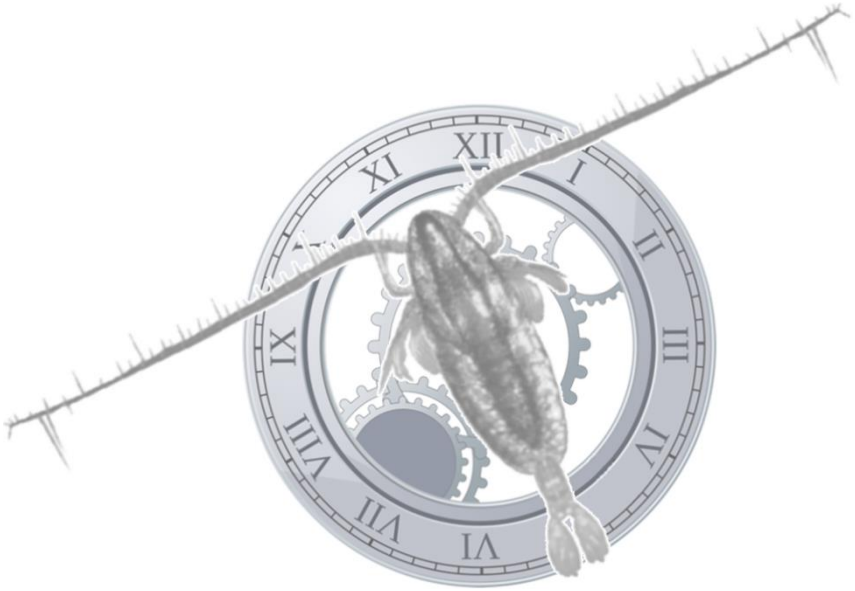
The molecular basis of diel and seasonal rhythmicity in the copepod *Calanus finmarchicus*

Die molekularen Grundlagen tages- und jahreszeitlicher Rhythmik in dem Copepoden *Calanus finmarchicus*



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Dedicated to my family

Table of contents

List of abbreviations

ADCP	acoustic Doppler current profiler
ANOVA	analysis of variances
AWI	Alfred-Wegener-Institute Helmholtz-Centre for Polar and Marine Research
C	carbon
cDNA	complementary DNA
Chl a	chlorophyll a
C/N ratio	carbon to nitrogen ratio
CO_2	carbon dioxide
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR associated protein 9
CTD	conductivity-temperature-depth profiler
CI-V	*Calanus finmarchicus* copepodid stage I-V
CVIf & CVIm	*Calanus finmarchicus* adult female (f) & male (m)
dB	decibel
DD	constant darkness
df	degrees of freedom
DNA	deoxyribonucleic acid
DVM	diel vertical migration
g	gram (µg & mg for micro- & milligram, respectively)
h	hour(s)
KCl	potassium chloride
kHz	kilohertz
L	liter(s) (µL & mL for micro- & milliliter, respectively)
LAW	lipid accumulation window
LD	light/dark cycle
LL	constant light
lxwxh	length x width x height
m	meter(s) (nm, µm & cm for nano-, micro- & centimeter, respectively)
M	moles per liter
min	minute(s)

mol	moles (µmol for micromoles)
mRNA	messenger RNA
MVBS	mean volume backscattering strength
N	nitrogen
n	number of replicates
n/a	not available
NI-VI	*Calanus finmarchicus* nauplius larvae stage I-VI
n.s.	not significant
O_2	oxygen
p	probability value
PAR	photosynthetic active radiation
PCR	polymerase chain reaction
PDF	pigment dispersing factor (insect equivalent of crustacean PDH)
psu	practical salinity units
RNA	ribonucleic acid
RNAi	RNA interference
R/V	research vessel
S2-cells	Schneider 2 cells derived from *Drosophila melanogaster*
SAMS	Scottish Association for Marine Science
SD	standard deviation
SEM	standard error of means
Sv	absolute backscatter volume
TALEN	Transcription Activator-Like Effector Nucleases
UTC	coordinated universal time
v/v	volume per volume
w/v	weight per volume
α	significance level
τ	period length
°C	degrees Celsius

Abbreviations of gene names are not included and can be found in the appendix. Generally, gene names and their abbreviations are written in italic lower case letters whereas protein abbreviations are written in capital letters.

Summary

The planktonic copepod *Calanus finmarchicus* inhabits an ecological key position in the northern Atlantic pelagic food web. It serves as a direct link from phytoplankton primary production to various higher trophic levels including predatory zooplankton, sea birds, marine mammals as well as several commercially important fish species like herring or Atlantic cod. Due to its ecological relevance, the biology of *C. finmarchicus* has been studied extensively and it is known that the copepods life is shaped by diel and seasonal rhythmicity. However, knowledge about the external factors and internal processes controlling these rhythms is limited. Endogenous clock mechanisms have been identified as potent tools for the regulation of diel and seasonal rhythmicity and numerous terrestrial species, but studies on marine organisms are very scarce. The best studied endogenous timer is the circadian clock, which uses gene/protein feedback loops to generate a rhythm with a period of ~24h length. The circadian clock has also proven to be central for the control of seasonal life cycles in various insect species, due to its ability to measure photoperiod (day length).

This dissertation investigates the rhythmic life of *C. finmarchicus* by combining analysis of clock gene expression with measurements of metabolic genes, physiological parameters, behavior, and population dynamics. It explores how the circadian clock affects the copepods diel phenotypical rhythmicity and how photoperiod, the circadian clock and other endogenous timing mechanisms may regulate the copepods seasonal life cycle. As climate change and rising ocean temperatures cause a poleward shift in the distribution of *C. finmarchicus*, the work further investigates how the extreme polar light conditions affect clock functioning and rhythmicity. The dissertation addresses these topics in the form of three publications focusing on: the circadian clock and diel rhythmicity (Publication I), seasonal rhythmicity and photoperiod (Publication II), and rhythmicity under polar light conditions (Publication III), respectively.

Publication I investigates the expression of circadian clock genes in *C. finmarchicus* and the species' diel phenotypic rhythmicity. Like many other marine organism, the copepod performs diel vertical migration (DVM). It is generally agreed that light is the dominant proximate cue for DVM, but rhythmic migrations also exist in deep sea habitats and during the polar night, showing that diel rhythmicity cannot be explained by light alone. Laboratory experiments found that many clock genes of *C. finmarchicus* show strong circadian expression rhythmicity that persisted under constant darkness and could also be found in a vertically migrating field population in the Scottish Loch Etive. The work further describes endogenous circadian rhythms in the copepods respiration and DVM

behavior. This strongly suggests that *C. finmarchicus* possesses a functioning circadian clock that is involved in the control of DVM.

Publication II addresses the seasonal life cycle of *C. finmarchicus*, which is characterized by feeding and development in surface waters in spring/summer followed by a phase of diapause in deeper waters in autumn/winter. Copepods were collected in Loch Etive over the 24h cycle at six seasonal time point. Analysis of diel and seasonal expression patterns of clock genes and metabolic genes were combined with investigation of lipid content and other physiological parameters, community composition and abundance, and vertical migration behavior. This integrative approach resulted in a highly detailed description of *C. finmarchicus*' life cycle and the most extensive field dataset on clock gene expression in a marine species to date. The work shows that diel clock gene cycling is confined to the phase of activity in surface waters and most likely ceases during diapause. While previous studies emphasized to role of lipid content and food availability for the seasonal timing of diapause, this work suggests that the initiation of diapause involves circadian clock based photoperiod measurement in interaction with other factors like lipid content and temperature. The data further indicates that the emergence from diapause could be controlled by an endogenous circannual timing mechanism.

Publication III explores the adaptive capacity of *C. finmarchicus*' circadian clock and its seasonal rhythmicity under the extreme light conditions in the high Arctic Kongsfjorden. Field samples were collected during the active life phase, early diapause and late diapause, representing times of near-permanent light (end of midnight sun), a clear day/night cycle, and permanent darkness (polar night), respectively. As for Loch Etive, samples were collected over the 24h cycle showing the clock genes cycling and DVM were during the active phase at the end of midnight sun, while rhythmicity ceased after the transition to diapause. Seasonal expression of metabolic genes further mostly resembled the patterns from Loch Etive. The work suggests that poleward distribution shifts of *C. finmarchicus* will have only minor effects on copepods circadian clock and diel rhythmicity. However, regulation of seasonal rhythmicity based on photoperiod measurement or a circannual timer could result in mismatch situations that may even be aggravated by climate change related shift in environmental timing.

In conclusion, the present dissertation expands the knowledge about the molecular timing mechanisms governing diel rhythmicity and the seasonal life cycle of the key copepod *C. finmarchicus*. In addition, this dissertation shows how external cues like light and photoperiod affect the copepods clock systems and its rhythmicity. The work provides an

example of how techniques well established in molecular biology and chronobiology of terrestrial model species can be applied to marine non-model organisms. It becomes evident that a detailed mechanistic knowledge about marine clock systems, especially in ecological key species like *C. finmarchicus*, will be crucial to understand marine rhythms of life and how these species will be affected by future climate change.

Zusammenfassung

Der planktonische Copepode *Calanus finmarchicus* hat eine Schlüsselposition im Nahrungsnetz des Nordatlantiks innen. Die Art schafft eine direkte Verbindung zwischen der Primärproduktion des Phytoplanktons und verschiedensten höheren trophischen Ebenen wie räuberischem Zooplankton, Seevögeln, Meeressäugern sowie mehreren kommerziell genutzten Fischarten wie Hering oder Kabeljau. Wegen seiner ökologischen Bedeutung wurde *C. finmarchicus* eingehend studiert und es ist bekannt, dass das Leben des Copepoden durch tages- und jahreszeitlich Rhythmen bestimmt wird. Das Wissen über die externen Faktoren und internen Prozesse, die diese Rhythmen kontrollieren, ist begrenzt. Es wurde gezeigt, dass endogene Uhren eine wichtige Rolle bei der Regulation tages- und jahreszeitlicher Rhythmen in zahlreichen terrestrischen Spezies spielen, während Untersuchungen zu marinen Arten selten sind. Der am besten untersuchte endogene Uhrmechanismus ist die zirkadiane Uhr, die mittels Rückkopplungsschleifen von Genen und Proteinen einen Rhythmus von ~24 Stunden erzeugt. Es wurde zudem nachgewiesen, dass die zirkadiane Uhr, wegen ihrer Fähigkeit die Photoperiode (Tageslänge) zu messen, zentral für die Kontrolle saisonaler Lebenszyklen in verschiedensten Insektenarten ist.

Diese Dissertation untersucht die Lebensrhythmen von *C. finmarchicus*, indem sie die Analyse der Expression von Uhrgenen mit Messungen von metabolischen Genen, Stoffwechselaktivität, physiologischer Parametern, Verhalten und Populationsdynamik kombiniert. Sie erforscht, wie die zirkadiane Uhr tageszeitliche phänotypische Rhythmen beeinflusst, und wie Photoperiode, die zirkadiane Uhr und andere endogene Uhrmechanismen den saisonalen Lebenszyklus des Copepoden regulieren könnten. Da der Klimawandel und steigende Meerestemperaturen das Verbreitungsgebiet von *C. finmarchicus* Richtung Pol verschieben, untersucht die Arbeit zudem, wie die extremen polaren Lichtverhältnisse die innere Uhr und Rhythmik des Copepoden beeinflussen. Die Dissertation behandelt diese Themen in Form von drei Publikationen, die sich jeweils auf die Aspekte zirkadiane Uhr und tageszeitliche Rhythmik (Publikation I), saisonale Rhythmik und Photoperiode (Publikation II), und Rhythmik unter polaren Lichtverhältnissen (Publikation III) konzentrieren.

Publikation I untersucht die Expression zirkadianer Uhrgene und die tageszeitliche phänotypische Rhythmik von *C. finmarchicus*. Wie viele andere marine Organismen zeigt der Copepode tageszeitliche Vertikalwanderungen (engl. diel vertical migration, DVM). Allgemein wird Licht als wichtigstes unmittelbares Signal für die Steuerung von DVM angesehen. Die Vertikalwanderungen geschehen jedoch auch in Tiefsee Habitaten und

während der Polarnacht, was zeigt, dass die tageszeitliche Rhythmik nicht durch Licht alleine erklärt werden kann. In Laborexperimenten wurde festgestellt, dass die Expression von Uhrgenen in *C. finmarchicus* eine starke tageszeitliche Rhythmik zeigt, die in konstanter Dunkelheit erhalten bleibt und zudem auch in einer Population im schottischen Loch Etive nachgewiesen wurde, die DVM zeigt. Die Arbeit beschreibt zudem endogene zirkadiane Rhythmen in der Respiration und der Vertikalwanderung der Copepoden. Dies deutet stark darauf hin, dass *C. finmarchicus* eine funktionstüchtige zirkadiane Uhr besitzt, die in die Kontrolle der tageszeitlichen Vertikalwanderung involviert ist.

Publikation II befasst sich mit dem saisonalen Lebenszyklus von *C. finmarchicus*. Dieser ist charakterisiert durch eine Phase des Fressens und Wachstums nahe der Oberfläche im Frühling/Sommer und eine Phase des Überwinterns (Diapause) in tieferen Wasserschichten im Herbst/Winter. An sechs saisonalen Zeitpunkten wurden im Loch Etive jeweils über den gesamten 24 Stunden Zyklus Copepoden gesammelt. Die tages- und jahreszeitlichen Expressionsmuster von Uhrgenen und Metabolischen Genen wurden analysiert und mit Untersuchungen des Lipidgehalt und anderer physiologischer Parameter, der Abundanz und Zusammensetzung der Copepodenpopulation und der Vertikalwanderungen kombiniert. Durch diesen integrativen Ansatz gelang eine hoch detaillierte Beschreibung des Lebenszyklus von *C. finmarchicus*, die zudem dem bisher umfangreichsten Feld-Datensatz zur Expression von Uhrgenen in einer marinen Spezies beinhaltet. Die Arbeit zeigt, dass die rhythmische Expression von Uhrgenen auf die aktive Phase nahe der Oberfläche beschränkt ist und sie in der Diapause höchstwahrscheinlich stoppt. Während frühere Studien die Bedeutung des Lipidgehalts und der Nahrungsverfügbarkeit für das saisonale Timing der Diapause betonten, legt diese Arbeit nahe, dass die durch die zirkadiane Uhr gemessene Photoperiode, in Interaktion mit anderen Faktoren wie Lipidgehalt und Temperatur, eine wichtige Rolle bei der Initiation der Diapause spielt. Die Daten deuten zudem darauf hin, dass das Erwachen aus der Diapause durch einen endogenen zirkannuellen (jahreszeitlichen) Uhrmechanismus kontrolliert werden könnte.

Publikation III erforscht die adaptive Kapazität von *C. finmarchicus*‘ zirkadianer Uhr und seiner saisonalen Rhythmik unter den extremen Lichtverhältnissen im hocharktischen Kongsfjord. Feldproben wurden währen der aktiven Lebensphase, sowie während der frühen und späten Diapause gesammelt. Die Phase repräsentierten jeweils Zeiten mit fast-permanentem Licht (Ende der Mitternachtssonne), einen klaren Tag/Nacht Zyklus, und permanente Dunkelheit (Polarnacht). Proben wurden wie im Loch Etive über einen 24 Stunden Zyklus gesammelt. Tageszeitliche Rhythmen in der Expression der Uhrgene

und DVM der Copepoden wurde in der aktiven Phase am Ende der Mitternachtssonne festgestellt, während die Rhythmik nach dem Übergang zur Diapause verloren ging. Die saisonalen Expressionsmuster der metabolischen Gene glichen zudem größtenteils den Mustern im Loch Etive. Die Arbeit legt nahe, dass die zirkadiane Uhr und tageszeitliche Rhythmik von *C. finmarchicus* nur in geringem Maße von einer Verschiebung des Verbreitungsgebiets des Copepoden Richtung Pol beeinflusst werden. Allerdings könnte eine Steuerung der saisonalen Rhythmik, die auf dem Messen der Photoperiode oder einem zirkannuellen Uhrmechanismus basiert, zu „Mismatch"-Situationen führen. Durch den Klimawandel bedingte saisonale Verschiebungen im Timing von Umweltparametern könnten die negativen Effekte solcher Situationen noch verstärken.

Zusammengefasst lässt sich sagen, dass die vorliegende Dissertation das Wissen über die Prozesse erweitert, die die tageszeitliche Rhythmik und den saisonalen Lebenszyklus der Schlüsselart *C. finmarchicus* regulieren. Zusätzlich zeigt die Dissertation, wie externe Signale wie Licht und Photoperiode die Uhrsysteme und Rhythmik des Copepoden beeinflussen. Die Arbeit liefert ein Beispiel dafür, wie Techniken, die in der molekularen Biologie und Chronobiologie für terrestrische Modelarten etabliert sind, auf marine nicht-Modelorganismen übertragen werden können. Die Arbeit macht deutlich, dass ein mechanistisches Verständnis mariner Uhrsysteme, speziell in ökologischen Schlüsselarten wie *C. finmarchicus*, essentiell ist, um marine Lebensrhythmen verstehen, und um zu verstehen, wie diese Arten durch zukünftige Klimaveränderungen beeinflusst werden.

1 General Introduction

In the following I like to introduce the major topics of the dissertation. It starts with a general description of the key species *Calanus finmarchicus* and its diel and seasonal rhythmicity. After that, the second major topic are endogenous clocks systems with an emphasis on the molecular functioning of circadian clocks, how they affect the diel rhythmicity of organisms, and how clock-based photoperiod measurement and circannual clock mechanisms can regulate seasonal life cycles. The third major aspect addresses the peculiarities of marine and polar rhythmic environments and the clock adaptations of organisms living under these conditions. The introduction ends with a description of the reasoning and the relevance of this dissertation, the main research questions, and how they were addressed.

1.1 The copepod Calanus finmarchicus

Calanus finmarchicus (Gunnerus, 1770) is a small crustacean from the subclass Copepoda. Copepods are divided into 10 orders with *C. finmarchicus* belonging to the order Calanoida (Ho 1990, Mauchline 1998). Calanoid copepods are mostly planktonic and they can be found in marine and freshwater habitats around the world. The order often dominates zooplankton in terms of biomass and abundance (Mauchline 1998), making them a crucial component of marine ecosystems.

Morphometrics, larval development and distribution range

Adult *C. finmarchicus* reaches a body length of 2.5-3.0 mm with the species growing bigger at higher latitudes under lower temperature (Melle et al. 2014, Leinaas et al. 2016). The body is divided into the barrel-shaped prosome, which carries a median naupliar eye, the antennas, mouthparts and swimming legs, as well as the slender urosome, which contains the genital somites, but carries no appendages except for the caudal remi (Marshall & Orr 1955) (Fig.1.1A). Many copepod species possess well-developed first antennae, but in the genus *Calanus* they typically exceed the entire body length of the animal. *C. finmarchicus* morphology shows large similarity to the congener species *C. glacialis* and *C. helgolandicus*. The three species partially overlap in distribution (Conover 1988, Mauchline 1998, Gabrielsen et al. 2012, Nielsen et al. 2014). Precise species identification requires the examination of appendage morphology or genetic analyses (Marshall & Orr 1955, Gabrielsen et al. 2012), but the color of antennae and somites (for *C. glacialis*) and the head shape (for *C. helgolandicus*) have been described as proxies for distinguishing *C. finmarchicus* from its congeners (Weydmann et al. 2014, Niehoff pers. comm.). Another *Calanus* species overlapping in distribution is *C. hyperboreus*, but this copepod is much larger in size and is therefore easy to identify.

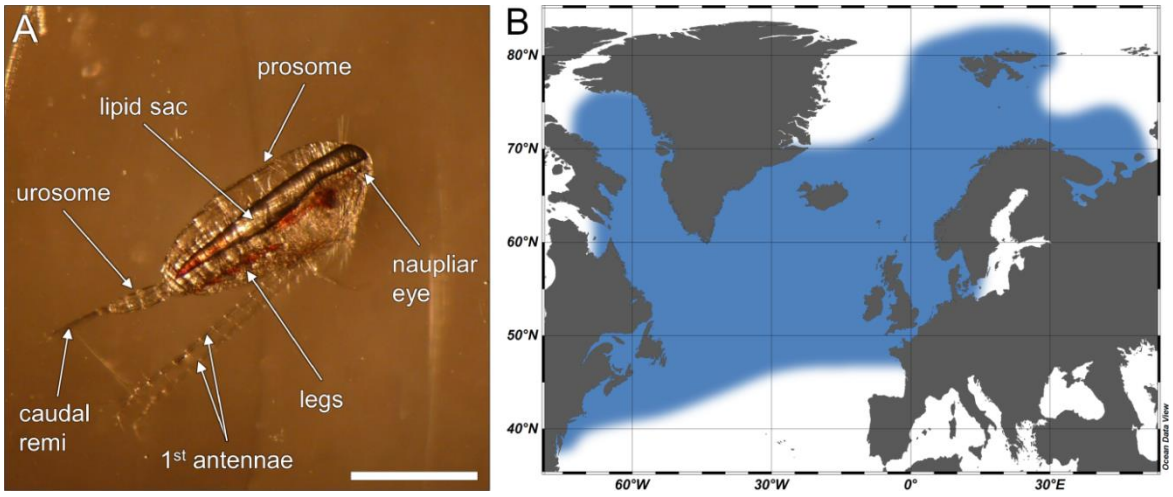

Figure 1.1: Morphology and Distribution of *C. finmarchicus*. (A) Lateral view of a *C. finmarchicus* (CV stage) is shown. Prominent morphological features are indicated. The white scale bar indicates 1 mm. (B) Distribution of *C. finmarchicus* in the northern Atlantic and in the Arctic. The map was created with Ocean Data View® based on data by Fleminger & Hulsemann (1977), Conover (1988), and Reygondeau & Beaugrand (2011).

The developmental cycle of *C. finmarchicus* starts with planktonic eggs that are fertilized internally and then released into the water column where they hatch after ~24h. The hatchlings molt through six nauplius larvae stages (NI-NVI) over a period of 10-20 days with the first feeding stage being the NIII stage (Campbell et al. 2001). The NVIs then molt into the first juvenile copepodid stage (CI), which is the first one resembling the adults in terms of body shape. The copepods develop trough five copepodid stages (CI-CV) with the number of prosome segments and leg pair increasing from two to five over the course of the molts. While the development time from CI to CV takes 10-15 days (Marshall & Orr 1955, Campbell et al. 2001), the duration of the CV, the final juvenile stage is highly flexible. Molting to the adult female or male stage (CVIf & CVIm, respectively) can happen already 10 days after reaching the CV stage, but the final molt may be delayed for periods of up to 300 days as *C. finmarchicus* can enter a phase of arrested development (diapause) in the CV stage (Saumweber & Durbin 2006, Wilson et al. 2016a) (see below). Generally, the development time is reduced by higher temperatures and food concentrations. However, while high food levels also lead to increased body size, higher temperatures have the opposite effect (Campbell et al. 2001).

C. finmarchicus inhabits oceanic and coastal waters throughout the northern Atlantic and may also be found in brackish habitats (Fig.1.1B) (Conover 1988, Helaouët & Beaugrand 2007, Hill 2009). The copepods live in the upper water column from the surface down to 500-1000 m, although vertical distribution can show strong variation over the diel and especially seasonal cycle (Hirche 1996a, Niehoff et al. 1999, Irigoien 2000) (see below). In shallower neritic habitats there vertical distribution may be limited by water depth. The

species covers a large latitudinal range from about 40°N up to 80°N, reaching down into the Gulf of Maine in the west and the waters around Great Britain and the North Sea in the east. The more southern distribution in the western Atlantic is probably related to the cold waters of the Labrador Current flowing south along the coast of the American continent as well as the warm water of the Gulf Stream spreading northward in the eastern Atlantic. At its northern edge of distribution, *C. finmarchicus* inhabits waters southwest of Greenland, parts of the Barent Sea and the western Fram Strait. The species northernmost resident population is assumed to live in Kongsfjorden, an Arctic fjord of the Svalbard archipelago and currents carry expatriates even further into the Arctic Ocean (Kwasniewski et al. 2003, Hirche & Kosobokova 2007).

Diel rhythmicity and zooplankton vertical migration

C. finmarchicus' life is shaped what is probably the largest daily migration of biomass on the planet, the diel vertical migration (DVM) of zooplankton and nekton in the world's oceans (Daase et al. 2008, Brierley 2014). Copepods and other zooplankton organisms ascent to shallow waters at sunset to feed on the phytoplankton, which consists of unicellular algae and photosynthetic bacteria that depend on light and thus grow near the surface. As the sun return in the morning the zooplankton retreats into deeper waters layers to seek refuge from visual predators that can only hunt during the day. Although many species performing DVM are only millimeters in size, the diel migrations can span several hundred meters (Brierley 2014). The DVM of *C. finmarchicus* covers a range of 50-100 m, but there are also reports diel migrations exceeding 200 m (Daase et al. 2008). The intensity of DVM in *C. finmarchicus* is strongly affected by life stage, season and environmental conditions like bottom depth or water column stratification (Dale & Kaartvedt 2000, Daase et al. 2008, Rabindranath et al. 2011). The daily cycle of migration and feeding is also reflected in copepod physiology as *C. finmarchicus* shows diel oscillations in respiration and digestive enzyme activity (Båmstedt 1988).

It is generally agreed that the ultimate reason for zooplankton DVM is the trade-off between feeding at the surface and predation pressure during the day (predator evasion hypothesis, Zaret & Suffern 1976). However, predation pressure (or the lack thereof) is often hard to sense until it is too late. In contrast, the change in light over the day/night cycle provides a very reliable proximate cue for the timing of DVM and the vertical migrations of most species are heavily influenced by ambient light conditions (Cottier et al. 2006, Brierley 2014). Other factors like predation pressure can also affect migration patterns, but the effects are small relative to light (Ringelberg & van Gool 2003, Hansson et al. 2007). There are two major assumptions on how light shapes DVM. While the "isolumen hypothesis" suggests that zooplankton organisms seek a depth of preferred

light intensity and follow this isolumen as its depth changes over the course of the day/night cycle), the “rate of change hypothesis” assumes that vertical migrations are triggered by fast change in light intensity as they occur at sunset and sunrise, respectively (Cohen & Forward Jr 2005). There is supporting evidence for both concepts and different zooplankton species may use different mechanisms, but there are also reports of DVM patterns, which cannot be explained by responses to light alone (Cohen & Forward Jr 2005). DVM was detected in deepwater habitats at the edge of from the mesopelagic to the bathypelagic zone (van Haren 2007, van Haren & Compton 2013). During their diel migration zooplankton organism retreated to depth below 1000 m where protons are no longer detectable. Despite the lack of an external cue, zooplankton rose from the deep at sunset. Similarly, rhythmic DVM was found in Arctic waters during the polar night where overall light intensity and its diel variation are extremely low (Berge et al. 2009, 2015). Diel change in sunlight may be detectable in surface waters during the polar night (Båtnes et al. 2013, Cohen et al. 2015) and vertical migration may also be supported by moonlight (Last et al. 2016), but for a large fraction of the day light is absent, raising the question on how zooplankton maintains diel rhythmicity. In habitats with a clear day/night cycle there are also DVM patterns, which cannot be explained by light alone. “Midnight sinking” describes a phenomenon where zooplankton rises to the surface at sunset, sinks to intermediate depth in the middle of the night and again migrates to the surface close to sunrise before retreating to daytime depth (Cohen & Forward Jr 2005). This behavior was also observed in *C. finmarchicus* and has been associated with a digestion phase after the initial feeding at sunset, followed by a second feeding phase (Simard et al. 1985). However other authors have shown that surface peak abundance is closely associated with sunset/sunrise independent of day length (Tarling et al. 2002). As digestion time should be constant, the author argued that *C. finmarchicus* was evading other vertically migrating predators (krill) that ascended to the surface around midnight. It was suggested that deep sea and polar DVM as well as midnight sinking are (partially) controlled by endogenous timing mechanisms that maintain diel rhythmicity in the absence of external cues (Cohen & Forward Jr 2005, van Haren & Compton 2013, Berge et al. 2015) and the nature of such endogenous clocks and how they can affect the rhythmic life of marine organism will be addressed in section 1.2 and 1.3.

Seasonal life cycle

The development of *C. finmarchicus* through the different life stages follows a distinct seasonal pattern. Adults spawn in spring with egg production being fueled by the phytoplankton bloom (Niehoff et al. 1999, Harris et al. 2000). After spawning, the adults

die and the new generation develops through the nauplius and copepodid stages in spring/summer feeding on surface phytoplankton (Marshall & Orr 1955, Meyer-Harms et al. 1999). After the bloom when the phytoplankton concentration is low the copepods may also feed omnivorously on protest and heterotrophic algae (Ohman & Runge 1994, Meyer-Harms et al. 1999). DVM in *C. finmarchicus* occurs mostly during this active phase with older copepodid stages showing the strongest vertical migration and generally staying deeper in the water column (Dale & Kaartvedt 2000, Daase et al. 2008). From the CIV stage on *C. finmarchicus* starts to accumulate large amounts of lipids in the form of wax esters (Kattner & Hagen 1995, Mauchline 1998). Lipids can account for more than 40% of copepod dry mass in the CV stage and are stored in a lipid sac in the prosome that can occupy more than half of the copepods body volume (Fig.1A) (Pasternak et al. 2001, Lee et al. 2006, Vogedes et al. 2010). In the CV stage, individuals either mature and molt to adults to spawn another generation, or they migrate down into deeper water layers were they enter a state of inactivity and developmental commonly referred to as diapause (Hirche 1996a). The seasonal timing of this descent can vary strongly between different populations, but is rather constant within a population between years (Marshall & Orr 1955, Melle et al. 2014). Diapause depth of *C. finmarchicus* depends on the environment with copepods staying at 400-1000 m in the open ocean, while in shallow coastal habitats they stay in the water column above the bottom and can also diapause in depth of ~100 m (Hirche 1996a, Clark et al. 2013). Diapausing CVs do not feed and have a reduced gut epithelium, meaning that, if food was present, they would be unable to process it (Hallberg & Hirche 1980, Hirche 1996a). The copepods show a metabolic reduction with the remaining energy costs being covered by the gradual mobilization of the wax esters stored in the lipid sac (Hirche 1996a, Ingvarsdóttir et al. 1999). Studies on *Calanus* have shown that these physiological changes happen after the copepods arrive at diapause depth (Head & Harris 1985, Freese et al. 2017). Diapause is not homogenous, but can be divided into a refractory and emergence phase, respectively. During the refractory phase development is arrested and energy expenditure is minimized. In the emergence phase the CVs start to develop their reproductive tissues and metabolic activity increases, leading to increased lipid utilization (Hirche 1996a,b, Baumgartner & Tarrant 2017). Emergence starts in early winter and thus well before the ascent to the surface in spring, although the final maturation of reproductive tissues, which consumes a large fraction of the lipids storages is closely associated with the final molt to the adult stage closely before the ascent (Tande 1982, Hirche 1996a,b, Rey-Rassat et al. 2002). Adult females generally largely outnumber males. The copepods mate during the ascent to the surface where they spawn to produce a new generation (Marshall & Orr 1955, Hirche 1996b).

Although *C. finmarchicus* diapause has been investigated in great detail since more than a century, the mechanisms controlling the initiation and termination of diapause are still poorly understood. The timing of diapause initiation and the number of generations produced per year differs largely between populations with high latitude copepods usually producing only 1 generation per year while there can be up to 3-4 generations at lower latitudes (Marshall & Orr 1955, Durbin et al. 2000, Walkusz et al. 2009, Melle et al. 2014). Even within populations it is common that one fraction of a generation enters diapause while the other fraction matures and produces one or two additional generations (Durbin et al. 2000, Tarrant et al. 2008). The currently dominant opinion is that diapause is initiated once the CV stages accumulate a critical amount of lipids considered sufficient to survive the time of starvation at depth (Rey-Rassat et al. 2002, Saumweber & Durbin 2006, Johnson et al. 2008, Tarrant et al. 2008, Maps et al. 2011).This concept is known as the “lipid accumulation window” (LAW) hypothesis (Rey-Rassat et al. 2002). It has also been proposed that the decrease in food concentration after the spring bloom triggers the descent to diapause (Hind et al. 2000, Wilson et al. 2016b). Photoperiod (day length) has also been proposed as a seasonal cue for *C. finmarchicus* diapause initiation, based on its central role in insects and other copepods (Grigg & Bardwell 1982, Miller et al. 1991, Marcus & Scheef 2010, Meuti & Denlinger 2013). There is supporting and contradicting evidence for all of these concepts, although the influence of photoperiod has so far received relatively little attention. Emergence and diapause termination is usually well synchronized within a given *C. finmarchicus* population even if several generations were produced that initiated diapause with several months of delay (Miller et al. 1991, Baumgartner & Tarrant 2017). This led to the assumption that emergence could be initiated either by the seasonal change in photoperiod (Miller et al. 1991, Speirs et al. 2005) or by an internal “hourglass” that measures time based either on the gradual depletion of lipid storages (Jónasdóttir 1999, Saumweber & Durbin 2006) or on a slow continuous development (Hind et al. 2000). As for the initiation, there is no clear understanding of the emergence process in *C. finmarchicus* and it is so far not possible reliably induce or terminate diapause in the laboratory (Baumgartner & Tarrant 2017).

Ecological relevance of the *Calanus* complex

C. finmarchicus and its congener species (*C. helgolandicus*, *C. glacialis*, *C. hyperboreus*) inhabit ecological key positions in the northern Atlantic and Arctic food web and often dominate zooplankton in terms of biomass (Smith & Schnack-Schiel 1990, Falk-Petersen et al. 2007, Atkinson et al. 2015). The copepods can consume large parts of the phytoplankton primary production (Hansen et al. 1990, Meyer-Harms et al. 1999), converting energy-poor algae into energy-rich wax esters. This makes *Calanus* a high

energy food source for various higher-level predators, ranging from amphipods to seabirds and baleen whales (Baumgartner et al. 2003, Steen et al. 2007, Kraft et al. 2013). Furthermore, the *Calanus* species are a crucial diet component for the larvae of several ecologically and commercially important fish species like Herring (*Clupea harengus*), Atlantic cod (*Gadus morhua*) and polar cod (*Boreogadus saida*) (Sundby 2000, Prokopchuk & Sentyabov 2006, Benoit et al. 2010). The copepods show latitudinal distribution shifts related to climate change and increasing ocean temperatures (Falk-Petersen et al. 2007, Reygondeau & Beaugrand 2011, Feng et al. 2017, Chivers et al. 2017). As the species differ largely in their nutritional content (Rey-Rassat et al. 2002, Falk-Petersen et al. 2009) changes in distribution or life history can have severe consequences for higher trophic levels (Falk-Petersen et al. 2007, Søreide et al. 2010, Perretti et al. 2017). Neither regulation of DVM nor diapause are completely understood and it is therefore unclear how the changing latitudinal environment will affect the rhythmic life of *C. finmarchicus* and consequently how this will impact the northern Atlantic and Arctic ecosystems.

1.2 Endogenous clocks

Life on earth is affected by a variety of rhythms like the day/night cycle caused by the earths own rotation, the lunar cycle with a period of 29.5 days, or the annual change of the season due to the planet tilted axis and its revolution around the sun. Most organisms have adapted to these rhythmic and predictable environmental changes by the evolution of endogenous clock, which enable them to anticipate the changes and thus maximize their fitness (Goldman et al. 2004, Mackey 2007, Yerushalmi & Green 2009, Tessmar-Raible et al. 2011, Dunlap & Loros 2016). The most prominent and best studied timing mechanism is the circadian clock, which creates an endogenous 24h rhythm. The initial characterization of this molecular clock by Jeffrey C. Hall, Michael Rosbash and Michael W. Young was honored with the 2017 Nobel Prize in Physiology or Medicine.

The circadian clock machinery

The creation of an endogenous 24h cycle is based on so-called clock genes. However, the types of involved clock genes and the ways in which they interact can differ strongly between organism groups, suggesting that circadian clock evolved independently in several different clades and also experienced major evolutionary diversification within these groups (Dunlap 1999 1999, Tauber et al. 2004, Christie et al. 2017, Kuhlman et al. 2017). The ubiquitous principle that all circadian clocks shared is the interaction of clock genes and their protein products via delayed feedback loops, resulting in oscillating gene activity with a period of ~24h (latin: *circa dies* = about a day).

The identification of molecular clock components started in the fruit fly *Drosophila melanogaster* (Bargiello et al. 1984, Reddy et al. 1984). Hence, the circadian clock in this model species is the most investigated one and will serve as an example here. At the center of the clock in *Drosophila* and almost all arthropods investigated so far are the clock genes *clock* (*clk*) and *cycle* (*cyc*) (Fig.1.2) (Tomioka & Matsumoto 2015). Their protein products CLK and CYC accumulate in the cytosol after sunrise and form a heterodimer, which is imported into the nucleus around midday where it acts as a transcription factor that binds to specific DNA sequences knowns as E-box elements (Mackey 2007, Strauss & Dircksen 2010, Tomioka & Matsumoto 2015). One or more E-boxes can be found in the promoter regions of numerous genes including the clock genes *period* (*per*) and *timeless* (*tim*) and the binding of the CLK/CYC heterodimer initiates their expression, which peaks at sunset (Mackey 2007, Matsumoto et al. 2007). The PER and TIM proteins accumulate in the cytosol during the night and enter the nucleus as heterodimer PER/TIM that inhibits CLK/CYC activity and thereby their own expression, forming the central feedback loop of the circadian clock (Mackey 2007). Another feedback loop is formed by the clock gene *clockwork orange* (*cwo*), which also peaks in expression at sunset with the CWO protein binding to E-boxes thereby preventing activation by CLK/CYC. *cwo* inhibits its own expression and is believed to increase oscillation amplitude in other clock genes (Lim et al. 2007, Richier et al. 2008, Zhou et al. 2016). The third feedback loop consists of the CLK/CYC-activated clock genes *vrille* (*vri*) and *PAR domain 1ε* (*pdp1ε*) that act on the expression of *clk* (Cyran et al. 2003). While VRI accumulates fast and inhibits *clk* expression, PDP1ε accumulates slower but then replaces VRI in the *clk* promoter region leading to increased expression. As a consequences *clk* but not *cyc* oscillates in the *Drosophila* clock with peak expression at sunrise. Several clock-associated genes like *doubletime* (*dbt*), *casein kinase 2 α* (*ck2α*), *protein phosphatase 2A* (*PP2A*) or *shaggy* (*sgg*) do not oscillate, but their proteins affect the phosphorylation state and the nucleus import of the core clock proteins like PER or TIM, thereby tuning the circadian clock to an endogenous period of ~24h (Bae & Edery 2006, Mackey 2007). The acting of the clock-associated genes can also evoke differences in oscillations between clock genes and their proteins, meaning that a clock protein can show circadian oscillations, although its gene is continuously expressed and vice versa (Reddy & Rey 2014, Thurley et al. 2017).

As the timing of the clock is not perfect, it needs to be entrained (synchronized) to the environmental day/night cycle on a regular basis. Light is by far the most reliable cue ("*Zeitgeber*") for this entrainment (Aschoff 1954) and its effect on the clock machinery of *Drosophila* and other arthropods is mediated by the protein of the gene *cryptochrome1*

(*cry1*), also known as *Drosophila*-like CRY (CRY-d) (Mackey 2007, Sandrelli et al. 2008). In the presence of (blue) light, CRY1 initiates the degradation of TIM (Fig.1.2). Without the stabilizing effect of TIM, PER also gets degraded, meaning that the PER/TIM heterodimer can only accumulate and act at night, ensuring a proper synchronization of the endogenous rhythm with the environment (Hardin 2005, Mackey 2007). Another environmental cue that can be used for clock entrainment is a diel change in temperature (Rensing & Ruoff 2002, Tataroglu et al. 2015). It is however important to note that circadian clocks in general are "temperature-compensated", meaning that they, unlike other physiological processes, run at a constant speed as ambient temperature change, thus maintaining a period length of ~24h (Kuhlman et al. 2017).

