The role of photoperiod in the entrainment of endogenous clocks and rhythms in Antarctic krill (*Euphausia superba*)

Die Rolle der Photoperiode in der Synchronisation von endogenen Uhren und Rhythmen im Antarktischen Krill (*Euphausia superba*)

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Dedicated to latecomers

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List of abbreviations

°C	degrees Celsius
°E	degrees of longitude East
°N	degrees of latitude North
°S	degrees of latitude South
µg	microgram
µl	microliter
µm	micrometer
µmol	micromole
AAD	Australian Antarctic Division
AGkrill	Arbeitsgruppe Krill
AIC	Akaike information criterion
AMLR	Antarctic Marine Living Resources
ATP	adenosine triphosphate
AWI	Alfred Wegener Institute
b	brain
bHLH	basic helix-loop-helix
C	carbon
cDNA	complementary deoxyribonucleic acid
Chl *a*	chlorophyll *a*
cm	centimeter
Cq	quantification cycle
CT	circadian time
Ct	cycle threshold
d	day
DD	constant darkness
DEH	Department of Environment and Heritage

DM	dry mass
DNA	deoxyribonucleic acid
DVM	diel vertical migration
e.g.	*exempli gratia*
E-box	enhancer box
edf	estimated degrees of freedom
EPB	Environment Protection and Biodiversity
es	eyestalks
EtOH	ethanol
F	Fisher's statistic
fdr	false discovery rate
FW	fresh weight
g	gram
GAM	generalized additive model
h	hour
i.e.	*id est*
IR	Infrared
KH_2PO_4	potassium dihydrogen phosphate
km	kilometer
K-W	Kruskal-Wallis test
l	liter
LD	light/dark cycle
LL	near-constant light
LM	linear model
m	meter
MDH	malate dehydrogenase
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar

mmol	millimole
mRNA	messenger ribonucleic acid
N	nitrogen
n	number of replicates
NADH	reduced nicotinamide adenine dinucleotide
n.s.	not significant
ng	nanogram
NRQ	normalized relative quantity
O_2	molecular oxygen
p	probability value
PACES	Polar Regions and Coasts in a Changing Earth System
PCR	polymerase chain reaction
PDF	pigment dispersing factor
pg	picogram
ppb	parts per billion
qPCR	quantitative polymerase chain reaction
R	correlation coefficient
r	retinae
R^2	coefficient of determination
RMT	rectangular midwater trawl
RNA	ribonucleic acid
RNAi	interference ribonucleic acid
rpm	rotation per minute
RSV	research survey vessel
s	second
SCN	suprachiasmatic nucleus
SE	standard error
SEM	standard error of the mean
SO	Southern Ocean
T	period of oscillation
t	student-*t* statistic

U	enzyme activity unit
UTC	universal time coordinated
ZT	*Zeitgeber* time
ε	extinction coefficient

Summary

Antarctic krill (*Euphausia superba*), hereafter krill, are key players in the ecosystem of the Southern Ocean. They are distributed all around Antarctica, and they are exceptionally abundant, representing the main link between primary producers and the higher trophic levels in the Antarctic marine food web. Due to their high ecological relevance, krill have been extensively studied in the field and in the laboratory, and it is known that their life-cycle is shaped by fundamental daily and seasonal rhythmic events. Actual knowledge about the external and internal factors involved in the regulation of rhythmic functions in krill is still quite limited but pivotal, especially in the context of future environmental changes driven by climate change.

One hypothesis is that the daily and seasonal rhythmic functions in krill might be regulated through the activity of so-called "endogenous" clocks. Endogenous clocks are molecular function units, which promote rhythmic oscillations in transcription, physiology and behavior at the daily and seasonal levels. Endogenous clocks can be entrained (i.e. synchronized) by rhythmic environmental cues, like the day/night cycle (i.e. photoperiod = day length) at the daily level, and the seasonal photoperiodic cycle at the seasonal level. The implications of endogenous rhythmicity (i.e. rhythmicity promoted by endogenous clocks) in the regulation of rhythmic biological functions are well documented among terrestrial species, but studies dealing with marine organisms are very scarce.

At the daily level, the best studied endogenous clock is the circadian clock, which is based on molecular feedback loops generating a rhythm with a period of approximately 24 h. Specific light-sensitive proteins promote the entrainment of the circadian clock with the day/night cycle, ensuring effective synchronization of rhythmic output functions according to daily recurring environmental changes. In krill, a circadian clock has been recently identified and characterized, and its influence on daily rhythms of metabolism and transcription has been demonstrated in the laboratory and in natural conditions. At the seasonal level, the regulation

of rhythmic functions is less well understood, also in terrestrial species. An endogenous circannual clock seems to be involved, but the molecular mechanisms underlying its functioning are still unclear. Due to its ability to measure changes in day length, the circadian clock might contribute to the seasonal entrainment of the circannual clock. In krill, a circannual rhythm (i.e. a rhythm promoted by a circannual clock) might be involved in the regulation of the seasonal shifts in sexual maturity and metabolic activity observed in the field in summer and winter.

During this dissertation, I investigated the involvement of endogenous clocks and rhythms in the regulation of rhythmic functions in krill at the daily and seasonal levels. Moreover, I also examined the role played by photoperiod in the entrainment of those clocks and rhythms. The work focused on three main research topics, which resulted in three publications: 1) the impact of the extreme seasonal photoperiodic cycle of the Southern Ocean on the activity of the circadian clock of krill at different times of the year (Publication I); 2) the involvement of an endogenous circannual rhythm and the role played by photoperiod in the regulation of the seasonal metabolic activity cycle of krill (Publication II); and 3) the involvement of the circadian clock and the role played by photoperiod in the regulation of diel vertical migration (DVM) in krill (Publication III).

In **publication I**, I investigated the activity of the circadian clock of krill in different simulated seasonal Antarctic light conditions. The extreme variability displayed by the seasonal photoperiodic cycle in the Southern Ocean might cause a problem for the photoperiodic entrainment of the clock in different seasons. Especially during summer and winter, when overt light/dark cues are missing, the clock might get disrupted and the clock output might become arrhythmic. Indeed, laboratory work demonstrated that under simulated mid-summer and mid-winter conditions, when overt photoperiodic cues were missing, the circadian clock of krill was arrhythmic, and the metabolic output was de-synchronized. Conversely, under simulated early-autumn and late-winter conditions, when overt photoperiodic cues were present, the circadian clock of krill was active, and the metabolic output was synchronized with the light/dark cycle. This suggested that major changes are occurring during the year in the entraining process of the circadian clock of krill, depending on the different seasonal light conditions to which krill are exposed.

In **publication II**, I investigated the involvement of an endogenous circannual rhythm in the regulation of the seasonal metabolic activity cycle of krill. Moreover, I also examined the role played by photoperiod in the entrainment of this rhythm. In response to the strong seasonal variability displayed by light and food availability in the Southern Ocean, krill display seasonal differences in metabolic rates, feeding activity and growth. During summer, when light and food availability is high, krill metabolic and feeding activity is enhanced, and krill growth rates are positive. During winter, when light and food conditions are low, krill metabolic and feeding activity is reduced, and krill show reduced growth or even shrinkage (i.e. reduction of size). It has been hypothesized that an endogenous rhythm entrained by the seasonal Antarctic light regime might be responsible for the regulation of the seasonal metabolic cycle of krill. Krill exposed to different long-term simulated natural seasonal light conditions, showed seasonal patterns of growth, enzyme activity and gene expression of key metabolic genes, which were also observed in krill exposed to constant darkness. The results strongly suggested the involvement of a circannual clock in the regulation of the seasonal metabolic cycle of krill. However, major differences were observed in the seasonal patterns of oxygen consumption, suggesting that exposition of krill to specific seasonal light cues might be necessary for the effective entrainment of the circannual clock.

In **publication III**, I investigated the involvement of an endogenous circadian rhythm in the regulation of krill diel vertical migration (DVM). Moreover, I also examined the role played by photoperiod in the entrainment of krill DVM. DVM is a mass migratory movement displayed by many zooplankton species worldwide. During the night, the animals come to the surface to graze on phytoplankton, while during the day they sink to deeper layers to escape from visual predators. The environmental factors involved in the regulation of DVM are photoperiod, food availability and presence/absence of predators. However, DVM occurs also in constantly dark environments (e.g. the deep sea and the Arctic ocean during the polar night), suggesting the involvement of an endogenous rhythm of regulation. Using krill exposed to different light/dark (LD) and constant darkness (DD) conditions, I found that krill DVM was driven by an endogenous rhythm, with krill moving upward during the light phase and downward during the dark phase. A similar rhythm was found in krill oxygen consumption, confirming the presence of an endogenous rhythm of activity associated with DVM. Rhythmic expression of clock genes related to the circadian clock was found in the

eyestalks of krill entrained to similar LD conditions, suggesting that an involvement of the circadian clock in the regulation of krill DVM would be possible. Major differences were observed among individual krill in the rhythmic regulation of DVM and oxygen consumption, suggesting that the circadian system of krill might display high degrees of individual plasticity.

In conclusion, this dissertation improves our knowledge about the mechanisms regulating daily and seasonal rhythmic functions in the Antarctic krill, *E. superba*. The implication of endogenous rhythmicity was demonstrated for krill DVM at the daily level, and for krill seasonal metabolic cycle at the seasonal level. Photoperiod proved to be a most fundamental factor for the entrainment of krill DVM and krill seasonal metabolic cycle, as well as for the modulation of the activity of the circadian clock of krill at different times of the year. This work provides an example of how techniques which have been developed to study the molecular biology and chronobiology of terrestrial model species can be applied to the study of ecologically relevant species in the marine environments. In the future, understanding the regulation of rhythmic functions in ecological key marine species like Antarctic krill will help us to understand how these species will adapt to environmental changes driven by climate change.

Zusammenfassung

Antarktischer Krill (*Euphausia superba*), im Folgenden Krill genannt, spielt eine Schlüsselrolle im Ökosystem des Südpolarmeeres. Er ist in den antarktischen Gewässern weit verbreitet, weist eine sehr hohe Abundanz auf und stellt die Hauptverbindung zwischen den Primärproduzenten und den höheren trophischen Ebenen im antarktischen marinen Nahrungsnetz dar. Aufgrund seiner hohen ökologischen Relevanz wurde Krill intensiv im Feld und im Labor untersucht. Es ist bereits bekannt, dass sein Lebenszyklus von täglichen und saisonalen Rhythmen geprägt ist. Das tatsächliche Wissen über die äußeren und inneren Faktoren, welche bei der Regulierung der rhythmischen Funktionen im Krill eine Rolle spielen, ist bislang jedoch noch sehr limitiert aber im Kontext zukünftiger Umweltveränderungen, die durch den Klimawandel ausgelöst werden, von zentraler Bedeutung.

Es wird angenommen, dass die täglichen und saisonalen Rhythmen in Krill durch die Aktivität sogenannter "endogener" Uhren reguliert werden. Endogene Uhren sind molekulare Funktionseinheiten, die rhythmische Oszillationen in Transkription, Physiologie und Verhalten auf täglicher und jahreszeitlicher Ebene erzeugen. Endogene Uhren können durch rhythmische Umgebungssignale wie den Tag/Nacht-Zyklus (d. h., Photoperiode = Tageslänge) auf der täglichen Ebene, sowie durch den jahreszeitlichen, photoperiodischen Zyklus, auf der saisonalen Ebene synchronisiert werden. Die Bedeutung der endogenen Rhythmik (d. h., der durch endogene Uhren erzeugten Rhythmik) für die Regulierung von biologischen Funktionen ist unter terrestrischen Spezies bereits gut dokumentiert. Studien, die sie sich in diesem Zusammenhang mit marinen Organismen befassen, sind dagegen eher selten.

Auf der täglichen Ebene ist die am besten untersuchte endogene Uhr die zirkadiane Uhr. Diese basiert auf molekularen Rückkopplungsschleifen, die einen Rhythmus mit einer Dauer von ungefähr 24 Stunden erzeugen. Spezifische, lichtempfindliche Proteine erzeugen die Synchronisation der zirkadianen Uhr mit dem Tag/Nacht-Zyklus, wodurch eine effektive

Anpassung der rhythmischen Funktionen (Output) an täglich wiederkehrenden Umweltveränderungen sichergestellt wird. Kürzlich wurde in Krill eine zirkadiane Uhr sowohl identifiziert als auch charakterisiert, und ihr Einfluss auf die tägliche Rhythmik im Metabolismus und der Transkription im Labor und unter natürlichen Bedingungen wurde nachgewiesen. Im Vergleich dazu ist die Regulation rhythmischer Funktionen auf der saisonalen Ebene nur wenig untersucht. Dies gilt auch für die weit besser untersuchten, terrestrischen Arten. Es scheint eine endogene zirkannuale Uhr daran beteiligt zu sein, jedoch sind die zugrunde liegenden molekularen Mechanismen bislang noch unklar. Aufgrund ihrer Fähigkeit, Änderungen in der Tageslänge zu messen, könnte die zirkadiane Uhr aber zur saisonalen Synchronisation der zirkannualen Uhr beitragen. In Krill könnte ein circannueller Rhythmus (d. h., erzeugt von einer zirkannualen Uhr) an der Regulation der im Feld beobachteten saisonalen Verschiebungen in der Geschlechtsreife sowie in der metabolischen Aktivität zwischen Sommer und Winter beteiligt sein.

Im Rahmen dieser Dissertation untersuchte ich die Beteiligung endogener Uhren und Rhythmen an der Regulierung rhythmischer biologischer Funktionen in Krill, sowohl auf täglicher als auch auf jahreszeitlicher Ebene. Darüber hinaus untersuchte ich die Rolle der Photoperiode bei der Synchronisation der beteiligten endogenen Uhren und Rhythmen. Die Arbeit konzentrierte sich auf drei Forschungsschwerpunkte, die in drei Publikationen mündeten: 1) die Auswirkungen des extremen, saisonalen photoperiodischen Zyklus im Südpolarmeer auf die Aktivität der zirkadianen Uhr in Krill in verschiedenen Jahreszeiten (Publikation I); 2) die Beteiligung eines endogenen zirkannuellen Rhythmus und die Rolle der Photoperiode bei der Regulation der saisonalen metabolischen Aktivität in Krill (Publikation II); und 3) die Beteiligung der zirkadianen Uhr sowie die Rolle der Photoperiode bei der Regulation der täglichen Vertikalwanderung (diel vertical migration = DVM) in Krill (Publikation III).

In **Publikation I** wurde die Aktivität der zirkadianen Uhr in Krill unter verschiedenen simulierten saisonalen antarktischen Lichtverhältnissen untersucht. Die extreme Variabilität des saisonalen photoperiodischen Zyklus im Südpolarmeer könnte ein Problem für die Synchronisation der inneren Uhr mit der Photoperiode in den verschiedenen Jahreszeiten darstellen. Besonders im Sommer und Winter, wenn ausgeprägte Hell/-Dunkelphasen fehlen,

kann die Uhr gestört und ihr Takt arrhythmisch werden. Tatsächlich zeigten Laborarbeiten, dass unter simulierten Sommer- und Winter-Bedingungen, durch das Fehlen von erkennbaren photoperiodischen Reizen sowohl die zirkadiane Uhr arrhythmisch als auch das metabolische Signal (Output) de-synchronisiert waren. Umgekehrt war unter simulierten Frühherbst- und Spätwinterbedingungen, wenn also erkennbare photoperiodische Reize vorhanden waren, die zirkadiane Uhr von Krill aktiv, und der metabolische Output war mit dem Hell-Dunkel-Zyklus synchronisiert. Dies deutet darauf hin, dass im Laufe des Jahres größere Veränderungen in der Synchronisation der zirkadianen Uhr von Krill auftreten, abhängig von den unterschiedlichen jahreszeitlichen Lichtverhältnissen, denen Krill ausgesetzt ist.

In **Publikation II** untersuchte ich die Beteiligung eines endogenen zirkannuellen Rhythmus bei der Regulation des saisonalen metabolischen Aktivitätszyklus von Krill. Darüber hinaus untersuchte ich die Rolle der Photoperiode bei der Synchronisation dieses Rhythmus. Als Reaktion auf die starke saisonale Variabilität, die sich durch die Verfügbarkeit von Licht und Futter im Südpolarmeer zeigt, weist Krill saisonale Unterschiede in Stoffwechselrate, Futteraktivität und Wachstum auf. Im Sommer, wenn die Verfügbarkeit von Licht und Futter hoch ist, sind sowohl Stoffwechselrate als auch Fressaktivität gesteigert und die Wachstumsraten sind positiv. Im Winter, wenn die Lichtbedingungen schwach und die Nahrungskonzentration gering sind, sind der Stoffwechsel und die Nahrungsaufnahme reduziert und es findet kein Wachstum statt oder es ist sogar negativ (d. h., die Tiere schrumpfen). Es wurde die Hypothese aufgestellt, dass ein endogener Rhythmus, der vom saisonalen antarktischen Lichtregime synchronisiert wird, für die Regulation der saisonalen metabolischen Aktivität von Krill verantwortlich sein könnte. Krill, der simulierten natürlichen Lichtbedingungen ausgesetzt war, zeigte saisonale Veränderungen im Wachstum, Enzymaktivität, Sauerstoffverbrauch und in der Genexpression, die auch beim Krill zu beobachten waren, der konstanter Dunkelheit ausgesetzt war. Diese Ergebnisse geben einen deutlichen Hinweis, dass eine zirkannuelle Uhr in der saisonalen Regulation der beobachteten Parameter involviert ist. Es wurden jedoch große Unterschiede in den jahreszeitlichen Mustern des Sauerstoffverbrauchs festgestellt zwischen Krill, die einem simulierten natürlichen saisonalen Lichtregime ausgesetzt wurden und Krill die sich in konstanter Dunkelheit befanden, was darauf hindeutet, dass für eine effektive

Synchronisation der zirkannualen Uhr in Krill, bestimmte jahreszeitliche Lichtreize notwendig sein könnten .

In **Publikation III** untersuchte ich die Beteiligung eines endogenen zirkadianen Rhythmus, sowie die Rolle der Photoperiode, bei der Regulation der täglichen Vertikalwanderung (DVM) in krill. DVM ist eine Massen-Wanderbewegung, die weltweit bei vielen Zooplanktonarten beobachtet werden kann. Während der Nacht kommen die Tiere an die Oberfläche um zu fressen, während sie zur Vermeidung von visuellen Räubern tagsüber in tiefere Wasserschichten sinken. Bei der Regulation von DVM spielen verschiedene Umweltfaktoren eine Rolle, wie die Photoperiode, die Verfügbarkeit von Nahrungsmitteln und die Anwesenheit/Abwesenheit von Predatoren. DVM tritt jedoch auch in Habitaten und Jahreszeiten von konstanter Dunkelheit (wie z. B. der Tiefsee und dem Arktischen Ozean während der Polarnacht) auf, was auf eine Beteiligung eines endogenen Regulationsrhythmus schließen lässt. Ich untersuchte Krill, der zuvor verschiedenen Hell/Dunkel (LD) Bedingungen und konstanter Dunkelheit (DD) ausgesetzt war. Hierbei zeigte sich, dass DVM in Krill von einem endogenen Rhythmus angetrieben zu sein scheint, wobei während der Lichtphase eine Aufwärts- und während der Dunkelphase eine Abwärtsbewegung festgestellt wurde. Ein ähnlicher Rhythmus wurde auch im Sauerstoffkonsum beobachtet, was das Vorhandensein eines endogenen Aktivitätsrhythmus im Zusammenhang mit der DVM bestätigt. Die rhythmische Expression von Uhr-Genen (die mit der zirkadianen Uhr assoziiert werden) in den Augenstielen von Krill, der ähnlichen LD-Bedingungen ausgesetzt war, legt nahe, dass eine Beteiligung der zirkadianen Uhr bei der DVM-Regulation in Krill möglich wäre. Bei den gemessenen Parametern wurden außerdem große Unterschiede zwischen den Individuen beobachtet, was auf einen hohen Grad an individueller Plastizität im zirkadianen System von Krill hindeutet.

Diese Dissertation verbessert unser Wissen über die Mechanismen zur Regulation der täglichen und saisonalen rhythmischen Funktionen in Antarktischem Krill, *E. superba*. Die Bedeutung der endogenen Rhythmik wurde für DVM auf der täglichen Ebene und für den saisonalen Stoffwechselzyklus auf der jahresezeitlichen Ebene gezeigt. Die Photoperiode erwies sich hier als der wichtigste Faktor für deren Synchronisation, sowie für die Modulation der Aktivität der zirkadianen Uhr zu verschiedenen Zeiten des Jahres. Diese

Arbeit liefert ein Beispiel dafür, wie Techniken, die entwickelt wurden, um die Molekularbiologie und Chronobiologie von terrestrischen Modellarten zu studieren, auf das Studium von ökologisch relevanten Arten in der marinen Umwelt angewendet werden können. In Zukunft wird das zunehmende Wissen über biologische Zeitgebungs-Funktionen in ökologisch wichtigen marinen Tieren wie dem antarktischen Krill außerdem helfen, die Auswirkungen des Klimawandels auf marine Ökosysteme zu verstehen.

1. General introduction

This dissertation collects the results of 4 years of laboratory research on the mechanisms underlying daily rhythms and seasonal cycles in Antarctic krill (*Euphausia superba*), one of the dominant zooplankton species in the Southern Ocean. The work was developed within the framework of the Helmholtz Virtual Institute PolarTime, which brought together partner institutions from Germany, Italy and Australia, with the main goal of investigating biological timing in polar pelagic key-species. In this introduction, I will present the major aspects related to the dissertation and clarify the objectives of the research, which was subdivided into three publications. First, I will provide a general description of the basic facts regarding the target organism, *Euphausia superba*, with special attention on those daily rhythms and seasonal cycles displayed by krill in the field, which were the subject of our investigation. After that, I will introduce the concept of endogenous regulation of rhythmic biological functions, with particular attention to the circadian clock and its involvement in the regulation of biological timekeeping at the daily and seasonal levels. This will lead into a short review of the main pre-existing findings related to the circadian clock in krill, with an emphasis on their ecological implications and open questions. Finally, I will present the overall research goal of the dissertation and introduce the specific research objectives of each single publication.

1.1. The Antarctic krill *Euphausia superba*

The Antarctic krill, *Euphausia superba* (Dana 1850), is a crustacean belonging to the order Euphausiacea, superorder Eucarida. The term ’krill’ comes from the Norwegian *kril* meaning “small fish”, and was originally introduced by North Atlantic whalers to describe the small crustaceans found in the stomachs of baleen whales (Nicol 1994). At present, the term refers to a group of about 85 species of pelagic shrimp-like crustaceans, also known as Euphausiids,

which are widespread in all the oceans of the world ranging from the tropics to the polar region (Siegel 2000, Nicol 2003). Among Euphausiids, three species are characterized by high abundance, and are therefore of great importance for the marine ecosystem and for human exploitation (Everson 2000): *Euphausia pacifica*, which is found in the North Pacific Ocean; *Meganyctiphanes norvegica*, which occurs mostly in the North Atlantic region; and *Euphausia superba*, the target of our study, which dominates the zooplankton communities in the Southern Ocean.

Morphology, development and distribution

Adult *Euphausia superba* (hereafter krill) can grow up to a maximum length of about 65 mm (Nicol & Endo 1999). Their body is surrounded by a calcified exoskeleton divided into a cephalothorax and an abdomen (Fig. 1.1A).

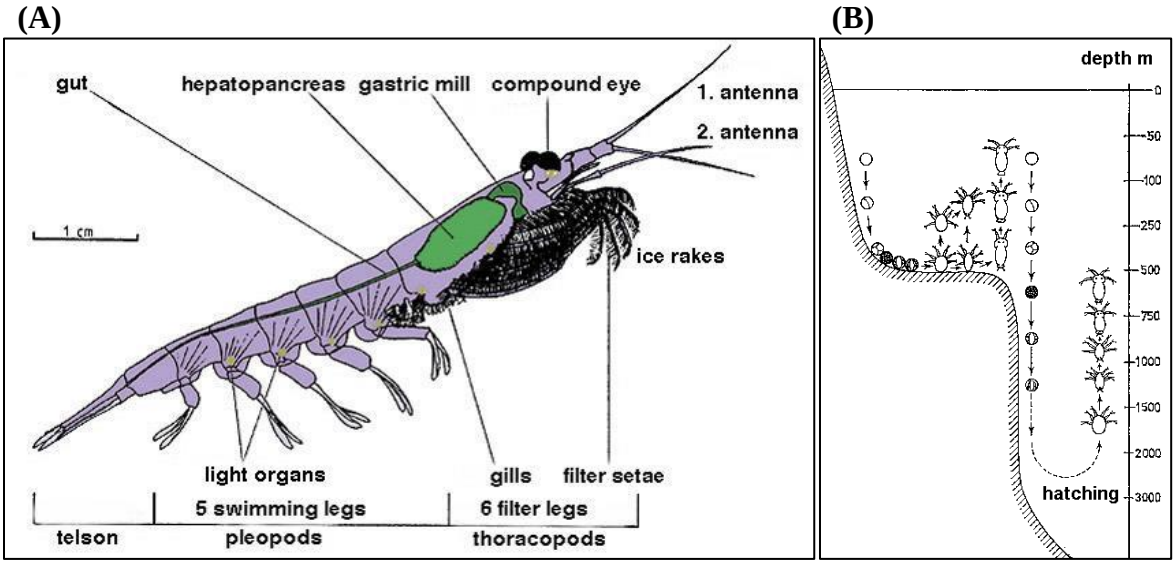

Figure 1.1 (A) Schematic representation of adult *Euphausia superba* Dana (colors are just to enhance contrast and do not correspond to the original). Author: Uwe Kils; license: CC BY-SA 3.0; unmodified. (B) Schematic representation of krill developmental ascent. Author: Uwe Kils; license: CC BY-SA 3.0; unmodified.

The cephalothorax carries the compound eyes, one pair of antennae and the thoracopods, which are used for filter feeding (filter legs). The gills are carried under the cephalothorax, which contains the digestive apparatus (composed by a gastric mill and a hepatopancreas) and the heart (not shown). In mature females, the external sexual organs (thelycum) are

carried under the cephalothorax at the intersection with the abdomen just after the gills, while the ovaries develop inside the cephalothorax. The gut stretches from the hepatopancreas into the abdomen, which is divided into six segments. The abdomen carries the pleopods, used for active swimming (swimming legs), and the body ends with a tail, created by the fusion of the last pair of pleopods (telson). In males, the first pleopods pair carries the sexual organs (petasma) together with the spermatophores. Krill possess luminous organs called photophores which are located close to the mouthparts, at the genitals (females) and at the base of the pleopods, and might be used for social interaction and camouflage.

During development, krill undergo several larval stages (Fig. 1.1B), known as nauplius, metanauplius, calyptopus and furcilia. Molts occur between each larval stage (sometimes also within), and each stage lasts between 8 and 15 days. The laid eggs sink for about 10 days, reaching in some cases as deep as 2000 m. After that, they hatch as nauplii, which have only one eye and no body segments or limb buds. The nauplii start the developmental ascent towards the surface and enter a metanauplius stage, where limb development begins. As larvae continue to rise, they further develop into calyptopes, which reach the surface and begin to feed. After three additional molts, larvae become known as furcilia, which possess movable compound eyes projecting from the edge of the carapace. Finally, furcilia develop into juveniles, which will become sexually mature adults during their second year (spring/summer) and begin to spawn at two years of age.

Krill are obligated schoolers, meaning that they mostly occur in large assemblages having a mean length of hundreds of meters, even if exceptionally long schools (up to 100 km) have been reported (Hamner & Hamner 2000). Krill density within a school may reach up to 100,000 individuals per cubic meter, but average densities are in the order of hundreds. Schooling is considered as a generic strategy implemented by krill to avoid individuals to be singled out by predators. However, it is unclear how schooling may represent an advantage with respect to large filter-feeding predators like baleen whales. Krill main food source is phytoplankton, but they can efficiently exploit also other energy sources like sea-bed detritus and other heterotrophic preys.

Antarctic krill display a circumpolar distribution (Fig. 1.2A), with a latitudinal range spanning from 51°S to 70°S, but more than half of the population is usually located in the

southwest Atlantic sector of the Southern Ocean and in the region of the West Antarctic Peninsula (Atkinson et al. 2004, Siegel 2016).

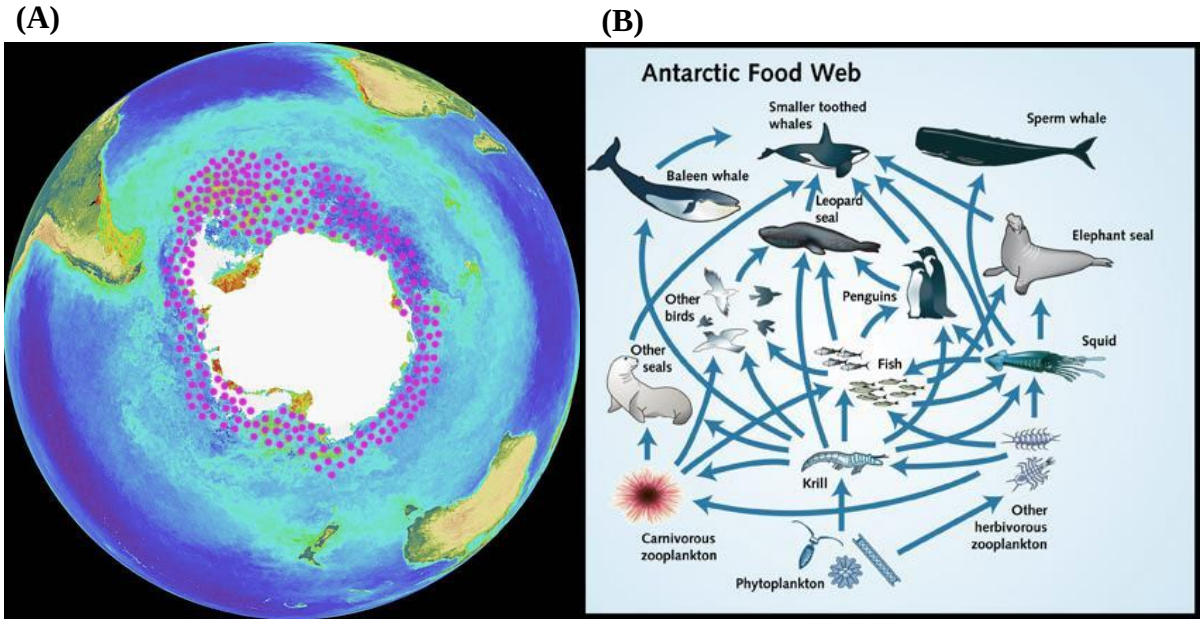

Figure 1.2: (A) Schematic representation of krill circumpolar distribution (purple dots represent krill). Author: Uwe Kils; license: CC BY-SA 3.0; unmodified. (B) Schematic representation of the Antarctic food web showing the central trophic role of krill as link between primary producers and higher trophic levels.

Krill are found mostly along the continental shelf break and slope, with the juveniles being preferentially located more inshore and the eggs and larvae more offshore (Siegel 2000, Siegel 2016). Usually, krill inhabit the upper 200 m of the water column, but in winter they are generally found in the deeper layers around 350 m and occasionally also as deep as 600 m (Marr 1962, Quetin & Ross 1991, Nicol 2006). Among the factors affecting krill distribution, sea surface circulation and winter sea-ice extent have often been implicated, but the only factor which seems to apply over the entire circumpolar range is food availability, with higher krill concentrations always associated with abundant food (Siegel 2005, 2016).

Ecological relevance and impact of climate change

Due to their distribution and abundance (it is generally agreed that krill represent 50% of the total zooplankton standing crop of the Southern Ocean) (Knox 1984), Antarctic krill are the dominant herbivores in the Antarctic food web (Fig. 1.2B), playing a key role in the energy

transfer from the primary producers (phytoplankton) to the higher trophic levels. In this sense, the status of the krill stock can affect the growth and survival of many other groups including fish, squids, seals, birds, penguins and whales. Two of the major krill feeding grounds in the Southern Ocean, the southwest Atlantic sector and the region of the West Antarctic Peninsula, are currently experiencing one of the most rapidly anthropogenic-driven warming on Earth, causing a 1°C increase of the surface summer temperature of the adjacent ocean since 1950 (Meredith & King 2005). There have been indications that the distribution and abundance of krill in the southwest Atlantic sector have already been altered significantly as a result of changes in primary productivity associated with the decline in sea ice (Atkinson et al. 2004). In addition, krill are the object of a developing fishery and concern has been expressed about the future sustainability of Antarctic krill fisheries in a changing environment (Schiermeier 2010). Krill's central position in the food web, the ongoing environmental changes in its habitat, and increasing commercial interest emphasize the urgency to understand the adaptability of krill to its environment.

Daily rhythms in krill: diel vertical migration

In the field, krill display daily rhythms in behavior, metabolism and transcription, which help them to coordinate their lifestyle with the changes in the environment. At the behavioral level, krill display diel vertical migration (DVM), swimming upwards towards the surface layers around sunset and downwards towards the deeper layers around sunrise (Quetin & Ross 1991) (Fig. 1.3A). DVM is a major adaptive strategy implemented by zooplankton species in marine and freshwater communities worldwide, and it is generally believed that it evolved in response to visual predator pressure, which would be higher in the surface layers during daytime (Brierley 2014). In the most common DVM pattern, called 'nocturnal', the organisms migrate towards the surface around sunset, where they spend most of the night feeding on phytoplankton, and migrate back towards the deeper layer around sunrise, to escape from visual predators (Hays 2003). Other DVM patterns have been observed, often in association with different modalities of predator-prey interaction, or with particular environmental conditions. For example, in the 'reverse' DVM pattern the animals migrate upwards during the day and downwards during the night. This has been associated with high levels of invertebrate predators that use tactile stimuli rather than vision to locate their prey

(Ohman et al. 1983, Neill 1990). Another option is the 'twilight' DVM pattern, where two following migrations are performed over the 24 h cycle, one around sunset and another around sunrise (Hays 2003). In this case, the 'midnight sink', has been related to a temporary activity decrease possibly driven by satiation or by an endogenous rhythm (Cohen & Forward 2005).

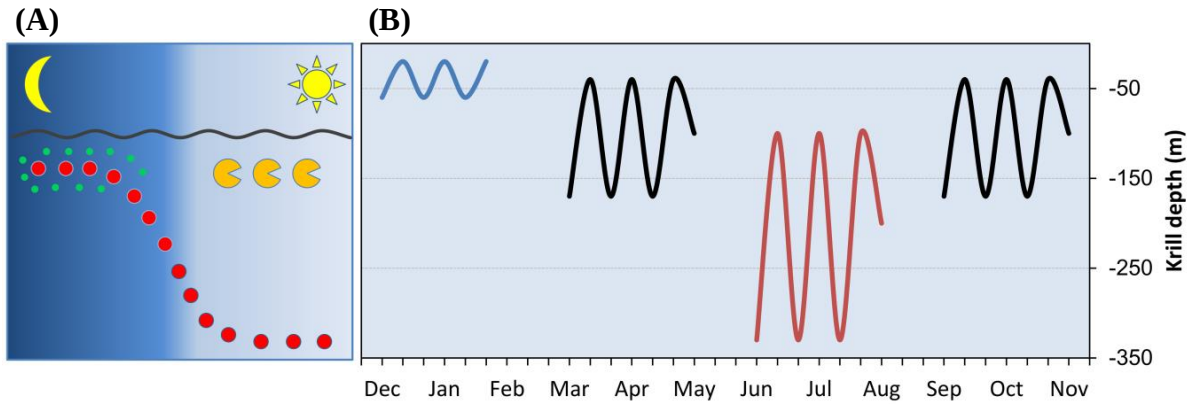

Figure 1.3: (A) Schematic representation of nocturnal DVM: krill (red dots) migrate to the surface at night to feed on phytoplankton (green dots), and sink towards the deeper layers during the day, to escape from visual predators (orange pac-men). (B) Seasonal variability of krill DVM patterns: in summer, krill DVM is restricted within the surface layers; in autumn and spring, it ranges between 50 and 150 m depth; in winter, krill go deeper, and DVM might range approx. between 100 and 350 m (modified after Siegel 2005).

Regarding the main proximate cues involved in DVM regulation, the debate is still open, even if light cues are generally considered to be the major driver, also due to the close association often observed between DVM ascent/descent and sunset/sunrise (Cohen & Forward 2009). However, food conditions and presence/absence of predators are often considered as additional regulatory factors involved in the process, and it has been showed that in the absence of predators some species stop performing DVM (Gliwicz 1986, Bollens & Frost 1989), and that in the presence of very high/very low food concentrations some other species display major alterations of their DVM patterns (Pearre 2003). Moreover, as we already mentioned in relation to the 'twilight' pattern, certain characteristics associated to DVM suggest the interplay of an internal (i.e. endogenous) timing mechanism that would support the rhythmic behavior even in the absence of external cues (Cohen & Forward 2005). Animals that migrate far below the detection limit of light (below 1000 m depth, for example) can still trigger their ascent in order to reach the surface exactly after sunset (van

Haren & Compton 2013). Similarly, rhythmic DVM was found in the Arctic at 80° latitude North during the Polar night, when overall changes in light intensity were extremely reduced (Berge et al. 2009). Even if biologically relevant low intensity light cues might be present during the Polar night (Cohen et al. 2015, Båtnes et al. 2015), and other light-related cues might come into play (e.g. moonlight) (Last et al. 2016), for a large fraction of the day light cues would still be virtually absent, raising the question on how would DVM be maintained in similar contexts.

In krill, 'nocturnal' DVM prevails, but other patterns have also been observed, suggesting that krill can flexibly adapt their DVM depending on the local food and predator conditions (Zhou & Dorland 2004, Cresswell et al. 2009). Moreover, krill DVM displays seasonal differences (Fig. 1.3B), being more pronounced in spring and autumn, when a clear day/night cycle is present, than in summer, when photoperiodic cues become weaker and food availability at the surface is high (Quetin & Ross 1991). In winter, krill might become more benthopelagic and live close to the bottom even at depths around 400 m (Kawaguchi et al. 1986). In this case, krill might perform extensive deep vertical migrations, remaining below 100 m during the night, and sinking down to around 300 m during the day (Taki et al. 2005, Siegel 2005). The influence of photoperiod and endogenous rhythmicity on krill DVM was investigated on freshly caught krill during summer by Gaten et al. (2008), who suggested that krill DVM might not be directly influenced by photoperiod, but rather by an endogenous timing system influenced by food availability and social interactions instead. The presence of daily rhythms in krill hemolymph sugar levels and oxygen consumption, which were higher in the laboratory during the dark phase, suggested a link between the daily rhythms of metabolic regulation and the nocturnal DVM pattern (Mezykowski & Rakusa-Suszczewski 1979, Teschke et al. 2011).

Seasonal cycles in krill: sexual maturity, metabolic activity and lipid utilization

The high-latitude environment of the Southern Ocean is characterized by strong seasonal fluctuations in day length, sea-ice cover, and primary production, which affect the dynamics of light and food availability over the year (Knox 2006) (Fig. 1.4A). Prolonged day length (up to 24 h) and absence of sea-ice cover in summer favor high irradiance at the sea surface, triggering elevated primary production, whereas in winter shortened day length (3–4 h) and

extended sea-ice cover cause significant reduction of irradiance at the sea surface, preventing primary production. In response to this, krill display seasonal cycles of metabolic activity, sexual maturity and lipid utilization, with low metabolic rates, sexual regression and high lipid utilization during winter, and high metabolic rates, sexual maturity and low lipid utilization during summer (Kawaguchi et al. 2007, Meyer et al. 2010, Meyer 2012) (Fig. 1.4B).

