UNIVERSITY NAME

DOCTORAL THESIS

Seasonal, Physiological and Genetic Functions in Antarctic Krill, *Euphausia superba*, at Different Latitudes in the Southern Ocean

---

Author:
Flavia HÖRING

Supervisor:
Dr. Bettina MEYER

A thesis submitted in fulfillment of the requirements
for the degree of Doktor der Naturwissenschaften

in the

Research Group Name
Department or School Name

March 10, 2019

Declaration of Authorship

I, Flavia HÖRING, declare that this thesis titled, "Seasonal, Physiological and Genetic Functions in Antarctic Krill, *Euphausia superba*, at Different Latitudes in the Southern Ocean" and the work presented in it are my own. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University.
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.
- Where I have consulted the published work of others, this is always clearly attributed.
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.
- I have acknowledged all main sources of help.
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself.

Signed:

_____

Date:

_____

"Thanks to my solid academic training, today I can write hundreds of words on virtually any topic without possessing a shred of information, which is how I got a good job in journalism."

Dave Barry

UNIVERSITY NAME

Abstract

Faculty Name
Department or School Name

Doktor der Naturwissenschaften

Seasonal, Physiological and Genetic Functions in Antarctic Krill, *Euphausia superba*, at Different Latitudes in the Southern Ocean

by Flavia HÖRING

The Thesis Abstract is written here (and usually kept to just this page). The page is kept centered vertically so can expand into the blank space above the title too...

Acknowledgements

The acknowledgments and the people to thank go here, don't forget to include your project advisor…

Contents

List of Figures

List of Tables

List of Abbreviations

LAH	**L**ist **A**bbreviations **H**ere
WSF	**W**hat (it) **S**tands **F**or

Physical Constants

Speed of Light $c_0 = 2.997\,924\,58 \times 10^8\,\mathrm{m\,s^{-1}}$ (exact)

List of Symbols

a	distance	m
P	power	W ($\mathrm{J\,s^{-1}}$)
ω	angular frequency	rad

For/Dedicated to/To my...

Chapter 1

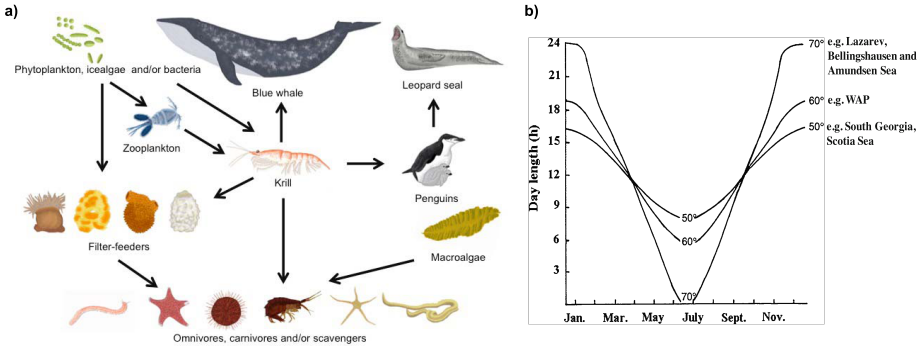
General Introduction

In this section, I would like to introduce the scientific background for this dissertation which studied seasonal, physiological and genetic functions in Antarctic krill, *Euphausia superba*, under different latitudinal light regimes. First, I will explain the importance of *E. superba* in the Southern Ocean ecosystem, the impact of fisheries, as well as the effects of climate change and potential phenological mismatches on this species. Afterwards, I will summarize findings from field studies that show that Antarctic krill has evolved pronounced seasonal cycles of physiology and behaviour to adapt to the highly variable environment in the Southern Ocean. Then, I will integrate information about the seasonal timing system in Antarctic krill including the role of environmental cues, the potential mechanisms of the endogenous timing system and the neuroendocrine control. In a final step, I will explain the gaps of knowledge, the research objectives of this dissertation, and the methodological approach.

Euphausia superba, a key organism in the Southern Ocean

With an estimated circumpolar biomass of 379 Mt (Atkinson et al., 2009), Antarctic krill (*Euphausia superba* Dana, 1850) belongs to one of the most abundant organisms on Earth. Antarctic krill is a key organism in the Southern Ocean food web linking primary production to higher trophic levels such as whales, penguins, birds and seals (Fig. 1.1). *Euphausia superba* is distributed over a large latitudinal range from approximately 50°S to more than 70°S (Hill et al., 2013). These different latitudinal habitats are characterized by extreme seasonal and regional fluctuations of photoperiod, primary production and sea ice extent (Quetin and Ross, 1991). Since

FIGURE 1.1: a) Simplified version of the Antarctic food web by Balana (2013) licenced under CC BY-NC-ND 3.0 ES; b) Latitudinal light regimes in different regions of the Southern Ocean by Meyer (2012) licenced under CC BY 4.0.



Antarctic krill is able to travel great distances within one year, either by active migration (Siegel, 1988) or passive transport within ocean currents (Thorpe et al., 2007), it seems to be highly flexible in adjusting it's phenology to both the high annual and regional variability of environmental factors in the different latitudinal habitats of the Southern Ocean.

Commercial interest in Antarctic krill and fishery are growing considering its high biomass, improved harvesting techniques and the increasing demand for newly developed krill products (Nicol et al., 2012). Antarctic krill is considered of high nutritional value when used as 'krill meal' in aquaculture (Yoshitomi et al., 2007) and for the production of dietary supplements and pharmaceutical products because of their suggested beneficial properties for human health (Tou et al., 2008). Krill fishery in the Southern Ocean is currently managed by the Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR). However, sustainable fishery's management is largely dependent on our current understanding of the Southern Ocean ecosystem and the correct prediction of krill abundances under changing environmental conditions in the future.

Climate change may pose another threat to Antarctic krill. It has been predicted that global warming and associated changes in chlorophyll concentration will reduce the favourable growth habitat of Antarctic krill, and thereby its biomass in the Southern Ocean (Hill et al., 2013). In the Southwest Atlantic Sector, it has been reported that Antarctic krill densities have declined associated with a southward shift of *E. superba*'s distribution and an increase in salp densities in that region (Atkinson et al., 2004, 2019). This shift has been explained by changes in sea ice extent (Atkinson et al., 2004) and anomalies of the Southern Annular Mode (Atkinson et al., 2019).

Changes in Antarctic krill biomass have been linked to the survival of upper-level predators such as penguins which indicates that climate change may lead to profound changes in the Southern food web (Trivelpiece et al., 2011).

Climate change effects in the Southern Ocean are especially relevant under the match/mismatch hypothesis that describes how the seasonal timing of a predator and the availability of its prey may affect recruitment, reproduction or survival of the predator (Durant et al., 2007). In particular, Antarctic krill may be influenced by temporal and spatial changes in phytoplankton distribution that do not match its seasonal requirements for recruitment or reproductive processes. However, these effects are difficult to predict, because the mechanisms and the flexibility of Antarctic krill's seasonal timing system are poorly understood.

Pronounced Seasonal Cycles of *E. superba* in the Field

Pronounced seasonal variations of growth, feeding, metabolic activity (Meyer et al., 2010), lipid turnover (Ericson et al., 2018; Hellessey et al., 2018), reproduction (Siegel, 2012) and gene expression (Seear et al., 2012) have been observed in Antarctic krill in the field (Fig. 1.2). Antarctic krill has evolved different overwintering strategies to cope with the conditions of near-constant darkness and low food availability in some regions of the Southern Ocean (Meyer, 2012). The by far most important strategy is the reduction of physiological functions consistent with a state of quiescence observed in Antarctic krill. Respiration is severely reduced during the winter season and may drop down to 30% of the summer rates (Meyer et al., 2010; Quetin and Ross, 1991). Moreover, a significantly lower activity of the metabolic key enzymes citrate synthase (Cullen et al., 2003; Meyer et al., 2002) and malate dehydrogenase (Meyer et al., 2010; Pape et al., 2008) was found in autumn and winter. In the course of winter-metabolic depression, low feeding activity has been observed, with indications that Antarctic krill can switch to alternative food sources like zooplankton or ice-algae (Atkinson et al., 2002; Meyer et al., 2010). As a consequence, growth rates of adult krill are extremely reduced during winter (Kawaguchi et al., 1986; Meyer et al., 2010), even shrinkage has been reported (Quetin and Ross, 1991). The seasonal accumulation of lipid stores and their utilisation during winter promotes survival of adult krill during periods of low food availability (Falk-Petersen et al., 2000; Hagen et al., 2001; Meyer et al., 2010; Quetin and Ross, 1991). Moreover, Antarctic krill

shows a pronounced seasonal cycle of maturity which is characterized by the regression of its external sexual traits towards winter and the sexual re-maturation towards spring (Kawaguchi et al., 2007). Gene expression analysis of seasonal Antarctic krill samples near the Antarctic Peninsula revealed an upregulation of genes related to feeding and digestion, respiration, motor activity, immunity and vitellogenesis in summer krill with respect to winter (Seear et al., 2012).

Antarctic krill is able to synchronize its seasonal life cycle to local photoperiod and food supply in the different latitudinal habitats of the Southern Ocean. Regional differences in the timing of reproduction (Spiridonov, 1995), growth (Kawaguchi et al., 2006), feeding activity and lipid storage (Schmidt et al., 2014), and gene expression (Seear et al., 2012) have been observed in the field. Spiridonov (1995) investigated the spawning season of Antarctic krill in different regions of the Southern Ocean and discussed that the reproductive timing is largely dependent on the variation of seasonal sea ice cover and the timing of phytoplankton blooms.

Kawaguchi et al. (2006) reported differences in the growth period of Antarctic krill examining the Southwest Atlantic Sector and the Indian Ocean sector. The authors suggested that the earlier timing of the phytoplankton bloom at lower latitudes might advance the growth period of Antarctic krill. Differences in the overwintering behaviour of Antarctic krill were observed by Schmidt et al. (2014) and Seear et al. (2012) in different latitudinal habitats of the Southern Ocean. Antarctic krill from the low-latitude region South Georgia had higher feeding activities and lower lipid stores during winter compared to the high-latitude region Lazarev Sea where Antarctic krill experienced near-constant darkness and the strongest limitation in food supply (Schmidt et al., 2014). Similar differences were found on the gene expression level, where winter Antarctic krill from South Georgia showed higher gene activities related to feeding, digestion and immunity with respect to krill from the Antarctic Peninsula (Seear et al., 2012).