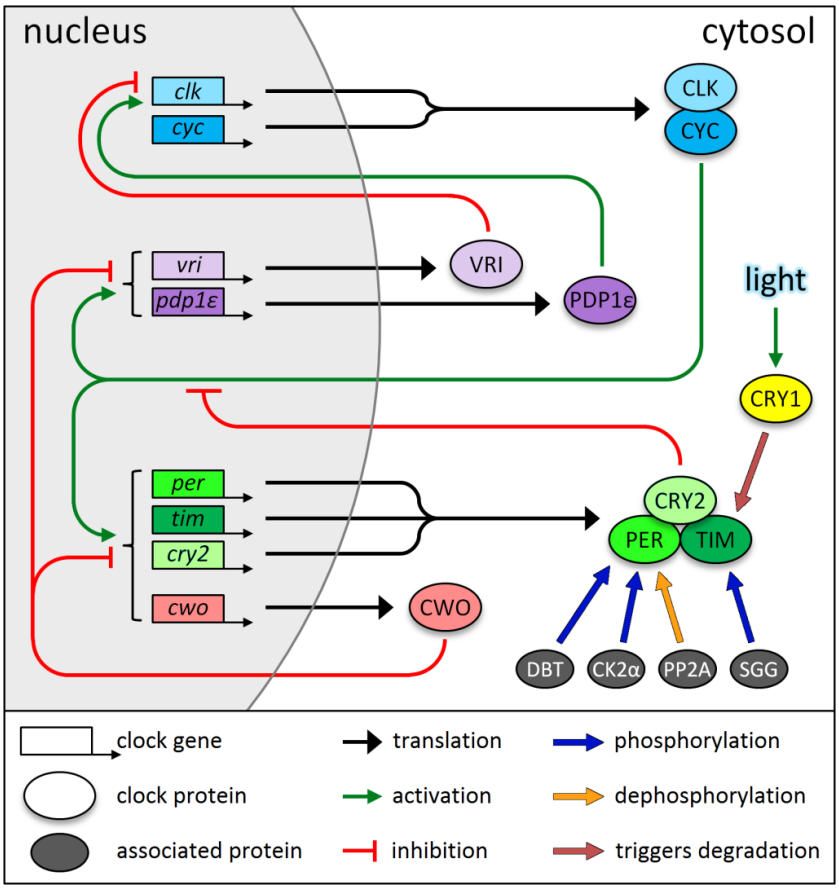

Figure 1.2: Generalized model of an arthropod circadian clock. Clock genes and their proteins interact with each via feedback loops. The clock is entrained by light via the protein CRY1. Accumulation and import of clock proteins into the nucleus are affected by clock-associated proteins. Note that *cry2* is not present in the clock of *Drosophila* described in the text, but is found in various other arthropod species. The model is simplified and does not include all molecular components of circadian timekeeping. The figure was created based on Hardin (2005), Mackey (2007), Allada & Chung (2010), and Goto (2013).

Although circadian clocks in other animals and especially other arthropods share great similarities, there are also some clear differences (Sandrelli et al. 2008, Tomioka & Matsumoto 2015). For example, *cyc* is continuously expressed in *Drosophila* while *cyc* expression oscillates in other dipteran species (Meireles-Filho & Kyriacou 2013). The most prominent difference between *Drosophila* and most other arthropods is the lack of the clock gene *cryptochrome2* (*cry2*) in the fruit fly. In contrast to CRY1, the CRY2 protein (also known as mammalian-like CRY, CRY-m) is not directly affected by light, but the

gene oscillates with peak expression at sunset and forms a complex with PER/TIM that then inhibits CLK/CYC (Zhu et al. 2005). Peak times of *cry2* expression differ between species with some showing peak highest expression during the day while in others *cry2* peaks at night (Gentile et al. 2009, Merlin et al. 2009, Teschke et al. 2011, Zantke et al. 2013). The fact that *cry2* is present in various species including *C. finmarchicus* and also in species which are believed to possess ancestral clock machineries suggests that the gene was lost in *Drosophila* (Reppert 2007, Zantke et al. 2013, Christie et al. 2013).

Circadian outputs and diel rhythmicity

As E-boxes are not confined to clock genes, but can be found of in the promoter regions of a variety of genes, the activation by the CLK/CYC heterodimer and its rhythmic inhibition by PER/TIM(/CRY2) and CWO creates a circadian output. Transcriptomic studies found that around 10% of transcripts can be expressed in a circadian fashion in animals, although it is likely that the expression of a large fraction of genes is not directly affected by clock components, but by signal cascades transducing the circadian rhythm (Panda et al. 2002, Albrecht 2006, Wu et al. 2014, Payton et al. 2017). The rhythmic expression of the clock machinery in higher organisms is usually confined to specific groups of clock neurons in an animals brain, often termed the ”central oscillator” (Zantke et al. 2013, Zhang et al. 2013, Kuhlman et al. 2017). Light entrainment of these cells within the brain can either happen directly via non-visual light perception by proteins like CRY1, or via signal transduction from the eye (visual light perception) (Yoshii et al. 2016). The orchestration of clock neurons as well as the communication of their circadian rhythms to other body parts is achieved direct neuron interaction as well as hormones. A central role in the organismic orchestration of diel rhythmicity is attributed to the pigment dispersing factor (PDF), which is known as pigment dispersing hormone (PDH) in crustaceans (Strauss & Dircksen 2010, Yoshii et al. 2016).

Circadian clocks affect a variety of diel rhythms in physiology and behavior. On the cellular level clock allow for a temporal separation of different metabolic pathways that can interfere with each other (Panda et al. 2002, Albrecht 2006, De Pittà et al. 2013, Thurley et al. 2017). For example, in the mussel *Mytilus californianus* mitochondrial energy production and cell growth are temporally separated (Chen & McKnight 2007, Gracey et al. 2008). Circadian rhythms in physiology include cycles in hormone levels like the “sleeping”-hormone melatonin (Feng & Bass 2016, Mendoza-Vargas et al. 2017, Kim et al. 2017) as well as diel cycles in the activity of metabolic and digestion enzymes (Trellu & Ceccaldi 1977, Mayzaud et al. 1984, Espinosa-Chaurand et al. 2017, Thurley et al. 2017). Behavioral rhythms do often present the most overt circadian output. These behavioral patterns can be very diverse and include swimming activity (Zhang et al. 2013,

Tosches et al. 2014), diel cycles of feeding (Nelson & Vance 1979, Stearns 1986, Santos et al. 2016), courtship behavior (Feng & Bass 2016) and also life cycle event like mating, spawning and hatching (Marcus 1985, Sakai & Ishida 2001, Sorek et al. 2014, Kaiser et al. 2016). There are furthermore several reports of zooplankton species performing circadian vertical migrations under constant darkness (Harris 1963, Enright & Hamner 1967, Cohen & Forward Jr 2005, Gaten et al. 2008). Circadian behavioral rhythms are not confined to one peak per 24h cycle, but also include bimodal patterns with peak activity often occurring at sunset and sunrise (Aschoff 1966, Kennedy et al. 2000, Klarsfeld et al. 2003, Gentile et al. 2009). Associated with diel patterns of physiological or behavior rhythmicity are often circadian cycles in metabolic activity and respiration (Mortola 2004, Teschke et al. 2011, Maas et al. 2016).

Photoperiod measurement and seasonal cycles

A striking feature of the circadian clock is the measurement of the environmental photoperiod, causing organisms to respond differently at different photoperiods (Goto 2013, Meuti & Denlinger 2013). This ability has often been related to the fact that the peak expression of many clock genes is closely associated with either sunset or sunrise and there are two major mechanistic concepts on how photoperiod measurement by the circadian clock is possible (Hut & Beersma 2011). The “external coincidence” model suggests a photosensitive phase at a certain point of the circadian cycle with the presence or absence of light during this phase triggering a long-day or short-day response, respectively (Bünning 1960). The photosensitive phase could thereby be characterized by high expression of one on several clock genes as it often occurs at sunset or sunrise. Alternatively, the “internal coincidence” model assumes the existence of two independent circadian oscillators (e.g. clock genes) with one of them peaking at sunset and the other one at sunrise (Pittendrigh 1960). In this model the phase difference and overlap of the two oscillators is used to infer photoperiod. There is supporting experimental data for both concepts suggesting that both mechanisms of photoperiod measurement are realized in nature (Davis 2002, Hut & Beersma 2011). Although photoperiod changes gradually over the course of the season, the transition between long-day and short-day response in organisms is mostly abrupt, indicating the existence of critical photoperiod at which the switch occurs (Watson & Smallman 1971, Hairston & Kearns 1995, Goldman et al. 2004, Salminen et al. 2015). This critical photoperiod can differ strongly between species and also within species often following a latitudinal gradient (Hairston & Kearns 1995, Tyukmaeva et al. 2011, Hut et al. 2013, Salminen et al. 2015) and it can also be shifted by other environmental factors like temperature (Watson & Smallman 1971, Hairston & Kearns 1995).

Another way in which circadian clocks can regulate seasonal timing is by photoperiodic entraining a circannual clock that creates an endogenous cycle of ~365 days (Randall et al. 1998, Goldman et al. 2004, Gwinner 2012). In this context photoperiod measured via the circadian clock is used as a source of information to entrain a circannual clock that keeps an annual rhythm with astonishing precision for several months or even years when kept at a constant photoperiod or even under constant darkness (Pengelley et al. 1976, Goldman et al. 2004). Although there are reports of circannual rhythms in several species including calanoid copepods (Conover 1965, Fulton 1973, Helm et al. 2013), the mechanistic nature of such endogenous long-range timing mechanisms remains largely enigmatic (Lincoln et al. 2006, Hazlerigg & Lincoln 2011).

1.3 Clocks in marine and polar organisms

While the clock mechanisms of model organism like *Drosophila* or mice have been extensively investigated in laboratory studies and are understood in great detail, the knowledge about clock systems in habitats that are difficult to access like the marine realm or the polar regions is very limited (Tessmar-Raible et al. 2011, Hut et al. 2013, Beale et al. 2016, Bulla et al. 2017). However, the differences in environmental rhythms and the often extreme light conditions in these environments pose a particular challenge for endogenous clock systems leading to unique types of adaptation.

Marine clock systems

Endogenous marine rhythms are known for a long time and clock gene sequences have been described in numerous species (Esterly 1917, Naylor 2010, Tessmar-Raible et al. 2011, Christie et al. 2013, Nesbit & Christie 2014, Sun et al. 2016, Perrigault & Tran 2017). However, expression patterns and clock machineries have only been characterized in a handful of marine organisms including microorganisms, corals, crustaceans, annelids, bivalves and fish (e.g. Teschke et al. 2011, Zantke et al. 2013, Zhang et al. 2013, Ottesen et al. 2013, Brady et al. 2016, Feng & Bass 2016, Perrigault & Tran 2017, Biscontin et al. 2017).

As in terrestrial chronobiology, a focus has been put on circadian clock systems, but marine organisms are exposed to several environmental rhythms aside of the diel cycle. The moon is the source of several marine cycles like the tidal cycle with a period of 12.4h that is of particular relevance in coastal and intertidal habitats (Tessmar-Raible et al. 2011). The importance of the tidal cycle in reflected in observations of circatidal rhythms in several species (Cronin & Forward 1979, Naylor 2010, Anderson et al. 2017). Kaiser et al. (2016) further have shown that circatidal rhythms in a species can be site-specific as

the local tidal regime is strongly affected by coast morphology. While some studies suggest that circatidal rhythms are generated from modulations of a circadian clock (Mat et al. 2014), there is strong evidence that circatidal clock can form an oscillator largely independent of the circadian clock (Zantke et al. 2013, Zhang et al. 2013). Yet there is so far no model describing the molecular interaction within a circatidal oscillator (Wilcockson & Zhang 2008, Tessmar-Raible et al. 2011, Bulla et al. 2017). The intensity of the tidal cycle itself changes with a semilunar period of 14.8 days due to the interaction of the gravitational forces of moon and sun, resulting in the strongest tidal amplitudes (spring tides) around full and new moon and weakest amplitudes (neap tides) in the first and third quarter of the lunar cycle. Circasemilunar rhythms are often related to reproduction and are especially frequent in species that either live in sub-/intertidal habitats or deposit their eggs there (Greeley & MacGregor 1983, Tessmar-Raible et al. 2011, Kronfeld-Schor et al. 2013, Kaiser et al. 2016, Raible et al. 2017). Finally, the cycle of changing moonlight intensity with a lunar period of 29.5 days does affect the behavior of marine species as well as their reproduction (Tessmar-Raible et al. 2011). The neritic annelid *Platynereis dumerilii* shows a circalunar cycle of maturation and spawning that is entrained by moonlight (Zantke et al. 2013) and circalunar rhythms have also been described in marine fish (Takemura et al. 2004). It was also shown that the light of the full moon can shift the vertical migration of zooplankton during the polar night from a diel to a lunar period (Last et al. 2016). The possibly most prominent example of marine rhythmicity is the synchronized mass spawning of corals. Once a year, a few nights closely after full moon, entire reefs of corals releases their gametes into the water column. This process is highly synchronized with different species spawning at different days and times of the night (Hoadley et al. 2011, Brady et al. 2016). This rhythm does also persist under constant conditions providing an impressive example of how circannual, circalunar and circadian clocks can interact in the marine environment.

One major feature of aquatic habitats compared to terrestrial ones is their three-dimensional structure where environmental parameters like temperature, oxygen content and especially light can show marked changes with depth. For *C. finmarchicus* and various other vertically migrating species this means that clock mechanisms not only have to stay in tune with the rhythmic environment, they also have to compensate for changes in this environment that are caused by the rhythmic movement of the organisms themselves. For example, if zooplankton organisms perform DVM by following an isolumen (Cohen & Forward Jr 2005), this would mean that they experience a minimal diel change in light intensity, raising the question on how these organisms entrain their circadian clock. Too little is known about marine clock systems to speculate on how they

maintain rhythmicity under such complex conditions. The attenuation of light intensity with depth also means that the subjective photoperiod perceived in deeper waters is shorter than near the surface, meaning that the clock of organisms using photoperiod measurement for seasonal timing may be not only adapted according to latitude but also to the species natural depth habitat (Miller et al. 1991, van Haren & Compton 2013). Furthermore, a problem exclusive to aquatic habitats is acidification due to increasing CO_2 levels. This can affect brain functioning and behavior of marine species (Munday et al. 2009, Nilsson et al. 2012), but it is yet completely unknown whether and how it may affect marine clock systems.

Polar clock systems

While cold temperatures are also found in alpine or deep sea habitats, what sets the polar regions apart from the rest of the world are the extreme seasonal oscillations in light conditions that result in a phase of permanent day in summer (midnight sun) and permanent night in winter (polar night). This poses a special challenge to circadian clocks that mostly rely on light as an entrainment cue (Beale et al. 2016). Clock investigations in terrestrial polar species have shown that circadian clocks may become arrhythmic during midnight sun and polar night in some species (Ohta et al. 2005, Lu et al. 2010, Kobelkova et al. 2015), while rhythmicity is maintained in others (Reierth et al. 1999, Ashley et al. 2014). For marine species it is known that DVM can persist throughout the polar night, although so far the relative importance of circadian clocks or weak diel changes in light conditions in winter is not known (Gaten et al. 2008, Berge et al. 2009, 2015, Cohen et al. 2015, Last et al. 2016).

Comparisons of high latitude and low latitude circadian clock systems are scarce, but studies on *Drosophila* species have shown that flies from high latitudes possess less robust clock that tend to become arrhythmic under constant darkness, while under constant light rhythmicity seems to be stronger than in low latitude flies (Menegazzi et al. 2017). High latitude flies showed weaker diel activity rhythms, but were better at maintaining circadian rhythmicity under very long photoperiods, suggesting special adaptations of the clocks to an extreme and variable light environment (Kauranen et al. 2016, Menegazzi et al. 2017, Kyriacou 2017). The lack of rhythmicity in constant darkness may be irrelevant in this context as the flies enter diapause in winter. Such latitudinal adaptations of circadian clock systems have raised concern about the adaptive capacity of low latitude clocks facing high latitude light regimes due climate change-induced poleward distribution shifts, either preventing those species from entering polar latitudes or leading to reduced fitness (Saikkonen et al. 2012). For the norther Atlantic and Arctic *Calanus* species this could mean that while the polar species *C. glacialis* and

C. hyperboreus may be forced to retreat poleward due to increasing temperatures, the boreal species *C. finmarchicus* and *C. helgolandicus* may struggle to replace them due to the extreme light conditions and the limited adaptive capacity of their circadian clocks (Falk-Petersen et al. 2007, Reygondeau & Beaugrand 2011, Saikkonen et al. 2012, Feng et al. 2017, Chivers et al. 2017). This would not only refer to the control of diel rhythmicity, but also to the timing of the seasonal life cycle that can be crucial for survival in polar environments (Søreide et al. 2010).

1.4 Research objectives

This dissertation is part of the project PolarTime, funded by the Helmholtz Association of German Research Centres. The project investigates the rhythmic life of polar pelagic key species and explores how they are affected by endogenous timing mechanisms and external cues. As outlined above, biological clocks play a central role in the life of terrestrial species, but their relevance for organisms and ecosystems in the marine environment has received relatively little attention. To understand how future environmental changes will affect marine systems, it is crucial to investigate timekeeping mechanisms in marine organisms, especially in key species like *C. finmarchicus* that have a strong impact on ecosystem functioning.

C. finmarchicus shows pronounced diel rhythmicity as well as a clear seasonal life cycle, with both of these rhythms being not well understood with regard to their mechanistic regulation. The species further occupies are large latitudinal range and is an integral part of the northern Atlantic food web. Due to climate change *C. finmarchicus* is experiencing a latitudinal distribution shift into polar habitats where it is exposed to extreme seasonal oscillations in photoperiod, making it an ideal object for the study of diel and seasonal rhythms as well as their endogenous control. The research objectives in this dissertation can be divided into three major complexes that focus on the following main questions:

1. Does *C. finmarchicus* possess a functioning circadian clock and how does this clock affect the species diel rhythmicity? (Publication I)

2. What characterizes the seasonal life cycle and diapause of *C. finmarchicus* on a molecular level and how is seasonality affected by endogenous clock mechanisms? (Publication II)

3. How do extreme polar light conditions affect clock functioning as well as the diel and seasonal rhythmicity of *C. finmarchicus*? (Publication III)

These topics were addressed by the combination of manipulative laboratory experiments with extensive diel and seasonal field sampling campaigns in Loch Etive, UK (56°N) and Kongsfjorden in Svalbard, Norway (79°N). An integrative approach was used to investigate rhythmicity manifests on different levels of organization and how these levels differ from and interact with each other. The applied methods range from the characterization of copepod vertical migrations and population structure in the field, to investigation of behavior and metabolic activity in the laboratory, the measurements of physiological parameters like body weight or lipid content. Gene expression patterns were investigated in great detail with a focus on the diel characterization of clock gene activity and the seasonal expression patterns of numerous metabolic genes. This work thus not only provides significant new insights into the diel and seasonal life of *C. finmarchicus*, but broadens the understanding of marine clock systems and their ecological relevance in general.

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2 Publication I

Circadian Clock Involvement in Zooplankton Diel Vertical Migration

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2.1 Summary

Biological clocks are a ubiquitous ancient and adaptive mechanism enabling organisms to anticipate environmental cycles and to regulate behavioral and physiological processes accordingly (Dunlap & Loros 2016). Although terrestrial circadian clocks are well understood, knowledge of clocks in marine organisms is still very limited (Tessmar-Raible et al. 2011, Zantke et al. 2013, Zhang et al. 2013, Ottesen et al. 2013). This is particularly true for abundant species displaying large-scale rhythms like diel vertical migration (DVM) that contribute significantly to shaping their respective ecosystems (Teschke et al. 2011). Here we describe exogenous cycles and endogenous rhythms associated with DVM of the ecologically important and highly abundant planktic copepod *Calanus finmarchicus*. In the laboratory, *C. finmarchicus* shows circadian rhythms of DVM, metabolism, and most core circadian clock genes (*clock*, *period1*, *period2*, *timeless*, *cryptochrome2*, and *clockwork orange*). Most of these genes also cycle in animals assessed in the wild, though expression is less rhythmic at depth (50-140 m) relative to shallow-caught animals (0-50 m). Further, peak expressions of clock genes generally occurred at either sunset or sunrise, coinciding with peak migration times. Including one of the first field investigations of clock genes in a marine species (Hoadley et al. 2011, Ottesen et al. 2013), this study couples clock gene measurements with laboratory and field data on DVM. While the mechanistic connection remains elusive, our results imply a high degree of causality between clock gene expression and one of the planet's largest daily migrations of biomass. We thus suggest that circadian clocks increase zooplankton fitness by

optimizing the temporal trade-off between feeding and predator avoidance, especially when environmental drivers are weak or absent (Zaret & Suffern 1976).

2.2 Results and Discussion

Diel vertical migration (DVM) in one of the most abundant and ecologically important marine copepods, *Calanus finmarchicus*, is paralleled by endogenous circadian rhythmicity at behavioral, physiological, and molecular levels. In the laboratory, copepods collected from an actively migrating field population showed endogenous rhythms of swimming, respiration, and core circadian clock gene oscillations under constant darkness. In the field, most clock gene oscillations mimicked laboratory findings, with some genes becoming less rhythmic in animals collected from depth. Peaks of gene expression follow sunset/sunrise, the periods of greatest vertical migrations over the solar day. Our data indicate that circadian timekeeping is an important component of DVM and particularly adaptive at maintaining migratory rhythmicity in habitats where the principle exogenous driver of DVM, light, is limited.

DVM of marine zooplankton is one of the most profound coordinated movements of organisms on the planet. It contributes fundamentally to ecological interactions in both marine and freshwater habitats (Hays 2003) and to global biogeochemical cycles (Steinberg et al. 2000). DVM also structures predator-prey interactions, since increased predation risk from visually hunting predators drives zooplankton to depths during the day, while at night they return to the surface to feed (Zaret & Suffern 1976). Current mechanistic knowledge of DVM suggests that diel light changes are the main environmental cue of migration behavior (Brierley 2014). However, paradoxically, DVM still occurs in deepwater habitats and at high latitudes during the winter where light is limited, suggesting alternative control mechanisms (Rudjakov 1970, Berge et al. 2009, van Haren & Compton 2013, Last et al. 2016).

In terrestrial organisms, endogenous temporal synchronization is achieved by a circadian clock cellular machinery involving an intricate network of gene/protein feedback loops that create a cycle of ~24h length (Mackey 2007). The clock is primarily entrained by light to ensure synchronization with the environment, and it is a potent tool of rhythm regulation controlling diel activity patterns (Aschoff 1954). However, studies addressing the role of molecular clock mechanisms in marine organisms are scarce (Teschke et al. 2011, Tessmar-Raible et al. 2011, Zantke et al. 2013, Zhang et al. 2013), primarily due to the non-model nature of most marine species and a lack of genetic resources. Furthermore, marine organisms are often difficult to maintain in the laboratory, and sampling them in

the field is often expensive and labor intensive. However, understanding marine clock mechanisms, especially in key ecological species, is crucial to predicting how the rhythmic life of these organisms may be affected by changes in environmental conditions (Falk-Petersen et al. 2007).

Copepods occupy a central position in marine pelagic food webs, providing an important energy source for their predators (Smith & Schnack-Schiel 1990). *C. finmarchicus* accumulates large lipid reserves (Falk-Petersen et al. 2009) and is the main link between phytoplankton and higher trophic levels in the North Atlantic, thereby sustaining one of the world's most productive fisheries (Prokopchuk & Sentyabov 2006). It is well recognized that *C. finmarchicus* undergoes DVM (Daase et al. 2008), and recently published transcriptomic resources (Lenz et al. 2014, Tarrant et al. 2014) make it an ideal model to examine the molecular clock machinery.

Vertical Migration in the Field

To determine DVM of copepods in their natural environment, an acoustic mooring was deployed in Loch Etive in the Bonawe deep (~145 m), UK (56°45´N, 5°18´W; Fig.A1.1). Acoustic Doppler current profilers (ADCPs) generated backscatter profiles as sound-scattering layers representing the vertical distribution of zooplankton biomass. The ADCP-generated data indicated clear DVM behavior of zooplankton, with near 24h periodicity during the field campaign (May 2015) (Fig.2.1). The main scattering layer was located in the upper 40 m depth at night, whereas during daytime this was typically between 40 and 80 m depth. The timing of the upward and downward migrations coincided closely with the time of local sunset (8:12 p.m.) and sunrise (4:24 a.m.).

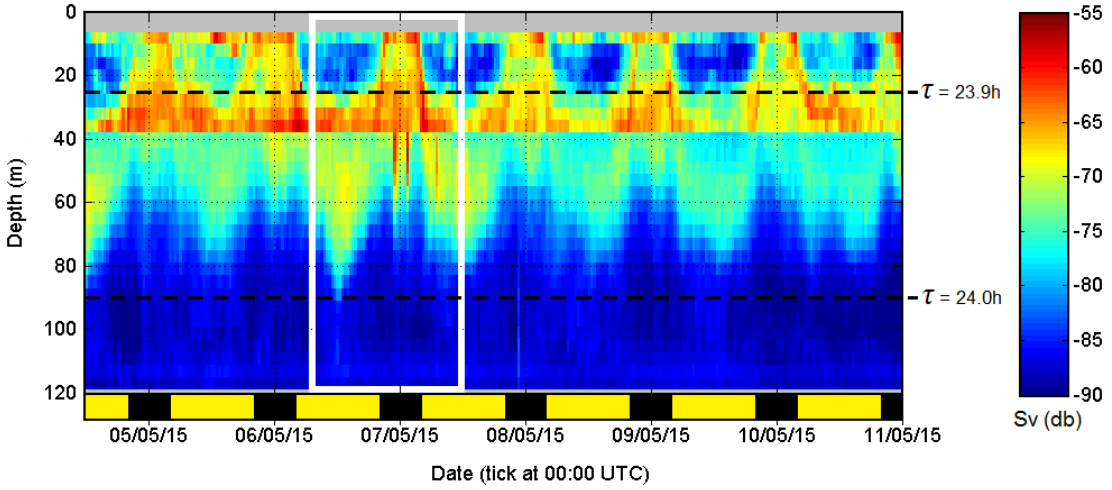

Figure 2.1: Backscatter Profile at Bonawe Deep, Loch Etive, in May 2015. DVM rhythms had periods (τ) of 23.9 and 24.0h at 25 and 90 m, respectively (TSA Cosinor analysis, May 4-11). Color bars indicate local sunrise/sunset; 28h field sampling is indicated by white box. The sharp backscatter change at ~38 m is a measuring artifact caused by the two acoustic profilers. Sampling site and water column characteristics are detailed in Fig.A1.1 and A1.2, respectively.

C. finmarchicus is the dominant zooplankton species in Loch Etive (Hill 2009). As such, the recorded DVM signals were assumed to primarily reflect the vertical migration of *C. finmarchicus*. This assumption was supported by net catches (data not shown) that established a high abundance of these animals in the water column during ADCP recordings.

Phenotypic Rhythmicity

DVM behavior and respiration were determined in *C. finmarchicus* collected from Loch Etive to investigate if the cyclic migrations observed in the field also persist under entrained and constant laboratory conditions. The animals were exposed to a simulated light-dark (LD) photoperiod (LD 16:8h) mimicking field conditions, followed by constant darkness (DD). The copepods exhibited 24h cycling in DVM under LD and near 24h rhythms under DD conditions, with clear downward movement in the subjective morning (Fig.2.2A, Tab.A1.1). These data clearly suggest an endogenous circadian regulation of DVM behavior. The rapid evening ascent and morning descent under LD, with light triggering a direct negative phototactic response, contrasted with the more gradual depth change and lower amplitude rhythm under DD, which dampened over time. Endogenous DVM rhythms have previously been described for zooplankton species, and several of these studies also reported lower amplitude DVM rhythms under DD (Enright & Hamner 1967, Cohen & Forward Jr 2005). While some of these studies found more robust endogenous rhythms of zooplankton DVM than detailed here, direct comparisons are not appropriate, as DVMs differ between species and life stages (Daase et al. 2008). Nevertheless, the persistence of DVM in copepods under constant darkness strongly suggests circadian clock involvement.

Swimming during vertical migration requires energy and is, therefore, accompanied by increased metabolic activity (Lampert 1989). Respiration experiments revealed that oxygen consumption under LD increased in *C. finmarchicus* during the late afternoon/early night, a pattern repeated over the subsequent 2 days under DD (Fig.2.2B, Tab.A1.1). While the peak respiration in the second night between the 2 DD days was phase delayed by ~8h towards the late night, peak respiration was once again in phase by the last night of the experiment, suggesting that the endogenous rhythm was still running on time. The delay initially observed under DD could be related to the transition from LD to constant darkness, constituting aftereffects suggested to reflect an adaption of the endogenous rhythm to unnatural changes in light regime (Pittendrigh 1960).

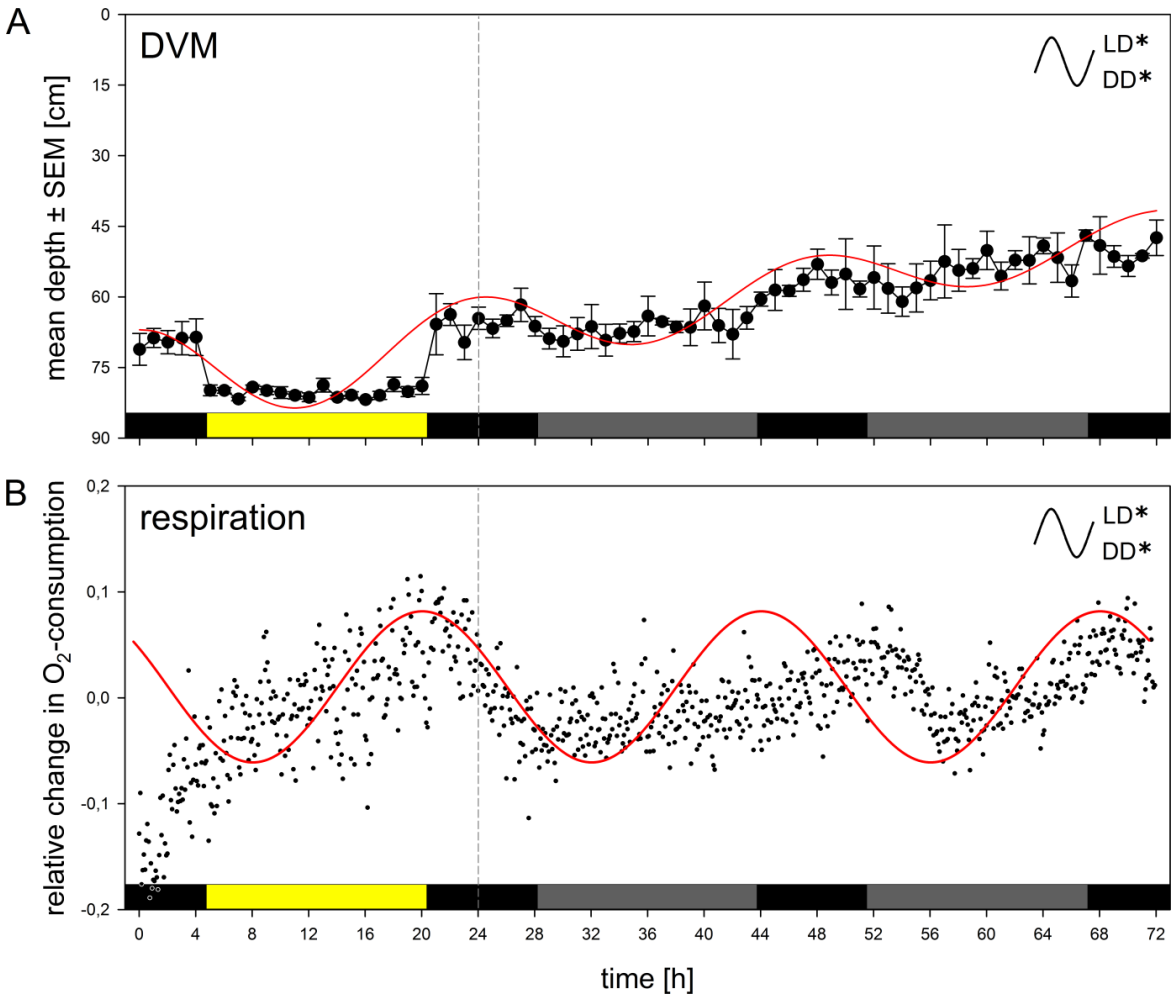

Figure 2.2: DVM and Respiration Rhythms in the Laboratory. (A) DVM. Depth of *C. finmarchicus* copepodid 5 (CV) stages in 90-cm DVM columns is shown. Data are derived from video recordings. Mean values (n = 4) ± SEM are shown. (B) Respiration. Mean values (n = 6) for each time point are shown. Due to the high sampling rate (5 min), error bars were removed for the sake of clarity. Color bars indicate (subjective) day and night. For both phenotypes, the first day with natural light/dark cycle (LD, photoperiod = 16h) and the two following days in constant darkness (DD) were analyzed separately, as indicated by the dashed gray line. Asterisks (*) indicate significant 24h rhythmicity. Sinusoidal curves (red) were fitted to illustrate the partially damped but still highly significant rhythms. For exact p-values, see Tab.A1.1.

The evening increase in respiration matches the time when the copepods undertake the energy demanding migration toward the surface (Lampert 1989), whereas the decrease in respiration toward sunrise may reflect passive copepod sinking or reduced energy costs for downward migration facilitated through negative buoyancy (Steele & Henderson 1998). Of relevance here is that respiration increases before the time of upward migration, indicating an endogenously regulated anticipatory process. Rudjakov (1970) hypothesized that DVM may actually be a result of an endogenous rhythm of metabolic activity that initiates upward migration around sunset, followed by passive sinking around sunrise. Overall, these data reveal that *C. finmarchicus* possesses an endogenous

rhythm of metabolic activity that matches DVM swimming behavior and is in line with previous findings (Båmstedt 1988).

Clock Gene Expression

To investigate the expression of clock genes under controlled conditions, copepods were collected in Loch Etive, and, as for DVM and respiration experiments, they were transferred to the laboratory where they were exposed to LD and DD conditions. Only core clock genes that interact via gene/protein feedback loops to create endogenous circadian rhythms were investigated (Mackey 2007). The results indicated strong 24h rhythmicity in the following six of eight core clock genes: *clock* (*clk*), *period1* (*per1*), *period2* (*per2*), *timeless* (*tim*), *cryptochrome2* (*cry2*), and *clockwork orange* (*cwo*). The two remaining core genes *cycle* (*cyc*) and *vrille* (*vri*) showed weak rhythmicity (Fig.2.3A-H, Tab.A1.2). Times of peak gene expression were closely associated with the time of sunset or sunrise, and they generally matched expression patterns of terrestrial model species (Richier et al. 2008, Merlin et al. 2009). Rhythmic gene expression persisted under DD, confirming the endogenous nature of the clock in *C. finmarchicus*.

The presence and rhythmic expression of a mammalian type *cry2* gene, which peaks in the evening, indicates a clock mechanism similar to the ancestral clock model known from the monarch butterfly *Danaus plexippus*, where *cry2* acts as a transcriptional repressor (Merlin et al. 2009). Laboratory studies in this insect found rhythmic *cry2* expression to peak in the early day, as with the Antarctic krill *Euphausia superba*, the water flea *Daphnia pulex*, and the marine annelid *Platynereis dumerilii* (Merlin et al. 2009, Teschke et al. 2011, Zantke et al. 2013, Bernatowicz et al. 2016). In contrast, *C. finmarchicus cry2* expression in the laboratory peaked at sunset (Fig.2.3F).

In addition to the core clock genes, expression was also measured in a suite of genes associated with the modification and localization of core clock proteins (*doubletime2*, *widerborst1*, *twins*, *casein kinase II* α, and *shaggy*) or light entrainment (*cryptochrome1*) (Harms et al. 2004). In accordance with previous findings, none of these clock-associated genes showed consistent circadian expression (Tab.A1.2) (Harms et al. 2004).

To investigate the functioning of a circadian clock in the field, we conducted a 28h sampling at Bonawe deep. Clock gene expression of *C. finmarchicus* was measured in two depth layers (5-50 m and 50-140 m). Generally, the expression patterns of the clock genes resembled those recorded in the laboratory (Fig.2.3I-O). However, gene rhythms were less overt in the field and the number of rhythmic genes was reduced, especially in copepods from the deeper layer (Tab.A1.2). Temperature changes and food availability can entrain clock activity (Vera et al. 2013, Rouyer & Chatterjee 2015), and it is possible

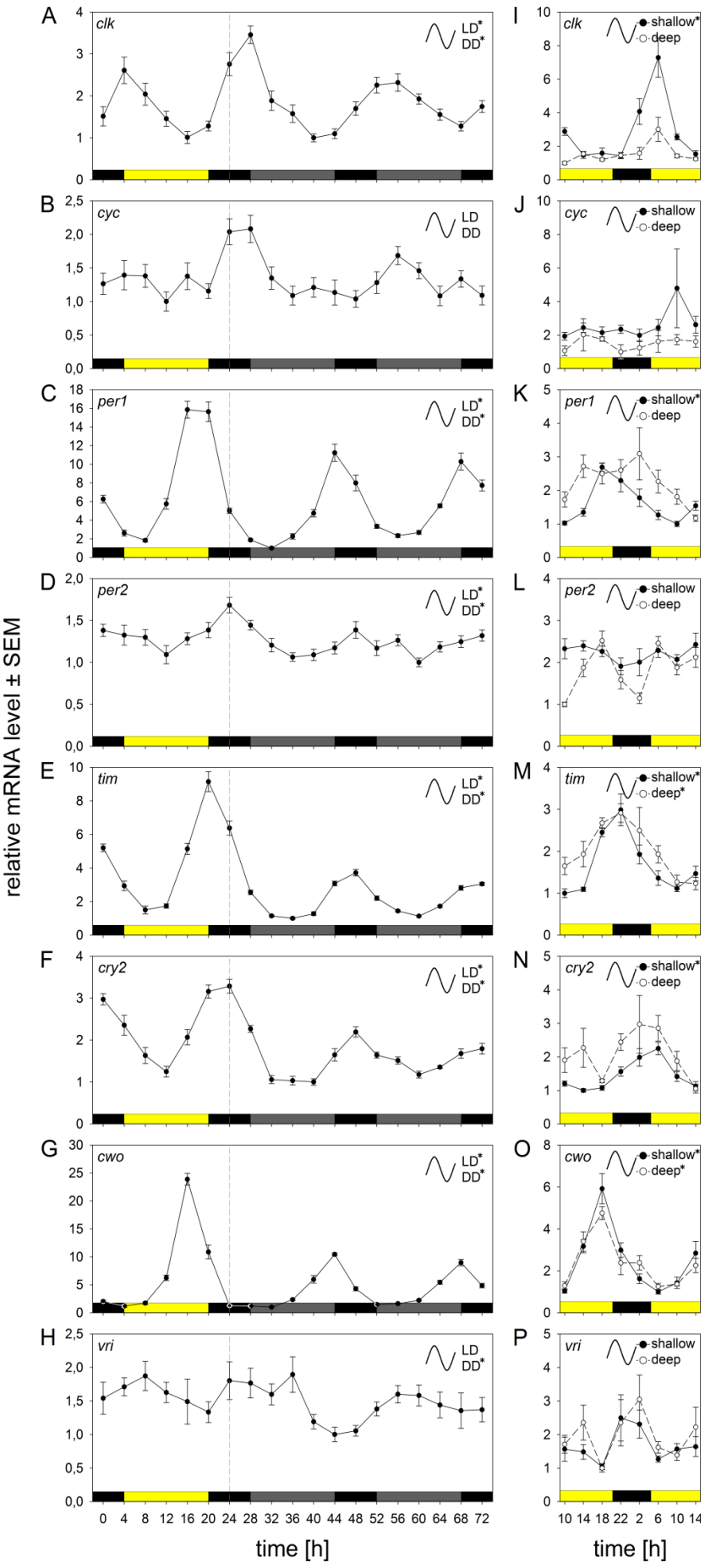

Figure 2.3: Diel Expression Patterns of Core Clock Genes in the Laboratory and in the Field. Expression patterns were recorded in *C. finmarchicus* CV stages and the investigated genes were as follows: *clock* (*clk*), *cycle* (*cyc*), *period1* (*per1*), *period2* (*per2*), *timeless* (*tim*), *cryptochrome2* (*cry2*), *clockwork orange* (*cwo*), and *vrille* (*vri*). Color bars indicate (subjective) day and night. (A-H) In the laboratory experiments, rhythm analysis of the clock genes *clock* (*clk*, A), *cycle* (*cyc*, B), *period1* (*per1*, C), *period2* (*per2*, D), *timeless* (*tim*, E), *cyrptochrome2* (*cry2*, F), *clockwork orange* (*cwo*, G), and *vrille* (*vri*, H) was done separately for LD (photoperiod = 16h) and DD intervals, as described in Fig.2.2. Per time point, *n* = 10 replicates were pooled from two identical experimental runs. (I-P) In the field, samples from 5-50 m (shallow) and 50-140 m (deep) were investigated for the same clock genes (photoperiod = 16h). *n* = 5 replicates per time point. Both laboratory and field data were analyzed for rhythmic expression using the R-package RAIN. Asterisks (*) indicate significant 24h rhythmicity. Mean values ± SEM are shown. Color bars indicate (subjective) day and night. For exact *p*-values, see Tab.A1.2.

that the vertical migration through layers of different temperature and phytoplankton concentration (Fig.A1.2) may have affected clock gene expression and resulted in more labile rhythms when compared with laboratory experiments. Further, the overall reduced rhythmicity at 50-140 m could reflect the physiological state of the copepods. At the time of the sampling, animals in the deep layer may already have started transitioning to seasonal diapause, a phase of inactivity in deep waters characterized by metabolic downregulation and without any known diel activity cycle (Hirche 1996, Hill 2009). Data collected later in the year (not shown) suggest that cyclic clock gene expression ceases during diapause. It is also noteworthy that the more labile gene rhythms at 50 to 140 m depth were mirrored by the weaker DVM signal acoustically recorded in this layer in Loch Etive (Fig.2.1), further suggesting a coupling between clock and DVM. Nevertheless, the existence of clock gene cycles in animals in the deeper layer shows that circadian clocks can operate under very low light intensities, providing an explanation for the observations of diel migrations in meso-/bathypelagic habitats (van Haren & Compton 2013) and at high latitudes during winter months (Berge et al. 2009, Last et al. 2016).

