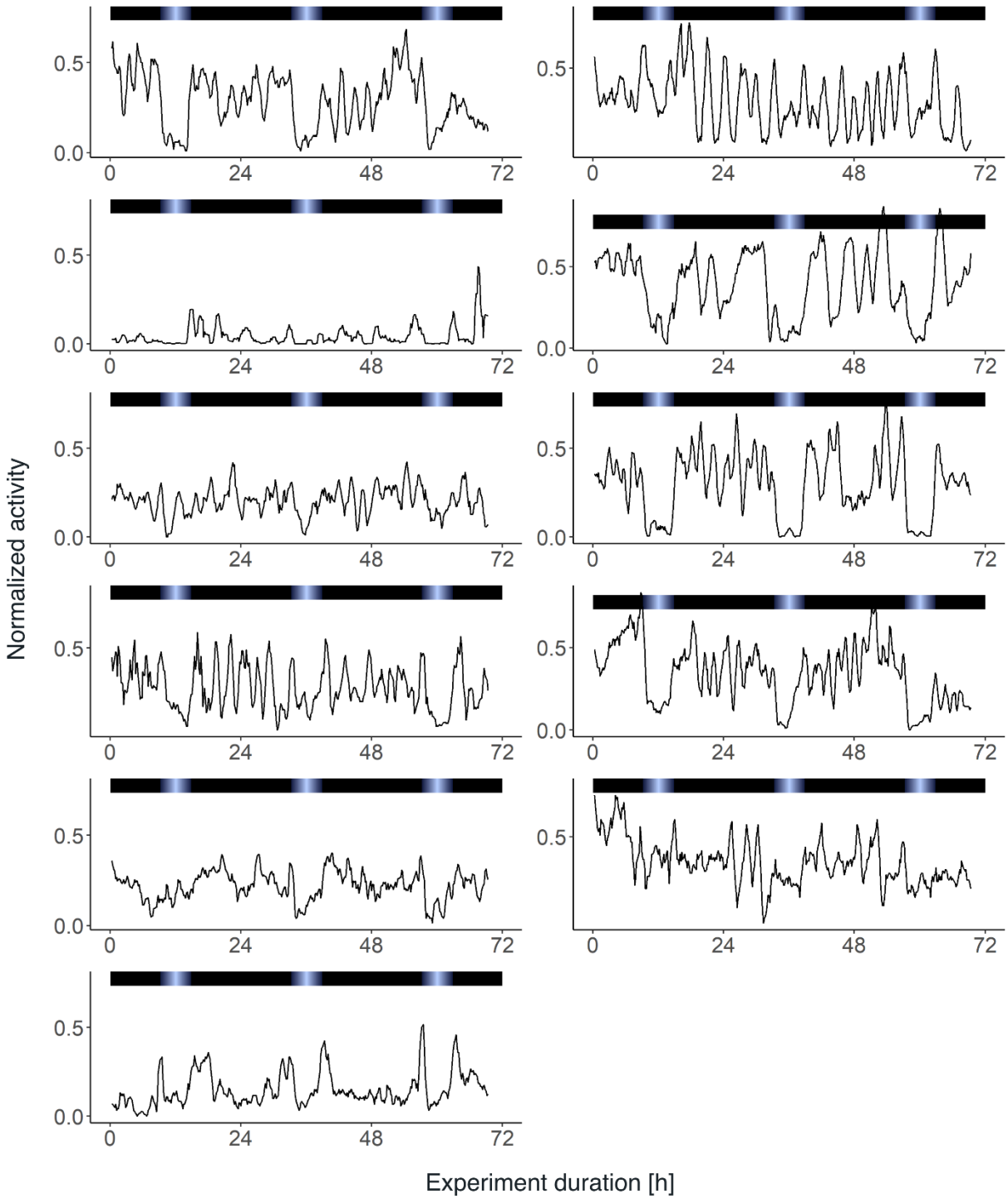


Figure S3: Swimming activity of individual krill under LD conditions.
Normalized swimming activity during experiment 1 under 5 days of simulated short-day conditions for each individual. Color bars at the top indicate the light regime provided.