Seasonal timing mechanisms in *E. superba*

The variable light, food, and temperature regimes may play a role in the regulation of *E. superba*'s seasonal cycles in the different latitudinal habitats of the Southern Ocean (Fig. 1.3). Controlled laboratory experiments were conducted to unravel the specific effects of these parameters on the different seasonal processes in Antarctic

FIGURE 1.2: Schematic representation of seasonal cycles of photoperiod and primary production in the Southern Ocean, and sexual maturity, respiration and lipid content of Antarctic krill following information from Meyer 2012, Arrigo et al. 2008, Kawaguchi et al. 2007, and Hellessey et al. 2018

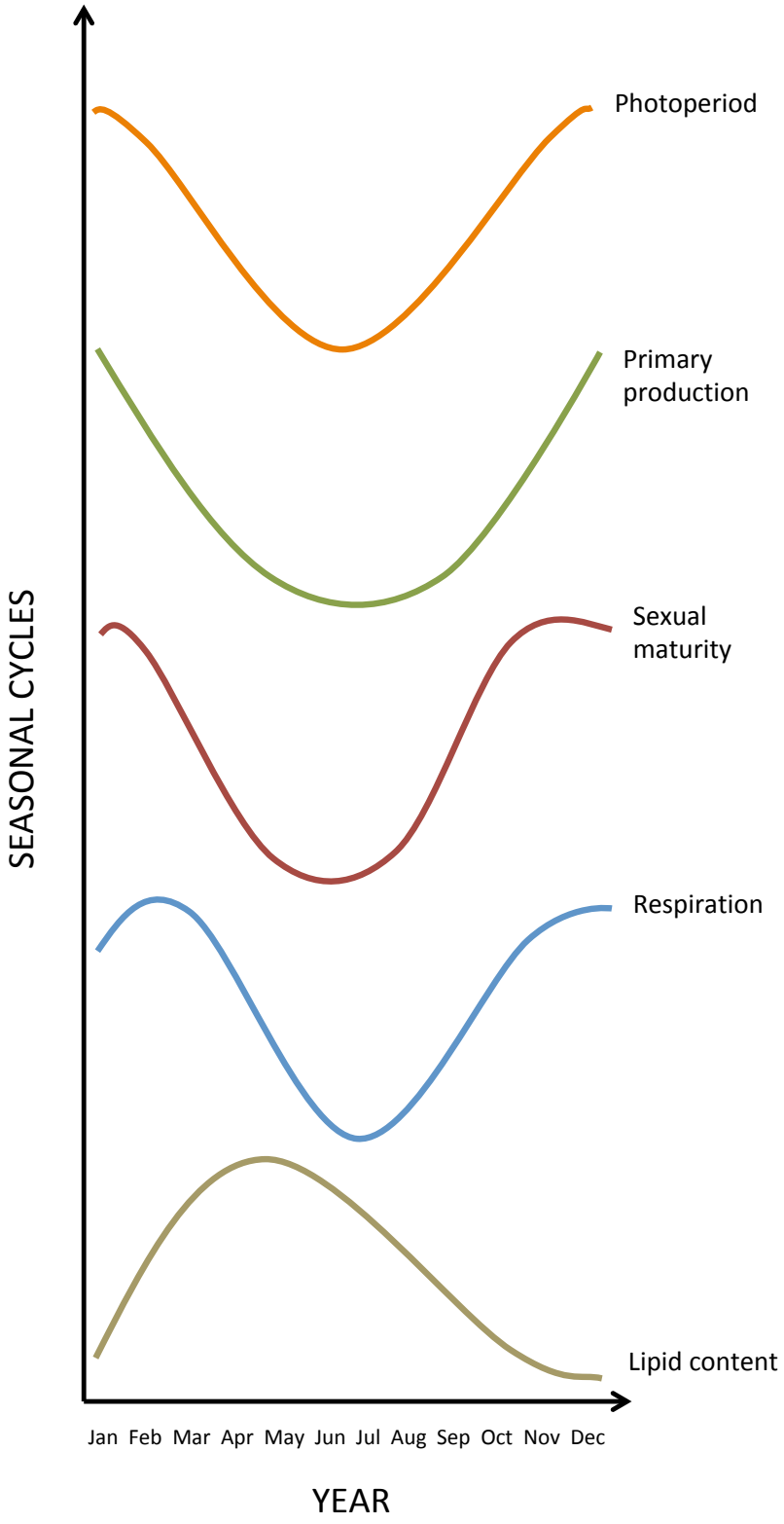
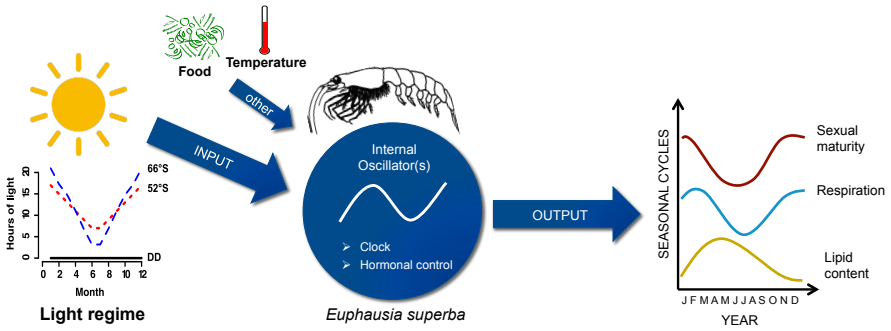


FIGURE 1.3: Schematic representation of the seasonal timing system of Antarctic krill.



krill. Higher water temperatures and high food supply were found to increase the growth rates of Antarctic krill (Brown et al., 2010; Buchholz, 1991), whereas seasonal changes of photoperiod modulated a seasonal growth pattern (Piccolin et al., 2018a). Kawaguchi et al. (2007) found that favourable feeding conditions accelerate the sexual maturation process of Antarctic krill. The initiation of sexual maturation and spawning were also observed when exposing Antarctic krill to long photoperiods (Hirano et al., 2003; Teschke et al., 2008). These long photoperiodic conditions also triggered an enhanced lipid catabolism that was suggested to be necessary for the maturation process (Teschke et al., 2008). During long-term laboratory experiments of constant food supply, different simulated light regimes flexibly adjusted the seasonal cycle of maturity in Antarctic krill (Brown et al., 2011). The metabolic activity of Antarctic krill was observed to be higher under long photoperiods similar to summer and autumn conditions compared to the simulated winter condition 'constant darkness' (Teschke et al., 2007), while the same study indicated that Antarctic krill might not be able to respond to high food concentrations under constant darkness. Although food supply and temperature have the potential to affect the respiration rates of Antarctic krill, these factors do not change the general seasonal pattern of metabolic activity (Brown et al., 2013). Recently, Piccolin et al. (2018a) confirmed in long-term experiments that a simulated annual light regime could trigger the seasonal cycle of metabolic activity in Antarctic krill. These seasonal photoperiodic effects on krill's metabolic cycle were also found on gene expression level (Piccolin et al., 2018a; Seear et al., 2009).

These studies reveal that light regime and seasonal changes in photoperiod are major cues that entrain the seasonal rhythms of growth, maturity, metabolic activity and gene expression in Antarctic krill. It has been suggested that these seasonal

rhythms are controlled by an endogenous timing system with photoperiod as timing cue (zeitgeber) (Brown et al., 2011, 2013; Piccolin et al., 2018a). An endogenous timing system (circannual clock) is characterized by the observation that seasonally rhythmic patterns persist, even if the actual zeitgeber is not present (Visser et al., 2010). Such evidence was found in Antarctic krill during long-term laboratory experiments where seasonal patterns of maturity, growth and metabolic activity were observed under constant darkness (Brown et al., 2011, 2013; Piccolin et al., 2018a).

In general, the molecular mechanisms of seasonal timing systems are poorly understood, but there are indications that the circadian (daily) clock may play role for photoperiodic time measurement and consequently the timing of seasonal life cycle events (Helm et al., 2013). In eukaryotes, the circadian clock functions as an approximately 24-h oscillator via interlaced transcriptional/posttranslational feedback loops that are synchronized by an environmental factor such as photoperiod (Mackey, 2007). In insects, circadian clock genes were found to play an important role for the seasonal photoperiodic timing of diapause (review by Goto, 2013; Meuti and Denlinger, 2013; Meuti et al., 2015), with exceptions (e.g. Emerson et al., 2009). The investigation of latitudinal clines of photoperiodism showed that insect populations generally showed higher critical photoperiods for the initiation of diapause at higher latitudes, and latitudinal adaptation of their photoperiodic response has been linked to clock gene polymorphisms (Hut et al., 2013). Photoperiodic plasticity may also be based on the differential expression of clock genes (Hodkova et al., 2003) depending on season and latitude. Moreover, it is speculated, if non-coding RNA or epigenetic modifications play a role in the regulation of circannual rhythms (Helm and Stevenson, 2014).

In Antarctic krill, molecular studies have analysed the functioning of its visual perception system and its circadian clock (Biscontin et al., 2016, 2017). Different opsin genes were identified in Antarctic krill that are important for the visual perception of light of different wavelengths (Biscontin et al., 2016). The circadian clock machinery of Antarctic krill was characterized by Biscontin et al. (2017) who found that the core clock resembled both insect's and mammalian circadian clock systems with a light mediated degradation mechanism that suggested light as the main zeitgeber. Controlled laboratory experiments revealed that the daily oscillations of clock genes in Antarctic krill varied under variable seasonal conditions of photoperiod and became arrhythmic under mid-winter and mid-summer conditions (Piccolin et

al., 2018b). Antarctic krill's circadian clock has not only been linked to the timing of daily rhythms of metabolic activity (De Pittà et al., 2013; Piccolin et al., 2018b; Teschke et al., 2011) and diel vertical migration (Gaten et al., 2008), but it is also suggested to play a role for the seasonal day length measurement and the regulation of seasonal rhythms in Antarctic krill based on the observation of seasonal patterns of clock gene expression in Antarctic krill (Piccolin et al., 2018a).

In crustaceans, circadian pacemakers (clocks) are located in the nervous system, in particular in retina of the eye, the eyestalk, the brain and the caudal photoreceptor (Arechiga and Rodriguez-Sosa, 2002; Rodríguez-Sosa et al., 2008) and may be linked to neuroendocrine control of seasonal rhythms. Seasonal life cycle events in decapods are mediated by various neuropeptides and signalling molecules that originate from the X-organ-sinus gland system of the eyestalk ganglia, brain, the thoracic ganglia, the Y-organ and the mandibular organ (Nagaraju, 2011). Important hormones comprise the 'CHH-superfamily' including the crustacean hyperglycaemic hormone, the moult-inhibiting hormone, the gonad/vitellogenesis-inhibiting hormone and the mandibular organ-inhibiting hormone that have multiple functions in carbohydrate and lipid metabolism, reproduction, moulting, osmoregulation and the regulation of methyl farnesoate synthesis from the mandibular organ (review by Webster et al., 2012). Other hormones that control moulting and reproduction in crustaceans include methyl farnesoate (Reddy et al., 2004), ecdysteroids and 'vertebrate-type' steroids (Lafont and Mathieu, 2007), and prostaglandins (review by Nagaraju, 2011).

In Antarctic krill, studies on the neuroendocrine control of seasonal processes are rare (Buchholz, 1991; Pape et al., 2008; Seear et al., 2012; Toullec et al., 2013). Buchholz (1991) studied changes in hemolymph titre of ecdysone-equivalents in the different moult stages of Antarctic krill. Pape et al. (2008) were not able to detect melatonin in Antarctic krill and therefore rejected the hypothesis that it played a role in regulating the seasonal metabolic cycle of Antarctic krill (Teschke et al., 2007). In the field, seasonal gene expression patterns of the neuropeptide neuroparsin and insulin-like peptides have been discussed in relation to the seasonal reproductive physiology of Antarctic krill (Seear et al., 2012). In the ice krill, Euphausia crystallorophias, various neuropeptide hormones were identified including members of the CHH superfamily (Toullec et al., 2013). The recently developed transcriptome

database may provide a source for *E superba*-specific target sequences of neuropeptides (Sales et al., 2017).

Research objectives

The current environmental changes in the Southern Ocean and the increasing commercial interest in Antarctic krill emphasise the need to better understand the adaptability of *E. superba* in different latitudinal regions of the Southern Ocean, especially under the aspect of potential mismatches in biological timing. It has not yet been investigated, if different latitudinal light regimes regulate the flexible seasonal physiology and behaviour of Antarctic krill in its diverse latitudinal habitats. In general, the seasonal timing system of Antarctic krill is not well understood including the potential involvement of the circadian clock and the neuroendocrine control of seasonal rhythms in Antarctic krill. Laboratory experiments that observe the seasonal cycle of Antarctic krill under controlled photoperiodic conditions over a period of multiple years and simulating different latitudinal light regimes are still lacking.

This dissertation aimed to understand the role of different latitudinal light regimes on the seasonal cycle of Antarctic krill focussing on (a) the molecular characterization of seasonal rhythms in different latitudinal habitats in the field (Publication 1), and (b) the investigation of seasonal rhythms under a two-year photoperiodic-controlled laboratory experiment with constant food supply (Publication 2 & 3). The following research objectives were addressed in the three different chapters of the dissertation:

1. Investigation of seasonal and regional differences in gene expression of summer and winter Antarctic krill from three different latitudinal regions: South Georgia (54°S), South Orkneys/Bransfield Strait (60°S-63°S) and Lazarev Sea (62°S-66°S) (Publication 1)

2. Analysis of seasonal cycles of growth, feeding, lipid metabolism and maturity of Antarctic krill under the simulated light regimes 52°S, 66°S, and constant darkness (Publication 2)

3. Characterization of seasonal expression patterns of genes involved in different metabolic processes, seasonal timing, reproduction, feeding and development

under the simulated light regimes 52°S, 66°S, and constant darkness (Publication 3)

A range of different methods were implemented to investigate the effect of latitudinal light regime on Antarctic krill. An RNAseq approach was used to characterize the seasonal and latitudinal gene expression differences in Antarctic krill in the field and to identify suitable seasonal target genes with focus on genes with potential regulatory functions in the seasonal cycle of Antarctic krill. For the first time, a two-year laboratory experiment was conducted that simulated different latitudinal light regimes and constant food supply. Antarctic krill from the laboratory experiments was investigated using morphometric and lipid content analysis, and gene expression data from custom designed TaqMan cards.