In summary, circadian clock gene expression in *C. finmarchicus* demonstrates pronounced rhythms that are well suited for evoking the observed rhythms in DVM and respiration. Expression patterns mostly persist in the field, strongly suggesting that the copepod possesses an endogenous clock that is also functioning under natural conditions.

Ecological Implications

The adaptive significance of a circadian clock underpinning DVM in *C. finmarchicus* and other vertically migrating organisms is clear. Primarily the clock would provide a mechanism for the copepods to anticipate the day/night cycle, thereby temporally adjusting behavioral functions, physiology, and gene expression accordingly. However, circadian clocks have also been implicated in the sensitivity to predator cues and avoidance behavior (Kennedy et al. 2000). Copepods and many other planktic organisms are prey to visual predators during the day (Fortier et al. 2001). The circadian clock would provide a mechanism for anticipating sunrise to return to deep, dark waters before sufficient light enables visual predation. For example, the sea urchin *Centrostephanus coronatus* shows an endogenous cycle in nocturnal foraging, which is closely tuned to the resting times of its predator, a diurnally active fish (Nelson & Vance 1979), increasing the urchin’s chance of survival and also maximizing the time it can spend foraging. Circadian clock involvement in vertical swimming may also explain midnight sinking behavior, which is characterized by a descent to intermediate depth in the middle of the night followed by a second upward migration closely before sunrise (Rudjakov 1970, Cohen & Forward Jr

2005). This behavior has been suggested to be an avoidance response to larger vertically migrating predators, which ascend later and descend earlier (Tarling et al. 2002). While predation risk can usually not be sensed until the predator is present, circadian clocks are highly suitable for controlling crepuscular activity patterns (Rudjakov 1970), and they could thus explain the two upward migrations at sunset and sunrise characteristic of midnight sinking.

Circadian clocks would also be adaptive for maintaining DVM rhythms in photoperiodically extreme environments, such as high latitudes during the polar night and the meso-/bathypelagic zone. In both these habitats, light as an entrainment cue is only temporarily available and/or extremely weak, and food levels are relatively constant over the course of the day (Khripounoff et al. 1998, Båtnes et al. 2013). Indeed, DVM occurrence in polar night habitats and the synchronized evening ascent of animals from the aphotic depths beyond 1,000 m support the hypothesis that DVM is underpinned by a circadian clock (Berge et al. 2009, van Haren & Compton 2013, Last et al. 2016). Interestingly, a recent study found that vertical migration shifted from diel (24h) to lunar day (24.8h) cycles under the influence of the full moon during the darkest part of the Arctic polar night (Last et al. 2016). This may indicate that, during the polar night, strong lunar light can either override endogenous rhythmicity or can act as an entrainment cue, lengthening the period of a circadian clock underlying the vertical migration pattern. Furthermore, *C. finmarchicus* digestive enzymes are probably produced before feeding to speed up digestion, thereby increasing the overall amount of food that can be consumed and digested while being at the surface for a limited time (Båmstedt 1988). A similar preparatory mechanism could be involved in the endogenous and light-entrained feeding rhythms in the copepod *Acartia tonsa* (Stearns 1986), as too the clock-controlled anticipatory enzyme production in the shrimp *Palaemon squilla* (Trellu & Ceccaldi 1977).

Circadian clocks have the capacity to regulate seasonal rhythmicity by measuring photoperiod (Meuti & Denlinger 2013). This can be achieved by a light-sensitive phase at the transition between day and night, which is associated with clock gene peak activity (external coincidence model, Bünning 1960). The presence or absence of light during this critical phase of the day/night cycle provides information about the photoperiod and, hence, season. Alternatively, peaks in clock gene activity might shift over the season following either sunset or sunrise, and the phase difference between these peaks would provide another measure of photoperiodic time measurement (internal coincidence model, Pittendrigh 1960). The seasonal life cycle of many insects is affected by photoperiod (Meuti & Denlinger 2013), as too are various aspects of copepod biology, including diapause, reproduction, activity, and feeding (Marcus & Scheef 2010). As with

many of its congeners, *C. finmarchicus* undergoes seasonal diapause fueled by its large lipid reserves (Falk-Petersen et al. 2009), where lipid content, food availability, and temperature are considered important regulators of this resting phase (Wilson et al. 2016). However, a clear understanding of the mechanisms initiating and terminating *Calanus* diapause is still missing, leading to the tantalizing suggestion that this critical life history transition may be underpinned by a circadian clock as an integral part in the timing of *C. finmarchicus*' annual cycle.

Conclusions

Our results provide a detailed description of clock gene expression in an ecologically important marine species combined with measurements of DVM and metabolic activity. *C. finmarchicus* shows robust clock gene cycling in the wild and endogenous 24h oscillations in the laboratory. The persistence of circadian rhythms in DVM and respiration under constant conditions suggests circadian clock involvement in the regulation of these processes. So far, the mechanistic link between clock rhythmicity and phenology remains elusive, where functional analyses of the clock machinery and its output pathways are now required. DVM has previously been shown to occur in the high Arctic during the polar night, in the aphotic depths beyond 1,000 m, and spontaneously as midnight sinking, all of which contradict the assumption of DVM being driven by purely exogenous cues. Given the ecological benefits offered by endogenous timekeeping, it seems likely that circadian clocks are extant in the regulation of vertical migration patterns. Furthermore, investigations of clock systems and DVM in marine phytoplankton and cyanobacteria (Shikata et al. 2013, Ottesen et al. 2013) have led to the suggestion that circadian DVM could exist even in these primordial organisms (Axmann et al. 2014). Our study provides a basis for better understanding the mechanisms of DVM and also for exploring the adaptive advantages of ancestral clock systems, which are hypothesized to have originated in the aquatic environment (Tauber et al. 2004).

2.3 Star★Methods

Contact for reagent and resource sharing

Further information and requests for resources and reagents, including the video material of the DVM experiment, the sequences of custom Taqman® probes/primers, and the RAIN rhythm analysis script, should be directed to and will be fulfilled by the lead author, Sören Häfker (shaefker@awi.de).

Table 2.1: Key resources

Reagent or resource	Source	Identifier
Chemicals, Peptides, and Recombinant Proteins		
RNA*later*®	Ambion	Cat#AM7021
RNeasy® Mini kit	QIAGEN	Cat#74104
TURBO DNA-*free*™ Kit	Ambion	Cat#AM1907
RevertAid H Minus Reverse Transcriptase	Invitrogen	Cat#EP0452
Taqman® low-density array card (custom designed)	Applied Biosystems	n/a
Deposited data		
Raw and analyzed data	this paper	https://doi.pangaea.de/10.1594/PANGAEA.875739
Oligonucleotides		
Taqman® primers/probes	this paper	n/a
Software and algorithms		
TSA Cosinor 6.3 package	Expert Soft Tech	n/a
R-package “RAIN”	(Thaben & Westermark 2014)	http://journals.sagepub.com/doi/10.1177/0748730414553029

Experimental model and subject details

All animal work was conducted in accordance with local legislation. All investigations were performed on CV life stages of the copepod *Calanus finmarchicus* (Gunnerus, 1770). Copepods were collected at the sampling site Bonawe deep in Loch Etive, Scotland (Fig.A1.1) and laboratory experiments were performed at the Scottish Association for Marine Science (SAMS) at in situ temperature (10°C). During the transfer to the laboratory (max. 1.5h) the copepods were kept dark and at in situ temperature. For the laboratory experiments filtered and UV-treated seawater was used that was pumped in from below a beach next to the institute. The water was adjusted to a salinity of 27.5 by adding Milli-Q water to match the conditions at the sampling site in ~50 m depth.

Laboratory copepods were exposed to an in situ photoperiod of 16h with a gradual change in light intensity and spectral compositions to simulate the natural conditions at Bonawe deep in a depth of ~50 m. From 4:00 (sunrise) on light intensity increased to ~5.5 Lux at noon measured right above the water surface. During this time color temperature shifted from initial 15460 K to 13780 K at noon. The decrease in the afternoon mirrored the morning increase resulting in complete darkness at 20:00 (sunset). To create these light conditions, a programmable LED-system was used (Mitras Lightbar oceanic blue / ProfiLux 3.1T control unit, both GHL Advanced Technology GmbH, Germany).

Method details

Study site characteristics

Loch Etive is a sea loch at the western coast of Scotland, UK (56°45´N, 5°18´W). It is connected to the open ocean by a sill with a width of 200 m and ~7 m water depth and has another sill with ~13 m depth further up the loch (Edwards & Edelsten 1977). Beyond the second sill there is the upper main basin with the deepest point of the loch (Bonawe deep, ~145 m) where all samplings were done (Fig.A1.1). The sills limit the water exchange leading hypoxic conditions in the deeper layers of the upper basin. Turnover events occur during the strongest spring tides in spring/autumn, but are irregular and only happen every few years (Edwards & Edelsten 1977).

During the sampling of the 28h field time series at Bonawe deep (6^{th}/7^{th} May 2015) the water column parameters salinity, temperature, oxygen concentration and Chlorophyll a (Chl a) fluorescence were recorded by a conductivity-temperature-depth (CTD) profiler (SBE 19plus V2 SeaCAT Profiler, Sea-Bird Electronics, USA). The water column was characterized by an approx. 5 m thick surface layer with a low salinity ≤20 psu (Fig.A1.2). From 5 m on salinity gradually increased to 27 at ~50 m and showed only a minor increase below this depth. Temperature from the surface to 26 m depth ranged between 8.3°C and 8.9°C. Below 26 m temperature sharply rose to a maximum of 12.2°C at 50 m depth before gradually decreasing to 10.4°C at 90 m and below (Fig.A1.2). The deeper layers of Bonawe deep were hypoxic during the sampling. From the surface to 26 m depth oxygen concentrations was ≥8.5 mg O_2*L^{-1} before sharply decreasing to 3.6 mg O_2*L^{-1} at 40-43mdepth (Fig.A1.2). Oxygen concentration then continued to gradually decreased to values ≤1.6 mg O_2*L^{-1} in 80 m depth and below. Chl a fluorescence was high in the upper 10 m (4-16 mg*m^{-3}), showed a second, much smaller maximum at 25 m and then quickly diminished with depth (Fig.A1.2). The conditions were similar in spring 2016 when animals for laboratory experiments on DVM and respiration were collected (data not shown).

Vertical migration in the field

A mooring was deployed close to Bonawe deep (depth: ~135 m) in March 2015 (Fig.A1.1). The mooring was equipped with two acoustic Doppler current profilers (ADCPs) pointing upward at 120 m and 45 m depth. The RDI 300 kHz ADCPs have been employed successfully in making biological observation of zooplankton migrations (Berge et al. 2009, Last et al. 2016). ADCP data were checked for quality using the RDI correlation index (a measure of signal to noise ratio) and absolute volume backscatter (Sv, measured in decibels, dB) was derived from echo intensity following the method

described in Deines (1999) with derived acoustic mean volume backscattering strength (MVBS). Acoustic data were analyzed via population mean TSA Cosinor analysis for backscatter rhythmicity in 25 m an 90 m depth (time series analysis [TSA] Cosinor 6.3 package). For the period 4th to 11th May 2015 significant backscatter rhythmicity could be detected in both, the shallow (45 m, τ = 23.9h, % model fit = 49.6) and the deep layer (125 m, τ = 24.0h, % model fit = 33.3). Tests on tidal (~12h) and lunar (24.8h) rhythms did not produce any significant rhythmicity.

Field time series

Samples were collected at Bonawe deep on the 6th/7th May 2015 starting at 11:00 and continuing in 4h intervals until 15:00 of the next day, resulting in a total of eight time points over a period of 28h. At each time point a WP2-net (200 µm mesh size, Hydro-Bios GmbH, Germany) was towed vertically through the water column to collect animals from 5-50 m depth and 50-140 m depth, respectively. Generally, the upper 5 m of the water column were excluded to avoid hypoosmotic stress for the copepods. Upon retrieval of the net, the sample was immediately (within 1 min) transferred into RNAlater® stabilizing solution (Ambion, UK) for later gene expression analysis (see below). A possible sample contamination by the congener species *C. helgolandicus* is unlikely due to its limited tolerance to low salinities and the brackish conditions in the loch (Hill 2009).

DVM experiment

To investigate the diel vertical migration (DVM) behavior, copepods were incubated in four so-called DVM-columns made out of acrylic glass (10*8*90 cm lxwxh, 7.2 L). Animals were collected on the 3rd June 2016, sorted, and per column 50 *C. finmarchicus* CV stages were incubated for a total of three days (LD-DD-DD, photoperiod = 16h). The columns were vertically divided into six 15 cm increments and each layer was filmed with a surveillance cameras equipped with filters excluding visible light (SKB140XP/SO, Sunkwang Electronics, South Korea). Infrared lights were used to illuminate the columns without disturbing the animals.

Copepod abundance per layer was then counted by three different persons from the recorded video material at 1h intervals. For every column, there was a certain fraction of copepods which was inactive and never left the bottom layer of the column. These animals were excluded from statistical analysis by determining the lowest number of copepods in the bottom layer over the course of the experiment for each column, respectively. This number was then defined as zero for the respective column. Copepods were not fed during the DVM experiments to avoid particle accumulation at the bottom, which could have affected vertical distribution. At the end of the experiment a vertical

oxygen profile was recorded using an oxygen tipping probe (PreSens GmbH, Germany). There was a weak (<6%), gradual decrease in oxygen from 9.27 mg O_2*L^{-1} near the surface to 8.75 mg O_2*L^{-1} close to the bottom.

Respiration experiment

Copepods collected on the 23rd June 2016 and sorted for *C. finmarchicus* CV stages were distributed to six glass bottles (305 mL) with filtered (0.2 µm) and UV-treated seawater which had been air-equilibrated for >1h (10 animals per bottles). Two additional bottles without animals served as controls. Bottles were closed tightly without any air bubbles inside and incubated for three days (LD-DD-DD, photoperiod = 16h). Oxygen content was measured using oxygen-sensitive sensor spots and monitoring equipment (OXY-4, PreSens GmbH, Germany). A moving average over 12h was calculated to remove the trend of gradually decreasing oxygen within the bottles and to reveal underlying rhythmic oscillations. A simple inverse correlation between oxygen content and animal oxygen consumption was assumed. As the moving average is based on comparing O_2-levels between time points, the resulting relative change in oxygen consumption is dimensionless. Data were binned to 1h intervals for rhythm analysis (see below).

Gene expression experiment

Copepods were collected on the 22nd May 2015 in 10-60 m depth. In the laboratory the animals were evenly distributed to 19 buckets filled with 20 L seawater. At midnight the sampling started by pouring the animals from the first, randomly chosen bucket through a sieve and fixing them in RNAlater®. Every 4h another bucket was sampled accordingly resulting in a total of 19 time points over a period of three days (72h). On the first experimental day (0-24h) the animals were exposed to a natural light/dark regime (LD, see above) while they were kept in constant darkness (DD) on the second and third day (24-72h). Copepods were fed with phytoplankton (Shellfish Diet 1800, Reed Mariculture, USA) in 4h intervals. A constant food concentration of200 mg C*L^{-1} was maintained to avoid starvation effects while not introducing a new *Zeitgeber*. The experiment was repeated in the same way (LD-DD-DD) with copepods collected on the 29th May 2015 and the data of both runs was pooled.

Gene expression analysis

Gene sequences were taken from an Illumina transcriptome of *C. finmarchicus* (Lenz et al. 2014). Core clock and associated genes had been previously annotated by Christie et al. (2013). Housekeeping genes were newly annotated from the respective transcriptome. All gene annotations were verified via blastn against NCBI database (see Tab.A1.2 for

accession numbers). They were then investigated for common protein domains via blastx and were checked for palindromic sequences and repeats via Oligoanalyzer 3.1 (http://eu.idtdna.com/calc/analyzer) and RepeatMasker 3.0 (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker). Binding regions for probes and primers were placed in sequence intersects that were specific for the respective genes (checked via blastn).

To measured gene expression, copepods were sorted in cooled RNAlater® (4°C) using dissecting microscopes. *C. finmarchicus* CV stages were pooled in groups of 15 copepods and RNA was extracted using the RNeasy® Mini kit (Quiagen, Netherlands). β-mercaptoethanol was added to the lysis buffer (0.14 M) as recommended for lipid-rich samples. DNA residues were removed with the TURBO DNA-free kit (Life Technologies, USA) and RNA was checked for concentration and purity (Nanodrop 2000 Spectrophotometer, Thermo Fisher Scientific, USA) as well as possible degradation (2100 Bioanalyzer / RNA 6000 Nano Kit, Agilent Technologies, USA). RNA was then converted to cDNA using RevertAid H Minus Reverse Transcriptase (Invitrogen GmbH, Germany). Gene expression was analyzed by real-time quantitative PCR (ViiATM 7, Applied Biosystems, USA) using custom-designed Taqman® low-density array-cards (Applied Biosystems, USA). The list of investigated genes included eight core clock genes, five clock-associated genes, one gene involved in clock entrainment via light, and 3 housekeeping genes (see Tab.A1.2). Gene expression levels were normalized against the geometric mean of the housekeeping genes *elongation factor 1* α, *RNA polymerase* and *actin* using the $2^{-\Delta\Delta CT}$-method developed by Livak & Schmittgen (2001). Housekeeping genes were chosen based on expression stability over the 24h cycle, expression level relative to other investigated genes and the findings of previous studies (Clark et al. 2013). For both experimental runs, five replicates were analyzed per time point. As there were no visible differences between the first and the second run, the datasets were pooled and treated as one resulting in n = 10 replicates per time point. For the 28h field time series, n = 5 replicates were analyzed per time point and depth. Shallow and deep samples were normalized against housekeeping genes together to ensure comparability of expression levels between depths.

Quantification and statistical analysis

Datasets of DVM, respiration and gene expression were investigated for 24h rhythmicity in RStudio (version 0.99.442, R Development Core Team 2013), using the RAIN-package. RAIN was specifically designed to detect (circadian) rhythms in biological datasets independent of waveform by using a non-parametric approach (Thaben & Westermark 2014). For the 28h field time series from May 2015, each depth (shallow/deep, n = 5, respectively) was analyzed separately as one dataset. In the

laboratory experiments (n = 10), the first 24h interval (LD) was analyzed separately from the following 48h interval (DD). The time point at midnight between the two intervals (LD/DD) was used in both analyses. Due to the limited computing capacity of RAIN and the large amount of data from the DVM (n = 4) and respiration experiments (n = 6), the mean values were used to analyses rhythmicity for the 48h DD interval of these experiments. Thus, to increase the confidence in the obtained results, each DD day in the DVM and respiration experiment was also analyzed individually using the respective replicates (see Tab.A1.1).

For the analyses of DVM and respiration data, an α of 0.05 was used (Tab.A1.1). For the gene expression analyses, a p-value < 0.001 was considered significant to account for the testing of multiple genes (Tab.A1.2). Graphs were created with SigmaPlot (v. 12.5).

Data and software availability

The mRNA sequences of the investigated genes can be found via the accession numbers summarized in Tab.A1.2. For the video material of the DVM experiment, the sequences of custom Taqman® probes/primers and the RAIN rhythm analysis script, please contact the lead author (shaefker@awi.de). Data of the DVM experiment (abundance counts), the respiration experiment (moving averages), and the gene expression data of the laboratory experiment and the field time series (raw CT-values) are accessible via PANGAEA (https://doi.org/10.1594/PANGAEA.875739).

2.4 Author contributions

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

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3 Publication II

Calanus finmarchicus seasonal cycle and diapause in relation to gene expression, physiology and endogenous clocks

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3.1 Abstract

The copepod *Calanus finmarchicus* plays a crucial role in the north Atlantic food web. Its seasonal life-cycle involves reproduction and development in surface waters before overwintering in diapause at depth. Although diapause has been studied for more than a century, the factors responsible for the initiation and termination of the dormant life-phase are still unclear. Endogenous clocks have been identified as potent tools for photoperiod measurement and seasonal rhythmicity in many terrestrial species. However, knowledge about such biological timing mechanisms in the marine realm remains scarce. We sampled a population of *C. finmarchicus* from a Scottish sea loch to characterize population dynamics, several physiological parameters, and diel and seasonal expression rhythms of 35 genes representing different metabolic pathways, including the circadian clock machinery. This generated a detailed overview of the seasonal cycle of *C. finmarchicus* including the most extensive field dataset on circadian clock gene expression in a marine species to date. Gene expression patterns revealed distinct clusters with different groups of genes upregulated at different phases of the copepods seasonal cycle. While diel clock cycling was restricted to the active spring/summer phase, many clock genes exhibited highest expression during diapause. Our results provide new insights into diapause on a physiological and genetic level. We further suggest that photoperiod, in interaction with other internal and external factors (lipid content, temperature, food availability) as well as endogenous clock mechanisms, plays an important role in the timing of diapause in *C. finmarchicus*.

3.2 Introduction

The calanoid copepod *Calanus finmarchicus* (Gunnerus, 1770) is an ecological key species in the Northern Atlantic (Runge 1988). The species shows high abundances between 40 and 80°N where it often dominates zooplankton biomass (Conover 1988, Kwasniewski et al. 2003, Helaouët & Beaugrand 2007). *C. finmarchicus* predominantly feeds on phytoplankton (Marshall & Orr 1955, Harris et al. 2000), and is itself an important energy source for higher trophic levels including several commercially important fish stocks (Sundby 2000, Prokopchuk & Sentyabov 2006). A key characteristic of *C. finmarchicus* is the accumulation of large lipid reserves for overwintering, making the copepod a nutritional food source for higher trophic levels (Falk-Petersen et al. 2009).

The seasonal cycle of *C. finmarchicus* initiates with adults from the overwintering stock spawning eggs prior to and during the spring bloom (Niehoff et al. 1999, Harris et al. 2000). The new generation develops through several nauplii (NI-NVI) and juvenile copepodid stages (CI-CV) before again reaching the adult female or male stage (CVIf or CVIm, respectively) (Marshall & Orr 1955). During the CIV and especially the CV stage the copepods accumulate large amounts of lipids, predominantly wax esters stored in a lipid sac (Falk-Petersen et al. 2009, Clark et al. 2013). From the CV stage on the life-cycle can progress in two different ways (Tarrant et al. 2014, 2016). Firstly, the CVs can migrate into deeper water where they enter a prolonged phase of inactivity commonly referred to as diapause (Hirche 1996a). Alternatively, copepods can remain in surface waters and continue development, molting into adults to produce a second generation of copepods. This second generation then again develops until the CV stage before either entering diapause or maturing to produce another generation. The timing of diapause initiation is not necessarily uniform within a population, with some copepods descending to diapause immediately, while other animals produce another generation that descends later in autumn (Miller et al. 1991, Johnson et al. 2008). While the number of generations and timing of descent is similar between years for any given population, there are geographic differences (Melle et al. 2014). *C. finmarchicus* in an Arctic fjord produces one generation per year, whereas a population in the Gulf of Maine produces up to three generations (Durbin et al. 2000, Walkusz et al. 2009).

During diapause, the copepods have reduced gut epithelium, show reduced metabolic activity and development is repressed (Hallberg & Hirche 1980, Hirche 1996a, Ingvarsdóttir et al. 1999). Diapause depth in the open ocean usually ranges from 400 m to 1000 m (Hirche 1996a), but in some coastal habitats (lochs and fjords) *C. finmarchicus* has been found to diapause close to the bottom at 100-150 m depth (Clark et al. 2013).

The copepods stay in diapause for several months before increasing metabolic activity and the development of gonadal tissues mark the emergence from diapause (Hirche 1996a,b). This usually starts in late autumn/early winter well before the copepods molt to adults and mate during their ascent to the surface (usually in February-April) (Marshall & Orr 1955, Hirche 1996a). While the seasonal cycle of *C. finmarchicus* and it congeners has been described in great detail with regard to their population dynamics (e.g. Plourde et al. 2001), and physiology (e.g. Clark et al. 2012, Freese et al. 2015, 2017), seasonal investigations on the genetic level are still rare (Tarrant et al. 2008, Clark et al. 2013) and the processes controlling the timing of the copepods annual life cycle are far from understood (Ji 2011, Baumgartner & Tarrant 2017).

Diapause initiation has been most prominently linked to lipid content (Rey-Rassat et al. 2002, Johnson et al. 2008, Tarrant et al. 2008, Pond et al. 2012) with a minimum lipid content required prior to diapause initiation to survive the time without feeding at depth (Saumweber & Durbin 2006, Maps et al. 2011). Alternatively, food availability has been proposed as an environmental cue, triggering diapause when phytoplankton concentrations decrease after the spring bloom (Hind et al. 2000, Wilson et al. 2016). Furthermore, the seasonal change of photoperiod (day length) has been proposed as a possible cue for diapause initiation (Grigg & Bardwell 1982, Miller et al. 1991). However, to date the trigger(s) of diapause remain unresolved (Tarrant et al. 2016, Baumgartner & Tarrant 2017).

While the same population can produce two or more generations entering diapause at different times of the year (e.g. Johnson et al. 2008), emergence from diapause is synchronous within a given population (Baumgartner & Tarrant 2017). An “hourglass” timer has been proposed that starts “ticking” with the initiation of diapause ultimately triggering emergence (Miller et al. 1991, Campbell et al. 2004). This hourglass mechanism could be based on decreasing lipid levels, the accumulation of hormones or continuous slow development (Hind et al. 2000, Irigoien 2004, Clark et al. 2012), but it does not account for the synchronized emergence of copepod from generations that entered diapause at different times. Further hypothesis suggests that the photoperiod could be a cue for synchronizing emergence from diapause (Miller et al. 1991, Tittensor et al. 2003, Speirs et al. 2005). There have been several model studies simulating *C. finmarchicus* diapause initiation and termination based on the abovementioned parameters (for overview see Baumgartner & Tarrant 2017), but it has so far not been possible to generate the seasonal cycle of diapause initiation and termination under controlled laboratory conditions. Whilst the significance of lipid content, food availability and temperature for *C. finmarchicus* seasonal timing has already received great attention

(Ingvarsdóttir et al. 1999, Rey-Rassat et al. 2002, Hassett 2006, Saumweber & Durbin 2006, Johnson et al. 2008, Clark et al. 2012, Pierson et al. 2013), research on the effects of photoperiod is still scarce (Miller et al. 1991, Johnson et al. 2008).

Many terrestrial organisms use a circadian clock to measure photoperiod and thus have a mechanism with which to predict cyclic seasonal change (Oster et al. 2002, Meuti & Denlinger 2013). The circadian mechanism that is also present in *C. finmarchicus*, consists of "clock genes" that interact via their protein products forming an intricate network of feedback loops, creating an endogenous rhythm of ~24h length (Latin: "*circa dies*": about a day) (Mackey 2007, Häfker et al. 2017). Although able to function independently, the clock is entrained on a regular basis by environmental cues, the most common of which is the light/dark cycle (Aschoff 1954). For many insects the circadian clock is crucial for measuring photoperiod, which is then used as cue for the entrainment of their seasonal life cycle (Oster et al. 2002, Meuti & Denlinger 2013). Also, many insects initiate or terminate diapause when surpassing a certain critical photoperiod marking the transition from one seasonal life phase to another (Sakamoto et al. 2009, He et al. 2009, Goto 2013, Salminen et al. 2015). Photoperiodic entrainment of seasonal diapause has also been demonstrated in marine and freshwater copepods (Einsle 1964, Watson & Smallman 1971, Marcus 1982) and was suggested for *C. finmarchicus* (Grigg & Bardwell 1982, Miller et al. 1991). However, the molecular and physiological processes underpinning photoperiodic time measurement and the pathways by which such timing mechanisms affect the seasonal life cycle and diapause of copepods, remain unexplored.

Here we behaviorally, physiologically and molecularly characterize *C. finmarchicus*' seasonal life cycle in a Scottish sea loch. Field sampling provided detailed insights into seasonal patterns of vertical migration rhythms, population dynamics, physiological parameters, and gene expression. We further present comprehensive data of diel and seasonal clock gene rhythmicity revealing endogenous timing mechanisms of *C. finmarchicus*. Our data suggests that photoperiod and/or a circannual clock may be involved in the initiation and termination of *C. finmarchicus* diapause.

3.3 Materials & Methods

Study site characteristics

The investigation took place in Loch Etive, a sea loch in western Scotland, UK (56°45´N, 5°18´W). A sill with a width of 200 m and a depth of ~7 m limits the exchange with the open ocean and the loch is further divided into an upper and a lower basin by a second sill with ~13 m depth (Edwards & Edelsten 1977, Nørgaard-Pedersen et al. 2006). All

sampling was conducted at the deepest point of the loch, Bonawe deep (~150 m) located in the upper basin (Fig.3.1). The deeper waters of the upper basin typically cycle from normoxic to hypoxic, with the sills limiting the water exchange with the open ocean. Overturning of these water masses happens irregularly every few years when spring tides in spring/autumn co-occur with low precipitation (Edwards & Edelsten 1977). To monitor the seasonal changes in the hydrography of the loch a mooring was deployed close to Bonawe deep (~135 m depth) equipped with equipped with temperature loggers (SBE 56, Sea-Bird Electronics, USA) attached in 10 m intervals. The mooring was deployed in March 2015, was out of the water for a few days in December for data extraction and maintenance and was ultimately retrieved in March 2016. Additionally, salinity, temperature, oxygen concentration, fluorescence-based chlorophyll a (Chl a) concentration and photosynthetic active radiation (PAR, 400-700 nm) were recorded of the water column by a conductivity-temperature-depth (CTD) profiler (SBE 19plus V2 SeaCAT Profiler, Sea-Bird Electronics, USA) equipped with a irradiance sensor (QSP-2300, Biospherical Instruments, USA) at midday and midnight during each seasonal time point. Except for PAR data, midday and midnight CTD hauls were pooled as they were similar.

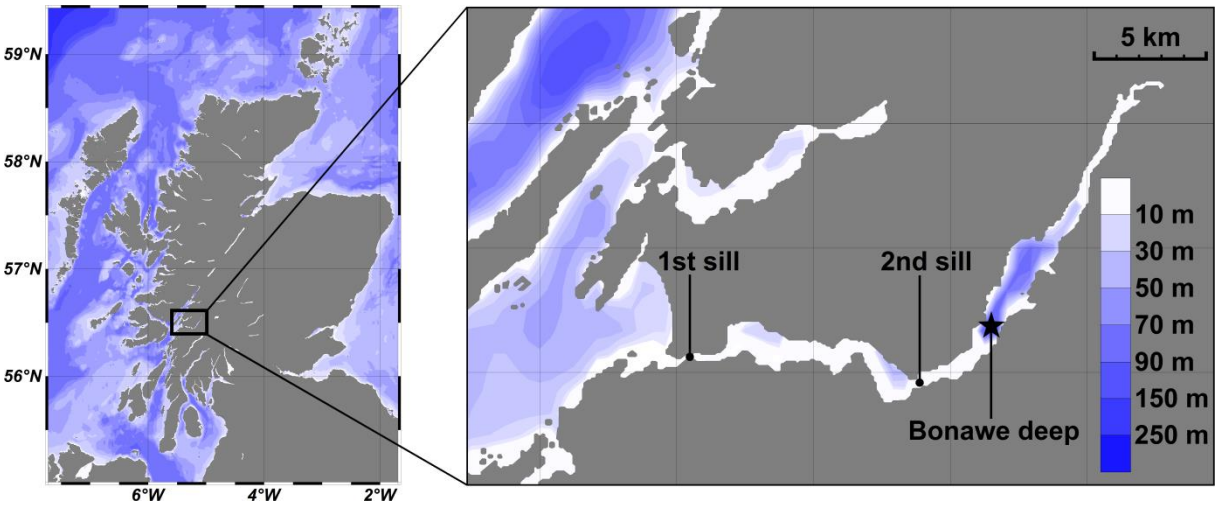

Figure 3.1: Sampling site characteristics. Loch Etive is a sea loch at the western coast of Scotland, UK (56°45´N, 5°18´W). Water exchange with the ocean is limited by two sills. All samplings as well as the mooring deployment were done at the deepest point of the loch, Bonawe deep (~150 m), at the Scottish Association for Marine Science (SAMS) permanent station RE5. Maps were created with Ocean Data View v. 4.7.4 (Schlitzer 2015).

Zooplankton vertical migration

To record vertical migration behavior in the loch, the mooring was furthermore equipped with two acoustic Doppler current profilers (ADCP, Teledyne RD Instruments, USA) pointing upwards at 45 m and 120 m depth. The 300 kHz ADCPs have been extensively used to monitor mesozooplankton migrations (Tarling et al. 2002, Cottier et al. 2006,

Brierley et al. 2006, Last et al. 2016). Acoustic data was checked for quality using the RDI correlation index (a measure of signal to noise ratio). Absolute backscatter volume (Sv, measured in decibel, dB) was derived from echo intensity according to Deines (1999) with derived acoustic mean volume backscattering strength (MVBS).

Field sample collection

Copepods were collected at Bonawe deep on 6 seasonal time points between May 2015 and March 2016 (Tab.3.1). The time period was chosen to consistently sample *C. finmarchicus* from the same seasonal cycle that started with hatching in April 2015 (Clark et al. 2013). All analyses were performed on the CV copepodid stage, which is the main overwintering stage and represents the bulk of the population over the year (Clark et al. 2013). Copepods were collected from the R/V *Calanus* (Scottish Association for Marine Science) using a WP2-net equipped with an opening/closing mechanism (200 µm mesh size / 50 µm cod end) hauled vertically through the water column. For all of samplings the water column was separated into two layers with 5-50 m representing the “shallow” sample and 50-140 m representing the “deep” sample. The upper 5 meters of the water column was excluded to avoid hypoosmotic stress of the animals due to the extremely low salinity close to the surface (Fig.3.2C). Community structure was determined with collections at midday and midnight and at both depths. Animals were fixed as bulk samples in 10% ethanol for later analysis of *C. finmarchicus* abundance, life stage composition and body length. It was assumed that sampling did not affect the stage composition as a mesh size of 200 µm has been shown to catch at least 95% of the smallest copepodid stage CI and 100% of the older stages (Nichols & Thompson 1991). For each collected sample, n = 200-300 *C. finmarchicus* individuals were staged and the length (from the tip of the head to the tip of the furca) of n = 100 CV stages was measured, respectively. Data for midday and midnight hauls was pooled for each seasonal time point and depth to prevent diel migration patterns from affecting seasonal observations. Additionally, animals collected at midday were sorted for *C. finmarchicus* CV stages using dissecting microscopes under dim red light and at *in situ* temperature and were then fixed in liquid nitrogen for later analyses of dry weight, C/N ratio, and lipid content (see below). A summary of samples collected can be found in Tab.3.2.

At each of the 6 seasonal time points, zooplankton samples were also collected over a period of 28h at 4h intervals with a total of 8 diel time points per seasonal time point. Sampling at each seasonal time point started between 10:00 and 11:00 and ended between 14:00 and 15:00 on the following day (Tab.3.1). The samples were immediately (within 1 min after retrieval) fixed as bulk samples in RNAlater® stabilizing solution (Ambion, UK). The bulk samples were later sorted at 2°C for *C. finmarchicus* CV stages

and for each depth layer, *n* = 5 replicates were collected per diel time point with 15-17 individuals pooled per replicate and stored at -20°C for later gene expression analyses (see below). Loch Etive represents a habitat of pure *C. finmarchicus* (as confirmed with population genetic studies, Søreide pers. comm.) and contamination of samples with the congener *C. helgolandicus* is highly unlikely as the species has limited tolerance to reduced salinities (Hill 2009).

Table 3.1: Diel time series sampling. Samples for gene expression analysis were collected at 8 diel time points in 4h intervals over a period of 28h at each seasonal time point. Times of sunset, sunrise and photoperiod at Bonawe deep were obtained from NOAA sunrise/sunset calculator. All times are given as UTC.

seasonal time point	gene expression sampling period	sunset	sunrise	photoperiod [h]
May 2015	6th May, 10:00 – 7th May 14:00	20:12	04:24	15.8
June	18th Jun, 10:00 – 19th Jun 14:00	21:15	03:23	17.9
August	5th Aug, 11:00 – 6th Aug 15:00	20:24	04:33	15.9
November	16th Nov, 11:00 – 17th Nov 15:00	16:11	08:04	8.1
January 2016	19th Jan, 11:00 – 20th Jan 15:00	16:22	08:41	7.7
March	3rd Mar, 10:00 – 4th Mar 14:00	18:01	07:05	13.1

Dry weight & carbon to nitrogen (C/N) ratio

Copepodid CVs were individually fixed in pre-weighed tin-caps in liquid nitrogen (*n* = 24 per seasonal time point and depth, Tab.3.2). They were then freeze dried and dry weighed was determined (XP6U Micro Comparator, Mettler-Toledo, USA). Afterwards, the C/N ratio of the individuals was determined via elemental analyzer (EuroEA, EuroVector, Italy).

Table 3.2: Collected samples. Listed samples were collected the same way at each seasonal time point. Sampling was always performed in two depth layers (5-50 m, 50-140 m) via vertical net hauls (see text). For each parameter, the time(s) of sampling, the number of collected replicates and sample handling are described. Only *C. finmarchicus* CV stages were used, except for abundance and stage composition where all copepodid life stages (incl. adults) were investigated.

parameter(s)	time(s)	*n* / depth layer	sample handling
abundance & stage composition	midday, midnight	2x 200-300 indv.	fixed in 10% methanol, sorted later
body size		2x 100 CVs	
lipid content	midday	5 (20 CVs / *n*)	sorted under red light, fixed in liquid N_2
dry weight & C/N ratio		24 (1 CV / *n*)	
gene expression	see Tab.3.1	5 per diel time point (15-17 CVs / *n*)	fixed in RNAlater®, sorted later

Lipid analysis

Total lipid content of CV copepodids was determined over the seasonal sampling period. *n* = 5 replicates were taken for each seasonal time point and depth, with 20 individuals pooled per sample (Tab.3.2). Total lipid was extracted following Folch et al. (1957).

Frozen copepods were initially homogenized in 4 mL chloroform:methanol (2:1 v/v) using a T10 ULTRA-TURRAX® disperser (IKA, Germany). The homogenate was then filtered through a pre-washed Whatman® N°1 filter (GE Healthcare, UK). After the addition of 1 mL KCl (0.88% w/v) the samples were thoroughly homogenized using a vortex mixer before being centrifuged at 400 g for 2 min for phase separation. After discarding the upper aqueous layer, the lower chloroform phase containing the total lipid extract was dried under a stream of oxygen free nitrogen. The samples were then desiccated under vacuum for 6h and total lipid mass [µg*indv.$^{-1}$] determined gravimetrically.

Gene selection

35 target genes as well as 3 housekeepers were selected for analysis including genes involved in the circadian clock machinery, lipid-, carbohydrate- and amino acid metabolism, aerobic/anaerobic energy metabolism, stress response and light perception. Sequences of relevant genes of interest from other crustaceans were used to browse a *de novo* transcriptome by Lenz et al. (2014) to identify respective gene sequences for *C. finmarchicus*. Clock genes had previously been annotated from the transcriptome (Christie et al. 2013a). The identities of the characterized sequences were verified via blastx using the NCBI online database. All measured genes, their physiological functions and their respective blastx top-hits are listed in Tab.A2.1.