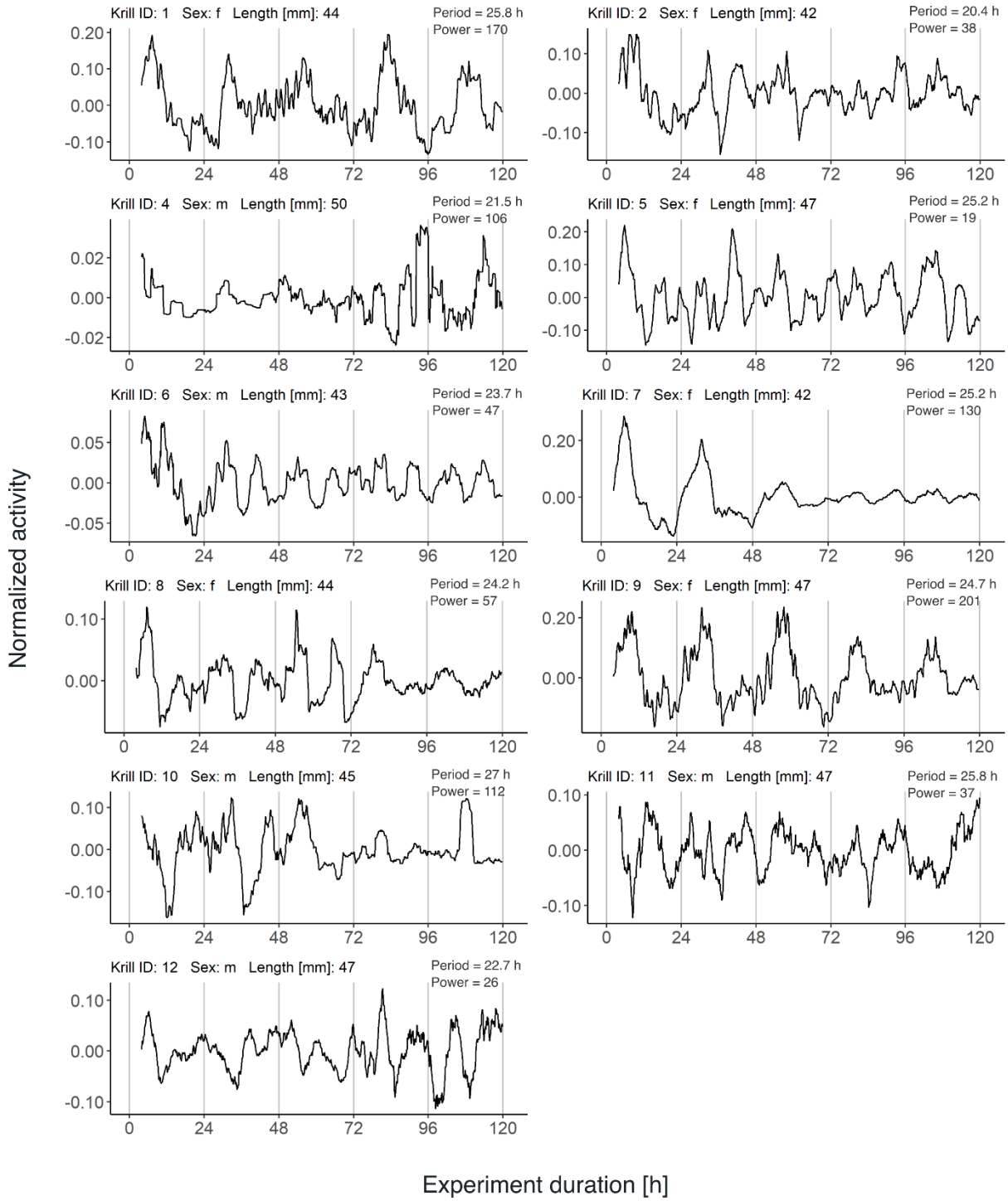


Figure S4: Swimming activity of individual krill under DD conditions.
Normalized and detrended swimming activity under 5 days of constant darkness (DD) after entrainment to simulated short-day conditions (experiment 1) for each individual. Period and power estimates from Lomb-Scargle Periodogram (LSP) analysis are detailed for each individual.