The findings of this dissertation improve our understanding of the effect of different latitudinal light regimes on seasonal cycles in Antarctic krill and their underlying molecular mechanisms.

Chapter 2

Seasonal gene expression profiling of Antarctic krill in three different latitudinal regions

Flavia Höring, Lars Harms, Cristiano De Pittà, Gabriele Sales, Christian Reiss, Bettina Meyer

Ready to be submitted to the journal Marine Genomics

Abstract

The key organism Antarctic krill, *Euphausia superba*, has evolved seasonal rhythms of physiology and behaviour to survive under the extreme photoperiodic conditions in the Southern Ocean. However, the molecular mechanisms generating these rhythms remain far from understood. The aim of this study was to investigate seasonal and regional differences in gene expression in three different latitudinal regions with variable photoperiodic conditions (South Georgia, South Orkneys/Bransfield Strait, Lazarev Sea) and to identify genes with potential regulatory roles in the seasonal life cycle of Antarctic krill. The RNAseq data were analysed (a) for seasonal differences between summer and winter field samples from each region, and (b) for regional differences within each season. In general, we found an upregulation of gene expression in summer krill in all regions with respect to winter. However, seasonal differences in gene expression were less pronounced in Antarctic krill from South Georgia where most genes related to metabolic, biological and regulatory processes were not found to be differentially expressed between summer and winter krill. We also identified genes with putative regulatory roles, for instance genes related to hormone

metabolism and signalling, reproductive and developmental processes. Our results suggest that Antarctic krill entered a state of metabolic depression and regressed development (so called winter quiescence) in South Orkneys/Bransfield Strait and Lazarev Sea region in winter. The winter quiescence seems to be less pronounced in the South Georgia region, most likely due to the milder seasonal conditions, the less extreme light regime in this low-latitude region, and hence food availability. These findings including the proposed target genes provide a basis for future laboratory studies of the molecular mechanisms of seasonal rhythms in Antarctic krill.

2.1 Introduction

Seasonal rhythms of physiology and behaviour are essential for the survival of marine organisms inhabiting regions with extreme seasonal changes of photoperiod (day length) like the Southern Ocean. Antarctic krill, *Euphausia superba*, holds a pivotal position in the Southern Ocean food web where it is a major link between primary production and higher trophic levels. It has been proposed that Antarctic krill may serve as a polar model organism to study the effects of climate change in the polar ecosystem of the Southern Ocean (Meyer, 2010). For that purpose, we want to understand the mechanisms of its seasonal life cycle including its flexibility under changing environmental conditions.

In the field, pronounced seasonal differences have been found in the Antarctic krill's body composition, metabolic activity, feeding, growth (Meyer et al., 2010) and maturity (Siegel, 2012). Survival in periods of near-constant darkness and low food availability is accomplished by different overwintering strategies. These include the accumulation of lipid reserves during summer and the reduction of metabolic activity, feeding activity and growth (Meyer, 2012) and sexual regression during winter.

Only few studies have investigated regional differences in the life cycle of krill such as the timing of reproduction (Spiridonov, 1995) and growth (Kawaguchi et al., 2006). Overwintering strategies seem to vary according to latitudinal habitat as krill near South Georgia was observed to have lower lipid stores and higher feeding activity in winter compared to higher latitudinal regions (Schmidt et al., 2014). Seasonal and regional differences in gene expression were found, for the first time, by Seear et al. (2012) who investigated seasonal effects near the Antarctic Peninsula (60°S) and spatial differences in winter comparing the Antarctic Peninsula and

South Georgia (54°S) region. This study concluded that genes involved in feeding and digestion, respiration, motor activity, immunity and vitellogenesis were upregulated in krill sampled in the Peninsula region during summer with respect to winter. The regional comparison of winter krill revealed an upregulation of genes related to feeding and digestion and immunity at South Georgia compared to the Peninsula region.

The seasonal cycle of Antarctic krill is influenced by different environmental factors such as light regime, food availability and/or temperature that may contribute to krill's flexible behaviour in different latitudinal regions. Controlled lab experiments have demonstrated the effect of temperature and food supply on krill growth (Buchholz 1991) and maturity (Kawaguchi et al. 2007). Photoperiod has been shown to affect feeding and metabolic activity (Teschke et al., 2007), growth (Brown et al., 2010), maturity (Brown et al., 2011) and gene expression (Seear et al., 2009) in Antarctic krill under laboratory conditions. Based on the photoperiodic studies, it has been suggested that seasonal rhythms in Antarctic krill are governed by an endogenous timing system with photoperiod as Zeitgeber. Recently, it has been confirmed in a two-year lab experiment that krill's seasonal rhythms seem to be affected by different latitudinal light regimes (Höring et al., 2018).

Studies on the molecular mechanisms of the endogenous timing system in Antarctic krill have mostly focused on daily rhythms, the circadian clock and the photoperception system in *E. superba* (Biscontin et al., 2016, 2017; De Pittà et al., 2013; Piccolin et al., 2018a). Biscontin et al. (2016) identified the opsin repertoire of Antarctic krill which may contribute to the perception of daily and seasonal changes in irradiance and spectral composition in the Southern Ocean. Antarctic krill possesses an ancestral circadian clock machinery with both insect- and vertebrate like features and a light mediated entraining mechanism (Biscontin et al., 2017). It has been suggested that krill's circadian clock does not only control daily rhythms in *E. superba*, but may also be involved in the timing of seasonal life cycle events (Piccolin et al., 2018b).

The flexibility of krill's seasonal cycle in different latitudinal regions and the underlying molecular mechanisms are still poorly understood. Current knowledge on the seasonal behaviour of Antarctic krill in the field is based on single observations and the analysis of few regions, whereas data from the winter season is generally less frequent. Even though extensive transcriptome studies have been conducted in

E. superba (Meyer et al., 2015; Sales et al., 2017), we still lack a comprehensive understanding of the molecular pathways that contribute to the regulation of seasonal rhythms in Antarctic krill in different latitudinal regions of the Southern Ocean.

This paper aims to investigate seasonal and regional differences in gene expression in Antarctic krill in three different latitudinal regions of the Southern Ocean: South Georgia (54°S), South Orkneys/Bransfield Strait (60°S-63°S) and Lazarev Sea (62°S-66°S). An RNA-seq approach is used to test for (1) seasonal differences in gene expression between summer and winter krill from each region, and (2) regional differences in gene expression between the three different regional krill samples from each season. The RNA-seq data is analysed with the goal to identify seasonal target genes with putative regulatory functions in the seasonal life cycle of Antarctic krill.

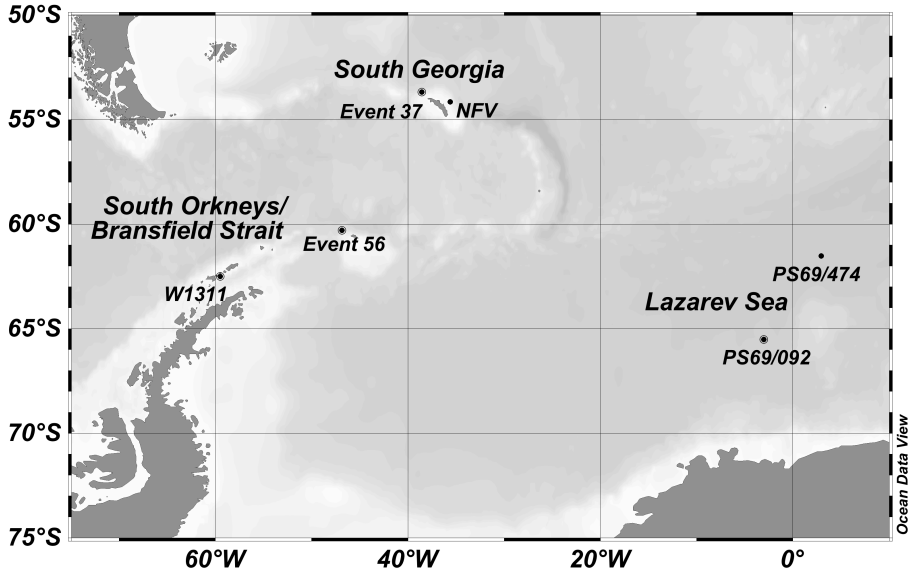
2.2 Methods

Sample collection and experimental design

Antarctic krill samples (*Euphausia superba*) were obtained from five different expeditions and from a Norwegian fishing vessel (Table I, Fig. 2.1. Sampling was carried out with a Rectangular Midwater Trawl (RMT8+1 for expeditions ANT23-2, ANT23-6 and JR15004, RMT8 for expedition JR260B), an Isaacs-Kidd Midwater Trawl (IKMT, expedition AMLR14) and a continuous pumping system (Norwegian fishing vessel). Snap-frozen Antarctic krill samples stored at $-80\,^{\circ}\mathrm{C}$ were transferred to the Alfred-Wegener-Institute, Bremerhaven, for molecular analysis.

The Antarctic krill originated from three different latitudinal regions: a) Lazarev Sea (62°S-66°S), b) South Orkneys/Bransfield Strait (60°S-63°S), and c) South Georgia (54°S), including summer and winter samples for each region. By visual inspection of the outer sexual organs, male petasma and female thelycum, adult males and females were identified. In total, 36 individuals were chosen for further analysis, with 6-7 individuals for each regional and seasonal sample including 3-4 females and 3 males (except the South Georgia winter sample, where solely males were available, and 5 males were analysed; see Table I for full sampling scheme).

FIGURE 2.1: Station map indicating station numbers (NFV - Norwegian fishing vessel) and the three studied regions



RNA extraction, library preparation and Illumina sequencing

RNA extraction was performed from frozen krill heads with the RNeasy Midi Kit (QIAGEN, Hilden, Germany). Frozen krill heads were cut on dry ice and transferred to 1.5 ml RLT lysis buffer in tissue homogenizing Precellys® tubes (CKMix Tissue Homogenizing Kit, Bertin corp., Rockville, MD, USA). Homogenization was carried out at 4 °C in a Precellys® homogenizer with the Cryolys® cooling system (Bertin corp.) with two runs for 15 s at 5000 rpm and 10 s break. Further steps of RNA extraction were carried out according to the manufacturer's protocol of the RNeasy Midi Kit. The quality and quantity of the RNA was inspected using the NanoDrop™2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the Agilent 2100 Bioanalyzer system (Agilent technologies, Santa Clara, CA, USA).

RNA samples were sent for sequencing to IGA Technology Services (Udine, Italy). cDNA libraries were performed with 1 µg to 2 µg RNA by using the TruSeq Stranded mRNA Sample Prep kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions. The poly-A mRNA was fragmented 3 minutes at 94 °C. 1X Agencourt AMPure XP beads (Agencourt Bioscience Cooperation, Beckman Coulter, Beverly, MA, USA) were used for every purification step. The RNA samples and final cDNA libraries were quantified with the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and quality tested by Agilent 2100 Bioanalyzer Nano assay. For

cluster generation on the flow cell, libraries were processed with cBot (Illumina, San Diego, CA, USA) following manufacturer's instructions. Sequencing was carried out on paired-end mode (2 × 100 bp) on HiSeq2500 (Illumina) with a targeted sequencing depth of about 80 million reads per sample. Raw data were processed with the software `CASAVA v1.8.2` (Illumina) for both format conversion and de-multiplexing.