The sequence information obtained via blastx was then used to identify common protein domains and the sequences were further checked for palindromic regions and repeats using the online tools Oligoanalyzer 3.1 (http://eu.idtdna.com/calc/analyzer) and RepeatMasker 3.0 (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker). This identified suitable binding regions for primers and probes. Based on this information, custom Taqman® low-density array-cards (Applied Biosystems, USA) were designed via the Applied Biosystems online tool (https://www.thermofisher.com/order/custom-genomic-products/tools/gene-expression/) with primer and probe binding regions placed in sequence intersects specific to the respective gene (checked via blastn against NCBI). Sequences of primers and probes were submitted to the PANGAEA online repository (https://doi.pangaea.de/10.1594/PANGAEA.884073).

Gene expression analysis

To measure the activity of the selected genes, RNA was extracted using the RNeasy® Mini kit (Quiagen, Netherlands) with β-mercaptoethanol (0.14 M) added to the lysis buffer as recommended for lipid-rich samples. The TURBO DNA-free kit (Life Technologies, USA) was used to remove genomic DNA and RNA was checked for possible degradation (2100 Bioanalyzer / RNA 6000 Nano Kit, Agilent Technologies, USA) as well as for

contaminations and overall RNA level (Nanodrop 2000 Spectrophotometer, Thermo Fisher Scientific, USA). RNA levels determined via Nanodrop were used as a proxy for overall gene expression activity. A thermocycler (T100 Thermal Cycler, Bio-Rad Laboratories, USA) was used to convert 2 µg RNA per sample to cDNA using RevertAid H Minus Reverse Transcriptase (Invitrogen, Germany). RNA samples were stored at -80°C between all processing steps and cDNA was stored at -20°C. Gene expression was the measured by real-time quantitative PCR (ViiATM 7, Applied Biosystems, USA).

Gene expression was normalized using the $2^{-\Delta\Delta CT}$-method (Livak & Schmittgen 2001) with housekeeping genes chosen based on expression stability, expression level relative to investigated genes and the findings of previous studies (Hansen et al. 2008, Tarrant et al. 2008, Clark et al. 2013, Fu et al. 2013, Häfker et al. 2017). For the investigation of seasonal expression patterns, genes were normalized against the geometric mean of the housekeeping genes *elongation factor 1* α (*ef1*α) and *ribosomal protein S13* (*rps13*) and all samples collected at the respective seasonal time points were pooled (6 seasonal time points, *n* = 40 replicates per gene, seasonal time point and depth). For the investigation of diel expression cycles, the 8 clock genes *clock* (*clk*), *cycle* (*cyc*), *period1* (*per1*), *period2* (*per2*), *timeless* (*tim*), *cryptochrome2* (*cry2*), *clockwork orange* (*cwo*), and *vrille* (*vri*) were normalized against the geometric mean of the housekeeping genes *rps13* and *RNA polymerase II* (*RNApoly*) and the 28h time series from each of the different seasonal time points were treated individually (8 diel time points, *n* = 5 replicates per gene, diel time point and depth).

Statistical analysis

For the analysis of DVM patterns backscatter rhythmicity of acoustic data from each seasonal sampling time point over the sampling period ± 3 days (7 days in total) for 25 m and 90 m depth was analyzed with TSA-Cosinor© (package 6.3, Expert Soft Technologies, France) and expressed as period length (τ) and % of model fit (Bingham et al. 1982).

All other statistical analyses were performed using RStudio (v. 0.99.442, R Development Core Team 2013). Graphics were created using SigmaPlot (v. 12.5) unless indicated otherwise. Seasonal patterns of body length, dry weight, C/N ration, lipid content, and overall RNA level were analyzed separately for shallow and deep samples via non-parametric Kruskal-Wallis ANOVAs on Ranks (α = 0.05) followed by Dunn's Multiple Comparison post-hoc tests (α = 0.05) provided in the "FSA" R-package. For each seasonal time point, a comparison between the shallow and the deep layer was made for all parameters using the Mann-Whitney U test (α = 0.05).

Seasonal patterns of gene expression were analyzed the same way via Kruskal-Wallis ANOVAs on Ranks and Mann-Whitney U tests, but with α = 0.0001 to account for the testing of multiple genes (35 genes in 2 depth layers over 6 seasonal time points resulting in 70 Kruskal-Wallis ANOVAs and 210 Mann-Whitney U tests). To identify reoccurring seasonal expression patterns, heatmaps and dedrograms of all genes were created for the shallow and the deep layer, respectively (R-packages “gplot” and “RColorBrewer”). For each depth genes were then grouped into four major clusters based on expression pattern similarities. Diel expression patterns of the 8 clock genes over the 28h time series collected at each seasonal time point were checked for 24h cycling with the R-package “RAIN” (Thaben & Westermark 2014), which uses a non-parametric approach to detect rhythmic oscillations independent of waveform. α = 0.001 was used to account for the testing of multiple genes (8 genes in 2 depth layers resulting in 16 RAIN analyses). Cycling was analyzed separately for each seasonal time point as well as for shallow and deep samples. The housekeeping genes used for normalization (*rps13*, *RNApoly*, *ef1*α) were excluded from all statistical analyses. All other non-clock genes were also checked for 24h rhythmicity, but none of them showed consistent diel patterns and thus data is not shown.

3.4 Results

Hydrography & primary production

Water temperature at Bonawe deep ranged between 8°C and 14°C over the course of the study. The temperature was most variable in the upper 60 meters with lowest temperatures in winter/spring and highest values at the end of the summer (Fig.3.2A,B). From May to October the temperature below 80 m ranged from 10.2°C to 10.8°C with minimal change with depth (<0.4°C), but in October/November temperature rose by ~1°C followed by a weak gradual warming that continued until the end of the study period. Salinity generally increased with depth and was stable at 27.5 psu below ~60 m throughout the study period. From May to August, salinity gradually decreased from 60 m toward the surface with a sharp decrease to 13-18 psu in the upper 5 m. From November to March the decrease in surface salinity was more pronounced, especially from January to March when salinity showed a sharp decrease at 20-30 m depth (Fig.3.2C). Oxygen concentration decreased with depth and largest changes occurred between 10 and 50 m, with an especially sharp decrease in January and March at 20-30 m depth (Fig.3.2D). According to the Chl a data, phytoplankton was concentrated in the upper 10 m of the water column with a second, weaker peak at ~25 m in May. The Chl a maximum in May (10.5 mg Chl a*m^{-3}) was followed by a sharp decrease until August (Fig.3.2E), while from November to March the phytoplankton abundance was very low (<1.5 mg Chl a*m^{-3}).

Midday PAR at 1 m depth varied between ~100 µmol photons*m^{-2}*s^{-1} in May-August and ~10 µmol photons*m^{-2}*s^{-1} in November/January, but was strongly affected by weather conditions (cloud cover) during the samplings. Generally, light intensity decreased rapidly with depth and was no longer detectable below 20 m at any seasonal time point (sensor detection limit: 1*10^{-12} µmol photons *m^{-2}*s^{-1}).

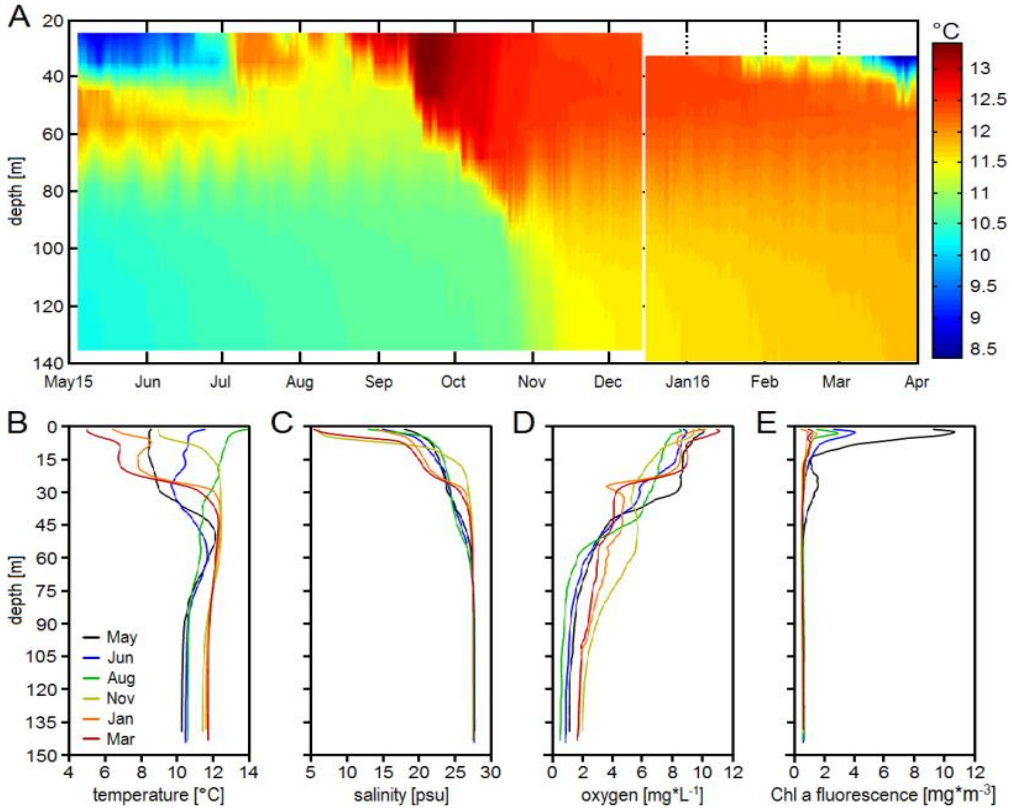

Figure 3.2: Environmental parameters. Water column characteristics at the sampling site Bonawe deep were monitored. (A) Seasonal change in temperature recorded from loggers attached in 10 m intervals to a mooring. Date labels indicate the 1st day of the respective month. CTD hauls additionally provided high resolution profiles of (B) temperature, (c) salinity, (D) oxygen concentration, and (E) chlorophyll a concentration for each seasonal time point.

Zooplankton diel vertical migration

Classical DVM was observed at the study site with animals migrating to the surface at night and retreating to deeper waters during the day. Times of upward/downward migration generally coincided with sunset/sunrise tracking the seasonal change in photoperiod (Fig.3.3A). In shallow waters (measured at 25 m), 24h backscatter rhythmicity could be detect at all seasonal sampling time points, while in the deeper layer (measured at 90 m) a diel backscatter rhythm was only found in May and June (Tab.3.3). Migrations stretched from the surface to ~70 m depth in May/June and became shallower afterwards (Fig.3.3B-D). In January/March animals did not migrate all the way to the surface but accumulated at ~30 m depth at night (Fig.3.3E).

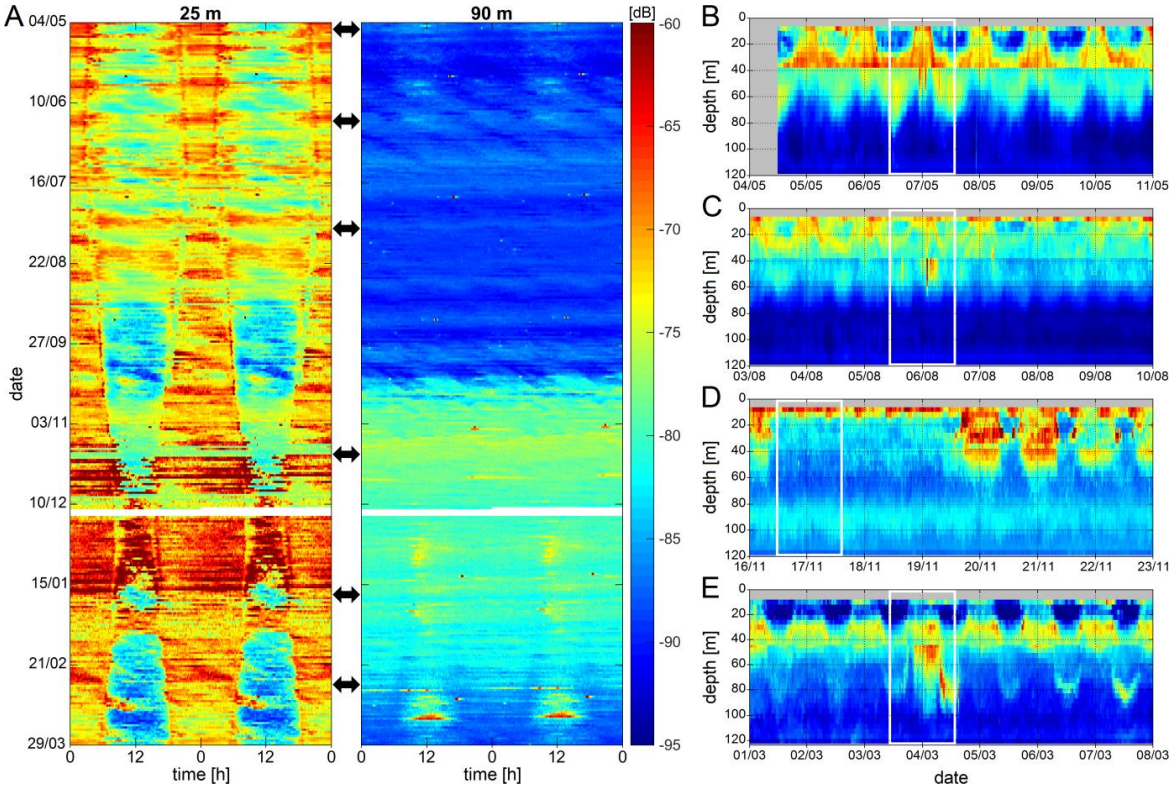

Figure 3.3: Diel vertical migration patterns at Bonawe deep, Loch Etive. Mean volume backscatter strength (MVBS, expressed in [dB]) is shown. (A) Diel change in MVBS at 25 m and 90 m, respectively. Data is double plotted. Arrows (↔) indicate seasonal sampling time points. The gap in December resulted from the temporary retrieval of the mooring for maintenance and data collection. (B-E) MVBS patterns recorded over 7 day periods around the seasonal sampling time points in May, August, November, and March, respectively. Date labels at 00:00h of the respective day. The times of the 28h sampling campaigns are indicated by white boxes. The sharp change in backscatter at ~38 m (particularly strong in May) is an artifact caused by the combination of data from two ADCPs at different depths.

Aside from DVM there appears to be another rhythm of backscatter detected at 25 m that corresponds to a period of 12.4h and that oscillates in intensity over a period of 28 (14) days (Fig.3.3A). Further, a seasonal accumulation of organisms was observed around 90 m depth from November to January (Fig.3.3A,E).

Table 3.3: Zooplankton backscatter rhythmicity over the course of the study. Data obtained via ADCP set to a frequency of 300 kHz. For each seasonal time point the oscillation period (τ) and fit were determined by TSA Cosinor analysis over a period of 7 days. n.s. indicates no significant rhythmicity.

	shallow (25 m)		**deep (90 m)**	
seasonal time point	**period (τ)**	**% model fit**	**period (τ)**	**% model fit**
May (04.–11.05.2015)	23.9	49.6	24.0	33.3
June (15.–22.06.2015)	24.1	52.7	23.6	16.8
August (03.–10.08.2015)	23.8	20.7	n.s.	–
November (09.–16.11.2015)	24.1	41.2	n.s.	–
January (19.–26.01.2016)	24.0	40.6	n.s.	–
March (01.–08.03.2016)	23.8	62.7	n.s.	–

C. finmarchicus population dynamics

Overall *C. finmarchicus* abundance in the shallow layer (5-50 m) was highest in May (453 indv.*m^{-3}) followed by August (147 indv.*m^{-3}) (Fig.3.4A), while in June and from November to March it was below 75 indv.*m^{-3}. In the deep layer (50-140 m), abundance gradually increased from a minimum in May (27 indv.*m^{-3}) to high abundances in November (220 indv.*m^{-3}) and January (197 indv.*m^{-3}) and then decreased to 75 indv.*m^{-3} in March (Fig.3.4B).

In the shallow layer, from May to August, early copepodid stages (CI-CIV) accounted for more than 60% of the *C. finmarchicus* population. The remaining population consisted of CVs and adults (mostly females) with the former outnumbering the latter (Fig.3.4A). By November, CVs made up ~60% of the shallow population and they stayed dominant until the end of the study, although in March the abundance of adults did again increase to ~35%. The deep layer was dominated by adult females (~65%) in May, but from June onwards until the end of the study almost the entire deep population (>95%) consist of CV stages (Fig.3.4B).

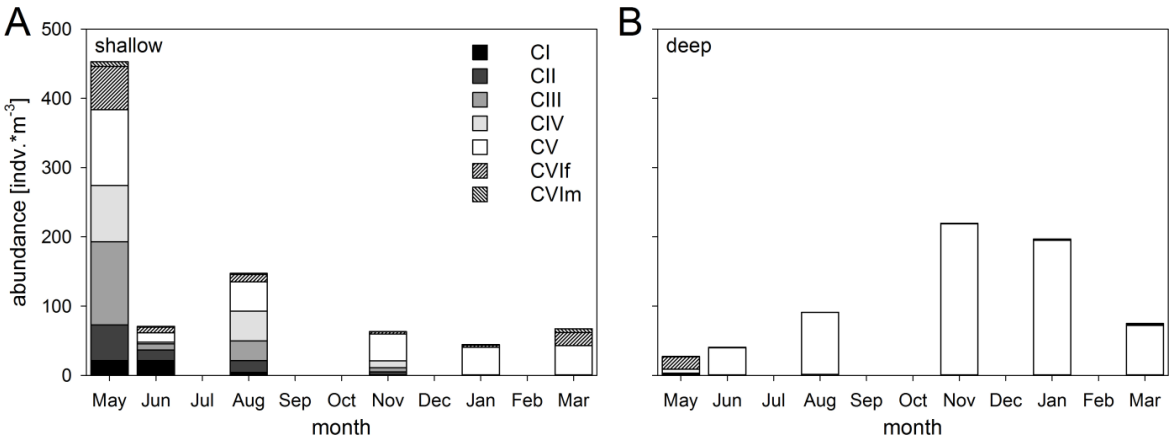

Figure 3.4: *C. finmarchicus* abundance and stage composition. Samples were collected at Bonawe deep via vertical net hauls in (A) the shallow layer and (B) the deep layer. Copepodid stages (CI-CV) and adults (CVIf, CVIm) were counted. At each seasonal time point samples were collected at midday and midnight and data was pooled to prevent DVM effects.

Body length, dry weight, C/N ratio and lipid content

CV stage body length was generally variable but still showed significant seasonal differences in both depth layers (Kruskal-Wallis $p < 0.05$, $df = 5$, respectively) (Fig.3.5A). In the shallow layer, CV body length decreased from ~2.9 mm in May/June to 2.7-2.75 mm from August to March. A similar pattern was observed in the deep layer, but with the major decrease between August and November. While shallow CVs were significantly bigger in May, deep CVs were bigger from August on (Mann-Whitney U test, $p < 0.05$, respectively).

Dry weight of the CV stage varied between 100 and 260 µg individual^{-1} and showed a significant seasonal change in both depths (Kruskal-Wallis $p < 0.05$, $df = 5$, respectively). In the shallow layer dry weight decreased from June to November followed by an increase towards January/March (Fig.3.5B). Dry weight of deep layer CVs peaked in August and then decreased to a minimum in January. In August, November, and March copepods from the deep layers had a significantly higher mass than those from the shallow layer (Mann-Whitney U test, $p < 0.05$, respectively).

The C/N ratio of individual CV stages changed significantly over the seasonal cycle with similar patterns in both depth layers (Kruskal-Wallis $p < 0.05$, $df = 5$, respectively). Changes were largest in the shallow layer with highest C/N values around 8 in June, August, and January, while the ratio decreased to 4.77 in November and 6.67 in March (Fig.3.5C). C/N ratios in the deep layer were significantly higher than in the shallow layer for all seasonal time points except January (Mann-Whitney U test, $p < 0.05$, respectively).

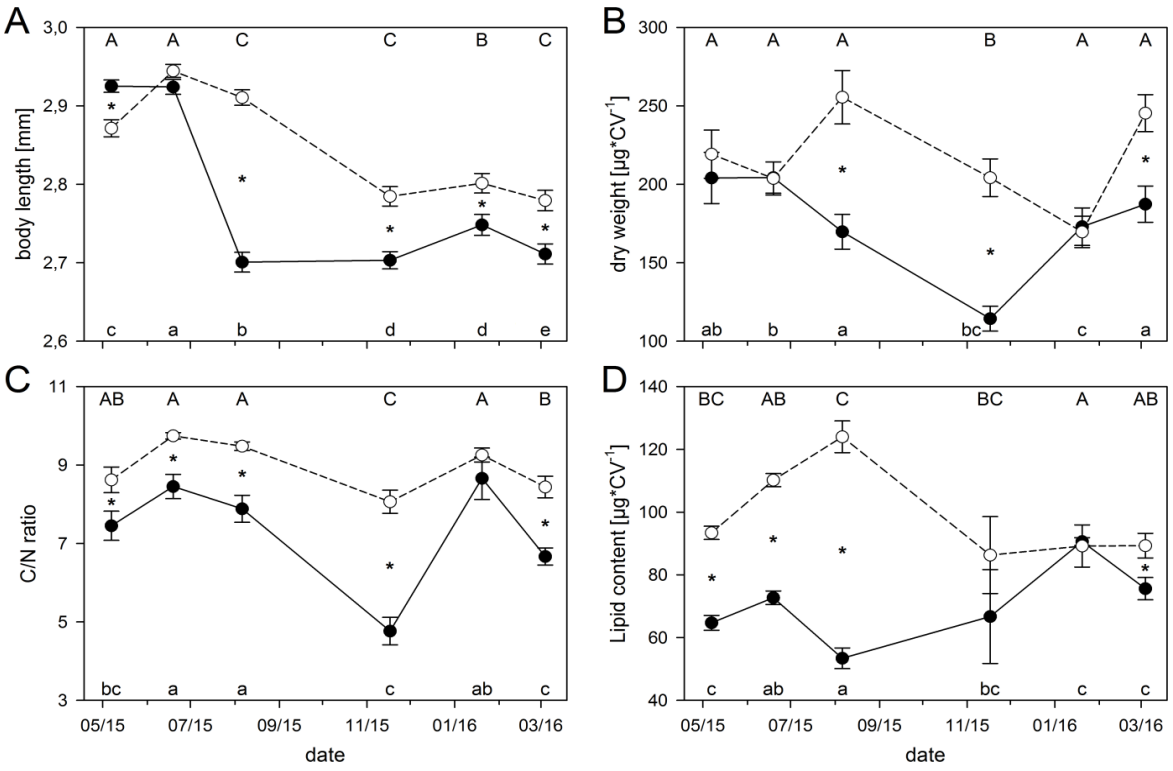

Figure 3.5: Body condition parameters. Changes in (A) body length, (B) dry weight, (C) C/N ratio, and (D) total lipid content of *C. finmarchicus* CV stages in the shallow layer (solid symbols and line) and the deep layer (open symbols, dashed line) are shown. Date labels indicate the 1st day of the respective month. Significant seasonal differences within depth layers were identified via Kruskal-Wallis ANOVA and are indicated by different capital letters for the shallow layer and different subscript letters for the deep layer, respectively. Significant differences between shallow and deep layer at each seasonal time point were identified via Mann-Whitney test and are indicated by an asterisk (*) between the symbols at the respective time points. For each depth layer and seasonal time point, the number of replicates measured was $n = 100$ for body length, $n = 24$ for dry weight, $n = 24$ for C/N ratio, and $n = 5$ for total lipid content. Mean values ± standard error (SE) are shown.

Significant seasonal changes in total lipid content of copepods from both the shallow and the deep layer were found (both Kruskal-Wallis $p < 0.05$, $df = 5$, respectively) (Fig.3.5D). In the shallow layer, total lipid content per individual CV stage varied from 53 µg in June to ~80 µg in January/March. In the deep layer, CV lipid content increased from 93 µg in May to 124 µg in August and was constant at ~90 µg from November on. The lipid content of CVs in the deep layers was higher than in the shallow layer from May to August and in March (Mann-Whitney U test, $p < 0.05$, respectively). Both depth layers showed high variability in lipid content in November.

Seasonal gene expression

Overall RNA levels in *C. finmarchicus* CVs were highest in May for both depths (Kruskal-Wallis $p < 0.05$, $df = 5$, respectively) and, with the exception of November, were generally higher in the shallow layer (Mann-Whitney U test, $p < 0.05$, respectively) (Fig.3.6). In the shallow layer, the RNA level gradually decreased from May to a minimum in November with a slight increase thereafter. In the deep layer, RNA levels decreased from May to a minimum in August and stayed constantly low thereafter.

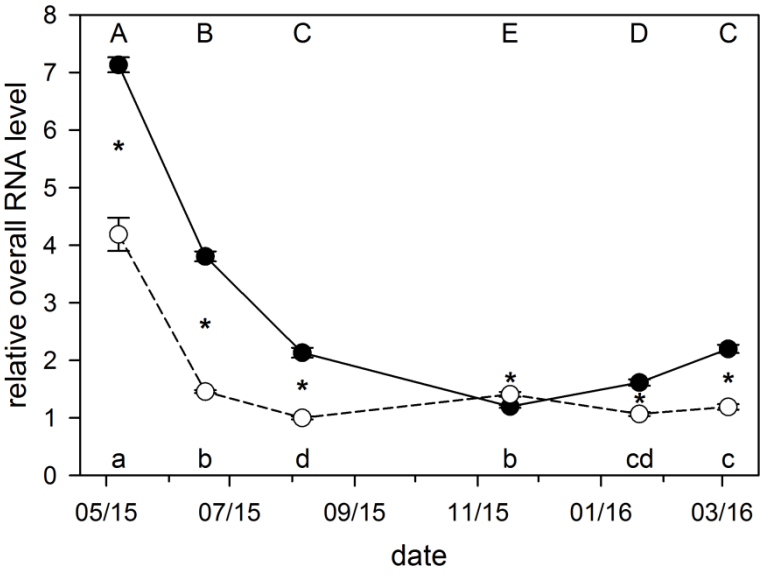

Figure 3.6: Overall RNA levels. Relative seasonal change of RNA content of *C. finmarchicus* CV stages in the shallow layer (solid symbols and line) and the deep layer (open symbols, dashed line) is shown. Date labels indicate the 1st day of the respective month. Statistical analyses and labelling were performed as described for Fig.3.5. For each depth layer and seasonal time point $n = 40$ replicates were measured. Mean values ± standard error (SE) are shown.

Almost all of the investigated genes showed significant seasonal expression changes in both depth layers (Kruskal-Wallis $p < 0.0001$, $df = 5$, respectively, Tab.A2.2). The Mann-Whitney U tests found numerous significant differences between the shallow and deep layer for various genes and seasonal time points ($p < 0.0001$, respectively, Tab.A2.2). The number of genes differing significantly in expression between shallow and deep layer varied with season. The number was lowest in May and November (11 and 15 genes, respectively) and highest in June and August (25 and 28 genes, respectively). Relative change in seasonal expression level was variable among genes, ranging from 1.6-fold

change for *citrate synthase* (*cs*) to 68-fold change for *chymotrypsin* (*chtrp*). An especially pronounced change was found for *elongation of very long fatty acids protein* (*elov*), which showed 5477-fold change in relative expression over the seasons. For 21 of the 35 investigated genes the maximum change was between 2- and 10-fold (Tab.A2.2).

Heatmap analyses of gene expression revealed several clusters of reoccurring seasonal patterns. In the shallow layer, there were 4 distinctive clusters (Fig.3.7A) with the first one (S_1) characterized by an expression minimum in November followed by an increase in January/ March. From May to August most of these genes showed elevated expression with levels similar to January/March and some genes displaying a peak in June. Genes in the S_1 cluster included among others *cs*, *malate dehydrogenase* (*mdh*), *cyclin B* (*cyclB*), *ecdysteroid receptor* (*ecr*) as well as clock-associated genes like *cryptochrome1* (*cry1*) and *casein kinase II* α (*ck2*α). The second cluster (S_2) showed consistent peak activity in May or June followed by a gradual or stepwise decrease toward lowest values in March. Genes in the S_2 cluster included *elov*, *phosphofructokinase* (*pfk*), *chtrp*, *glutamate dehydrogenase* (*gdh*) as well as the clock genes *cyc*, *per2*, and *cwo*. The third cluster (S_3) showed no clear seasonal patterns and consisted of the genes *phosphoenolpyruvate carboxykinase* (*pepck*), *widerborst* (*wbt1*) and the clock gene *vrille* (*vri*). The fourth shallow layer cluster (S_4) was characterized by an expression peak in November, minimum values in March and consistently low expression in the remaining months. Representatives of the S_4 cluster were *hemocyanin* (*hc*), *ferritin* (*fer*), *couch potato* (*cpo*), *arginine kinase* (*argk*), an opsin (*ops*), and the clock genes *per1*, *tim* and *cry2*.

In the deep layer, heatmap analysis identified four distinctive clusters (Fig.3.7B). In the first cluster (D_1), expression was high in May, largely reduced from June until November and then increased again to initial levels in March. The cluster composition shared large similarity with the S_1 cluster of the shallow layer and included the genes *cs*, *mdh*, *cyclB*, *ecr*, *cry1*, and *ck2*α. Comparing D_1 and S_1, expression was similar between depths in May, November and March while expression was higher in the shallow layer in June, August and January (Tab.A2.2). The second cluster of the deep layer (D_2) was characterized by peak expression in May followed by a sharp decline to low levels in June/August and thereafter. The genes of the D_2 cluster (including *elov*, *pfk*, and *chtrp*) were all found in the S_2 cluster of the shallow layer. Individual gene comparison of D_2 and S_2 revealed higher expression in the shallow layer from May to November with the difference between depths gradually decreasing (Tab.A2.2). Genes of the third deep layer cluster (D_3) all showed peak expression in June followed by a sharp or gradual decrease and often low expression in May. The D_3 cluster included *pepck*, *pigment dispersing hormone receptor* (*pdhr*), and the clock genes *cyc*, and *vri* that were found in

the S_2 and S_3 clusters of the shallow layer. Expression of individual genes was similar between deep and shallow layer for most of the study period, with some genes showing higher expression in the deep layer from June to November (Tab.A2.2). The fourth cluster of the deep layer (D_4) was characterized by and high expression levels from June to November and low expression in May and March. Most genes of the D_4 cluster were found in the S_4 cluster of the shallow layer (incl. *hc*, *fer*, *cpo*, *argk*, *ops*, *per1*, *tim*, and *cry2*), while a few like *3-hydroxyacyl-CoA dehydrogenase* (*hoad*) and *clk* were found in the S_2 cluster. Expressions were similarly low in the D_4 and the S_4 cluster in May and March, but most genes showed much higher expression levels in the deep layer from June to January compared to the shallow layer (Tab.A2.2).

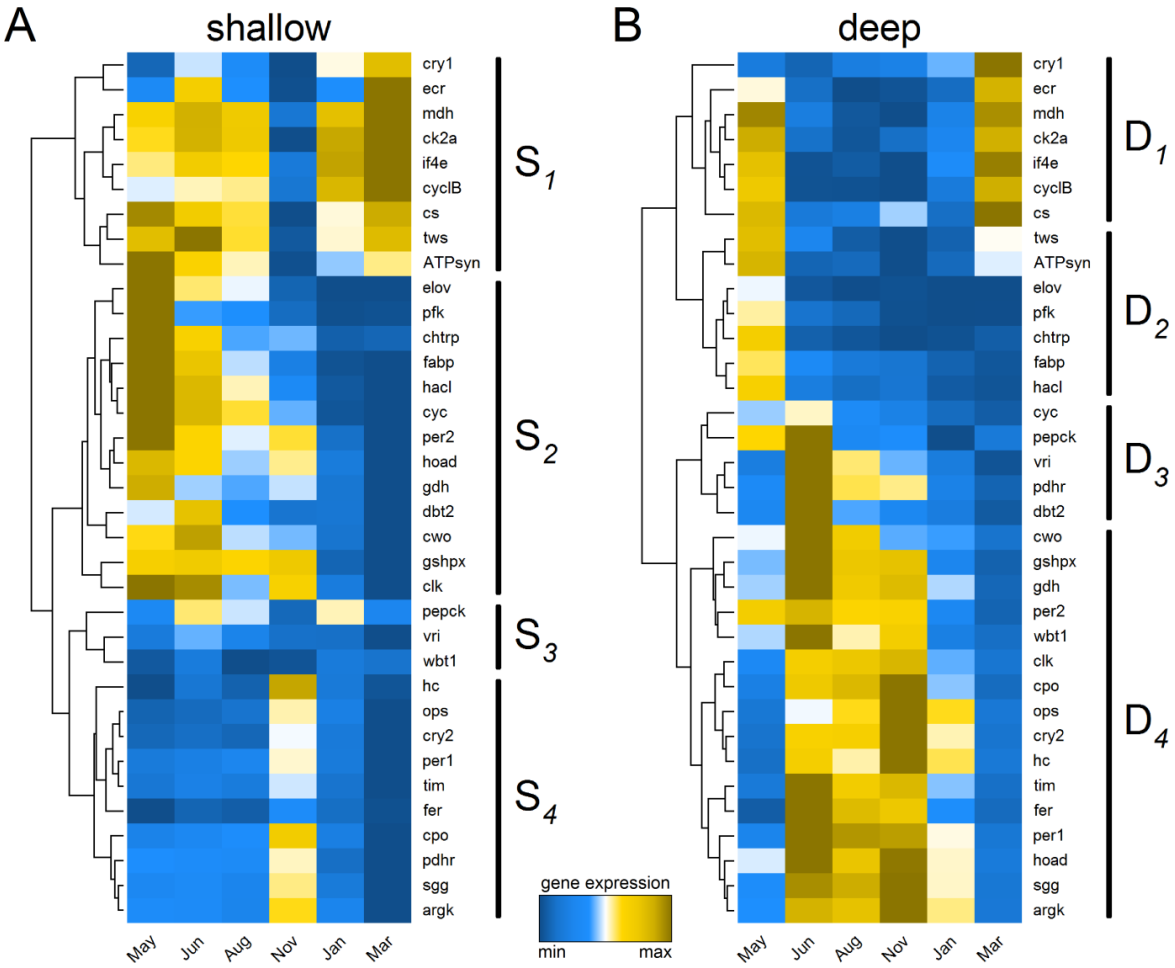

Figure 3.7: Seasonal gene expression patterns. (A) Shallow and (B) deep layer gene expression heatmaps and clusters are shown. Gene clusters were defined based on patterns similarities. Gene expression of both depth layers was normalized individually for each gene, meaning that relative expression levels are comparable between months and depth layers but not between genes. A detailed overview of expression differences between months and depths layers is given in Tab.A.2. Graphic was created with R.

Diel clock gene expression

The clock genes *clk*, *per1*, *tim*, *cry2*, and *cwo* showed a similar seasonal pattern. Diel (24h) expression cycling was mostly confined to the shallow layer from May to August (Tab.3.4). In the deep layer, 24h cycling (if present) was almost exclusively found in in May. From May to August, *clk* showed peak activity just after sunrise in May/Jun and around midnight in August (Fig.3.8A). In contrast, the diel expression of *per1*, *tim*, and *cwo* typically peaked around sunset (Fig.3.8B,C,E). The diel cycle of *cry2* was variable with peak expression occurring around sunrise in May/June but in the middle of the night in August (Fig.3.8D). The clock genes *cyc*, *per2*, and *vri* showed generally little 24h cycling and no consistent diel patterns over the seasonal cycle (graphs not shown).

Table 3.4: Diel cycling in clock gene expression. *p*-values of "RAIN" rhythm analysis are shown with *p*-values indicating significant 24h cycling ($p < 0.001$) printed bolt. For the sake of clarity, *p*-values > 0.05 are not shown (–).

		Rhythm analysis (24h, α = 0.001)					
gene	**depth**	**May**	**Jun**	**Aug**	**Nov**	**Jan**	**Mar**
clock (*clk*)	shallow	**<0.001**	**<0.001**	0.016	–	–	–
	deep	–	–	–	–	–	–
cycle (*cyc*)	shallow	–	0.023	–	–	–	–
	deep	–	**<0.001**	–	–	–	–
period1 (*per1*)	shallow	**<0.001**	0.005	**<0.001**	–	–	–
	deep	0.027	0.014	–	–	–	–
period2 (*per2*)	shallow	–	–	–	–	–	–
	deep	0.005	–	–	–	–	–
timeless (*tim*)	shallow	**<0.001**	0.023	**<0.001**	–	–	0.050
	deep	**<0.001**	–	–	–	–	–
cryptochrome2 (*cry2*)	shallow	**<0.001**	0.007	**<0.001**	–	0.005	–
	deep	–	–	–	–	–	–
clockwork orange (*cwo*)	shallow	**<0.001**	**<0.001**	**<0.001**	–	–	0.005
	deep	**<0.001**	–	0.004	–	–	–
vrille (*vri*)	shallow	–	–	0.002	**<0.001**	–	–
	deep	–	0.018	–	–	–	–

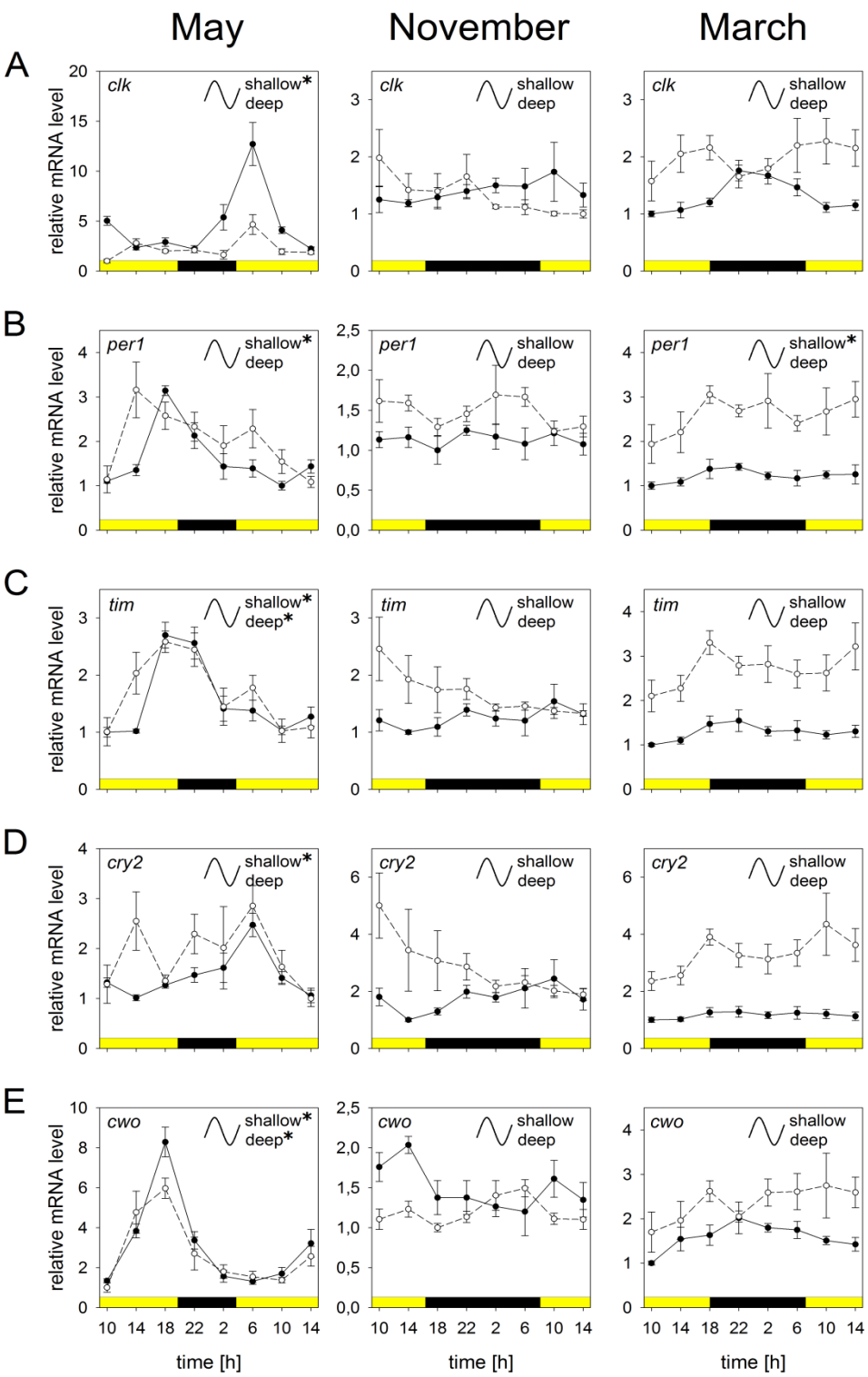

Figure 3.8: Diel clock gene expression patterns. Relative mRNA levels of (A) *clock*/*clk*, (B), *period1*/*per1*, (C) *timeless*/*tim*, (D) *clockwork orange*/*cwo*, and (E) *cryptochrome2*/*cry2* are shown for the months May, November and March in the shallow (solid symbols and line) and in the deep layer (open symbols, dashed line). Color bars indicate day/night. For each gene, depth layer, and diel time point n = 5 replicates were measured. Mean values ± standard error (SE) are shown. Gene expression was normalized individually for each month, meaning that relative expression levels are comparable between depth layers but not between months or genes. All genes were checked for rhythmic gene activity using the R-Package "RAIN" and significant 24h cycling is indicated by asterisks (*). Information about the cycling of other clock genes and months is presented in Table 3.4.