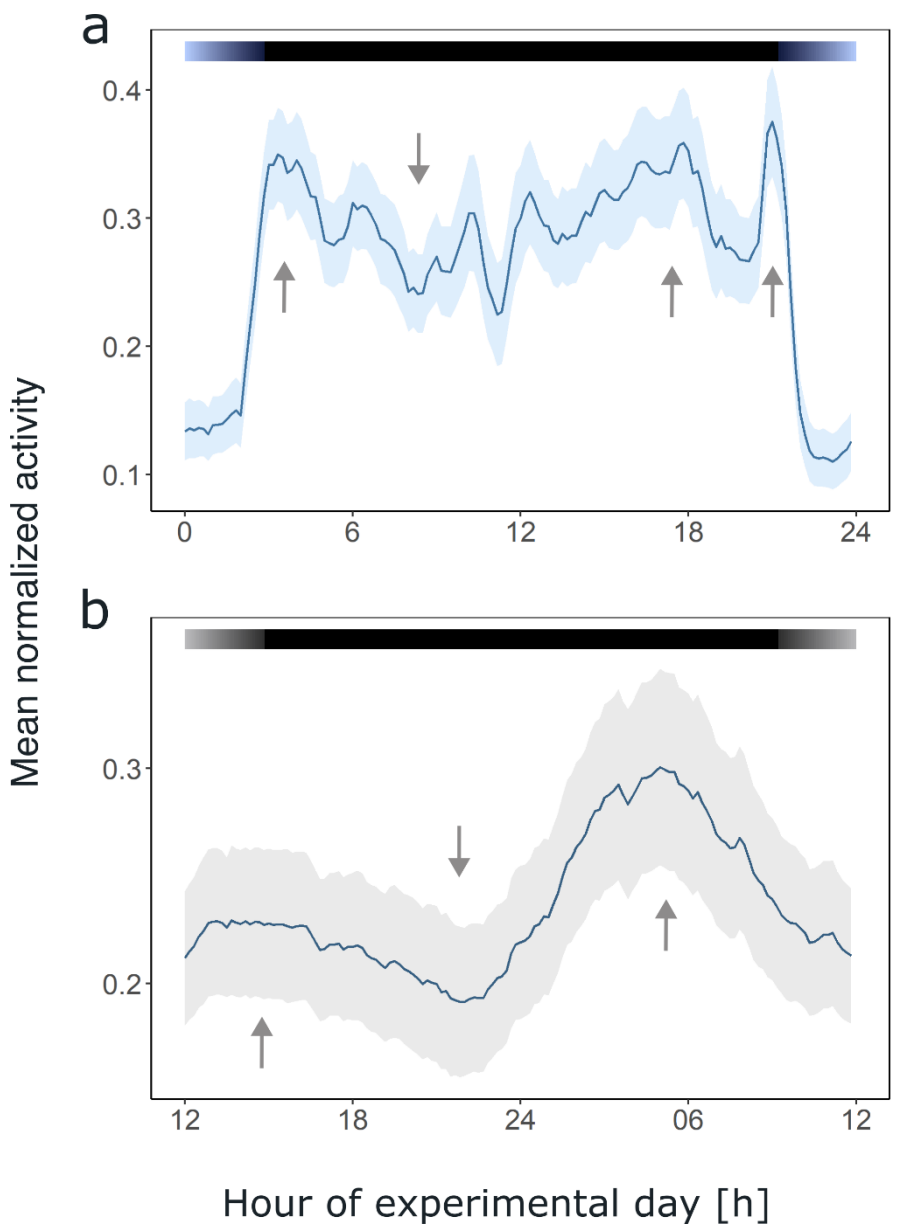


Figure S5: Comparison of daily swimming activity between LD and DD conditions. Group mean swimming activity of rhythmic individuals over an average day under 3 days of simulated short-day conditions (A) and 5 days of constant darkness (B)during the short day treatment of experiment 1. Color bars at the 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..