Quality control and analysis of RNA-seq data

The programme `BBDuk` from `BBMap package v36.38` (Bushnell, 2016) was used for the removal of adapter sequences and quality trimming of reads (set parameters: ktrim=r, k=23, mink=11, hdist=1, tpe tbo, qtrim=r, trimq=10, minlen=36). The quality of the trimmed reads was checked with the programme `FastQC v0.11.5` (Andrews, 2017). Since the `FastQC` reports indicated the presence of reads encoding ribosomal RNA, these reads were removed using the software `SortMeRNA v2.1` (Kopylova et al., 2012). Transcript abundance in each sample was estimated by aligning the processed paired-end reads to the *E. superba* reference transcriptome (Meyer et al., 2015) using the software `Trinity v2.4.0` (Grabherr et al., 2011) with the abundance estimation method `RSEM v1.2.26` (Li and Dewey, 2011) and the alignment tool `Bowtie2 v2.2.5` (Langmead and Salzberg, 2012). As reference, we chose the transcriptome by Meyer et al. (2015) instead of the recently developed KrillDB transcriptome (Sales et al., 2017), because preliminary alignment tests yielded approximately 10% higher alignment rates to the transcriptome by Meyer et al. (2015). Both transcript expression matrix of non-normalized counts and matrix of TMM-normalized expression values were calculated. Mapping rates to the reference transcriptome had a mean ± SD of 69.22 ± 3.37%. Differential gene expression was analysed with `edgeR` (Robinson et al., 2010). Significant differentially expressed genes (DEGs) were identified using a false discovery rate (FDR) cutoff value of 0.001 and a minimum absolute Log2 fold change (log2FC) of 2. Pairwise comparisons included seasonal comparisons within each region and regional comparisons in both summer and winter between regions (Fig. 2). The PtR script was used to do a principal component analysis (PCA) of all differentially expressed transcripts. Both PC 1 and PC 2 were correlated to season accounting for overall 46.89% variation in the dataset (supplementary Figure S1), but did not show correlation to region, sex or sample processing.

To annotate the DEGs, local blastx searches against the protein UniProt databases Swiss-Prot and UniRef90 (Boutet et al., 2007) with a cutoff E value of 10^{-9} were performed using `BLAST+ v.2.5.0` (Camacho et al., 2009). From the 1929 DEGs, 693 genes could be annotated resulting in an annotation rate of 35.93%. Additional annotation information were retrieved from the UniProt website (https://www.uniprot.org/). To aim for a crustacean-specific annotation and functional characterization, we chose to do a manual categorization of the annotated genes rather than focussing on the enrichment of gene ontology (GO) terms. Thereby, we were also able to improve the functional characterization of DEGs for the regional and seasonal comparisons where only few DEGs were found (Table II). Using the information from the UniProt website and crustacean-specific literature, if available, the annotated genes were inspected and sorted manually into categories (supplementary Table S1). For selected genes of interest, the annotation was reviewed performing blastx searches against NR using the web interface on https://blast.ncbi.nlm.nih.gov/Blast.cgi (Johnson et al., 2008). For contigs HACF01031034, HACF01033533, HACF01010344 and HACF01005894, improved annotation results were added to the annotation table (supplementary Table S1). For Fig. 3 and supplementary Figure S2 , category normalization was carried out by dividing the DEG counts per category for upregulated genes per sample by the size of the respective category (total count of DEGs within the same category). Normalized categories are shown in normalized units ranging from 0 to 1 indicating a higher importance of categories with increasing values towards 1. Top categories were defined for values higher or equal to 0.2.

Data archiving

Raw sequences and the transcript expression matrix of non-normalized counts have been deposited in the ArrayExpress database at `EMBL-EBI` (`www.ebi.ac.uk/arrayexpress`) under accession number `E-MTAB-7467`.

2.3 Results

Seasonal comparisons of gene expression in three different latitudinal regions

Highest differential gene expression was found in the seasonal pairwise comparisons in the three regions with 295 to 1121 DEGs which were mostly upregulated in summer krill (Table II). Most DEGs were found in the winter-summer krill comparison of the South Orkneys/Bransfield Strait region, followed by Lazarev Sea and South Georgia. Krill from the South Georgia region had the lowest seasonal differences in gene expression compared to the other regions.

The highest functional variety of DEGs was found to be upregulated in summer krill from the South Orkneys/Bransfield Strait region (Fig. 3a). The 19 top categories comprised bioluminescence (1), detoxification (0.92), proteolysis (0.91), metabolism related to bioactive lipids (0.9), digestion (0.87), hormone metabolism (0.83), visual perception (0.79), receptor-related proteins (0.78), amino acid metabolism (0.75), carbohydrate and lipid metabolism (0.74 each), development and reproduction (0.69 each), immune response and dephosphorylation (0.67 each), transport (0.63), transcriptional regulation (0.6), translation (0.43) and cytoskeleton (0.21). Thereof, the first 8 categories were particularly distinct for South Orkneys/Bransfield Strait summer krill with respect to the other two studied regions.

For Lazarev Sea summer krill, 13 top categories were found: amino acid metabolism and transcriptional regulation (0.5 each), energy metabolism (0.45), reproduction (0.43), development (0.41), carbohydrate and lipid metabolism (0.4 each), muscle development & regulation and dephosphorylation (0.33 each), transport (0.29), immune response (0.27), cytoskeleton (0.26) and proteolysis (0.2). Compared to summer krill from the other two studied regions, the categories energy metabolism and muscle development & regulation had highest values in Lazarev Sea summer krill.

For South Georgia summer krill, only three top categories were identified: translation (0.64), immune response (0.33) and transcriptional regulation (0.2). Compared to the other two regions, the category translation was most pronounced in South Georgia summer krill. Most genes related to other metabolic, regulatory and biological processes were not differentially expressed in South Georgia krill.

Compared to summer krill, only few DEGs were found to be upregulated in winter krill from the three regions (Fig. 3b). Only one top category was found for winter

krill from each studied region: protein folding (0.67) for South Orkneys/Bransfield Strait, visual perception (0.29) for Lazarev Sea, and energy metabolism (0.2) for South Georgia.

Detailed `edgeR` results of the seasonal differential expression analysis can be found in the supplementary Table SII.

2.3.1 Detailed analysis of categories including genes with putative seasonal regulatory functions

With the aim to look for target genes with potential seasonal regulatory functions in Antarctic krill, we chose the following 7 categories for further investigation: metabolism related to bioactive lipids, hormone metabolism, visual perception, receptor-related proteins, development, reproduction, dephosphorylation and transcriptional regulation. The analysis was not restricted to the samples where these categories were enriched, but all identified DEGs within these categories were inspected. Selected members of these categories are shown in Table III.

Genes within the category metabolism related to bioactive lipids were enriched in the South Orkneys/Bransfield Strait summer krill. Some of these genes were also found in Lazarev Sea summer krill. These genes had predicted functions in sphingolipid metabolism, such as the biosynthesis of ceramide and sphingosine, and in the biosynthesis of phosphatidylcholine.

The category hormone metabolism was found to be enriched in the South Orkneys/Bransfield strait summer krill. However, few genes within this category were also upregulated in summer krill from the other two regions. Genes within category hormone metabolism had predicted functions in steroid metabolism, thyroxine metabolism, retinoic acid biosynthesis, octopamine biosynthesis, taurine biosynthesis and the breakdown of bioactive peptides.

Genes within the category visual perception were enriched and upregulated in the South Orkneys/Bransfield Strait summer krill and in Lazarev Sea winter krill. This category comprised genes related to signal transduction, visual pigment biogenesis and eye development.

The category receptor-related proteins was enriched in the South Orkneys/Bransfield Strait summer krill. Two genes of this category were also found to be upregulated in the South Orkneys/Bransfield Strait and South Georgia winter krill. These genes

coded for different receptor-related proteins with predicted functions in the formation of receptor complexes and various signalling pathways and regulatory processes, such as the Wnt and insulin signalling pathway.

The category development was found to be enriched in South Orkneys/Bransfield Strait and Lazarev Sea summer krill. Three genes of this category were upregulated in the winter krill of the three studied regions. The genes had predicted functions in cell differentiation and proliferation, nervous system development, pigmentation, metamorphosis and the regulation of other development processes.

The category reproduction was enriched in South Orkneys/Bransfield Strait and Lazarev Sea summer krill. A few DEGs within this category were also upregulated in South Georgia summer krill. This category included DEGs with putative functions as the lipid transporter vitellogenin and in the metabolism of reproduction-related hormones, in particular prostaglandin biosynthesis and juvenile hormone esterase-like carboxyesterases.

The category dephosphorylation contained only three DEGs which coded for putative phosphatases. The category was enriched in South Orkneys/Bransfield Strait and Lazarev Sea summer krill.

The category transcriptional regulation was found to be enriched in summer krill of the three studied regions. One gene within this category was identified in South Georgia winter krill. These DEGs coded for proteins with putative RNA binding activity and regulatory functions in transcription.

Regional comparisons of gene expression within each season (summer and winter)

Less genes were differentially expressed in the regional pairwise comparisons in summer (82 to 234 DEGs) and winter (73 to 174 DEGs) (Table II). A plot showing the results of the regional comparisons can be found in the supplementary material (Fig. S2).

Comparing the three regional summer samples from South Orkneys/Bransfield Strait, Lazarev Sea and South Georgia, least DEGs were found within the comparison South Orkneys/Bransfield Strait vs. South Georgia krill. Only few upregulated DEGs from the South Orkneys/Bransfield Strait latitudinal region could be annotated compared to the other regions in summer. In the South Orkneys/Bransfield Strait summer krill the category visual perception was enriched with respect to

Lazarev Sea and South Georgia summer krill (0.21 each). Lazarev Sea summer krill showed two enriched top categories with respect to South Orkneys/Bransfield Strait summer krill only: energy metabolism (0.25) and reproduction (0.26) comprising vitellogenin-like genes only. South Georgia summer krill had two top categories with respect to Lazarev Sea summer krill only: cytoskeleton (0.32) and translation (0.68).

Comparing the three regional winter samples, only few DEGs could be annotated. The category carbohydrate metabolism (0.2) was found to be enriched for the South Georgia winter krill with respect to Lazarev Sea winter krill only.

Detailed `edgeR` results of the regional differential expression analysis can be found in the supplementary Table SIII.

2.4 Discussion

Generalisations and methodological discussion

This study investigated seasonal and regional differences in gene expression in Antarctic krill in three different latitudinal regions of the Southern Ocean (Lazarev Sea: 62°S-66°S, South Orkneys/Bransfield Strait: 60°S-63°S, and South Georgia: 54°S). Seasonal differences between summer and winter krill were generally found to be more pronounced than regional differences in summer or winter. Most differentially expressed genes were found to be upregulated in summer krill indicating that Antarctic krill entered a less active state during winter in all studied regions. However, these seasonal differences in gene expression seemed to be less distinct in the low-latitude region South Georgia.

Differences in the seasonal gene expression pattern between the three tested regions may reflect an adaptive behaviour of Antarctic krill to the environmental conditions that krill is exposed to in the different habitats. The highest variety of functionally enriched genes was found in the South Orkneys/Bransfield Strait region which indicates that summer krill was in a highly active condition in this region with respect to winter. In particular, Antarctic summer krill from South Orkneys/Bransfield Strait were characterized by the upregulation of genes related to bioluminescence, detoxification, metabolism related to bioactive lipids, digestion,

hormone metabolism, visual perception and receptor-related proteins. The upregulation of amino acid, lipid and carbohydrate metabolism and the biological categories reproduction and development in both South Orkneys/Bransfield Strait and Lazarev Sea summer krill with respect to winter supports the assumption that krill enters a state of metabolic depression and regressed development during winter in these regions (so called winter quiescence). However, genes related to energy metabolism were found to be upregulated in Lazarev Sea summer krill only. This may point to stronger seasonal differences in energy metabolism in the high-latitude region Lazarev Sea. In contrast, most genes related to metabolic, biological and regulatory processes were not found to be differentially expressed in the comparison of summer and winter krill from South Georgia which may be a response to the less extreme winter conditions in this low-latitude region (Meyer et al., 2017).