3.5 Discussion

This study has generated one of the most comprehensive investigations of *C. finmarchicus*' life cycles to date. The multi-level approach revealed seasonal rhythms of DVM, population dynamics, physiology, gene expression as well as clock rhythmicity. Initial discussion will focus on seasonal changes in DVM followed by a detailed characterization of the three distinct phases in the life cycle of *C. finmarchicus*: (i) activity/development (shallow layer, May-October), (ii) diapause (deep layer, June-November), and (iii) emergence (November-March). All phases show specific patterns of physiology, gene expression and clock rhythmicity (Fig.3.9). Finally, observed patterns of circadian clock rhythmicity and lipid content will be discussed in relation to photoperiod and possible regulatory mechanisms of diapause initiation/termination.

Diel vertical migration

DVM showed 24h rhythmicity throughout the study and the times of zooplankton ascent and descent in Loch Etive coincided with sunset and sunrise and shifted over the seasons according to photoperiod (Fig.3.3, Tab.3.1), with shoaling of DVM in autumn/winter. This supports the general assumption that DVM is synchronized by light. The observed patterns were primarily attributed to *C. finmarchicus*, the dominant zooplankton species in Loch Etive (Mauchline 1987, Hill 2009) and in our net catches. However, in autumn/winter, while the majority of *C. finmarchicus* were diapausing in the deep layer (Fig.3.4), other zooplankton species like the large predatory copepod *Paraeuchaeta norvegica* or the chaetognath *Parasagitta elegans* might have contributed to the DVM pattern, observed at this time of the year (Mauchline 1987). Light intensity in Loch Etive decreased rapidly with depth due to the river input of humic compounds (Wood et al. 1973) and PAR data suggests that at any seasonal time point light was undetectable for *C. finmarchicus*, and probably most zooplankton organisms, below 20 m depth (Båtnes et al. 2013, Miljeteig et al. 2014, Cohen et al. 2015). Daytime backscatter was strongest at 40-60 m depth, suggesting that the ascent to the surface at sunset was not necessarily triggered by light, but by internal cues like hunger or endogenous clock systems. In March, a pronounced cline due to freshwater runoff (Fig.3.2B-D) prevented zooplankton from ascending above 30 m depth (Fig.3.3E), meaning that they spend the entire 24h cycle in constant darkness and still showed synchronized DVM. This strongly suggests an endogenous regulation of zooplankton DVM (Enright & Hamner 1967, Cohen & Forward Jr 2005, Häfker et al. 2017).

The 12.4h backscatter cycle observed in the shallow water (<30 m) is reflective of the tidal flushing over the second sill into the upper basin of Loch Etive. In addition an overt

semi-lunar cycle (~14.7 days) is seen in both the acoustic backscatter and water temperature data representative of the effects of the spring/neap tidal cycle (Fig.3.2A & 3.3A), from May to September. It remains unclear whether these backscatter cycles were a result of zooplankton advection, a direct behavioral response to tidal currents or were affected by underlying circatidal/circasemilunar clock mechanisms, as described for several marine species (Tessmar-Raible et al. 2011).

Seasonal life cycle of *C. finmarchicus*

Activity and development phase

Spring and summer in the shallow layer were characterized by *C. finmarchicus* early copepodid stages, high metabolic activity and diel rhythmicity. The first generation (G_1) sampled in May/June were the offspring of the overwintering stock. One fraction of the G_1 generation descended to diapause whereas the other fraction matured and spawned a second generation (G_2), which was sampled in August. The presence of a second generation was underlined by a decrease in body length, dry weight and lipid content of the shallow layer copepodid stages from June to August (Fig.3.5). This finding was surprising as previous investigations in Loch Etive reported only one generation per year (Hill 2009) and may be liked to nutrient enrichment from increasing fin-fish aquaculture in the loch in the last few years. The descent of the G_1 and the G_2 generation to diapause occurred around June and October, respectively. While the descent of the G_1 generation is mostly reflected in the abundance data from the shallow and deep layers in June/August (Fig.3.4), the descent of the G_2 generation becomes evident from the backscatter increase at depth in October (Fig.3.3A, 90 m). *C. finmarchicus* abundance in net samples collected in the shallow and deep layer for physiological and genetic analyses support the suggestion that a major relocation of biomass (i.e. of the G_1 generation) to the deep layer already occurred in June. According to seasonal migration data of *C. finmarchicus*' congener *C. glacialis*, the descent of the G_2 generation in October seems to be associated with an increase in water temperature (Kosobokova 1999, Niehoff & Hirche 2005) (Fig.3.2A).

From May to August, gene expression patterns indicate high metabolic activity and an active development of the copepods in the shallow layer (Fig.3.7A S_1-S_4). In the S_1 cluster high expression was found in genes associated with metabolic activity like *mdh* or *cs* (Meyer et al. 2002, 2010, Freese et al. 2017), translation activity (*initiation factor 4E*, *if4E*), molting (*ecr*) and tissue development (*cyclB*). The S_2 gene cluster showed high expression during the phase of activity and development and especially in May, i.e. the time when the phytoplankton concentration peaked (Fig.3.2E). Many S_2 genes are

involved in feeding-related processes such as digestion (*chtrp*), the channeling of carbohydrates (*pfk*) and nitrogen compounds (*glutamate dehydrogenase*, *gdh*) into the citric acid cycle as well as in the synthesis of lipid reserves (*elov*, *fatty acid-bind protein* (*fabp*)). High expression levels were further found in *hydroxyacyl-CoA lyase* (*hacl*), an enzyme which catabolizes the α-oxidation of consumed fatty acids in the peroxisomes (Casteels et al. 2007). It thus seems that expression of several S_2 genes can be affected by ambient food conditions. Genes in the S_3 and S_4 cluster showed low expression levels from May to August, suggesting that they play a minor role during the phase of activity and development. The clock genes *per1*, *tim*, and *cry2* were also found in the S_4 cluster.

The strong diel cycling of clock genes in the shallow layer from May to August (Fig.3.8, Tab.3.4) indicates a functioning circadian clock coordinating diel rhythms in behavior (e.g. DVM) and physiology. The diel expression of the clock genes mostly resembles the patterns previously reported for *C. finmarchicus* (Häfker et al. 2017) and other arthropod species (Richier et al. 2008, Merlin et al. 2009) (Fig.3.8). No coincidence was seen in the peak expression of clocks genes and the seasonal change in times of sunrise/sunset suggesting that photoperiod measurement may be achieved via an “external coincidence” model (Bünning 1960, Pittendrigh 1960). This proposes a photosensitive phase around sunset or sunrise associated with peak activity of one or more clock genes. While 24h cycling was only found in clock genes but not in any other genes, it is probable that the clock also controls diel physiological rhythms such as respiration (Häfker et al. 2017) or enzyme activity associated with nocturnal feeding (Båmstedt 1988). Circadian control by the clock does not necessarily result in the diel expression of metabolic genes, but can also affect a smaller number of downstream genes that then trigger cellular signal cascades regulating metabolic processes (Ceriani et al. 2002, Panda et al. 2002). These cascades may influence protein synthesis or enzyme activity (Panda et al. 2002, Reddy & Rey 2014, Thurley et al. 2017) explaining the lack of diel cycles in metabolic gene expression. Interestingly, several clock genes (*clk*, *cyc*, *per2*, *cwo*) grouped in the S_2 cluster showed a clear change in expression level from May to August corresponding to the change in phytoplankton availability. However, diel clock rhythmicity was not affected by these seasonal changes (Tab.3.4), suggesting that the clock can maintain a robust 24h rhythm even when environmental conditions and overall clock gene expression levels change.

Diapause phase

C. finmarchicus typically diapauses at the CV stage (Marshall & Orr 1955, Hirche 1996a), which dominated the deep layer from June to March. Some copepods of the G_1 generation already descended to diapause in May as indicated by the presence of lipid

rich CV stages in the deep layer at this time. The descent of the G_2 generation in October was accompanied by a decrease in deep layer average CV body length and dry weight (Fig.3.5A,B).

According to gene expression levels in May and June, the physiological “switch” to diapause took place after the descent to the deep layer, confirming the findings of previous studies on *Calanus* (Head & Harris 1985, Freese et al. 2017). Diapausing copepods were characterized by high expression of genes in the D_3 and D_4 clusters, suggesting that the diapause phase lasted from June until after November (Fig.3.7B). The low expressed genes in the D_1 and D_2 clusters were related to metabolic activity, development and food processing (e.g. *mdh*, *cyclB*, *chtrp*) as well as molting (*ecr*), and lipid synthesis (*elov*, *fabp*) (Fig.3.7B), which is in line with previous findings in diapausing *C. finmarchicus* (Tarrant et al. 2008, Aruda et al. 2011, Clark et al. 2013).

Genes in the D_3 cluster showed highest expression in June, suggesting an association with the initiation of diapause (e.g. *pepck*, *pdhr*). High expression of *pepck*, the rate-limiting enzyme of gluconeogenesis, suggests that during the transition to diapause, copepods accumulated glycogen storages that were thereafter slowly converted to wax ester lipids. This may explain why the lipid content of deep layer CVs increased from May to August despite low phytoplankton concentrations (Fig.3.5D). The gene *pdhr* is involved in reducing the sensitivity to light (Strauss & Dircksen 2010). The high *pdhr* expression in June might be associated to the transition to diapause as light can trigger arousal in *Calanus* (Miller et al. 1991, Morata & Søreide 2013). The exact role of D_3 cluster genes in diapause initiation remains unknown and warrants closer investigation.

The D_4 cluster contained several genes typically associated with overwintering such as *cpo*, which is linked to diapause in insects (Christie et al. 2013b, Salminen et al. 2015) or *hoad*, which is a key enzyme of lipid catabolism (Hassett et al. 2010). Other upregulated genes are involved in blood oxygen supply (*hc*), anaerobic energy production (*argk*), or in the protection from oxidative stress (*gshpx*, *fer*). Elevated expression of *fer* in deep diapausing *C. finmarchicus* has already been described (Tarrant et al. 2008, Aruda et al. 2011) and is intuitive considering that hypoxia can cause oxidative stress (Chandel et al. 2000). However, while the oxygen concentration was constantly low in the deep layer of Loch Etive (Fig.3.2D), expression in the D_4 cluster was only elevated from June to November/January, suggesting that the observed changes were directly associated with diapause and possibly a response to the reduced oxygen and ATP demands due to metabolic reduction (Ingvarsdóttir et al. 1999).

Considering the metabolic reduction during diapause (Ingvarsdóttir et al. 1999), it is surprising that the major decrease in the lipid content and C/N ratio of deep layer CVs happened between August and November, possibly attributable to the descent of the G_2 generation. The second generation descended in October, therefore spending less time in diapause than the G_1 generation and thus needing fewer lipids to survive. The difference in lipid content between G_1 and G_2 generation would explain the high variability in lipid levels of *C. finmarchicus* CVs sampled in November as well as the reduction in the deep layer C/N ratio (Fig.3.5C,D). Further explanation for the loss of lipids in these animals could be the higher metabolic rates associated with the warm temperatures in Loch Etive compared to the North Atlantic (Ingvarsdóttir et al. 1999, Maps et al. 2014).

Rhythmic diel clock gene expression was lost with the physiological transition to diapause (Fig.3.8, Tab.3.4), but was present in the deep layer in May, before the physiological transition to diapause. This suggests that the rhythmic output of the clock was actively "switched off" during diapause and that arrhythmicity was not just a consequence of the lack of light at depth. As gene expression was determined from pooled individuals, the possibility that individual clocks remained rhythmic during diapause cannot be discounted. Although clock genes showed no diel cycling during diapause (Fig.3.8, Tab.3.4), several clock and clock-associated genes showed seasonal patterns of elevated expression during diapause from June to November (Fig.3.7B). The reasons for these apparently contradictive results are unclear.

Emergence phase

Gene expression (Fig.3.7) and increasing overall RNA levels (Fig.3.6) indicate that emergence from diapause occurred after the sampling in November and continued until the end of the study in March. The expression of genes associated with metabolic activity and developmental processes increased in the S_1 and D_1 clusters, whereas the expression of genes related to diapause in the D_3 and D_4 clusters decreased. There was no increase in the expression of food-associated genes in the S_2 and D_2 clusters, as phytoplankton levels were still low in March.

Despite the upregulation of genes related to developmental processes, lipid levels stayed constant from November to March (Fig.3.5D). A large fraction of storage lipids is used for gonad development (Rey-Rassat et al. 2002). This energetically costly and time-consuming process starts several months before the ascent to the surface (Tande 1982, Hirche 1996b, Jónasdóttir 1999). However, the major part of gonad maturation takes place during and after the molt to the adult stage (Tande 1982, Rey-Rassat et al. 2002), explaining why lipid content and C/N ratio of CV stages showed little change during

emergence (Fig.3.5C,D). The variance in the expression of individual genes stayed consistently low during the emergence phase (Fig.A2.1), suggesting that the copepods were all in the same physiological state. This supports the hypothesis that emergence was synchronized within the population, with all copepods resuming development and ascending to surface waters synchronously, even though the G_1 and G_2 generations entered diapause with a delay of 3-4 months (Miller et al. 1991, Tittensor et al. 2003, Speirs et al. 2005, Baumgartner & Tarrant 2017).

Diel clock gene expression remained arrhythmic during diapause emergence of the CV stage (Fig.3.8, Tab.3.4). The ascent to surface waters take place after the molt to the adult stage (Miller et al. 1991, Hirche 1996a), implying that the clock will be "switched on" in the adult stage after the final molt. CV stages present in the shallow layer in November/January showed no clock gene cycling although light levels should have been sufficient to entrain the clock (Tab.3.4). The lack of clock cycling and similarities in seasonal clock gene expression patterns in the shallow and the deep layer from November to March suggest that these shallow layer CVs were not remnants of the active surface population, but rather diapausing copepods that had been advected to the surface (Fig.3.8). An active ascent in late autumn offers no ecological advantage and thus a passive transport from the deep layer seems most likely (Durbin et al. 1997) and is plausible as there was a partial exchange of Loch Etive deep water at this time. This exchange was supported by wind-forced mixing by the storm "Barney" during the time of the November sampling. Furthermore, the associated temperature increase below 60 m from October to December as a consequences of the partial overturning of the loch (Fig.3.2A,B), could have reduced the density of storage wax esters and hence increased copepod buoyancy (Visser & Jónasdóttir 1999, Pond & Tarling 2011, Pond et al. 2012). The fact that the exposure to light at the surface did not "re-activate" clock gene cycling underpins the suggestion that the circadian clock is actively "switched off" in diapausing CV stages (Tab.3.4).

Mechanisms of diapause initiation

As highlighted in the introduction, the regulation and timing of diapause is still poorly understood and hence subject to debate (Baumgartner & Tarrant 2017). Currently, the most accepted hypothesis for the initiation of diapause is the "lipid accumulation window" (LAW) hypothesis (Rey-Rassat et al. 2002). The concept assumes a time window during which the copepods accumulate lipids towards a certain threshold representing the minimum amount needed to survive the time of diapause. In this context, information about lipid status is possibly mediated via lipid-derived hormones (Irigoien 2004, Pond et al. 2012). The copepods that reach the "lipid-threshold" within the time window enter

diapause while the others molt to adults and reproduce (Rey-Rassat et al. 2002) or may enter diapause with insufficient lipid reserves, leading to an early depletion and emergence (Maps et al. 2014). However, our seasonal lipid data is not supportive of this nor has it been possible to initiate diapause in the laboratory when copepods were fed *ad libitum* (Baumgartner & Tarrant 2017). The continuous increase in lipid content in the deep layer CV copepods from May to August and the evidence that G_1 and G_2 generations entered diapause with different amounts of lipids raise doubts about the exclusive role of a certain lipid threshold in triggering diapause. In addition, the inability to accumulate sufficient lipid reserves indicates poor food conditions. If copepods then mature and reproduce under these conditions, their offspring would also have to face the same problem, potentially resulting in low survival rates (Søreide et al. 2010). In Loch Etive, this would affect the copepods in the shallow layer in November/January. However, gene expression data and clock rhythmicity imply that physiologically the animals had been, or were still, in diapause (Fig.3.7 & 3.8, Tab.3.4). Further, the considerable amount of lipids found in shallow and deep layer copepods in November/January (Fig.3.5D) argue against an early emergence due to insufficient lipid reserves.

An alternative hypothesis for the initiation of diapause assumes that the descent to diapause depth is related to low food concentrations (Hind et al. 2000). However, in Loch Etive, all copepods of the G_1 generation experienced the same food conditions, not explaining why one fraction of the generation entered diapause while the others matured and produced a second generation.

In various insects and several copepods the timing of diapause is controlled via photoperiod measurement by the circadian clock. In Loch Etive, the diel cycling of clock genes during the phase of activity and development shows the potential for photoperiod measurement in *C. finmarchicus* (Tab.3.4). In insects and other copepods diapause is mostly initiated in response to short photoperiods (Watson & Smallman 1971, Hairston & Kearns 1995, Meuti & Denlinger 2013, Salminen et al. 2015). In Loch Etive, the G_1 generation entered diapause in June implying an extremely long critical photoperiod close to 18h (Tab.3.1). Although such long critical photoperiod have been reported (Hut et al. 2013), it seems unlikely that diapause initiation of *C. finmarchicus* in Loch Etive is based exclusively on photoperiod.

The triggers of diapause initiation in *C. finmarchicus* are still unknown, but probably involve the interaction of multiple internal and external factors (Miller et al. 1991, Johnson et al. 2008). Sufficient lipid reserves are crucial for surviving diapause, so that a critical minimum threshold of lipids is required before entering diapause (Rey-Rassat et al.

2002). However, photoperiod is a more reliable cue for the timing of the seasonal cycle that can trigger diapause in insects with high seasonal precision (Goto 2013, Meuti & Denlinger 2013). Hence, by the time a critical lipid threshold is reached, photoperiod might determine whether copepods enter diapause or mature to produce another generation. Further, temperature can modulate photoperiod-dependent diapause initiation in copepods (Watson & Smallman 1971, Marcus 1982, Hairston & Kearns 1995). In spring/summer surface waters gradually warm and high temperatures might increase the likelihood of *C. finmarchicus* diapause initiation at a given photoperiod. It is however also possible that the evasion of surface waters into deeper layers is a direct response to unfavorable temperature conditions (Kosobokova 1999, Niehoff & Hirche 2005).

Diapause triggers in *C. finmarchicus* might already be determined in an earlier life stage (Johnson et al. 2008). In the freshwater cyclopoid copepod *Diacyclops navus* for example, the photoperiod experienced during the CI stage determines if diapause is initiated in the CIV stage (Watson & Smallman 1971).

The existence of different clock gene alleles could explain the large differences in diapause timing between geographically distinct *C. finmarchicus* populations and also between generations within the same population (Durbin et al. 2000, Walkusz et al. 2009, Melle et al. 2014). Marcus (1984) found different critical photoperiods in geographically separated populations of the same copepod species and there are reports of different *tim*-alleles in *Drosophila* that modulate the diapause response to a given photoperiod (Sandrelli et al. 2007). The presence of different clock gene alleles among *C. finmarchicus* populations could lead to differences in the critical photoperiod, meaning that depending on the present alleles a given day length can initiate diapause in one population while copepods elsewhere mature and reproduce. The strong selective pressure on accurate diapause timing should lead to allele frequency differences among populations even if some genetic exchange exists between them. Different alleles within a population would further explain why one fraction enters diapause while another fraction matures to produce a second generation (e.g. Durbin et al. 2000, Tarrant et al. 2008, this study). If both generations overwinter successfully, the respective alleles should be able to coexist within the population. Such genetically determined phenological diversity may be identified by comparing allele frequencies of genes involved in the clock and in light perception between different populations or generations of the same population. Generally, if diapause timing in *C. finmarchicus* or other ecological key species is to some extend determined genetically, this raises concerns about the adaptability to environmental changes like a shift in the seasonal timing of the phytoplankton bloom (Søreide et al. 2010, Leu et al. 2011).

Mechanisms of diapause termination

In the open ocean *C. finmarchicus* diapauses at depths between 400 and 1000 m (Hirche 1996a) where seasonal changes of environmental parameters are minimal or absent. The potential lack of seasonal cues for triggering emergence has led to the proposal of an hourglass model based on the gradual depletion of wax esters (Jónasdóttir 1999, Saumweber & Durbin 2006) mediated by lipid-derived hormones (Irigoien 2004, Pond et al. 2012) or based on continuous slow development (Hind et al. 2000). However, our results from Loch Etive contradict this view. Lipid levels in diapausing *C. finmarchicus* CVs were constant and little depleted during the phase of emergence from November to March and the G_1 and G_2 generations descended with different amounts of lipids and a delay of 3-4 months (Fig.3.5D), making an hourglass-based synchronized emergence unlikely.

The repeatedly reported synchronized emergence in *C. finmarchicus* indicates a response to a reliable seasonal cue like photoperiod. However, although *Calanus* is very sensitive to light, PAR data reveal that light levels in the deep layer of Loch Etive were well below the copepods detection limit (Båtnes et al. 2013, Miljeteig et al. 2014), with no rhythmic circadian clock gene expression at this time (Tab.3.4). While a clock-based photoperiod measurement or hourglass timers therefore appear unsuitable for triggering emergence, the observed seasonal rhythmicity could be explained by a circannual clock that creates an endogenous rhythm with a period of ~365 days. Such endogenous annual rhythms have been described in many different species and although their molecular nature remains largely unresolved it is agreed that photoperiod must play a crucial role in the entrainment of such long-range timing mechanisms (Randall et al. 1998, Goldman et al. 2004, Lincoln et al. 2006). For *C. finmarchicus* the photoperiodic entrainment of the circannual clock before descent would ensure synchronized emergence as the G_1 and the G_2 generations experienced different photoperiods and thus their circannual rhythms should be synchronized in spite of them descending at different times. Circannual rhythms can run under constant conditions with astonishing precision over several years (Goldman et al. 2004) and they could also trigger emergence in the open ocean where light or other seasonal cues at diapause depth may be missing. Evidence from studies on other *Calanus* species indicates that circannual timing exists in this genus (Conover 1965, Fulton 1973). A putative circannual clock may share some components with the circadian clock, as several clock genes like *tim*, *per1/2*, and *cry2* were upregulated in diapause and their expression decreased after the onset of emergence, although the animals resided in constant darkness and diel rhythmicity ceased (Fig.3.7B).

It is further noteworthy that the expression of the opsin gene *ops* was highest in the deep layer in November suggesting an increased sensitivity to light (Fig.3.7B). Similarly, the expression of *cry1*, the gene considered responsible for the light entrainment of the circadian clock, increased during emergence. While copepods experienced constant darkness in the deep layer, Hill (2009) reported a gradual shoaling of *C. finmarchicus* diapause depth from January to March in Loch Etive, i.e. after the onset of emergence. Similar reports also exist for *Calanus* in the open ocean (Hirche 1997, Bandara et al. 2016). This was suggested to represent an ascent to depth where photoperiod measurement becomes possible thus aiding the synchronization of the emergence process within the population (Speirs et al. 2005). A rhythmic circadian clock may not be essential for this as photoperiod measurement may also be achieved without it (Emerson et al. 2009, Goto 2013). Although the exact regulatory mechanisms are still unclear, the combined insight from literature and our own results suggest that photoperiod – either directly or as an entrainment cue for a circannual clock – plays an important role in *C. finmarchicus* emergence from diapause.

Conclusions

Our results describe the seasonal life cycle of *C. finmarchicus* consisting of a phase of activity and development in surface waters from May to October, diapause in deep waters from June to November, and a phase of emergence from November to March ending with the ascent to the surface (Fig.3.9). Two generations of copepods were produced in the surface waters and descended to diapause in June and October, respectively. All phases of the seasonal cycle were characterized by distinct patterns of gene expression with several genes (e.g. *elov*, *fer*, *tim*, *ecr* or *cyclB*) being potential proxies for the genetic determination of diapause status. While a circadian clock regulated 24h cycles in copepods actively developing in surface waters, diel clock gene cycling was absent in diapause and during emergence, although many clock genes showed highest expression during diapause.

Seasonal patterns of physiology and gene expression in *C. finmarchicus* exclude lipid content and food as exclusive cues for the initiation or termination of diapause. Similarly, photoperiod alone cannot explain the observed seasonal diapause pattern. It is likely that the interactions of multiple external (photoperiod, temperature, food concentration) and internal (circadian clock, lipid content, hormones) factors determine whether the copepods enter diapause or mature to produce another generation. The synchronized emergence from diapause in spite of a lack of light at depth suggests a control mechanism involving an endogenous circannual clock entrained via photoperiod measurement before the descent to diapause depth (Fig.3.9).

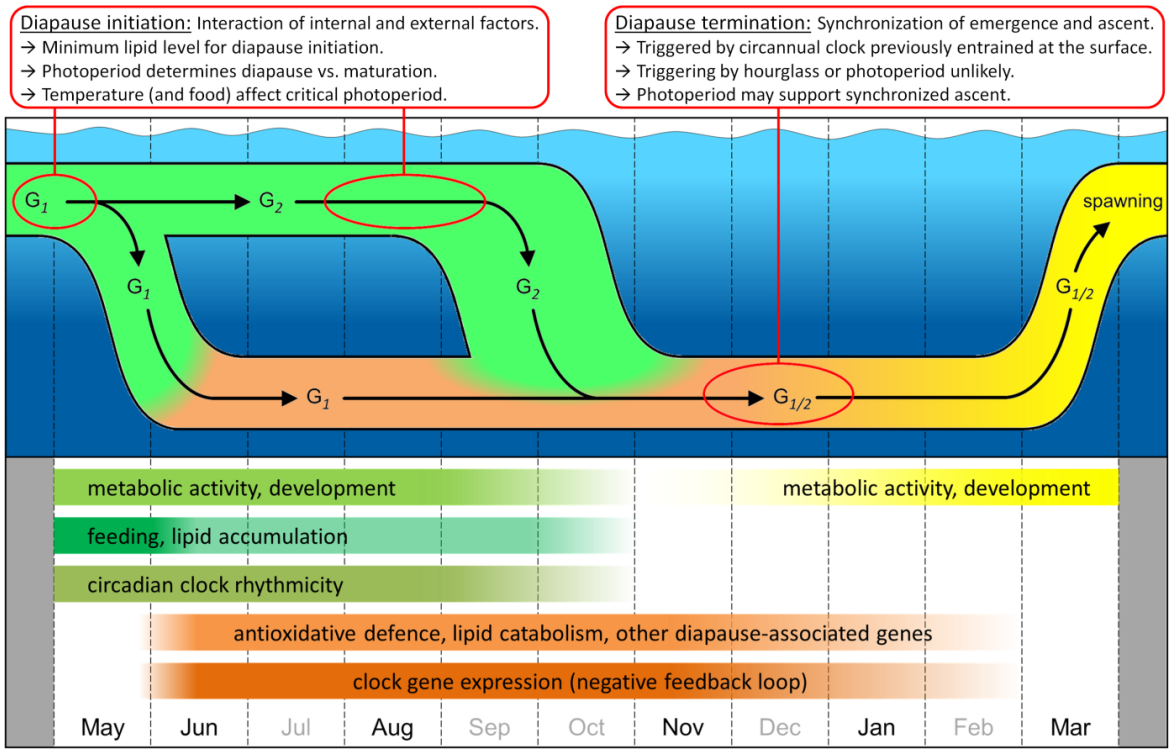

Figure 3.9: *C. finmarchicus*' seasonal life cycle in Loch Etive. A schematic depiction of population dynamics, physiological changes, and potential diapause regulation mechanisms is shown. The spawning of the overwintering stock happened in April 2015 before the start of our study and is not shown. Colors indicate different life phases as well as the metabolic processes and gene groups characterizing the respective phases (green = activity/development, orange = diapause, yellow = emergence/ascent). Months printed gray were not sampled. Graphic was created with MS Powerpoint.

However, a mechanistic understanding of the factors controlling diapause remains elusive and can only now be gained from laboratory experiments (Baumgartner & Tarrant 2017). Considering our findings, combining the manipulation of external factors with the detailed monitoring of physiological and genetic parameters, could shed new light on the mechanisms of diapause regulation in *C. finmarchicus*.

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4 Publication III

Adaptive capacity of *Calanus finmarchicus* diel and seasonal rhythmicity in relation to circadian clock functioning in extreme polar photoperiods

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4.1 Abstract

Climate change and increasing temperatures cause poleward distribution shifts in many marine organisms including the northern Atlantic key zooplankton species *Calanus finmarchicus*. The copepod shows diel cycles of vertical migration and feeding, a seasonal life cycle with diapause in winter and it possesses a functioning circadian clock. Endogenous clock mechanisms control various aspects of rhythmic life and are heavily influenced by environmental light conditions. Here we explore how the extreme seasonal change in photoperiod (day length) in a high Arctic fjord affect circadian clock functioning as well as diel and seasonal cycles in *C. finmarchicus*. Clock gene expression measured in the active life phase at the end of midnight sun, in early diapause when photoperiod was ~12h and in late diapause during the polar night show that while the clock maintains diel rhythmicity under extremely long photoperiods, it becomes arrhythmic during diapause, probably not because of a lack of light, but in association with the physiological changes in diapause. Seasonal expression analyses of 35 genes show distinct patterns for each investigated life phase. *C. finmarchicus* is able to maintain diel rhythmicity under extreme polar photoperiods and marine species may be generally less affected by extreme photoperiods than terrestrial ones due to the attenuation of light with depth and sea-ice cover. However, seasonal life cycle timing by clock-based photoperiod measurement could lead to temporal mismatches that limit the distribution or reduce the fitness of species not adapted to polar photoperiods.

4.2 Introduction

The copepod *Calanus finmarchicus* is a key zooplankton species in the northern Atlantic food web that provides a crucial trophic between primary production and commercially important fish stocks (Runge 1988, Sundby 2000, Prokopchuk & Sentyabov 2006). The species' inhabits a large latitudinal range from ~40°N up to 80°N and often dominates zooplankton biomass (Conover 1988, Kwasniewski et al. 2003, Helaouët & Beaugrand 2007). *C. finmarchicus* performs diel vertical migration (DVM), meaning that the copepods migrate to surface waters at night to feed on phytoplankton and retreat to deeper water layers during the day to hide from visual predators (Dale & Kaartvedt 2000). *C. finmarchicus* further shows diel cycles of feeding as well as respiration and enzyme activity (Båmstedt 1988). The copepods seasonal life cycle is characterized by distinct physiological phases starting with the spawning and active development in surface waters in spring/summer when phytoplankton is abundant (Hirche 1996a, Baumgartner & Tarrant 2017). During this time the animals develop through five larval stages as well as five juvenile copepodid stages (CI-CV) and accumulate a considerable amount of energy rich lipids (Marshall & Orr 1955, Falk-Petersen et al. 2009, Clark et al. 2013). The lipid-rich CV stages migrate into deeper water layers where they enter diapause, a phase of overall reduced activity and fasting that lasts until the forthcoming spring (Hirche 1996a, Ingvarsdóttir et al. 1999). While *C. finmarchicus* CVs at lower latitudes may molt directly into adults and produce up to three generations per year before entering diapause (Durbin et al. 2000), at higher latitudes the species spawns only one generation per year that then enters diapause in the CV stage (Walkusz et al. 2009, Melle et al. 2014). The emergence from diapause starts in winter at depth, indicated by increasing metabolic activity and the development of reproductive tissues (Hirche 1996b, Ingvarsdóttir et al. 1999). Ultimately, the copepods molt to adults and ascent to surface waters to spawn a new generation that can then benefit from the spring phytoplankton bloom (Hirche 1996a, Baumgartner & Tarrant 2017). Although both, the seasonal cycle of *C. finmarchicus* as well as its diel cycle of activity and migration have been described in great detail, the mechanisms controlling diel and seasonal rhythmicity are still poorly understood (Baumgartner & Tarrant 2017, Häfker et al. 2017).

A potent molecular tool for the regulation of diel and seasonal cycles is the circadian clock, which generates an endogenous rhythms of ~24h length (latin: "*circa dies*": about a day), thus enabling organism to anticipate the rhythmic changes of the day/night cycle. The molecular machinery of circadian clocks is based on so-called clock genes and their protein products that interact with each other in feedback loops with the genes ultimately inhibiting their own transcription (Mackey 2007). The expression patterns of most clock

genes show diel rhythms with peak levels often being associated with sunset or sunrise (Meireles-Filho & Kyriacou 2013). Several clock-associated genes regulate and delay the accumulation of the clock proteins as well as their import into the nucleus, resulting in a cycle period of ~24h (Allada & Chung 2010). Although this cycle persists under constant darkness, the clock is not perfectly precise and needs to be entrained (synchronized) by external cues on a regular basis to stay in tune with the environment. Light is by far the most common cue used for this entrainment (Aschoff 1954). Circadian clocks affect organisms on various organizational levels reaching from diel rhythms of gene expression and enzyme activity to the control of the sleep/wake cycle to the regulation of DVM in fish and zooplankton including *C. finmarchicus* (Brierley 2014, Dunlap & Loros 2016, Häfker et al. 2017).

Another crucial feature of circadian clocks is the ability to measure photoperiod (day length) and thus to provide information about the seasonal cycle (Hazlerigg & Wagner 2006, Goto 2013). Various arthropods including several copepod species use changes in photoperiod as a cue for the timing of seasonal life cycle events like reproduction or diapause (Watson & Smallman 1971, Marcus & Scheef 2010, Meuti & Denlinger 2013). For *C. finmarchicus*, the involvement of clock-based photoperiod measurement in seasonal timing and diapause has been proposed, but is discussed controversially (Grigg & Bardwell 1982, Miller et al. 1991, Baumgartner & Tarrant 2017).

Circadian clocks have been explored to great detail in terrestrial model species like *Drosophila* and mice, but investigations of marine clock systems are still scarce (Tessmar-Raible et al. 2011, Zantke et al. 2013, Zhang et al. 2013). This is particularly true for high latitude systems with the most poleward study on marine clocks conducted at 65°S (Teschke et al. 2011). However, the polar regions pose a special challenge to circadian clocks due the extreme seasonal changes in photoperiod reaching from permanent light in summer (midnight sun) to permanent darkness in winter (polar night). There are several studies reporting diel rhythms in marine organism during midnight sun or the polar night (Dale & Kaartvedt 2000, Berge et al. 2009, Last et al. 2016, Tran et al. 2016), but the mechanistic basis of these rhythms remains completely unknown. Also, the productive period at high latitudes is short, meaning that precise timing of seasonal life cycle events like the emergence from diapause can be crucial for survival and population size (Søreide et al. 2010). In the face of climate change and increasing ocean temperatures *C. finmarchicus* and many other marine species have experienced a poleward distribution shift (Perry et al. 2005, Reygondeau & Beaugrand 2011, Chivers et al. 2017). As a consequence the circadian clock of the originally boreal *C. finmarchicus* is exposed to the more extreme polar light conditions that were shown to affect circadian

clock functioning and rhythmicity in various species (Reierth & Stokkan 1998, Cottier et al. 2006, Lu et al. 2010, Benoit et al. 2010, Beale et al. 2016). This could affect copepod fitness, population size and distribution (Søreide et al. 2010, Saikkonen et al. 2012). Here we investigate how the strong seasonal changes in photoperiod in an Arctic fjord affects the diel rhythmicity as well as the seasonal life cycle of *C. finmarchicus* with respect to gene expression, physiology and behavior and discuss possible consequences of latitudinal distribution shifts for copepod fitness and ecosystem functioning.

4.3 Materials & Methods

Study site characteristics

Kongsfjorden is an Arctic fjord situated in the western part of the Svalbard archipelago (Fig.4.1). Due to its depth of >300 m and the lack of a sill at the fjord entrance can be influenced by the influx of Arctic and Atlantic water masses (Svendsen et al. 2002, Cottier et al. 2005). Data on diel changes in surface photosynthetically active radiation (PAR, measured at 385-695 nm in Ny-Ålesund) were obtained from the database of the Baseline Surface Radiation Network (BSRN) (Fig.4.2).

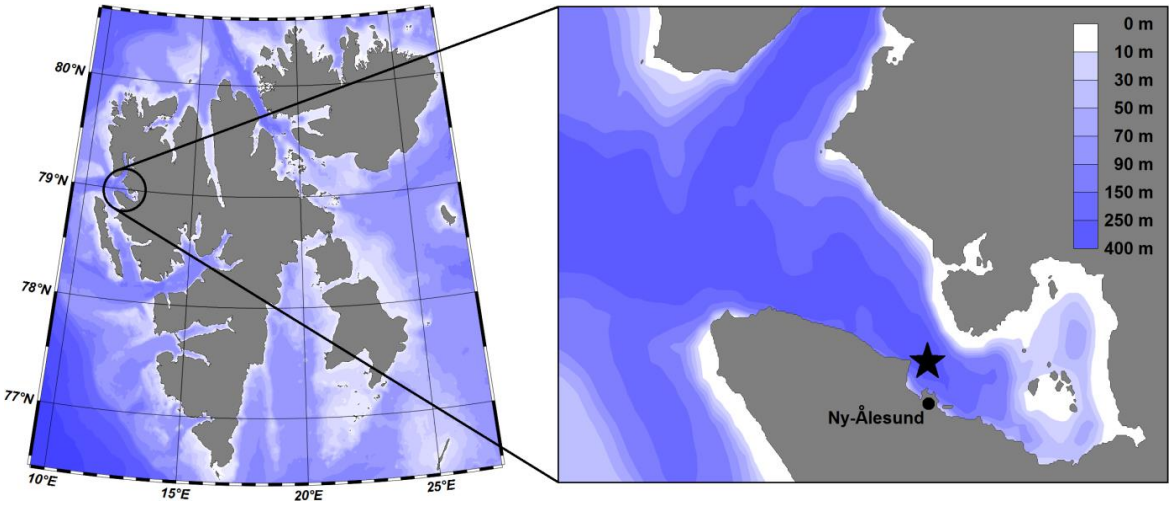

Figure 4.1: Kongsfjorden and the Svalbard archipelago. Samples were collected at the sampling site KB3 (★). Copepods in early and late diapause (September 2014 and January 2015, respectively) were collected and sorted on board of R/V *Helmer Hanssen*, while active copepods (August 2016) were collected on board of R/V *Teisten* and were sorted at the Kings Bay Marine Laboratory in Ny-Ålesund.

Copepod sampling

Calanus finmarchicus CV stages were collected at Kongsfjorden permanent sampling station KB3 (78°57´N, 11°57´E) on board of R/V *Helmer Hanssen* (The Arctic University of Norway, Tromsø) in September 2014, January 2015 and on board of R/V *Teisten* (Kings Bay AS, Ny-Ålesund) in August 2016 (Tab.4.1). In September 2014 and August 2016 a WP3 net (Ø = 1 m, mesh size = 1000 µm) was used whereas in January 2015 a

WP2 net (Ø = 0.5 m, mesh size = 200 µm) was used. Different net types were not expected to have an effect as both types should sample the relatively large *C. finmarchicus* CV stage efficiently (Nichols & Thompson 1991, Kwasniewski et al. 2003). In September 2014 and January 2015 (early and late diapause, respectively), the net was opened ~10 m above the bottom, towed vertically and closed at 200 m depth. Due to drifting of the boat, the bottom depth at the sampling site varied, but was mostly between 340 and 355 m, with 2 exceptions (285 m, 315 m). In August 2016 (active phase) the net was lowered to 150 m, opened, and then towed to the surface. For each life phase, a diel time series was created by collecting samples in 4h intervals over a period of 20-24h. While in January 2015 net failure resulted in the loss of the first diel time point, weather conditions in August 2016 forced an interruption of the sampling resulting in a gap in the diel time series (Tab.4.1).