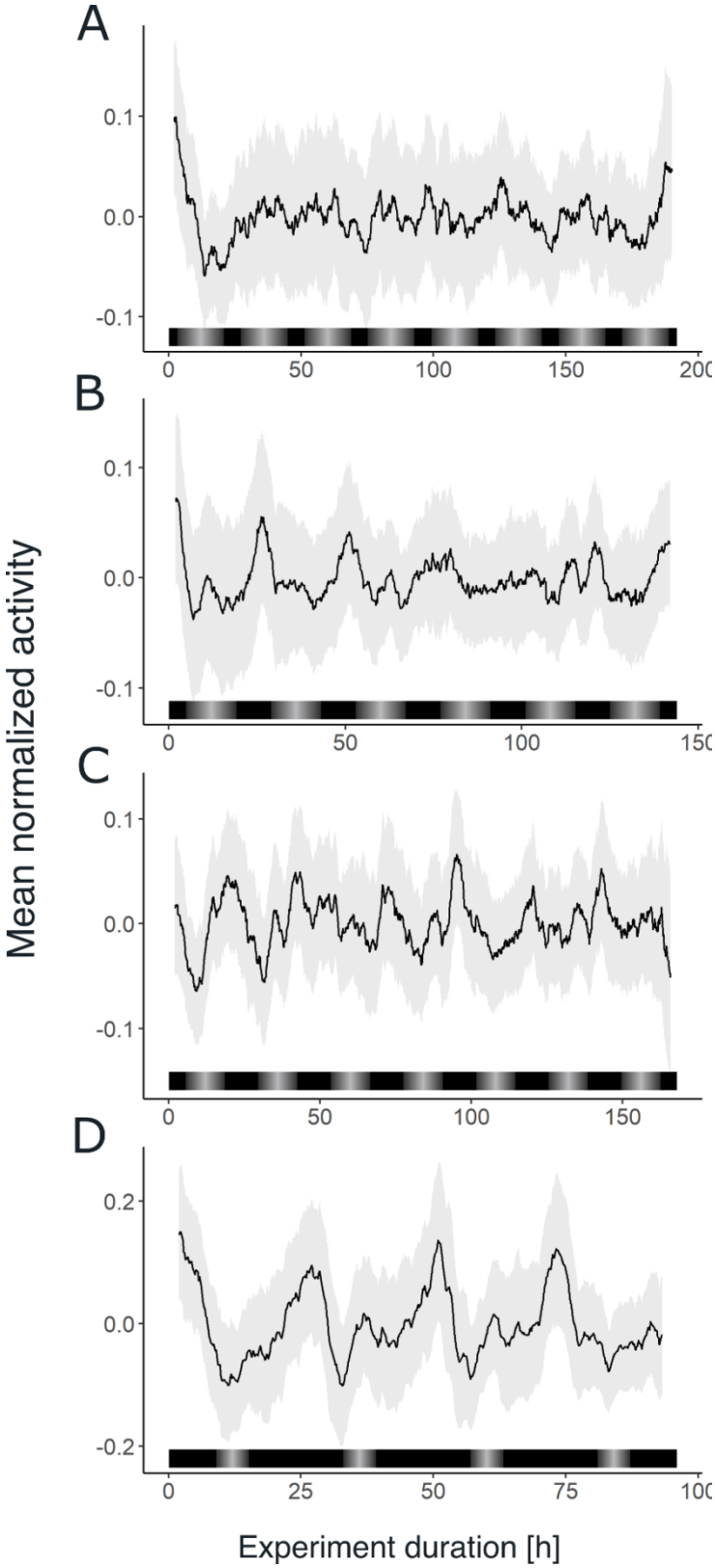


Figure S6: Group swimming activity under DD conditions across seasons.
Group mean swimming activity of rhythmic individuals under DD conditions after individuals have been sampled from their natural environment during summer (A), late summer (B), autumn (C), and winter (D) for experiment 2. Grey shading indicates the s.e.m..