We also identified a variety of candidate genes with likely roles in the metabolism related to bioactive lipids, hormone metabolism, visual perception, as receptor-related proteins, in development, reproduction, dephosphorylation and transcriptional regulation. The selected genes may serve as target genes for future studies of seasonal rhythms in Antarctic krill.

This paper partly confirms results from a microarray study by Seear et al. (2012) who found an upregulation of genes involved in feeding and digestion, respiration, motor activity, immunity and vitellogenesis in Antarctic krill near the Antarctic Peninsula (60°S) in summer. By contrast, the present paper investigated as novel aspect the seasonal gene expression profiles of Antarctic krill from three different latitudinal regions in the Atlantic sector of the Southern Ocean. In particular, it adds seasonal gene expression data for summer and winter krill from the high-latitude region Lazarev Sea and the low-latitude region South Georgia. Moreover, the present study used a different molecular approach, RNA-seq, focussing on putative regulatory processes of seasonal rhythms in Antarctic krill and the identification of potential seasonal target genes. In contrast to the study by Seear et al. (2012), we did not find strong differences in the direct regional comparisons of the summer or winter krill samples, such as the upregulation of genes related to digestion and immunity in the South Georgia region in winter (Seear et al., 2012). Moreover, we observed a more diverse pattern of gene expression related to energy metabolism and respiration in the three studied regions.

These differences may have been caused by different methodological constraints

of our RNAseq study. A limited number of Antarctic krill samples were available for sequencing. The sequenced Antarctic krill originated from the field and were sampled under highly variable conditions. Social cues, feeding condition, migratory behaviour of krill, and abiotic factors that cannot be controlled may have affected the gene expression profile detected in the samples. Moreover, differences in sampling time and station coordinates, variable sampling techniques on the different vessels and unknown parameters such as age or moulting stage of the studied individuals may have introduced variation in the dataset. To partly compensate for this variation, we used high replicate numbers for differential gene expression analysis in this RNAseq study.

In this study, the generally low level of upregulated genes in winter krill cannot directly explain the winter behaviour of Antarctic krill in some regions. For instance, the Bransfield Strait is a food-rich overwintering ground for Antarctic krill where large vertical migrations have been observed (Bernard et al., 2018; Reiss et al., 2017). Hence, gene expression needs to be activated in winter to allow for this behaviour. However, our data analysis focussed on significant differences in seasonal gene expression. It mainly revealed genes that are important for Antarctic krill in its more active summer condition, where gene expression is apparently much larger with respect to winter. But the expression of genes below the detection level of our statistical methods may still allow for winter feeding and migration behaviour.

Potential influences on Antarctic krill's seasonal and regional gene expression

Our seasonal gene expression results from South Orkneys/Bransfield Strait and Lazarev Sea agree with observations in Antarctic krill from the field: a reduced metabolic activity and regressed development in winter and an enhanced metabolism, development, reproductive activity and gene expression in summer (Meyer et al., 2010; Siegel, 2012). In contrast, metabolic depression in winter and enhanced expression of reproduction- and development related genes in summer were not observed in the South Georgia krill. Yet, generally higher gene expression was also found in the South Georgia summer krill related to other processes such as translation, immune response and transcriptional regulation. These differences in seasonal gene expression discovered in krill from the South Georgia region may reflect the flexible overwintering behaviour of Antarctic krill found in this region (Schmidt et al., 2014).

Variable factors such as water temperature, reproductive timing, food availability and light regime may have influenced the regional differences observed in seasonal gene expression in Antarctic krill in this study.

However, regional differences in seasonal water temperature cannot explain why the least seasonal differences in gene expression were found in the South Georgia region. Largest seasonal differences in water temperature are observed around South Georgia, where temperature may rise above 4°C in summer and remains around 0°C in winter (Whitehouse et al., 1996). In contrast, seasonal water temperatures are more stable in the Lazarev Sea and close to the Antarctic Peninsula ranging between -1.8°C and -0.1°C (Meyer et al., 2010, and station data), but krill from these regions showed the largest differences in seasonal gene expression in our study.

The variable reproductive timing of Antarctic krill according to region and sea ice conditions (Spiridonov, 1995) may explain why in our study less seasonal differences in gene expression were observed in the Lazarev Sea compared to South Orkneys/Bransfield Strait. Summer krill samples from Lazarev Sea were obtained in the end of December, when sea ice was melting and the phytoplankton bloom just started to develop (Meyer et al., 2010). Thus, the krill from the Lazarev Sea was probably only in preparation of the spawning season. On the contrary, the South Orkneys/Bransfield Strait krill was caught in an ice-free region in the beginning of February, where krill was in the middle of the spawning period and probably in a more active condition than in Lazarev Sea. The high metabolic demand of the South Orkneys/Bransfield Strait summer krill is reflected by the high proportion of detoxification genes found in this region.

Annual feeding conditions may have especially affected the different behaviour of Antarctic krill in the lower-latitudinal region South Georgia, where krill is exposed to less extreme winter conditions compared to the other two regions. South Georgia is ice-free throughout the year and prolonged periods of phytoplankton blooms occur in this area. In winter, Antarctic krill from South Georgia was observed to be feeding on phytoplankton and seabed detritus, and contained lower lipid stores compared to Antarctic krill from Bransfield Strait and Lazarev Sea (Schmidt et al., 2014). Therefore, seasonal differences in gene expression may be less pronounced in that area.

There are indications that light regime and an endogenous timing system may play a major role in controlling the flexible behaviour and life cycle of Antarctic krill

in different latitudinal regions of the Southern Ocean. Recently, a two-year lab experiment has shown that the latitudinal light regime (photoperiod, the day length) affected seasonal cycles of growth, maturity, feeding and lipid content of Antarctic krill (Höring et al., 2018). Seasonal patterns of growth, feeding and maturity were also observed under constant darkness which indicated the presence of an endogenous timing system that was most likely entrained by light regime prior to the experiments. Critical photoperiods for female maturity were found to be higher under the simulated high-latitude light regime which pointed to a flexible seasonal timing system in Antarctic krill under different latitudinal photoperiods. Piccolin et al. (2018b) demonstrated the effect of photoperiod on the seasonal cycle of growth, enzyme activity and oxygen consumption in Antarctic krill. The authors linked the results to the seasonal expression of circadian clock genes and suggested their involvement in the seasonal timing mechanism in Antarctic krill.

These findings reveal that photoperiod is an important zeitgeber for Antarctic krill that seems to entrain its seasonal timing system under the variable photoperiodic conditions in the Southern Ocean. The less pronounced seasonal cycle of Antarctic krill observed around South Georgia may therefore be partly controlled by the less extreme seasonal light conditions in that region and krill's endogenous clock. The photoperiodic seasonal timing system may be complemented by other factors such as food supply as explained above.

Target genes and their putative functions in Antarctic krill

We identified regulatory genes with multiple functions that may play an important role in the control of seasonal physiology and behaviour in Antarctic krill. These target genes were selected from annotated genes with putative regulatory functions that were differentially expressed between summer and winter krill in this study. The functional roles of these genes still need to be validated in Antarctic krill in future laboratory experiments. Moreover, controlled laboratory experiments may be conducted to test if these genes are rhythmically expressed under different light regimes and if they are effectively involved in the regulation of seasonal life cycle events in Antarctic krill. Thus, our study establishes a basis for future laboratory studies to further elucidate the molecular mechanisms of seasonal rhythms in Antarctic krill. In the following, we will describe the potential functional roles of our proposed seasonal target genes.

We identified several genes that are involved in the metabolism of bioactive lipids. The biosynthesis pathway of sphingolipids such as ceramide and sphingosine play a key role in the regulation of these bioactive compounds. Bioactive lipids have been shown to mediate stress-related responses and processes such as cell proliferation and differentiation, apoptosis and inflammation (Hannun and Obeid, 2008). Ceramide is also involved in the induction of protein dephosphorylation by activating Ser-Thr phosphatases such as PP2A (Chalfant et al., 2004), potentially affecting insulin signalling and metabolism (Hannun and Obeid, 2008). The metabolism of bioactive lipids may therefore be involved in the regulation of growth, metabolism and immune response in Antarctic krill.

Target genes with putative functions in the metabolism of different hormones may play a role in the regulation of hormone levels in Antarctic krill. These included for example genes with functions in steroid, thyroxine, retinoic acid and octopamine metabolism and the breakdown of bioactive peptides. In crustaceans, ecdysteroids and vertebrate-type steroids mediate the regulation of moulting and reproduction (Lafont and Mathieu, 2007). Thyroxine and retinoic acid might have similar functions as the insect juvenile hormone, such as the regulation of development and reproduction (Laufer and Biggers, 2001). In the European hamster, the thyroid hormone metabolism has been associated with the seasonal timing of reproduction (Sáenz de Miera et al., 2014) and it remains to be clarified if thyroxine possesses a similar function in Antarctic krill. Octopamine affects heart beat and behaviour in lobsters (Battelle and Kravitz, 1978; Kravitz, 1988). We also identified two genes involved in the breakdown of bioactive peptides: neprilysin-1 which was found to inactivate the circadian neurotransmitter pigment dispersing factor (Isaac et al., 2007); and neuroendocrine convertase 1, a prohormone processing enzyme that was found to play a role in the reproduction processes in abalone (Zhou and Cai, 2010).

For visual perception, we identified arrestin, which is an important component of the visual transduction system (Montell, 2012), and carotenoid isomerooxygenase, a key enzyme for the biogenesis of visual pigments (Voolstra et al., 2010).

Receptor-related proteins play an important role for signal transduction in the nervous system and may have regulatory roles in various seasonal processes in Antarctic krill such as growth and metabolism. These candidate genes include the adiponectin receptor which is known to regulate insulin secretion, glucose and lipid

metabolism in Drosophila (Kwak et al., 2013), and supports the maintenance of skeletal muscle fiber in crustaceans (Kim et al., 2016). The prolow-density lipoprotein receptor-related protein 1 may have multiple functions in Antarctic krill such as in development, cellular lipid homeostasis, endocytosis and the regulation of signalling pathways (Franchini and Montagnana, 2011). The leucine-rich repeat-containing G-protein coupled receptor 4 may be involved in the Wnt/β-catenin signaling pathway and development (Carmon et al., 2011) and the regulation of circadian rhythms of plasma lipids (Wang et al., 2014). Integrins form cell-surface-adhesion receptors with functions for instance in development, immune response and signalling (Harburger and Calderwood, 2009). The translocon-associated protein (TRAP) subunit gamma has been suggested to contribute to cellular homeostasis during stress responses such as glucose deprivation (Yamaguchi et al., 2011) and may therefore play a similar role in the winter quiescence of Antarctic krill. The Guanine nucleotide-binding protein subunit beta-2-like 1(alias RACK1) has been found be involved in developmental processes (Vani et al., 1997), maturation (Ron and Mochly-Rosen, 1994), and immune response in crustaceans (Jia et al., 2016).

As candidate gene for the future investigations of reproductive processes in Antarctic krill, we propose the lipid transport molecule vitellogenin. It is an essential component in the process of egg maturation (Krishnan et al., 2008), but may also be required for other processes with high energy demand such as growth and moulting. The hormone-like prostaglandins have been related to the regulation of ovarian maturation in crustaceans (Wimuttisuk et al., 2013). We also identified transcripts closely related to juvenile hormone esterase-like carboxylesterases which may potentially degrade and thereby inactivate methyl farnesoate in crustaceans (Lee et al., 2011). Methyl farnesoate was found to promote both reproductive maturation and moulting in crustaceans (Reddy et al., 2004).