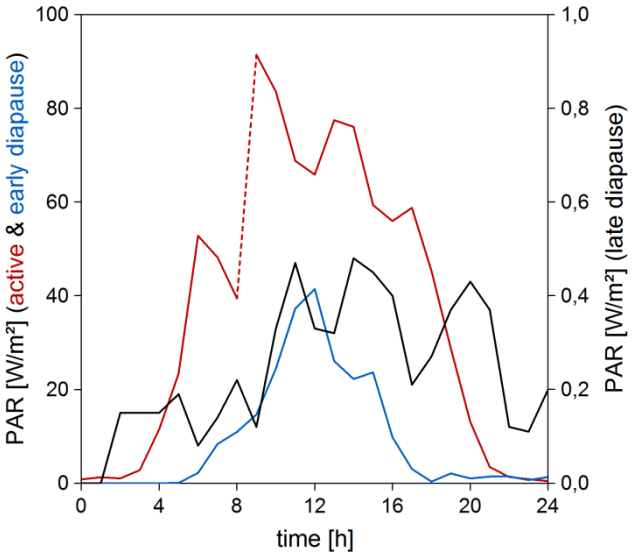

Figure 4.2: Diel change in surface PAR during the samplings. The sampling during the active phase had to interrupted due to bad weather conditions (see also Tab.4.1) and hence light data from 25/08 and 27/08 is shown (gap indicated by dashed line).

After net retrieval, copepods for gene expression analysis were fixed as bulk samples immediately in RNAlater® (Ambion, UK) and later sorted for *C. finmarchicus* CV stages in RNAlater at 4°C using a dissection microscope. Copepods for analyses of lipid content and C/N ratio were collected once at midday for each life phase and were sorted alive using dissecting microscopes either on board (September/January) or at the Kings Bay Marine Laboratory, Ny-Ålesund (August) and were fixed in liquid nitrogen. The exposure to light was minimizes (red light used for sorting) and copepods were kept at *in situ* temperature before and during sorting. To avoid contamination of samples with the congener species *C. glacialis*, samples were sorted based on sizes classes by Kwasniewski et al. (2003) and considering the fact that reddish antenna are indicative of *C. glacialis* (Nielsen et al. 2014). Sorting of copepods for lipid and C/N analyses took max. 1h.

Table 4.1: Life phase samplings in Kongsfjorden. Samplings were performed in September 2014 (early diapause), January 2015 (late diapause) and August 2016 (active phase). Samples for gene expression were collected according to the diel time series. Samplings for dry weight, C/N and lipid analyses were performed at midday for each life phase. *For the early diapause sampling at 08:00, the net closing mechanism failed and the entire water column was sampled.

life phases	diel time series (no° of diel time points)		depth
active phase	25/08 00:00 - 25/08 08:00 + 27/08 12:00 - 28/08 00:00	(3+4)	surface-150 m
early diapause	26/09 00:00 - 27/09 00:00	(7)	200 m-bottom*
late diapause	13/01 04:00 - 14/01 00:00	(6)	200 m-bottom

Gene expression analysis

A total of 35 genes were investigated based on their involvement in different metabolic processes including the circadian clock machinery, energy and lipid metabolism, digestion, stress response and light perception. A detailed gene list is given in Tab.A3.1. Gene sequences were identified from a *C. finmarchicus* transcriptome (Christie et al. 2013a, Lenz et al. 2014) using annotated sequences from other crustaceans. All obtained gene sequences verified and checked for palindromic sequences and repeats as described by Häfker et al. (2017). Gene sequences were used to design custom Taqman® low-density array-cards (https://www.thermofisher.com/order/custom-genomic-products/tools/gene-expression/, Applied Biosystems, USA) and designed primer and probe sequences were checked via blastn against NCBI for gene specificity. NCBI accession numbers of genes identified from the transcriptome as well as Taqman primer and probe sequences can be accessed via PANGAEA (https://doi.pangaea.de/10.1594/PANGAEA.884073).

RNA of *C. finmarchicus* CV stages was extracted using the RNeasy® Mini kit (Quiagen, Netherlands) with 0.14 M β-mercaptoethanol added to the extraction buffer. For each sample, 15 copepods were pooled. Genomic DNA residues were removed with the TURBO DNA-free kit (Life Technologies, USA). Extracted RNA was checked for degradation (2100 Bioanalyzer / RNA 6000 Nano Kit, Agilent Technologies, USA) and was investigated for contaminations and RNA concentration (Nanodrop 2000 Spectrophotometer, Thermo Fisher Scientific, USA). 2 µg RNA per sample were converted to cDNA using RevertAid H Minus Reverse Transcriptase (Invitrogen, Germany). Finally, samples were distributed to Taqman® cards and gene expression was measured via real-time quantitative PCR (ViiATM 7, Applied Biosystems, USA).

Gene expression was normalized according to Livak & Schmittgen (2001) using the $2^{-\Delta\Delta CT}$-method. For investigations of diel expression patterns, each of the diel time series from each of the three life phases (active phase, early diapause, late diapause) was normalized individually against the geometric mean of the housekeeping genes

elongation factor 1 α, *RNA polymerase II* and *actin* (6-7 diel time points per life phase, *n* = 5 replicates per diel time point and gene). For life phase comparisons, each of the three diel times series was pooled, resulting in gene expression data for three life phases (active, early diapause, late diapause) that was normalized against the geometric mean of *elongation factor 1* α and *RNA polymerase II* (3 life phases, *n* = 30-35 replicates per life phase and gene). Housekeeping genes were chosen based on expression stability and the results of previous studies (Hansen et al. 2008, Tarrant et al. 2008, Clark et al. 2013, Fu et al. 2013, Häfker et al. 2017).

Dry weight, C/N ratio and lipid content

For the determination of dry weight and C/N ratio, *n* = 24 individual copepods per life phase were freeze dried in pre-weight tin caps. The dry weight was then measured gravimetrically. C/N ratio was afterward measured via elemental analyzer (EuroEA, EuroVector, Italy).

Lipid samples were measured according to Folch et al. (1957). 20 CV stages were pooled for each sample with *n* = 5 replicates per life phase. Frozen copepods were homogenized in 4 mL chloroform:methanol (2:1 v/v) (T10 ULTRA-TURRAX® disperser, IKA, Germany). The homogenate was then filtered through pre-washed Whatman® N°1 filter (GE Healthcare, UK). 1 mL KCl (0.88% w/v) was added and the homogenate was thoroughly vortexed and centrifuged at 400 g for 2 min to induce phase separation. The upper aqueous phase was discarded and the remaining lower phase was dried under a stream of nitrogen gas. Finally, samples were freeze dried for 6h and total lipid content [µg*indv.$^{-1}$] was determined gravimetrically (XP6U Micro Comparator, Mettler-Toledo, USA).

Zooplankton diel vertical migration

The vertical distribution of zooplankton at Kongsfjorden station KB3 was determined via a mooring equipped with two acoustic Doppler current profilers (ADCP, Teledyne RD Instruments, USA) at 90-100 m depth, one of which facing upward and the other one downward. The set up with 300 kHz ADCPs to monitor vertical zooplankton migrations has be used repeatedly and produced convincing results (Cottier et al. 2006, Berge et al. 2009, Last et al. 2016). Quality of data was checked via the RDI correlation index, which analyses signal to noise ratio. Absolute backscatter volume (Sv, measured in decibel, dB) was recorded and converted to mean volume backscattering strength (Deines 1999). Rhythmicity of DVM (expressed as period length τ and % of model fit) was assessed for 85 m and 180 m depth using TSA-Cosinor© (package 6.3, Expert Soft Technologies, France) according to Bingham et al. (1982). Data for the time of seasonal samplings in September 2014 (early diapause), January 2015 (late diapause) and August 2016

(active) was analyzed over a period of 7 days that included the time of the respective sampling. As the ADCPs were not in place during the sampling in August 2016 due to maintenance, DVM rhythmicity was analyzed for the last 7 days before the ADCPs were retrieved (14.08.16-20.08.16) and for the first 7 days after the deployment of the ADCPs (30.08.16-05.09.16).

Statistics

Statistical analyses were done in RStudio (v. 0.99.442, R Development Core Team 2013). Diel time series of clock gene expression we checked for 24h cycling using the R-package "RAIN" (Thaben & Westermark 2014). A significance level of $\alpha = 0.001$ was used to account for the number of investigated genes (8 clock genes tested). Life phase differences in gene expression, dry weight, lipid content, and C/N ratio were identified via Kruskal-Wallis ANOVAs on Ranks followed by Dunn's Multiple Comparison post-hoc tests provided in the "FSA" R-package. For gene expression analyses $\alpha = 0.0001$ was used (35 genes tested) whereas for dry weight, lipid content and C/N ratio the significance level was $\alpha = 0.05$. Graphs were created via SigmaPlot (v. 12.5).

4.4 Results

Diel clock gene expression

24h cycling of clock gene expression was mostly confined to the active life phase in surface waters in August (Fig.4.3). During the active phase when photoperiod was close to 24 hours, the clock genes *period1* (*per1*), *timeless* (*tim*), *cryptochrome2* (*cry2*) and *clockwork orange* (*cwo*) showed significant 24h cycling. During early diapause in September the photoperiod was close to 12 hours and diel expression cycling at depth below 200 m was confined to the genes *clock* (*clk*) and *period2* (*per2*). In late diapause during the polar night (January) 24h rhythmicity was completely absent. The clock genes *cycle* (*cyc*) and *vrille* (*vri*) did not shown significant cycling in any of the life phases.

In the active phase, cycling clock genes showed maximum expression in the afternoon with peak times reaching from close to noon (*cwo*) to around 16:00 (*per1*, *tim*) to sunset (*cry2*) (Fig.4.3). In early diapause, *clk* and *per2* both showed minimum expression before sunrise, but while *clk* reached maximum expression around noon, expression of *per2* gradually increased and peaked at 20:00 after sunset.

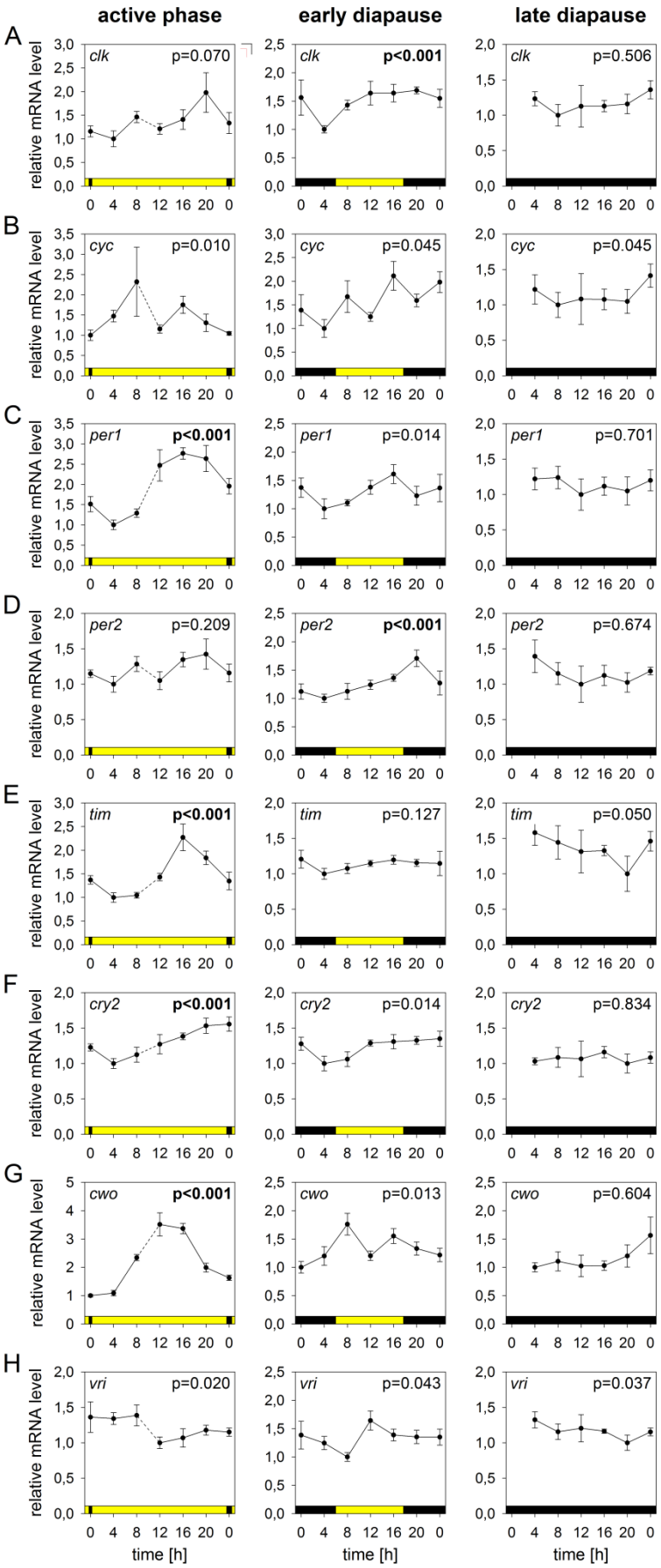

Figure 4.3: Diel clock gene expression. Diel expression changes of the clock genes *clock* (*clk*), *cycle* (*cyc*), *period1* (*per1*), *period2* (*per2*), *timeless* (*tim*), *crytochrome2* (*cry2*), *clockwork orange* (*cwo*) and *vrille* (*vri*) are shown. Color bars indicate day (yellow) and night (black). The sampling during the active phase had to interrupted due to bad weather conditions (see also Tab.4.1) and hence data from 25/08 and 27/08 is shown (gap indicated by dashed line). Genes were investigated for 24h rhythmicity via "RAIN"-analysis and *p*-values are shown. Significant 24h cycling ($p < 0.001$) is indicated by bold letters. Mean values ± standard error of means (SEM) are shown.

Life phase differences in gene expression

Most of the investigated genes showed clear expression differences between life phases (Tab.A3.1). The majority of genes peaked in expression during the active phase including most of the clock genes (e.g. *clk*, *per2*, *cwo*) and several clock-associated genes (e.g. *cry1*, *doubletime2* (*dbt2*)) (Fig.4.4). Other genes elevated during the active phase were involved in food digestion and metabolization (*chymotrypsin* (*chtrp*), *phosphofructokinase* (*pfk*), *glutamate dehydrogenase* (*gdh*), *2-hydroxyacyl-CoA lyase* (*hacl*)), lipid accumulation (*fatty-acid binding protein* (*fabp*), *elongation of very long fatty-acids protein* (*elov*)).

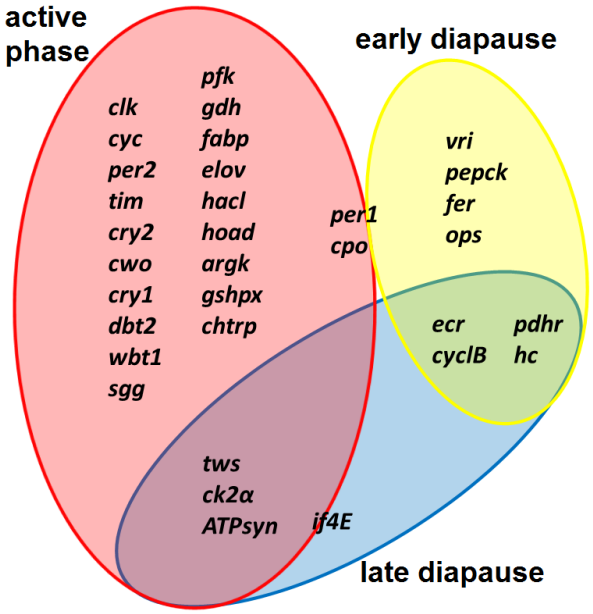

Figure 4.4: Life phase-dependent changes in gene expression. Life phases of *C. finmarchicus* CV stages were compared via Kruskal-Wallis ANOVA on Ranks and Dunn's post-hoc test. The circles indicate the life phases during which the respective genes showed elevated expression. For a detailed characterization of individual gene patterns see Tab.A3.1.

In early diapause, peak expression was shown by the genes *vri* (clock gene), *phosphoenolpyruvate carboxykinase* (*pepck*, gluconeogenesis), *ferritin* (*fer*, antioxidative defense) and by an opsin gene (*ops*, light perception) (Fig.4.4). None of the investigated genes was exclusively highly expressed in late diapause. However, the clock-associated genes *twins* (*tws*) and *casein kinase II* α (*ck2*α) as well as the *eukaryotic translation initiation factor 4E* (*if4E*, translation) and the *ATP synthase γ subunit* (*ATPsyn*, mitochondrial energy production) showed high expression during both late diapause and the active phase. In contrast, the genes *ecdysteroid receptor* (*ecr*, molting), *cyclin B* (*cyclB*, cell cycle), *pigment-dispersing hormone receptor* (*pdhr*, light perception) and *hemocyanin* (*hc*, blood oxygen transport) were upregulated in early and late diapause (Fig.4.4).

Dry weight, C/N ratio and lipid content

Dry weight and C/N ratio and total lipid content of CV stages differed significantly between life phases ($p < 0.05$, $df = 2$, respectively). Dry weight was highest during early diapause (mean ± standard error of means (SEM): 504 ± 6 µg, $n = 24$) compared to the active phase (183 ± 3 µg, $n = 22$) and late diapause (155 ± 5 µg, $n = 22$) when dry weights were similarly low (Fig.4.5A). The C/N ratio was lowest in the active phase (4.73 ± 0.05, $n = 22$) and showed a maximum during early diapause (8.94 ± 0.05, $n = 24$) before decreasing to intermediate levels in late diapause (6.86 ± 0.05, $n = 22$) (Fig.4.5B). Lowest lipid levels were measured in active CVs (47 ± 3 µg*indv.$^{-1}$, $n = 5$). Lipid content was highest during early diapause (208 ± 6 µg*indv.$^{-1}$, $n = 5$) and at intermediate levels in late diapause (96 ± 6 µg*indv.$^{-1}$, $n = 5$) (Fig.4.5C).

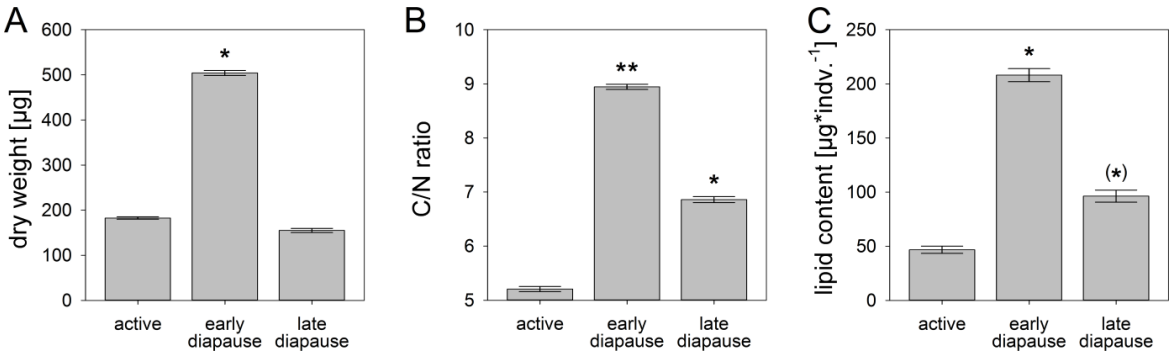

Figure 4.5: Life phase-dependent changes in physiology. (A) Dry weight, (B) C/N ratio and (C) lipid content of *C. finmarchicus* CV stages were analyzed via Kruskal-Wallis ANOVA on Ranks and Dunn's post-hoc test. *p*-values of Kruskal-Wallis ANOVAs are shown and significant differences between life phases are indicated by different numbers of asterisks (✱). Lipid content in late diapause was not significantly different from the other life phases, which is why the asterisk is put in bracket. Mean values ± standard error of means (SEM) are shown.

Zooplankton diel vertical migration

During the active life phase of the copepods in August MVBS was strongest below 200 m depth. A strong backscatter signal was also found above 50 m depth, but only in early/mid August before the sampling, while backscatter close to the surface was lower at the end of August after the sampling (Fig.4.6A). Backscatter rhythmicity at 85 m depth had a period of $\tau = 24.3$h (11.1% model fit) and $\tau = 23.7$h (14.0% model fit) before and after the August sampling, respectively. The period of backscatter rhythmicity at 180 m was $\tau = 22.9$h (17.0% model fit) before the August sampling and $\tau = 25.1$h (20.6% model fit) afterwards. In September (early diapause) a diel backscatter rhythm reaching from the surface down to beyond 200 m depth was visible (Fig.4.6B). Backscatter rhythmicity had a period of $\tau = 23.7$h (38.7% model fit) at 85 m and a period of $\tau = 24.4$h (43.4% model fit) at 180 m. In January (late diapause) no significant backscatter rhythmicity could be

detected at 85 m and at 180 m depth, although the graph suggests a weak diel change in backscatter in the upper 70-80 m (Fig.4.6C).

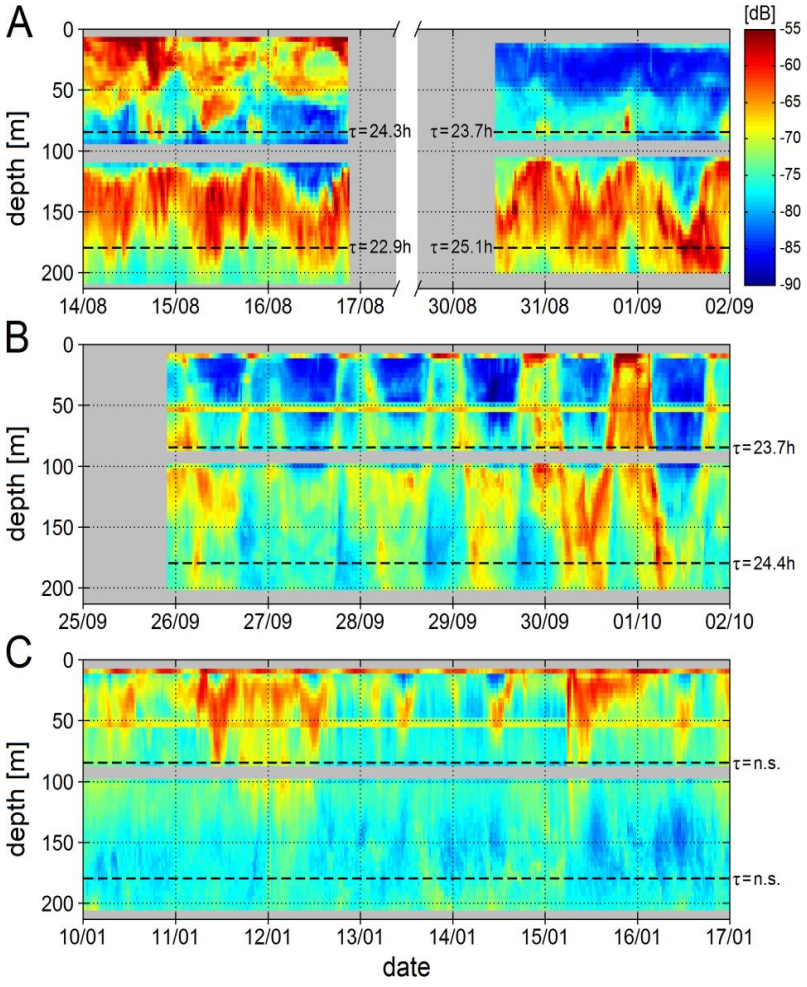

Figure 4.6: Backscatter profiles at Kongsfjorden station KB3. Backscatter measured (A) before/after the active phase sampling in August 2016 (25/08-27/08), (B) during the early diapause sampling in September 2014 (26/09-27/09), and (C) during the late diapause sampling in January 2015 (13/01-14/01). Period lengths (τ) of significant backscatter rhythmicity are shown for 85 m and 180 m depth. Values in brackets indicate secondary significant period lengths. n.s. indicates no significant rhythmicity. Note that backscatter rhythmicity before/after the active phase sampling in August was analyzed over 7 days the sampling and 7 days after the sampling, respectively. Date ticks are at 00:00h of the respective day.

4.5 Discussion

Diel rhythmicity

Our study provides new insights into the diel rhythmicity and adaptability of the boreal zooplankton key species *Calanus finmarchicus* in a polar environment and under extreme light conditions. During the active life phase in August, when copepods were developing and feeding in surface waters, the diel cycling in clock gene expression was strongest. The clock genes *per1*, *tim*, and *clockwork orange* showed the most pronounced diel expression cycles with peak activity in the afternoon, closely resembling laboratory and field data of an active *C. finmarchicus* population in Loch Etive, UK (56°N, photoperiod = 16h) (Häfker et al. 2017). The clock gene expression patterns of the active phase were recorded in August when midnight sun ended and photoperiod was close to 24h. Classical terrestrial chronobiology shows that while clock rhythmicity usually persists under constant darkness (DD), the constant presence of light (LL) does often disrupt diel

rhythmicity (Ohta et al. 2005, Oren et al. 2015, Kobelkova et al. 2015). There are several reports of a lack of diel rhythmicity during midnight sun (Reierth & Stokkan 1998, Lu et al. 2010, Benoit et al. 2010) and DVM patterns have also been reported to become arrhythmic during this time (Cottier et al. 2006, Wallace et al. 2010). However, surface PAR nevertheless showed a clear diel cycle of light intensity (Fig.4.2) that persisted over the entire period of midnight sun (Wallace et al. 2010). ADCP data further suggests that although initially weak, rhythmic DVM started around the time of the August sampling as also shown in previous studies (Fig.4.6A) (Cottier et al. 2006). This indicates that the diel light change was strong enough to evoke behavioral rhythmicity. Furthermore, light intensity attenuates with water depth and active phase copepods were collected down to 150 m depth, meaning that the subjective photoperiod perceived by the copepods in Kongsfjorden was possibly shorter than the actual time between sunrise and sunset (Miller et al. 1991, van Haren & Compton 2013). This would explain why *per1*, *tim* and *cwo*, which typically peak close to sunset (Richier et al. 2008, Meireles-Filho & Kyriacou 2013, Häfker et al. 2017), showed highest expression in the afternoon (Fig.4.3C,E,G). The shorter subjective photoperiod at depth could support clock rhythmicity during midnight sun enabling *C. finmarchicus* as well as other polar marine species to maintain a 24h cycle even under extremely long photoperiods. The presence of sea-ice would shorten the subjective photoperiod in the marine environment even further.

Samples of active copepods were collected in August, at the end of the midnight sun period and the question remains whether *C. finmarchicus* does maintain clock rhythmicity over the entire summer period of permanent (although oscillating) light. Net catch data of *C. finmarchicus* during midnight sun shows rhythmic DVM strongly suggesting a functioning clock (Dale & Kaartvedt 2000). However, ADCP data suggests that DVM just resumed diel rhythmicity by the time of the sampling in August (Fig.4.6A), while it was probably arrhythmic beforehand (Cottier et al. 2006, Wallace et al. 2010). In this context, Cottier et al. (2006) argued that rhythmic vertical migration may persists on the individual level, but is desynchronized within the zooplankton community, resulting in arrhythmic overall migration patterns, possibly driven by hunger and/or predator avoidance. For our analysis we pooled 15 CVs per replicate and still found clear diel clock gene cycling, showing that at least on the level of gene expression the *C. finmarchicus* population at the end of midnight sun was synchronized in time. Arrhythmic DVM during midnight sun thus may result from a desynchronization among different species while individuals of the same species stay synchronized. Alternatively, migration behavior could become decoupled from the rhythmic clock during midnight sun resulting in the desynchronization of DVM. While this could be an adaption to the permanent risk of visual predation during

midnight sun (Cottier et al. 2006, Benoit et al. 2010), maintaining clock rhythmicity can be important for the temporal orchestration of different physiological processes that can interfere with each other (Panda et al. 2002, Albrecht 2006).

Cycling clock gene expression during early diapause in September (photoperiod = ~12h) was limited to the clock genes *clk* and *per2* and in late diapause during the polar night 24h rhythmicity was completely absent (Fig.4.3). Diapause samples were collected below 200 m where light levels are much lower than in the surface waters sampled during the active phase. However, cycling of clock genes was still present during early diapause, indicating that even at this depth the diel cycle of light intensity (Fig.4.2) could be perceived to maintain 24h rhythmicity. This is in line with the high light sensitivity of *Calanus*, which enables the copepods to detect light levels as low as 10^{-7}-10^{-6} µmol protons*m^{-2}*s^{-1} (Båtnes et al. 2013, Miljeteig et al. 2014). ADCP data supports this assumption as rhythmic DVM in September was reaching down beyond 200 m (Fig.4.6B). Although this most likely reflected the vertical migration of euphausiid crustaceans and not DVM of the diapausing *C. finmarchicus* (Berge et al. 2014), it shows that diel rhythmicity existed in the deeper layers of the fjord at this time. It is thus likely that the reduction of clock gene cycling in early diapause is associated with the physiological transition to a phase of inactivity, rather than a mere consequence of insufficient light levels. In contrast, during late diapause in January the diel light cycle was very weak (Fig.4.2) and light intensities at >200 m depth are below the detection level reported for *Calanus* (Båtnes et al. 2013, Miljeteig et al. 2014, Cohen et al. 2015). This is also reflected in the observation that although DVM was statistically not detectable and rhythmic diel change in backscatter was visible only in the upper ~80 m of the water column (Fig.4.6C). Rhythmic migrations during the polar night were most likely performed by euphausiids, which stay active in winter and are only slightly less light sensitive than *Calanus* (Cohen et al. 2015, Darnis et al. 2017). It is thus not possible to exactly determine whether the lack of diel clock gene cycling in late diapause was related to diapause physiology or to the lack of a diel light cue for clock entrainment.

Seasonal cycle

C. finmarchicus showed strong differences in gene expression between life phases. The majority of genes were upregulated during the active life phase in August (Fig.4.4). High expressions of *ATPsyn*, a key component of the mitochondrial respiration chain, as well as *if4E*, which is involved in protein synthesis, indicate high metabolic activity matching the high respiration rates reported for this life phase (Marshall & Orr 1958, Ingvarsdóttir et al. 1999). Several upregulated genes are involved in the digestion and metabolization of food (*pfk*, *gdh*, *hacl*, *chtrp*) and the accumulation of lipid storages (*fabp*, *elov*), reflecting

that this is the time when the copepods are feeding at the surface and are building up large lipid storages for the time of diapause (Casteels et al. 2007, Falk-Petersen et al. 2009, Clark et al. 2013). This was also visible in the strong difference in dry weight, lipid content and C/N ratio of active copepods that were still feeding in surface waters compared to animals in early diapause that had just descended to deep waters (Fig.4.5). The upregulation of *glutathione peroxidase* (*gshpx*) and *arginine kinase* (*argk*) suggest an increased protection from oxidative stress (*gshpx*) and an increased capacity for anaerobic energy production (*argk*). This is surprising as in *C. finmarchicus* protection from oxidative stress is typically associated with diapause (Tarrant et al. 2008, Aruda et al. 2011) and also anaerobic metabolism would rather be expected in deeper water layers that tend to be more hypoxic. A possible explanation may be related to the short polar productive period of phytoplankton. A limited time window for growth and lipid accumulation means that the copepods have to feed and grow as much and as fast as possible, resulting in a high energy turnover that could support oxidative stress and increase the need for additional anaerobic energy sources. Additionally, the high light levels during the active phase increase predation risk (Fortier et al. 2001) and escape responses depend on short but intense bursts of energy most likely fueled by anaerobic energy storages. Other genes that are typically associated with diapause but showed upregulation during the active phase were *couch potato* (*cpo*) and *3-hydroxyacyl-Coa dehydrogenase* (*hoad*). While *cpo* is involved in the regulation of diapause in insects (Christie et al. 2013b, Salminen et al. 2015), *hoad* is a key enzyme for the mobilization of lipid storages and was described as upregulated during *Calanus* diapause (Hassett et al. 2010, Freese et al. 2017). Nevertheless, the decrease of dry weight, lipid content and C/N ratio from early to late diapause shows that a significant fraction of storage wax esters was metabolized during diapause (Fig.4.5). The low expression of *hoad* in diapause thus may reflect that energy demands during this phase are reduced and that lipid storages are depleted rather slowly. The definitive reasons for the expression patterns of *cpo* and *hoad* remain unknown and more detailed latitudinal comparisons of *C. finmarchicus* populations will be needed to evaluate whether the observed patterns are a special adaptation to the polar environment.

Most clock genes and clock-associated genes showed highest expression during the active phase matching the time of strongest clock rhythmicity (Fig.4.3 & 4.4). It seems reasonable that clock genes show highest expression at the time when the clock is actually “ticking”, although the exact network of interactions within the *C. finmarchicus* circadian clock machinery yet needs to be unraveled.

Genes upregulated in early diapause in September represent a variety of metabolic processes (Fig.4.4). *fer* is part of the copepods antioxidative defense machinery and its high expression in diapause matches previous reports (Tarrant et al. 2008, Aruda et al. 2011). Upregulation of *pepck*, a key enzyme of gluconeogenesis, suggests the accumulation of glycogen during the transition to diapause. While wax ester lipids are the typical form of long-term energy storages in *C. finmarchicus* and related species (Clark et al. 2012, Mayzaud et al. 2015), glycogen has a higher density (Ford et al. 1983, Visser & Jónasdóttir 1999) thus possibly easing the descent from surface waters to diapause depth. Expression of *fabp*, an enzyme involved in lipid synthesis, was higher in early than in late diapause, indicating that glycogen storages may get converted to wax esters upon arrival at diapause depth. *pdhr* and a gene encoding an opsin (*ops*) were also upregulated in early diapause and both genes are involved in light perception. *pdhr* is a receptor for pigment dispersing hormone (PDH), which reduces the eyes sensitivity to light (Strauss & Dircksen 2010). Light can cause arousal in *Calanus* (Miller et al. 1991, Morata & Søreide 2013) and the upregulation of *pdhr* may ease the transition to diapause. The nature of the opsin is unknown, meaning that its increase expression does not necessarily reflect increased light sensitivity, but could also indicate a change in responsiveness to specific wavelengths related to the change in light composition during the descent to diapause depth.

Notably, the clock gene *vri*, which did not show any diel cycling during any life phase (Fig.4.3), peaked in expression during early diapause. In *Drosophila vri* is part of a separate negative feedback loop (Mackey 2007), but its mechanistic role within the *C. finmarchicus* clock in unknown. Possibly, it may be involved in the transition to diapause and/or the cessation of clock rhythmicity.

None of the investigated genes was exclusively upregulated in late diapause (Fig.4.4). High expression levels of *ATPsyn* and *if4E* suggest an increase in metabolic activity indicative of the emergence from diapause. Resumption of activity in January and preparation for the ascent to surface waters was further supported by the upregulation of *cyclB*, which is involved in the cell cycle and tissue development, most likely representing the formation of reproductive tissues that starts in the late phase of diapause (Tande 1982, Hirche 1996b, Jónasdóttir 1999). Also, the copepods were preparing for the final molt as indicated by the high expression of *ecr*, a molting hormone receptor. Hence, the emergence process which ends with the molt to the adult stage and the ascent to the surface is initiated well before the start of the phytoplankton bloom, which in Kongsfjorden happens around April/May (Leu et al. 2006).

Ecological implications

C. finmarchicus shows a persistent northward shift in its latitudinal distribution range that has been associated with climate change and increasing ocean temperatures (Reygondeau & Beaugrand 2011). Similar shifts have also been observed in numerous other marine species (Perry et al. 2005, Chivers et al. 2017). These distribution shifts allow species to stay within their optimal thermal window, but for terrestrial species it has been argued that the extreme photoperiods experienced at higher latitudes could limit poleward range expansions (Saikkonen et al. 2012). Studies on drosophilid flies from different latitude show that high latitude species can maintain circadian rhythmicity under extremely long photoperiods whereas lower latitude species become arrhythmic (Kauranen et al. 2016, Menegazzi et al. 2017). The most likely reason for this limitation is that the strong seasonal changes in photoperiod could exceed the adaptive capacity of circadian clocks of species adapted to lower latitudes (Hut et al. 2013, Kyriacou 2017). Latitudinal range limitation would force species to live under suboptimal temperature conditions reducing overall fitness. Alternatively, if species prioritize temperature over clock functioning and shift to latitudes with light regimes beyond their adaptive capacity, this would result in an impairment of diel cycles in physiology and behavior, also resulting in a loss of fitness. In consequence, species can show reduced growth or population size. This would be particularly severe in ecological key species like *C. finmarchicus* that are a food source for a variety of higher trophic levels. However, our data on clock gene cycling during the active phase at the end of midnight sun suggests that in terms of diel rhythmicity *C. finmarchicus* seems to be able to cope with the extreme polar photoperiods. As the copepods do diapause in winter and not show any diel rhythmicity, the lack of light during the polar night is probably of lesser importance for *C. finmarchicus* (Hirche 1996a). The question remains whether these patterns are exclusive to *C. finmarchicus* or may be related to the discussed effects of water depth and sea-ice on environmental light conditions and subjective photoperiod. The existence of rhythmic DVM and growth during the polar night suggest that the Arctic marine species may be well adapted to the strong reduction of light levels in winter (Berge et al. 2009, 2015, Last et al. 2016), but the phytoplankton bloom and the growth season of zooplankton in polar habitats typically occur during midnight sun (Daase et al. 2013). DVM is one of the most important phenomena shaping pelagic species interactions (Brierley 2014) and thus more detailed investigations of diel marine rhythms during midnight sun will be needed to evaluate how poleward migrations of boreal species will affect future polar pelagic ecosystems.

Circadian clock are not only central in regulating diel rhythmicity, but are also important for the control and timing of seasonal life cycles due to their ability to measure photoperiod (Hazlerigg & Wagner 2006, Goto 2013). In polar habitats seasonal timing can be crucial as low light levels and sea-ice cover can limit the time of phytoplankton primary production to a very short period (Niehoff et al. 2002, Leu et al. 2006, Søreide et al. 2010). The timing of diapause initiation and termination in *C. finmarchicus* has been investigated in great detailed, but is still discussed controversially (Baumgartner & Tarrant 2017). While influences of copepod lipid content and food availability are most prominently addressed (Hind et al. 2000, Rey-Rassat et al. 2002, Maps et al. 2014, Wilson et al. 2016), the potential effects of photoperiod on diapause have received less attention (Grigg & Bardwell 1982, Miller et al. 1991). Nevertheless, there are several reports of photoperiod controlling copepod diapause timing (Watson & Smallman 1971, Marcus & Scheef 2010, Meuti & Denlinger 2013). The timing of diapause initiation in *C. finmarchicus* as well as the number of generations per year vary strongly between populations and latitudes (Durbin et al. 2000, Walkusz et al. 2009, Melle et al. 2014). In *Drosophila*, clock gene alleles were identified that differ between populations and caused flies to initiate diapause under different photoperiods (Sandrelli et al. 2007). A similar genetic diversity could explain the differences in diapause timing between *C. finmarchicus* populations. The timing of emergence is of particular importance as the copepods heavily rely on the phytoplankton bloom to fuel reproduction and development of the new generation (Richardson et al. 1999, Niehoff et al. 2002). Emergence starts well before phytoplankton is available and is usually well synchronized within a population (Hirche 1996a,b, Ingvarsdóttir et al. 1999, Baumgartner & Tarrant 2017). This led to the hypothesis that emergence timing is based on photoperiod measurement (Tittensor et al. 2003, Speirs et al. 2005). Alternatively, emergence by hourglass timers based on decreasing lipid content or slow continuous development have been proposed (Hind et al. 2000, Irigoien 2004, Saumweber & Durbin 2006), but these model struggle to explain synchronized emergence in habitats where *C. finmarchicus* produces several generations per year that all initiate diapause at different times. The lack of clock gene cycling in late diapause during the polar night as well as the low light levels make clock-based photoperiod measurement and emergence at this time highly unlikely. The synchronized emergence could however be based on a circannual clock that had been entrained by the circadian clock and photoperiod while the copepods were still active in surface waters (Goldman et al. 2004, Lincoln et al. 2006). Circannual life cycle timing has been described in *C. finmarchicus*' high Arctic congener *C. hyperboreus* and other calanoid copepods (Conover 1965, Fulton 1973). Such long-range timing mechanisms can run with impressive precision and could enable a synchronized emergence of the copepods

(Goldman et al. 2004). If diapause timing in *C. finmarchicus* involves endogenous clocks and possibly habitat-specific clock gene alleles, this could hinder the adaptation to the seasonal environmental cycle of newly occupied habitats. Furthermore, it would increase the vulnerability to changing environmental conditions as exemplified in a study by Søreide et al. (2010). The authors investigated the influence of the timing of sea-ice breakup and phytoplankton bloom on the population dynamics of *C. finmarchicus* and *C. glacialis* in an Arctic fjord. They found that while a sea-ice breakup two months earlier than usual caused a similar shift in the phytoplankton bloom, the timing of copepod emergence and reproduction stayed the same, once again suggesting an endogenous timer independent of acute external stimuli. This caused a mismatch as food levels were already decreasing as the newly hatched copepods developed. As a consequence, the biomass of both *C. finmarchicus* and *C. glacialis* was much lower than in the previous year, also affecting higher trophic levels (Søreide et al. 2010). Endogenous clock can help to maximize fitness under stable, predictable conditions, but they may become detrimental in polar habitats that can show strong interannual variability in environmental parameters (Laxon et al. 2003, Kahru et al. 2011).