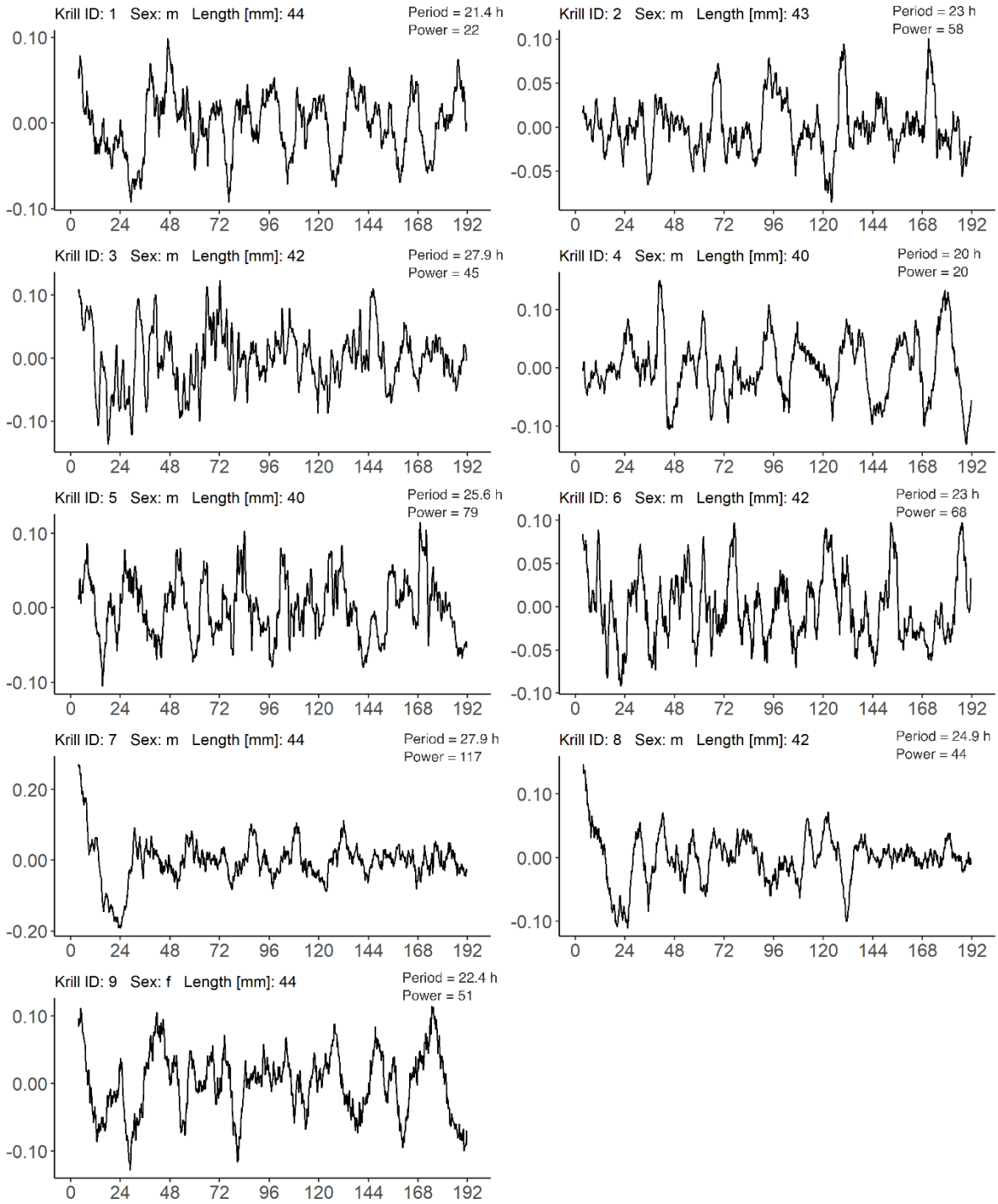


Figure S7: Individual swimming activity under DD conditions of krill sampled in Summer.

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.