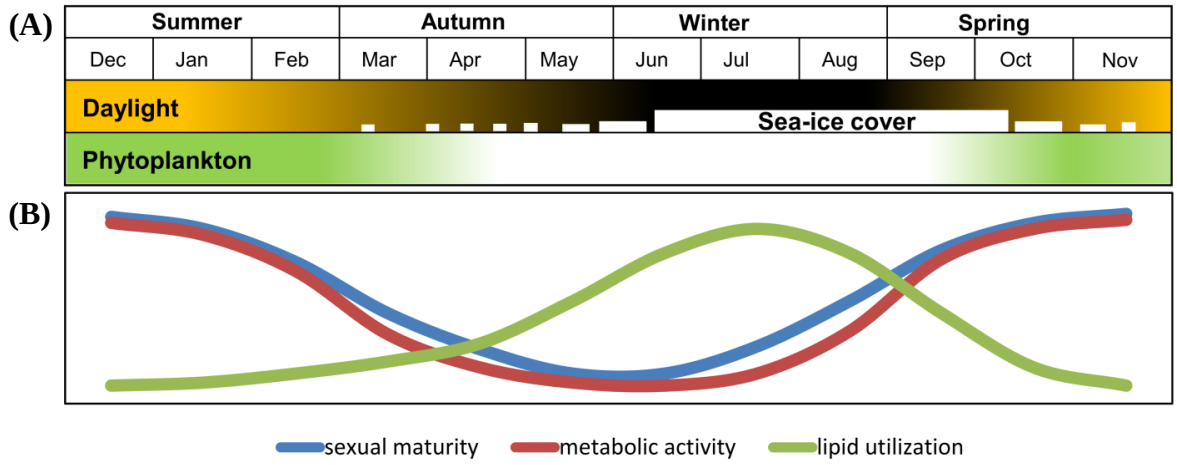

Figure 1.4: (A) Schematic representation of seasonal cycles of light, sea-ice and phytoplankton availability in the Southern Ocean. (B) Corresponding seasonal cycles of krill sexual maturity, metabolic activity and lipid utilization (modified after Kawaguchi et al. 2007, Meyer et al. 2010, Meyer 2012).

These cycles have been generally interpreted as part of a comprehensive over-wintering strategy implemented by krill to save energy during the food-depleted season (Meyer 2012). Field observations usually showed that krill maturity and metabolic status correlated well with food concentrations in the surface layers, suggesting that food availability was the main driver. However, Kawaguchi et al. (1986) and Torres et al. (1994) observed that seasonal changes in krill metabolic activity did not always correlate with changes in food availability, suggesting a different mechanism of regulation. Indeed, following laboratory analyses showed that krill response to high food conditions during winter was influenced by changes in light regime, and that prolonged photoperiods could stimulate increased feeding activity (Atkinson et al. 2002, Teschke et al. 2007, Meyer et al. 2010). Similarly, krill sexual maturity could be advanced under prolonged photoperiods, while sexual regression could be advanced under shortened photoperiods (Teschke et al. 2008; Brown et al. 2011). Moreover, seasonal

changes in krill sexual maturity and metabolic activity were observed in the laboratory also when krill were maintained under constant conditions for months or even years (Thomas & Ikeda 1987; Kawaguchi et al. 2007; Brown et al. 2013), leading to the hypothesis that krill seasonal cycles were regulated by an endogenous timing system entrained by the seasonal Antarctic light regime (Meyer et al. 2010).

1.2. Endogenous rhythmicity and the circadian clock

Life on Earth is characterized by many rhythmic events, among all the daily day/night cycle and the yearly cycle of the seasons. Most living organisms have evolved endogenous timing systems, often referred to as endogenous clocks, to synchronize their daily lifestyle and seasonal life cycle with the rhythmic changes in the environment (Goldman et al. 2004, Yerushalmi & Green 2009). In general, an endogenous timing system can be represented as a molecular oscillator, which can be entrained (i.e. synchronized) by rhythmic environmental cues (*Zeitgebers*) and can promote rhythmic output functions at the metabolic, physiological and behavioral levels (Fig. 1.5).

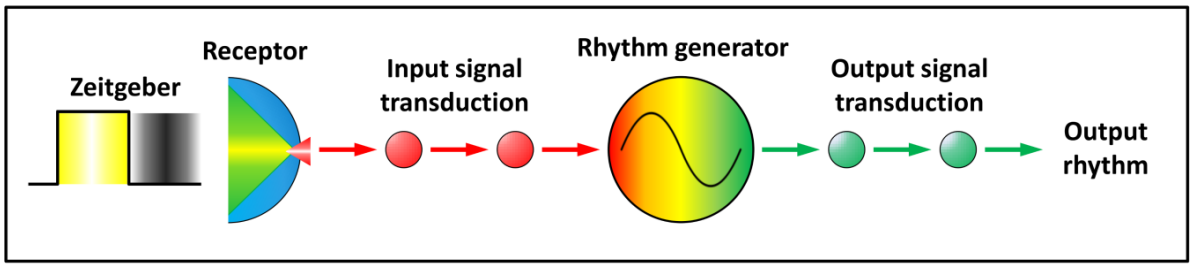

Figure 1.5: Schematic representation of an endogenous timing system. The environmental cue (also called *Zeitgeber*; in this case the light/dark cycle) is captured by the corresponding receptor (in this case an optic receptor, e.g. the retina of the eye). An input signaling cascade is started which reaches the central oscillator (rhythm generator). The oscillator gets synchronized and releases the output signaling cascade, which finally regulates the rhythmic output function.

The most studied endogenous clock is the circadian clock, which synchronizes daily rhythms of metabolism, physiology and behavior accordingly to the day/night cycle (Dunlap 1999). The circadian clock is based on the rhythmic expression of so called “clock” genes, and has been found in virtually all groups of organisms, from cyanobacteria to plants, animals and

fungi. The differences observed among the clock genes and the ways in which they interact suggest that the circadian clock has evolved independently in the different groups, experiencing major evolutionary diversification (Dunlap 1999, Tauber et al. 2004). The most well characterized circadian clock is that of the fruit fly *Drosophila melanogaster*, which will be used as an example in the next section.

The circadian clock in *Drosophila*

At the center of *Drosophila*'s circadian clock there is a set of transcriptional feedback loops (Allada 2003), consisting of sequence-specific DNA binding proteins that stimulate transcription of their own repressors (therefore often referred to as 'negative' feedback loops) (Fig. 1.6). The role of the activator is played by the DNA-binding CLOCK/CYCLE[1] (CLK/CYC) heterodimer, which binds to E-box sequences in target promoters and activate gene expression accordingly (Hardin 2005). In the principal feedback loop, CLK/CYC activates the transcription of the clock genes *period*[2] (*per*) and *timeless* (*tim*), with mRNA levels peaking around dusk. PER and TIM proteins accumulate and dimerize in the cytoplasm during the early night, and translocate into the nucleus by the middle of the night. The temporal delay is achieved through the phosphorylation of PER and TIM regulated by a set of protein kinases including DOUBLETIME (DBT) and SHAGGY (SGG), and protein phosphatases including PROTEIN PHOSPHATASE 2A (PP2A) among others (Mackey 2007). Once in the nucleus, the PER-TIM-DBT complex inhibits the DNA-binding ability of the CLK/CYC heterodimer, possibly through DBT-mediated phosphorylation of CYC, suppressing the transcription of *per* and *tim* during the late night (Allada & Chung 2010). Following this, *per* and *tim* transcript levels decrease reaching a trough around dawn. A new cycle of transcriptional activation is then started during the early day, after the ubiquitin ligase SUPERNUMERARY LIMBS (SLIMB) has triggered the degradation of phosphorylated PER, relieving repression upon CLK/CYC (Allada & Chung 2010). A similar feedback loop is present at the core of the circadian clock with minor differences also in mammals (Shearman et al. 2000).

[1] Protein names are usually reported in capital letters
[2] Gene names are usually reported in lowercase and italics

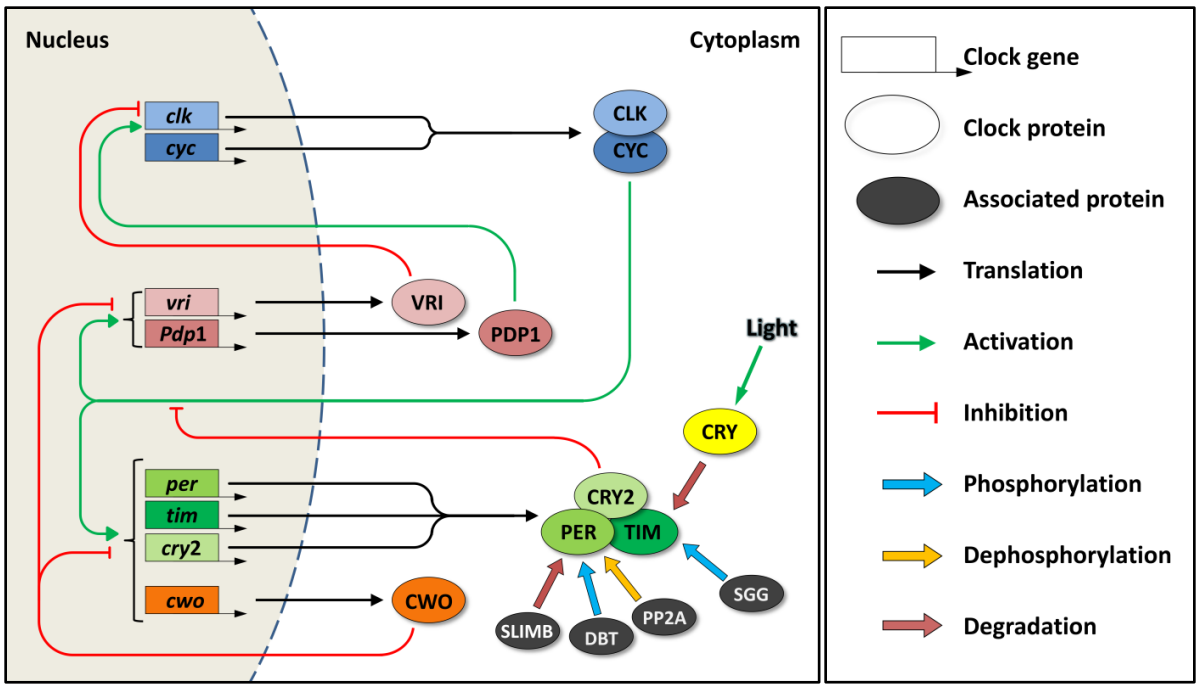

Figure 1.6: Schematic representation of the circadian clock in *Drosophila*. The principal *per*/*tim* feedback loop is represented together with the additional *vri*/*Pdp*1 and *cwo* loops, closely interacting with each other. The clock is entrained by light via activation of CRY and degradation of TIM. Please note that *cry*2 is not present in *Drosophila*, but is present in other arthropods species including the monarch butterfly *Danaus plexippus* (Reppert 2007) and the Antarctic krill (Biscontin et al. 2017). This model is simplified and does not include all molecular components of circadian timekeeping. The figure was created based on Hardin (2005), Reppert (2007), and Allada & Chung (2010).

The principal feedback loop, also called the *per*/*tim* loop, creates the 24 h oscillations in transcriptional activation/repression functions which are at the base of the generation of daily output rhythms operated by the circadian clock (Allada 2003, Hardin 2005, Allada & Chung 2010). In the absence of environmental cues, these oscillations typically persist with a period of approx. 24 h (therefore the name of *circadian* clock, from the Latin *circa dies* meaning "about a day"). In order to get synchronized with the rhythmic events in the environment, the clock needs to be informed by specific environmental cues. Even if different kind of cues, including food and temperature, are known to affect the synchronization of the clock, by far the most reliable cue for the regulation of daily rhythms is represented by the day/night cycle, and light can be considered as the main *Zeitgeber* for the circadian clock. The effect of light on the clock of *Drosophila* is mediated by the blue-light photoreceptor CRYPTOCHROME (CRY) (Sandrelli et al. 2008). In the presence of blue light, CRY promotes the degradation of TIM and the destabilization of the PER/TIM heterodimer, causing further degradation of

PER. Due to this light-induced effect, PER/TIM can only accumulate during the night, providing proper synchronization between the endogenous oscillation and the day/night cycle in the environment.

Two additional interdependent molecular feedback loops involving CLK/CYC activation have been identified in *Drosophila*, which may play an important role in the regulation of the phase and the amplitude of the core oscillator and the output rhythms (Allada & Chung 2010). In the first case, CLK/CYC activate the transcription of two basic leucine zipper transcriptional factors, the activator *Par domain protein* 1 (*Pdp*1), and the repressor *vrille* (*vri*), which in turn can regulate activation/repression of *clk* and *cyc*. Since PDP1 accumulation is usually delayed respect to VRI, *clk* and *cyc* transcripts oscillates in antiphase with respect to *per* and *tim*, peaking in the early day. The function of this feedback loop is still unclear. In the second case, CLK/CYC activates a bHLH (basic helix-loop-helix) repressor called *clockwork orange* (*cwo*). CWO specifically binds the CLK/CYC target E-box thus repressing CLK/CYC-mediated transcription. Additional roles of CWO as activator have also been suggested. In general, loss of *cwo* results in altered molecular and behavioral rhythms, characterized by lower amplitude and longer period, suggesting that this additional feedback loop might be required to promote robust rhythmicity.

Most features of the *Drosophila* clock are conserved also in other organisms, including arthropods and mammals, making it a good starting point to understand how endogenous rhythms can be promoted at the molecular level. However, some differences are present in the kind of clock genes which are involved and in the way they interact with each other. One major difference between the circadian clock in *Drosophila* and the circadian clock in mammals and other arthropod species is the presence of an additional cryptochrome, called *cryptochrome*2 (*cry*2). In contrast to CRY, CRY2 is not affected by light and is not involved in the light-entrainment of the clock. *Cry*2 oscillates with a peak of expression at sunset (like *per* and *tim*), and CRY2 forms a complex with PER/TIM to inhibit CLK/CYC (Zhu et al. 2005). *Cry*2 has been found in species which are believed to possess an ancestral form of the circadian clock, and the absence of *cry*2 in *Drosophila* has been interpreted as a subsequent loss happened during evolutionary times (Reppert 2007).

Clock localization, light-entrainment and output regulation

The oscillator cells (i.e., those cells in which the circadian feedback loops are active) can be localized by searching for rhythmic clock gene expression within the different tissues of an organism. In *Drosophila*, different clusters of "clock" neurons have been identified in the brain, but other oscillators have been found also in the head and in the body (Hardin 2005). The different oscillators may interact with each other, but they may also act autonomously, depending on the tissue and on the biological function involved. Light-entrainment generally occurs through the light-induced activation of a photoreceptor, which then directly or indirectly alters the activity of one or more components within the target oscillator. In *Drosophila*, external (compound eye and ocelli) and internal (Hofbauer-Buchner eyelet) photoreceptors contribute together with the blue light photoreceptor CRY to the light-dependent entrainment of the clock (Hardin 2005). When the clock is entrained, synchronized daily oscillations are elicited at the molecular level, which propagate beyond the framework of the circadian feedback loop and affect large portions of the transcriptome. In animals, possibly due to the effect of clock-regulated signaling cascades, around 10% of all transcripts display circadian-related oscillations over the 24 h cycle (Panda et al. 2002). At the top of the cascades are those transcripts which are directly regulated by elements of the clock (clock-regulated transcripts), for example through CLK/CYC-mediated activation. However, the identification of downstream elements and processes leading to the rhythmic phenotypes (e.g. rhythmic behavior) is much more complex. In *Drosophila*, specific clock neurons in the brain rhythmically release a neuropeptide called pigment dispersing factor (PDF), which is required for the regulation of locomotor activity rhythms (Hardin 2005). The mechanisms involving PDF are still not fully understood, but they might include the coordination of downstream target motor neurons, as well as a feedback influence on the molecular loops within the clock neurons themselves (Mezan et al. 2016).

A great variety of physiological and behavioral processes are affected by the circadian clock at the daily level. In the cells, temporal orchestration of basic homeostatic processes which may interfere with each other can be regulated in a circadian manner (Panda et al. 2002). At a higher level of complexity, circadian rhythms might be found in the circulating levels of biologically relevant molecules, for example the hormone melatonin, which has been related to the regulation of the sleep-wake cycle in mammals (Cajochen et al. 2003), and might play

important roles for circadian regulation also in other species, including crustaceans (Mendoza-Vargas et al. 2017). Possibly the most evident output of the circadian clock is represented by the regulation of rhythmic behaviors, which can include locomotor activity rhythms, rhythms of feeding and fasting, rhythms of sleep and wake, rhythms related to reproduction (courtship behaviors, mating events, spawning events and hatching). Rhythmic circadian behaviors are not restricted to periods of 24 h, but they might include also bimodal patterns with 12 h periods, for example with the locomotor activity rhythms showing a first peak in the early morning followed by a second peak later in the evening (Aschoff 1966). Circadian cycles of metabolic activity and respiration have often been observed in association with the daily rhythms in physiology and behavior (Mortola 2004, Maas et al. 2016).

Photoperiodic time-measurement and the regulation of seasonal responses

Another kind of biological rhythms which can be associated with the activity of the circadian clock are those phenomena related with the increasing and/or shortening of the day length, like flowering in plants and diapause (a physiological state of dormancy and development reduction used to survive unfavorable environmental conditions) in insects (Song et al. 2015, Goto 2013). Even if the involvement of the circadian clock in the regulation of short - vs. long-day responses might be intuitively linked with its ability to track the duration of the day, the exact mechanism by which this should happen is still unclear, and two models have been proposed. In the "external coincidence" model, the presence/absence of light cues during a specific photosensitive phase of the circadian cycle (e.g. during the evening) would trigger long/short-days responses accordingly (Bünning 1960), possibly through the interaction with the clock genes at their times of maximum/minimum expression (sunset/sunrise). Alternatively, in the "internal coincidence" model, two independent circadian oscillators interact, one peaking at sunset and the other at sunrise (Pittendrigh 1960). The phase difference/overlap between these two oscillators would be then used to infer photoperiod. Supporting evidence for both models has been produced, suggesting that both ways of measuring photoperiod are realized in nature (Davis 2002, Hut & Beersma 2011).

Another kind of biological rhythms, called "circannual" rhythms, might also be linked to the activity of the circadian clock. These rhythms display a period of approx. 365 days, are

endogenous (i.e., they persist in the absence of external cues) and can be entrained by environmental cues. For example, the seasonal cycles in reproduction and hibernation observed in mammals and birds have been often interpreted as circannual rhythms (Gwinner 2012). In a putative circannual model, the photoperiodic information collected by the photoperiodic clock (which might be the circadian clock) is used to entrain the endogenous circannual clock, which then regulates the seasonal output accordingly. The conceptual model is similar to that described for the circadian clock (Fig 1.5), with the addition of an "event counter", which is able to accumulate photoperiodic-related information over multiple days and create the seasonal signal (Dolezel 2015). However, even if several reports of circannual-related rhythms have been registered in different species, the mechanism of the circannual clock remains mostly unknown.

One aspect which might be shared between long/short day responses and circannual rhythms is the presence of a critical photoperiodic threshold which needs to be crossed in order to elicit the process. The threshold can differ depending on the species and the biological process involved, and also within the same species depending on the latitude, or in response to changes in other environmental factors like food availability and temperature (Goldman et al. 2004).

1.3. The circadian clock in Antarctic krill

Most of the actual knowledge about the circadian clock comes from study on terrestrial model organisms like *Drosophila* and mouse. Not much has been investigated in non-model marine organisms, and even less is known about circadian regulation in high-latitude pelagic zooplankton like krill. Terrestrial and marine organisms are exposed to different rhythmic environmental cues (e.g. the tidal rhythm in the marine environment), which may determine major differences in their timekeeping functions (Tessmar-Raible et al. 2011). High-latitude organisms are exposed to extreme seasonal changes in photoperiod, ranging from near-constant darkness during winter to near-constant light during summer, which may represent a challenge for the regulation of circadian functions throughout the year (Williams et al. 2015). Krill are mostly pelagic, and they might not be much affected by tidal rhythms, but due to

DVM they are exposed to daily changes in light spectral composition and light intensity which go beyond the simple photoperiodic signal registered at the surface. Being distributed roughly between 50°S and 70°S, krill can experience major changes in the photoperiodic cycle depending on the time of the year, and at the southernmost extreme of their distribution they might be exposed to periods of near-constant light during summer and near-constant darkness during winter.

First reports of clock gene activity in krill

First reports of circadian clock gene activity in Antarctic krill can be found in Mazzotta et al. (2010). The authors isolated a krill orthologue of the mammalian-like *cry*2 gene, Es*cry*2[3], and investigated daily patterns of expression of both the gene and the related protein (EsCRY2) in krill collected over the 24 h cycle during a summer day in Antarctica (Mazzotta et al. 2010). Es*cry*2 showed daily fluctuations in the mRNA levels, peaking in the early morning (06:00), but no oscillations were observed in the corresponding protein. The presence of a *cry*2 gene in krill suggested that the circadian feedback loop was similar to the ancestral form found in the monarch butterfly (Reppert 2007), and the 24 h oscillation in the mRNA levels suggested that the clock was active, even if no clear light/dark cycle was present at the time of sampling (mid-summer, the sun was never completely below the horizon). However, the daily profile of Es*cry*2 expression was not in agreement with previous findings in the honeybee, *Apis mellifera*, and in the monarch butterfly, *Danaus plexippus*, where the peak of expression had been found during the dark phase (Rubin et al. 2006, Zhu et al. 2008). In the absence of clear light/dark cues, entrainment of the clock may follow the rhythms of alternative *Zeitgebers*, for example the daily changes in light intensity and/or in light spectral composition (Roenneberg & Foster 1997). Since Es*cry*2 oscillations did not show any apparent link with the daily cycle of light intensity, the authors suggested a major involvement of light spectral composition (Mazzotta et al. 2010).

[3] The use of the prefix 'Es' in front of a gene or protein name indicates that this is the specific orthologue form isolated in *Euphausia superba*.

Involvement of the clock in the regulation of krill rhythmic output functions

Following the first reports of clock gene activity in krill, laboratory analyses were conducted to determine if an endogenous rhythm was present at the molecular level, and whether this could be related to the regulation of rhythmic output functions in krill physiology. Teschke et al. (2011) investigated daily patterns of Es*cry*2 expression under simulated long-day conditions (16 h light: 8 h darkness, or LD 16:8) and constant darkness (DD), and showed that daily oscillations were present in both cases, demonstrating the endogenous nature of the clock (Teschke et al. 2011). Moreover, krill displayed associated 24 h fluctuations in oxygen consumption, with a peak during the dark phase, and in the activity of key enzymes involved in carbohydrate metabolism, suggesting an involvement of the clock in the regulation of the rhythmic output at the physiological level (Teschke et al. 2011). The endogenous oscillation of Es*cry*2 in DD demonstrated an unexpected period shorter than 24 h (usually endogenous oscillations show a period slightly longer than 24 h, but Es*cry*2 displayed 18 h oscillations), which was discussed as a possible adaptation of krill's circadian clock to the strong photoperiodic fluctuations experienced by krill over the year (Teschke et al. 2011). The metabolic-related enzymes displayed 12 h oscillations, which were interpreted as a bimodal or "circasemidian" output of the clock. The increase of oxygen consumption observed during the dark phase suggested a link between clock-controlled respiration and the regulation of krill DVM in the field, as it had been suggested by Gaten et al. (2008). A microarray analysis of krill transcriptome over the daily cycle was then conducted by De Pittá et al. (2013), using field samples coming from the same summer catch used by Mazzotta et al. (2010). A significant proportion of the transcriptome (8 %) displayed daily oscillations with either 24 h or 12 h period, creating a chronological progression of biochemical and physiological events throughout the 24-hour, with the breakdown of energy-yielding nutrients and energy storage pathways specifically activated in the early morning, and glycogen mobilization, gluconeogenesis and fatty acids catabolism activated in the evening and throughout the night (De Pittá et al. 2013). This suggested that the clock was involved in the regulation of daily cycles of transcription in krill during summer.

Functional characterization of the circadian clock in krill

To collect the increasing information regarding krill transcriptome (Clark et al. 2011, Meyer et al. 2015, Martins et al. 2015), two different online databases were created (Hunt et al. 2017, Sales et al. 2017). By screening those databases, Biscontin et al. (2017) and Hunt et al. (2017) identified the putative krill orthologues of the principal clock components, and Biscontin et al. (2017) further defined their role within the circadian feedback loop, comparing them with known circadian models in mammals, insects and crustaceans (Fig. 1.7A).

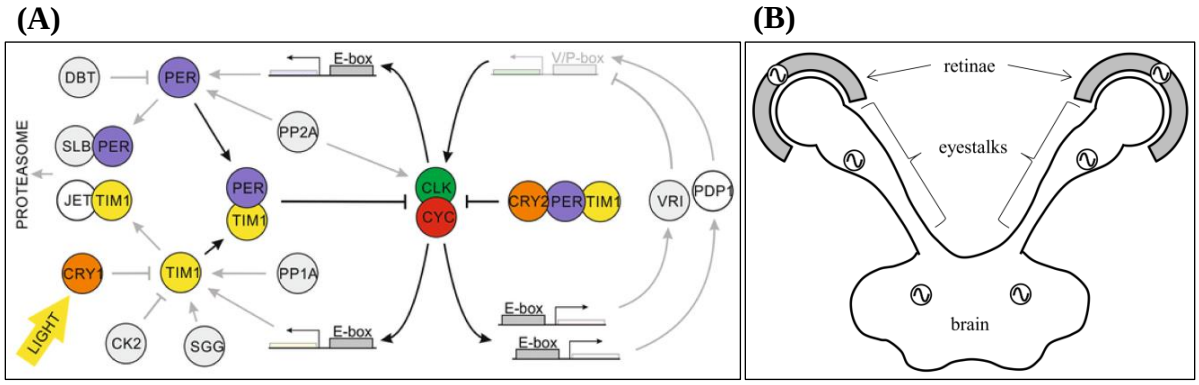

Figure 1.7: (A) The circadian clock model proposed for krill. The two principal feedback loops are represented together. CRY2, PER and TIM interact to suppress CLK/CYC activation. CRY1 is activated by light and causes degradation of TIM, releasing PER/TIM suppression from CLK/CYC (modified after Biscontin et al. 2017). (B) Schematic representation of putative oscillator centers in the head of a generic crustacean. Oscillator cells are located in the retinae of the compound eyes, in the eyestalks and in the brain (modified after Strauss & Dircksen 2010).

The krill clock proteins CLK (EsCLK) and CYC (EsCYC) were identified as the putative positive elements of the principal feedback loop, as described for *Drosophila*, whereas the krill clock proteins PER (EsPER), TIM (EsTIM) and CRY2 (EsCRY2) were identified as the putative negative elements, as described for the monarch butterfly *Danaus plexippus*. The suppression function was carried over mostly by EsCRY2 and EsPER, with EsTIM playing a stabilizing role. The specific contribution of EsPER to the suppression process represented a major difference with respect to the monarch butterfly model, where PER only promotes CRY2 entry into the nucleus. In this sense, the circadian feedback loop of krill displayed features of both *D.plexippus*, where CRY2 is the main suppressor, and *Drosophila*, where this function is mostly carried by the PER/TIM heterodimer. In krill, EsPER and EsTIM

interacted with various kinases. In particular, EsPER interacted with DBT (EsDBT), and EsTIM possibly with CASEIN KINASE 2 (EsCK2) and EsDBT. The krill blue-light photoreceptor CRY (EsCRY) was identified as the putative light-sensitive element responsible for the light-dependent entrainment of the clock, possibly via interaction with EsTIM, as described in *Drosophila*. Despite the strong variability in annual day length that characterizes the high-latitude regions, the conservation of this synchronization mechanism suggested the persisting pivotal role of light as a *Zeitgeiber* also in krill (Biscontin et al. 2017). The light-entrainment of the clock in krill might be related to the daily cycles of expression recently observed in the mRNA levels of three krill opsins, *rhodopsin*1 (Es*rh*1), *rhodopsin* 3 (Es*rh*3) (two visual-related opsins) and *peropsin* (Es*rrh*) (a non-visual opsin), which respond to different wavelength in the light spectrum, and displayed different peaking times during the day (Biscontin et al 2016). The oscillator cells in krill have not yet been localized, but following the circadian model proposed for crustaceans by Strauss & Dircksen (2010), they might be located in specific structures within the head (retinae of the compound eyes, eyestalks and brain) and also along the body (caudal photoreceptor) (Fig. 1.7B). The involvement of the hormone melatonin in the regulation of clock output functions was investigated in krill collected in summer and winter respectively, but no detectable levels of the hormone could be detected, suggesting that melatonin might not play a major role in the regulation of rhythmic output functions in krill (Pape et al. 2008).

1.4. Research objectives

This dissertation is part of the PolarTime project, funded by the Helmholtz Association of German Research Centers. The project had the main goal of investigating biological timing functions in polar pelagic key-species, with a special focus on how these can be affected by the interplay between endogenous mechanisms and environmental cues. In order to understand the rationale behind the project, we need to consider that A) major changes are already taking place in relation to global warming and climate change within the Polar regions, with a potential negative effect for the future conditions of high-latitude species in general; B) in the marine context, polar pelagic key species like Antarctic krill play a

fundamental role supporting the food web and the environmental cycles of nutrients and basic elements like C and N: an unfavorable impact of climate change on those key species would therefore affect the whole ecosystem; and C) biological rhythms and cycles play a central role in the adaptive success of polar pelagic key species like krill, especially in the regulation of DVM and the seasonal life cycle: the synchronization between endogenous and external factors might get affected by environmental changes driven by climate change, creating a "match-mismatch" scenario with potential negative effects for the krill population.

During my work, I focused mostly on the following three aspects:

1) The performance of the circadian clock of krill at different times of the year in response to the extreme seasonal photoperiodic cycle of the Southern Ocean. Is the circadian clock of krill active throughout the year? Is the regulation of rhythmic output functions changing throughout the year? What's the role played by the light/dark cycle at different times of the year? (Publication I, accepted for publication by the Journal of Crustacean Biology)

2) The endogenous regulation of krill seasonal metabolic cycle and the role of photoperiod as main *Zeitgeber*. Is krill seasonal metabolic cycle regulated by an endogenous circannual rhythm? What is the role played by photoperiod in the regulation of krill seasonal metabolic cycle? May the circadian clock (or elements of the circadian clock) be implicated in the timekeeping process? (Publication II, submitted for publication to Frontiers in Physiology – Aquatic Physiology)

3) The endogenous regulation of krill DVM and the role of photoperiod as main *Zeitgeber*. Is krill DVM regulated by an endogenous rhythm? What is the role of photoperiod in the regulation of krill DVM? May the circadian clock be involved? (Publication III, in preparation for submission to the Journal of Experimental Marine Biology and Ecology)

Experimental work was conducted exclusively in the laboratory, where we applied different long- and short-term simulations of different photoperiods, in combination with different

analytical methods including behavioral monitoring (for DVM), the measurement of basic physiological parameters (growth, oxygen consumption), and the measurements of other metabolic-related parameters at the molecular level (enzyme activity, gene expression). The activity of the clock was monitored by measuring daily and seasonal variations in the mRNA levels of expression of core clock genes participating in the circadian feedback loop (e.g. Es*per*, Es*clk*, Es*tim*, Es*cry*2). The involvement of endogenous rhythmicity at the daily and seasonal levels was tested by monitoring the persistence of the target rhythmic functions (e.g. the 24 h oscillation of clock gene expression, the 24 h pattern of DVM, and the seasonal pattern of metabolic activity) in the absence of external entraining cues (constant darkness, constant temperature and constant food conditions). All laboratory experiments were performed at the Antarctic krill Research Aquarium at our partner institution, the Australian Antarctic Division (AAD) in Hobart, Tasmania. Physiological measurements with living krill were performed there as well. All molecular analyses (enzyme activity, gene expression) were performed at the AGKrill laboratory at the Alfred Wegener Institute in Bremerhaven, Germany.

This work provides significant new insights into the mechanisms regulating daily and seasonal rhythmicity in Antarctic krill, and contributes to our general understanding about circadian regulation in polar pelagic key species, and its implications for the ecology of the polar environments.

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2. Publication I

Photoperiodic modulation of circadian functions in Antarctic krill *Euphausia superba* Dana, 1850 (Euphausiacea)

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2.1. Abstract

An endogenous circadian clock influences metabolic output rhythms in the Antarctic krill (*Euphausia superba* Dana, 1850), a key species in the Southern Ocean ecosystem. Seasonal changes in photoperiod in Antarctica, ranging from midnight sun (24 h light) during mid-summer to very short days (3–4 h light) during mid-winter, represent a challenge for the synchronization of the krill circadian clock. We analyzed clock gene activity and clock output functions in krill exposed to different light conditions during a long-term photoperiodic simulation in the laboratory. In simulated early-autumn (light/dark or LD 16:8) and late-winter (LD 8:16) conditions, the circadian clock of krill was functional and the metabolic output was synchronized to the light/dark cycle, the clock genes Es*per* and Es*clk* peaked in antiphase around simulated dusk/dawn and most metabolic-related genes showed upregulation around simulated dusk. In contrast, in simulated mid-summer (light/light or LL) and mid-winter (LD 3:21) conditions, the synchronization of the circadian clock and the metabolic output appeared to be weaker, with clock gene expression becoming arrhythmic and upregulation of metabolic genes occurring at different times during the day. Early-autumn and late-winter photoperiodic cues in the laboratory thus seem to be sufficient to entrain the krill clock and promote metabolic synchronization, whereas mid-winter and mid-summer photoperiodic cues seem to be insufficient for krill entrainment. Krill in the field may overcome the seasonal lack of overt photoperiodic cycle occurring during mid-summer and mid-winter by using alternative light-related *Zeitgebers* (i.e., varying light intensity rather than the presence or absence of light) to promote basic homeostatic rhythms over 24 h.

2.2. Introduction

Antarctic krill, *Euphausia superba* Dana, 1850 (hereafter krill), are key players in the Southern Ocean (SO) ecosystem in terms of distribution, abundance, and trophic relevance (Quetin & Ross 1991). During the last decades, due to warming in the Atlantic sector of the SO and increasing interest in krill by commercial fisheries, concerns have been raised about

the well-being of the krill population (Atkinson et al. 2004), and efforts to understand the interaction between krill and their environment have been intensified.

The high-latitude environment of the SO is characterized by strong seasonal fluctuations in day length, sea-ice cover, and primary production, which affect the dynamics of light and food availability over the year (Knox 2006). Prolonged day length (up to 24 h) and absence of sea-ice cover in summer favor high irradiance at the sea surface, triggering elevated primary production, whereas in winter shortened day length (3–4 h) and extended sea-ice cover cause significant reduction of irradiance at the sea surface, preventing primary production.

In response, krill display seasonal cycles of metabolic activity and sexual maturity, with lower metabolic rates and sexual regression during winter and higher metabolic rates and sexual maturity during summer (Kawaguchi et al. 2007, Meyer et al. 2010). Moreover, krill display seasonal changes in their vertical distribution and diel vertical migration (DVM) behavior, being generally shallower in summer compared to winter, and performing a more pronounced DVM in spring-autumn compared to summer-winter (Quetin & Ross 1991, Taki et al. 2005).

The mechanisms underlying these rhythmic functions in krill are still debated. Investigations on the influence of light regime and food availability on the seasonal cycles of metabolic activity and sexual maturity suggested that light might play a prominent role (Atkinson et al. 2002, Teschke et al. 2008). Even though light conditions are generally considered to be the main proximate cue for zooplankton DVM (Cohen & Forward 2009), other factors like food availability, predation pressure, and social interactions may play a major role in krill (Gaten et al. 2008). Laboratory analyses have indicated that krill rhythmic functions may persist also in the absence of external environmental stimuli, suggesting the interplay with an internal (i.e. endogenous) timing system, possibly a circadian clock (Teschke et al. 2007, Gaten et al. 2008, Brown et al. 2013).

The circadian clock is a molecular oscillator based on the rhythmic expression of clock genes, which free-runs with a period of approximately 24 h. The clock can be synchronized to the day/night cycle by the interaction with light cues (Dunlap 1999) and can promote

output rhythms in metabolism, physiology, and behavior at the daily and seasonal levels (Allada & Chung 2010, Dardente et al. 2010, Goto 2013). It has been suggested that the circadian clock in krill might be implicated in the regulation of DVM (Gaten et al. 2008), metabolic activity (Teschke et al. 2011), and transcription (De Pittá et al. 2013), but actual knowledge about krill clock functions is still limited. The recent identification of the most known (from model organisms) set of clock genes in the transcriptome of krill and the molecular characterization of krill circadian feedback loop have opened new opportunities to study the clock at the molecular level (Biscontin et al. 2017, Hunt et al. 2017).

For high latitude organisms like krill, the strong seasonality of the photoperiodic cycle might represent a limitation for the activity of the circadian clock and the entrainment of daily rhythms, especially at those times of the year when photoperiodic cues get weaker (mid-winter) or even disappear (mid-summer). To overcome this problem, high-latitude organisms may either suppress their clock functions and use other mechanisms to entrain daily rhythms when necessary (Reierth et al. 1999, van Oort et al. 2005, Lu et al. 2010), or preserve a functional clock and switch to alternative *Zeitgebers* (entraining cues) when photoperiodic conditions become insufficient (Ashley et al. 2013, 2014).

The aim of this study was to investigate the impact of the seasonal photoperiodic cycle of the Southern Ocean on the activity of the circadian clock and the regulation of circadian functions in Antarctic krill, with particular emphasis on light patterns typical of those times of the year when photoperiodic cues might be strongly reduced (mid-winter, day length below 3–4 h) or even missing (mid-summer, when the sun never goes below the horizon). We exposed krill in aquaria to a long-term photoperiodic simulation in the absence of other *Zeitgebers* and examined clock gene activity and the regulation of daily output rhythms at four simulated seasonal light regimes corresponding to mid-summer (midnight sun, 24 h light), early-autumn (long day, 16 h light), mid-winter (very short day, 3 h light) and late-winter (short day, 8 h light). To monitor the activity of the clock, we analyzed daily patterns of expression of the clock genes Es*clk* and Es*per*, the krill homologues of the canonical *Drosophila* clock genes *clock* and *period* (Dunlap 1999), and to monitor changes in clock output functions, we analyzed daily patterns of regulation of a selection of genes controlling rate-limiting steps in carbohydrate, lipid, and energy metabolism.