Summary

C. finmarchicus in the high Arctic Kongsfjorden maintained diel clock gene cycling under extremely long photoperiod at the end of midnight sun, but clock rhythmicity diminished with the initiation of diapause. Gene expression differences between life phases reflect clock rhythmicity as well as metabolic activity and feeding during the active phase and the emergence process in late diapause.

The circadian clock of *C. finmarchicus* seems to be capable of maintaining diel rhythmicity under the extreme polar photoperiods. However, in the variable polar environment seasonal life cycle timing based on clock mechanisms and photoperiod measurement may cause mismatch situations that could lead to reduced fitness and stock size. Thus, while polar marine species like *C. glacialis* and *C. hyperboreus* may be pushed back in distribution by increasing temperatures, boreal species like *C. finmarchicus* could struggle to take their place due to the limited adaptive capacity of their endogenous clock systems (Saikkonen et al. 2012). This could lead to food limitation and restructuring of the Arctic ecosystem (Falk-Petersen et al. 2007, Weydmann et al. 2017). To predict such ecological changes, it is essential to reach a better understanding of marine clock systems, especially in key species like *C. finmarchicus*.

4.6 Acknowledgments

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5 General Discussion

The dissertation provides new insights into the diel and seasonal rhythmicity of the northern Atlantic key copepod *Calanus finmarchicus* and explores the molecular basis for the regulation of these rhythms. Referring to the initial research questions stated in the general introduction, in the following, I will first discuss the acquired information about the circadian clock machinery of *C. finmarchicus* and what will be necessary to reach a detailed understanding of its molecular functioning (Question 1). Second, I explore how the circadian clock affects the copepods diel rhythmicity and how this manifests in diel vertical migration patterns (Question 1). The third section addresses the genetic and physiological characteristics of *C. finmarchicus*' seasonal life cycle and diapause at different latitudes and discusses the role of clock mechanisms in seasonal timing (Question 2 & 3). In the fourth section I discuss how climate change, latitudinal distribution shifts and extreme light conditions may affect clock functioning and diel and seasonal rhythms in *C. finmarchicus* and other species and what consequences this could have for marine ecosystems (Question 3). The discussion will end with a summary of the central findings of this work, their relevance for *C. finmarchicus* and marine ecosystems and suggestions for future directions in marine chronobiological research.

5.1 The putative circadian clock of *Calanus finmarchicus*

This work shows that the *C. finmarchicus* possesses a circadian clock that is functional under constant darkness as well as under extreme photoperiods. From the obtained clock gene expression data I will in this section infer potential molecular functions of individual clock genes, comparing them to clock systems of other arthropods and marine species. Based on these insights I will give examples of future experiments to further unravel the mechanistic functioning of the copepods circadian clock and the ways in which it is affected by the environment. The clock gene repertoire of *C. finmarchicus* closely resembles what is known from other arthropod species like *Drosophila* (Christie et al. 2013a), although the presence of a mammalian-like *cryptochrome2* (*cry2*) gene suggests an ancestral clock system as described for the Antarctic krill *Euphausia superba* or the monarch butterfly *Danaus plexippus* (Reppert 2007, Biscontin et al. 2017). While the gene *PAR domain 1ε* (*pdp1ε*) was lacking in the original description of *C. finmarchicus* clock genes, a recent re-analysis of the dataset suggests that *pdp1ε* also exists in the copepod (Christie et al. 2013a, Christie et al. 2017). A peculiarity is the presence of two different *period* (*per*) gene transcripts in *C. finmarchicus* (Christie et al. 2013a). There are several reports of species lacking certain clock genes (Sandrelli et al. 2008, Tomioka & Matsumoto 2015, Christie et al. 2017), but in crustaceans multiple isoforms of core clock

genes have so far only been reported for *timeless* (*tim*) in the Norway lobster *Nephrops norvegicus* (4 isoforms, Sbragaglia et al. 2015) and the water flea *Daphnia pulex* (8 isoforms, Tilden et al. 2011).

clock* & *cycle

Highest *clk* expression occurred at sunrise as also reported for D*rosophila*, mosquitos and the crustaceans *N. norvegicus* and *D. pulex* (Richier et al. 2008, Gentile et al. 2009, Sbragaglia et al. 2015, Bernatowicz et al. 2016). Although patterns may differ in other species with peak *clk* expression occurring in the afternoon (Meireles-Filho et al. 2006, Zantke et al. 2013) or in the middle of the night (Payton et al. 2017a), the *clk* expression patterns of *C. finmarchicus* is quite typical for arthropods. *cyc* expression showed no clear diel oscillations, although laboratory data shows some similarities to *clk* expression (Fig.2.3B). It has been repeatedly observed that either only *clk* or only *cyc* shows circadian oscillations (Rubin et al. 2006, Reitzel et al. 2010, Hoadley et al. 2011, Meireles-Filho & Kyriacou 2013, Meuti et al. 2015). As the proteins of *clk* and *cyc* (CLK and CYC) can only act as transcription factors, by forming a heterodimer (CLK/CYC), it is likely that the oscillation of one of the genes is sufficient to maintain circadian transcriptional activation of other genes. In *C. finmarchicus*, circadian regulation seems to focus on the expression of *clk* rather than on *cyc*.

period1/2*, *timeless* & *cryptochrome2

The peak activity of *period1* (*per1*), *timeless* (tim), and *cryptochrome2* (*cry2*) *in C. finmarchicus* at sunset or closely after sunset resembles patterns observed in many other species (Fig.2.3). While this expression pattern is typical for *per* and *tim*, several different peak times were previously reported for *cry2*, indicating that the role of *cry2* in the circadian clock could differ between species (Richier et al. 2008, Merlin et al. 2009, Teschke et al. 2011, Zantke et al. 2013). In *C. finmarchicus cry2* expression strongly resembles the patterns of *per1* and *tim*, suggesting that, like in e.g. the honey bee *Apis mellifera*, the proteins of *per1*, *tim* and *cry2* act together as a heterodimer (PER1/TIM/CRY2) that inhibits CLK/CYC (Rubin et al. 2006). However, the role of *period2* (*per2*) remains unclear. The gene showed weak but consistent circadian oscillations in the laboratory, but was mostly arrhythmic in the field (Fig.2.3D,L & 4.3D, Tab.3.4). The expression of both *per1* and *per2* should be initiated via CLK/CYC binding to E-boxes in the genes promoter regions, meaning that the different expression patterns could result from different numbers of E-boxes or differences in E-Box sequences that affect CLK/CYC binding (Matsumoto et al. 2007). Alternatively, if *per2* is located in a chromosomal region with higher chromatin condensation or gene sequences are methylated differently, this could affect *per2*'s transcription resulting in weaker oscillation

patterns (Weinstock et al. 2006, Kouzarides 2007). Finally, it is also possible that the expression of *per1* and *per2* differed between tissues and that this could not be resolved in the investigation due to the analysis of whole animals. It is noteworthy that *per2* showed clear diel cycling in the copepods from Kongsfjorden that had just descended to diapause below 200 m depth (Fig.4.3D, Tab.3.4). Similarly, *per2* expression in the deep layer of Loch Etive in May increased in the afternoon to a maximum at sunset before decreasing during the night (Fig.2.3L). The crustaceans, for which different clock gene isoforms were reported, inhabit a broad environmental range and it has been suggested that different clock gene isoforms may be expressed depending on environmental conditions (Tilden et al. 2011, Sbragaglia et al. 2015, Bernatowicz et al. 2016). Therefore, it seems possible that *per2* in *C. finmarchicus* may support clock rhythmicity under conditions where light levels are low. Mammals possess two or three *per* genes and their proteins form a heterodimer with the proteins of two mammalian *cry* genes (Zylka et al. 1998, Mackey 2007). In *C. finmarchicus* the PER2 protein could either replace PER1 or could associate with the PER1/TIM/CRY2 protein heterodimer and thereby affect its stability and/or core import.

clockwork orange

The clock gene that showed the strongest and most consisted diel oscillations in *C. finmarchicus* was *clockwork orange* (*cwo*) (Fig.2.3G,O & 4.3G). Its involvement in the circadian clock is a relatively new discovery (Matsumoto et al. 2007, Kadener et al. 2007, Lim et al. 2007) and like *per*, *tim*, and *cry2* its transcription is initiated via an E-Box, explaining that it showed a similar expression pattern with a peak at sunset. The CWO protein replaces the CLK/CYC protein heterodimer at the E-box thereby repressing transcription (Zhou et al. 2016). A dysfunctional *cwo* gene results in low-amplitude oscillations in clock gene expression as well as reduced rhythmicity and period lengthening under DD (Matsumoto et al. 2007, Kadener et al. 2007, Lim et al. 2007, Richier et al. 2008). Hence, *cwo* is believed to support circadian clock robustness by producing high-amplitude oscillations in clock gene activity and behavioral rhythms. *C. finmarchicus* often experiences low light conditions or even constant darkness in its marine habitat as shown by the rapid attenuation of light intensity with depth in Loch Etive where light is absent below ~20 m. This also applies to oceanic habitats where diel vertical migration is performed down to depth of >1400 m (van Haren 2007, van Haren & Compton 2013). The vertical migration of the copepods through stratified water layer further affects the diel light cycle, which the animals experience and the migration is often accompanied by pronounced diel changes of environmental parameters like temperature, salinity or oxygen concentration (Fig.3.2). All these parameters have the potential to

affect clock rhythmicity (Rensing & Ruoff 2002, Vera et al. 2013, Tataroglu et al. 2015, Adamovich et al. 2017, Depping & Oster 2017) and the strong oscillations of *cwo* could support robust diel cycling of clock genes under these variable conditions not only in *C. finmarchicus*, but also in other vertically migrating marine species. To my knowledge, this is the first time *cwo* expression was investigated in a marine organism and more research is needed to see whether the pronounced *cwo* oscillations described here are a specific feature of *C. finmarchicus*' circadian clock or may be generally adaptive in marine species.

vrille

Surprisingly, no clear diel oscillations were found for *vri* (Fig.2.3H & 4.3H), although the gene is rhythmic in other species (Cyran et al. 2003, Richier et al. 2008, Gentile et al. 2009). The corresponding protein (VRI) typically inhibits the expression of *clk* before the gene gets activated by PDP1ε. The gene *clk* showed clear circadian expression indicating that either the VRI protein shows diel rhythmicity in activity/abundance or that *clk* oscillations are mostly regulated via *pdp1ε*, which was only recently identified in *C. finmarchicus* and could thus not be investigated in this dissertation (Christie et al. 2017).

Clock-associated genes

Clock-associated genes showed no diel oscillations and from gene expression alone it is difficult to assess the ways in which the protein products of these genes affect the core clock machinery. With the exception of the protein phosphatase 2A subunits *twins* (*tws*) and *widerborst* (*wbt*), clock-associated genes are typically described as arrhythmic in arthropods (Mackey 2007, Sandrelli et al. 2008, Allada & Chung 2010). While the genes *tws* and *wbt1* did not any circadian oscillations in *C. finmarchicus*, it is still possible that their corresponding proteins show oscillations in abundance and/or phosphatase activity. Thus the role and importance of clock-associated genes in the copepod remains obscure. However, laboratory experiments that were performed at SAMS in Scotland and were not included in the publications indicate that at least the associated gene *doubletime* (*dbt*) is important for proper circadian clock functioning, as a disruption of this clock component caused a lengthening of the endogenous clock period. Freshly caught *C. finmarchicus* from Loch Etive were kept for 24h in seawater containing 5 µM of PF-670462 (abcam, UK), a chemical inhibitor of casein kinase 1 proteins including the protein product of the *dbt* gene (DBT). Afterwards, animals were transferred to normal seawater and clock gene expression was measured over the following 48h in 4h intervals. During and after the chemical incubation the copepods were kept in DD. Clock gene expression rhythmicity was checked for periods from 24h to 32h. The chemical treatment resulted in an overall dampening in *C. finmarchicus* clock gene oscillations as well as a clear period

lengthening by 4-8h (Fig.5.1), indicating an important role of *dbt* in maintaining clock rhythmicity. It is further interesting that the effect differed between genes with *clk* and *per2* showing more pronounced period lengthening while no obvious effect on period length was observed for *cry2*. A detailed mechanistic understanding of clock gene interactions will be needed to explain these differences. In the intertidal isopod *Eurydice pulchra* treatment with Pf-670462 caused a lengthening of the circadian period as well as reduced diel rhythmicity (Zhang et al. 2013) and in the marine polychaete *Platynereis* dumerilii the addition of the chemical caused a complete cessation in clock gene rhythmicity (Zantke et al. 2013). These similar responses to the chemical treatment support the findings reported for *C. finmarchicus*.

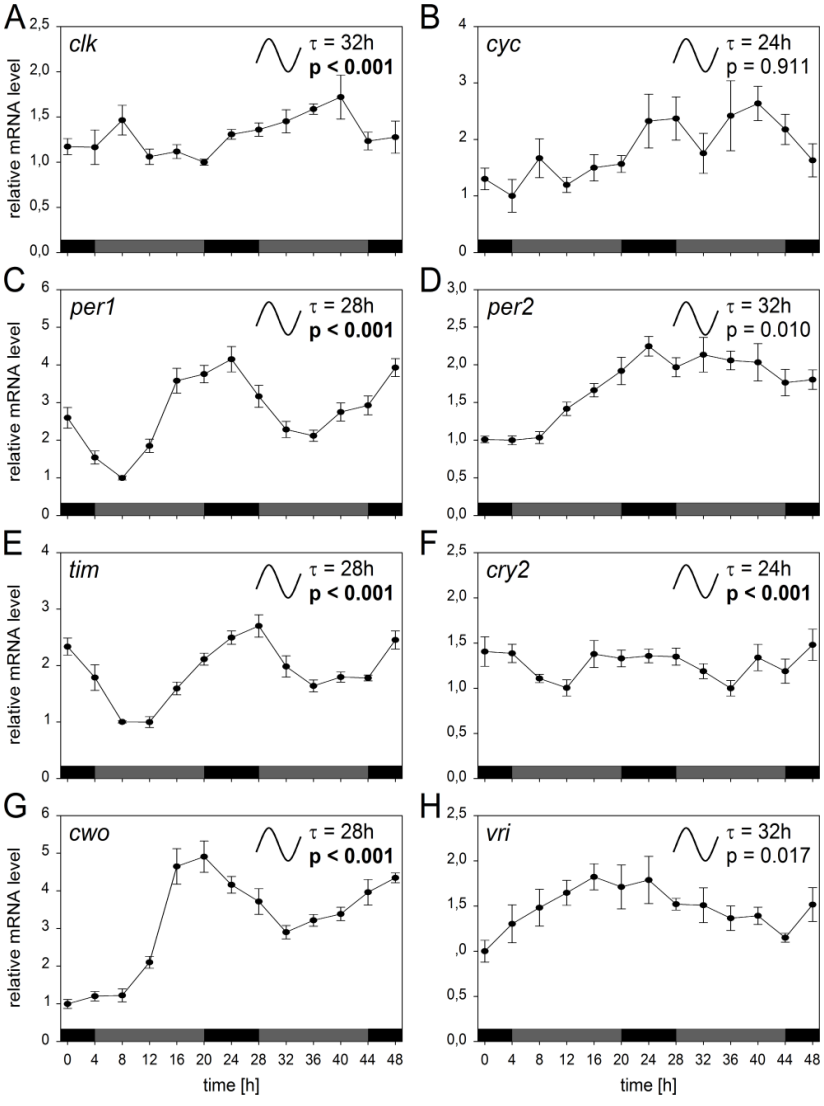

Figure 5.1: Clock gene patterns in *C. finmarchicus* after treatment with the casein kinase 1 inhibitor PF-670462. Expression of the clock genes (A) *clock*, (B) *cycle*, (C) *period1*, (D) *period2*, (E) *timeless*, (F) *cryptochrome2*, (G) *clock-work orange*, and (H) *vrille* is shown. Copepods were kept in DD. Black/grey color bars indicate subjective day/night. Gene rhythmicity was tested for period lengths (τ) between 24h and 32h using the R-package "RAIN" (Thaben & Westermark 2014). *n* = 5 replicates were measured per time point and mean values ± SEM are shown. Bold p-values indicate significant rhythmicity for the respective period. See Fig.2.3A-H for clock gene patterns without chemical treatment.

Future approaches to unravel *C. finmarchicus*' circadian clock machinery

The gene expression data reported here for *C. finmarchicus* provides first insights into diel changes in clock gene activity and into the potential adaptability of the copepods

circadian clock under variable environmental conditions. However, to understand how individual clock genes and proteins interact to produce circadian rhythmicity, research has to be expanded to other methodologies than the ones applied in the PhD project. The most direct way to investigate how an individual gene affects the clock is by disabling the respective gene and there are several possible approaches to achieve this. RNA interference (RNAi) means that double-stranded RNA of a desired gene is introduce into a cell where it causes the degradation of the mRNAs of the desired gene. For RNAi, double-stranded RNA is often directly injected into the organism, making it difficult to apply on small species like *C. finmarchicus*. However, studies have shown that it is possible to perform RNAi by feeding organisms bacteria that express the desired double-stranded RNA and that this approach can also work in marine crustaceans (Sarathi et al. 2008, Payton, et al. 2017b and references therein). While bacteria may be too small for *C. finmarchicus* to feed on effectively (Meyer-Harms et al. 1999), unicellular algae have been successfully used as a vector for the bacteria to perform RNAi in oysters (Payton et al. 2017b) and a similar non-invasive approach could also be applied to the copepod. A different well-established method of identifying clock gene functions is the use of *Drosophila*-derived S2-cell cultures (Rosato et al. 2001, Yuan et al. 2007, Zhu et al. 2008, Zhang et al. 2017). By introducing one or more clock genes or gene sections (e.g. promoter regions) via plamid transfection in combination with a reporter gene (usually luciferase), it can be investigated how the presence of specific clock proteins or light affects the expression of other clock genes. Although this approach is based on highly artificial conditions, it has been crucial for the development of mechanistic clock models (Rosato et al. 2001, Yuan et al. 2007) and was also successfully applied to the krill *E. superba* (Biscontin et al. 2017). While RNAi and S2-cells focus on short-term effects of clock manipulation, the creation of mutants, in which specific clock genes are dysfunctional, makes it possible to investigate the consequences of these mutations over the entire life cycle. Such mutations can be introduced by genome editing systems like TALEN (transcription activator-like effector nucleases) or CRISPR/Cas9 that produce DNA double strand breaks in specific gene sequences (Gaj et al. 2013). The cellular repair mechanisms that fix such breaks introduce additional bases, causing a shift in the reading frame that makes the respective gene dysfunctional. If such genetic tools are used on zygotes, the mutation will be present throughout the developing organism and is heritable. TALEN as well as transgenesis have been successfully used on eggs of the polychaete *P. dumerilii* (Backfisch et al. 2013, Bannister et al. 2014, Zantke et al. 2014). The polychaetes eggs are of similar size as the ones of *C. finmarchicus* (100-150 µm), meanings that such genetic methods may also be applicable to the copepod (Marshall & Orr 1955, Zantke et al. 2014). *C. finmarchicus* can be maintained in aquaculture

throughout its entire life cycle (Jensen et al. 2006) and the creation of clock mutants for this species could help to understand how its diel rhythmicity changes through the different life stages and how it may effects life cycle events like the initiation/termination of diapause.

While the approaches mentioned above all focus on gene expression, clock proteins are also a crucial part of the circadian machinery. Investigations of diel changes in the abundance and cellular localization of clock proteins would thus be highly beneficial to unravel clock component interactions and feedback loop functioning. For this it will be necessary to develop antibodies of *C. finmarchicus* clock proteins for immunostaining of individual genes or proteomic analyses (Wagner et al. 2005). Aside from the choice of analytical techniques, it is also important to get a general understanding of the environmental parameters that affect the circadian clock of *C. finmarchicus*. Light is considered the dominant *Zeitgeber* in most species (Aschoff 1954), but temperature, oxygen levels and food availability have all been shown to entrain circadian clocks in different organisms (Rensing & Ruoff 2002, Vera et al. 2013, Tataroglu et al. 2015, Adamovich et al. 2017, Depping & Oster 2017) and vertically migrating copepods can experience diel changes in all these parameters. In *Drosophila*, light and temperature were shown to act synergistically in the entrainment of the circadian clock (Yoshii et al. 2009). Whether such environmental changes can support clock rhythmicity in *C. finmarchicus* or rather obstruct it, is unclear. It is for example noteworthy that *cry2* peaked in the evening in laboratory experiments and in Kongsfjorden, but showed a morning peak during the May sampling in Loch Etive. In mice *cry2* was shown to be involved in the entrainment of the circadian clock via cycles of oxygen concentration (Adamovich et al. 2017). Oxygen concentration in Loch Etive shows a pronounced decrease with depth (Fig.3.2D) and gene expression patterns could indicate that in the field *cry2* of *C. finmarchicus* is entrained but factors other than light. Furthermore, the potential effects of ocean acidification on clock systems are completely unknown due the chronobiological focus on terrestrial species. It is therefore necessary to not only investigate the response to light but also to other environmental factors that could influence clock systems and diel rhythmicity in *C. finmarchicus*.

Taken together, the characterization of clock gene patterns in *C. finmarchicus* under laboratory and field conditions showed that the copepod possess a robust circadian clock with high amplitude oscillations in gene activity. This, together with the fact that the species can be reared in the laboratory, makes *C. finmarchicus* a potential model species for chronobiological investigations of zooplankton and vertically migrating marine species in general. Future work will depend on laboratory experiments to develop a mechanistic

model of the copepods circadian clock as well as on field investigations that describe in which way the natural environments modulates the clocks rhythmicity.

5.2 The clock's impact on diel rhythmicity

The circadian rhythmicity in *C. finmarchicus* also manifested itself on the phenotypic level in the form of diel oscillations in oxygen consumptions as well as endogenous diel vertical migration (DVM). However, a circadian rhythm was not found in any of the investigated metabolic genes, raising the question how the circadian output by the clock is mediated within the organism. While it can only be speculated about the circadian regulation of cellular processes and the mechanisms evoking diel phenotypic rhythmicity, circadian rhythms in physiology and behavioral patterns like DVM provide clear ecological benefits. Thus it will be important to explore the pathways through which the circadian clock regulates cellular processes, physiology, and behavior.

The lack of metabolic gene oscillations and potential signaling pathways

Considering the strong oscillations of clock genes found in *C. finmarchicus*, it is surprising that none of the investigated metabolic genes showed consistent diel cycling neither in the laboratory nor in the field. Studies found 5-20% of the transcriptome in other species to be expressed in a circadian manner (Panda et al. 2002, De Pittà et al. 2013, Montenegro-Montero & Larrondo 2015, Payton et al. 2017a). This often included genes responsible for metabolic key steps like *phosphofructokinase* (*pfk*, glycolysis), *phosphoenolpyruvate carboxykinase* (*pepck*, gluconeogenesis) or *malate dehydrogenase* (*mdh*, citric acid cycle) that were also measured in *C. finmarchicus* (Akhtar et al. 2002, Panda et al. 2002). As the metabolic genes were measured in parallel with the clock genes, a methodological bias is unlikely and the obtained diel patterns can be considered correct. There are two possible explanations for the lack of circadian gene expression in *C. finmarchicus*. A very simple explanation would be that circadian rhythmicity of metabolic genes is present in *C. finmarchicus*, but was not detected due to an "unlucky" choice of investigated genes. Alternatively, it is possible that the circadian regulation of gene expression is limited to a relatively small number of output genes triggering cellular cascades, which result in a circadian regulation of enzyme activity via phosphorylation, methylation or other protein modifications (Ceriani et al. 2002, Panda et al. 2002, Reddy & Rey 2014). Metabolic enzymes often have long half-lives making fast regulation over the diel cycle difficult. In contrast, the half-life of regulatory proteins that act as messenger can be much shorter allowing for fast regulation of metabolic pathways (Thurley et al. 2017). Going from the cellular to the histological level, the circadian expression of clock genes is mostly limited to the "central oscillator", a group of neurons in the organism's

brain (Kuhlman et al. 2007, Sandrelli et al. 2008). Circadian signal cascades within these cells can be transmitted to other brain regions via synaptic interaction and through signaling molecules like the pigment dispersing hormone PDH (equivalent to PDF in insects), which is central for the coordination of clock neurons in the central oscillator and is also involved in clock entrainment as well as circadian output (Yoshii et al. 2016, Mezan et al. 2016). In *Drosophila* a neuronal pathway has been identified that starts in the clock neurons of the central oscillator and ultimately results in a circadian output of locomotor activity (King et al. 2017). Fly mutants with a dysfunctional gene for PDF showed reduced circadian locomotor activity, suggesting that PDF could be involved in activity regulation (King et al. 2017). The distribution of PDH was also described for the brain of *C. finmarchicus* (Sousa et al. 2008) and although the related receptor gene *pigment dispersing hormone receptor* (*pdhr*) showed no diel expression rhythm, the brain regions in which PDH is localized could harbor clock neurons and the regions may be involved in the transmission of the cellular circadian rhythm to the organismic level.

Circadian phenotypic rhythmicity and ecological benefits

The endogenous rhythms of respiration and DVM found in *C. finmarchicus* clearly indicate that these processes are at least partially regulated by the circadian clock (Fig.2.2). Circadian rhythms of respiration have been described in several marine species and they have been mostly linked to behavioral activity rhythms (Livingston 1971, Aguzzi et al. 2003, Teschke et al. 2011, Maas et al. 2016). *C. finmarchicus* DVM can span more than hundred meters (Daase et al. 2008) and the ascent at sunset comes with considerable energetic costs, while passive sinking could reduce the energy needed for the descent (Lampert 1989, Steele & Henderson 1998). The increase in respiration rate around sunset and the decrease towards sunrise could thus be explained by active upwards swimming and passive sinking, respectively. The fact that respiration started to increase before sunset is possibly related to anticipatory processes that prepare the copepod for the ascent and feeding at the surface. If the ascent is supported by energy production from anaerobic sources like L-arginine phosphate, the anticipatory increase in respiration could reflect the aerobic creation of these energy storages. Furthermore, some species including crustaceans possess circadian rhythms of feeding and enzyme activity (Saito et al. 1976, Trellu & Ceccaldi 1977, Mayzaud et al. 1984, Stearns 1986, Espinosa-Chaurand et al. 2017). In these studies digestive enzyme activity was often seen to anticipate feeding. A diel cycle with highest digestive enzyme activity around sunset was also described in *C. finmarchicus* and the enzyme pattern was matched by an evening increase in respiration (Båmstedt 1988). It is therefore possible that an increase in respiration before sunset is related to a preparation of digestive enzymes in

anticipation of feeding at night. The feeding time in shallow waters in limited, meaning that food should be processed as fast as possible to maximize consumption. If the production of digestive enzymes was only initiated in response to feeding, this would increase the time needed to process the food resulting in lower overall consumption, meaning that anticipatory enzyme production would provide a significant ecological advantage for *C. finmarchicus*. It was furthermore suggested that endogenous patterns of DVM could be the direct result of circadian rhythms of metabolic activity characterized by high activity at sunset and low activity at sunrise, resulting in an active ascent and passive descent, respectively (Rudjakov 1970). Circadian regulation of DVM behavior was not only found in *C. finmarchicus*, but also in several other species, suggesting that the endogenous control of migration behavior is ecologically adaptive (Harris 1963, Enright & Hamner 1967, Sournia 1975, Cohen & Forward Jr 2005, Gaten et al. 2008, Tosches et al. 2014). For planktonic organisms like *C. finmarchicus* the migration to the surface at sunset is time consuming and it may thus be beneficial to initiate migration before light levels have decreased to an extent that allows for safe foraging. If DVM was performed according to the "isolumen hypothesis", it is questionable whether small planktonic species would be able to keep up with the rapid shoaling of this layer of preferred light intensity at sunset. Additionally, actively searching for constant light conditions throughout the day could impair circadian clock functioning and thus other rhythmic processes like e.g. digestive enzyme production (see above). The involvement of a circadian clock in DVM further reduces predation risk as the copepods can anticipate the morning increase in light intensity and thus descend before light levels become sufficient for visual predation (Nelson & Vance 1979, Fortier et al. 2001). Similarly, midnight sinking behavior does not follow the change in light levels, indicating an endogenous regulation. It has been proposed that this descent to intermediate depth around midnight is a response to predation from larger vertically migrating organisms (Tarling et al. 2002). A circadian regulation of DVM would be especially adaptive in habitats where light as an entrainment cue is scarce or only temporarily available. Such conditions exist in deep sea habitats, at high latitudes during the polar night, and also in the waters of Loch Etive where light penetration is poor. In Loch Etive, light is absent below ~20 m depth, but ADCP data shows that copepods performing DVM reside at 40-80 m during the day (Fig.3.3). This means that the ascent must be triggered endogenously. While hunger could theoretically act as a trigger, a circadian regulation is much more likely as vertical migrations were highly synchronized. In the open ocean DVM can be performed down to depth of more than 1400 m during the day (van Haren 2007, van Haren & Compton 2013). As light is typically undetectable below 1000 m even at noon and in the clearest oceanic waters, this deep sea DVM must rely on endogenous

timing mechanisms. In high latitude habitats the diel change in light intensity during the polar night is extremely weak. Nevertheless, a high degree of diel rhythmicity including DVM was reported during the Arctic winter (Berge et al. 2009, 2015). Sea birds and other large predatory zooplankton species feed on other zooplankton organisms during the polar night, indicating that DVM is adaptive even when overall light levels are very low (Berge et al. 2015, Last et al. 2016). Under these conditions sunlight is only detectable for a short period around noon, meaning that for the rest of the day organisms have to rely on endogenous timers for the regulation of their diel cycle.

Future approaches to characterize copepod diel rhythmicity

Large-scale screening approaches will be needed to understand how the circadian clock of *C. finmarchicus* affects the organizational levels of gene expression, protein abundance, and enzyme activity. Transcriptomics have been successfully used to identify genes under circadian control in various organisms including marine crustaceans (Akhtar et al. 2002, De Pittà et al. 2013, Oren et al. 2015, Sbragaglia et al. 2015, Payton et al. 2017a). Similarly, proteomic and metabolomic approaches could be used to identify potential circadian signal proteins as well as circadian changes in metabolite concentrations and related metabolic pathways (Wagner et al. 2005, Eckel-Mahan et al. 2012). While screening approaches provide a detailed overview about cellular component and processes under circadian regulation, they are also expensive and require a lot of manpower and methodological knowledge to analyze the large amount of data produced. Furthermore, they provide only limited insights into the mechanistic regulation of phenotypic rhythmicity. In contrast, manipulative approaches like RNAi or TALEN (see section 5.1) provide detailed insights into how individual genes affect animal's rhythmicity on different levels reaching from gene expression all the way to metabolic activity and behavior. They thus may be used after an initial screening for genes that could be involved in circadian regulation. Incubation experiments can also be easy but effective tools to learn more about the regulation of metabolic rhythms or behaviors like DVM. Exposing *C. finmarchicus* to diel cycles in temperature, oxygen and food concentration or a combination of these factors and monitoring the respiration and activity could identify *Zeitgeber*s other than light. The addition of chemical risk cues from copepod predators could further add an additional level of ecological complexity and the use of cue from predators that also perform DVM could help to understand what evokes midnight sinking behavior (Tarling et al. 2002). To better understand in which way light trigger DVM, animal incubation columns could be illuminated from above, from below or with diffuse light. This would give indication about whether DVM is a direct result of activity or possibly related to diel rhythms of phototaxis or geotropism.

Concluding this section, it becomes evident that while the circadian clock seems to have an important role in regulating the phenotypic rhythmicity of *C. finmarchicus*, the cellular and physiological pathways that transmit the circadian signal are still unknown. In contrast, the ecological benefits that the copepods gains from a circadian regulation are overt and allow for an optimal timing of DVM as well as maximization of food consumption. While screening tools like transcriptomics will be essential to identify genes and other cellular components involved in the regulation of diel rhythmicity, manipulative approaches will allow for the characterization of individual genes and circadian signal pathways. Incubation experiment will further be needed to gain a better understanding of the environmental factors that affect diel rhythmicity and should also be combined with field studies to verify the laboratory finding under natural conditions.

5.3 The seasonal life cycle of *C. finmarchicus* and endogenous clock involvement

In the past, the seasonal life cycle and diapause of *C. finmarchicus* has been investigated in great detail with regard to population dynamics and physiological characteristics like lipid content and enzyme activity (Plourde et al. 2001, Hassett 2006, Johnson et al. 2008, Clark et al. 2012). However, the mechanisms controlling the copepods life cycle are still poorly understood. Knowledge about seasonal gene expression in *C. finmarchicus* is scarce as previous studies mostly focused on comparisons of animals from two different depths or life phases (Tarrant et al. 2008, Hassett et al. 2010, Aruda et al. 2011, Unal et al. 2013, Tarrant et al. 2016), while seasonal cycles were rarely addressed (Clark et al. 2013). This dissertation provides the first detailed characterization of seasonal gene expression in *C. finmarchicus*. By combining gene expression data with investigations of clock rhythmicity, physiological characteristics, vertical migration behavior and population dynamics, an integrative picture of the copepods seasonal cycle could be created that also allows for latitudinal comparisons. Based in these findings, it is now possible to develop hypotheses about the mechanistic regulation of *C. finmarchicus* diapause and to propose experimental approaches to tests these hypotheses.

Characteristics of *C. finmarchicus*' life cycle phases

The obtained data suggest that the seasonal life cycle of *C. finmarchicus* is characterized by three phases: (1) activity and development in surface waters, (2) diapause in deep waters, and (3) emergence and reproduction, with each phase being characterized by distinct patterns of gene expression (Fig.3.9). Active copepods in surface water showed an upregulation of genes related to metabolic activity, feeding and the accumulation of lipids. This applies to the *C. finmarchicus* sampled in the shallow layer of Loch Etive from

May to August as well as to the copepods sampled in August in Kongsfjorden. The animals showed strong diel clock gene cycling as well as clear DVM (Fig.3.8 & 4.3, Tab.3.4). While high expression of the lipid-related genes *elongation of very long fatty acids protein* (*elov*) and *fatty acid-bind protein* (*fabp*) was previously described in active *C. finmarchicus* (Tarrant et al. 2008, Aruda et al. 2011), monitoring the expression in the field suggests that these and other genes involved in food processing could respond directly to ambient phytoplankton concentrations (Fig.3.2E & 3.7). Furthermore, several other genes indicative of *C. finmarchicus* activity could be identified. This includes genes like *mdh* or *initiation factor 4E* (*if4E*) that showed pronounced changes in expression during the initiation of diapause and during emergence and could serve as life phase indicator in future studies. Gene expression also confirmed that the physiological switch to diapause happens after the decent to deep waters (Head & Harris 1985, Freese et al. 2017) as did the upregulation of *ferritin* (*fer*) in diapausing copepods (Tarrant et al. 2008, Aruda et al. 2011). While the elevated expression of *fer* in deep waters of Loch Etive could be considered a response to reduced oxygen conditions, the seasonal time series in combination with the expression of the genes *arginine kinase* (*argk*, anaerobic metabolism) and *hemocyanin* (*hc*, blood oxygen transport) shows that the expression is directly related to the phase of diapause and possibly a response reduced energy demands associated with an increase in oxidative stress (Ingvarsdóttir et al. 1999). Also, the same pattern of *fer* expression was found in Kongsfjorden where the decrease in oxygen with depth is much weaker. A completely new observation is the upregulation of *pepck* in early diapause, as described for Loch Etive as well as Kongsfjorden. Although the definite reasons for this remain unclear, this could indicate a buoyancy-related new mechanism that supports the descent to diapause. Lipids are low in density and it has been proposed that their buoyancy could hinder the descent of copepods to diapause depth (Visser & Jónasdóttir 1999, Pond & Tarling 2011, Clark et al. 2012). The initial accumulation of glycogen, which has a higher density than lipids (Ford et al. 1983), could therefore aid downward migration. Due to the pressure and low temperatures at depth, wax ester lipids undergo a phase transition to a solid state with higher density, allowing the copepods to remain neutrally buoyant (Pond & Tarling 2011, Clark et al. 2012). The glycogen storages may then get converted to wax esters thus explaining why lipid content of deep copepods in Loch Etive kept increasing from May to August in spite of a lack of food (Fig.3.5D). Although it has previously been suggested that *C. finmarchicus* diapause can be divided into different phases (Tande 1982, Hirche 1996a), this is the first time this assumption could be confirmed on the genetic level. While emergence from diapause was hardly detectable in terms of physiological parameters like lipid content, gene expression showed clear changes in late winter signaling the end of diapause (Fig.3.7).

Genes like *ecdysteroid receptor* (*ecr*) or *cyclin B* (*cyclB*) could be monitored in future studies to determine the time at which emergence starts in different habitats and latitudes to get a better understanding of *C. finmarchicus* diapause patterns and the environmental conditions under which emergence happens.

Life cycle differences between Loch Etive and Kongsfjorden

The seasonal cycles of copepods from Loch Etive and Kongsfjorden show that both populations share large similarities. DVM is most pronounced in copepods developing in surface waters, although the almost permanent presence of light in August in Kongsfjorden probably reduced the intensity of vertical migrations (Fig.3.3 & 4.6). Lipid patterns were also similar with low levels in active copepods and highest levels in early diapause that then gradually decreased towards emergence (Fig.3.5D & 4.5C). Gene expression was characterized by the upregulation of genes related to metabolic activity and lipid accumulation in active animals in both populations, followed by pronounced switch in gene expression pattern with the initiation of diapause (Fig.3.7 & 4.4). Both populations also showed an upregulation of genes like *ecr* and *cyclB* that marked the phase of emergence. Differences were however observed in genes like *argk, couch potato* (*cpo*) and *3-hydroxyacyl-CoA dehydrogenase* (*hoad*) that were upregulated during diapause in Loch Etive, but peak in active copepods in Kongsfjorden. The high expression of *argk* in active *C. finmarchicus* in Kongsfjorden could be related to the larger vertical distance covered by DVM in the fjord as well as to the overall high light levels. Elevated *argk* expression could indicate the accumulation of larger anaerobic energy storages. These could be used to either provide additional energy for the ascent to surface waters at sunset and could also fuel burst energy production during escape responses. The high overall light levels during the Arctic increase the risk of predation and the upregulation of *argk* could account for this. *cpo* is typically associated with diapause in insects) (Christie et al. 2013b, Salminen et al. 2015), making it surprising to see the gene upregulated in active copepods in Kongsfjorden. It seems possible that this observation is connected to the differences in the timing of diapause and in the environmental conditions in Loch Etive and Kongsfjorden. In *Drosophila*, six different *cpo* transcripts were identified in flies from different latitudes and the authors suggested that these transcripts are one reason for the different diapause patterns observed between populations (Schmidt et al. 2008). Only one transcript was investigated in *C. finmarchicus* and this was identified in copepods collected in the Gulf of Maine at ~44°N (Christie et al. 2013b). This could mean that there might be other *cpo* transcripts in Kongsfjorden that are upregulated during diapause. *hoad* encodes a key enzyme for the metabolization of lipids and its expression is typically upregulated in diapause (Hassett

2006, Hassett et al. 2010, Freese et al. 2017). This matches the seasonal pattern observed in Loch Etive, but in Kongsfjorden *hoad* showed highest expression in active copepods whereas it was low during diapause. There is no clear explanation for this observation. Possibly, the low expression of *hoad* in diapause in Kongsfjorden relative to Loch Etive reflects the fact that *C. finmarchicus* resides in deeper, colder waters in the Arctic, causing a stronger reduction of metabolic activity and lower rates of lipid metabolization (Ingvarsdóttir et al. 1999, Clark et al. 2012, Maps et al. 2014).