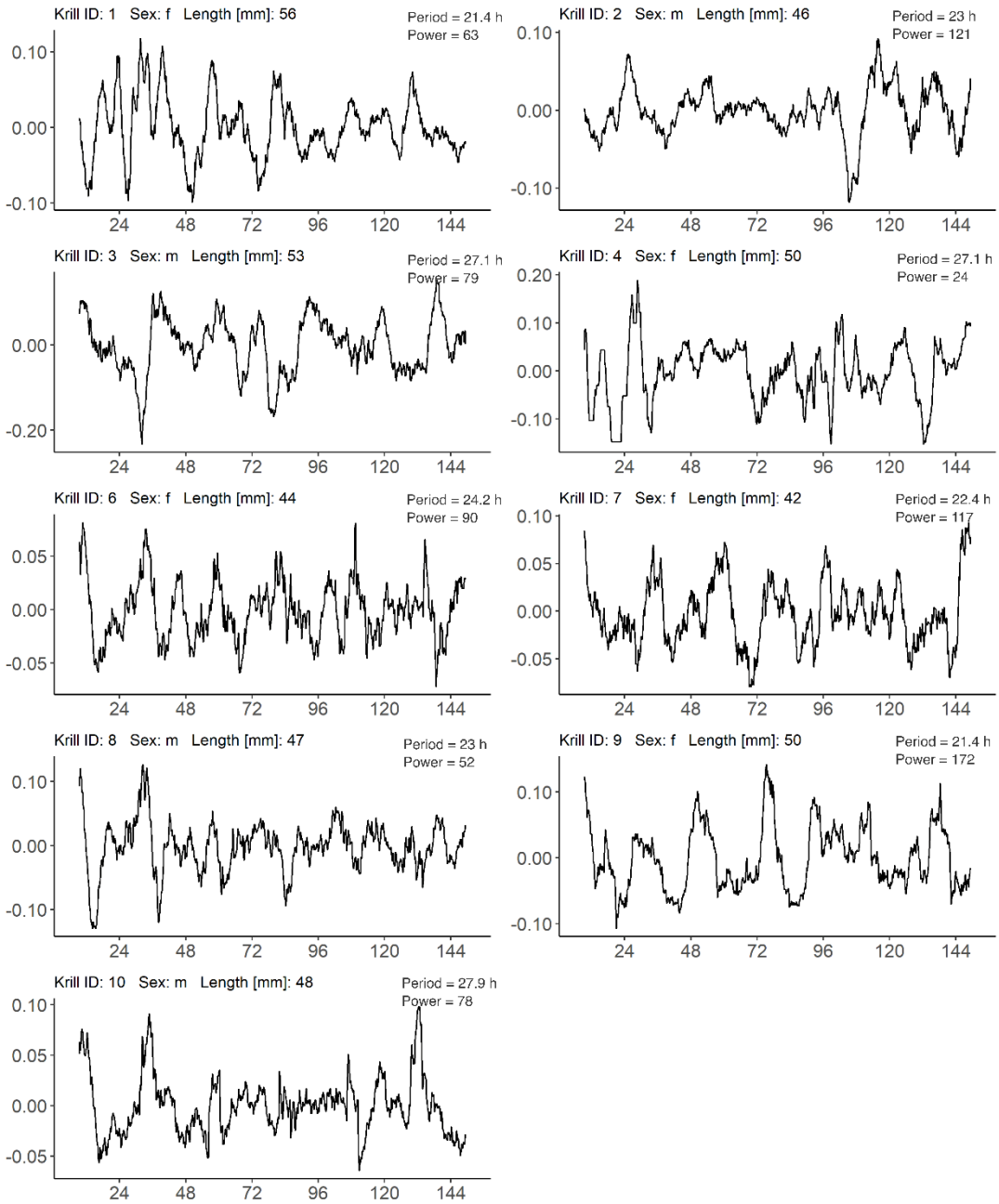


Figure S8: Individual swimming activity under DD conditions of krill sampled in Late Summer.

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.