We propose target genes that may affect various developmental and growth-related processes during the seasonal cycle of Antarctic krill. These genes coded amongst others for the blastula protease 10 (alias SpAN), which has been functionally described during sea urchin embryogenesis (Lepage et al., 1992) and may also play a role for cell differentiation in Antarctic krill. Carbohydrate sulfotransferase 11 (alias chondroitin 4-sulfotransferase 1) has been linked to the Wnt signalling pathway affecting developmental processes such as cell proliferation (Nadanaka et al., 2008). From structural similarities, fibrocystin-L (gene PKHD1) is proposed to play

a role for cell proliferation, adhesion and repulsion (Onuchic et al., 2002). Potential candidate genes for nervous system development comprise for instance glycoprotein 3-alpha-L-fucosyltransferase A which catalyzes the glycosylation of neural-specific proteins (Yamamoto-Hino et al., 2010) and neurotrophin 1, a secreted protein with regulatory functions in the nervous system (Zhu et al., 2008). Laccase 2 is a phenoloxidase gene that has been related to cuticle tanning in insects (Arakane et al., 2005) and may have a similar function in Antarctic krill, but may also be involved in immune response in crustaceans (Clark and Greenwood, 2016). We also found crustacyanin-A2 subunit which is known to generate the colouration of the lobster shell (Cianci et al., 2002). Krüppel homolog 1 seems to be linked to juvenile hormone during metamorphosis in Drosophila (Minakuchi et al., 2008), but may also regulate development in an independent pathway in crustaceans (Miyakawa et al., 2018). The role of krüppel may be versatile as effects have also been observed on vitellogenin expression in the fat body and ovarian maturation and growth in insects (Song et al., 2014) and on fat metabolism in nematods (Zhang et al., 2009).

Dephosphorylation by phosphatases represents another important step in the post-translational regulation of proteins. We found for instance the serine/threonine-protein phosphatase 2A (PP2A) which may contribute to a variety of processes including ovarian maturation (Zhao et al., 2017), visual perception (Wang et al., 2008) and circadian timing (Pegoraro and Tauber, 2011).

On the transcriptional level, we found for example a gene coding for the CREB-binding protein, which is a transcriptional coactivator affecting circadian behavioural activity (Maurer et al., 2016), postembryonic development (Roy et al., 2017) and eye development (Kumar et al., 2004) in insects and may therefore have similar functions in Antarctic krill.

Application to future studies of seasonal rhythms

This study provides further understanding of the gene expression profiles behind the flexible seasonal behaviour of Antarctic krill in different latitudinal regions of the Southern Ocean. It further discusses the potential environmental factors that may affect the observed regional differences in seasonal gene expression. Our data suggests

that a number of genes related to sphingolipid metabolism, hormone metabolism, visual perception, receptor-related proteins, reproduction, development, dephosphorylation and transcriptional regulation may have regulatory functions in krill's seasonal physiology. This study provides a basis for future laboratory studies where the effect of different environmental factors such as light regime or food supply on the expression of these seasonal candidate genes may be tested.

Genes related to insulin and the juvenile hormone like signalling pathway in crustaceans may be of special interest. Insulin signalling (Sim and Denlinger, 2013) and the absence of juvenile hormone has been related to reproductive diapause and associated metabolic processes in insects (Liu et al., 2017) and may have a similar role in Antarctic krill for the preparation of winter quiescence.

Even though seasonal differences in clock gene expression could not be detected in this study, genes involved in the circadian clock and downstream pathways may still be appropriate for the investigation of seasonal rhythms in Antarctic krill. Clock genes were found to affect photoperiodic diapause in insects (Ikeno et al., 2010) and a potential seasonal role has also been suggested for Antarctic krill (Piccolin et al., 2018b). However, a seasonal timing system independent of the circadian clock may also exist (Bradshaw et al., 2006) and other levels of seasonal control may comprise non-coding RNAs or epigenetic modifications (Helm and Stevenson, 2014).

2.5 Conclusion

This study examined seasonal and regional differences in gene expression in Antarctic krill from three latitudinal regions of the Southern Ocean (Lazarev Sea, South Orkneys/Bransfield Strait, South Georgia) with the additional goal to identify target genes with putative regulatory functions in the seasonal cycle of Antarctic krill. The studied regions were characterized by different latitudinal light regimes with more extreme annual changes of photoperiod and therefore more severe winter conditions experienced by Antarctic krill in higher latitudinal regions such as Lazarev Sea. We found a downregulation of most differentially expressed genes in the winter samples indicating that Antarctic krill entered a less active state in winter. However, seasonal differences in gene expression seemed to be less pronounced in Antarctic krill from the South Georgia region compared to the South Orkneys/Bransfield Strait and Lazarev Sea region. In the South Georgia krill, the seasonally differential

expression of most genes related to metabolic, biological and regulatory processes was missing. This may be explained by a less pronounced seasonal cycle of Antarctic krill in this low-latitude region that is characterized by less extreme light conditions, milder winters with no sea ice coverage and enhanced food availability. We propose that seasonal gene expression may be partly governed by a photoperiodic timing system that may influence the flexible behaviour and physiology of Antarctic krill in different latitudinal regions of the Southern Ocean. Moreover, we identified target genes with potential regulatory roles in the seasonal cycle of Antarctic krill including processes of growth, reproduction and metabolism. These genes are functionally linked to different regulatory pathways such as hormone and sphingolipid metabolism, and juvenile hormone like and insulin signalling pathways and may serve as starting point for understanding the molecular mechanisms of seasonal rhythms in Antarctic krill.

2.6 Acknowledgements

We thank So Kawaguchi, Patti Virtue and our cooperation partners from the British Antarctic Survey and Southwest Fisheries Science Center (NOAA) for the provision of Antarctic krill samples from different regions and seasons for this study. Mathias Teschke is acknowledged for his advice during preliminary laboratory work.

Funding was provided by the Helmholtz Virtual Institute "PolarTime" (VH-VI-500: Biological timing in a changing marine environment — clocks and rhythms in polar pelagic organisms), the PACES (Polar Regions and Coasts in a changing Earth System) programme (Topic 1, WP 5) of the Helmholtz Association, and the ministry of science and culture (MWK) of Lower Saxony, Germany (Research Training Group IBR "Interdisciplinary approach to functional biodiversity research").

2.7 References

Chapter 3

Light regime affects the seasonal cycle of Antarctic krill (*Euphausia superba*): impacts on growth, feeding, lipid metabolism, and maturity

Flavia Höring1,2, Mathias Teschke1, Lavinia Suberg1, So Kawaguchi3,4, and Bettina Meyer1,2,5

1 Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Section Polar Biological Oceanography, Am Handelshafen 12, 27570 Bremerhaven, Germany

2 Carl von Ossietzky University of Oldenburg, Institute for Chemistry and Biology of the Marine Environment (ICBM), Carl-von-Ossietzky-Str. 9-11, 26111 Oldenburg, Germany

3 Australian Antarctic Division, Department of the Environment and Energy, Channel Highway, Kingston, Tasmania 7050, Australia

4 Antarctic Climate and Ecosystems Cooperative Research Centre, University of Tasmania, Private Bag 80, Hobart, Tasmania 7001, Australia

5 Helmholtz Institute for Functional Marine Biodiversity (HIFMB) at the University of Oldenburg, 26111 Oldenburg, Germany

Can. J. Zool. 96: 1203–1213 (2018) dx.doi.org/10.1139/cjz-2017-0353

3.1 Abstract

Light regime is an important zeitgeber for Antarctic krill (*Euphausia superba* Dana, 1850), which seems to entrain an endogenous timing system that synchronizes its life cycle to the extreme light conditions in the Southern Ocean. To understand the flexibility of Antarctic krill's seasonal cycle, we investigated its physiological and behavioural responses to different light regimes and if an endogenous timing system was involved in the regulation of these seasonal processes. We analysed growth, feeding, lipid content, and maturity in a 2-year laboratory experiment simulating the latitudinal light regimes at 52°S and 66°S and constant darkness under constant food level. Our results showed that light regime affected seasonal cycles of growth, feeding, lipid metabolism, and maturity in Antarctic krill. Seasonal patterns of growth, feeding, and maturity persisted under constant darkness, indicating the presence of an endogenous timing system. The maturity cycle showed differences in critical photoperiods according to the simulated latitudinal light regime. This suggests a flexible endogenous timing mechanism in Antarctic krill, which may determine its response to future environmental changes.

3.2 Introduction

Concerns are growing about the impact of global warming on the Antarctic marine ecosystem. The observed changes in sea-ice extent and zooplankton distribution may lead to trophic mismatches and thereby profound changes in the Southern Ocean food web (Atkinson et al. 2004; Steinberg et al. 2015). To be able to predict future changes, we need to better understand the adaptive potential of polar key organisms such as the Antarctic krill (Euphausia superba Dana, 1850) (Meyer 2010).

Antarctic krill's success in the Southern Ocean likely originates from its ability to synchronize its life cycle to local photoperiod and food supply. It has evolved seasonal patterns of growth, lipid turnover, metabolic activity (Meyer et al. 2010), and maturation (Kawaguchi et al. 2007) that bring an evolutionary advantage to survive in an environment with strong seasonal fluctuations of sea-ice extent, photoperiod, and primary production. These seasonal patterns seem to vary according to latitudinal region, as it has been observed that Antarctic krill near South Georgia (54°S) had lower lipid stores and higher feeding activities in winter com- pared with regions at higher latitudes where near-constant dark- ness during winter limits food supply

(Schmidt et al. 2014). However, the mechanisms shaping these seasonal rhythms remain poorly understood.

Photoperiod seems to play a major role in the modulation of the seasonal rhythms of Antarctic krill. Laboratory experiments revealed that photoperiod affected seasonal patterns of growth (Brown et al. 2010), maturity (Hirano et al. 2003; Teschke et al. 2008; Brown et al. 2011), feeding, and metabolic activity (Teschke et al. 2007). It is not yet clear if light regime also promotes acclimatization to the varying seasonal conditions in different latitudinal habitats of Antarctic krill.

An endogenous timing system may be involved in the regulation of seasonal rhythms in Antarctic krill. Seasonal patterns of maturity were observed to persist under constant darkness (Brown et al. 2011), indicating an endogenous timing system that maintained the rhythm even if the zeitgeber (environmental cue) was absent (= concept of a biological clock). Recent studies suggest that Antarctic krill possesses a circadian clock that regulates its daily metabolic output rhythms and is entrained by photoperiod (Mazzotta et al. 2010; Teschke et al. 2011). However, it is unknown if the circadian clock is also involved in the timing of seasonal events in Antarctic krill.

This study aims to investigate the effect of different light regimes on growth, feeding, lipid metabolism, and maturity in Antarctic krill, as well as the involvement of an endogenous timing system in the modulation of seasonal rhythms. We analyse a unique data set from multiyear laboratory experiments simulating different latitudinal light regimes (52°S, 66°S, constant darkness) and constant food supply over 2 years. We will test (i) if light regime stimulates seasonal patterns of growth, feeding, lipid metabolism, and maturity; (ii) if different latitudinal light regimes cause different seasonal patterns; and (iii) if seasonal patterns persist under constant darkness indicating an endogenous timing system.

3.3 Materials and Methods

3.3.1 Antarctic krill collection and maintenance prior to the experiments

Antarctic krill were caught with a rectangular mid-water trawl (RMT 8) on 12 February, 2013 (66°47′S, 65°8′E) during the voyage V3 12/13 of RSV Aurora australis and on 15 January, 2015 (65°31′S, 141°23°E) during voyage V2 14/15. The sampling

methods are described in detail by King et al. (2003). The sampled Antarctic krill arrived at the Australian Antarctic Division aquarium in Hobart on 22 February, 2013 and on 25 January, 2015, respectively. For acclimation and for keeping of Antarctic krill until the start of the experiments, they were transferred to 800 L tanks (temperature 0.5 °C) that simulated the natural light regime at 66°S. A detailed description of the Antarctic krill aquarium facility and the simulated light regime can be found in Kawaguchi et al. (2010).

3.3.2 Photoperiodic-controlled laboratory experiments

Long-term laboratory experiments were conducted over a period of 2 years starting in January 2015. Three different light regimes were tested, simulating (1) natural light conditions at 52°S, (2) natural light conditions at 66°S, and (3) constant darkness (DD) (Figs. 1a, 1b). For each treatment, 250 Antarctic krill were transferred from the 800 L acclimation tanks to a 250 l experimental tank connected to a recirculating chilled seawater system with a constant water temperature of 0.5 °C. For the initial experimental set-up, Antarctic krill collected in 2013 were used (tanks A, B, E, F).