2.3. Materials and methods

Sampling and laboratory maintenance of experimental krill

Specimens of *E. superba* were collected in East Antarctica between 65°19'S, 125°37'E (17 September 2007) and 64°08'S, 119°16'E (9 October, 2007) in the upper 200 m of the water column using a rectangular midwater trawl (RMT 8) during voyage V1 07/08 of RSV *Aurora Australis*. All krill were immediately transferred to 200 l tanks located in a constant-temperature room at 0°C and supplied with a continuous flow of chilled seawater. Individuals were kept in conditions of dim light and in the absence of food, and dead animals and molts were removed daily from the tanks. After arrival in Hobart, Tasmania (17 October 2007), krill were transported from the ship to the krill research aquarium at the Australian Antarctic Division (AAD) in Kingston, Tasmania, and transferred to a 1670 l holding tank connected to a 8000 l chilled seawater recirculation system.

Temperature of seawater in the tank was maintained constant at 0.5°C. Water was continuously recirculated through an array of mechanical and biological filters and constantly monitored for quality following King et al. (2003). Light in the aquarium was provided by fluorescent tubes covered with a gel filter (Lee Roll 131 Marine Blue; ARRI, Munich, Germany). A PC-controlled timer and dimming system (winDIM v4.0e; EEE, Lisbon, Portugal) provided a light regime similar to that occurring in the Southern Ocean at 66° latitude south (66°S) and 30 m depth. A sinusoidal annual cycle with monthly variations of photoperiod and daily variation of light intensity was calculated by assuming continuous light and a maximum light intensity of 100 lux at the surface of the tank (equal to 1% light penetration to 30 m depth) during summer midday (December at 66°S). The system was adjusted every month to reflect SO conditions.

Krill were fed daily with a mixture of living algae at the final concentration of 1.5×10^4 cells ml^{-1} of the pennate diatom *Phaeodactylum tricornutum* Bohlin, 1897, 2.2×10^4 cells ml^{-1} of the chlorophyte *Pyramimonas gelidicola* (McFadden, Moestrup & Wetherbee, 1982), and 2×10^4 cells ml^{-1} of the cryptophyte *Geminigera cryophila* (Taylor & Lee) Hill, 1991. Instant algae were added to yield final concentration of 1×10^4 cells ml^{-1} of *Thalassiosira*

weissfloggii (Fryxell & Hasle, 1977) (1200TM; CCMP1051/TWsp.; Reed Mariculture, Campbell, CA, USA), 5.1 x 10^4 cells ml^{-1} *Isochrysis sp.* (1800TM; Reed Mariculture), and 4.8 x 10^4 cells ml^{-1} *Pavlova sp.* (1800TM; Reed Mariculture). Krill also received 2 g per tank per day of nutritional supplements (1 g of Frippak #1 CAR; 1 g of Frippak #2 CAR; INVE; Nonthaburi, Thailand). After the phytoplankton mix was added, water flow in the tank was shut off for 2 h to enable krill to feed on the algal mixture. This feeding regime has been used successfully in several experiments trials at the AAD prior to this study, and maintains krill in good conditions, with low mortality and high feeding rates, in long-term laboratory experiments (King et al. 2003, Kawaguchi et al. 2010). Animals were fed *ad libitum*, thus keeping feeding conditions always at their optimum. Dead individuals and molts were removed from the tank daily.

Experimental setup and sampling of krill in the laboratory

The experiment started at the beginning of December 2009. 300 adult krill of mixed sexes were collected at random from the holding tank and transferred to a cylindrical 100 l tank connected to the aquarium's recirculating seawater facility. The tank was placed into a black lightproof plastic container to create a separate light compartment and enable complete control over light conditions throughout the experiment. Light inside the container was provided by fluorescent tubes (Osram L18W/640 Cool White) covered with a gel filter (Lee Roll 131 Marine Blue; ARRI) simulating light as attenuated to 30 m depth in the ocean. The duration of the light phase and light intensity were regulated using the same PC-controlled system as for the holding tank. The duration of the light phase was adjusted every month to mimic seasonal changes of natural day length at 66°S (Fig. 2.1). Light intensity at the surface of the tank was 0 lux during the dark phase and 100 lux during the light phase. The transition between light and dark phases was gradual to simulate natural dawn and dusk conditions. Temperature was held constant around 0.5°C throughout the experiment. The krill were fed with the same phytoplankton mixture and the same procedure described for the holding tank. A fixed volume of food was added to the tank daily to reach a feeding optimum (*ad libitum*) and keep daily food availability constant throughout the experiment.

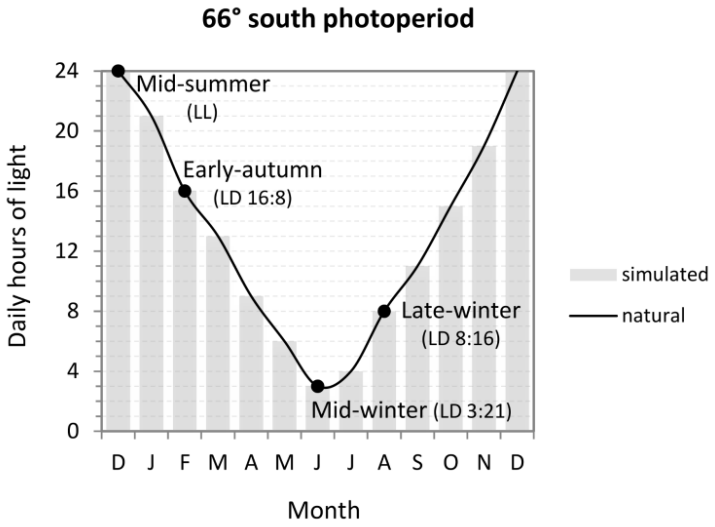

Figure 2.1: Laboratory simulation of the natural photoperiodic cycle occurring at 66° latitude South in the Southern Ocean. Black solid line indicates the natural course of the photoperiodic cycle at 66°S. Gray-shaded bars indicate monthly changes in day length in the simulation. Black dots indicate time-points of collection of 24 h samples.

We sampled four 24-hour time-series (Fig. 2.1): December 2009 (simulated mid-summer conditions, near-constant light or LL), February 2010 (simulated early-autumn conditions, 16 h light/8 h darkness or LD 16:8), June 2010 (simulated mid-winter conditions, 3 h light/21 h darkness or LD 3:21), and August 2010 (simulated late-winter conditions, 8 h light/ 16 h darkness or LD 8:16). Mid-summer (LL) and mid-winter (LD 3:21) conditions were selected to investigate the effect of light regime towards the extremes of the seasonal cycle, whereas early-autumn (LD 16:8) and late-winter (LD 8:16) were selected to investigate the effect of long and short days, respectively. Moreover, early-autumn and late-winter represent turning points in the seasonal life cycle of krill (processes related to overwintering and sexual regression start in early-autumn, whereas processes related to termination of quiescence and sexual maturity begin in late-winter), and it was therefore deemed worthwhile to investigate the effect of the clock during those times of the year. Sampling was conducted always at the beginning of the third week of the corresponding month. During sampling, food supply was interrupted and six animals were randomly collected every 3 h, starting at 06:00 and ending at 06:00 on the following day. Sampling during dark phases was conducted under dim red light. All animals were immediately frozen in liquid nitrogen and stored at –80°C.

Molecular analyses

Total RNA for gene expression analyses was extracted from krill heads. For each time-point, six frozen heads were dissected on dry ice and individually transferred in separated Precellys® tubes (Bertin Instruments; Montigny-le-Bretonneux, France) containing the TRIzol® reagent (ThermoScientific; Waltham, MA, USA). Tissue homogenization was performed using a Precellys®24 tissue homogenizer (Bertin Instruments) connected to a Cryolys cooling element; the process was carried out at 4°C. The homogenates were removed from the tubes and treated with chloroform/isopropanol for phase separation and precipitation. The RNA pellets were washed twice with 75% ethyl alcohol and resuspended in RNase-free water. We checked RNA concentration and purity using a NanoDrop™2000 UV-Vis spectrophotometer (ThermoScientific), and RNA integrity using an Agilent 2100 Bioanalyzer system (Agilent Technologies; Santa Clara, CA, USA). To prevent genomic contamination, all samples were treated with the TURBO DNA-Free kit from Ambion (ThermoScientific).

After DNA removal, for each sample 2 µg of total RNA were retro-transcribed to cDNA using the RevertAid H Minus Reverse Transcriptase kit from Invitrogen (ThermoScientific) to a final volume of 50 µl per sample (40 ng/µl). Gene expression was measured using custom-designed TaqMan® Low-Density Array Cards (ThermoScientific). Primers for qPCR analysis were designed around sequences of interest using the Custom TaqMan® Assay Design Tool (ThermoScientific). The primer sets for the clock genes Es*clk* and Es*per* and for the metabolic genes *glycogen synthase* (*gys*), *phosphofructokinase*-6 (*pfk*6), *citrate synthase* (*cs*), *hydroxyacyl-coenzyme* A *dehydrogenase* (*hadh*), *acetyl-coenzyme* A *carboxylase* (*acc*), and ATP *synthase* (*atp*) were designed using the sequences available online at http://krilldb.bio.unipd.it (Sales et al. 2017) (Tab. A1.1). For each sample we used 20 µl of cDNA (40 ng/µl), 30 µl of RNase-free water and 50 µl of TaqMan® Gene Expression Master Mix (ThermoScientific), for a final volume of 100 µl. The cards were analyzed using a ViiA™ 7 Real-Time PCR System (ThermoScientific). Standard curves were conducted to verify the efficiency of each primer pair on the card.

Normalization and relative quantification

The levels of transcription of all genes were normalized and quantified following the $2^{-\Delta\Delta Ct}$ method implemented by the ddCtExpression-method function of the ddCT R-package (Zhang et al. 2015). To select the most stable genes to use in the normalization procedure, we measured 24 h patterns of gene expression of five different candidate reference genes in the head of adult krill in the four different photoperiodic conditions considered and used Genorm (Vandesompele et al. 2002), Bestkeeper (Pfaffl et al. 2004), and Normfinder (Andersen et al. 2004) to identify reference genes that did not show a sinusoidal expression during the 24 h period.

Our candidate reference genes were *ubiquitin carboxyl-terminal hydrolase* 46 (*usp*46), *ryanodine receptor* (*ryr*), *elongation factor* 1-*alpha* (*ef*1a), *ribosomal protein* S13 (*rps*13), and *ribosomal protein* L32 (*rpl*32). *Usp*46 and *ryr* had showed constant mRNA levels during the daily 24 h cycle in a previous experiment conducted in LD/DD conditions (data not shown) and in previous experiments on daily patterns of expression of opsin-related mRNAs in the head of adult krill (Biscontin et al. 2016). *Ef*1a, *rps*13 and *rpl*32 had showed constant mRNA levels in previous experiments involving manipulation of photoperiod in the lepidopteran *Plutella xylostella* (Linnaeus, 1758) (Fu et al. 2013). Here, *usp*46 and *rps*13 showed constant mRNA expression levels in all simulated photoperiodic conditions (Fig. A1.1). We therefore used a combination of *usp*46 and *rps*13 as a reference for normalization in the ddCT package. Daily changes in mRNA levels were then calculated relative to the daily average for each target gene.

Statistical analysis

We used the R-package "rain" (Thaben & Westermark 2014) to test for the presence of 24 h rhythmicity in the daily patterns of gene expression. The package fits the data to sinusoidal or sawtooth curves having the required period (24 h or 12 h in our case) and returns a probability value (p) indicating the likelihood of the fit and the phase of the fitted curve (corresponding to the time-point where the amplitude of the oscillation is maximal). P-values were corrected for multiple testing using the Bonferroni method implemented by the package. Since the "rain" software is designed specifically to detect sinusoidal and sawtooth

waves, more complex or less structured rhythmic expression profiles might sometimes be overlooked. To overcome this limitation, when we failed to detect a significant fit but the expression profile showed clear changes over the 24 h, we used the Wilcoxon-Mann-Whitney non-parametric test implemented by R to put in evidence significant differences between peaks and troughs of gene expression.

Ethics Statement

All animal work was conducted according to relevant national and international guidelines. Krill catches, welfare, and experimentation were based on permission of the Department of Environment and Heritage (DEH) of the Australian Government, and were conducted in accordance with the Antarctic Marine Living Resources Conservation Act 1981 (permit 06_09_2220) and the Environment Protection and Biodiversity Conservation Act 1999 (permit: WT2007-1480).

2.4. Results

Clock gene expression

To monitor changes in the activity of the circadian clock of krill in the different photoperiodic conditions, we analyzed the daily patterns of expression of the clock genes Es*clk* and Es*per*, which are the krill homologs of the *Drosophila* core clock genes *clock* and *period*. Due to the opposite roles played by *clock* and *period* within the circadian molecular feedback loop (*clock* is a promoter, *period* a suppressor), the daily patterns of expression of these two clock genes usually show an antiphase relationship, with *period* peaking around dusk and *clock* peaking around dawn (Dunlap 1999, Hardin 2005).

In krill exposed to near-constant light (mid-summer conditions, LL), the expression patterns of Es*clk* and Es*per* did not show a clear antiphase relationship (Fig. 2.2A,B). Both genes showed a peak during the first half of the 24 h cycle, at CT 6 (CT = Circadian Time, indicating time intervals in the absence of Zeitgeber starting at 00:00), and a trough during the second half of the 24 h cycle, at CT 15. The daily profiles of expression looked irregular

and the variability between biological replicates (standard error mean) was high. This indicates that in LL the circadian molecular feedback loop did not work properly and that no synchronization was present among the clocks of different individual krill.

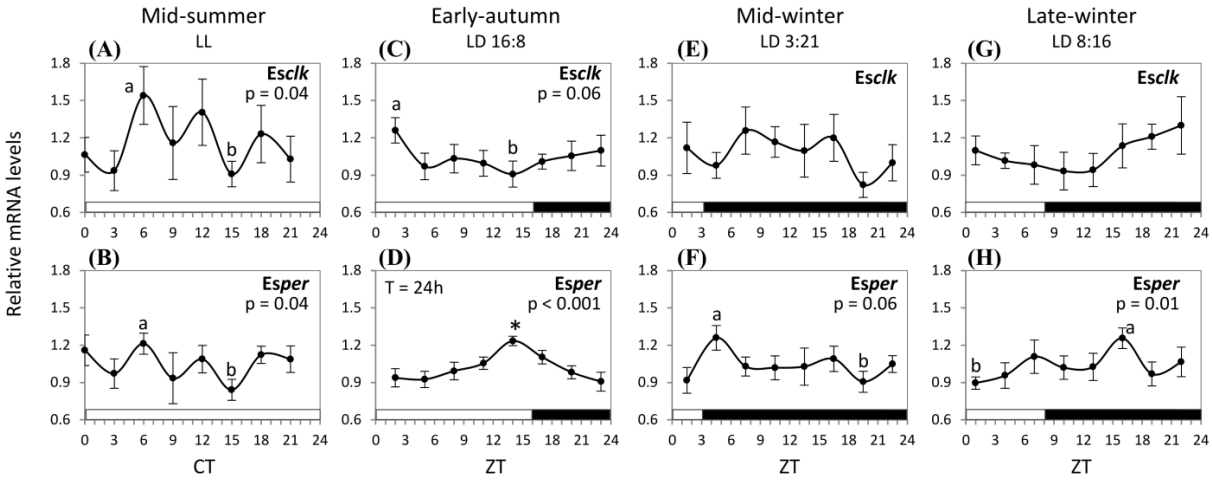

Figure 2.2: Daily patterns of expression of Es*clk* and Es*per* in mid-summer (A, B), early-autumn (C, D), mid-winter (E, F), and late-winter (G, H). Data points represent means ± SEM (n = 6). Significant differences in probabilities between peaks (a) and troughs (b), and for significant 24 h rhythmicity (T = 24 h) are reported; asterisk indicates phase of oscillation. White/black bars at the bottom of the graphs indicate photoperiod (white, light phase; black, dark phase). ZT, Zeitgeber Time; ZT 0 corresponds to 04:00 h in early-autumn, 10:30 h in mid-winter, and 08:00 h in late-winter; CT, Circadian Time; CT 0 corresponds to 00:00 h in mid-summer.

In krill exposed to long-day conditions (early-autumn, LD 16:8) the expression pattern of Es*clk* and Es*per* showed a clear antiphase relationship (Fig. 2.2C,D), with Es*per* showing 24 h rhythmicity with the phase of the oscillation set towards the end of the light phase (ZT 14; ZT = Zeitgeber Time, indicating the time intervals from the beginning of the light phase), and Es*clk* showing a well-defined daily pattern with a peak of expression at the beginning of the light phase (ZT 2). The daily profiles of expression looked regular, and the variability between biological replicates was low, especially for Es*per*. This indicates that in LD 16:8 the circadian molecular feedback loop was working properly and the clocks of different individual krill were synchronized. The occurrence of peaks and troughs of expression at the beginning and end of the light phase indicates that photoperiod was acting as an entraining cue.

In krill exposed to very short-day conditions (mid-winter, LD 3:21) the expression patterns of Es*clk* and Es*per* did not show a clear antiphase relationship (Fig. 2.2E,F). The mRNA levels of Es*clk* were high around ZT 7–8, whereas Es*per* showed a peak at ZT 4–5 at the beginning of the dark phase. The daily profile of expression of Es*clk* was irregular, and the variability between replicates was high, whereas the profile of Es*per* was more regular and the variability was lower. Even though the expression pattern of Es*per* showed some synchronization with the LD cycle, the absence of a clear antiphase relationship between Es*clk* and Es*per* and the irregular expression profile of Es*clk* indicated that in LD 3:21 the circadian molecular feedback loop was not working properly, and that the synchronization among clocks of different individual krill was not tight.

In krill exposed to short-day conditions (late-winter, LD 8:16), the expression patterns of Es*clk* and Es*per* (Fig. 2.2G,H) were similar to those observed in long-day conditions (early-autumn or LD 16:8, Fig. 2C,D). In particular, Es*clk* shows almost the same pattern with mRNA expression levels rising during the dark phase, peaking at the end of the dark phase (ZT 22), dropping during the light phase and reaching a trough at the beginning of the dark phase (ZT 13). Es*per* showed low mRNA levels at the beginning of the light phase with a trough at ZT 1, followed by the mRNA levels rising towards the end of the light phase and during the first half of the dark phase, reaching a peak at ZT 16. The profiles of expression of both genes looked regular, and the variability between biological replicates was low, indicating that in LD 8:16 the clock in krill was active and synchronized to the photoperiodic cycle.

Metabolic gene expression

To investigate potential changes in the daily patterns of regulation of the metabolic output in the different photoperiodic conditions, we paralleled our clock gene expression analysis with that of a selection of key metabolic genes including *glycogen synthase* (*gys*), *phosphofructokinase*-6 (*pfk*6) and *citrate synthase* (*cs*), which play prominent roles in carbohydrate metabolism, *hydroxyacyl*-CoA *dehydrogenase* (*hadh*) and *acetyl*-CoA *carboxylase* (*acc*), which play a fundamental role in the metabolism of fatty acids, and ATP *synthase gamma subunit* (*atp*), which regulates the synthesis of ATP.

Daily rhythms of metabolic regulation were present in simulated mid-summer conditions (LL), but the different pathways showed upregulation at different times of the day and the oscillations in the mRNA expression levels were characterized by different periods and different phases (Fig. 2.3A,B,C).

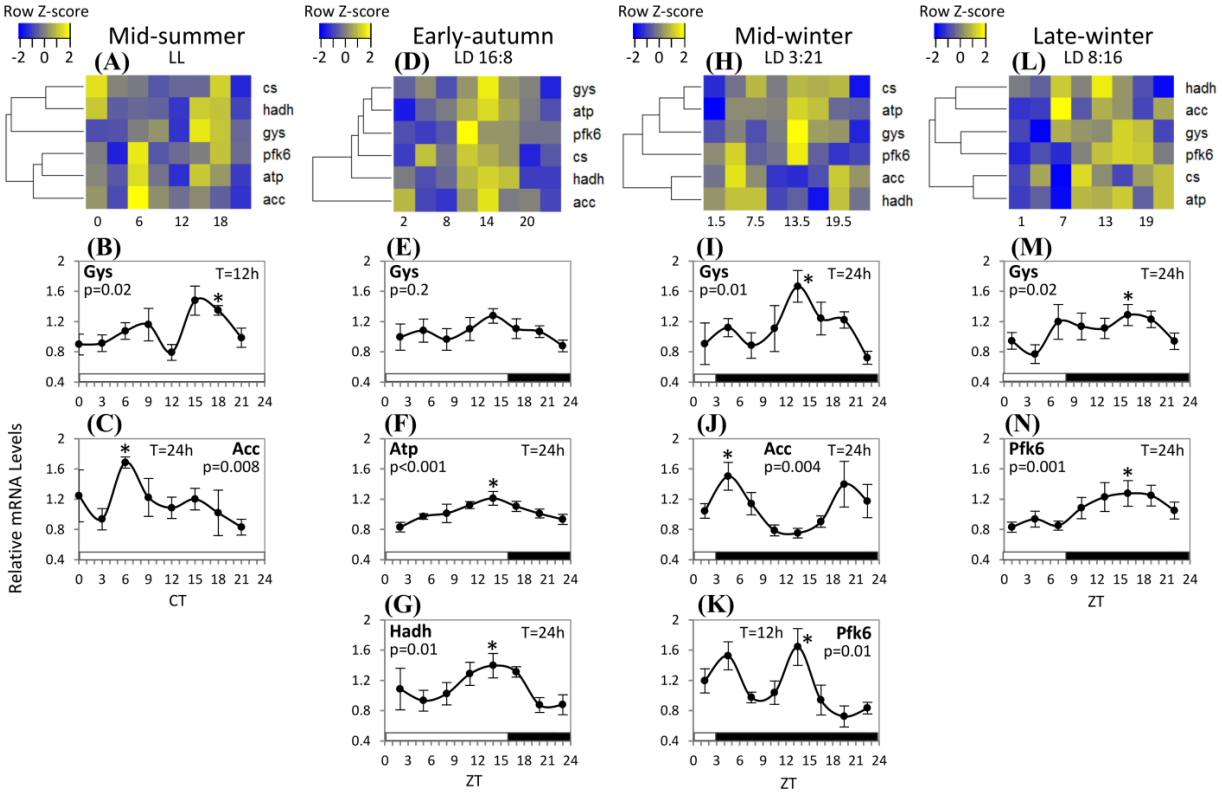

Figure 2.3: Daily expression signatures of metabolic genes in mid-summer (A–C), early-autumn (D–G), mid-winter (H–K) and late-winter (L–N). Heatmaps and dendrograms, based on Euclidean distances, show the expression levels of metabolic genes during the 24 h cycle and are represented with a color-coded scale; yellow and blue represent high and low expression levels, respectively. Graphs show daily expression profiles with significant 12 h (T = 12 h) or 24 h (T = 24 h) rhythmicity. Data points represent means ± SEM (n = 6). Asterisks indicate phase of oscillation. White/black bars at the bottom of the graphs indicate photoperiod (white, light phase; black, dark phase). ZT, Zeitgeber Time; ZT 0 corresponds to 04:00 h in early-autumn, 10:30 h in mid-winter, and 08:00 h in late-winter; CT, Circadian Time, CT 0 corresponds to 00:00 h in mid-summer. The names of genes are provided in Tab. A1.1.

Genes involved in the citric acid cycle (*cs*), glycogen synthesis (*gys*), and beta-oxidation of fatty acids (*hadh*) showed upregulation mostly during the second half of the 24 h cycle, at CT 15–18, whereas genes involved in glycolysis (*pfk*6), ATP production (*atp*), and fatty acids synthesis (*acc*) showed upregulation mostly at CT 6 during the first half of the 24 h cycle.

Among the genes showing significant daily rhythmicity, *gys* was characterized by a 12 h period of oscillation with phase set around CT 18 (Fig. 2.3B), whereas *acc* showed a 24 h period with phase set around CT 6 (Fig. 2.3C).

Metabolic regulation seemed to be synchronized to the light/dark (LD) cycle in simulated early-autumn conditions (LD 16:8). All pathways showed upregulation at the end of the light phase (ZT 11–14–17; Fig. 2.3D). This was particularly evident for the genes involved in the synthesis of ATP (*atp*; Fig. 2.3F) and in the beta-oxidation of fatty acids *(hadh*; Fig. 2.3G), which showed 24 h rhythmic expression with low mRNA levels at the beginning of the LD cycle (ZT 2–5), increasing during the light phase, peaking at the end of the light phase (ZT 14), and then dropping again during the dark phase towards the end of the LD cycle. A very similar pattern was also present for *gys*, even if it is not statistically significant (Fig. 2.3E).

Daily rhythms of metabolic regulation were present in simulated mid-winter conditions (LD 3:21), but the different pathways showed upregulation at different times of the day and the oscillations in the mRNA expression levels were characterized by different periods and different phases (Fig. 2.3H,I,J,K). Genes involved in carbohydrate metabolism (*gys*, *cs*, and *pfk*6) and ATP production (*atp*) showed upregulation mostly during the second half of the LD cycle, around ZT 13.5, whereas genes involved in fatty acids metabolism (*acc* and *hadh*) showed upregulation mostly during the first half of the LD cycle, around ZT 4.5. Among the genes showing significant daily rhythms of regulation, *gys* was characterized by a 24 h period of oscillation with the phase set at ZT 13.5 (Fig. 2.3I), whereas *acc* showed a significant 24 h period with the phase set at ZT 4.5 (Fig. 2.3J). An intermediate pattern was present for *pfk*6, which showed significant 12 h rhythmicity with the phase set at ZT 13.5 and a second peak at ZT 4.5 (Fig. 2.3K).

Metabolic regulation seemed to be synchronized to the light/dark (LD) cycle in simulated late-winter conditions (LD 8:16), with the upregulation of the metabolic genes taking place mostly during the dark phase, from ZT 7–10 until ZT 16–19 (Fig. 2.3L). This was particularly evident for the genes involved in the synthesis of glycogen (*gys*, Fig. 2.3M) and glycolysis (*pfk*6, Fig. 2.3N), which showed 24 h rhythmic expression with low mRNA levels during the light phase, increasing during the dark phase and peaking in the middle of the dark phase, at ZT 16.

2.5. Discussion

While photoperiod during our long-term simulation was adjusted every month to mimic natural conditions, food availability and temperature were held constant throughout the experiment. We observed different levels of modulation of krill circadian clock, depending on the time of the year when the samples had been taken. Krill sampled in February and August (simulated early-autumn and late-winter conditions), when photoperiodic conditions were not extreme (LD 16:8 and LD 8:16, respectively), displayed a functional circadian clock. Positive (Es*clk*) and negative (Es*per*) clock components showed rhythmic daily patterns peaking in antiphase with respect to each other, as in the *Drosophila* circadian feedback loop (Dunlap, 1999; Hardin, 2005). The occurrence of peaks and troughs of expression at the transition between light and dark phases (simulated dawn and dusk) indicated that the photoperiodic cycle was playing a major role in the entrainment of the clock. Alternatively, in krill sampled in December and June (simulated mid-summer and mid-winter conditions) when photoperiodic conditions were extreme (LL and LD 3:21 respectively), the circadian clock seemed to be disrupted, clock gene expression was irregular and the antiphase relationship between positive and negative clock components was not present anymore. Moreover, the clocks seemed not to be synchronized among individual krill, as if a proper entraining cue was missing. Similar disruptive effects of extreme light conditions on the activity of the circadian clock have been observed before in insects (Sauman & Reppert 1996) and mammals (Ohta et al. 2005), and even if recent findings suggested that high-latitude *Drosophila* species might have evolved specific clock features to mitigate such problem (Kyriacou 2017, Menegazzi et al. 2017), our results indicated that in krill exposure to extreme photoperiodic conditions in the laboratory can lead to the de-synchronization of the clock.

Different levels of modulation were observed at the different times of the year in the regulation of the metabolic output, in agreement with the corresponding clock activity. In simulated early-autumn and late-winter conditions, the metabolic output showed synchronization to the LD cycle, with upregulation at the transition between light and dark phase (in simulated early-autumn; Fig. 2.4B) and during the dark phase (in simulated late-

winter, Fig. 2.4D), suggesting the presence of higher levels of activity during the simulated night. In contrast, in simulated mid-summer and mid-winter conditions the daily patterns of regulation of the main metabolic pathways seemed to lack a clear temporal synchronization, with some pathways showing upregulation during the first half of the 24 h cycle, and some other showing upregulation during the second half (Fig. 2.4A,C). Moreover, the period of the oscillations was heterogeneous, being in some cases 24 h and 12 h in others. The emergence of 12 h rhythmicity might be interpreted as circasemidian output related to the clock (Teschke et al. 2011, De Pittá et al. 2013), but also as ultradian output, with no direct connection with the clock (Lloyd & Stupfel 1991), suggesting a tendency towards the occurrence of "around the clock" patterns of activity (Bloch et al. 2013).

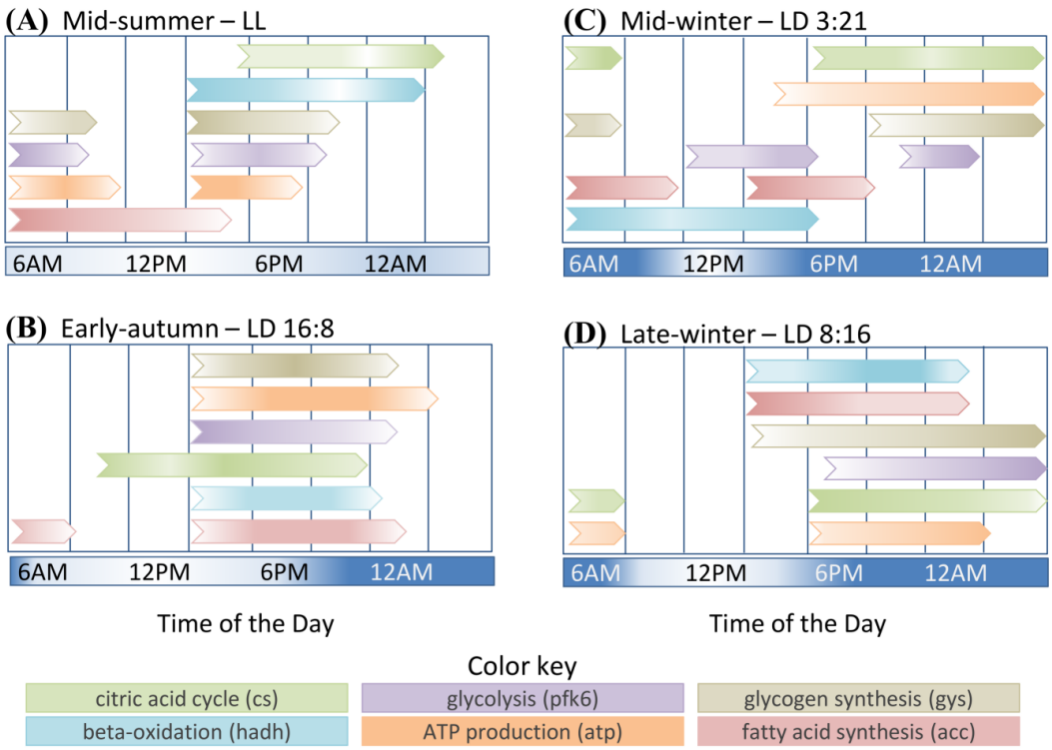

Figure 2.4: Schematic representation of the daily regulation of main metabolic pathways in mid-summer (A), early-autumn (B), mid-winter (C), and late-winter (D) based on gene expression data of key-metabolic genes. The different metabolic processes are indicated by gradient-colored arrows showing the time of the day corresponding to upregulation of key metabolic genes. The lengths of the arrows and darkness of the colors indicate intervals and intensity of upregulation. Time of the day is indicated at the bottom of each figure, where an indicative representation of light intensity is also shown. Each pathway is associated to one color in the color key, with the corresponding gene indicated in parenthesis.

The synchronization of krill circadian clock and metabolic output with the LD cycle observed in simulated early-autumn and late-winter photoperiodic conditions were in agreement with previous observations of rhythmic clock gene activity and oxygen consumption in krill exposed to similar LD conditions in a short-term, laboratory photoperiodic simulation (Teschke et al. 2011), which also indicated the presence of higher metabolic activity during the dark phase (simulated night). This suggested that during autumn and spring photoperiodic cues alone could play a major role as *Zeitgeber* for the entrainment of krill circadian clock, possibly promoting the daily rhythms of activity generally observed in the field at those times of the year (i.e., DVM and higher activity during the night; Ross et al. 1996).

Conversely, the loss of synchronization observed in simulated mid-summer photoperiodic conditions was in apparent contrast with previous reports of rhythmic clock gene expression and rhythmic transcriptional output in krill collected during the midnight sun in Antarctica, when the sun was never below the horizon (Mazzotta et al. 2010, De Pittá et al. 2013, Biscontin et al. 2017). Because a clear 24 h cycle of light intensity was present in the field at that time of the year (De Pittá et al. 2013), we hypothesize that krill circadian functions were being entrained by alternative rhythmic light cues related to light-intensity. Entrainment of the clock during the midnight sun by means of alternative *Zeitgebers* has been reported before for other high-latitude organisms (Williams et al. 2015), and it might represent an adaptation to preserve homeostatic regulation of basic physiological processes at times of the year when overt photoperiodic cues are missing (Sharma 2003, Vaze & Sharma 2013). In that case, due to the major loss of rhythmicity in the environment, even if basic clock functions might be preserved, the daily rhythms of activity might get uncoupled from the clock and lose their synchronization (Bloch et al. 2013). A similar process might be related to the de-synchronization of krill DVM observed during mid-summer.

Field data describing krill clock activity and circadian regulation during mid-winter are missing, but a similar concept of clock entrainment by alternative light cues might apply also during that time of the year. Rhythmic zooplankton DVM has been reported during winter in the Arctic at 80°N (Berge et al. 2009) in association with changes in the phase and height of the moon (Last et al. 2016), whereas daily rhythms of activity have been observed in the

scallop *Chlamys islandica* (Müller 1776), during three consecutive winters at 79°N, possibly entrained by low-intensity light cues (Tran et al. 2016). Field observations conducted during the Polar night at 79°N revealed the presence of low-intensity rhythmic light cues (Cohen et al. 2015) that might have the potential to entrain circadian rhythms during mid-winter (Båtnes et al. 2015). Because corresponding information on clock gene activity is missing, it is still unclear whether these wintertime rhythmic activities would be promoted by alternative light cues in a direct way (masking effect), or if an involvement of the clock should be considered instead (Tran et al. 2016). Our results suggest that severely reduced photoperiodic cues in krill might be insufficient for the entrainment of the circadian clock during mid-winter. It will be of particular interest investigating the influence of other light-related cues (e.g., light intensity and spectral composition).

We developed a conceptual model (Fig. 2.5) that links the seasonal modulation of krill circadian functions with the seasonal changes in photoperiod, sea-ice cover, phytoplankton abundance, and predator pressure in the environment. Predation pressure from visual predators is mainly concentrated during the day during autumn and spring, when a regular day/night cycle is present. At the same seasons, phytoplankton blooms are present in the surface layers, which are not covered by sea-ice at all times. Robust rhythmic regulation of krill metabolic activity promoted by the clock might be of great advantage, allowing krill to anticipate the light/dark cycle by implementing synchronized adaptive behaviors (i.e., DVM) at the population level. In mid-summer, when the sun never goes below the horizon, visual predators may occur at all times. At the same time, the surface layers are rich in food and free from sea-ice cover, and krill are in their seasonal phase of high metabolic activity with high energy demand to fuel reproduction. We therefore hypothesize that the costs related to predation when krill are in the surface layers are compensated by the benefits related to increased energy uptake and reproductive output. Less robust rhythmic regulation of the daily schedules might be more advantageous, allowing krill to switch to an “around the clock” activity mode and stay closer to the surface at all times to maximize food intake. The reduction of day length in mid-winter, together with the increase of sea-ice cover would significantly limit the amount of light reaching the surface layers, preventing primary production. By that time, krill have entered their seasonal phase of quiescence, characterized by reduced metabolic rates and feeding activity, and sexual regression. Even if visual

predation might still be concentrated in the surface layers during a restricted time of the day, there would be no reason for krill to expose themselves in the food-depleted surface layers at any time. Less robust rhythmic regulation might therefore be advantageous, allowing krill to remain quiescent in the deeper layers.

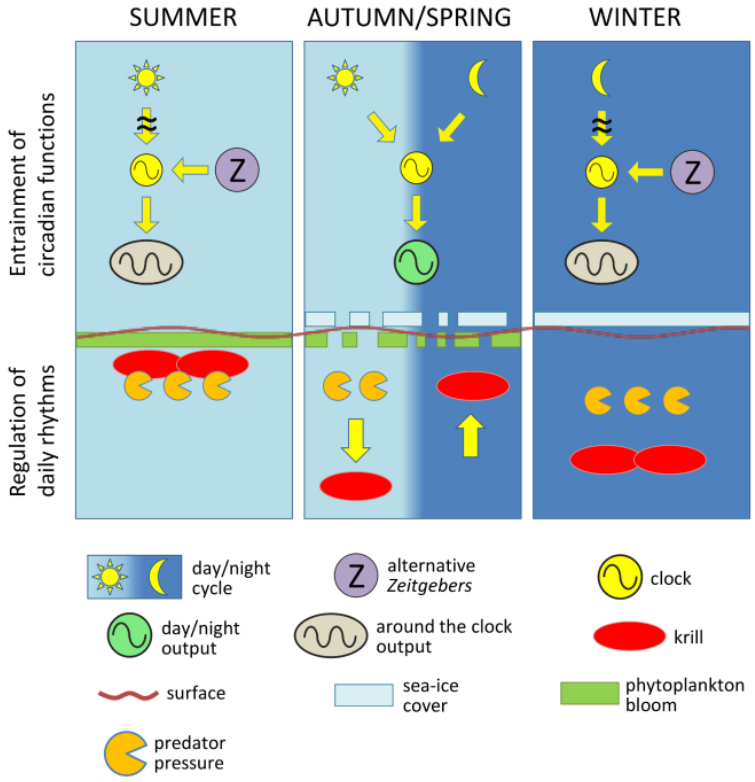

Figure 2.5: Conceptual model of photoperiodic-dependent modulation of circadian functions in Antarctic krill in different seasons. Photoperiod is the main *Zeitgeber* for the clock in autumn/spring and the daily output rhythms are synchronized to the LD cycle. Daily rhythms of predation are present and krill show DVM being active at the surface during the night. Photoperiod in summer/winter is insufficient to entrain the clock and alternative *Zeitgebers* come into play, promoting "around the clock" output rhythms. Daily rhythms of predation are less predictable and krill DVM gets altered (shallower in summer-deeper in winter).

Photoperiodic cues may thus play a major role for the entrainment of krill circadian functions during spring and autumn, when a regular day/night cycle is present. This might promote synchronous DVM and upregulation of metabolic activity during the night, allowing krill to evade visual predation by resting in the deeper layers during the day. During mid-summer and mid-winter, when the day/night cycle is strongly biased towards full light or full dark,

photoperiodic cues might not be sufficient to entrain krill circadian functions. Basic clock functions necessary for homeostatic regulation might nevertheless still be promoted by alternative *Zeitgebers* related to the daily cycle of light intensity in mid-summer and to low-intensity light cues in mid-winter. The absence of overt photoperiodic cues might at the same time promote the uncoupling of krill DVM and the emergence of "around the clock" activity patterns, which could be more suitable in a less rhythmic environment.