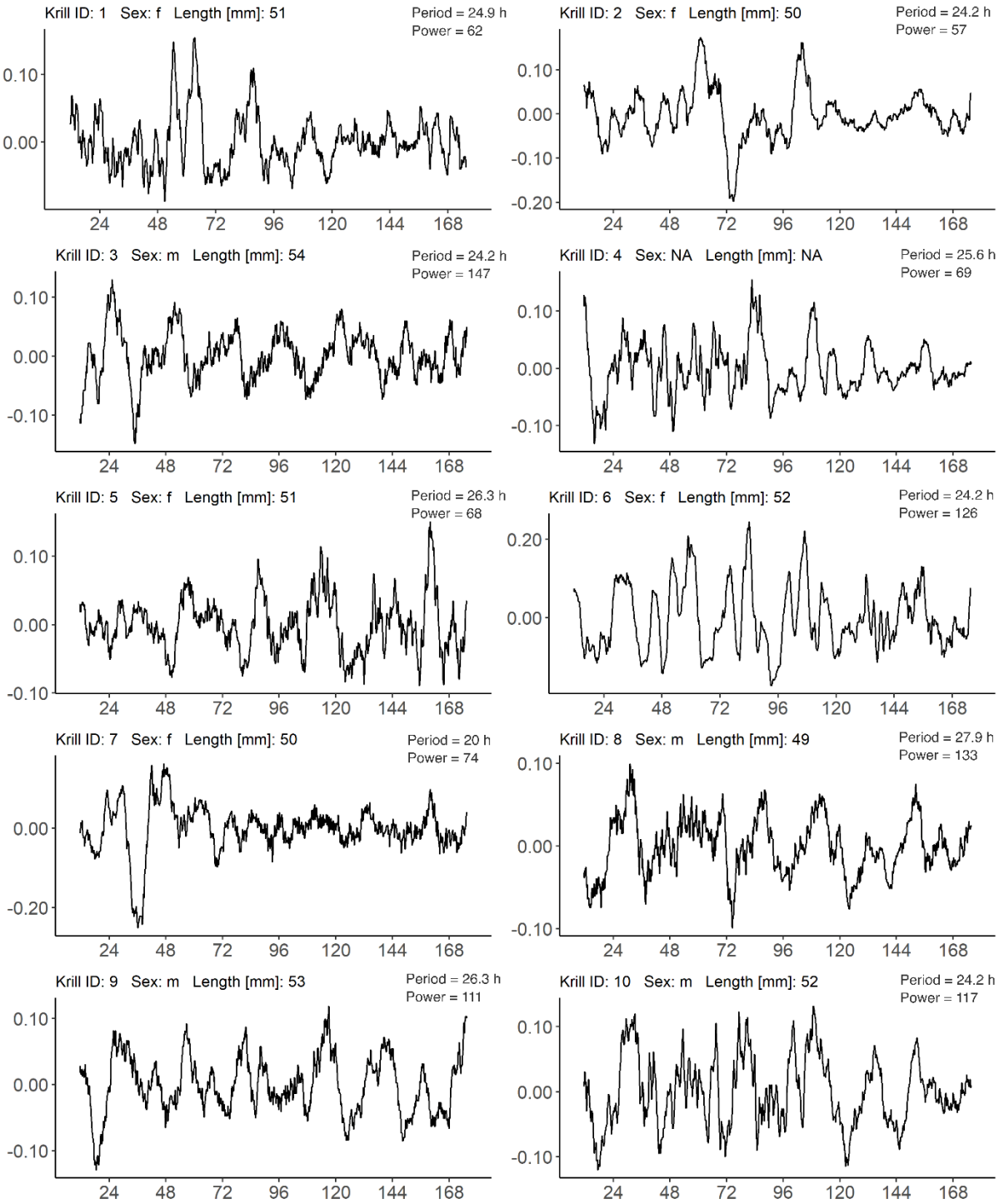


Figure S9: Individual swimming activity under DD conditions of krill sampled in Autumn.

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.