However, due to increased mortality in tank A (treatment DD), an additional tank for treatment DD (tank K) was set up in the beginning of March 2015 using freshly caught Antarctic krill collected in 2015. The three different light conditions were simulated within black lightproof plastic containers, one for each experimental tank, using twin fluorescent tubes (Osram L18W/640 Cool White) with a marine blue gel filter (Marine Blue 131; ARRI Australia Pty. Ltd.). Light adjustment under treatments 52°S and 66°S was carried out using a PC-controlled timer and dimming system (winDIM version 4.0e; EEE, Portugal) with a maximum light intensity of 100 lx (photon flux = 1.3 µmol m^{-1} s^{-2}) during midday in January (corresponds to 1% light penetration at 30 m depth). According to the light regime, photoperiod and light-intensity profiles were adjusted at the beginning of each month for each treatment. The simulated light-intensity profiles for each treatment and month can be found in Supplementary Table S1.1

The food level was held constant to remove that effect from our experiments because we solely wanted to identify the effect that light regime had on the seasonal cycle of Antarctic krill. Antarctic krill were fed daily between the hours of 0830 and 0930 and the water flow in the tanks was turned off for approximately 2

h to ensure feeding. The food comprised three live laboratory-cultured algae (final concentrations were 1.5×10^4 cells mL^{-1} of *Phaeodactylum tricornutum* Bohlin, 1897, 2×10^4 cells mL^{-1} of *Geminigera cryophila* (D.L. Taylor and C.C. Lee) D.R.A. Hill, 1991, 2.2×10^4 cells mL^{-1} of *Pyramimonas gelidicola* McFadden, Moestrup and Wetherbee, 1982), three types of commercial algal paste (1×10^4 cells mL^{-1} of *Thalassiosira weissflogii* (Grunow) G. Fryxell and Hasle, 1977 "TW 1200TM", 5.1×10^4 cells mL^{-1} of Isochrysis Parke, 1949 "Iso 1800TM", 4.8×10^4 cells mL^{-1} of Pavlova Butcher, 1952 "Pavlova 1800TM"; Reed Mariculture, USA), and two types of prawn hatchery feeds (0.5 g of FRiPPAK FRESH #1CAR, 0.5 g of FRiPPAK FRESH #2CD; INVE, Thailand). Antarctic krill under treatment DD were fed in dim red light. Moults and dead Antarctic krill were removed regularly from the tanks.

Antarctic krill sampling of 6–10 individuals per tank and month was carried out in the middle of each month during midday starting in February 2015 (for treatment DD in dim red light). Due to different rates of mortality in the tanks, the sampling scheme had to be adjusted during the course of the experiment (Table 1) to assure sampling over the whole experimental period. Due to the problem with increased mortality under treatment DD mentioned above, we decided to sample tanks A and K sequentially to ensure the completion of the experiment over the 2-year period.

Live Antarctic krill was inspected under a stereomicroscope and the sex was determined. Pictures of the carapace and the sexual organs (female thelycum and male petasma) were taken with a Leica DFC 400 camera system (Leica Microsystems, Germany). Car- apace length (tip of the rostrum to posterior notch) and digestive gland length (longest axis through carapace) were determined from the pictures within the Leica `DFC Camera` software `version 7.7.1` (Leica Microsystems, Switzerland).

After visual inspection, the sampled Antarctic krill was immediately frozen in liquid nitrogen. Frozen samples were stored at $-80\,^{\circ}C$.

The first inspection of the sex ratio within the experimental tanks revealed that females dominated, with proportions of 71%– 85% per tank.

3.3.3 Growth analysis

Carapace length was used as a proxy for growth in the experiments. Antarctic krill were sampled randomly from each experimental tank; thus, a general trend observed in the carapace length data are assumed to display the general trend of growth.

The data analysis was performed in `RStudio version 1.0.136` (RStudio Team, 2016). Before the modelling process, a Pearson's product moment correlation was conducted to determine a potential difference in growth pattern between male and female Antarctic krill; thus, the need for separate models for each sex. Due to the strong correlation ($r = 0.82$, $p < 0.001$) between males and females, based on the mean carapace length for each sex across all treatments, data from both sexes were combined ($n = 617$). To investigate the long-term trend (variable "time") and the seasonal variability (variable "month") of Antarctic krill growth for each "treatment" (light regime), a generalized additive mixed model (GAMM) with a Gaussian distribution was used. An additive model was chosen over a linear one to resolve the nonlinear relationship of the response and explanatory variables. The GAMM takes the structure as specified by Hastie and Tibshirani (1987) and was fitted using the gamm function in the mgcv package (Wood 2006). Random effects for "tank" were included in the model to account for potential dependencies between individuals from the same tank. Prior to the modelling 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 the autocorrelation can result in biased estimates of model parameters (Panigada et al. 2008). As autocorrelation was neither detected, nor evident in residual analysis during model validation, no temporal autocorrelation term was included in the final model.

Smoothed terms were fitted as regression splines (variable "time"), apart for the variable "month", which was modelled using cyclic cubic regression splines, setting knots manually between 1 (January) and 12 (December) to account for the circular nature of this term. Differences in temporal pattern between the three light regimes (52°S, 66°S, DD) were implemented using the by- argument of the gamm function, which allows for the creation of separate smoothers for each level of the treatment factor (light regime) over the temporal variables "month" and "time". Hence, separate parameter estimates for the temporal variables are obtained for each treatment level. To avoid overfitting, the smooth function of the variable "month" was manually restricted to $k = 5$. Model selection was conducted using manual stepwise-backward selection based on Akaike's information criterion (AIC) (Akaike 1981). If the addition of a term led to an AIC decrease of >2 per degree of freedom, or an increase of the adjusted R2, or if the term was significant, then the term was included in the model. Model fit was examined by residual analysis.

3.3.4 Feeding Analysis

The feeding index (%) was calculated as digestive gland length $\times$ (carapace length)-1 $\times$ 100. Data of males and females were combined because of the strong correlation of monthly mean values (Pearson's product moment correlation, $r = 0.95$, $p < 0.001$). To investigate a temporal pattern in the feeding index of Antarctic krill for each treatment, a GAMM was employed as described above (section Growth analysis). The smooth function of the variable "time" was manually restricted to $k = 6$.

3.3.5 Lipid content analysis

Every 3 months from April 2015 to July 2016, six replicate samples from each treatment were tested for their lipid content. Lipids were extracted from the carapace, which was separated from the frozen samples with a scalpel on dry ice prior to extraction. Lipid extraction was performed with dichloromethane:methanol (2:1, v:v) according to the method described by Hagen (2000). Lipid content was determined gravimetrically and was calculated in percentage of dry mass. One data point (sample code "Jan16_E04") was removed due to the negative value of lipid content that indicated incorrect measurement for that individual.

Lipid content differed between male and female Antarctic krill (Pearson's product moment correlation of pooled monthly mean values, $r = 0.26$, $p = 0.62$); therefore, statistical analysis was performed separately for each sex. Data for males were not sufficient for robust modelling and only females were considered for this analysis ($n = 83$). Only one tank for each time point and treatment was available, therefore a mixed model to resolve a potential tank effect could not be employed. For treatment DD, five samples were available from a second tank, but these were not sufficient for the inclusion of a random effect. Therefore, a generalized additive model (GAM) was employed to examine the temporal pattern of female Antarctic krill lipid content, following the protocol described in section Growth analysis. The smooth function of the variable "time" was manually restricted to $k = 6$. Because the variable "month" was not significant, it was excluded from the final model.

3.3.6 Maturity Analysis

The maturity stage of the sampled Antarctic krill was assessed by analysing pictures of the external sexual organs according to Makarov and Denys (1980) and Thomas

and Ikeda (1987). A maturity score was assigned using the method of Brown et al. (2010, 2011). Due to the ordinal characteristic of the maturity scores, Pearson correlation of monthly mean values could not be per- formed with the data set. Therefore, we visually inspected the relationship between maturity score and hours of light in males and females. Seasonal maturity scores differed between male and female Antarctic krill (Fig. 2); therefore, statistical analysis was performed on females only (n = 493), as there were not sufficient data to allow for modelling males separately. To investigate the temporal pattern of maturity of female Antarctic krill for each treatment, a GAMM was employed as described in section Growth analysis. Because model residuals were autocorrelated, an auto- regressive correlation structure of the order 1 was added, which improved model fit and resolved the dependencies between residuals. Maturity scores are represented as whole numbers and take values between 3 and 5. Therefore, the GAMM was initially modelled using a Poisson distribution with a logarithmic link function between predictor and response. Due to overdispersion, a negative binomial GAMM had to be used. The smooth function of the variable "time" was manually restricted to k = 6.

To examine differences in the critical photoperiod between latitudinal light regimes 52°S and 66°S, a logistic regression was used. As only full maturity was investigated, maturity scores <5 were set to zero and full maturity (score = 5) was set to one in all samples, resulting in a data set of zeros and ones. The relationship between full maturity of female Antarctic krill and photoperiod was modelled with a binomial generalized linear mixed model (GLMM) with a logit function between predictor and response and an interaction term for factor "treatment" and continuous variable "hours of light". The model was fitted using the glmer function from the lme4 library. To account for dependencies between individuals from the same tank, random effects for "tank" were included in the model. Model fit was assessed by constructing a receiver operating characteristic (ROC) curve using the pROC package in R, where the area under the curve (AUC) indicates the goodness of fit (Boyce et al. 2002). Values below 0.7 are considered poor and 1.0 represents a perfect fit (Cumming 2000). The critical photoperiod (= photoperiod, when the probability to be fully mature is 50%) was predicted from the 95% confidence intervals.

3.3.7 Data archiving

Processed data have been uploaded to the database PANGAEA and can be accessed under https://doi.pangaea.de/10.1594/PANGAEA.885889.

3.4 Results

3.4.1 Growth Analysis

Carapace length ranged from 8.1 to 19.02 mm with a mean (±SD) of 11.71 mm (±1.61 mm) across the whole data set. The GAMM (model M1; Table 2) revealed significant seasonal and interannual patterns in growth, which were similar across all treatments (Figs. 3a, 3b). Shrinkage was observed in the beginning of the experiments. A significant seasonal variability with shrinkage to- wards austral winter (June to August) and growth towards austral summer (December to February) was observed under treatments 52°S and DD (not significant under treatment 66°S).

3.4.2 Feeding

The feeding index data ranged from 25.15% to 66.09% with a mean (±SD) of 42.00% (±6.58%).

The GAMM revealed significant changes in the feeding index over time (model M2; Table 2). We observed an increase of the feeding index throughout the experimental period in all treat- ments and a final stagnation in treatments 52°S and DD (Figs. 4a, 4b). The seasonal trend differed between treatments. In treatment 52°S, the feeding index strongly increased during the autumn period (March to May) with a subsequent decrease and stabilization during the rest of the year. The seasonal trend in treatment 66°S was very weak and will therefore not be described further. In treatment DD, the feeding index increased over a longer period (March to July) and decreased during the rest of the year.

3.4.3 Lipids

The lipid content data of males and females ranged from 2.53% to 57.75% with a mean (±SD) of 17.04% (±9.12%). The GAM considering female lipid content data only (model M3; Table 2) revealed significant differences in temporal variability of

lipid content be- tween the experimental treatments (Fig. 5). Even though the variable "month" was not significant, a resembling seasonal pattern was observed in the interannual trend under treatment 66°S with an increase towards austral winter and a decrease towards austral summer. The increase of lipid content during the second winter was much stronger than the first winter. No significant patterns were found for treatments 52°S and DD.

3.4.4 Maturity

Implementing the negative binomial GAMM for female maturity (model M4; Table 2), we found a significant seasonal cycle of maturity under treatments 52°S, 66°S, and DD with sexual regression towards austral winter and sexual re-maturation towards austral spring and summer (Figs. 6a, 6b). Significant interannual patterns differed between treatments. In treatments 52°S and 66°S, a slight decrease of maturity over the whole study period was observed. The interannual pattern in treatment DD showed that sexual regression was only completed during the first winter of the experiments.