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3. Publication II

The seasonal metabolic activity cycle of Antarctic krill (*Euphausia superba*): evidence for a role of photoperiod in the regulation of endogenous rhythmicity

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3.1. Abstract

Antarctic krill (*Euphausia superba*), a key species in the Southern Ocean, reduce their metabolism as an energy saving mechanism to survive during the Antarctic winter. Although the adaptive significance of this seasonal metabolic shift seems obvious, the driving factors are still unclear. In particular, it is debated whether the seasonal metabolic cycle is driven by

changes in food availability, or if an endogenous timing system entrained by photoperiod might be involved. In this study, we used different long-term photoperiodic simulations to examine the influence of light regime and endogenous rhythmicity on the regulation of krill seasonal metabolic cycle. Krill showed a seasonal cycle of growth characterized by null-to-negative growth rates during autumn-winter and positive growth rates during spring-summer, which was manifest also in constant darkness, indicating strong endogenous regulation. Similar endogenous cycles were observed for the activity of the key-metabolic enzyme malate dehydrogenase (MDH) and for the expression levels of a selection of metabolic-related genes, with higher values in spring-summer and lower values in autumn-winter. On the other side, a seasonal cycle of oxygen consumption was observed only when krill were exposed to simulated seasonal changes in photoperiod, indicating that light-related cues might play a major role in the regulation of krill oxygen consumption. The influence of light-regime on oxygen consumption was minimal during winter when light-phase duration was below 8 h, while it was maximal during summer when light-phase duration was above 16 h. Significant upregulation of the krill clock genes Es*clk*, Es*cry*2 and Es*tim*, as well as of the circadian-related opsins Es*rh*1a and Es*rrh*, was observed after light-phase duration had started to decrease in early autumn, suggesting the presence of a signaling cascade linking specific seasonal changes in the Antarctic light regime with clock gene activity and the regulation of krill metabolic dormancy over the winter.

3.2. Introduction

Antarctic krill *(Euphausia superba*, hereafter krill*)*, a shrimp-like crustacean species, plays a central role in the Southern Ocean ecosystem, being both a major grazer of marine phytoplankton and a critical food item for whales, seals, birds and fish, thereby linking primary production to higher trophic levels. Its circumpolar distribution shows a latitudinal range from 51°S to 70°S with more than 50% of Southern Ocean krill stocks located in the southwest Atlantic sector and the region of the West Antarctic Peninsula (Atkinson et al. 2004). These areas are currently experiencing some of the most rapid anthropogenic-driven warming on Earth, resulting in a 1°C increase of the surface summer temperature of the

adjacent ocean since 1950 (Meredith & King 2005). There have been indications that the distribution and abundance of krill in the southwest Atlantic sector have already been altered significantly as a result of changes in primary productivity associated with the decline in sea ice (Atkinson et al. 2004). In addition, krill are the object of a developing fishery and concern has been expressed about the future sustainability of Antarctic krill fisheries in a changing environment (Schiermeier 2010). The central position of krill in the food web, the ongoing environmental changes in its habitat, and increasing commercial interest emphasize the urgency to understand the adaptability of krill to its environment.

The Southern Ocean pelagic environment is characterized by extreme seasonal changes in environmental factors such as day length (photoperiod), light intensity, sea ice extent and food availability. Almost complete darkness in winter, when most of the Southern Ocean is covered by sea ice, alternates with near constant daylight in summer. This, in turn, means almost no food in the water column during winter with less than 0.1 µg l^{-1} Chl *a* and primary production lower than 1 mg C m^{-2} d^{-1}, in contrast to massive phytoplankton blooms of > 1000 mg C m^{-2} d^{-1} in spring and early summer resulting in available Chl *a* concentrations of more than 10 µg l^{-1} (Atkinson et al. 2002, Vernet et al. 2012). Consequently, biological timing that ensures regulation of krill's physiology and behaviour in reaction to annual fluctuations of biologically significant factors is particularly advantageous and likely to be a major feature determining the success of krill in the Southern Ocean. Many high latitude species have developed circannual (approximately a year) oscillations that enable them to anticipate and prepare for forthcoming environmental changes and synchronize seasonal events (e.g. reproduction) to environmental fluctuations (Jørgensen & Johnsen 2014).

Indeed, krill has evolved a reproductive seasonal cycle with a peak of full sexual maturity and reproduction in the favorable summer months (December-January) and a trough (regression of sexual maturity) during winter months (May-June) (Kawaguchi et al. 2007). In the laboratory, this cycle can be maintained independently without direct control of factors such as food, light or temperature (Thomas & Ikeda 1987, Kawaguchi et al. 2007, Brown et al. 2013), indicating that krill passes through a fundamental inherent seasonal transition in maturity development. Other experiments showed that light conditions of prolonged photoperiod can force krill into maturity while shortened photoperiods force animals into

regression of maturity faster than under a natural light-dark regime (Teschke et al. 2008, Brown et al. 2011). These experiments strongly indicate that the overt cycle of maturity represents an endogenous annual rhythm (circannual), controlled by an endogenous timing system in krill (endogenous clock) in which photoperiod acts as a main Zeitgeber (entraining cue), synchronizing the clock with the natural year.

Similarly, krill shows a cycle of metabolic regulation throughout the year, with highest metabolic activity during the summer months (December-January) and quiescent-like reduced metabolic activity during winter (May-June). This process is thought to represent a major overwintering mechanism during times when food availability is low (Quetin & Ross 1991, Cullen et al. 2003, Meyer 2012), however, the underlying mechanisms leading to this seasonal rhythm are still not fully understood.

A central question is whether such metabolic shifts observed in krill over the seasons simply reflect a change in ingestion rate (i.e. food availability), or whether these are the result of an endogenous adaptive seasonal cycle driven by photoperiod, similar to what has been shown for the maturity cycle. A compilation of data from different investigations on the seasonal metabolic activity of krill in different regions of the Southern Ocean (Fig. 3.1A) and the corresponding cycles of photoperiod and Chl *a* concentration (Fig. 3.1B,C) demonstrate the correlation of these parameters and imply that both, the seasonal cycle of photoperiod, and food availability in the environment have the potential to play a prominent role as modulating factor for seasonal changes in metabolic rates of krill.

Long-term experiments testing the effect of photoperiod on krill in the laboratory demonstrated that feeding and metabolic activity were affected by different simulated light-dark cycles (LD) irrespective of food supply, suggesting that simulations of prolonged photoperiod can force animals into a state of increased metabolic activity and vice-versa (Teschke et al. 2007). Moreover, molecular analyses (Seear et al. 2009) revealed differential gene expression of target genes (i.e. those involved in metabolism) in response to photoperiodic changes, indicating a signalling cascade that link the photoperiod cue to the target response. However, despite these studies, up to date experimental evidence for the causative connection between the cycle of physiological and metabolic activity of krill throughout the year and the seasonal course of photoperiod is still elusive.

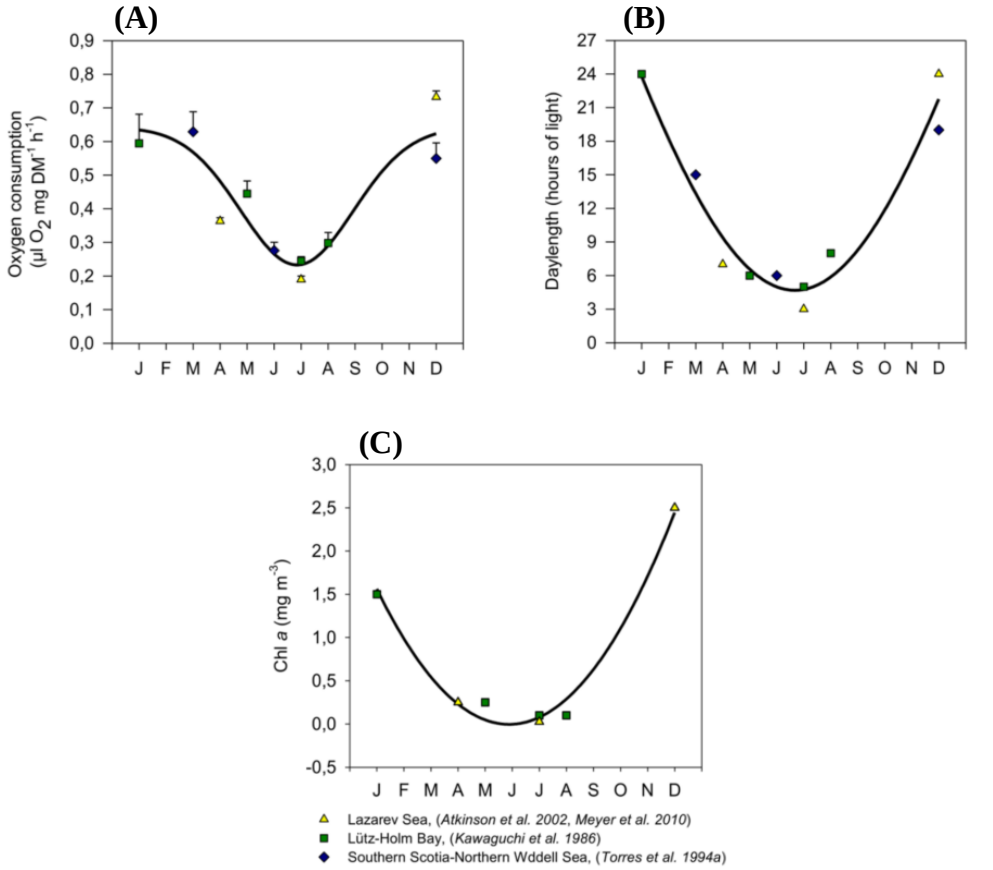

Figure 3.1: Seasonal respiration rates of adult krill from different study sites in the Southern Ocean (A), and the corresponding photoperiods (B) and Chl *a* concentrations (C) at those sites during measurements, modified after Meyer et al. 2010. Values in (A) were expressed as means ± SEM (Jan: n=3, Mar: n=2, Apr: n=16, May: n=5, Jun: n=3, Jul: Atkinson/Meyer et al.: n=17; Kawaguchi et al. 1986: n=5, Aug: n=3, Dec: Atkinson/Meyer et al. 2010: n=8; Torres et al. 1994: n=3). For respiration rates (A), photoperiods (B) and Chl *a* concentrations (C) a non-linear regression curve was fitted (A: R = 0.926; p = <0.0001; n = 65, B: R = 0.974; p = 0.0003; n = 10, C: R = 0.992; p = 0.0034; n = 7). Note that no Chl *a* values were available for Torres et al. (1994).

The aims of this study were firstly to examine whether and to what extent different physiological life cycle-parameters of krill are synchronized to the seasonal cycle of photoperiod, and secondly to investigate the endogenous response mechanism to changes in photoperiod. To implement these aims, we exposed krill to long-term simulations of different photoperiodic cycles and we monitored seasonal changes in growth, oxygen consumption, enzyme activity and gene expression.

3.3. Materials and methods

Ethics Statement

All animal work has been conducted according to relevant national and international guidelines. Krill catches, welfare and experimentation were based on permission of the Department of Environment and Heritage (DEH) of the Australian Government and were conducted in accordance with the Antarctic Marine Living Resources Conservation Act 1981 (AMLR, permit number: 06_09_2220) and the Environment Protection And Biodiversity Conservation Act 1999 (EPB, permit number: WT2007-1480).

Collection of animals in the field and maintenance in the laboratory

Euphausia superba were collected during the voyage V1 07/08 of RSV *Aurora Australis* by means of several Rectangular Midwater Trawls (pelagic net, RMT 8) in the upper 200 m of the water column between 65°19´S, 125°37´E (September 17, 2007) and 64°08´S, 119°16´E (October 9, 2007). After collection, krill were maintained on board in 200 l tanks on board with continuous supply of seawater in 0°C laboratory, with dim light and virtually no food, other than the small amount contained within the ambient water flow. After arrival in Hobart, Tasmania, krill were transferred to the Australian Antarctic Division (AAD) marine research aquarium and kept in a 1670 l holding tank connected to a 8000 l chilled seawater recirculation system. Water temperature was maintained at 0.5°C, light was provided by fluorescent tubes and controlled by a computer-regulated timer system which provided a simulation of the natural photoperiodic cycle occurring at 66°S. For a more detailed description of the aquarium see King et al. (2003) and Kawaguchi et al. (2010).

Experimental setup

Experimental tanks. The experiment started in December 2009 by separating approx. 900 adult krill of mixed sex from the holding tank into three cylindrical 100 l tanks (300 krill each), filled with 0.5°C seawater. The seawater supply was connected to the re-circulating sea-water facility of the aquarium to guarantee identical water quality and temperature for all

experimental stock throughout the study. Each tank was surrounded by a black lightproof plastic container with a sliding door at the front to create a separate light compartment. Light inside the containers was provided by fluorescent tubes (Osram L18W/640 Cool White) covered with a gel filter (ARRI, Marine Blue 131) simulating light attenuation at 30 m depth. Photoperiod and light intensity were controlled through separate channels by the same PC-controlled timer system used for the rest of the aquarium.

Feeding. All experimental tanks were exposed to the same feeding regime as used for the rest of the aquarium population. Krill were fed daily with a mixture of living algae at the final concentration of 1.5 x 10^4 cells ml^{-1} of the pennate diatom *Phaeodactylum tricornutum*, 2.2 x 10^4 cells ml^{-1} of the chlorophyte *Pyramimonas gelidicola* and 2 x 10^4 cells ml^{-1} of the cryptophyte *Geminigera cryophila*. Instant algae were added to yield final concentrations of 1 x 10^4 cells ml^{-1} of *Thalassiosira weissfloggii* (1200TM, CCMP1051/TWsp., Reed Mariculture, USA), 5.1 x 10^4 cells ml^{-1} *Isochrysis sp.* (1800TM, Reed Mariculture, USA) and 4.8 x 10^4 cells ml^{-1} *Pavlova sp.* (1800TM, Reed Mariculture, USA). Krill also received 1 g per tank per day of nutritional supplements (0.5 g of Frippak #1 CAR, 0.5 g of Frippak #2 CAR, INVE, Thailand).). After the phytoplankton mix was added, water flow into the tank was shut off for 2 h to enable krill to feed on the food mixture. This feeding regime has been used successfully in several experimental trials at the AAD prior to this study and it has been found to guarantee good condition of krill in long-term laboratory experiments with low mortality and high feeding rates (King et al. 2003, Kawaguchi et al. 2010). Feeding during dark phases was conducted under dim red light. Feeding was suspended on the day of each sampling until sampling was complete.

Light treatments. The three tanks were exposed to one of the following long-term photoperiodic treatments respectively: 1) LD treatment, simulation of the natural annual course of Antarctic photoperiod corresponding to 66°S; 2) DD treatment, constant darkness; and 3) LD ½ treatment, simulation of the annual course of Antarctic photoperiod corresponding to 66°S, but shortened into a 6 months period (Tab. 3.1). In the LD and LD ½ treatments, a midday maximum of 100 lux at the surface of the tanks was set during light phases. The transition between light and dark phases happened gradually to simulate sunrise and sunset. Both photoperiodic simulations started at a light/dark ratio in agreement with that

of the holding tank from which they originated, ensuring optimal acclimation transition into the experimental treatments. Photoperiod in LD was then adjusted at the beginning of each month while photoperiod in LD ½ was changed every two weeks.

Table 3.1: Daily duration of the light phase (hours) in the different experimental months and different experimental treatments.

month	LD	LD 1/2	DD
00_Dec	24	24	0
01_Jan	21	16	0
02_Feb	16	9	0
03_Mar	13	3	0
04_Apr	9	8	0
05_May	6	15	0
06_Jun	3	24	0
07_Jul	4	—	0
08_Aug	8	—	0
09_Sep	11	—	0
10_Oct	15	—	0
11_Nov	19	—	0
12_Dec	24	—	0

Oxygen consumption and body length measurements

To examine changes over time in the overall metabolic activity of krill under the different light regimes, oxygen consumption of three individual krill was determined every month in each tank. Krill were incubated individually for 24 h in 2 l bottles, which were completely filled with filtered seawater (0.1 µm pore size), sealed with parafilm and placed back into the corresponding experimental tanks to maintain the experimental temperature at 0.5°C. One bottle of the same volume without krill was used as control for each tank. At the end of the incubation time, three subsamples were siphoned out from each incubation bottle and transferred into separate 50 ml Winkler bottles using a glass tube, according to Atkinson et al. (2002). Oxygen concentration was measured after immediate fixation for Winkler titrations as described in Meyer et al. (2002), using a 702 SM Titrino (Metrohm). The

decrease in oxygen concentration for all experiments was < 10%, which did not affect krill behavior and hence respiration rates (Johnson et al. 1984). Oxygen consumption was calculated in µl O2 *mg^{-1} DM * h^{-1}.After incubation, the body length of each krill was measured from the anterior tip of the rostrum to the posterior end of the uropods, excluding their terminal setae. The animals were then individually snap-frozen in liquid nitrogen and stored at -80°C for enzyme activity measurements (see below).

Malate dehydrogenase (MDH) activity measurements

MDH is a key metabolic enzyme which plays a major role in the citric acid cycle as well as in other aspects of overall metabolism and has been used previously as a proxy for overall krill metabolic rate (Donnelly et al. 2004, Teschke et al. 2007). To analyze MDH activity (MDH; EC 1.1.1.37), the fifth abdominal segment of the frozen animals was used. The dissection took place on a chilled stage to avoid thawing. The tissue was homogenized in pre-weighed 2 ml tubes containing ceramic beads of 1.4 and 2.8 mm diameter (Precellys®) in ice-cold deionized water at a concentration of 100 mg fresh weight (FW) ml^{-1}, which corresponds to a dilution of 1:10. Homogenization was performed using the Precellys® 24 homogenizer with two agitation intervals of 15 s at 5000 rpm and one pause of 10 s between intervals. A constant temperature of 4°C within the homogenization chamber was maintained using a Precellys® cooling module pre-filled with liquid nitrogen. The homogenates were centrifuged for 15 min at 14000 rpm (4°C) and the supernatants were then transferred into new reaction tubes and stored at -80°C until analysis. MDH activity was determined according to Teschke et al. (2007) in a 96-well plate in technical triplicates. To 180 µl reaction buffer (0.1 M KH2PO4, pH 7), 6.7 µl NADH (7 mM) and 6.7 µl homogenate (diluted 1:30 in reaction buffer) were added. The reaction was started with 6.7 µl Oxalacetate (12 mM) and monitored at 25°C, 340 nm for 5 min. The activity was expressed as U g FW-1 (= µmol min-1 g FW-1) using the extinction coefficient ε340 = 6.22 l mmol-1 cm-1.

Gene expression measurements

***Sampling*.** For the gene expression measurements krill were sampled from the LD and DD treatments in December (2009), February, June and August (2010), at the beginning of the

third week of the corresponding month. In order to average out the effect of daily variability, 6 animals were collected every 3 hours over the 24 h cycle (06:00, 09:00, 12:00, 15:00, 18:00, 21:00, 00:00 and 03:00). During sampling feeding was suspended and during dark phases sampling was conducted under dim red light. The sampled animals were immediately snap-frozen in liquid nitrogen and stored at -80°C for molecular analyses.

***RNA extraction and cDNA synthesis*.** Total RNA was extracted from krill heads to determine relative levels of expression of target genes. Frozen krill heads were cut off on dry ice and immediately transferred in Precellys® tubes containing the TRIzol® reagent. The tissue was homogenized at 4°C using a Precellys®24 tissue homogenizer (Bertin Instruments) connected to a Cryolys cooling element. The homogenate was removed from the Precellys® tubes and treated with chloroform/isopropanol for phase separation and precipitation. The RNA pellet was washed two times with 75% EtOH and resuspended in RNase-free water. Total RNA was checked for concentration and purity using a NanoDrop™2000 UV-Vis Spectrophotometer (ThermoScientific) and for integrity using an Agilent 2100 Bioanalyzer system (Agilent Technologies). To prevent genomic contamination, all samples were treated with the TURBO DNA-Free kit from Ambion (ThermoScientific). After DNA removal, for each sample 2 µg of total RNA were retro-transcribed to cDNA using the RevertAid H Minus Reverse Transcriptase kit from Invitrogen (ThermoScientific) to a final volume of 50µl per sample (40 ng/µl).

***Primer design and qPCR*.** Gene expression was measured using custom-designed TaqMan® Low-Density Array Cards (ThermoScientific). Primers for qPCR analysis were designed around sequences of interest using the Custom TaqMan® Assay Design Tool (ThermoScientific). To examine the effect of the seasonal photoperiodic cycle on krill metabolic gene expression, we investigated seasonal changes in the expression levels of 6 genes involved in carbohydrate metabolism (*cs*, *pfk*6), lipid synthesis (*acc*), amino acid metabolism (*gldh*), protein synthesis (*ef*1a) and energy metabolism (*atp*) (Tab. A2.1). Moreover, to investigate the involvement of the putative light-entrained timekeeping mechanism at the molecular level, we included in the analysis 3 genes related to the krill circadian clock (Es*clk*, Es*cry*2, Es*tim*) and 3 genes related to krill light perception (Es*rh*1a, Es*rh*6 and Es*rrh*) (Tab. A2.1). All sequences used for primer design are available online in

the recently published krill database at http://krilldb.bio.unipd.it (Sales et al. 2017). For the LD treatment and the February DD sample, all collected krill were analyzed (6 krill per 8 time points, total n = 48), whereas for the December, June and August DD samples, due to high analytical costs and budget limitation, only 4 krill at 4 time-points (06:00, 12:00, 18:00 and 00:00) were used, for a total n = 16. For each biological replicate we mixed 20 µl of cDNA (800 ng), 30 µl of RNase-free water and 50 µl of TaqMan® Gene Expression Master Mix (ThermoScientific), for a final volume of 100µl which was loaded on the cards. The cards were analyzed using a ViiA™ 7 Real-Time PCR System (ThermoScientific). Due to technical failures affecting one card run (ineffective sealing of the card prior to analysis), 6 additional biological replicates could not be included in the final analysis, giving us the following final sample sizes: Dec in DD, n = 16; Dec in LD, n = 47; Feb in DD, n= 48; Feb in LD, n = 48; Jun in DD, n = 16; Jun in LD, n = 43; Aug in DD, n = 16; Aug in LD, n = 48. Standard curves were conducted to calculate the efficiency of each primer pair on the card.

***Normalization and relative quantification*.** The levels of transcription of the target genes were normalized and quantified using the modified $2^{-\Delta\Delta Ct}$ method proposed by Hellemans et al. (2007). This method represents an improvement of the classic $2^{-\Delta\Delta Ct}$ method, as it takes into account gene-specific amplification efficiency and allows for combination of multiple reference genes. To select the most stable genes to use in the normalization procedure, we tested 3 candidate reference genes using Normfinder (Andersen et al. 2004). Our candidate reference genes were *ubiquitin carboxyl-terminal hydrolase* 46 (*usp*46), *ribosomal protein* S13 (*rps*13) and *ribosomal protein* L32 (*rpl*32) (Tab. A2.1). *Usp*46 had showed constant mRNA levels in previous analyses of krill exposed to different LD/DD conditions (Biscontin et al. 2016), while *rps*13 and *rpl*32 had showed constant mRNA levels in previous experiments involving manipulation of photoperiod in the Lepidoptera *Plutella xylostella* (Fu et al. 2013). Following our stability analysis, *usp*46 was selected as the most stable reference gene candidate, showing a variability of ≤ 0.25 Cq (quantification cycle) in the LD treatment and ≤ 0.98 Cq in the DD treatment, and was therefore used as the reference in the normalization procedure. Normalized relative quantities (NRQs) were calculated by selecting as a baseline the sample showing the lowest Cq value among both treatments.

Statistical analyses

All data analyses were conducted using RStudio version 1.0.136 (RStudio Team 2016). We conducted separate analyses for: 1) the temporal patterns of body length, MDH activity and oxygen consumption in LD and DD; 2) the temporal pattern of oxygen consumption in LD ½; 3) the relationship between light-phase duration and oxygen consumption in LD and LD ½; and 4) the seasonal changes in gene expression in LD and DD.

***Comparison of temporal patterns of body length, MDH activity and oxygen consumption in the LD and DD treatments*.** To investigate differences in the temporal patterns of krill body length, MDH activity and oxygen consumption between LD and DD, a generalized additive model (GAM) with a Gaussian distribution was used. An additive model was chosen over a linear one to resolve the non-linear relationship of the response variables over time. The GAM took the structure as specified by Hastie and Tibshirani (1987) and was fitted using the *gam* function in the "mgcv" package (Wood 2011). Prior to the modeling process, temporal autocorrelation was examined using the *acf* function in R. Time series are often subject to latitudinal dependencies between data points and not accounting for autocorrelation can result in biased estimates of model parameters (Peres-Neto 2009). Although in some cases autocorrelation was found, it was resolved during the modeling process by the explanatory variables and was not evident in the residuals during model validation. Therefore, no temporal autocorrelation term was included in the final model. Smoothed terms (continuous, non-linear variables) were fitted as regression splines (variable: experimental *month*). In order to avoid overfitting, the smoothing parameters were manually restricted to $k \leq 6$ when necessary. Differences in temporal patterns between the two treatments (LD, DD) were implemented using the *by*-argument, which allows for the creation of separate smoother functions for each level of the *treatment* factor over the temporal variable *month*. Hence, separate parameter estimates for variable *month* for each *treatment* level were obtained. In order to test whether the temporal patterns between treatments were significantly different, the fit of the model with separate smoother functions for each *treatment* level was compared to one with a single smoother function for variable *month*, using the *anova* function and F-statistic. In addition, Akaike Information Criterion (AIC) (Akaike 1981) was consulted to check whether a model with separate functions for each

treatment level enhanced model fit. In the case of oxygen consumption, one extreme observation was excluded from the analysis, as it significantly improved the model fit. Model fit was examined by means of residual analysis.

Temporal pattern of oxygen consumption in LD ½. The temporal pattern of oxygen consumption in LD ½ was modeled as described for the temporal patterns of body length, MDH activity and oxygen consumption in the LD and DD treatments. However, due to the skewed distribution of the response variable, a gamma distribution was used. Furthermore, the number of knots was manually set to $k = 4$. The treatment LD ½ was modeled separately rather than included together with LD and DD due to 1) different data coverage in relation to the other two treatments (only three experimental months overlapped between LD ½ and the other two treatments, and the compressed light cycle in LD ½ only covered half of the total length of the time series for LD and DD); and 2) potentially different effects of the treatments (while LD and DD related to differences in light condition over the annual cycle, LD ½ tested the response of krill to quickly changing light conditions). To compare oxygen consumption between LD and LD ½ in the overlapping months (Jan-Mar-Jun), we used the non-parametric Wilcoxon Rank Sum test implemented by the *wilcox.test* function in R.

Relationship between light-phase duration and oxygen consumption in LD and LD ½. As the relationship between light and oxygen consumption for LD and LD ½ was found to be non-linear, a GAM was implemented as described for the temporal patterns of body length, MDH activity and oxygen consumption in the LD and DD treatments. Again, a single extreme observation was removed as it significantly improved the model fit. In the model, the smoother function for *light* was included together with an interaction term for *treatment* (levels: LD and LD ½), which considered the different relationships between oxygen consumption and light for each of the two treatments (LD and LD ½). Differences between the treatments were investigated using AIC and the *anova* function on the model fits as described for the temporal patterns of body length, MDH activity and oxygen consumption in the LD and DD treatments.

Seasonal changes in gene expression in LD and DD. To analyze changes in gene expression among seasonal samples in LD and DD, we used the non-parametric Kruskal-Wallis (K-W) Rank Sum test implemented by the *kruskal.test* function in R. The K-W test

does not assume normality and works well with unequal sample sizes. To correct for multiple testing, we applied the false discovery rate (fdr) method implemented by the *p.adjust* function in R. The false discovery rate method is a less conservative correction method compared to the familywise error rate method implemented for example by the Bonferroni correction, meaning that it provides a greater statistical power at the cost of a less stringent control over Type I errors. To further characterize the seasonal expression patterns we did post-hoc pairwise comparisons between seasonal samples (within treatment) using Dunn's method (Dunn 1961) implemented by the *kwAllPairsDunnTest* function in the "PMCMRplus" package (Pohlert 2018), using the Holm method (Holm 1979) to correct the p-values. Finally, to examine in more detail the effect of light on gene expression in the different seasons, we used the non-parametric Wilcoxon Rank Sum test implemented by the *wilcox.test* function in R to test differences between LD and DD within the different months (Dec-Feb-Jun-Aug), using Holm's method to correct the p-values.

3.4. Results

Temporal patterns of krill body length in LD and DD

In both treatments (LD and DD) there were significant changes in krill body length over time (Tab. 3.2, M1), but no significant differences were observed for the model fit between the two treatments (Model fit comparison with Anova: $F = 2.4$; $p = 0.07$). In fact, the temporal pattern was very similar between both treatments, with a slight decrease in krill body length occurring during the first half of the experiment, followed by an intense increase occurring during the second half (Fig. 3.2A,B). At the beginning of the experiment (month 0, Dec) the average length of individual krill in LD was 34.84 ± 0.86 mm, in agreement with that in DD (35.6 ± 1.09 mm). After that, in LD krill body length remained constant until month 3 (Mar, mean body length = 35.31 ± 0.4 mm), then decreased during months 4, 5 and 6 (Apr, May, Jun) and reached a minimum in month 7 (Jul, mean body length = 27.75 ± 1.25 mm). In DD, krill body length started decreasing already during month 1, 2, 3 and 4 (Jan, Feb, Mar and Apr), reaching a minimum in month 5 (May, mean body length = 29.48 ± 1.27 mm). After the minimum was reached, in LD krill body length increased steeply during months 8, 9, 10

and 11 (Aug, Sep, Oct and Nov), reaching maximum values in month 12 (Dec, mean body length = 47.61 ± 1.72 mm), whereas in DD the increase in body length started in month 6 (Jun) and proceeded at a slower pace until month 12 (Dec), reaching a final mean value of 44.49 ± 1.03 mm.

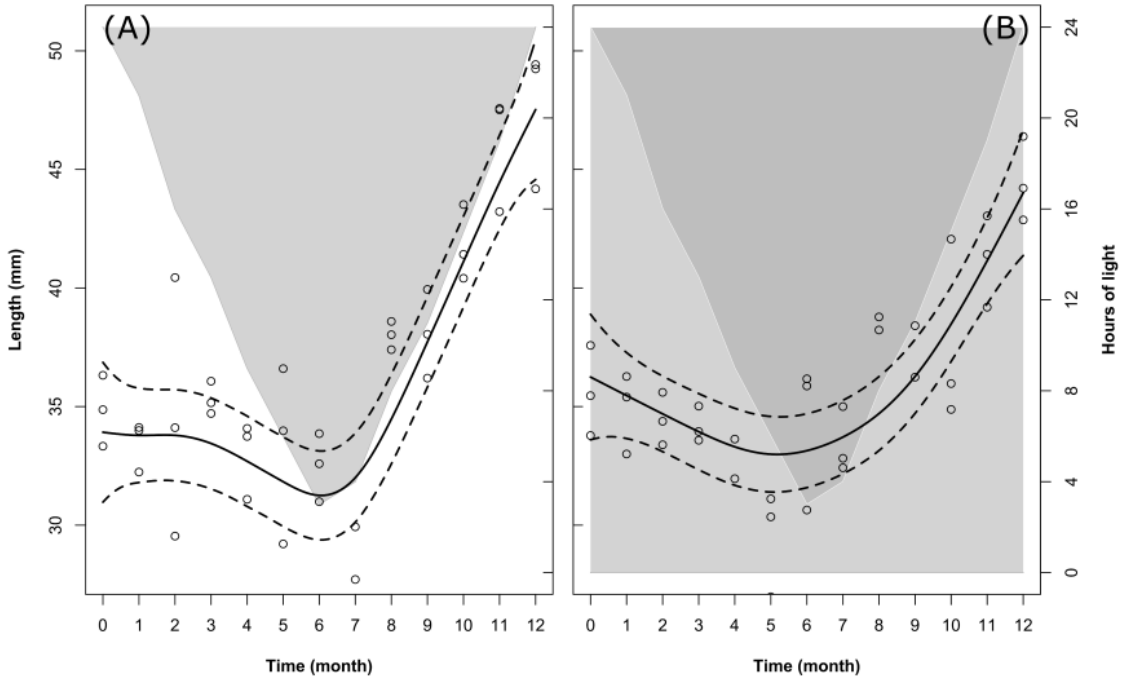

Figure 3.2: Changes in krill body length over time in the LD (A) and DD (B) treatments. Solid dark lines represent the models fit; dotted lines represent confidence intervals around the fit; circles represent individual measurements. In LD (A), light-grey shaded area vs. white area represents temporal changes in the duration of dark phase vs. light phase. In DD (B), dark-grey shaded area vs. light-grey shaded area represents temporal changes in the duration of dark phase vs. subjective light phase.

Temporal patterns of oxygen consumption

LD and DD treatments. The changes over time in krill oxygen consumption were significant in LD but not in DD (Tab. 3.2, M2). This was confirmed also by the model fit comparison, which showed that the two model fits were significantly different from each other (Model fit comparison with Anova: F = 6.59; p = 0.014). The temporal pattern in LD was characterized by a decrease in oxygen consumption during the first half of the experiment followed by an increase during the second half (Fig. 3.3A).

Table 3.2: GAM statistics for parametric coefficients (estimates, standard errors (SE), F- or t-values and p-values), explained variance (Deviance or R^2) and non-parametric terms (smooth) (estimated degrees of freedom (edf), F-statistic and p-values). M1: body length over time (LD-DD); M2: oxygen consumption over time (LD-DD); M3: oxygen consumption over time (LD ½); M4: oxygen consumption over light-phase duration (LD-LD ½); M5: MDH activity over time (LD-DD)

Intercept M1	**Estimate**	**SE**	**t-value**	**p-value**	**Deviance**
Length	36.04	0.4	100.4	≤0.001	68.50%
Smooth	**edf**		**F-value**	**p-value**	
Month LD	4.04		20.1	≤0.001	
Month DD	2.82		12.33	≤0.001	
Intercept M2	**Estimate**	**SE**	**t-value**	**p-value**	**Deviance**
Oxygen	0.3	0.018	16.79	≤0.001	43.10%
Smooth	**edf**		**F-value**	**p-value**	
Month LD	3.69		5.01	0.003	
Month DD	1		0.69	0.41	
Intercept M3	**Estimate**	**SE**	**t-value**	**p-value**	**Deviance**
Oxygen	0.88	0.005	179	≤0.001	62.60%
Smooth	**edf**		**F-value**	**p-value**	
Month LD 1/2	2.08		5.17	0.027	
Intercept M4	**Estimate**	**SE**	**t-value**	**p-value**	**Deviance**
Oxygen	0.3	0.015	20.56	≤0.001	69.60%
Smooth	**edf**		**F-value**	**p-value**	
Light LD	5.21		10.93	≤0.001	
Light LD1/2	1.05		1.17	0.3	
Intercept M5	**Estimate**	**SE**	**t-value**	**p-value**	**Deviance**
MDH	202.1	4.34	46.55	≤0.001	39.10%
Smooth	**edf**		**F-value**	**p-value**	
Month LD	4.63		5.96	0.0003	
Month DD	3.43		2.5	0.053	

Oxygen uptake was maximal from month 0 (Dec, mean oxygen consumption = 0.39 ± 0.03 µl O2 *mg-1 DM * h-1) until month 3 (Mar, 0.39 ± 0.06 µl O2 *mg-1 DM * h-1), then decreased and reached a minimum in month 7 (Jul, 0.03 ± 0.02 µl O2 *mg-1 DM * h-1). After that, oxygen consumption remained low during month 8 (Aug, 0.08 ± 0.01 µl O2 *mg-1 DM * h-1), then quickly recovered back to maximal levels already by month 9 (Sep, 0.41 ± 0.08 µl O2 *mg-1 DM * h-1), remaining high until the end of the experiment (month 11, Nov, 0.37 ± 0.02 µl O2 *mg-1 DM * h-1; month 12 is missing).

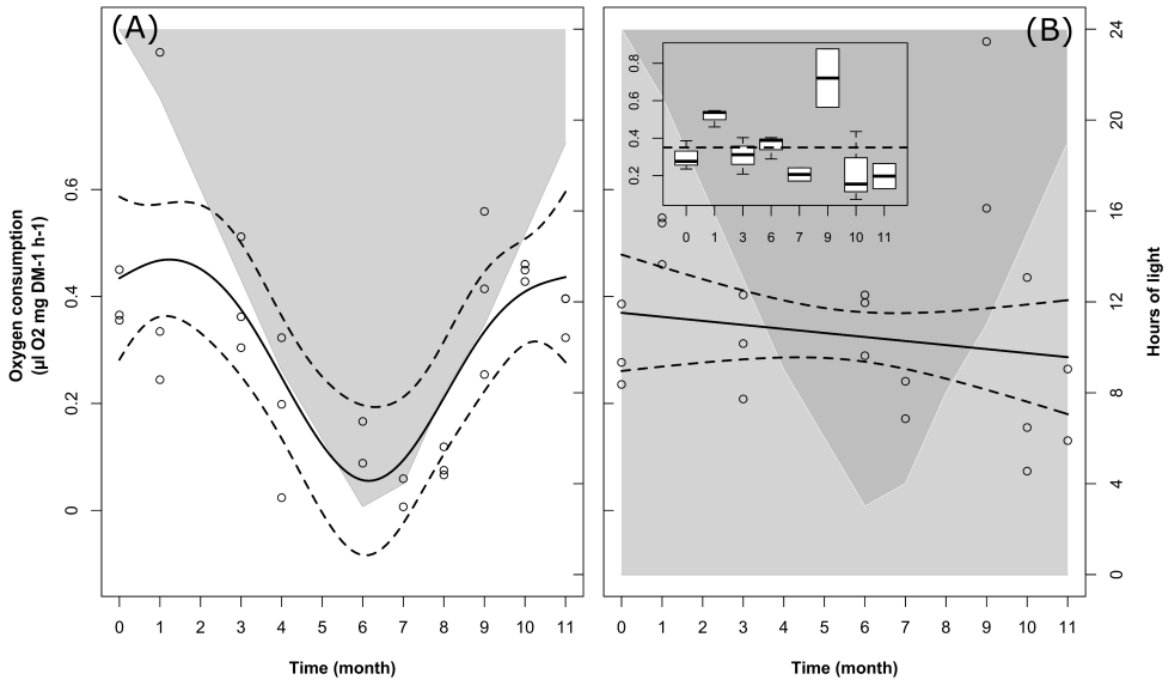

Figure 3.3: Changes in krill oxygen consumption over time in the LD (A) and DD (B) treatments. Solid dark lines represent the models fit; dotted lines represent confidence intervals around the fit; circles represent individual measurements. In LD (A), light-grey shaded area vs. white area represents temporal changes in the duration of dark phase vs. light phase. In DD (B), dark-grey shaded area vs. light-grey shaded area represents temporal changes in the duration of dark phase vs. subjective light phase. In DD (B), upper-left small pane represents monthly changes in oxygen consumption relative to the treatment mean (dotted line).