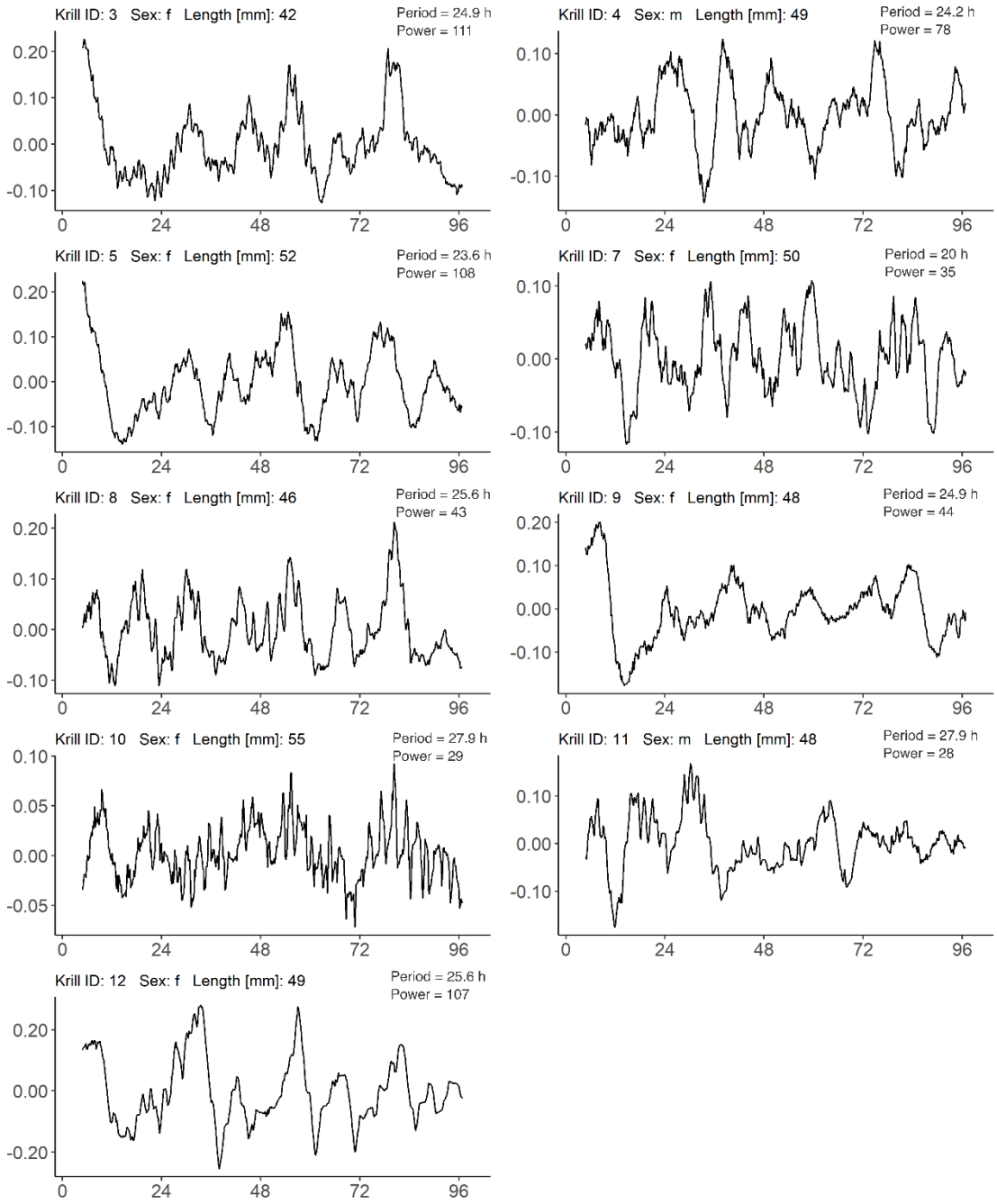


Figure S10: Individual swimming activity under DD conditions of krill sampled in Winter.

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.

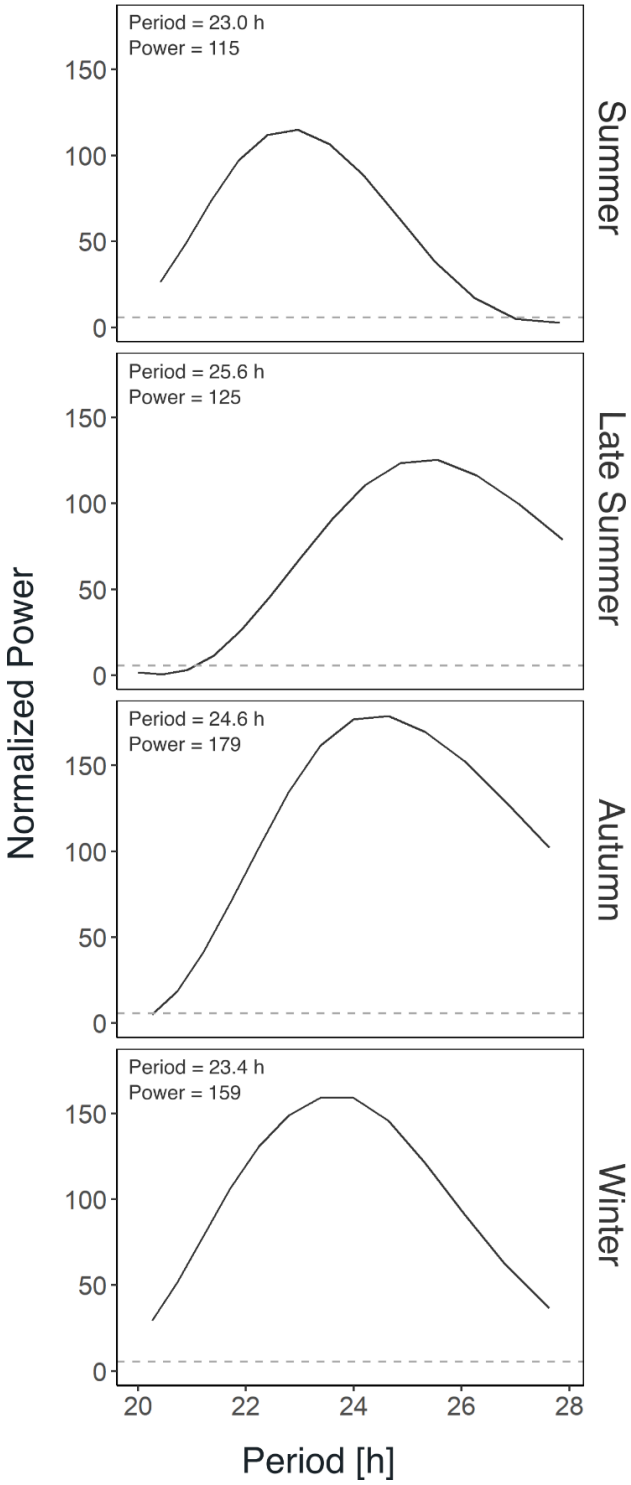


Figure S11: Rhythm analysis of group swimming activity under DD conditions across seasons. Periodogram derived from Lomb-Scargle Periodogram analysis (LSP) of group mean swimming activity under DD conditions after individuals have been sampled from their natural environment in different seasons for experiment 2 (see fig. S6).