The binomial GLMM (model M5; Table 2) suggests that the variable "hours of light" significantly affects female maturity in treatments 52°S and 66°S. The interaction term between "hours of light" and "treatment" was marginally not significant. When investigating the critical photoperiod at the probability of 50%, differences between the treatments were found (Fig. 7). For treatment 52°S, the critical photoperiod was estimated as 12.5 h of light with 95% confidence intervals (11.86, 13.22). For treatment 66°S, an estimate of 14.76 h of light with 95% confidence intervals (13.3, 16.3) was found.

3.5 Discussion

We present findings from the first 2-year laboratory experiments investigating the effect of light regime and the biological clock on the seasonal cycle of Antarctic krill.

The observed seasonal cycles of growth, feeding, lipid metabolism, and maturity under the simulated latitudinal light regimes suggest that light regime is an essential zeitgeber for Antarctic krill. The occurrence of a pronounced lipid cycle under treatment 66°S and the observed differences in critical photoperiods for the maturation cycle indicate that Antarctic krill may respond flexibly to different latitudinal light

regimes. This may represent an adaptive mechanism to the extreme light regimes in the Southern Ocean and ensure survival of Antarctic krill in different latitudinal habitats, especially during winter. Moreover, seasonal patterns of growth, feeding, and maturity persisted under constant darkness indicating the presence of an endogenous timing system modulating these rhythms. High food supply does not suppress endogenously driven seasonal rhythms of growth, feeding, lipid metabolism, and maturity.

The following considerations should be taken into account when interpreting the findings of this study. Due to limits in space and costs for the long-term laboratory experiments and variable mortality rates in the tanks, we had to adjust the experimental set-up and sampling scheme accordingly. This led to a sampling design with replication in experimental units over the full study period for treatment 52°S only. Carapace length, digestive gland length, and maturity data from treatment 66°S and partly treatment DD, as well as the lipid content data set, may be regarded as pseudoreplicated (Colegrave and Ruxton 2018) because the replication in experimental units over the full study period is incomplete. We have included the random effect "experimental tank" in our models, where appropriate, during statistical analysis of the data to account for a potential tank effect as far as possible. How- ever, we cannot exclude that differences in tank and replicate number may have influenced the results of our tests.

To interpret the response of Antarctic krill to constant darkness over the full 2-year period, we combined data from two different cohorts of Antarctic krill. The "new" cohort was acclimated to the laboratory conditions for 1 year, before sampling started. Preliminary analysis revealed similar trends in both cohorts under constant darkness, which supports our assumption that both cohorts responded similarly to the treatment.

Moreover, we decided to solely analyse a reduced data set for lipid content because frozen Antarctic krill samples from the 2-year experiments are very valuable and can be used for multiple analyses. The reduced data set is adequate to display the pronounced seasonal lipid cycle under the high latitudinal light regime, but it may be insufficient to test for weaker patterns in the other treatments. Since potential differences in the male pattern were indicated and the number of males was too low to conduct a separate analysis, we decided to analyse females only for lipid content and maturity.

Moreover, we presume that the observations made in the first few months of the experiment represent a general period of acclimation to the experimental conditions. It may explain the strong shrinkage, suppressed lipid accumulation, and a general similarity of the data under all treatments in the beginning of the experiments.

Our observation of a seasonal cycle of growth confirms findings by Brown et al. (2010) that suggest growth is influenced by light regime, independently of food supply. For the first time, we show that Antarctic krill's growth cycle is endogenous and persists under constant darkness. The observed shrinkage in autumn and winter in this study may be partly related to the maturity cycle. Females have been observed to shrink during sexual regression (Thomas and Ikeda 1987) and Tarling et al. (2016) suggested that it might be explained by morphometric changes due to the contraction of the ovaries. On the other hand, the shrinkage may reflect an overwintering mechanism (Quetin and Ross 1991). This is sup- ported by our observation of significant seasonal shrinkage under constant darkness where we did not find a pronounced maturity cycle over the 2-year period.

The seasonal increase of feeding in autumn, which was observed under treatment 52°S, may represent an inherent strategy to be able to accumulate enough lipid stores for winter (Hagen et al. 2001; Meyer et al. 2010). These results partly agree with the short-term study by Teschke et al. (2007) who observed higher clearance rates under autumn and summer light conditions compared with constant darkness, suggesting enhanced feeding activity under light conditions of prolonged day length. The comparability of both studies may be limited because we solely used a morphometric index as a measure of feeding activity. The feeding index may be biased by the strong shrinkage that occurred in the beginning of our experiments, which could have masked a suppressed feeding activity in the first months. In our long-term study, the seasonal feeding trend under treatment DD resembled the other treatments with a shift of peak feeding activity towards winter that may indicate an endogenous control of seasonal feeding activity in Antarctic krill. The general increase of feeding index during the experiments suggests that Antarctic krill is able to make use of food supply throughout the whole experimental period. This observation may also indicate a flexible feeding behaviour of Antarctic krill (Atkinson et al. 2002) that has also been observed in the field in winter (Quetin and Ross 1991; Huntley et al. 1994; Schmidt et al. 2014).

In our study, we observed a seasonal pattern of lipid content under treatment

66°S that may be stimulated by the high latitudinal light regime. It resembles the lipid cycle observed in the field with highest values of lipid content in autumn and lowest values in early spring (Hagen et al. 2001; Meyer et al. 2010). This is the first study that shows the possible influence of light regime on the lipid cycle in Antarctic krill. The accumulation of lipid re- serves may be adjusted according to the latitudinal light regime, which may explain the differences observed in the field with higher lipid stores found in regions at higher latitudes (Schmidt et al. 2014). We also observed a match of the period of lipid depletion and re-maturation, which supports the assumption that lipid stores may be used for the maturation process (Teschke et al. 2008).

The effect of light regime on the maturity cycle (Hirano et al. 2003; Teschke et al. 2008; Brown et al. 2011) is confirmed by our study. The endogenous cycle of maturity under constant darkness has been observed in short-term experiments before (Thomas and Ikeda 1987; Kawaguchi et al. 2007; Brown et al. 2011). We show that this pattern does not persist during the second year under constant darkness and suggest that the zeitgeber photoperiod is required for the entrainment of the maturity cycle over longer periods. Results from former experiments (Hirano et al. 2003; Brown et al. 2011) indicate that Antarctic krill's maturity cycle may be entrained by the timing of two contrasting photoperiods (peak and trough light regimes).

To study potential differences in the physiological response of Antarctic krill to different latitudinal light regimes, we used the critical photoperiod (defines the day length when 50% of the population shift from one state to another, here maturity) as an indicator to determine the time of the year that is a turning point in the seasonal cycle. However, using critical photoperiod, we cannot give rise to any conclusion regarding the mechanism of entrainment of these rhythms. We observed that the critical photo- period for maturity differed between latitudinal light regimes, being higher under the high latitudinal light regime. An increase of critical photoperiod with latitude has also been found in insects in relation to diapause (Bradshaw and Holzapfel 2007; Tyukmaeva et al. 2011; Hut et al. 2013). Organisms with higher critical photo- periods have an adaptive advantage under the extreme seasonal changes of photoperiod at higher latitudes where they have to prepare early enough to ensure survival during winter. Specifically, a higher critical photoperiod for maturity implies that Ant- arctic krill is able to undertake the critical stage of sexual regression

and re-maturation during the time of the year when photoperiods are longer compared with regions at lower latitudes. In regions with extreme changes of photoperiod and severe winter conditions, this adaptive mechanism may ensure that Antarctic krill prepares early enough for winter and keeps up energy- saving mechanisms long enough.

Antarctic krill's flexibility in adjusting its photoperiodic response to a wide range of latitudinal light regimes may be advantageous under future climate change, as a southward migration trend of Antarctic krill to higher latitudes at the western Antarctic Peninsula has been reported (Ross et al. 2014). Still, changes in sea-ice dynamics, such as the timing of sea-ice formation or melt, may lead to mismatches in the timing of critical life-cycle events (Clarke et al. 2007). For instance, an earlier phytoplankton bloom associated with earlier sea-ice melt may influence the survival and reproductive success of Antarctic krill. Therefore, its potential to adapt to future environmental changes may also depend on its genetic flexibility in adjusting its photoperiodic response and the timing of critical life-cycle events (Bradshaw and Holzapfel 2007).

Our findings support the assumption of a circannual timing system synchronized by light regime in Antarctic krill (Meyer 2011). The modulation of seasonal rhythms of growth, feeding, lipid metabolism, and maturity happen independently of constant food supply, indicating an inherent mechanism in Antarctic krill that regulates the timing of these processes according to the light regime. Photoperiod may play a significant role in the initiation of neuroendocrine cascades (on–off mechanism) in Antarctic krill, as it has been found to be the primary signal initiating diapause, migration, or reproduction in other arthropods (Bradshaw and Holzapfel 2007). It remains to be clarified if the photoperiodic time measurement inducing seasonal events in Antarctic krill is related to the circadian clock (Hut et al. 2013; Meuti et al. 2015) or represents an independent circannual timing system. Using light regime as a seasonal zeitgeber makes ecologically sense because it is a more reliable cue than food availability. The intensity of the initiated seasonal physiological processes may be regulated in the field by the interaction with other factors such as food or temperature. High food quality and quantity were found to advance growth (Ross et al. 2000; Atkinson et al. 2006) and maturation (Quetin and Ross 2001) in Antarctic krill. We pro- pose that this effect is restricted to specific seasonal periods that are determined by the response of Antarctic krill's endogenous timing system

to the exposed latitudinal light regime.

This study has high relevance for future modelling approaches of Antarctic krill densities in the Southern Ocean, especially under the aspect of climate change. Recent Antarctic krill models have focussed on intraspecific food competition (Ryabov et al. 2017) or have been conducted on a conceptual basis (Groeneveld et al. 2015). The incorporation of light regime into dynamic models may significantly improve the predictability of growth, energy budget, and reproduction in Antarctic krill. Recently, a coupled energetics and moult-cycle model has been developed for Antarctic krill that considered resource allocation based on the seasonal cycles of growth and maturity (Constable and Kawaguchi 2018). Further research on the phenology and biological clock of Antarctic krill will help to better understand its adaptive potential to environmental changes.

3.6 Conclusion

This study aimed to investigate the impact of light regime on Antarctic krill's phenology and the role of its endogenous timing system. Our observations suggest that light regime affects seasonal cycles of growth, feeding, lipid metabolism, and maturity under constantly high food supply. Antarctic krill possesses an endogenous timing system that maintains seasonal rhythms un- der constant darkness and is most likely entrained by light regime. Varying critical photoperiods under different latitudinal light regimes indicate that this timing system is flexible, allowing Antarctic krill to adjust its physiological and behavioural responses to the extreme light conditions in the Southern Ocean.

3.7 Acknowledgements

We thank the staff at the Australian Antarctic Division (namely R. King, T. Waller, A. Cooper, and B. Smith) for their advice and the maintenance of the Antarctic krill during the long-term experiments in the Antarctic krill aquarium. Sincere thanks go to F. Piccolin and F. Müller for their help in setting up the experiment and their collegial support. M. Vortkamp is acknowledged for her help in the laboratory during lipid content analysis. This study 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), the ministry of science and culture (MWK) of Lower Saxony, Germany (Research Training Group "Interdisciplinary approach to functional bio- diversity research" (IBR)), and Australian Antarctic Program Project #4037. Additional funds were made available via the PACES (Polar Regions and Coasts in a changing Earth System) programme (Topic 1, WP 5) of the Helmholtz Association.

3.8 References

> see original publication

Appendix A

Frequently Asked Questions

A.1 How do I change the colors of links?

The color of links can be changed to your liking using:

`\hypersetup{urlcolor=red}`, or

`\hypersetup{citecolor=green}`, or

`\hypersetup{allcolor=blue}`.

If you want to completely hide the links, you can use:

`\hypersetup{allcolors=.}`, or even better:

`\hypersetup{hidelinks}`.

If you want to have obvious links in the PDF but not the printed text, use:

`\hypersetup{colorlinks=false}`.