In contrast to the temporal patterns observed in LD, no significant changes over time were detected in oxygen consumption in the DD treatment (Fig. 3.3B, large pane), possibly due to great variability between months (Fig 3.3B, small pane). At the beginning of the experiment (month 0, Dec), mean oxygen consumption was lower compared to the same month in LD (0.29 ± 0.04 µl O2 *mg-1 DM * h-1). In month 1 (Jan), we observed a sudden increase up to

and above LD maximum levels (0.51 ± 0.03 µl O2 *mg-1 DM * h-1). After that, oxygen consumption decreased to 0.3 ± 0.05 µl O2 *mg-1 DM * h-1 in month 3 (Mar) and fluctuated back to 0.36 ± 0.04 µl O2 *mg-1 DM * h-1 by month 6 (Jun). Values dropped again in month 7 (Jul, 0.21 ± 0.04 µl O2 *mg-1 DM * h-1) and then increased suddenly in month 9 (Sep), again up to above LD maximum levels (0.72 ± 0.11 µl O2 *mg-1 DM * h-1). After that, oxygen consumption dropped back to LD minimum-like values in month 11 (Nov, 0.22 ± 0.1 µl O2 *mg-1 DM * h-1) and month 12 (Dec, 0.19 ± 0.06 µl O2 *mg-1 DM * h-1).

LD ½ treatment. In the LD ½ treatment there were significant changes in krill oxygen consumption over time (Tab. 3.2, M3). Oxygen consumption decreased during the first half of the experiment and slightly increased during the second half (Fig. 3.4, dotted line).

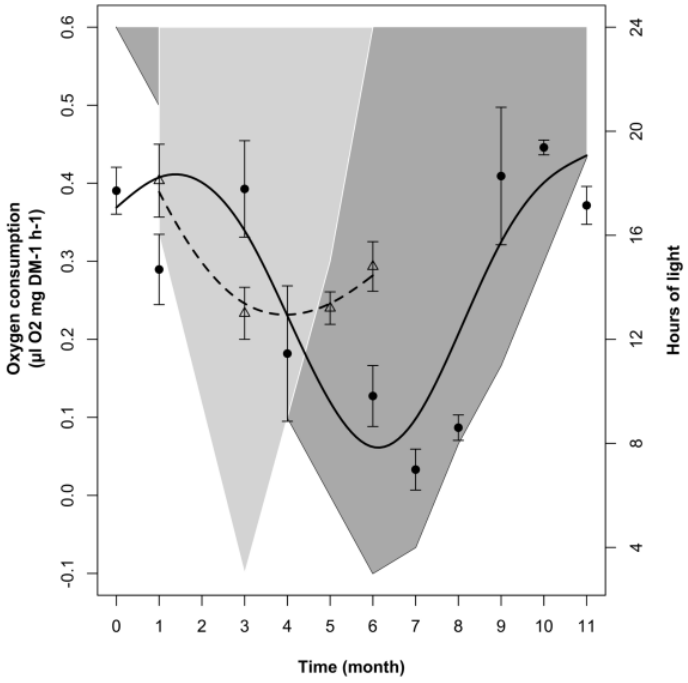

Figure 3.4: Changes in krill oxygen consumption over time in the LD ½ treatment (dotted line represents the model fit; empty triangles represent monthly average ± SEM, n = 3) compared to the LD treatment (solid line represents the model fit, black dots represent monthly average ± SEM, n = 3). Light-grey shaded area represents changes in dark phase duration in LD ½, dark-grey shaded area vs. white area represents dark phase vs. light phase duration in LD.

Oxygen consumption was maximal in month 1 (Jan, 0.41 ± 0.04 µl O2 *mg-1 DM * h-1; month 0 is missing), then dropped to minimum values in month 3 (Mar, 0.23 ± 0.03 µl O2

*mg-1 DM * h-1). After that, oxygen consumption remained low in month 5 (May, 0.24 ± 0.02 µl O2 *mg-1 DM * h-1) and finally showed a small increase in month 6 at the end of the experiment (Jun, 0.29 ± 0.03 µl O2 *mg-1 DM * h-1). The comparisons between LD and LD ½ mean oxygen consumption levels in months 1 (Jan), 3 (Mar) and 6 (Jun) did not show significant differences, nevertheless the two treatments showed significant differences in the way oxygen consumption was influenced by light-phase duration in the different months (see below).

Relationship between light-phase duration and oxygen consumption in LD and LD ½

The relationship between oxygen consumption and light-phase duration was significant in LD but not in LD ½ (Tab. 2, M4). This was confirmed also by the model fit comparison, which showed that the two model fits were significantly different from each other (Model fit comparison with Anova: F = 60.559; p = 0.001). In LD (Fig. 3.5, solid line), oxygen consumption levels were constantly minimal when light-phase duration was between 3 and 8 h (light phase never went below 3 h).

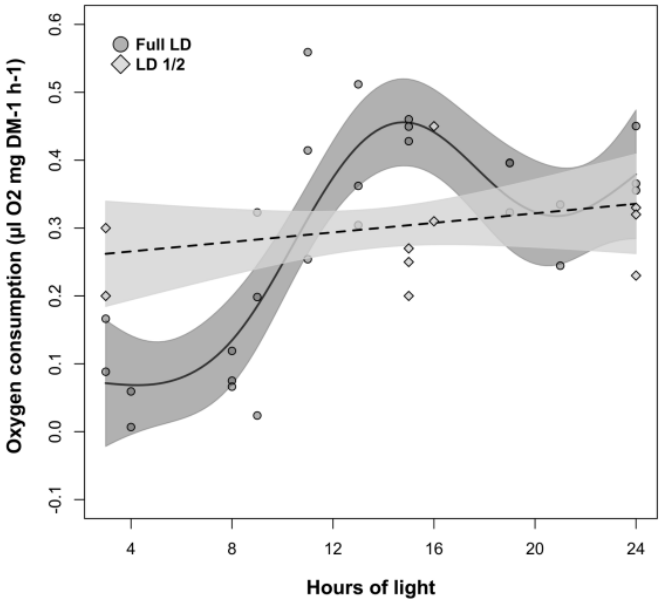

Figure 3.5: Changes in krill oxygen consumption over light-phase duration in the LD and LD ½ treatments. For LD, solid line represents the model fit, dark-grey shaded area represents confidence interval around the fit, and dark-grey shaded circles represent individual measurements. For LD ½, dotted line represents the model fit, light-grey shaded area represents confidence interval around the fit, and light-grey shaded squares represent individual measurements.

Between 8 and 16 h of light, oxygen consumption levels displayed a strong positive relationship with light-phase duration. Maximal oxygen consumption levels were observed when light-phase duration was about 16 h. Further increase in light-phase duration beyond 16 h did not result in a further increase in oxygen consumption, but in a small contraction instead. However, in the LD ½ treatment no clear relationship between the two variables could be observed (Fig. 3.5, dotted line).

Temporal patterns of MDH activity in LD and DD

Changes in MDH activity over time were significant in LD and marginally non-significant in DD (Tab. 2, M5). Nevertheless, the model fit comparison showed that the two model fits were not significantly different from each other (Model fit comparison with Anova: $F = 1.11$; $p = 0.36$) (Fig. 3.6A,B).

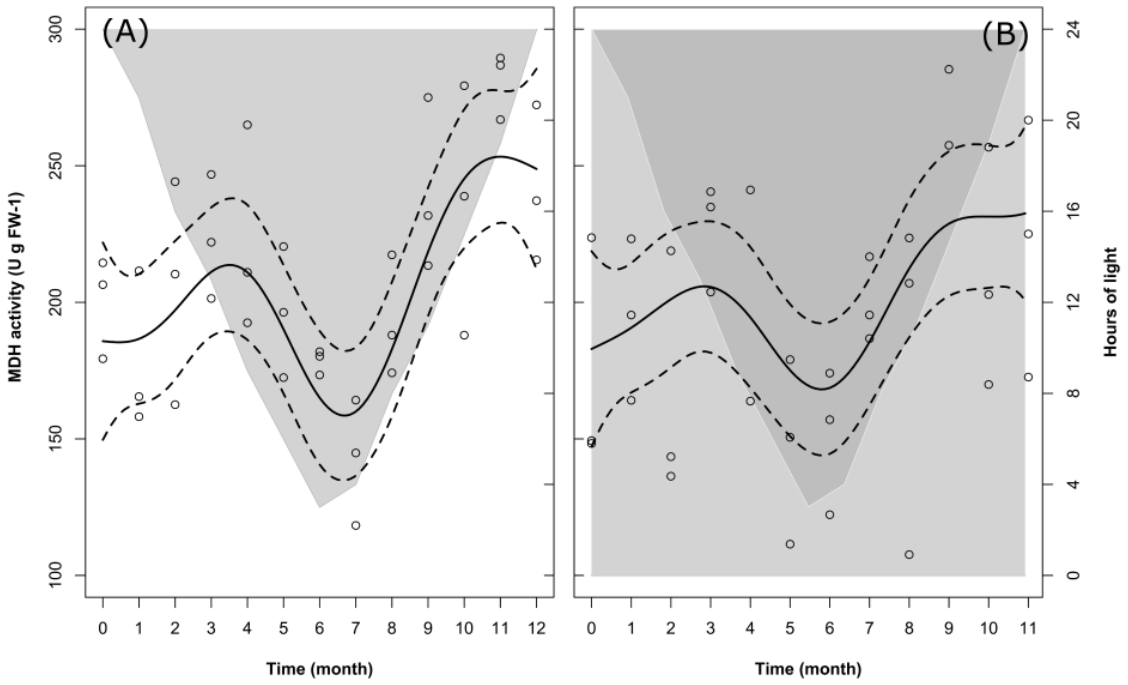

Figure 3.6: Changes in krill MDH activity over time in the LD (A) and DD (B) treatments. Solid dark lines represent the model fit; dotted lines represent confidence interval around the fit; circles represent individual measurements. In LD (A), light-grey shaded area vs. white area represents temporal changes in the duration of dark phase vs. light phase. In DD (B), dark-grey shaded area vs. light-grey shaded area represents temporal changes in the duration of dark phase vs. subjective light phase.

In both treatments we observed a similar seasonal trend characterized by at first, an increase in MDH activity between month 0 (Dec) and month 3 (Mar), then a drop between month 3 and month 6-7 (Jun-Jul), and then a second increase between month 6-7 and month 9-11 (Sep-Nov). The values then remained stable until the end of the experiment (month 12 in LD, month 11 in DD, 12 is missing).

In LD, mean MDH activity at the beginning of the experiment (month 0, Dec) was 200.09 ± 10.63 U g FW-1, slightly higher than in DD at the same time of the year (173.79 ± 24.95 U g FW-1). In both treatments, the first maximum was reached in month 3 (Mar), with comparable mean values of 223.4 ± 13.13 U g FW-1 in LD and 226.37 ± 11.43 U g FW-1 in DD. After that, the winter minimum was reached in month 7 (Jul) in LD (142.44 ± 13.3 U g FW-1), whereas it was reached two months earlier (month 5, May) in DD (147.01 ± 19.59 U g FW-1). Similarly, the second maximum was reached in month 11 (Nov) in LD (281.05 ± 7.13 U g FW-1) and in month 9 (Sep) in DD (271.4 ± 13.91 U g FW-1).

Seasonal changes in gene expression in LD and DD

***Metabolic-related genes*.** In the LD treatment, all metabolic genes showed significant seasonal differences in their expression levels (Tab. 3.3), and all of them showed significant upregulation in December and August compared to June ($p \leq 0.005$, Fig. 3.7). Also, significant upregulation was generally observed in February compared to June ($p \leq 0.008$, Fig. 3.7). Moreover, *atp* and *gldh* showed significant upregulation in December compared to February ($p \leq 0.004$, Fig. 3.7C-E), *pfk*6 showed significant upregulation in August compared to December ($p \leq 0.04$, Fig. 3.7B), and *pfk*6 and *atp* also showed significant upregulation in August compared to February ($p \leq 0.001$, Fig 3.7B,C). In the DD treatment, 5 of the 6 tested genes showed significant seasonal differences (Tab. 3.3). *Cs* and *acc* showed significant upregulation in December compared to February ($p \leq 0.02$, Fig. 3.7A,D); *atp*, *acc*, *gldh* and *ef*1a showed significant upregulation in December compared to June ($p \leq 0.02$, Fig. 3.7C-F); *ef*1a showed upregulation in December compared to August ($p \leq 0.02$, Fig. 3.7F), and also in February compared to June ($p \leq 0.001$, Fig. 3.7F); *cs* showed upregulation in August compared to February ($p \leq 0.01$, Fig. 3.7A); and *cs*, *atp* and *gldh* showed upregulation in August compared to June ($p \leq 0.04$, Fig. 3.7A,C,E).

Table 3.3: Summary of results of Kruskal-Wallis (K-W) tests for differences in metabolic gene expression among seasonal samples in LD and DD. P-values were corrected using the false discovery rate (fdr) method. When K-W test was significant, post-hoc pairwise comparisons between seasonal samples (Dec-Feb, Dec-Jun, Dec-Aug, Feb-Jun, Feb-Aug, Jun-Aug) were calculated after Dunn, and the p-values were corrected after Holm. Non-significant p-values (p ≥ 0.05) are indicated as n.s.

LD	K-W	Dec-Feb	Dec-Jun	Dec-Aug	Feb-Jun	Feb-Aug	Jun-Aug
cs	< 0.001	n.s.	< 0.001	n.s.	< 0.001	n.s.	< 0.001
pfk6	< 0.001	n.s.	< 0.001	0.04	< 0.001	< 0.001	< 0.001
atp	< 0.001	< 0.001	< 0.001	n.s.	< 0.001	< 0.001	< 0.001
acc	< 0.001	n.s.	0.002	n.s.	< 0.001	n.s.	0.005
gldh	< 0.001	0.004	< 0.001	n.s.	0.008	n.s.	< 0.001
ef1a	< 0.001	n.s.	< 0.001	n.s.	< 0.001	n.s.	< 0.001
DD	**K-W**	**Dec-Feb**	**Dec-Jun**	**Dec-Aug**	**Feb-Jun**	**Feb-Aug**	**Jun-Aug**
cs	0.009	0.02	n.s.	n.s.	n.s.	0.01	0.04
pfk6	n.s.	—	—	—	—	—	—
atp	0.03	n.s.	0.02	n.s.	n.s.	n.s.	0.02
acc	0.008	< 0.001	0.01	n.s.	n.s.	n.s.	n.s.
gldh	0.02	n.s.	0.02	n.s.	n.s.	n.s.	0.02
ef1a	< 0.001	n.s.	< 0.001	0.02	< 0.001	n.s.	n.s.

For gene-name abbreviations please see Tab. A2.1

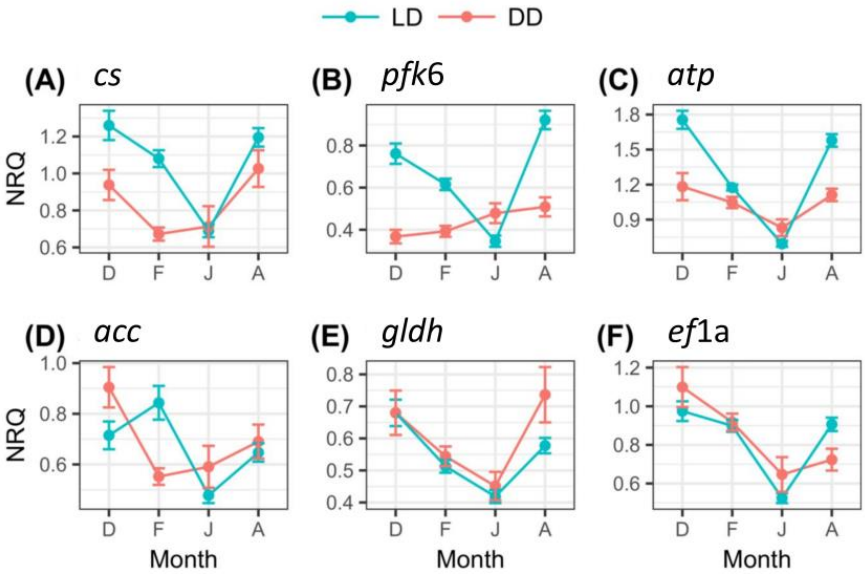

Figure 3.7: Seasonal changes of metabolic genes expression in LD and DD. For each month (D = December, F = February, J = June, A = August), mean NRQ ± SEM are reported. Sample sizes are as follows: Dec in DD, n = 16; Dec in LD, n = 47; Feb in DD, n= 48; Feb in LD, n = 48; Jun in DD, n = 16; Jun in LD, n = 43; Aug in DD, n = 16; Aug in LD, n = 48. For gene name abbreviations please see Tab. A2.1.

Moreover, comparing the expression levels between LD and DD at each seasonal sampling time (Dec-Feb-Jun-Aug), we observed that *pfk*6 and *atp* showed significant upregulation in December in LD (Tab. 3.4, Fig. 3.7B,C), *cs*, *pfk*6, *atp* and *acc* showed significant upregulation in February in LD (Tab. 3.4, Fig. 3.7A-D), and *pfk*6 and *atp* showed significant upregulation in August in LD (Tab. 3.4, Fig. 3.7B,C). No significant differences between LD and DD were observed in June (Tab. 3.4).

Table 3.4: Summary of results of Wilcoxon Rank Sum tests on differences in gene expression between the LD and DD treatment at the four seasonal sampling times (Dec, Feb, Jun, Aug). P-values were corrected after Holm. Non-significant p-values ($p \geq 0.05$) are indicated as n.s.

LD-DD	Dec	Feb	Jun	Aug
cs	n.s.	< 0.001	n.s.	n.s.
pfk6	< 0.001	< 0.001	n.s.	< 0.001
atp	0.001	0.01	n.s.	< 0.001
acc	n.s.	0.004	n.s.	n.s.
gldh	n.s.	n.s.	n.s.	n.s.
ef1a	n.s.	n.s.	n.s.	n.s.
Es*clk*	n.s.	< 0.001	n.s.	0.001
Es*cry2*	n.s.	< 0.001	n.s.	n.s.
Es*tim*	n.s.	< 0.001	n.s.	n.s.
Es*rh1a*	0.02	< 0.001	n.s.	n.s.
Es*rh6*	n.s.	n.s.	n.s.	n.s.
Es*rrh*	n.s.	< 0.001	n.s.	n.s.

For gene-name abbreviations please see Tab. A2.1

Clock and light-related genes. In the LD treatment, all clock and light-related genes showed significant seasonal differences in their expression levels (Tab. 3.5). Upregulation was generally observed in February compared to December, June and August ($p \leq 0.001$, only Es*rh*6 did not show upregulation in February, Fig. 3.8). Es*rh*6 showed significant upregulation in June compared to December ($p = 0.01$, Fig. 3.8E), while Es*rrh* showed significant upregulation in December compared to June ($p \leq 0.001$) and August ($p = 0.03$), and in August compared to June ($p = 0.003$, Fig. 3.8F). In DD, only Es*rh*1a showed significant seasonal differences (Tab. 3.5), with significant upregulation in December compared to February ($p = 0.01$), June ($p = 0.002$) and August ($p = 0.02$, Fig. 3.8D).

Table 3.5: Summary of results of Kruskal-Wallis (K-W) tests for differences in clock and light-related gene expression among seasonal samples in LD and DD. P-values were corrected using the false discovery rate (fdr) method. When K-W test was significant, post-hoc pairwise comparisons between seasonal samples (Dec-Feb, Dec-Jun, Dec-Aug, Feb-Jun, Feb-Aug, Jun-Aug) were calculated after Dunn, and the p-values were corrected after Holm. Non-significant p-values ($p \geq 0.05$) are indicated as n.s.

LD	K-W	Dec-Feb	Dec-Jun	Dec-Aug	Feb-Jun	Feb-Aug	Jun-Aug
Es*clk*	< 0.001	< 0.001	n.s.	n.s.	< 0.001	< 0.001	n.s.
Es*cry*2	< 0.001	< 0.001	n.s.	n.s.	< 0.001	< 0.001	n.s.
Es*tim*	< 0.001	0.001	n.s.	n.s.	< 0.001	< 0.001	n.s.
Es*rh*1a	< 0.001	< 0.001	n.s.	n.s.	< 0.001	< 0.001	n.s.
Es*rh*6	0.01	n.s.	0.01	n.s.	n.s.	n.s.	n.s.
Es*rrh*	< 0.001	< 0.001	< 0.001	0.03	< 0.001	< 0.001	0.003
DD	**K-W**	**Dec-Feb**	**Dec-Jun**	**Dec-Aug**	**Feb-Jun**	**Feb-Aug**	**Jun-Aug**
Es*clk*	n.s.	—	—	—	—	—	—
Es*cry*2	n.s.	—	—	—	—	—	—
Es*tim*	n.s.	—	—	—	—	—	—
Es*rh*1a	0.01	0.01	0.002	0.02	n.s.	n.s.	n.s.
Es*rh*6	n.s.	—	—	—	—	—	—
Es*rrh*	n.s.	—	—	—	—	—	—

For gene-name abbreviations please see Tab. A2.1

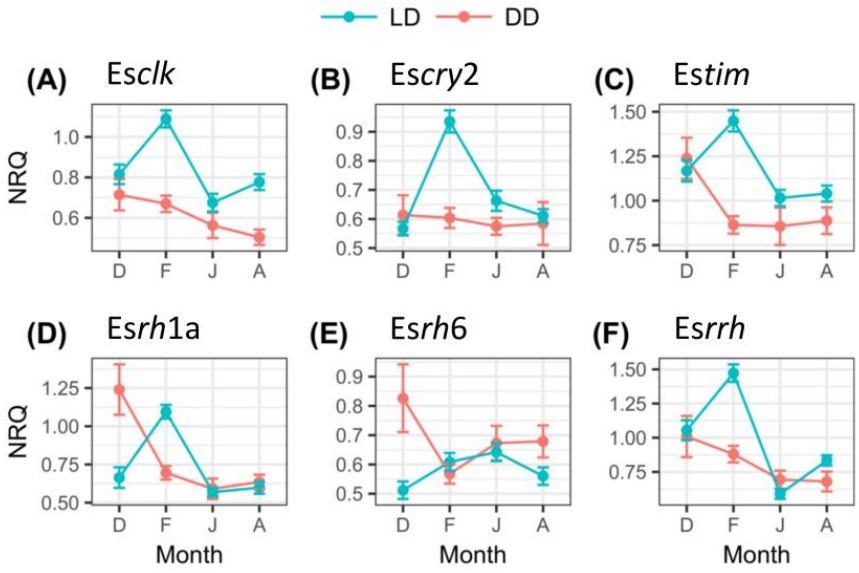

Figure 3.8: Seasonal changes of clock and light-related genes expression in LD and DD. For each month (D = December, F = February, J = June, A = August), mean NRQ ± SEM are reported. Sample sizes are as follows: Dec in DD, n = 16; Dec in LD, n = 47; Feb in DD, n= 48; Feb in LD, n = 48; Jun in DD, n = 16; Jun in LD, n = 43; Aug in DD, n = 16; Aug in LD, n = 48. For gene name abbreviations please see Tab. A2.1.

Comparing the expression levels between LD and DD at each seasonal sampling time (Dec-Feb-Jun-Aug), we observed general upregulation in February in LD (Tab. 3.4, only Es*rh*6 did not show significant differences, Fig. 3.8). Moreover, Es*rh*1a showed significant upregulation in DD in December (Tab. 3.4, Fig. 3.8D), while Es*clk* showed upregulation in August in LD (Tab. 3.4, Fig. 3.8A). No significant differences were observed between LD and DD in June (Tab. 3.4).

3.5. Discussion

Influence of food availability on the regulation of krill seasonal metabolic cycle

During our LD simulation, krill displayed clear seasonal cycles of growth, enzyme activity, oxygen consumption and metabolic gene expression, with a trough in June-July at simulated 66°S midwinter light conditions and a peak in December-January at simulated 66°S midsummer light conditions. This was in agreement with field observations showing that krill undergo a period of winter dormancy to save energy during the food-depleted season, while they enhance their activity during summer to take advantage of the abundant food resources and trigger reproduction (Stepnik 1982, Quetin & Ross 1991, Meyer 2012). Early observations by Ikeda and Dixon (1982) showing that laboratory krill starved for over 200 days did not die but responded with body shrinkage instead, suggested that major physiological changes related to krill winter dormancy might be directly regulated by food availability. However, Kawaguchi et al. (1986) and Torres et al. (1994) later observed that seasonal changes in krill metabolic activity did not always correlate with changes in food availability, suggesting a different mechanism of regulation. Indeed, following laboratory experimentations showed that krill response to high food conditions during winter could be influenced by changes in light regime, and that prolonged photoperiods could stimulate increased feeding activity (Atkinson et al. 2002, Teschke et al. 2007, Meyer et al. 2010). This suggested that krill metabolic cycle was not directly regulated by seasonal changes in food availability, but rather by changes in feeding activity stimulated by the Antarctic light regime (Meyer at al. 2010). The results obtained during our LD simulation under constant food conditions indicated that food availability was not the main factor influencing krill metabolic

cycle and supported the hypothesis that the seasonal light-regime was playing a major role instead.

Endogenous regulation of krill metabolic cycle

Based on the observation that different light regimes could affect the feeding activity of krill during winter (Teschke et al. 2007), Meyer et al. (2010) hypothesized the presence in krill of an inherent (i.e. endogenous) seasonal metabolic cycle triggered by photoperiodic cues. This hypothesis was further investigated by Brown et al. (2013), who showed that krill oxygen consumption increased after winter, independently from light regime, food availability and temperature, suggesting the presence of an endogenous seasonal rhythm which had been entrained prior the commencement of the experiment. Indeed, the seasonal cycles of growth, enzyme activity and gene expression observed during our DD simulation strongly supported this hypothesis, indicating that fundamental processes related to krill growth and metabolism were following an inherent seasonal cycle irrespective of light and food conditions.

However, krill oxygen consumption, which is generally used as a proxy for overall metabolic activity, showed a seasonal cycle only in LD. Oxygen consumption represents the sum of multiple physiological processes occurring together, including basal metabolism, swimming activity, growth, feeding and reproduction (Clarke & Morris 1983). Therefore, the correlation between the seasonal cycles of oxygen consumption, growth and enzyme activity that we observed in LD was in some ways to be expected. Conversely, the missing correlation of oxygen consumption observed in DD would suggest that the seasonal dynamics of some oxygen-demanding process was altered by the prolonged exposure to constant darkness. Considering the endogenous seasonal patterns displayed in DD by krill growth and by the expression profile of *ef*1a, a gene involved in protein synthesis and cell growth and proliferation, we might suggest that growth-related processes were not directly affected. Similarly, considering the endogenous cycles displayed in DD by MDH activity and by the expression profiles of *cs* (involved in the Citric Acid Cycle) and *atp* (involved in ATP synthesis), we might suggest that basal carbohydrate and energy metabolism were also not directly affected. However, this would be opposed by the different response displayed in LD and DD by the metabolic gene *pfk*6, coding for the rate-limiting step of the glycolytic

pathway, which showed a clear seasonal profile only in LD. In this case, we might hypothesize that basal krill metabolic functions related to glycolysis were indeed being altered under prolonged darkness, possibly leading to the different oxygen consumption pattern observed in DD. In fact, even if glycolysis is generally considered as an oxygen-independent metabolic pathway, it may still contribute to overall aerobic metabolism since its end products (pyruvate and reducing power) can be used to fuel the Citric Acid Cycle, which is a typical oxygen-dependent process.

Even if no overt seasonal cycle of oxygen consumption was observed in DD, the comparison between the LD and LD ½ treatments suggested that the interplay of a seasonal (i.e. endogenous) factor should not be excluded *tout court*. In fact, despite that clear temporal changes in oxygen consumption were observed in both treatments, significant differences were present in the relationship between light-phase duration and oxygen consumption between the two treatments. In particular, this seemed to be related to the different rate of change of oxygen consumption observed in LD ½, which did not compensate for the rate of advance in the photoperiodic cycle, especially in May and June when oxygen consumption was still minimal despite the fact that a significant increase in light-phase duration had already taken place (from 3 h in March to 15 h in May and 24 h in June).

Photoperiodic entrainment of krill seasonal metabolic cycle

Following the hypothesis that krill seasonal metabolic cycle was regulated by an endogenous timekeeping mechanism (i.e. a circannual clock) entrained by light regime (Meyer et al. 2010, Brown et al. 2013), the lack of seasonality observed for oxygen consumption in DD might be interpreted as a lack of proper photoperiodic entrainment. In fact, in order to achieve effective circannual synchronization, the onset of a seasonal response might require exposition to specific photoperiodic cues during a determined time of the year, which in the case of a winter-related response may be represented by the gradual shortening of day length at the end of summer (Woodfill et al. 1994). Since our DD treatment was initiated early in December (midsummer), this would have deprived krill of those late summer/early autumn light cues which might have been necessary to entrain their circannual clock and elicit proper orchestration of the winter-related responses, possibly causing an uncoupling among the

different oxygen-demanding processes. This would be in agreement with the different seasonal response observed by Brown et al. (2013), who initiated their DD treatment later in autumn thus allowing for proper krill entrainment during the previous summer.

The presence of specific photoperiodic thresholds (or conditions) for the timing of krill seasonal metabolic responses was suggested also by the light-oxygen relationship observed in the LD treatment. In fact, metabolic down-regulation towards winter was initiated only after the light phase duration had been reduced below 16 h, corresponding to an early autumn light regime, whereas metabolic up-regulation occurred only after light phase duration had exceeded 8 h, corresponding to a late winter light regime. A similar season-specific interaction with light regime was also displayed by metabolic gene expression. In fact, while the DD expression levels of *pfk*6, *cs* and *acc* in February were already minimal and comparable with June (i.e. winter-like), those in LD were still maximal and comparable with December (i.e. summer-like), suggesting that early autumn light conditions might represent a threshold also at the transcriptional level. Conversely, an anticipatory (e.g. endogenous) mechanism seemed to take place at the molecular level at the end of winter, as metabolic gene expression increased to summer-like values already by August, when oxygen consumption was still minimal. This suggested that while the initiation of krill winter dormancy might be regulated in a top-down manner, with the environmental changes (i.e. light regime) triggering the physiological responses, the termination of krill winter dormancy might be regulated in a bottom-up manner, with the physiological responses being triggered by the endogenous factors. A similar concept was suggested also by Brown et al. (2011) regarding the regulation of krill seasonal sexual maturity cycle, and might therefore represent a general mechanism underlying krill seasonal timekeeping.

Involvement of circadian clock genes

Photoperiodic time-measurement can be achieved through the interaction between appropriate light cues and an internal timekeeping mechanism (i.e. a photoperiodic clock), which can track changes in day length over a period of time and stimulate the season-specific response (Dolezel 2015). Even if the nature of the photoperiodic clock remains elusive, various evidences have been collected indicating the involvement of elements of the

circadian clock. The circadian clock is a molecular oscillator based on rhythmic clock gene expression, which can be entrained by light cues and can stimulate daily rhythms in metabolism and behavior (Dunlap 1999). Being able to track day length, the circadian clock has the potential to play also a role in photoperiodic time-measurement, and the link between clock gene expression and the regulation of seasonal responses has indeed been demonstrated in mammals and insects (Hazlerigg 2010, Goto 2013). In krill, the circadian clock has been involved in the regulation of daily rhythms in behavior, metabolism and transcription (Gaten et al. 2008, Teschke et al. 2011, De Pittá et al. 2013), but a putative clock involvement in the regulation of krill seasonal strategies has not yet been investigated.

During our simulation, the krill clock genes Es*clk*, Es*cry*2 and Es*tim* showed upregulation in autumn in LD, suggesting a specific link between clock-related activity and light regime at that time of the year. Interestingly, photoperiodic-dependent regulation of *tim*, *cry*2 and *clk* was found to play a major role in the induction of seasonal responses in the Drosophilid fly, *Chymomyza costata*, (Stehlík et al. 2008) and in the bean bug, *Riptortus pedestris*, (Ikeno et al. 2011, Ikeno et al. 2013), suggesting that a similar process may also take place in krill.

Since the light input to the photoperiodic clock might be transmitted by elements of the phototransduction cascade (Tamaki et al. 2013), we also monitored seasonal changes in the expression levels of the krill opsins Es*rh*1a, Es*rh*6 and Es*rrh*, which had previously shown rhythmic expression at the daily level (De Pittá et al 2013, Biscontin et al. 2016). Interestingly, Es*rh*1a and Es*rrh* showed LD autumn upregulation in a similar way to the clock genes, suggesting the presence of a signaling cascade linking the photoperiodic cue to the putative molecular timing mechanism.

Ecological implications for krill in the field

Due to the marked seasonal photoperiodic cycle at high-latitudes, regulation of seasonal phenology via photoperiodic time-measurement would probably represent the most intuitive timekeeping system for krill, allowing for reliable synchronization of major life-cycle events with the seasonal time course in the environment (Gwinner 2012). However, the seasonal photoperiodic signal might vary significantly across the broad latitudinal range of krill distribution (approx. from 51°S to 70°S), also considering that individual krill might be

capable of actively and/or passively travelling across many degrees of latitude and move among different habitats (open ocean, continental shelf, sea-ice marginal zone, deep ocean sea floor, etc..) within and between seasons (Thorpe et al. 2007; Reiss et al., 2017). This would suggest that a flexible timekeeping system would be preferable, allowing to adjust the seasonal responses depending on the local conditions (Visser et al. 2010, Helm et al. 2013). Schmidt et al. (2014) showed that winter food uptake (used as an indicator of overall metabolic activity) differed significantly between krill collected at similar latitudes (60-65°S) in different habitats in the Lazarev Sea (ice-covered and food-depleted deep oceanic area) and the Bransfield Strait (ice-free and productive shelf area). Since the samples had been collected under similar photoperiodic conditions, the authors suggested that additional environmental cues (e.g. small changes in light intensity or food "smell") were playing a major role in adjusting the seasonal response of krill to the local food conditions (Schmidt et al. 2014). We definitely agree that the complex interaction of many factors should be taken into consideration when trying to determine the mechanisms underlying the regulation of the seasonal phenology of krill in different ecological contexts. Nevertheless, we would suggest more caution in ruling out the causative role of photoperiod in this particular case, as due to thick sea-ice cover at the time of sampling the actual light conditions experienced by krill in the surface layers in the Lazarev Sea (where lower feeding was observed) might have been much poorer than those in the Bransfield Strait, which was free of sea-ice (Schmidt et al. 2014).

The influence of photoperiod might become less stringent when krill move towards lower latitudes, or alternatively the photoperiodic threshold (if present) might show a latitudinal cline, for example getting longer (in terms of hours of light) at higher latitudes (Helm et al. 2013). Seear et al. (2012) found no differences between the expression levels of genes involved in respiration and motor activity in krill collected during winter at the Antarctic Peninsula (62°S) and South Georgia (54°S), suggesting that both populations were in a similar state of quiescence despite the different seasonal photoperiodic cycle at the two locations. In the South Georgia region, where more favorable growth and feeding conditions were present, krill showed up-regulation of genes involved in feeding, digestion, and immunity, suggesting that different food conditions might have had an impact (Seear et al. 2012). A hierarchy of external signals might therefore interact with photoperiod to modulate

the seasonal life-cycle of krill, with food availability being most likely the major one among them. To understand the interplay between light and food conditions as environmental *Zeitgebers*, experiments have to be conducted combining different photoperiods and different food levels at the same time. To take into account the seasonal effect, such experiments should be repeated at different times of the year.

Two of the major krill feeding grounds in the Southern Ocean, the southwest Atlantic sector and the region of the West Antarctic Peninsula, are currently experiencing rapid anthropogenic-driven warming (Meredith & King 2005), and the abundance of krill in these areas has already been altered significantly as a result of changes in primary productivity associated with sea-ice decline (Atkinson et al. 2004). In this context, the implications for krill to entrain their seasonal responses through photoperiod might become problematic, as krill photoperiodic-driven metabolic cycle might have evolved to anticipate seasonal changes in phytoplankton blooms and optimize the match with food sources at the end of winter. Phytoplankton dynamics might be affected by climate change, delineating a "match-mismatch" scenario in which krill seasonal timing might need to be rescheduled, with potential negative effects on the krill population and on the rest of the ecosystem.

3.6. Conclusions

Our results supported the hypothesis that krill seasonal metabolic cycle was not regulated by changes in food availability, but rather by an endogenous timing system entrained by photoperiod instead. Endogenous regulation was observed in relation to krill growth, enzyme activity and gene expression. However, photoperiodic entrainment seemed to play a major role in the regulation of krill oxygen consumption. We hypothesize that specific light cues presented to krill during autumn might be necessary for the entrainment of the seasonal metabolic cycle. The light input might be collected by specific elements of krill phototransduction cascade and transmitted to an internal photoperiodic clock. The nature of such clock still remains elusive, but we suggest that elements of krill circadian clock might be involved.

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4. Publication III

Endogenous regulation of DVM in Antarctic krill (*Euphausia superba*) and its link with photoperiod and circadian clock activity

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4.1. Abstract

Diel vertical migration (DVM) in Antarctic krill seems to be promoted by an endogenous rhythm, but the underlying mechanism of regulation and the involvement of the light/dark (LD) cycle are still unclear. We monitored krill DVM in the laboratory in different LD and DD (constant darkness) conditions, using a custom-designed vertical tank together with an

infrared camera system. Since DVM might be associated with an endogenous rhythm of activity, we monitored also daily changes in oxygen consumption in krill exposed to similar LD and DD conditions. Moreover, to investigate the possible involvement of the circadian clock in the regulatory process, we measured daily patterns of clock genes activity in krill exposed to LD. In LD, krill displayed a clear DVM pattern, with high correlation with the light/dark cycle, which persisted also in DD. DVM showed a "reverse" phasing, with upward migration during the (subjective) light phase and downward migration during the dark phase, which might have been entrained by food cues prior to our experiment (from Feb 2013 until Nov 2016), since krill had been usually fed during the day, between 06:00 and 12:00. A similar LD-DD rhythm was found also for krill oxygen consumption, with higher rates during the (subjective) light phase and lower rates during the dark phase, suggesting that an endogenous rhythm of activity was promoting the observed DVM pattern. However, only few animals displayed extensive upward migration reaching the top of the tank, and approximately 30% of the investigated individuals displayed rhythmic oxygen consumption. Endogenous regulation of DVM might have been more robust within few rhythmic individuals, which might have influenced the other members of the group through social interactions. The krill clock genes Es*per*, Es*cyc*, Es*vri* and Es*dbt* displayed 24 h rhythmicity in the eyestalks of krill sampled in LD, with upregulation during the dark phase, suggesting that an involvement of the circadian clock in the regulation of the observed output rhythms (DVM and oxygen consumption) is plausible.

4.2. Introduction

Diel vertical migration (DVM) is a common rhythmic behavior typically found in numerous aquatic species around the world (Brierley 2014). In the most common DVM pattern, individuals migrate from the deeper layers to the surface around dusk (ascent) and sink back towards the deeper layers around dawn (descent). Other migratory patterns have also been described, such as the reverse pattern, where the animals migrate upward during the day and downward during the night, ant the twilight pattern, where the animals perform two consecutive migrations, one at sunset and another at sunrise (Lampert 1989). DVM

represents one of the largest mass-migratory movements on our Planet, and play a fundamental role in the shaping of marine and freshwater communities, as well as in the regulation of global-scale biogeochemical cycles, in particular in the sequestration process of N and C from the surface to the deeper layers of the oceans (Hays 2003).

It is generally agreed that the ultimate cause of DVM is to protect individuals from being eaten by visual predators in the surface layers during daytime (Lampert 1989). Due to the strong correlation between ascent/descent of animals and sunset/sunrise, changes in light conditions are considered to be the main proximate factor involved in the regulation of DVM (Ringelberg & van Gool 2003). Other proximate factors affecting DVM include food availability (Pearre 2003) and the presence/absence of predators (Bollens & Frost 1991, Neill 1990, Ohman et al. 1983). However, growing experimental evidence indicates that in order to fully understand DVM it is necessary to consider also the involvement of an underlying endogenous rhythm of activity, whose regulation is not yet fully understood (Cohen & Forward Jr 2005).

Antarctic krill, *Euphausia superba* (hereafter krill), are one of the dominant zooplankton species in the Southern Ocean (Ross et al. 1996). Due to their wide distribution and extremely high biomass, krill play a central role in the Antarctic food web and contribute significantly to the shape of the ecosystem (Quetin & Ross 1991). Field observations generally confirm the presence of DVM in krill (Godlewska & Klusek 1987), but the migratory pattern seems to be strongly affected by local factors like predator pressure and food availability (Cresswell et al. 2009, Zhou & Dorland 2004), and the question arises whether or not the changes in light conditions can be considered as the main proximate factor for DVM in krill.

With regard to this, Tarling & Johnson (2006) discussed the effect of stomach fullness on the swimming activity of tethered krill and concluded that feeding activity would affect daily patterns of vertical migration and eventually contribute to the development of multiple DVM cycles over the 24 h cycle. Similarly, Gaten et al. (2008) discussed the effect of photoperiod (i.e. the day/night cycle) on the regulation of rhythmic vertical swimming activity in freshly caught krill and concluded that changes in light conditions may not represent the main *Zeitgeber* for krill DVM, but they did not exclude the possibility that an endogenous timing

system was involved. However, both studies monitored the swimming activity of isolated single individuals, which in a highly social animal like krill may represent a strong constrain to the development of natural patterns of behavior (Hamner & Hamner, 2000).

Recent investigations on DVM in another high-latitude zooplankter from the Arctic, the calanoid copepod *Chalanus finmarchichus*, showed that DVM was regulated by an endogenous rhythm which persisted also in constant darkness (DD) (Häfker et al. 2017). In association with this, the copepods showed comparable endogenous rhythms in oxygen consumption and clock gene expression, strongly suggesting the presence of a link between the activity of the circadian clock, the daily rhythm of activity and DVM. The circadian clock is a molecular oscillator based on the rhythmic expression of clock genes, which can be synchronized by the day/night cycle and promote daily output rhythms in behavior and physiology (Dunlap 1999). In Antarctic krill, clock genes have been isolated and characterized (Biscontin et al. 2017, Mazzotta et al. 2010), and it has been suggested that the activity of the clock might be implicated in the regulation of daily rhythms of metabolic activity (Teschke et al. 2011) and transcription (De Pittà et al. 2013).

In this study, we investigated the effect of photoperiod and the involvement of the circadian clock in the regulation of DVM in small groups of Antarctic krill ($n \approx 40$) exposed to light/dark (LD) and constant darkness (DD) conditions in the laboratory. Moreover, we monitored daily rhythms of oxygen consumption in individual krill, which had been entrained to similar LD and DD conditions. Finally, we examined daily patterns of clock gene expression in krill entrained to LD. The objectives were 1) to verify if krill DVM was regulated by and endogenous rhythm of activity; 2) to investigate the entraining role of photoperiod; and 3) to examine the possible involvement of the circadian clock in the regulation of DVM.

4.3. Materials and methods

Experimental krill

All animal work has been conducted according to relevant national and international guidelines. Krill catches, welfare and experimentation were based on permission of the Department of Environment and Heritage (DEH) of the Australian Government and were conducted in accordance with the Antarctic Marine Living Resources Conservation Act 1981 (AMLR, permit number: 06_09_2220) and the Environment Protection And Biodiversity Conservation Act 1999 (EPB, permit number: WT2007-1480).

Collection of krill in the field. Antarctic krill (*Euphausia superba*) were collected in East Antarctica (66°47'S, 65°08'E) (Feb 12, 2013 at 17:43 UTC) in the upper 30 m of the water column using a Rectangular Midwater Trawl (RMT 8) during voyage V3 12/13 of RSV *Aurora Australis*. Upon collection, all krill were immediately transferred to 200 l tanks located in a constant-temperature room at 0°C and supplied with a continuous flow of chilled seawater. The animals were kept in conditions of dim light and in the absence of food, and dead animals and molts were removed daily from the tanks. After arrival in Hobart, Tasmania (Feb 22, 2013), krill were transported from the ship to the krill research aquarium at the Australian Antarctic Division (AAD) in Kingston and transferred to a 1670 l holding tank connected to a 8000 l chilled seawater recirculation system.

Maintenance in the laboratory. In the aquarium, the temperature of seawater in the holding tank was maintained constantly at 0.5°C. Water was continuously recirculated through an array of mechanical and biological filters and constantly monitored for quality following King et al. (2003). Light in the aquarium was provided by fluorescent tubes covered with a gel filter simulating light attenuation at 30 m depth in natural conditions (ARRI, Marine Blue 131). A PC-controlled timer and dimming system (winDIM v4.0e, EEE, Portugal) ensured a light regime similar to that occurring in the Southern Ocean at 66° latitude south (66°S) and 30 m depth. A sinusoidal annual cycle with monthly variations of photoperiod and daily variation of light intensity was calculated by assuming continuous light and a maximum light intensity of 100 lux at the surface of the tank (equal to 1% light penetration to 30 m depth)

during summer midday (December at 66°S). The system was adjusted every month to reflect Southern Ocean conditions.

Krill were fed daily with a mixture of living algae at the final concentration of 1.5 x 10^4 cells ml^{-1} of the pennate diatom *Phaeodactylum tricornutum*, 2.2 x 10^4 cells ml^{-1} of the chlorophyte *Pyramimonas gelidicola* and 2 x 10^4 cells ml^{-1} of the cryptophyte *Geminigera cryophila*. Instant algae were added to yield final concentration of 1 x 10^4 cells ml^{-1} of *Thalassiosira weissfloggii* (1200TM, CCMP1051/TWsp., Reed Mariculture, USA), 5.1 x 10^4 cells ml^{-1} *Isochrysis sp.* (1800TM, Reed Mariculture, USA) and 4.8 x 10^4 cells ml^{-1} *Pavlova sp.* (1800TM, Reed Mariculture, USA). Krill also received 2 g per tank per day of nutritional supplements (1 g of Frippak #1 CAR, 1 g of Frippak #2 CAR, INVE, Thailand). After the phytoplankton mix was added, water flow in the tank was shut off for 2 h to enable krill to feed on the food mixture. This feeding regime has been used successfully in several experiments trials at the AAD prior to this study, and it guarantees good condition of krill in long-term laboratory experiments with low mortality and high feeding rates (Kawaguchi et al. 2010, King et al. 2003). Animals were fed *ad libitum*, thus keeping feeding conditions always at its optimum. Dead individuals and molts were removed from the tank on a daily basis.

Experimental design

All experiments were performed at the AAD krill research aquarium during November 2016. At that time of the year, the photoperiod in the aquarium was regulated to simulate October light regime at 66°S in the Southern Ocean, with a light/dark cycle of 12 h light:12 h darkness (LD 12:12). Lights were switched on at 06:00 and off at 18:00, reaching the midday maximum light intensity of 46 Lux measured at the surface of the holding tank. The same photoperiodic cycle (LD 12:12) was applied to determine the influence of light-regime on daily rhythms of krill DVM, oxygen consumption and clock gene expression, and will be hereafter denoted as LD. In addition, to verify the presence of endogenous regulation, we repeated the observations when krill was exposed to constant darkness, a condition which will hereafter be denoted as DD.

Vertical migration measurements. To investigate the presence of circadian rhythms in krill DVM, we performed two experimental runs. In run 1 we monitored for 48 h the vertical swimming activity of a group of krill (n = 45) in LD, while in run 2 we monitored for 72 h the vertical swimming activity of another group of krill (n = 41) exposed to LD (for the first 24 h) and DD (for the following 48 h). For each run, adult krill of mixed sexes were randomly collected from the holding tank and transferred into the experimental tank for DVM monitoring. Before starting, we allowed krill to adapt to the new tank conditions for three days. From the moment when the krill were transferred into the experimental tank until the end of each run, no food was offered. At the end of each run, krill were removed from the experimental tank, transferred into a temporary holding tank and finally put back into the main holding tank with the rest of the population. No krill died during the experimental runs.

The DVM experimental tank was a transparent acrylic cylindrical tank (200 cm height x 50 cm diameter) filled with chilled and filtered seawater connected to the recirculating water system of the aquarium (Fig. 4.1). The cylinder was placed inside a square tank (198 cm height x 55 cm length x 55 cm depth), which was also filled with chilled and filtered seawater connected to the recirculating water system. This provided thermal isolation of the experimental tank from the rest of the aquarium, with a constant inner temperature of 0.5 ± 0.1°C and no vertical temperature gradients. The whole system (cylinder and square tank) was located in a separated lightproof compartment of the aquarium, with complete control over light conditions. Light inside the compartment was provided by fluorescent tubes and filters as already described for the rest of the aquarium. The light regime was controlled using a separate channel of the same PC-controlled system as mentioned for the rest of the aquarium.

To monitor krill DVM, the experimental tank was divided into five sectors of 40 cm height (Fig. 4.1). Each sector was monitored by a separate camera (SJ4000; SJCAM®, Shenzhen Hongfeng Century Technology LTD, Shenzhen, China) with infrared (IR) filter removed. Light for video capture during dark phases was provided by six IR illuminators (Camera2000 Limited, emitted wavelength: 850 nm), placed at increasing heights, three on each side of the tank, perpendicularly to the direction of the cameras to avoid backlight effects. To attenuate the residual dim red glowing produced by the illuminators, we shaded them with acrylic

filters (LUXACRYL®-IR black 1698; ttv GmbH, Illertissen, Germany). To avoid differential exposition to IR during light and dark phases, the IR illuminators were let on at all times during the experiment. To avoid condensation of water vapor on the surface of the tank, we used continuous directed jets of compressed extra-dry air. Videos were recorded at 7 frames/second using the Multi2 software (Computer System Department, University of Murcia). Due to krill transparency and poor IR penetration into the water column, automated tracking software failed to detect krill swimming in the background. Therefore, the determination of DVM was performed manually by two independent observers, who counted the number of krill present in the different height sectors at regular time-intervals (30 min).

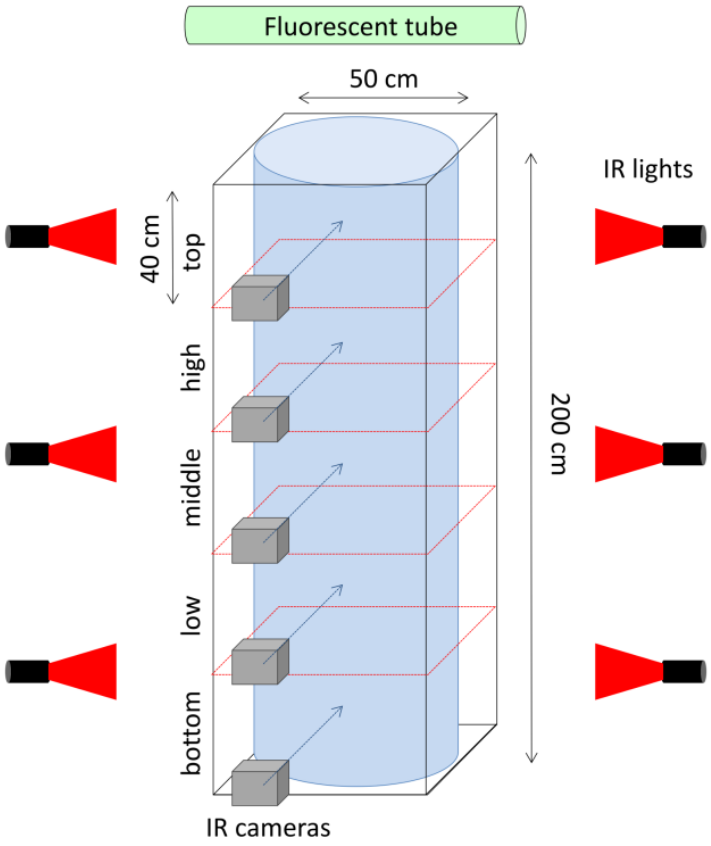

Figure 4.1: Experimental setup used for the determination of krill DVM. In light blue, the inner cylinder where the krill were free to swim. The cylinder was placed into a squared "water jacket", represented as a transparent frame. Both tanks were filled with chilled and filtered seawater, connected to the circulating water facility of the aquarium. Temperature inside the cylinder was maintained stable around 0.5 °C. Five IR cameras were placed at regular height intervals, covering the entire height of the cylinder. Perpendicular to the cameras, six IR illuminators (three on each side) provided infrared light to film during dark phases. Light during light phases was provided by a fluorescent tube placed above the tank.

To estimate mean krill depth at each time interval, we further sub-divided each sector into three height steps, for a total of 15 increasing height steps (total column height/n° of steps = 200 cm /15 steps = 13.33 cm/step) and we assigned to every krill found within the same height step the corresponding individual height (step n° x 13.33 cm), which was finally converted to individual depth (200 cm – individual height level). To test for the presence of 24 h patterns in mean krill depth we used the R package "rain" (Thaben & Westermark 2014). The package takes the mean krill depth estimated at each consecutive time-interval and fits it to a sinusoidal or sawtooth wave having the requested period (in our case, 24 h), giving a p-value indicating the likelihood of the fit (significant 24 h oscillations were detected when $p \leq 0.05$). We repeated the test using the krill count data produced by each of the two independent observers, and we obtained the same results.

Oxygen consumption measurements. To investigate the relationship between krill DVM and krill activity rhythms, we examined daily patterns of oxygen consumption in individual krill exposed for 48 h to LD (run 1) and DD (run 2) conditions respectively. For each run, 7 krill were used, which had been starved for about 6 h prior the incubation to avoid interaction with digestive processes. Each animal was incubated separately in a 2 l Schott glass bottle filled with oxygen-saturated chilled and filtered (0.2 µm) seawater. Three bottles filled with filtered seawater but without animals served as control. The bottles were sealed and placed into the holding tank, ensuring constant temperature (0.5°C) throughout the experiment. In run 2, to provide DD conditions the bottles were wrapped into black lightproof plastic bags. Decrease in oxygen saturation was monitored in parallel using a 10-channel fiber optic oxygen transmitter (Oxy-10 Mini, PreSens®, Germany) in combination with type PSt3 sensors (limit of detection 0.03% of oxygen saturation, corresponding to 15 ppb, PreSens®, Germany) and the Oxy-10 software (PreSens®, Germany). Calibration of the system was achieved by bubbling nitrogen (for the 0% oxygen reference value) and air (for the 100% oxygen reference value) for 20 minutes into each Schott bottle. Oxygen saturation was recorded every 15 minutes. At each time-interval, the values obtained from each bottle with krill were normalized against the mean values obtained from the control bottles.

Due to krill oxygen consumption, oxygen saturation showed a general decreasing trend over time. In order to examine the presence of daily fluctuations in the individual oxygen

consumption rates, we first applied a linear fit assuming a constant consumption rate, and then checked the presence of 24 h oscillations in the model residuals. To apply the linear model, we used the *lm* function in R (RStudio version 1.0.136, RStudio Team 2016), and to extract the residuals we used the *residuals* function. To check the presence of a temporal pattern in the distribution of the residuals, we applied a generalized linear model (GAM) using the *gam* function in the "mgcv" R package (Wood 2010). When the GAM displayed significant changes in oxygen consumption over time ($p \leq 0.05$), we used the "rain" package (Thaben & Westermark 2014) to test the presence of 24 h oscillations as described for DVM.

Clock gene expression. To investigate the possible involvement of the circadian clock in the regulation of krill DVM, we analyzed daily changes in the expression levels of 10 clock genes (Tab. A3.1), which have been recently isolated and characterized in krill (Biscontin et al. 2017). The selected genes included the krill core clock genes *clock* (Es*clk*), *cycle* (Es*cyc*), *period* (Es*per*), *timeless* (Es*tim*) and *cryptochrome*2 (Es*cry*2), which are involved in the principal circadian feedback loop, and showed rhythmic 24 h patterns of expression in different light conditions in the field (Biscontin et al. 2017, Mazzotta et al. 2010) and in the laboratory (Teschke et al. 2011). Moreover, we also included the krill clock genes *clockwork orange* (Es*cwo*) and *vrille* (Es*vri*), which are involved in the secondary feedback loops in *Drosophila* (Allada & Chung 2010), *doubletime* (Es*dbt*) and *shaggy* (Es*sgg*), which code for protein kinases involved in the phosphorylation of *period* and *timeless* in *Drosophila* (Mackey 2007), and the *nuclear receptor* E75 (Es*e*75), which codes for a nuclear receptor which has been involved in the repression of *clock* and in the regulation of the circadian output in *Drosophila* (Kumar et al. 2014).

To measure clock genes expression, 200 adult krill of mixed sexes were sampled randomly from the holding population and separated into three 200 l tanks filled with chilled and filtered seawater and connected to the recirculating water facility of the aquarium (water temperature = 0.5°C). Each tank was located into a separate lightproof compartment, ensuring complete light isolation from the rest of the aquarium. Inside each compartment, light was provided by fluorescent tubes as described for the rest of the aquarium and controlled through separate channels of the same PC-controlled system. The light/dark cycle was set to LD 12:12, with lights switched on at 06:00 and off at 18:00, and a maximum

intensity of approx. 50 Lux at the surface of the tank at midday. After the krill were transferred into the tanks, we gave them one week of acclimation to the new tank conditions and we stopped feeding them to avoid entraining cues related to food. At the end of the acclimation period, we started our time-series sampling, which lasted 80 h (Fig. 4.2). We started on day 1 at 02:00, and we sampled 3 krill from each tank every 4 h until 10:00 on day 4. During day 1, all tanks were exposed to LD, while during day 2, 3 and 4 they were exposed to DD. Sampling during dark periods was conducted under dim red light.

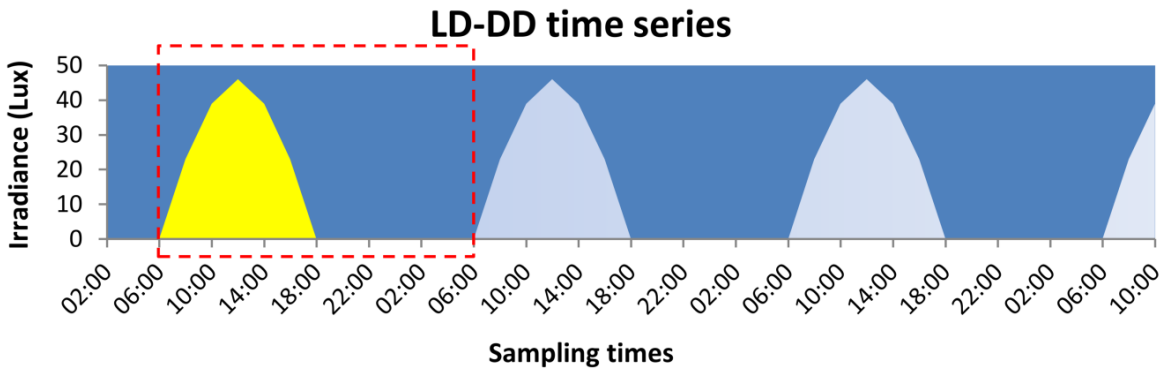

Figure 4.2: Schematic representation of the light-regime during collection of samples for clock genes expression measurements. During day 1 (highlighted in the red box), the animals were exposed to LD conditions, with lights-on at 06:00 and lights-off at 18:00, and a maximum light intensity of 46 Lux at midday. During day 2 and day 3, the animals were exposed to constant darkness. The light phase in day 1 is represented in yellow, the subjective light phases in day 2 and 3 are represented in light blue, and the dark phases are represented in blue.

Following the anatomical localization of the major circadian pacemaker centers in a generic crustacean proposed by Strauss & Dircksen (2010), we analyzed clock gene expression in two different target tissues: the eyestalks and the brain (Fig. 4.3A; retinae were not included because due to pigment contamination we did not manage to extract clean RNA from them). Immediately after sampling, we separated the heads from the rest of the bodies, using a chilled petri dish and cutting in a skewed angle directly behind the eyes to avoid contamination with stomach tissue (Fig. 4.3B). The heads were immediately placed in RNA later and incubated for 24 h at 4°C. After that, each head was removed from the RNA later and brain and eyestalks were dissected. All unnecessary surrounding tissue was removed, leaving only the eyes connected through the eyestalks to the brain (Fig. 4.3C). Eye retinae were removed cutting at the transition between dark-pigmented and white tissue (Fig.

4.3C,D). Eyestalks were separated from the brain cutting them at the root (Fig. 4.3D), and the brain was cleaned from leftover tissue and chitin. Eyestalks and brain were immediately and separately placed in fresh RNA later, incubated for 24 h at 4° C and then frozen at -80° C until further analyses.

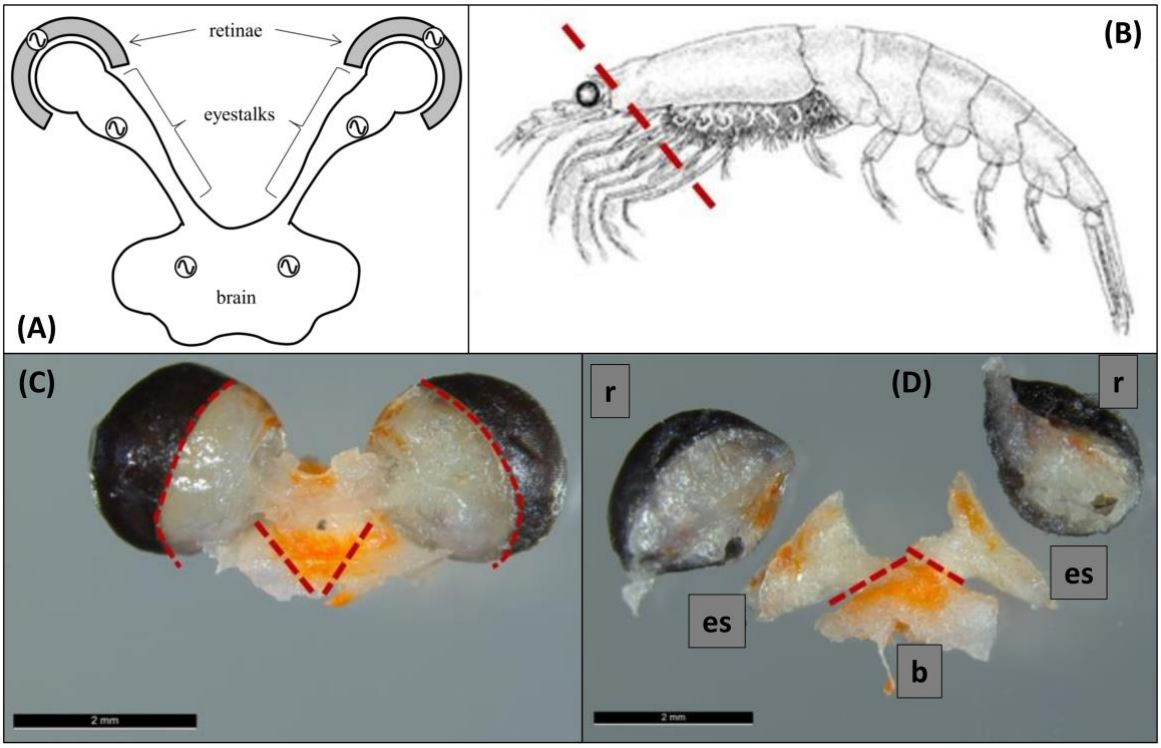

Figure 4.3: (A) Schematic representation of the putative localization of circadian oscillator centers in the head of a generic crustacean, modified after Strauss & Dircksen (2010). Putative pacemaker centers are located in the retinae of the compound eyes, in the eyestalks and in the brain. (B) Drawing of krill, side view, with indication of the line of dissection used to separate the head from the rest of the body. (C) Detail of krill brain-eyestalk-eyes complex, with indication of dissection lines used to separate the retinae from the eyestalks, and to remove leftover tissue around the brain. (D) Detail of krill brain-eyestalk-eyes complex, with retinae (r) separated from the eyestalks (es), and indication of dissection line used to separate the eyestalks from the brain (b).

RNA was extracted from individual eyestalks and brains using phenol-chloroform phase separation followed by in-column RNA purification using the direct-Zol™ RNA MircoPrep kit (Zymo Research, USA). Tissue homogenization was conducted using a PrecellysR 24 homogenizer (Bertin Technologies, France) connected to a Cryolys cooling element to keep temperature at 4°C. Homogenates were treated with phenol-chloroform (Sigma-Aldrich, USA) for phase separation. The upper aqueous phase containing the RNA was mixed with an

equal volume of 100% molecular biology grade ethanol and processed with the direct-Zol™ RNA MicroPrep kit (Zymo Research, USA) as recommended by the manufacturer. To remove genomic DNA contamination, a DNase step was added during the purification procedure, as recommended by the manufacturer. Pure RNA was eluted in 15 µl of nuclease-free water. RNA concentration and purity were assessed using a Nanodrop™ 2000 Spectrophotometer (ThermoScientific, MA, USA), while RNA integrity was examined using an Agilent™ 2100 Bioanalyzer system (Agilent Technologies, CA, USA) and the RNA 6000 Nano Kit (Agilent Technology) according to manufacturer's instructions.

Due to high analytical costs and time limitations, only seven time-points (from 06:00 on day 1 to 06:00 on day 2; see red box in Fig. 4.2), which had been collected under LD conditions, were finally retro-transcribed to cDNA and used in the gene expression analysis. Moreover, due to RNA extraction failures (too less RNA was obtained), 13 brain samples and 8 eyestalks samples were not included in the analysis, giving the following final sample sizes: A) for brain: time-point 1, 06:00, n = 7; time-point 2, 10:00, n = 6; time-point 3, 14:00, n = 8; time-point 4, 18:00, n = 7; time-point 5, 22:00, n = 8; time-point 6, 02:00, n = 8; time-point 7, 06:00 (day 2), n = 8 (total n = 50); B) for eyestalks: time-point 1, 06:00, n = 7; time-point 2, 10:00, n = 6; time-point 3, 14:00, n = 8; time-point 4, 18:00, n = 8; time-point 5, 22:00, n = 9; time-point 6, 02:00, n = 9; time-point 7, 06:00 (day 2), n = 9 (total n = 55).

For each sample, 1 µg of total RNA was retro-transcribed to cDNA using the RevertAid H Minus Reverse Transcriptase kit from Invitrogen (ThermoScientific) to a final volume of 50 µl per sample (20 ng/µl). Gene expression was measured using custom-designed TaqMan® Low-Density Array Cards (ThermoScientific). Primers for qPCR analysis were designed around sequences of interest using the Custom TaqMan® Assay Design Tool (ThermoScientific). The primer sets for the clock genes were designed using the sequences published in Biscontin et al. (2017) (Tab. A3.1). For each sample, we used 20 µl of cDNA (corresponding to 400 ng of total RNA), 30 µl of RNase-free water and 50 µl of TaqMan® Gene Expression Master Mix (ThermoScientific), for a final volume of 100 µl. The cards were analyzed using a ViiA™ 7 Real-Time PCR System (ThermoScientific). Standard curves were conducted to verify the efficiency of each primer pair on the card.

For the normalization of the expression levels of the target clock genes, we used a combination of internal (endogenous) and external (exogenous) controls. The internal control was represented by the housekeeper gene *ubiquitin carboxyl-terminal hydrolase* 46 (*usp*46), which had been used before in similar clock gene expression studies with krill exposed to similar LD conditions and showed no significant oscillations over the 24 h cycle. The primers and probes for *usp*46 were designed as described for the clock genes around the sequence of interest which can be found online at http://krilldb.bio.unipd.it (Sales et al. 2017) (Tab. A3.1). The external control was represented by two exogenous target sequences (hereafter called spike20 and spike25) which had been selected before from a human transcript plasmid library and used for the determination of clock gene expression levels in krill caught during summer in Antarctica (Biscontin et al. 2017). For each spike, 1.5 µg of DNA were transcribed to RNA using the MAXIscript™ T3 Transcription Kit (Thermo Fisher Scientific, USA). After transcription, residual DNA was removed adding 1 µl of TURBO DNase™ and incubating for 15 min at 37°C. The transcripts were further purified with the RNA Clean & Concentrator™-5 kit (Zymo Research, Irvine, USA) and finally eluted in 10 µl of nuclease-free water. The concentration and purity of the spikes were measured using the Nanodrop™ 2000 Spectrophotometer (ThermoScientific, MA, USA) and the integrity was checked with the Agilent™ Bioanalyzer 2100 (Agilent Technologies, CA, USA) and the RNA 6000 Nano Kit (Agilent Technology). The purified spikes were added to each sample at a constant concentration (10 pg) before cDNA synthesis. The primers and probes for spike20 and spike25 were designed as described for the other genes around the sequence of interest (Tab. A3.1).

The levels of transcription of the target genes were normalized and quantified using the modified $2^{-\Delta\Delta Ct}$ method proposed by Hellemans et al. (2007). This method represents an improvement of the classic $2^{-\Delta\Delta Ct}$ method, as it takes into account gene-specific amplification efficiencies and allows for combination of multiple reference genes. As reference, we used a combination of *usp*46 (internal) and spike20 (external), which had showed more stable results compared to spike25. Normalized relative quantities (NRQs) were calculated by using as a baseline the sample showing the lowest Cq (quantification cycle) value among all time-points and between both tissues. The presence of 24 h oscillations in the mean expression levels of the target clock genes was finally tested using the R package “rain” (Thaben &

Westermark 2014) in a similar way as described for DVM and oxygen consumption. Moreover, when the expression levels between brain and eyestalks appeared to be significantly different (at a specific time-point), we compared them using the non-parametric Mann-Whitney-Wilcoxon t-test implemented in R.

4.4. Results

Vertical migration

LD conditions. In run 1, we monitored vertical migration of a group of 45 individual krill exposed to LD conditions over 48 h. In this experimental run, a clear 24 h rhythm in the vertical distribution of krill across the different height sectors of the experimental tank was observed. At 06:00, when the light phase started (Fig. 4.4A,B; ZT = 0; ZT: Zeitgeber time), most of the animals (78%) were lying on the bottom of the tank (‘floor’ sector) in an apparently inactive state. As light intensity increased, an increasing number of krill left the bottom and actively migrated to the upper sectors. The peak of upward migration happened shortly after the midday maximum of light intensity (ZT 6), when most krill (67%) had left the bottom and were distributed across all height sectors of the tank. Thereafter, krill started gradually to descent back towards the lower sectors (ZT 6–12), and shortly after the lights were off (ZT 12) most of the animals were back at the bottom of the column. The peak of downward migration happened shortly before the middle of the dark phase (ZT 18), when almost all krill were lying on the bottom (‘floor’ sector) in an apparently inactive state. A similar pattern of DVM was observed also in day 2. The rhythmic pattern of krill vertical distribution in the different height sectors was confirmed statistically by the clear 24 h oscillation detected in mean krill depth using the “rain” algorithm (Fig 4.4B, $p < 0.001$). In both days, mean krill depth was shallower during the second half of the light phases, after midday light intensity maximum (ZT 6–12 and ZT 30–36), while it was deeper during the second half of the dark phases (ZT 18–24 and ZT 42–48).

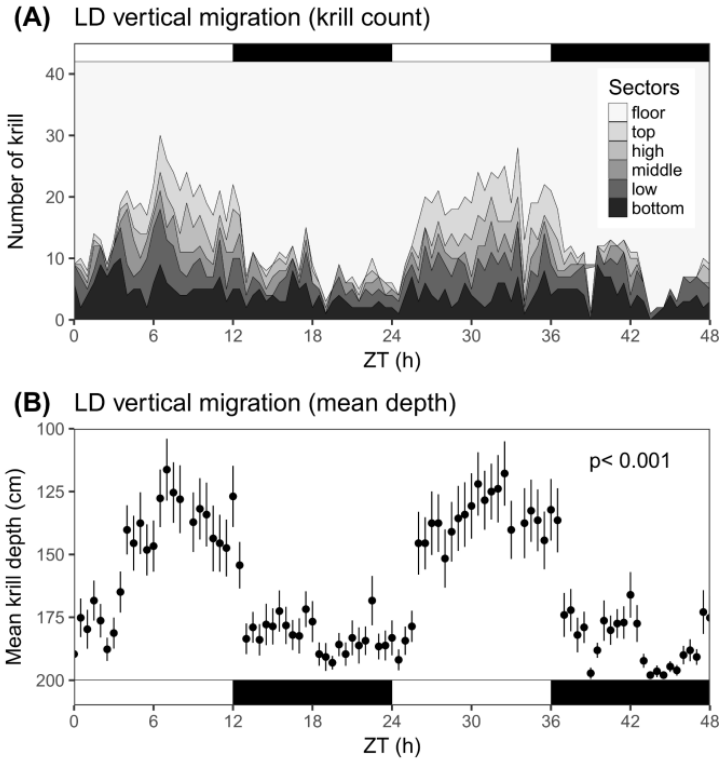

Figure 4.4: Krill DVM patterns in LD conditions. (A) Staked areas graph reporting changes in number of krill at the different time intervals in the different height sectors of the tank. A schematic representation of photoperiod is given above the graph, where white rectangles represent light phases, and black rectangles represent dark phases. ZT = Zeitgeber Time, measuring the time intervals (h) from the beginning of the first light phase (ZT = 0, corresponding to 06:00). (B) Dot chart representing changes in mean krill depth over time. Each point represents the mean of the estimated depths of 45 individual krill (n = 45), with associated error bars representing the standard error of the mean (SEM). The results of the *rain* test are reported ($p < 0.001$), indicating significant 24 h rhythmicity. A schematic representation of photoperiod is given below the chart, where white rectangles represent light phases, and black rectangles represent dark phases. ZT = Zeitgeber Time, measuring the time intervals (h) from the beginning of the first light phase (ZT = 0, corresponding to 06:00).

LD and DD conditions. In run 2, a second group of krill (n = 41) was monitored for 72 h. During the first 24 h the experimental tank was exposed to LD conditions, while during the following 48 h it was exposed to DD (Fig. 4.5A,B). During day 1 (LD), we observed a similar DVM pattern as described for LD conditions (outlined above), with upward migration during the light phase and downward migration during the dark phase. In day 2 (first day in DD), upward migration started during the subjective light phase and continued until the first half of the dark phase (ZT 24–42). After that, krill migrated back towards the bottom and remained there until the beginning of the next subjective light phase (ZT 48).

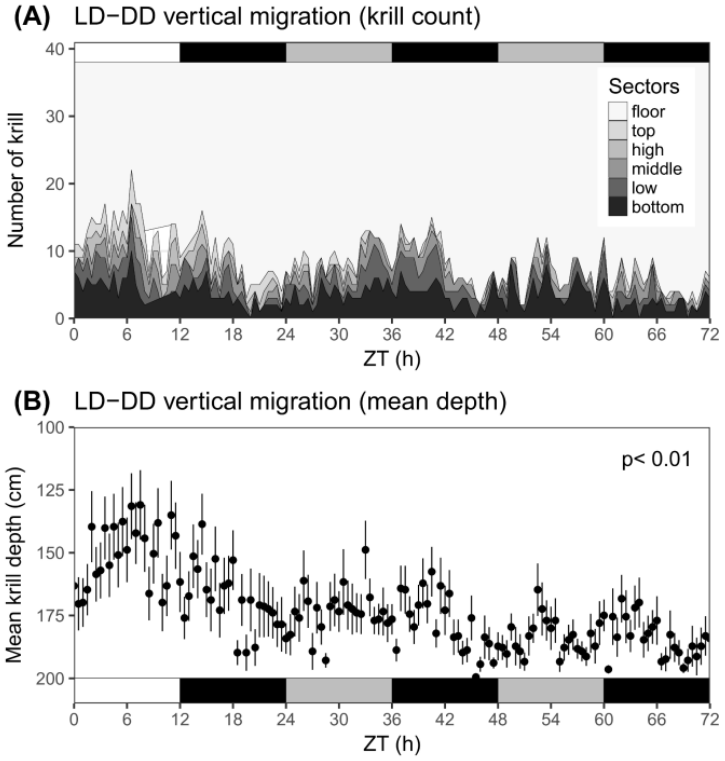

Figure 4.5: Krill DVM patterns in LD-DD conditions. (A) Staked areas graph reporting changes in number of krill at the different time intervals in the different height sectors of the tank. A schematic representation of photoperiod is given above the graph, where white rectangles represent light phases, grey rectangles represent subjective light phases, and black rectangles represent dark phases. ZT = Zeitgeber Time, measuring the time intervals (h) from the beginning of the initial light phase (ZT = 0, corresponding to 06:00). (B) Dot chart representing changes in mean krill depth over time. Each point represents the mean of the estimated depth of 41 individual krill (n = 41), with associated error bars representing the standard error of the mean (SEM). The results of the *rain* test are reported ($p < 0.01$), indicating significant 24 h rhythmicity. A schematic representation of photoperiod is given below the chart, where white rectangles represent light phases, grey rectangles represent subjective light phases and black rectangles represent dark phases. ZT = Zeitgeber Time, measuring the time intervals (h) from the beginning of the initial light phase (ZT = 0, corresponding to 06:00).

During day 3, no krill reached the top of the tank. However, krill were more active in the lower sectors during the subjective light phase, in particular during the second half (ZT 54–60), while they were less active during the dark phase, in particular during the second half (ZT 66–72). Temporal changes in krill mean depth (Fig. 4.5B) displayed significant 24 h oscillation over the entire experimental run (LD-DD) ($p < 0.01$), even if DVM was generally attenuated during day 2 and 3 (DD) compared to day 1 (LD), and during day 3 (2^{nd} day in DD) compared to day 2 (1^{st} day in DD).

Oxygen consumption

LD conditions. In run 1, we monitored oxygen consumption of 7 individual krill exposed to LD conditions for 48 h (Fig. 4.6). Due to a technical failure associated with one channel of the Oxy-10 mini oxygen detector, one individual was removed from the analysis (channel 2). Of the remaining 6 individual krill, two of them displayed significant 24 h oscillation of oxygen consumption (channels 5 and 7, $p < 0.001$).

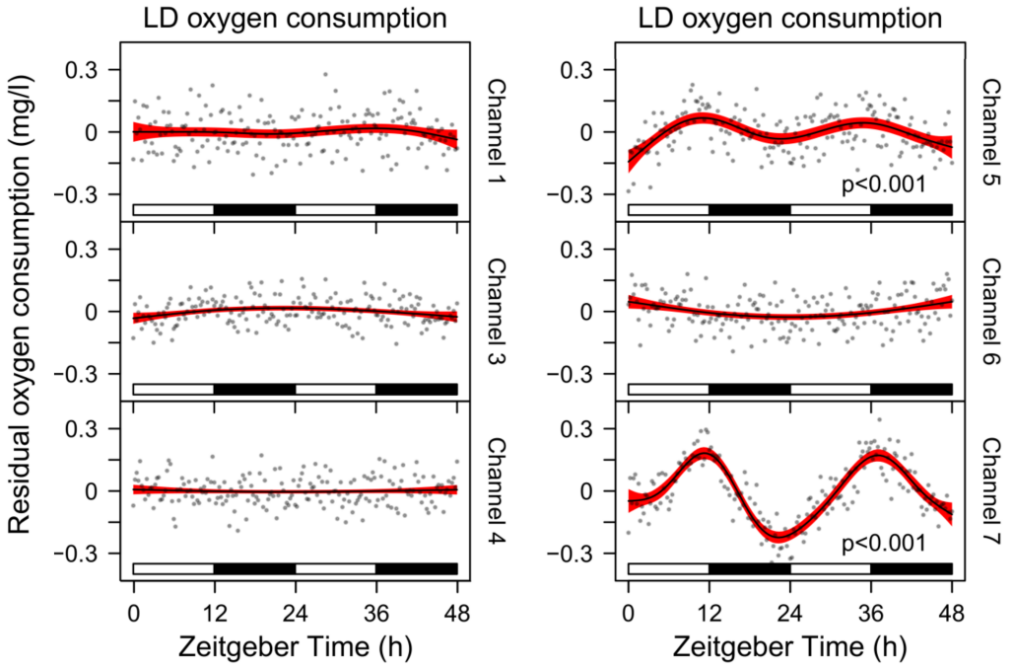

Figure 4.6: Daily patterns of oxygen consumption in individual krill exposed to LD conditions. Each channel corresponds to one animal. Please note that due to technical failure we removed channel 2 from the analysis. For each channel, the black solid line represents the model fit (GAM) calculated on the residuals of the linear model (LM). Red-shaded areas represent the confidence interval around the model fit (GAM). Grey dots represent the residuals (the differences calculated between observed and predicted values at each time interval) of the linear model (LM) used to feed the GAM. Below each graph, a schematic representation of the light/dark cycle is reported, with white rectangles representing light phases, and black rectangles representing dark phases. Significant 24 h rhythmicity ($p < 0.001$), is reported below the corresponding graph for channel 5 and channel 7. Zeitgeber Time: time intervals (h) from the beginning of the initial light phase (ZT = 0, corresponding to 06:00).

Both individuals showed the same pattern, with oxygen consumption increasing during the light phases (ZT 0–12 and ZT 24–36) and decreasing during the dark phases (ZT 12–24 and ZT 36–48). Oxygen consumption was highest at the transition between light and dark phases (ZT 12 and ZT 36), while it was lowest at the transition between dark and light phases (ZT 0,

ZT 24 and ZT 48). The remaining 4 individual krill did not show significant 24 h oscillations, and oxygen consumption appeared to be more or less constant throughout the experimental run (channels 1, 3, 4 and 6).

DD conditions. In run 2, we monitored oxygen consumption of 7 individual krill exposed to DD conditions for 48 h (Fig. 4.7). Also in this case, only two individual krill displayed significant 24 h oscillations of oxygen consumption (channels 4 and 7, $p < 0.001$).

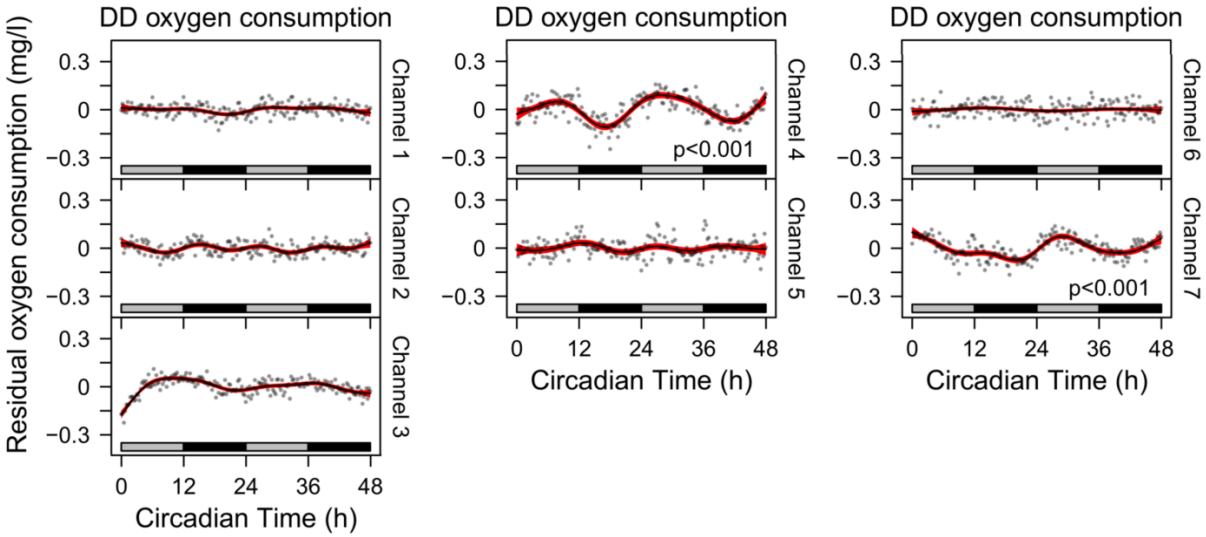

Figure 4.7: Daily patterns of oxygen consumption in individual krill exposed to DD conditions. Each channel corresponds to one animal. For each channel, black solid line represents the model fit (GAM) calculated on the residuals of the linear model (LM). Red-shaded areas represent the confidence interval around the model fit (GAM). Grey dots represent the residuals (the differences calculated between observed and predicted values at each time interval) of the linear model (LM) used to feed the GAM. Below each graph, a schematic representation of the light/dark cycle is reported, with grey rectangles representing subjective light phases, and black rectangles representing dark phases. Significant 24 h rhythmicity ($p < 0.001$), is reported below the corresponding graph for channel 4 and channel 7. Circadian Time: time intervals (h) from the beginning of the experiment in the absence of *Zeitgebers* (CT = 0, corresponding to 06:00).

In channel 4, oxygen consumption displayed a clear fluctuation, with higher values during the subjective light phases (ZT 0–12 and ZT 24–36) and lower values during the dark phases (ZT 12–24 and ZT 36–48). In channel 7, oxygen consumption decreased gradually during day 1 (ZT 0–24), then increased again at the beginning of day 2 (ZT 24) and displayed a similar oscillation like channel 4, with higher consumption during the subjective light phase (ZT 24–36) and lower consumption during the dark phase (ZT 36–48). In the remaining five channels (channels 1, 2, 3, 5, and 6) no significant 24 h oscillations were detected.

Clock gene expression

We measured daily patterns of gene expression of 10 clock genes (Tab. A3.1) in krill collected every 4 h during 24 h under LD conditions, starting at 06:00 in day 1 and ending at 06:00 in day 2, for a total of 7 time-points (Fig. 4.2, red box). The gene expression measurements were conducted using total RNA extracted from two different tissues, the eyestalks and the brain. In the eyestalks (Fig. 4.8A), upregulation of clock genes occurred mostly during the dark phase (ZT 16–24). The clock genes Es*per*, Es*cyc*, Es*vri* and Es*dbt* showed significant 24 h oscillations (Fig. 4.8C-F), with Es*per* peaking at ZT 16, Es*dbt* peaking at ZT 12, and Es*cyc* and Es*vri* peaking at ZT 20. Also in the brain (Fig. 4.8B) upregulation of clock genes occurred mostly during the dark phase, at ZT 16 and ZT 20. Es*tim* and Es*cry*2 showed a different pattern with upregulation during the light phase, at ZT 4. We did not detect any significant 24 h oscillations in the brain, but the expression profile of Es*per* was very similar to the one in the eyestalks (Fig. 4.8C), suggesting similar temporal regulation. The expression levels of Es*dbt* were consistently higher in the brain than in the eyestalks throughout the 24 h period ($p \leq 0.05$ for all time-points excluding ZT 12; Fig. 4.8F). Minor differences between the expression levels of Es*cyc* and Ess*gg* in the brain and eyestalks were detected (Fig. 4.8D,G). Es*cyc* was upregulated in the brain at ZT 0, ZT 8 and ZT 16 ($p \leq 0.05$), while Ess*gg* was upregulated in the eyestalks at the end of the dark phase, at ZT 20 and ZT 24 ($p \leq 0.05$).

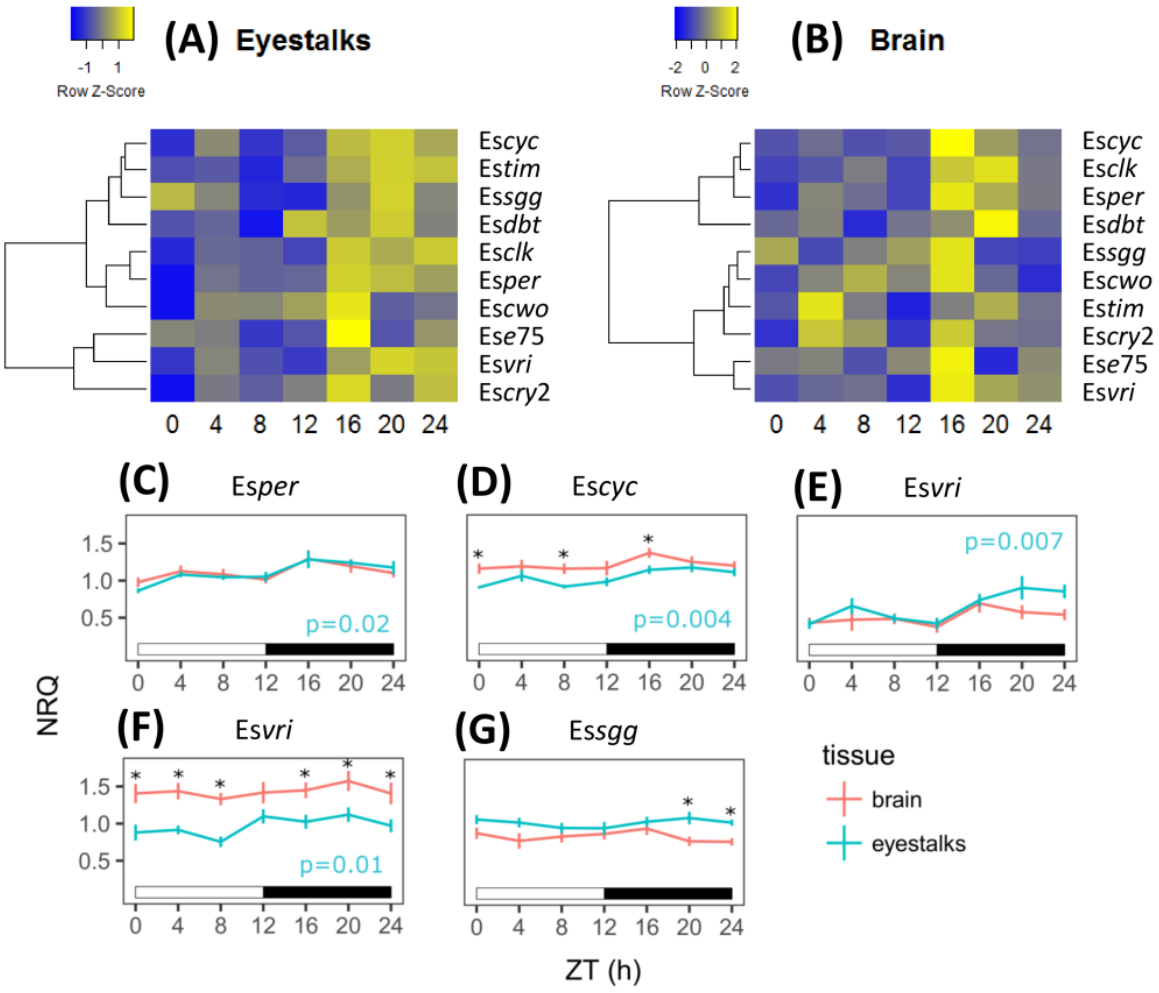

Figure 4.8: Daily patterns of clock genes expression in krill exposed to LD conditions. (A,B) Heatmaps representing daily patterns of up (yellow) and down (blue) regulation of clock genes in krill eyestalks (A) and brain (B) over the 24 h cycle. Dendrograms are based on Euclidean distances. (C-G) Line-graphs representing daily profiles of expression in the eyestalks (blue) and in the brain (red) of krill, for those genes showing significant 24 h rhythmicity (C-F), and/or significant differences between tissue at one or more specific time-points (D,F,G). P-values are reported for those genes showing significant 24 h rhythmicity in the eyestalks. Asterisks indicate time-points where significant ($p < 0.05$) differences were detected in the levels of expression between eyestalks and brain. ZT = Zeitgeber Time, measuring the time intervals (h) from the beginning of the light phase (ZT = 0, corresponding to 06:00). NRQ = Normalized Relative Quantity, representing the level of target genes expression relative to the level of expression of the reference used for normalization.

4.5. Discussion

Endogenous regulation of DVM in krill and its link to the light/dark cycle

In a previous study on freshly caught krill, Gaten et al. (2008) showed that krill DVM did not display high correlation with the light/dark cycle, suggesting that an endogenous timing system entrained by food cues and/or by social interactions might play a major role. However, the authors tested DVM in individual krill and could not verify the implications of group dynamics (Gaten et al. 2008). Here, we examined krill DVM in a different setup, with small groups of krill (n = 45 and 41) monitored together within the same experimental tank for over 48 h in LD (run 1) and 72 h in LD-DD (run 2). During run 1, in LD conditions (12 h light: 12 h darkness; lights-on at 06:00, lights-off at 18:00), krill showed a clear pattern of vertical migration according to the dominant light/dark cycle, being shallower during the light phase and deeper during the dark phase. During the experimental runs, no other *Zeitgeber* related to food or temperature was introduced, and light-regime was the only external factor which could have been related to the rhythmic patterns. When we exposed krill to DD conditions (run 2), the rhythmic vertical migration pattern was still detected, suggesting that it was being regulated by an endogenous rhythm. In DD, the amplitude of the oscillation was attenuated and the period was slightly phase-shifted, suggesting that the LD cycle was playing a major role in the stimulation and synchronization of the endogenous DVM rhythm.

The different results obtained by Gaten et al. (2008) might be related to the different experimental setup. In fact, the authors monitored vertical swimming activity of isolated individual krill, which might have affected natural rhythmic behavior in a highly social species like krill (Hamner & Hamner 2000). Another explanation could be related to the different entraining conditions of the experimental krill. In fact, we used laboratory krill which had been entrained to simulated late-spring (October) Southern Ocean light conditions at 66°S, while Gaten et al. (2008) used fresh krill collected during summer (January and February) at two different locations at different latitudes (around 60°S and 52°S respectively), where krill might have been exposed to different light-regimes and different overall environmental conditions. Heterogeneous entrainment of krill coming from different

areas might have contributed to the confounding of the results, which might have been already affected by high variability due to the individual-based monitoring setup. Moreover, seasonal differences have been observed in krill DVM in the field (Quetin & Ross 1991, Siegel 2005), which might have interfered with the measurements of Gaten et al. (2008). During summer, krill DVM might get de-synchronized at the population level with individual krill performing multiple unsynchronized migrations over the 24 h cycle, as it has already been hypothesized for other high-latitude zooplankton species in the Arctic (Cottier et al. 2006). The rhythmic individual swimming activity with periods shorter than 12 h observed by Gaten et al. (2008) would be in agreement with the hypothesis of multiple individual migrations in krill during summer.

A major aspect which resulted from our observations was the reverse DVM pattern displayed by krill in LD conditions, where the animals moved upward during the light phase and downward during the dark phase. Following what has been observed before in krill in the field (Quetin & Ross 1991, Zhou & Dorland 2004), we would have expected to observe the nocturnal DVM pattern, with upward migration during the dark phase and downward migration during the light phase. This is the most common zooplankton DVM pattern, which inspired the predator avoidance hypothesis as the ultimate cause of DVM (Brierley 2014). Following this hypothesis, the animals would migrate downwards during the day, in order to reduce the risk of being predated by visual-oriented predators, while they would migrate upward during the night, driven by the necessity to search for food (Hays 2003). Nevertheless, different DVM patterns have been observed (e.g. the twilight pattern, where two consecutive migrations happen around dusk and dawn, and the reverse pattern, which was observed for krill in the present study and for larval krill in later winter (Meyer et al. 2017)), in relation to different environmental conditions (e.g. food availability, presence/absence of predators) and different modalities of predator-prey interaction (Neill 1990, Ohman et al. 1983). In krill, DVM seems to be flexible with respect to food conditions and presence/absence of predators (Cresswell et al. 2009). In this sense, the reverse DVM pattern observed in LD during the present study might be related to the artificial laboratory conditions in which krill had been maintained prior our experiments. In the laboratory, food availability is kept at a constant optimal level throughout the year to guarantee maximum krill survival rates (Kawaguchi et al. 2010, King et al. 2003), and feeding occurs mostly

during the day, when the technical staff of the aquarium is on duty. Moreover, due to the artificial absence of predators, krill are no longer subject to any kind of environmental pressure during the day. Artificial food conditions and absence of predators might therefore have led to the development of the reverse DVM patterns observed in the present study.

Relationship between krill DVM and krill oxygen consumption

The persistence of DVM in the absence of external light cues might be related to the presence of an endogenous rhythm of activity (Cohen & Forward 2005). In the calanoid copepod *Calanus finmarchicus*, DVM patterns were observed in the laboratory together with rhythmic oxygen consumption, with upward migration during the dark phase, and higher oxygen consumption at the transition between light and dark phase (Häfker et al. 2017). The increase in oxygen consumption during DVM was associated with increasing swimming activity in the vertically migrating euphausiid *Euphausia pacifica* (Torres & Childress 1983). In this study, krill oxygen consumption was measured over the 24 h cycle to investigate the presence of an underlying activity rhythm, which might have been connected to the DVM patterns. Due to the size of the DVM tank, we were not able to monitor oxygen consumption directly in the migrating krill, and we had to use individual krill incubated in a smaller volume of water instead. A similar method was used before to study the relationship between DVM and oxygen consumption in *C. finmarchicus*, and apparently it did not affect the final outcome (Häfker et al. 2017).

In LD and DD, the daily patterns of oxygen consumption displayed major differences among individual krill, and most of the individuals did not show any significant 24 h rhythm. However, 30% of the individuals displayed clear 24 h rhythms, with increasing oxygen consumption during the (subjective) light phases and decreasing consumption during the dark phases. The rhythmic animals displayed similar phases of oscillation but different amplitudes. All individuals came from the same holding population, and had been entrained to similar light and food conditions prior to the experiments. They showed all a similar level of activity and overall good conditions, and the incubation procedure was the same for all of them. Therefore, the different individual outcomes might not be related to differences in the entrainment, or in the physiological status of the animals, or in the experimental setup. Even

if only the minority of the individuals displayed rhythmic oxygen consumption, the oscillations persisted in DD, showing consistent synchronization with the (subjective) light/dark cycle, suggesting the presence of a common endogenous mechanism of regulation entrained by photoperiod, as previously proposed by Teschke et al. (2011). Nevertheless, such mechanism seemed to be functional only in a smaller proportion of the individuals examined. We hypothesize that major differences at the molecular level might occur between rhythmic and non-rhythmic animals, possibly in relation to specific elements involved in the circadian clock (e.g. different clock gene alleles) and/or to other clock-related processes (e.g. light entrainment and output regulation). When we tested for 24 h oscillations using mean oxygen consumption instead of individual oxygen consumption ($n = 6$ for LD; $n = 7$ for DD), both experimental conditions displayed significant rhythmicity ($p < 0.001$; data not shown), with higher consumption during the (subjective) light phases and lower consumption during the dark phases. When testing for a rhythmic output within a population (or within a sample), we must be aware that major differences might be present among individuals, and that a relatively small number of rhythmic animals might mask the final outcome and create on average a rhythmic output even when most of the animals were arrhythmic. This should be considered when future analyses of circadian output rhythms will be conducted.

The daily patterns of oxygen consumption observed in the rhythmic animals suggested a connection with DVM. Rhythmic individuals displayed higher oxygen consumption during the light phases, and migrating animals moved upward during the light phases. This suggested the presence of an endogenous rhythm of activity underlying DVM, in agreement with previous observations conducted on the calanoid copepod *C. finmarchicus* by Häfker et al. (2017) using a similar experimental setup. However, rhythmic oxygen consumption was displayed only by the minority of krill, while DVM seemed to affect the entire group. Even at times of maximum upward migration, most of the animals were either lying on the bottom, or swimming in the lower sectors, while only few animals actually migrated to the higher sectors, demonstrating strong rhythmicity. Therefore, major differences among individual krill might have been present also in the DVM tank, and some animals might have been more rhythmic than others, but this could not be demonstrated as we did not monitor the movements of the single individuals within the group. We hypothesize that a few highly rhythmic animals might have influenced the activity patterns of the other less rhythmic

members of the group, promoting DVM within the experimental tank. Entrainment of rhythmic functions through social interactions might be relevant in a highly social species like krill, as it has been recently demonstrated for honeybees (Fuchikawa et al. 2016). This would also help to understand the difficulties encountered by Gaten et al. (2008) when they tried to assess DVM using individual krill.

Involvement of the circadian clock in the regulation of krill DVM

If an endogenous rhythm of activity was involved in the regulation of krill DVM, it might have been promoted by the circadian clock (Ewer et al. 1992). To investigate this, we analyzed daily patterns of clock gene expression in krill exposed to LD 12:12 (same light-regime as for DVM and oxygen consumption). The animals came from the same holding population and had been exposed to the same entraining conditions (light, food, temperature) prior and during the experiments. Clock gene expression was determined in two different tissues, eyestalks and brain, where putative circadian oscillators might be located (Strauss & Dircksen 2010). Within both tissues, clock gene expression was generally higher during the dark phase, suggesting a link with the light/dark cycle. Rhythmic clock gene expression was detected only in the eyestalks, where Es*per*, Es*cyc*, Es*vri* and Es*dbt* displayed significant 24 h oscillations in their mRNA levels. Es*per* and Es*dbt* peaked towards the beginning of the dark phase, while Es*cyc* and Es*vri* peaked later towards the end of the dark phase. During the 24 h cycle, the levels of clock gene expression were generally comparable between the eyestalks and the brain. Only Es*dbt* displayed consistently higher mRNA levels in the brain, while Es*cyc* and Es*gg* displayed only minor differences at specific times of the day. The amplitude of the oscillations was generally low, never above 1-fold change in mRNA levels, suggesting a weak circadian clock. Similar reduced fold-changes had been previously reported for Es*clk*, Es*per* and Es*tim*, while increased amplitudes had been observed for Es*cyc* and Es*cry*2 (Biscontin et al. 2017, Mazzotta et al. 2010, Teschke et al. 2011).

The presence of rhythmic clock genes expression in the eyestalks suggested that a link between the activity of the circadian clock and the regulation of an endogenous rhythm of activity leading to DVM was plausible in krill. The oscillation phases of DVM and oxygen consumption were in opposition to the oscillation phase of clock genes expression, but this

should not imply the involvement of a different mechanism of regulation. In mammals, different phenotypic phasing is often observed between diurnal and nocturnal species, but this is usually not a result of different phasing within the oscillators, which display always the same oscillation phase independently from the phenotype (Mrosovsky & Hattar 2005). Changes in the input pathways upstream from the oscillator (e.g. light input) and/or modifications in the output pathways downstream from the oscillator seem to play the most prominent role in the phase-regulation of the phenotypic responses (Chiesa et al. 2010, Smale et al. 2003). Therefore, even if during our study krill displayed a reverse DVM pattern, possibly in response to the artificial conditions of the aquarium, an involvement of the clock might still be possible, and the opposite phasing observed between the oscillator (clock genes) and the rhythmic output (DVM, oxygen consumption) might be related to differences in the regulation of upstream/downstream processes.

Ecological implications for krill in natural conditions

Endogenous regulation of DVM in krill might be adaptive for different reasons. First of all, in the case of the most common nocturnal DVM pattern, the endogenous clock would help krill to anticipate changes in the light/dark cycle (i.e. sunrise and sunset), allowing them to leave the surface layers before sunrise. Similarly, it would trigger the upward migration in such a way that the animals would reach the surface briefly after sunset (Cohen & Forward 2005). However, nocturnal DVM might not be the most favorable strategy when specific conditions related to light regime, food availability and presence/absence of predators are present. In these cases, it might be more advantageous for krill to develop a flexible response and temporarily shift their DVM patterns during a different time of the day (Cresswell et al. 2009). The endogenous clock might also help krill to entrain DVM when overt photoperiodic cues are missing. Field observations suggested that krill DVM might get shallower and de-synchronized during mid-summer, at times of midnight sun (Cisewski & Strass 2016, Siegel 2005), but it might still be synchronized in mid-winter, even if during that time krill move to the deeper layers, where light penetration might be severely reduced (Siegel 2005). When photoperiodic cues are virtually missing, the entrainment of the clock might still be promoted by alternative light-related cues, and especially during mid-winter a major role might be

played by low-intensity light cues or by alternative light sources, for example moonlight (Båtnes et al. 2015, Cohen et al. 2015, Last et al. 2016).

4.6. Conclusions

An endogenous rhythm entrained by photoperiod was regulating krill DVM and krill oxygen consumption during the present study. Krill displayed a reverse pattern of migration, with the animals migrating upward during the light phase, which was possibly due to the artificial conditions of the laboratory, where food was presented mostly during the day and predators were always absent. Rhythmic oxygen consumption was observed only in the minority of the investigated animals, with higher levels during the light phase, in agreement with the observed DVM pattern in the laboratory. This suggested that an endogenous rhythm of activity might underlie krill DVM. Social interactions between rhythmic and non-rhythmic animals might then promote DVM at the group level. The involvement of the clock in the regulation of krill DVM might be possible, since clock genes expression in the eyestalks was rhythmic. However, the phase-opposition observed between clock genes expression on one side (peaking during the dark phase), and DVM and oxygen consumption on the other side (peaking during the light phase), suggested the presence of upstream/downstream processes involved in the regulation of the phasing of the phenotypic output. Flexible regulation of clock output functions might allow krill to successfully entrain their daily rhythms in different specific environmental contexts.

4.7. Acknowledgements

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expression analysis. We thank Sina-Katharina Wohlgemüht and Aaron Wolley for their help in the manual determination of krill DVM. We finally thank Cristiano Bertolucci for providing the Multi2 software used for DVM video recording, and for his advices regarding the setup of the camera system. This work was funded by the Helmholtz Virtual Institute ‘‘PolarTime’’ (VH-VI- 500: Biological timing in a changing marine environment – clocks and rhythms in polar pelagic organisms) and contributes to the PACES (Polar Regions and Coasts in a Changing Earth System) program (Topic 1, WP 5) of the Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, and Australian Antarctic Science Program Project no. 4037 (Experimental krill biology: Response of krill to environmental change).

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5. General discussion

This dissertation provides new insights into the regulation of daily and seasonal rhythms in the Antarctic krill, *Euphausia superba*, a key species in the Southern Ocean, and explores the environmental (i.e. photoperiod) and endogenous (i.e. circadian clock) factors, which are implicated in the regulation of these rhythms. The discussion will be divided into four sections. In the first section, I will consider the implications of photoperiod and the circadian clock in the regulation of rhythmicity in krill at the daily level, focusing on the daily patterns of DVM and oxygen consumption described in publication III. In the second section, I will discuss the photoperiodic-dependent modulation of the clock in different simulated seasonal light regimes, and its implications in the regulation of output rhythms at the metabolic level, focusing on the daily patterns of clock– and metabolic-genes expression described in publication I. In the third section, I will discuss the involvement of photoperiod and the circadian clock in the regulation of endogenous rhythmicity at the seasonal level, focusing on the seasonal metabolic activity cycle described in publication II. At the end of each section, I will summarize the main findings of the corresponding publication in relation to the initial research objectives stated in the general introduction (section 1.4), and discuss future directions and outlooks related to the topic. In the final section, I will consider the ecological implications of the findings for krill in the field, and discuss the possible adaptive advantages for krill of having an endogenous clock for the regulation of their rhythmic functions at the daily and seasonal levels.

5.1. Photoperiodic entrainment and endogenous regulation of krill DVM

Krill take part in the mass diel migratory movement known as diel vertical migration (DVM) of the zooplankton worldwide (Brierley 2014). During DVM, animals migrate vertically in the water column in a synchronous way, causing a daily rhythmic mass displacement of entire populations across different depth levels. DVM has a broad ecological impact on the

shaping of marine communities and plays a major role in the biogeochemical cycles regulating the exchange of C and N between the atmosphere and the deep ocean. In the most common DVM pattern, called “nocturnal”, the animals migrate upwards at sunset and spend the night at the surface, while they migrate downwards at sunrise and spend the day in the deeper layers (Lampert 1989). Other common DVM patterns are i) the “twilight” pattern, where two consecutive migrations occur over the 24 h cycle, one around sunset and another around sunrise (Cohen & Forward 2005a), and ii) the “reverse” pattern, where animals migrate upwards at sunrise to spend the day at the surface, and sink downward at sunset to spend the night in deeper layers (Tester et al. 2004).

The ultimate cause for DVM is generally believed to be the avoidance of predators. The animals sink down during the day to escape from visual predators, and come back to the surface at night to graze on phytoplankton (Hays 2003). Among the proximate environmental cues involved in the regulation of DVM, light is generally considered to be the most prominent one (Ringelberg & van Gool 2003), due to the close association of the upward/downward movements with sunset and sunrise. Food cues can also play a major role, and it has been demonstrated that in many cases the control exerted by food might override the one exerted by light (Pearre 2003). The presence of DVM in environments where no overt light/dark cycle occurs, for example in the deep sea (van Haren 2007, van Haren & Compton 2013), or in the Arctic during the Polar night (Berge et al. 2009), suggests that an endogenous rhythm of activity might be involved in the regulatory process (Cohen & Forward 2005b). Indeed, recent laboratory analyses demonstrated that DVM in the calanoid copepod *C. finmarchicus* was promoted by an endogenous rhythm, entrained by photoperiod, suggesting a major implication of the activity of the circadian clock in the regulatory process (Häfker et al. 2017).

In krill, DVM is usually present in natural conditions (Godlewska & Klusek 1987, Quetin & Ross 1991), but different DVM patterns have been observed depending on the season and the region. In spring and autumn, when light conditions in the Southern Ocean are not extreme, krill DVM patterns are mostly nocturnal, with krill rising to the surface during the night and sinking to the deeper layers during the day (Quetin & Ross 1991). During summer, when the sun never goes below the horizon, krill might stop performing synchronized DVM (Cisewski

& Strass 2016, Siegel 2005), and multiple shallow individual migrations might occur instead, as already observed for copepods in the Arctic (Cottier et al. 2006). During winter, when extremely reduced light conditions are present, also in association with thick sea-ice cover, krill might move to deeper layers (200-300 m depth) (Siegel 2005). Even if light penetration to those depths might be virtually prevented, krill might still perform DVM, possibly under the influence of an endogenous rhythm (Cisewski & Strass 2016, Siegel 2005). Moreover, krill DVM seems to be strongly affected by local factors like predator pressure and food availability (Cresswell et al. 2009, Zhou & Dorland 2004).

The implication of photoperiod and the involvement of an endogenous rhythm of activity in the regulation of krill DVM were investigated by Gaten et al. (2008), by monitoring daily changes in vertical swimming activity of freshly caught krill exposed to light/dark (LD) and constant darkness (DD) conditions. Only the minority of the examined individuals displayed a certain degree of rhythmicity, no correlation was observed with the light/dark cycle, and a period of about 12 h was observed instead (Gaten et al. 2008). The authors concluded that photoperiod was not acting as the main *Zeitgeber* for krill DVM, and that an endogenous rhythm entrained by food and/or by social cues might have been involved instead. However, due to the seasonal and regional differences displayed by krill DVM, the results from Gaten et al. (2008) might have been masked by underlying seasonal and/or regional factors, since their experiments were conducted with fresh krill collected during summer at two different locations. Moreover, they monitored DVM using single animals, which in a highly social species like krill might have prevented the development of a fully natural behavior (Hamner & Hamner 2000). In a similar study with fresh krill caught during summer, Tarling & Johnson (2006) investigated the effect of stomach fullness on the swimming activity of tethered krill. The animals exhibited different levels of swimming activity in relation to the different degrees of stomach fullness, and individuals with full to half-full stomachs spent a significant amount of time in a so-called “parachute” mode, in which swimming activity was interrupted and krill were sinking towards the bottom (Tarling & Johnson 2006). The calculated downward distance covered by a satiated “parachuting” krill ranged between 9 m and 43 m, which would be in agreement with the krill DVM range estimated using fishing depths and catch rates data from Japanese commercial trawling operations across the Scotia Sea during summer (Siegel 2005, Taki et al. 2005). Once the stomach is empty, krill would

return back to the surface to feed again, promoting multiple individual short-range migrations over the 24 h cycle, in agreement with observations of unsynchronized DVM during summer in the Arctic (Cottier et al. 2006).

In publication III, we examined the role of photoperiod and the involvement of the circadian clock in the regulation of krill DVM in controlled laboratory conditions (LD-DD). To improve social interactions during the experiment, we monitored DVM using small groups (n ≈ 40) of krill, which had been previously entrained to LD conditions. Since an endogenous activity rhythm might underlie krill DVM, we also monitored changes in oxygen consumption over the 24 h cycle in individually incubated krill exposed to similar LD-DD conditions. Moreover, to examine the possible involvement of the circadian clock, we also measured daily patterns of clock genes activity in krill exposed to LD.

We observed a clear DVM pattern in LD, displaying a high degree of correlation with the light/dark cycle, with krill migrating upward during the light phase and downward during the dark phase ("reverse" DVM). The DVM pattern persisted also after two days in DD, even if attenuated, indicating the presence of an endogenous regulatory rhythm with a period of about 24 h. During upward migration, only few animals reached the top of the experimental tank, while the majority of them displayed less extensive migrations, suggesting differences among individual krill in the intensity of DVM. Oxygen consumption displayed also an LD-DD rhythm, with higher levels during the light phase and lower levels during the dark phase, in agreement with the DVM pattern observed during the experiment. Also in this case, major differences were present among individual krill, and only the minority (30%) of the individuals displayed a clear rhythm. The daily expression patterns of the krill clock genes *period* (Es*per*), *cycle* (Es*cyc*), *vrille* (Es*vri*) and *doubletime* (Es*dbt*)[1] oscillated over the 24 h in the eyestalks of krill in LD, showing high correlation with the light/dark cycle, with upregulation during the dark phase.

The persistence of DVM in DD indicated the presence of an endogenous regulatory rhythm, and the tight relationship of upward/downward migration with the light/dark cycle in LD indicated that photoperiod was possibly acting as the main *Zeitgeber*. However, the "reverse"

[1] The use of the prefix 'Es' in front of a gene or protein name indicates that this is the specific orthologue form isolated in *Euphausia superba*.

DVM pattern might have been promoted by food cues prior to our experiment. In fact, during their captivity in the aquarium (February 2013–November 2016), krill had been exposed to food mostly during the day. This, in association with the artificial absence of predators, might have determined the phasing of DVM observed during the experiment. In LD and DD, oxygen consumption oscillated in agreement with DVM, suggesting a link between these two processes and a common endogenous rhythm of regulation. In another vertically migrating Euphausiid, *Euphausia pacifica*, increasing oxygen consumption showed positive correlation with increasing swimming activity (Torres & Childress 1983), suggesting that increasing swimming during DVM might account for the increase in oxygen consumption. Unfortunately, we did not monitor krill swimming activity during our oxygen consumption measurements, but a similar relationship between oxygen consumption and swimming activity might be present also during krill DVM. If that would be true, an endogenous rhythm of swimming activity might underlie DVM in krill, as already proposed for the calanoid copepods, *Calanopia americana* (Cohen & Forward 2005b) and *Calanus finmarchicus* (Häfker et al. 2017).

Only few animals displayed robust oxygen consumption rhythms and extensive upwards migrations, suggesting the presence of major individual differences in the regulation of these endogenous rhythms. All animals had been entrained to the same light and food conditions prior to the experiments, and showed similar overall good physiological conditions at the beginning of the measurements. Therefore, the different individual outcomes might have been determined by differences occurring at the molecular level, possibly in relation to elements of the individual clock (e.g. different clock genes alleles, different regulation of input/output pathways). In mouse, individual differences in circadian activity rhythms correlated positively with differences in the molecular oscillator within the suprachiasmatic nucleus (SCN) (Evans et al. 2015), while in human children, individual differences of circadian cortisol release rhythms displayed additive genetic variance, suggesting the involvement of specific heritable genetic factors (van Hulle et al. 2012). Alternatively, individual krill might be able to switch between rhythmic and non-rhythmic activity patterns in relation to changes in the social context. In the honeybee, *Apis mellifera*, individual plasticity in circadian rhythms can be socially modulated, with individuals switching between rhythmic and arrhythmic activity patterns depending on the role played within the colony

(Bloch 2010). This plasticity might involve the re-organization of the individual clock, through an entrainment operated by social interactions (Fuchikawa et al. 2016, Shemesh et al. 2007). Considering the high level of sociability displayed by krill in the field (Hamner & Hamner 2000), similar processes of social modulation of circadian rhythmicity might take place also in krill.

The rhythmic activity displayed by the clock genes in krill exposed to LD suggested that an involvement of the circadian clock in the regulation of the daily patterns of DVM and oxygen consumption is possible. In krill, the resetting of the clock might work in a similar way as in *Drosophila*, where the light-sensitive protein CRYPTOCHROME[2] (CRY) promotes the degradation of the clock protein TIMELESS (TIM) during the light phase (Biscontin et al. 2017). Together with the clock protein PERIOD (PER), TIM is involved in the suppression of the transcriptional activation promoted by the heterodimer CLOCK/CYCLE (CLK/CYC), which regulates the transcription of the clock genes *period* (*per*) and *timeless* (*tim*). Following TIM degradation, suppression on CLK/CYC is released, and transcription of *per* and *tim* gets activated. Therefore, the mRNA levels of *per* and *tim* usually increase during the light phase and reach their peak at the beginning of the dark phase (Dunlap 1999). In agreement with this, during our experiment the levels of expression of Es*per* in the eyestalks of krill exposed to LD increased during the light phase and reached a peak at the beginning of the dark phase, suggesting that the circadian feedback loop of krill was actively ticking.

In conclusion, krill DVM seems to be regulated endogenously. DVM entrainment in krill seems to be complex, involving the interplay of different factors like food availability, the presence of predators and social interactions. During my investigations, krill DVM displayed good correlation with the light/dark cycle, suggesting a *Zeitgeber* role for photoperiod. However, the observed "reverse" migratory pattern suggested a major role of food conditions in the shaping of DVM prior to the experiment. The different individual outcomes in the rhythms of oxygen consumption and in the extension of the DVM patterns indicated that some animals might display more robust rhythmicity than others. In a highly social species like krill, social interactions between rhythmic and non-rhythmic individuals might play a

[2] Conventionally, protein names are indicated in capital letters, while gene names are indicated in lowercase and italics.

fundamental role in the regulation of rhythmicity (e.g. DVM) within the population. The implication of the circadian clock in the regulation of krill DVM is plausible, but our knowledge in this direction is still very limited.

Future work should focus in two different directions. On one side, the differences observed in the regulation of rhythmic output functions among individual krill should be investigated in more detail. Preliminary results indicated that similar differences in rhythmic oxygen consumption might be present among individual krill also in natural conditions, and also in other species such as the calanoid copepod *C. finmarchicus* from the Arctic, suggesting that this might be a common trait for high-latitude zooplankton species (Meyer, personal communication). Individuals displaying more robust rhythmicity should be compared to those displaying less robust rhythms, also at the molecular level including the genotypic analysis of elements related to the clock. The interactions between rhythmic and non-rhythmic animals should be investigated, to examine if individual plasticity of circadian rhythms might be regulated through social interactions. On the other side, the interplay between endogenous and environmental factors in the regulation of krill DVM should be examined in more detail, in different seasons and regions in the field, and in simulated natural conditions in the laboratory. Also, the involvement of the circadian clock should be addressed more specifically, by applying more powerful, reverse-genetics manipulative approaches (e.g. RNAi = interference RNA) to the functional analysis of single clock genes relative to DVM. Additional laboratory observations with krill incubated under different light and food conditions, separately and in combination, would help to understand the relative impact of different environmental cues on krill DVM.

5.2. Photoperiodic modulation of circadian functions in krill

Due to the extreme seasonal variability displayed by the light/dark cycle in Polar regions, high-latitude species like krill might face a problematic situation when using photoperiod to entrain their circadian functions at different times of the year. During mid-winter and mid-summer, when near-constant darkness and near-constant light conditions are present, some aspects related to circadian rhythmicity, for example the implementation of strict diurnal or

nocturnal patterns of activity, might become less advantageous, and might therefore be suppressed (Bloch et al. 2013, Lu et al. 2010). Conversely, some other aspects related to circadian rhythmicity might be advantageous independently form external conditions, for example the temporal coordination among internal homeostatic processes, and might therefore be preserved also in the absence of an overt light/dark cycle (Vaze & Sharma 2013, Williams et al. 2015).

The processes leading to the seasonal modulation of circadian functions in high-latitude organisms are currently debated, and different mechanisms have been implicated. One hypothesis is that the activity of the circadian clock might be seasonally modulated, promoting different levels of rhythmicity at different times of the year, in relation to different photoperiodic conditions (Reierth & Stokkan 1998, Reierth et al. 1999). Alternatively, the activity of the clock might be constant, but the clock control of output pathways might be seasonally modulated, leading to seasonal differences in rhythmic output functions (Williams et al. 2015, Steiger et al. 2013) In Arctic zooplankton, DVM patterns become arrhythmic at times of midnight sun during summer, suggesting seasonal modulation of circadian functions (Cottier et al. 2006), and a similar process might take place also in krill (Cisewski & Strass 2016). Conversely, daily cycles of transcriptions have been observed in krill during summer, suggesting that some circadian functions might be preserved also in the absence of an overt light/dark cycle (De Pittà et al. 2013).

In publication I, we analyzed the activity of the circadian clock and the regulation of metabolic output rhythms in krill exposed to different simulated seasonal photoperiodic conditions. We entrained krill to a long-term simulation of the seasonal photoperiodic cycle in the Southern Ocean (66°S), and examined daily patterns of clock– and metabolic gene activity at four seasonal time-points, corresponding to mid-summer (near-constant light; LL), early-autumn (16 h light: 8 h darkness; LD 16:8), mid-winter (3 h light: 21 h darkness; LD 3:21) and late-winter (8 h light: 16 h darkness; LD 8:16).

In simulated early-autumn (LD 16:8) and late-winter (LD 8:16) conditions, clock gene activity was rhythmic, and the metabolic output was synchronized with the light/dark cycle, with up-regulation of key-metabolic genes occurring during the dark phase. Conversely, in simulated mid-summer (LL) and mid-winter (LD 3:21) conditions, clock gene activity was

arrhythmic, and up-regulation of key-metabolic genes occurred at different times during the day. In a previous study, krill exposed to similar non-extreme photoperiodic conditions in the laboratory (LD 16:8) showed rhythmic clock gene activity, in association with a rhythmic output at the metabolic level (Teschke et al. 2011). This, together with the present results, suggests that overt light/dark cues might be able to entrain the circadian clock in krill and promote the synchronization of metabolic output functions during the 24 h. Conversely, the disruption of the clock observed in simulated extreme mid-summer (LL) and mid-winter (LD 3:21) photoperiodic conditions was in contrast to previous reports of rhythmic clock gene expression and rhythmic transcriptional output in krill caught in the Southern Ocean during midnight sun (Biscontin et al. 2017, De Pittà et al. 2013, Mazzotta et al. 2010).

For temperate model organisms like *Drosophila* and the common mouse, *Mus musculus*, near-constant light can be deleterious for the activity of the circadian clock and for the regulation of rhythmic output functions (Ohta et al. 2005, Sauman and Reppert 1996). However, high-latitude species might have evolved specific clock features to cope with extreme environmental light conditions (Kyriacou 2017, Menegazzi et al. 2017). According to the present results, near-constant light conditions seem to lead to the disruption of the clock of krill, in the absence of other *Zeitgebers*. However, this does not imply that in the field the circadian clock of krill will get disrupted during summer. In the natural environment, alternative *Zeitgebers* might be present, which might entrain the clock when overt photoperiodic cues are missing (Williams et al. 2015). During mid-summer, even if the sun never sets, rhythmic cues related to changes in light intensity and/or changes in light spectral composition might be used by krill to promote rhythmic clock gene activity and rhythmic regulation of basic homeostatic functions (Biscontin et al. 2017, De Pittà et al. 2013, Mazzotta et al. 2010). A similar concept might apply also during mid-winter, when low-intensity light cues might be able to synchronize basic krill circadian functions despite the extreme reduction of overall light availability (Båtnes et al. 2015, Cohen et al. 2015).

In conclusion, our results highlight the plasticity of the circadian clock of krill in response to the extreme seasonal photoperiodic cycle of the Southern Ocean. During autumn and spring, the overt photoperiodic cycle might function as main *Zeitgeber* and promote rhythmic clock activity, contributing to the synchronization of daily output rhythms in the regulation of

metabolism at the transcriptional level. Conversely, during mid-summer and mid-winter, the extreme photoperiodic conditions might be insufficient for the entrainment of the clock. In this case, alternative *Zeitgebers* (e.g. light intensity, light spectral composition) might come into play, allowing for the entrainment of the clock and for the temporal coordination of basic internal homeostatic processes.

Future work should focus on the validation of the generated findings in krill in the field. Clock genes activity and metabolic genes regulation should be investigated over the 24 h in different seasons, in association with acoustic records of DVM and environmental data regarding light-regime, food availability and presence/absence of predators. The effect of light intensity and light spectral composition on the circadian system of krill should be examined in more detail, using laboratory experiments with krill exposed to different light intensity cycles and light color cycles over a 24 h period. In this context, it is worthwhile to take into account the investigation of the spectral sensitivity range of krill conducted by Frank & Widder (1999).

5.3. Endogenous regulation of krill seasonal metabolic cycle

The epipelagic environment of the Southern Ocean is characterized by extreme seasonal fluctuations in light and food availability, and sea-ice extent (Knox 2006). In summer, the increasing day length together with enhanced sunlight intensity and the absence of sea-ice cover, promote high levels of irradiance in the surface layers. Primary production is thus enhanced, and phytoplankton is available for grazers like krill. In the summer season, krill display high levels of metabolic and feeding activity, growth, and sexual maturation (Kawaguchi et al. 2007, Meyer 2012). In winter, decreasing day length together with reduced sunlight intensity and the presence of thick sea-ice cover, promote low levels of irradiance in the surface layers, leading to very low primary productivity. Therefore, in the winter season krill display low levels of metabolic and feeding activity, reduced or even negative growth rates, and sexual regression (Kawaguchi et al. 2007, Meyer 2012).

The seasonal cycles of light and food availability in the environment show significant relationships with the seasonal shifts in krill physiology. However, seasonal changes in food availability do not always correlate with seasonal changes in krill metabolic and feeding activity, suggesting that light-regime might play a more prominent role instead of food (Kawaguchi et al. 1986, Torres et al. 1994). Indeed, laboratory investigations indicated that krill feeding and metabolic activity could be influenced by photoperiod irrespective of food supply, suggesting that prolonged photoperiods could stimulate feeding and metabolic activity, and vice-versa (Teschke et al. 2007). In addition, prolonged photoperiods were able to advance krill sexual maturity, while shortened photoperiods advanced krill sexual regression (Brown et al. 2011, Teschke et al. 2008). Moreover, long-term laboratory simulations with krill exposed to constant food, temperature and darkness demonstrated that the seasonal changes in krill physiology could persist also in the absence of external seasonal cues, suggesting the presence of an underlying endogenous circannual rhythm (Brown et al. 2013, Kawaguchi et al. 2007, Thomas & Ikeda 1987).

Circannual rhythms are widespread among living organisms that face recurrent changes in their environment on the seasonal level. Circannual regulation of physiological functions related to reproduction and overwintering might be highly adaptive in high-latitude organisms, which alternate between favorable environmental conditions during summer and unfavorable environmental conditions during winter (Gwinner 2012). An endogenous circannual clock might be responsible for the regulation of circannual rhythms, in a similar way as the circadian clock has been implicated in the regulation of circadian rhythms (Goldman et al. 2004). However, even if the molecular mechanism of the circadian clock is well characterized, this is not the case for the circannual clock (Hazlerigg & Lincoln 2011, Lincoln et al. 2006). It is hypothesized that the circadian clock, with its ability to track changes in photoperiod, might play a role in the photoperiodic entrainment of the circannual clock (Goldman et al. 2004, Gwinner 2012). Evidence supporting an involvement of the circadian clock in the regulation of seasonal biological functions has been described in mammals and birds (Dardente et al. 2010), and circadian clock genes have been implicated in the regulation of photoperiodism (i.e. physiological responses to changes in day length) in insects (Goto 2013).

In publication II, we investigated the presence of an endogenous circannual rhythm in the regulation of the seasonal metabolic cycle of krill. Krill were exposed to long-term simulations of different seasonal photoperiodic cycles in the absence of other *Zeitgebers* (e.g. food availability, temperature), and seasonal changes of different physiological parameters including growth, oxygen consumption, enzyme activity (Malate Dehydrogenase = MDH) and gene expression were monitored.

Under simulated natural Antarctic light conditions at 66°S, krill displayed clear seasonal changes in growth, enzyme activity, oxygen consumption and gene expression, with low values during the simulated winter months, and high values during the simulated summer months. This was in agreement with field observations, indicating that krill undergo a period of metabolic quiescence during winter, followed by an increase of metabolic activity and growth during spring and summer (Meyer 2012, Quetin & Ross 1991, Stepnik 1982). The temporal changes displayed by krill growth, metabolic activity and transcription correlated well with the simulated seasonal changes in light regime. Considering that food supply was maintained at a constant high level throughout the experiment, this suggested that photoperiod was playing a main *Zeitgeber* role. In constant darkness (DD), similar seasonal cycles were observed for growth, enzyme activity and gene expression, suggesting the involvement of an endogenous circannual rhythm, as already proposed by Meyer et al. (2010) and Brown et al. (2013).

However, oxygen consumption, which represents the sum of multiple physiological processes including basal metabolism, swimming activity, growth, and feeding activity (Clarke & Morris 1983, Torres & Childress 1983), did not display a seasonal pattern in DD, suggesting a more direct implication of the seasonal light regime in the regulatory process. In a previous study, Teschke et al. (2007) investigated the effect of prolonged (12 weeks) darkness on krill feeding activity at constant high food availability and demonstrated that feeding was reduced in DD compared to LD conditions. During winter, when light conditions are very scarce in the Southern Ocean, krill display a general tendency to reduce all energy-demanding processes such as metabolic activity and growth (Meyer 2012), and most likely also the swimming activity. A correlation between oxygen consumption pattern and krill swimming activity was suggested before for DVM on the daily level (see section 5.1).

Therefore, processes related to krill feeding and/or swimming activity might have been altered during the DD simulation, leading to a different seasonal oxygen consumption pattern compared to the natural light regime simulation.

The lack of a seasonal cycle of oxygen consumption observed during our DD simulation was in contrast with previous findings by Brown et al. (2013). Using krill exposed to similar simulated long-term DD conditions, the authors demonstrated that krill oxygen consumption increased after winter independently from light regime, and suggested the involvement of an endogenous circannual rhythm (Brown et al. 2013). The difference between our results and those of Brown et al. (2013) might have been determined by different entraining conditions experienced by krill prior the initiation of the DD simulation. In order to achieve effective entrainment, the circannual clock might require exposition to a specific portion of the seasonal photoperiodic cycle. In female sheep, the circannual rhythm of reproductive neuroendocrine activity is effectively entrained by long-day photoperiodic cues perceived during summer (Woodfill et al. 1994). When these are missing, the circannual rhythm gets altered or even dampened (Woodfill et al. 1994). Similarly, the absence of specific summer-related light cues experienced by krill at the beginning of our DD simulation (December) might have affected the entrainment of the circannual rhythm, possibly leading to the altered seasonal orchestration of the metabolic cycle. This might not have been a problem for Brown et al. (2013), who initiated their DD treatment later in early autumn (February), possibly allowing krill to be exposed to proper summer light cues leading to effective circannual entrainment, and to the "correct" seasonal response. This would imply that at a certain time between mid-summer and early autumn, the corresponding light conditions in the Southern Ocean play a fundamental role for the entrainment of krill circannual clock. Interestingly, under simulated natural light conditions (66°S), oxygen consumption started to decrease only after light-phase duration had been reduced below 16 h, corresponding to simulated early autumn (February) natural light conditions.

To investigate the involvement of the circadian clock in the entrainment of the circannual clock, we analyzed seasonal patterns of clock genes expression under simulated natural light conditions (66°S) and DD. Interestingly, the krill clock genes *clock* (Es*clk*), *cryptochrome*2 (Es*cry*2) and *timeless* (Es*tim*), showed up-regulation under simulated early autumn

(February) conditions, suggesting a specific link between clock-related activity and light-regime at that time of the year. Since specific elements of the phototransduction cascade might participate in the light input to the photoperiodic clock (Tamaki et al. 2013), we also monitored seasonal changes in the expression levels of different krill opsins, and we found up-regulation for the krill opsins *rhodopsin*1a (Es*rh*1a) and *peropsin* (Es*rrh*) under simulated early autumn (February) light conditions. Es*rh*1a and Es*rrh* had previously shown rhythmic expression in krill over the 24 h cycle, suggesting an implication in the input pathway to the circadian clock (Biscontin et al. 2016, De Pittà et al. 2013).

In conclusion, our results supported the hypothesis that the seasonal life cycle of krill is regulated by an endogenous circannual rhythm entrained by the seasonal Antarctic light regime. Endogenous regulation was observed in relation to krill growth, enzyme activity and gene expression. However, photoperiodic entrainment seems to play a major role in the regulation of krill oxygen consumption, which displayed a seasonal pattern only under simulated light conditions. We hypothesize that long-day photoperiodic cues perceived by krill during summer might be necessary for the effective entrainment of the circannual rhythm. The seasonal light input might be collected by specific elements of krill phototransduction cascade and transmitted to an internal photoperiodic clock. The nature of such clock still remains elusive, but we suggest that elements of krill circadian clock might be involved.

Future work should focus on the determination of the specific seasonal light cues, which are necessary to promote the entrainment of the circannual clock. The utilization of DD to interrupt the simulated natural seasonal Antarctic light regime at different times of the year, in particular during summer and autumn, might be useful to verify the presence of specific photoperiodic thresholds linked to the seasonal metabolic cycle. Also, the involvement of an endogenous circannual rhythm of swimming activity should be investigated in more detail, monitoring krill swimming activity in different seasons, both in natural and in simulated laboratory conditions. The effect of different latitudinal light regimes should also be taken into account for future investigations, since krill distribution displays a broad latitudinal range (approx. 50°–70°S). Krill located further north (50°–60°S) are exposed to less extreme

seasonal light conditions, and might display less intense seasonal responses possibly driven by other environmental factors like food availability (Meyer 2012, Schmidt et al. 2014).

Preliminary results from long-term laboratory simulations with krill exposed to different latitudinal light regimes indicated that the seasonal cycles of lipid utilization and sexual maturity might be affected, suggesting the presence of a latitudinal cline in the photoperiodic threshold (Höring, personal communication). Conversely, Seear et al. (2012) observed comparable expression levels of genes involved in respiration, motor activity and vitellogenesis in krill captured during winter at two different latitudes in South Georgia (54°S) and around the Antarctic Peninsula (62°S). Currently, a comparison of the transcriptomic signature of krill collected at different latitudes and in different seasons is being processed, which will provide further insights into the mechanisms leading to the regulation of seasonal responses in krill in different areas.

5.4. Ecological implications for krill in natural conditions

In this section I will provide an ecological interpretation for the findings generated during the PhD project, by relating them to the daily and seasonal events characterizing the environment of the Southern Ocean. I will first consider the ecological implications of endogenous rhythmicity at the daily level, focusing on the findings related to the implication of endogenous rhythmicity in krill DVM, presented in publication III. Then, I will examine the ecological implications of the seasonal photoperiodic modulation of krill circadian functions discussed in publication I. Finally, I will focus on the ecological implications of the endogenous regulation of krill seasonal metabolic cycle presented in publication II.

Krill DVM

In publication III, it was demonstrated that an endogenous rhythm might underlie krill DVM, suggesting that the circadian clock might be involved in the regulatory process. For krill in the field, an endogenous regulation of DVM would be advantageous for different reasons. First, considering the nocturnal DVM pattern, it would help krill in the deeper layers to

remain synchronized with the day/night cycle at the surface, triggering upward migration at the appropriate time of the day in order to reach the surface briefly after sunset. Similarly, it would help krill at the surface to trigger their downward migration appropriately, in order to sink towards the deeper layers briefly before sunrise (Cohen & Forward 2005b). The involvement of the circadian clock would also help krill to orchestrate their internal physiological processes accordingly to the rhythmic changes in behavior and metabolism associated to DVM, promoting higher levels of activity when krill are at the surface and lower levels of activity when krill are in the deeper layers. Since the circadian clock seems to be highly suitable also for the regulation of crepuscular activity patterns (Rudjakov 1970), it might be involved in the regulation of twilight DVM, triggering the first descent to intermediate depth in the middle of the night, and then stimulating the second upward migration closely before sunrise (Cohen & Forward 2005a). Field data from Japanese fishing vessels suggested that during winter krill might perform extensive DVM in the deeper layers, moving approx. between 100 and 350 m depth (Siegel 2005, Taki et al. 2005). At this time of the year and at those depths light conditions might be extremely poor. Therefore, an endogenous rhythm of activity promoted by the circadian clock might be fundamental for the regulation of such migratory patterns.

The implication of the light/dark cycle in the regulation of krill DVM seemed to be complex. During our experiments, a clear correlation was observed between upward/downward migrations and light/dark phases, suggesting a *Zeitgeber* role for photoperiod. However, the reverse phasing displayed during the DVM experiments suggested also an involvement of food as *Zeitgeber*. Therefore, we suggest that krill DVM might be regulated through the interplay between endogenous (i.e. the circadian clock) and environmental (i.e. light regime, food availability, predator dynamics) factors, as previously proposed by Gaten et al. (2008) and Cresswell et al. (2009). The flexibility of krill to switch between different *Zeitgebers* in response to changes in the environment might be necessary for the successful entrainment of krill DVM in different seasons and regions.

Seasonal modulation of krill circadian functions

In publication I, we investigated the effect of the seasonal Antarctic light regime on the regulation of krill circadian functions at different times of the year, and we concluded that when light conditions are not extreme (early-autumn and late-winter), photoperiod might be sufficient for the entrainment of the clock and for the synchronization of the metabolic output rhythm. Conversely, when light conditions are extreme (mid-summer and mid-winter), photoperiod might not be sufficient to entrain the clock, and the synchronization of the metabolic output rhythm might get lost. However, this does not imply that in krill in the field the clock is disrupted during mid-summer and mid-winter. Even when external conditions are not rhythmic, circadian regulation might still be preserved to ensure the temporal coordination of internal metabolic and physiological processes (Sharma 2003, Vaze & Sharma 2013). In this case, additional *Zeitgebers* might contribute to the entrainment of the clock when photoperiod gets extreme in natural conditions (Williams et al. 2015). In the Svalbard ptarmigan, *Lagopus mutus hyperboreus*, daily activity rhythms get de-synchronized during summer, but persist during winter, suggesting the involvement of the circadian clock and the occurrence of alternative *Zeitgebers* at that time of the year (Reierth & Stokkan 1998, Reierth et al. 1999). However, not all high-latitude species might display this ability to switch between different *Zeitgebers* at different times of the year. In the reindeer, *Rangifer tarandus*, the circadian clock seems to be completely suppressed, and the daily rhythms of activity briefly displayed around the equinoxes seem to be regulated directly by the masking effect of light (Lu et al. 2010, van Oort et al. 2005).

In krill, flexible entrainment of circadian functions might allow for rhythmic organization at different times of the year, including summer and winter. Photoperiodic-driven circadian rhythmicity might be mostly adaptive during spring and autumn, when an overt light/dark cycle is present, allowing for DVM entrainment in orchestration with daily rhythms of metabolism and physiology. Conversely, during summer and winter, when overt photoperiodic cues are missing, other environmental factors might play the *Zeitgeber* role for the circadian clock. In this case, some krill rhythmic functions might remain under the control of the clock, while other rhythmic functions might get uncoupled and start to follow other external factors. For example, high phytoplankton abundance during summer might

attract krill towards the surface at all times, overriding the control exerted by the clock on DVM, and promoting multiple shallow vertical migrations via hunger/satiation (Tarling & Johnson 2006). At the same time, the clock might continue to exert its control on the regulation of basic homeostatic rhythms also during summer (De Pittá et al. 2013). Therefore, as already suggested in the previous paragraph, circadian regulation in krill seems to be highly flexible, allowing for the temporal orchestration of metabolism, physiology and behavior over the 24 h cycle in different seasonal and regional contexts.

Endogenous regulation of krill seasonal metabolic cycle

In publication II, we investigated the impact of the seasonal Antarctic light regime on the regulation of endogenous rhythmicity in krill at the seasonal level. The results strongly suggested the involvement of an endogenous circannual rhythm entrained by photoperiod in the regulation of krill seasonal metabolic cycle. Endogenous regulation of krill seasonal phenology would ensure reliable synchronization of fundamental krill life-cycle traits (e.g. reproduction, dormancy) with the seasonal changes in light and food availability in the environment of the Southern Ocean. Food availability might not be the best seasonal *Zeitgeber*, because primary production in the Southern Ocean is too unpredictable, with major inter-annual changes in terms of phytoplankton bloom initiation, duration and intensity (Moore & Abbott 2000). Instead, photoperiod is a stable parameter, and would therefore be a reliable timing cue, allowing krill to entrain their seasonal life-cycle in order to anticipate the seasonal shifts in primary production. However, the photoperiodic cycle differs significantly across the latitudinal range of krill distribution (50°–70°S) (Siegel 2016), and for krill living at lower latitudes the influence of photoperiod might be less stringent, causing a latitudinal cline in the intensity of krill seasonal responses.

Indeed, krill feeding activity was significantly lower in winter compared to summer in the Lazarev Sea (60°–65°S), but did not display significant seasonal changes at South Georgia (53–54°S) (Schmidt et al. 2011, 2014). However, winter feeding activity differed significantly also between krill collected at similar latitudes (60–65°S), but within different habitats in terms of ice-cover and food availability, such as the Lazarev Sea (ice-covered, low food deep oceanic area) and the Bransfield Strait (ice-free, high productive shelf area),

suggesting a prominent role of food conditions, independently from light regime (Schmidt et al. 2014). At the transcriptional level, genes involved in respiration and motor activity were significantly down-regulated in winter at the Antarctic Peninsula (62°S) and in South Georgia (54°S), suggesting a similar seasonal pattern of gene expression independent from the latitude. Conversely, in winter genes involved in feeding and digestion were up-regulated in South Georgia (54°S), while they were down-regulated at the Antarctic Peninsula (62°), suggesting a different seasonal gene expression pattern in relation to the different latitudes (Seear et al. 2012). Different feeding conditions might occur in winter between these two locations, which might have caused the different expression levels observed for genes related to feeding and digestion (Seear et al. 2012). Therefore, food conditions interact with photoperiod in the modulation of krill seasonal responses in different habitats and at different latitudes, allowing for the adjustment of different physiological functions (e.g. feeding and metabolism) accordingly to the prevailing environmental conditions.

In the context of climate change, two of the main krill feeding grounds in the Southern Ocean, the southwest Atlantic sector and the region of the West Antarctic Peninsula, have recently experienced a rapid anthropogenic-driven warming (Meredith & King 2005). The abundance of krill within these areas decreased significantly during the last decades, possibly as a result of changes in phytoplankton bloom dynamics associated with sea-ice decline (Atkinson et al. 2004). This suggested that krill might be affected by the rapid environmental changes driven by climate change, and raised concerns regarding the future conditions of the krill population, also considering the increasing commercial interest of krill fisheries (Schiermeier 2010). Due to the increasing anthropogenic-driven warming and hence reduced sea ice cover, phytoplankton dynamics in the Southern Ocean might get significantly altered, while the seasonal photoperiodic cycle will remain the same. This might cause a "match-mismatch" scenario between krill's seasonal life-cycle (entrained by photoperiod) and the seasonal patterns of food availability in the environment (affected by climate change) (Helm et al. 2013). However, the impact of global warming on phytoplankton might vary significantly on a regional scale (Deppeler & Davidson 2017), and krill might adapt due to migration to new feeding grounds. In that case, the flexibility displayed by krill to regulate their daily and seasonal rhythms using different environmental cues (e.g. photoperiod, light intensity, food availability) might facilitate their adaptation to future habitat changes.

5.5. Final remarks and outlooks

This dissertation highlighted the role of endogenous clocks in the regulation of daily rhythms and seasonal cycles in the Antarctic krill, *Euphausia superba*, and discussed A) the involvement of photoperiod as main entraining cue, and B) the implication of the circadian clock as underlying molecular oscillator. Endogenous rhythmicity seems to play a fundamental role in krill, being involved in the regulation of DVM at the daily level, and in the regulation of major physiological shifts in growth and metabolic activity at the seasonal level. Photoperiod seems to be the most relevant external cue used by krill to synchronize their daily and seasonal rhythms to the environmental cycles. However, the dissertation demonstrates that also food availability can play a major role as *Zeitgeber*. In krill, the circadian clock displays the potential to participate in the regulation of rhythmic functions, both at the daily and seasonal levels.

As general take-home message for the future, in order to understand the regulation of biological rhythms in Antarctic krill, it will be necessary not only to look at the direct effect of proximate environmental cues (e.g. light and food availability), but also to take into account the role played by the internal endogenous clocks. Information about clock functioning should be further implemented into individual- and population-based models, to predict the impact of environmental changes driven by climate change on the regulation of fundamental krill's life-cycle traits. Increasing knowledge about endogenous clocks in marine species like krill, which might possess an ancestral form of the clock compared to terrestrial organisms, could also provide new insights into the evolutionary history of biological timekeeping functions, and lead to a better understanding of the adaptive value of endogenous clocks in different environments.

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Erklärung des Autors

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Erklärung gemäß §12 (2) b) der Promotionsordnung der Fakultät V (Mathematik & Naturwissenschaften) der Carl von Ossietzky Universität Oldenburg (Datum: 05.09.2014).

Ich erkläre hiermit,

1. dass die vorliegende Dissertation selbstständig verfasst wurde und alle genutzten Quellen angegeben wurden.
2. dass Teile der Dissertation bereits veröffentlich wurden. Der Status zur Veröffentlichung der Publikationen I, II und III ist am Anfang der jeweiligen Abschnitte (Sektionen 2, 3 und 4) angegeben.
3. dass die Dissertation weder in ihrer Gesamtheit noch in Teilen einer anderen Hochschule vorgelegt wurde.
4. dass der akademische Grad Dr. rer. nat angestrebt wird.
5. dass die Leitlinien guter wissenschaftlicher Praxis der Carl von Ossietzky Universität Oldenburg befolgt wurden.
6. dass beim Verfassen der Dissertation keinerlei kommerzielle Beratungsdienste in Anspruch genommen wurden.

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(Fabio Piccolin)

Appendix

A1 Supplementary material to Publication I

Table A1.1: List of sequences of primers and probes used to amplify all genes (clock genes, metabolic genes, housekeeper genes) included in Publication I.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe (5'-3')
Es*clk*	GGCCTCAGTTGGTACGAG AAATG	AATTTCCATTCTATACTGTG CCTTGATGT	TTGGCTCCAGAATCAT
Es*per*	TGAGGGTAAATTCAACAA TAAATGGAATACATCT	GAGTAACATCAACATTTTCC AACCAACT	ACCACCCAATTTTG
***pfk*6**	AGTTATAACAAGAGTTAA GCAGCAGTGT	CCCTGACATGCCAAAAATG CA	ATACCGACCAAAATCA
gys	GTTAGCCATGAGGCTGCA AATAAAG	AACTCTTCTACTGATGCACC ACATT	TCGGTGGCATCTACC
cs	ATGTCTCGCTGCTGCTATC TC	TCTTCACTAGTTATTGTTGA TGCCAGAA	CAAGTCCTCAAAATTC
hadh	CCGATTATGTTGGCCTCGA TGTATT	GGTCTTGAAAACTGGATTA TCCGGAAA	TCACGCTCCATTCCAC
acc	TGCAGACGCCTCAAGTTG A	GTTTGTTAGCGCCACAGTTT GT	AACAACGCCAGACCTT
atp	GTCAAGAACATCCAGAAG ATCACTCA	GCTTCAACTCCCTTTCAGCT CTT	CTGCTGCCAAGTTTGC
***usp*46**	TGGAACTGGTATTAACAG AGGACACT	ACTGCATCGTCATCAAAGA GCA	AAGAGCCACAGATTTT
***rps*13**	TGCTCAGGTTCGCTTTGTC A	CTCTGGGATATCTGGAGCA AGTC	CACACGCAGGATTTT

Es*clk* = *Euphausia superba* orthologue of the clock gene *clock*
Es*per* = *Euphausia superba* orthologue of the clock gene *period*
*pfk*6 = *phosphofructokinase*-6
gys = *glycogen synthase*
cs = *citrate synthase*
hadh = *hydroxyacyl*-CoA *dehydrogenase*
acc = *acetyl*-CoA *carboxylase*
atp = ATP *synthase gamma subunit*
*usp*46 = *ubiquitin carboxyl-terminal hydrolase* 46
*rps*13 = *ribosomal protein* S13.

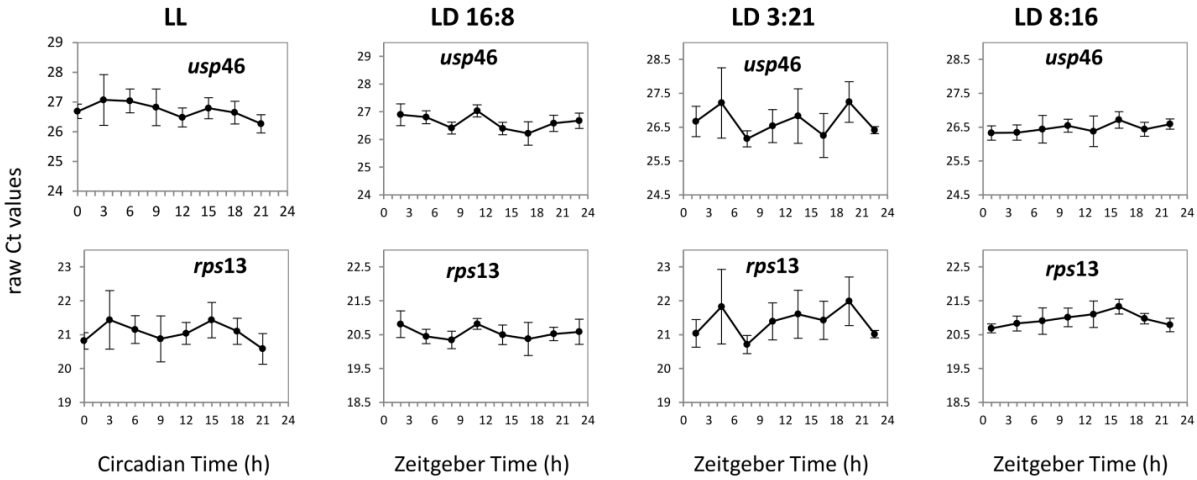

Figure A1.1: Changes in raw Ct (cycle threshold) values of candidate reference genes *usp*46 (*ubiquitin carboxyl-terminal hydrolase* 46) and *rps*13 (*ribosomal protein* S13) along the 24h cycle in the different photoperiodic conditions. LL = near-constant light (mid-summer); LD 16:8 = long day (early-autumn); LD 3:21 = near-constant darkness (mid-winter); LD 8:16 = short day (late-winter). Circadian Time 0 corresponds to 00:00 in mid-summer. Zeitgeber Time 0 corresponds to 04:00 in early-autumn, 10:30 in mid-winter and 08:00 in late-winter.

A2 Supplementary material to Publication II

Table A2.1: Summary information of genes included in Publication II. Sequences available at http://krilldb.bio.unipd.it/

Gene Name	Short Form	Forward Primer (5'-3')	Reverse Primer (5'-3')	Internal Probe (5'-3')	Sequence ID
citrate synthase	*cs*	ATGTCTCGCTGCTGCTATCTC	TCTTCACTAGTTATTGTTGATGCCAGAA	CAAGTCCTCAAAATTC	ESS076337
ATP *synthase subunit gamma*	*atp*	GTCAAGAACATCCAGAAGATCACTCA	GCTTCAACTCCCTTTCAGCTCTT	CTGCTGCCAAGTTTGC	ESS129614
phosphofructokinase-6	*pfk*6	AGTTATAACAAGAGTTAAGCAGCAGTGT	CCCTGACATGCCAAAAATGCA	ATACCGACCAAAATCA	ESS011313
acetyl-CoA *carboxylase*	*acc*	TGCAGACGCCTCAAGTTGA	GTTTGTTAGCGCCACAGTTTGT	AACAACGCCAGACCTT	ESS011644
glutamate dehydrogenase	*gldh*	CTGTTCAATCCTTGTCAAGATGCTT	TGTACTGGCACTGGCTTATATTGC	CCAAGCAACAATACCC	ESS028087
elongation factor 1-alpha	*ef*1a	TGGGCAAGGAAAAGATCCACATC	CTTGCCGGAGTCGACATGA	CCAACCACCACAATCG	ESS116888
clock	Es*clk*	GGCCTCAGTTGGTACGAGAAATG	AATTTCCATTCTATACTGTGCCTTGATGT	TTGGCTCCAGAATCAT	ESS034513
cryptochrome 2	Es*cry2*	CAGTGCTCAAGAACTTCCCAACTAA	GCGTCCTATGACACATTTAGACTGT	ACTGCACCAGAAAAT	ESS118469
timeless	Es*tim*	CAAGACAAAGCGAGATGGCATTT	AGGGTTGGAAGAAGGTTTTGTGAAA	TCGGCGTTCACTCTTC	ESS040526
rhodopsin 1a	Es*rh1a*	TCATTGATAAGCATTGGGCCAACT	GCCAAGGACATAGTGCCACAT	TCCCACCTGTGAACCC	ESS058227
rhodopsin 6	Es*rh6*	GTGTATGCCATTAGTCATCCCAAGT	ACTGAGCCATGGCATTTTCGT	CCGTGCTGCCCTCTAT	ESS092680
peropsin	Es*rrh*	CTGCTGTTGGTGCCATGATC	TGTGATGCACTACTTTCTCTTAGATTCAG	CTCCACAGGAACTTCT	ESS008171
ribosomal protein S13	*rps*13	TGCTCAGGTTCGCTTTGTCA	CTCTGGGATATCTGGAGCAAGTC	CACACGCAGGATTTT	ESS060818
ribosomal protein L32	*rpl*32	TCAAGCCTAACTGGCGTAAGC	TGACCCTTGAAGCGACGAC	CCTGTTGTCAATACCC	ESS059136
ubiquitin carboxyl-terminal hydrolase 46	*usp*46	TGGAACTGGTATTAACAGAGGACACT	ACTGCATCGTCATCAAAGAGCA	AAGAGCCACAGATTTT	ESS079224

The use of the prefix 'Es' in front of a gene name indicates that this is the specific orthologue form isolated in *Euphausia superba*.

A3 Supplementary material to Publication III

Table A3.1: Primer sequences used for the analysis of daily patterns of expression of clock genes in krill exposed to LD. Forward (fwd) and reverse (rev) primers are reported for each gene, together with the accession number of the corresponding sequence used for primer design. *Usp*46 was used as internal control during normalization, together with *spike*20 and *spike*25, which were added as external controls.

Target	Primer	Sequence (5'-3')	Accession number
Es*clock*	fwd	GGCCTCAGTTGGTACGAGAAATG	ESS034514
	rev	AATTTCCATTCTATACTGTGCCTTGATGT	
Es*cycle*	fwd	GCAGGATCAGATTGTGCGTCAA	ESS133965
	rev	TGCTATCTACACAGGAAGCTCTTCT	
Es*period*	fwd	TGAGGGTAAATTCAACAATAAATGGAATA CATCT	ESS133963
	rev	GAGTAACATCAACATTTTCCAACCAACT	
Es*timeless*	fwd	CAAGACAAAGCGAGATGGCATTT	ESS040526
	rev	AGGGTTGGAAGAAGGTTTTGTGAAA	
Es*cryptochrome2*	fwd	CAGTGCTCAAGAACTTCCCAACTAA	FM200054
	rev	GTCCTATGACACATTTAGACTGT	
Es*clockwork orange*	fwd	AAAACTTTGATAAACAAAACCTCTTTCAT C	ESS049812
	rev	GAGGGAGCTCATGACATGTGT	
Es*vrille*	fwd	GAAGTAGCTACACTTAAATACCTGTTGGT	ESS123359
	rev	CAAAACTATTCTAACGAGATCCATCGGA	
Es*e75*	fwd	CAGTCTGCTTCTGCTTCAACCT	ESS094384
	rev	GCCTTCTGACGGTGCTCTAC	
Es*doubletime*	fwd	AAAGAATAGAGCTTCAATATGTATATATT TAAAACAAAGT	ESS096455
	rev	TGAAAACAAGAAAAATTATAGAATCTTCT ATCCTAGATAAGG	
Es*shaggy*	fwd	GGTGGGTTGCGGAACATTG	ESS074789
	rev	TGGTCCACCACTGCCA	
usp-46	fwd	TGGAACTGGTATTAACAGAGGACACT	ESS079224
	rev	CTGCATCGTCATCAAAGAGCA	
spike20	fwd	TGCAATGATGATAACCGTTCCCTTTAA	XM_017004857.1
	rev	CCAGATATGCTTGAATTGGATCACCT	
spike25	fwd	GCTGGGACCTAGTGTCAAGTAC	XM_011537537.1
	rev	TGGAGTAACCATGCTAGATTAAAGAAATA CAATT	

The use of the prefix 'Es' in front of a gene name indicates that this is the specific orthologue form isolated in *Euphausia superba*.