Seasonal patterns of clock gene expression and rhythmicity

In both Loch Etive and Kongsfjorden cyclic expression of circadian clock genes was confined to the phase of activity in surface waters and the time just after the descent to diapause (Fig.3.8 & 4.3, Tab.3.4). Intuitively, this could be attributed to the lack of light in deeper water layers. However, clock rhythmicity was also found in deep waters in early diapause and it is actually possible that diel clock gene cycle persisted on the individual level throughout diapause, and was just not detected due the pooled analysis of desynchronized individuals. Clock genes were found to be rhythmically expressed in diapausing mosquitos kept under short day conditions (Meuti et al. 2015) and overwintering bears kept under constant light conditions still showed circadian rhythms of activity and body temperature (Jansen et al. 2016). The lack of clock rhythmicity in *C. finmarchicus* advected to the shallow layer of Loch Etive in autumn/winter does strongly suggest, that with the transition to diapause clock gene cycling was actively “switched off”, but individual-based clock gene measurements in diapausing copepods will be needed for a definitive answer. Considering the lack of clock rhythmicity in diapausing copepods, it is surprising that the overall expression levels of many clock genes (*clk*, *per1/2*, *tim*, *cry2*, *cwo*) were elevated during diapause in Loch Etive (Fig.3.7). Most of these genes are part of negative feedback loops within the circadian clock machinery and their proteins should accumulate in the darkness, inhibiting their own transcription (Mackey 2007, Matsumoto et al. 2007). In Kongsfjorden, most clock genes were upregulated in active animals matching the time at which the genes show diel rhythmicity. However, Loch Etive patterns were consistent over several seasonal time points, making an error unlikely. So far, it can only be speculated about the meaning of these seasonal changes in clock gene expression. If individual clocks are rhythmic but desynchronized within the population, the increased expression may support robust cycling under constant darkness, although the ecological benefits are unclear. Alternatively, the seasonal clock gene expression patterns could reflect an involvement in an unknown annual timing mechanism.

Endogenous clocks in *C. finmarchicus* diapause timing

As described in the general introduction (see section 1.1), the factors that control diapause and the seasonal life cycle of *C. finmarchicus* are poorly understood. The “lipid accumulation window” (LAW) hypothesis assumes that diapause is initiated when the lipid content of copepods exceeds a certain threshold and that animals that do not reach this threshold within a given time either molt to adults and reproduce or may enter diapause with insufficient lipid reserves leading to premature emergence (Rey-Rassat et al. 2002, Maps et al. 2014). However, this hypothesis is contradicted by the patterns of lipid content detailed in publication II as well as by the fact that is has so far not been possible to induce diapause in laboratory copepods fed *ad libitum* (Baumgartner & Tarrant 2017). Food limitation has also been proposed as diapause trigger (Hind et al. 2000, Wilson et al. 2016), but does not explain why in some population one fraction of animals enters diapause while the others mature, although they experienced the same food conditions (Durbin et al. 2000, Tarrant et al. 2008). In insects a common cue used for diapause timing is photoperiod (Goto 2013, Meuti & Denlinger 2013). The diel cycling in clock genes with defined peaks at sunrise and sunset suggests that *C. finmarchicus* possesses the ability to measure the environmental photoperiod via its circadian clock. The peaks times of clock genes in the field did however not consistently shift with changes in photoperiod, but seemed to be rather fixed with morning peaks around 6:00 and evening peaks around 18:00. This was especially evident from the patterns from Kongsfjorden in August 2016 (Fig.4.3). It has to be considered that samples were collected between 0 and 150 m and the subjective sunset may shift forward due to the attenuation of light with depth, but *per1* and *tim* peak at 16:00 and *cwo* even earlier. This suggests that photoperiod measurement in *C. finmarchicus* is based on the “external coincidence” model (Bünning 1960) that postulates a light sensitive phase, characterized e.g. by peak expression of one or more clock genes (Hazlerigg & Wagner 2006). Photoperiod measurement based on the “internal coincidence” model would be characterized by two expression peaks coinciding with sunrise/sunset and shifting according to the seasonal cycle of changing photoperiod (Pittendrigh 1960), but such seasonal shifts were not visible in the data. While in insects and other copepods photoperiodic diapause initiation typically happens under short day conditions (Watson & Smallman 1971, Hairston & Kearns 1995, Meuti & Denlinger 2013, Salminen et al. 2015), diapause of the first *C. finmarchicus* generation in Loch Etive was initiated in June when days were longest. This would suggest that a photoperiod of ~18h does still evoke a short day response. Similar critical photoperiods have been reported from other species and critical photoperiods generally increase with increasing latitude (Hut et al. 2013). Furthermore, the subjective photoperiod at depth may also be shorter than the time

between sunrise and sunset. Although photoperiod seems to be a suitable cue for the initiation of diapause in *C. finmarchicus*, it has to be considered that sufficient lipid reserves are essential for the copepods to survive the time of overwintering. It is thus likely that a combination of factors is responsible for diapause initiation. This could mean that once the lipid storages of the copepods exceed a critical threshold that is considered sufficient for diapause, the photoperiod at this time determined whether the animals descend to deeper waters or mature and reproduce. Furthermore, temperature does affect both copepod diapause induction at a given photoperiod (Watson & Smallman 1971, Hairston & Kearns 1995) as well as the rate of lipid consumption in diapausing *C. finmarchicus* (Pierson et al. 2013, Maps et al. 2014). It is thus possible that the critical lipid content for diapause initiation and the critical photoperiod change in response to ambient temperatures. Situations in which one fraction of a generation enters diapause while the other animals mature could be explained in two possible ways. First, if individuals differ in their rates of lipid accumulation in surface waters, they would reach the lipid threshold necessary for diapause at different times and would thus experience different photoperiods. Alternatively, it is possible that the response to a given photoperiod differs between individuals. Critical photoperiods can differ between populations of the same species (Marcus 1984, Hut et al. 2013) and it was shown that in *Drosophila* this is related to the presence of different alleles of the clock gene *tim* (Sandrelli et al. 2007). The existence of different alleles within a population would explain why a given photoperiod evokes diapause in one fraction of a generation while it triggers maturation in the other fraction. If both alleles are ecologically adaptive, they should persist and result in patterns as observed in Loch Etive and in other populations where different generations of copepods enter diapause at different times of the year (Durbin et al. 2000, Tarrant et al. 2008). *C. finmarchicus* populations can be distinguished genetically (Smolina 2015) and differences in clock gene alleles could also explain the many different patterns of seasonal diapause timing observed throughout the northern Atlantic. There is a high evolutionary pressure on proper diapause timing (Søreide et al. 2010), meaning that even if genetic exchange between populations exist, newly introduced alleles should be eliminated quickly, if they are maladaptive.

With regard to the emergence of *C. finmarchicus* from diapause, an “hourglass” timer has been proposed that triggers emergence when a defined period of time passed after the initiation of diapause (Miller et al. 1991, Campbell et al. 2004). Such a timer could be based on decreasing lipid levels or slow continuous development (Jónasdóttir 1999, Hind et al. 2000, Irigoien 2004, Saumweber & Durbin 2006). However, the fact that the two copepod generations in Loch Etive descended 3-4 months apart from each other and the

observation that copepods showed little change in lipid content during emergence contradict the first assumption (Fig.3.5D & 3.9). Furthermore, seasonal gene expression showed that diapause consisted of distinct phases that make slow continuous development unlikely. The constant darkness in the deep layer of the loch and the fact that in the open ocean *C. finmarchicus* diapauses at depth of up to 1000 m (Hirche 1996a) suggest that photoperiod is not a suitable emergence cue either. This leaves a circannual clock as the most probable mechanism for diapause emergence in *C. finmarchicus*. An endogenous rhythm of ~365 days could initiate emergence at a specific time of the annual cycle (e.g. in early winter) independent of the presence of a seasonal cue at this time. Circannual rhythms can run with high precision for several years under constant conditions allowing for a synchronized emergence process and they have been described in various species and it is generally believed that photoperiod is the dominant cue for their entrainment (Goldman et al. 2004). A link to the circadian clock has been suggested, but the mechanisms creating such long-term endogenous cycles are largely unknown (Lincoln et al. 2006). Laboratory emergence patterns of field collected *Calanus* suggest the circannual emergence could also exist in this genus (Conover 1965, Fulton 1973). In *C. finmarchicus* photoperiodic entrainment of the circannual cycle could happen before the descent to diapause when the circadian clock is still rhythmic. If two or more generation exist in a population that descent at different times, the different photoperiod experienced by these generations before descent would ensure a synchronization of the entire population and consequently emergence. In this context, it is intriguing that the expression of several circadian clock genes increased during diapause in Loch Etive and decreased again after the initiation of emergence (Fig.3.7). Assuming an involvement of the circadian clock in circannual regulation, the presence of different clock gene alleles as discussed for diapause initiation could also result in differences in the circannual timing of emergence between populations.

Future approaches to investigate diapause timing in *C. finmarchicus*

This dissertation provides many new insights into the seasonal cycle and diapause of *C. finmarchicus*, but still a mechanistic understanding of the regulation of diapause is missing. Based on the presented findings, future research should use laboratory incubation experiments in which conditions of light, temperature and food availability are modified to identify the parameters that lead to the initiation of diapause in *C. finmarchicus*. During these experiments lipid content, the expression of indicator genes like *fer*, *mdh* or *if4E* as well as clock rhythmicity should be monitored to determine critical lipid thresholds and the diapause status of the animals. This could result in a method to reliably induce *C. finmarchicus* diapause in the laboratory. Performing such investigations

on populations that differ in seasonal timing and collecting field samples from the respective populations would further help to understand how environmental factors shape diapause timing in a given habitat. Sequence analyses of clock genes in copepods from different populations or different generations from the same population could reveal allele variations that might explain differences in critical photoperiods. Addressing the mechanisms of diapause in emergence experimentally is more difficult as it means that copepods have to be kept in diapause under laboratory conditions. *C. finmarchicus* does typically not enter diapause in the laboratory and the handling stress during the field collection of diapausing copepods usually triggers awakening (Miller et al. 1991, Tarrant et al. 2008, 2016). If it becomes possible to induce diapause in the laboratory, long-term incubation of copepods under constant photoperiods or even constant darkness could grant insights into potential circannual timing. For this, it would be interesting to see how the photoperiod used to induce diapause affects the length of the resting phase. Creating a phase response curve of diapausing *C. finmarchicus* exposed to different photoperiod could further show whether photoperiod measurement is involved in emergence or not. For long-term experiments it is further recommended to monitor lipid content and gonad maturation to account for potential hourglass timers based on lipid depletion or slow continuous development. A general problem hard to overcome in laboratory experiments is the change in pressure than *C. finmarchicus* experiences during its descent to diapause. Gene expression data shows that diapause is initiated after the descent and it cannot be outruled that the increase in pressure and its effects on lipid density do affect copepod physiology and thus contributes to diapause initiation. Although long-term incubations under pressure are probably not possible, monitoring the changes in diapause indicator genes over shorter periods of exposure to water pressures experienced at overwintering depth in e.g. Loch Etive and a comparison with parallel control incubations under atmospheric pressure could give indication about the contribution of this factor to diapause (Zarubin et al. 2016). Additionally, a detailed monitoring of the environmental conditions that trigger diapause in the field will make it easier to resemble these conditions in the laboratory, leaving pressure as the only major variable differing between laboratory and field.

In summary, the seasonal investigations of *C. finmarchicus* showed that the copepods life cycle is characterized by the three phases of (1) activity and development, (2) diapause, and (3) emergence. Circadian clock rhythmicity was limited to the active phase in surface waters and gene expression data revealed several new aspect of the copepods seasonal cycle like the possible role of glycogen during the initiation of diapause. Although no definite factors responsible for the initiation and termination of diapause could be

identified, the obtained data suggests an interaction of photoperiod, lipid content and temperature responsible for diapause initiation, while emergence could be triggered by a circannual clock mechanism. Laboratory experiments manipulating several environmental factors in combination with long-term incubation will be necessary to reveal the relative contribution of these factors. The large variability in diapause timing may be addressed by the field sampling of different populations followed by laboratory characterizations of diapause timing and clock gene sequences.

5.4 *C. finmarchicus* rhythmicity at high latitudes and ecological consequences

The center of distribution for *C. finmarchicus* is the northern Atlantic, but over the past decades the species' latitudinal range has shifted northward into Arctic waters (Reygondeau & Beaugrand 2011, Wilson et al. 2015, Chivers et al. 2017). This shift has been attributed to increasing ocean temperatures and an increased Atlantification of polar waters (Falk-Petersen et al. 2007, Chivers et al. 2017) and similar poleward distribution shifts have been observed in many other marine species including various planktonic organisms and several commercially important fish stocks (Corten 2001, Hays et al. 2005, Perry et al. 2005, Chivers et al. 2017). High latitude environments pose a special challenge to endogenous clock systems due to the extreme seasonal variations in light conditions. This raises the question how this will affect diel and seasonal rhythmicity in boreal species like *C. finmarchicus* when their distributions shift poleward. The findings of this dissertation indicate that the extreme polar light conditions have only minor effects on the copepods circadian clock functioning and diel rhythmicity, and that this could be a general pattern in marine species due to the nature of their aquatic habitat. However, latitudinal shifts could result in a mismatch of seasonal timing, if based on endogenous clock mechanisms and photoperiod measurement. Such situations may further be aggravated by changes in environmental parameters like the timing of the sea-ice breakup. This could have severe consequences for the fitness of *C. finmarchicus* and hence its biomass, with major impacts on the pelagic ecosystems the copepod inhabits. To predict the copepods fitness in polar waters, it will be necessary to assess the adaptive capacity of *C. finmarchicus'* clock mechanisms and to explore special adaptations in the clock systems of the polar congeners *C. glacialis* and *C. hyperboreus*.

Clock functioning and diel rhythmicity under polar light conditions

Diel clock gene patterns of copepods collected in Kongsfjorden during the active summer phase show that *C. finmarchicus* does maintain circadian clock rhythmicity under photoperiods close to 24h. However, several terrestrial polar species were reported to

become arrhythmic during midnight sun (Reierth & Stokkan 1998, Lu et al. 2010). ADCP data on zooplankton DVM in the fjords of Svalbard showed arrhythmicity from ~May until late August with synchronized vertical migrations resuming thereafter (Cottier et al. 2006, Wallace et al. 2010). It is thus possible that by the time of the sampling in late August the circadian clock of *C. finmarchicus* had just regained rhythmicity after a period of arrhythmicity in summer. However, rhythmic DVM of *C. finmarchicus*, based on net catch analyses, has been reported during midnight sun (Dale & Kaartvedt 2000). A diel cycle of light intensity persists throughout summer (Wallace et al. 2010) and the subjective photoperiod experienced by marine organisms decreases with depth due to the attenuation of light (Miller et al. 1991). Thus the photoperiod experienced by the copepods was probably shorter than at the surface and it seems possible that the circadian clock of *C. finmarchicus* stayed rhythmic throughout the midnight sun period. If clock rhythmicity was maintained, the cessation of rhythmic DVM in summer needs to be investigated. The reports of arrhythmic vertical migrations are mostly based on ADCP recordings that describe the distribution of biomass (as backscatter) in the water column but do not resolve the migrations of specific species or individuals. Findings by Cottier et al. (2006) suggest that vertical migrations are present in summer, but are desynchronized, resulting in no diel change in net backscatter patterns that could be recorded via ADCP. The authors argued that this could be the results of the combined effects of hunger and predation risk. Considering the rhythmic clock gene expression of *C. finmarchicus* and the pooling of copepods for analysis it seems possible that different zooplankton species are desynchronized during midnight sun, while synchronization persist within the species. This seems however questionable as predators that feed only on specific species could still find their prey in high density and in the presence of light, eliminating the advantage of synchronized DVM. In contrast, a desynchronization of individuals of the same species would result in low prey densities and ineffective foraging for the predator. If the DVM patterns of *C. finmarchicus* individuals are desynchronized during midnight sun while their circadian clocks are “ticking” in synchrony, this could mean that migration behavior is decoupled from clock rhythmicity and is primarily driven by other factors like hunger at this time. Maintaining clock rhythmicity is probably still beneficial for coordination of different metabolic processes over the diel cycle (Panda et al. 2002, Albrecht 2006).

The reduced rhythmicity in clock gene expression in early diapause and the complete absence of rhythmicity in late diapause during the polar night mirrors the patterns observed in Loch Etive. Light levels below 200 m in September (early diapause) were probably well above the copepods detection limit (Båtnes et al. 2013), supporting the

findings from Loch Etive, which indicate that the cessation of clock rhythmicity in diapause is related to physiological changes and not the result of a lacking entrainment cue. Thus the lack of light during the polar night has probably little direct effects on the clock of diapausing *C. finmarchicus*.

Adaptability of *C. finmarchicus*' diel rhythmicity to extreme photoperiods

The change in subjective photoperiod with depth does affect all marine organisms and this could mark a general difference between marine and terrestrial polar systems. Clock adaptability to extreme photoperiods has been considered a limiting factor for the poleward range expansion of terrestrial species (Saikkonen et al. 2012). This problem may be less severe in marine species due to the attenuation of light with depth that results in shorter, less extreme photoperiods in summer. Following this logic, the time of polar night in winter should result in a cessation of rhythmicity due to the lack of light. However, shallow DVM was detected during the sampling in Kongsfjorden in January (Fig.4.6C). Other studies report similar migration patterns during the polar night (Berge et al. 2009, Darnis et al. 2017) as well as diel activity cycles in benthic invertebrates in shallow waters (Berge et al. 2015, Tran et al. 2016). This indicates that the weak diel change in light levels during the polar night is sufficient to evoke synchronized diel rhythmicity in marine environments and rhythmicity may further be supported by other cues like moonlight (Last et al. 2016). It seems possible that the diel rhythmicity of marine species, that show poleward shift in distribution due to e.g. increasing temperatures, is less affected by the extreme polar light conditions than are terrestrial species, easing the occupation of new high latitude habitats. Nevertheless, a latitudinal distribution shift results in a change of the environmental photoperiod to which the circadian clock system has to adapt. *C. finmarchicus* inhabits a wide latitudinal range reflecting a high variability in diel rhythmicity (Conover 1988, Helaouët & Beaugrand 2007, Melle et al. 2014). However, it is unknown whether this reflects the general plasticity of the circadian clock, or whether it is the result genetic population differences like the presence of different clock gene alleles (Sandrelli et al. 2007). If the latter is true, this could slow down the occupation of high latitude environments as it cannot be predicted how long it would take until copepods with suitable clock gene alleles establish in a polar habitat. Thus the adaptive capacity of the circadian clock of *C. finmarchicus* and other marine species is a crucial feature to predict future changes in distribution and diel rhythmicity in polar habitats.

Clock-based seasonal timing under polar light conditions

Photoperiod is a major cue for the timing of seasonal life cycles (Goto 2013, Meuti & Denlinger 2013) and a distribution shift to higher latitudes with more extreme seasonal

changes in photoperiod could result in the improper timing of life cycle event. For numerous insect species it was shown that the critical photoperiod, at which diapause is initiated, is specific for any given population and increases with latitude (Hut et al. 2013). Species that rely on photoperiod measurement for seasonal timing could thus initially struggle to expand their distribution to higher latitudes as their seasonal cycles may no longer match the environmental annual cycle. At high latitudes the phytoplankton bloom is short but intense due to sea-ice cover and the strong seasonal change in illumination (Leu et al. 2006, 2011). This means that precise seasonal timing is crucial to benefit from the available food as much as possible (Søreide et al. 2010). If photoperiod measurement is involved in the seasonal timing of diapause initiation and termination in *C. finmarchicus*, the rigidity of this mechanism could become a disadvantage. The phytoplankton bloom occurs later at higher latitudes (Leu et al. 2011), meaning that boreal copepods could emerge and reproduce or enter diapause too early to optimally benefit from primary production. If differences in the seasonal timing in *C. finmarchicus* populations are based on differences in clock gene alleles as proposed in publication II and III (see sections 3.5 & 4.5), the lack of alleles ensuring proper seasonal timing in newly occupied high latitude habitats could prevent the copepods from completing their seasonal reproduction cycle successfully. The strong selective pressure on precise seasonal timing would however mean that as soon as alleles suitable for the habitats emerge, they will likely spread fast within the population. In this context, it is unclear in which way the warming of polar marine environments will affect the success of *C. finmarchicus* in its new high latitude habitat. The warming of polar seas has led to a shift in seasonal environmental timing with sea-ice breakup and peak phytoplankton production occurring up to two months earlier than a decade ago (Leu et al. 2011, Kahru et al. 2011). This may benefit species like *C. finmarchicus* that enter the Arctic from boreal habitats were blooms typically occur earlier, meaning that less adaptation in seasonal timing is needed. However, the trend of warming and sea ice reduction is expected to continue (Stroeve et al. 2012) with the timing of phytoplankton blooms shifting even further. Thus populations that seem adapted to polar conditions like the *C. finmarchicus* population in Kongsfjorden would experience further environmental shifts, forcing them to adjust their seasonal timing. Photoperiod-based seasonal regulation of life cycle events does probably not allow for immediate responses. The plasticity of seasonal timing in *C. finmarchicus* and its congener *C. glacialis* seems to be limited as interannual variability shows that both an earlier sea-ice breakup as well as an unusually long persistence of ice can result in a seasonal mismatch and low copepod biomass for the respective year (Søreide et al. 2010, Leu et al. 2011). Sea-ice conditions are highly variable between years, posing a challenge to seasonal timing mechanisms that rely on

predictable parameters like photoperiod (Laxon et al. 2003). The emergence from diapause in *C. finmarchicus* starts well before the copepods ascent to the surface and reproduce (Tande 1982, Hirche 1996a,b, Rey-Rassat et al. 2002). Thus *C. finmarchicus* has to initiate the emergence process without information about the timing of phytoplankton availability for the respective year, meaning that the capacity to respond to the sea-ice breakup spontaneously is probably very limited. Native polar marine species may have developed adaptation mechanisms to this inter-annual variability. Therefore, comparing the mechanisms of seasonal timing between boreal species like *C. finmarchicus* and polar species like *C. glacialis* and *C. hyperboreus* could grant valuable insights into the seasonal timing in a polar marine environment.

Effects of climate change and ecological consequences

Arctic marine ecosystems will likely face future changes in community composition due to increasing temperatures and the stronger input of Atlantic water (Falk-Petersen et al. 2007). While polar species like *C. glacialis* and *C. hyperboreus* will have to retreat further poleward to avoid unfavorable temperatures, boreal species like *C. finmarchicus* will become more dominant (Reygondeau & Beaugrand 2011, Feng et al. 2017). Boreal species often represent a lower quality food source for higher trophic levels than polar ones, as reflected in the pronounced differences in lipid content between *C. finmarchicus* and its Arctic congeners (Conover & Huntley 1991, Falk-Petersen et al. 2009). Additionally, the findings discussed in this dissertation suggest that the potential use of endogenous timing mechanisms for the regulation of diel and seasonal rhythmicity in *C. finmarchicus* could limit the copepods adaptability to high latitude environments. Hence, poleward expansions of distribution range could be slowed down or even confined by the capacity of clock systems. This means that if the energy-poor *C. finmarchicus* replaces its polar congeners, its abundance and/or biomass may be even lower than expected based on phytoplankton availability. Thus the shift of Arctic zooplankton communities to systems dominated by *C. finmarchicus* will most likely reduce the energy available to higher trophic levels and could have severe consequences for the abundance and distribution of species preying on *Calanus* (Falk-Petersen et al. 2007, Steen et al. 2007, Jakubas et al. 2017). This could also have negative effects on commercially important fish species for which *Calanus* species are an essential part of their diet (Prokopchuk & Sentyabov 2006, Benoit et al. 2010). A reduction in copepod biomass in Arctic pelagic ecosystems would further affect the biogeochemical cycling of carbon. Both DVM and the seasonal vertical migration of *Calanus* contribute significantly to the biological carbon pump and an impairment of these rhythms under the polar environmental conditions would reduce the amount of carbon sequestered to the ocean depth (Plourde & Runge 1993, Steinberg et

al. 2000, Darnis & Fortier 2012, Jónasdóttir et al. 2015). To predict how climate change will affect Arctic marine ecosystems and biogeochemical fluxes it is thus essential to reach a better understanding of the mechanisms controlling diel and seasonal rhythmicity in key species like *C. finmarchicus*.

Future approaches to investigate the adaptive capacity of *Calanus* clock systems

The sections 5.1 and 5.3 discussed how the factors and processes controlling diel and seasonal rhythmicity in *C. finmarchicus* could be identified. To investigate how these timing mechanisms function in polar environments, it will be crucial to explore the limits of their adaptability. Circadian clock entrainment capacity could be investigated by exposing copepods to extremely long or short photoperiod and determining the photoperiods at which long-term diel rhythmicity (e.g. clock gene cycling or swimming activity) can no longer be maintained. By exposing copepods to permanent light with diel oscillations in light intensity it could further be possible to resemble midnight sun and determine whether the circadian clock and DVM stay rhythmic under these conditions. The adaptive capacity of seasonal timing mechanisms is difficult to address in the laboratory as it would require long-term incubations of copepods adapted to a given seasonal regime and exposing them to an altered seasonal environment of photoperiod and possibly temperature. Monitoring animal fitness, clock gene allele frequencies, the seasonal timing of diapause, and how these parameters might change over several generations could grant insights into the copepods ability to adapt to polar environments as well as into its capacity to cope with inter-annual variability.

Generally, it is highly recommended to compare the entrainment capacity of clocks between populations from different latitudes (e.g. from Loch Etive and Kongsfjorden) as the critical photoperiod of a species can change strongly with latitude (Hut et al. 2013). To identify special adaptations of clock systems in polar marine species it would further be highly beneficial to compare *C. finmarchicus*' timing systems to those of *C. glacialis* and *C. hyperboreus*, which are native to polar habitats. Studies on *Drosophila* species from different latitudes showed that the circadian clocks of high latitude fly species have a weaker endogenous rhythmicity and are less light sensitive (Menegazzi et al. 2017, Kyriacou 2017). While this caused arrhythmic behavior under constant darkness, rhythmicity under constant light was more robust. Weaker circadian rhythmicity was repeatedly reported for high latitude terrestrial species (Hut et al. 2013, Kauranen et al. 2016, Vaze & Helfrich-Förster 2016), but it is unclear if this pattern also exists in the marine realm. Exploring mechanistic differences in the circadian clocks and the seasonal timing systems of different *Calanus* species and determining their robustness and

responsiveness to environmental parameters could reveal adaptations specific to the polar environment.

In conclusion, this section suggests that *C. finmarchicus* possesses a robust circadian clock that is probably able to maintain rhythmicity over the phase of midnight sun. As clock rhythmicity in *C. finmarchicus* generally seems to cease in diapause, the lack of light during polar night has probably little effect on the copepod. The attenuation of light with depth could support diel rhythmicity in aquatic environments during midnight sun, leading to the general suggestions that poleward distribution shifts of *C. finmarchicus* will likely not be hindered by impairments in circadian clock functioning and diel rhythmicity. This may also apply to other marine organisms and could be a general difference between aquatic and terrestrial systems. The involvement photoperiod-related endogenous timing in the regulation of seasonal rhythmicity could result in mismatch situations and reduced copepod fitness under the polar light regime. This could reduce the overall productivity of Arctic pelagic ecosystems with the effects potentially aggravated by climate change. It is therefore crucial to determine the adaptive capacity of the endogenous timing systems of *C. finmarchicus* and its Arctic congeners.

5.5 Summary and future directions

The present dissertation highlights the role of endogenous timing mechanisms in marine species by the example of the northern Atlantic key zooplankton species *Calanus finmarchicus*. Coming back to the initial research objectives stated in the general introduction (see section 1.4), it could be shown that *C. finmarchicus* possesses an endogenous circadian clock that affects diel rhythms of metabolic activity as well as vertical migration behavior, thus raising the awareness of the relevance of endogenous timing for the regulation of large-scale ecological rhythms like DVM. The dissertation further provides one of the most detailed characterizations of the seasonal life cycle of *C. finmarchicus* to date and underlines the importance of considering photoperiod and endogenous timing mechanisms as factors for the regulation of seasonal rhythmicity. The work from the high Arctic Kongsfjorden shows that the copepods circadian clock is robust under extremely long photoperiods, but also raises concerns about the adaptive capacity of diel and especially seasonal rhythmicity of this boreal species to the polar environment. This dissertation illustrates the enormous potential of applying molecular methods well established for terrestrial model species to marine organisms of high ecological relevance, and of combining such measurements with integrative analysis of physiological parameters and field investigations. It does however also emphasize the limitations of the current knowledge about the rhythmicity regulation in marine species

like *C. finmarchicus*, as illustrated by the still existing lack of understanding of the exact mechanisms controlling diapause timing in the copepod.

It becomes evident that a mechanistic knowledge about marine clock systems is the basis for understanding how clocks shape the rhythmic phenotypes of individuals. In this context it is also crucial to explore how clock systems will respond to changes in environmental parameters like light, temperature or CO_2. While investigations of the molecular functioning of clock systems will depend on detailed laboratory investigations and manipulative approaches, field studies will be essential to verify the laboratory finding under natural conditions and to assess the environmental influence on individual rhythmicity and large-scale patterns such as DVM or seasonal life cycles. By implementing the obtained knowledge about clock functioning in the natural environment into population models of *C. finmarchicus* and other marine species, it will then be possible to make projections on how environmental changes will affect an organism's rhythmicity and interactions with other species. The ecological key position of *C. finmarchicus* in combination with its strong diel and seasonal rhythmicity and the changing nature of its environment make the copepod a prime candidate for future investigations of marine clock systems.

5.6 References

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Erklärung des Autors

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Ich erkläre hiermit,

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

(Sören Häfker)

Appendix

A1 Supplemental material to Publication I

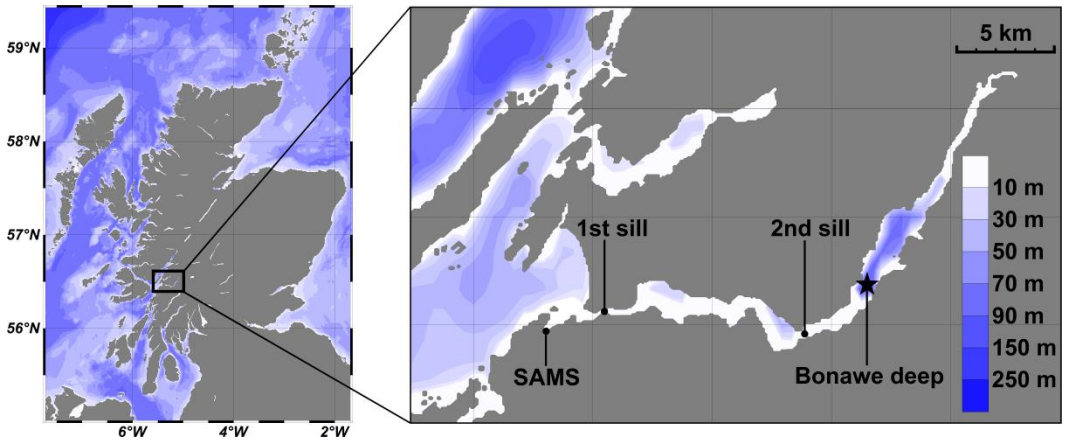

Figure A1.1: Geographical characteristics of the study area. Related to Fig.2.1. Loch Etive is a sea loch at the western coast of Scotland, UK (56°45´N, 5°18´W). Water exchange with the ocean is limited by two sills. All samplings as well as the mooring deployment were done at the deepest point of the loch, Bonawe deep (~145 m), at the Scottish Association for Marine Science (SAMS) permanent station RE5. Laboratory studies were conducted at SAMS, in close proximity to Loch Etive. Maps were created with Ocean Data View (v. 4.7.4, Schlitzer 2015).

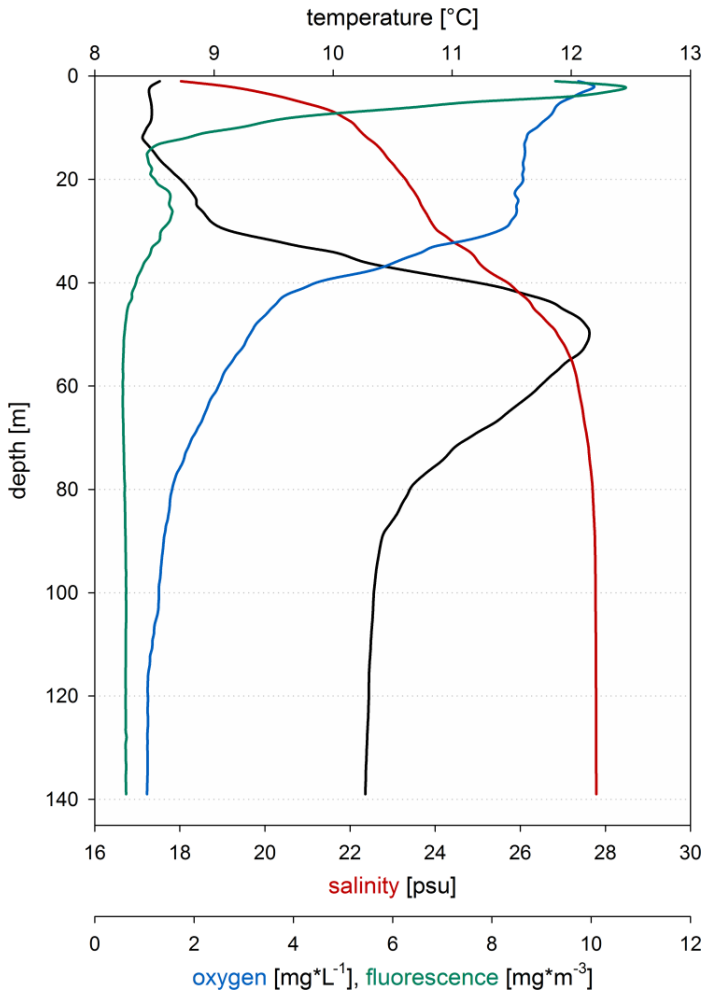

Figure A1.2: Water column characteristics at the study site. Related to Fig.2.1. Data was pooled from three CTD hauls conducted during the 28h field sampling campaign on the 6th/7th May 2015 at Bonawe deep. Mean values of the pooled hauls are shown.

Table A1.1: Statistical rhythm analysis of DVM and respiration. Related to Fig.2.2. Analysis was performed with the R-package "RAIN" (Thaben & Westermark 2014). Bold values indicate significant 24h rhythmicity ($p < 0.05$). A subscript m ($_m$) indicates results derived from mean value analysis.

	Rhythm analysis (24h, **α = 0.05**)			
Phenotype	LD	DD	DD - day1	DD - day2
DVM	**< 0.001**	**0.039$_m$**	**< 0.001**	**0.002**
respiration	**< 0.001**	**< 0.001$_m$**	**< 0.001**	**< 0.001**

Table A1.2: List of investigated genes. Related to Fig.2.3. Gene sequences were obtained from Lenz et al. 2014 and Christie et al 2013. Genes were normalized against the geometric mean of the housekeeping genes elongation factor 1 α, RNA polymerase and actin. Rhythm analysis was performed with the R-package "RAIN" [S2]. Bold values indicate significant 24h rhythmicity ($p < 0.001$). For the sake of clarity, p-values > 0.05 are not shown (–).

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

A2 Supplemental material to Publication II

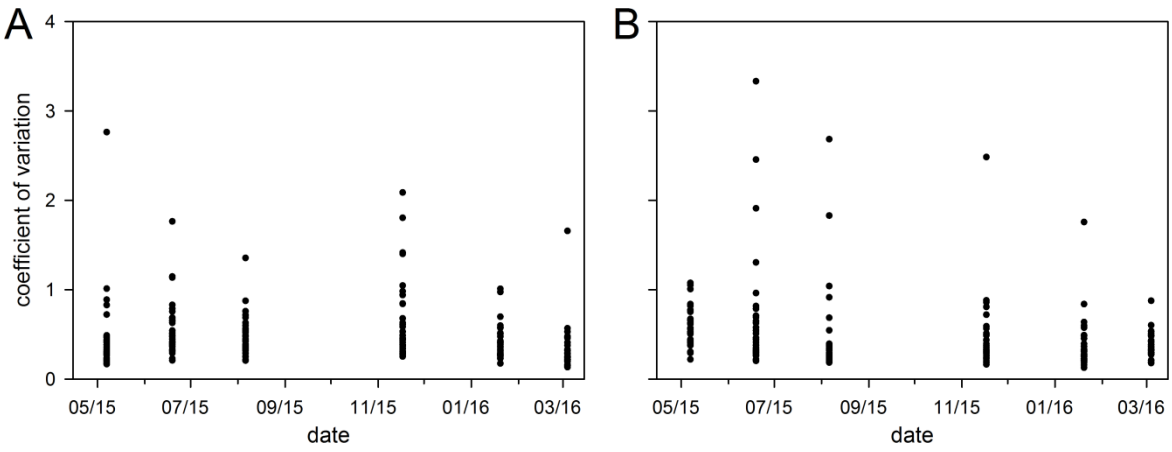

Figure A2.1: Seasonal change in gene expression variance. Related to Fig.3.7. Coefficients of variation (SD divided by mean) in (A) the shallow layer and (B) the deep layer for all genes are shown. Date labels indicate the 1st day of the respective month. Each dot represents one gene at the respective seasonal time point. Note that while some genes show increased variation at specific seasonal time points (e.g. November/shallow, June/deep), the variance for most of the genes stayed consistently low throughout our study. This applies also to the phase of emergence in January/March 2016.

Table A2.1: List of investigated genes. Related to Fig.3.7. Gene names, abbreviations and physiological functions are shown. Gene sequences were taken from a *C. finmarchicus de novo* transcriptome (Christie et al. 2013, Lenz et al. 2014). All annotations were verified via blastx against NCBI database and the respective top-hit as well as the e-value and sequence identity are shown. Housekeepers are listed at the bottom.

Calanus finmarchicus				**NCBI top-hit**		**Comparison**	
Gene	**Abbrev.**	**Function**	**Accession No°**	**Species**	**Accession No°**	**e-value**	**Identity**
clock	*clk*	clock gene	GAXK01092177.1	*T. domestica*	BAJ16353.1	6e-152	61%
cycle	*cyc*		GAXK01131751.1	*A. gambiae*	XP_556301.3	8e-177	48%
period1	*per1*		GAXK01127710.1	*M. siamensis*	BAI47546.1	6e-96	32%
period2	*per2*		GAXK01015947.1	*R. maderae*	AGA01525.1	9e-88	41%
timeless	*tim*		GAXK01195225.1	*D. erecta*	XP_001968572.1	0.0	38%
cryptochrome2	*cry2*		GAXK01199676.1	*S. invicta*	AGD94517.1	0.0	80%
clockwork orange	*cwo*		GAXK01116566.1	*L. decemlineata*	AKG92774.1	8e-52	34%
vrille	*vri*		GAXK01130166.1	*C. marinus*	AFS34627.1	3e-44	61%
cryptochrome1	*cry1*	clock light input	GAXK01107177.1	*A. mississippiensis*	XP_006275665.1	0.0	64%
doubletime2	*dbt2*	clock-associated	GAXK01058829.1	*O. mykiss*	CDQ75112.1	2e-174	77%
casein kinase II α	*ck2α*		GAXK01065631.1	*P. nana*	AII16523.1	0.0	91%
PP2A subunit twins	*tws*		GAXK01019902.1	*S. paramamosain*	AFK24473.1	0.0	89%
PP2A subunit widerborst	*wbt1*		GAXK01125267.1	*C. gigas*	EKC28886.1	0.0	75%
shaggy	*sgg*		GAXK01013351.1	*C. floridanus*	XP_011268375.1	0.0	85%
phosphofructokinase	*pfk*	glycolysis	GAXK01138511.1	*A. aegypti*	XP_001652300.1	0.0	65%
phosphoenolpyruvate carboxykinase	*pepck*	gluconeogenesis	GAXK01051558.1	*H. laboriosa*	KOC67158.1	0.0	60%
citrate synthase	*cs*	citric acid cycle	GAXK01175199.1	*L. salmonis*	ACO12464.1	0.0	82%
malate dehydrogenase	*mdh*		GAXK01175724.1	*L. salmonis*	ACO12495.1	8e-162	76%
glutamate dehydrogenase	*gdh*	nitrogen metabolism	GAXK01100997.1	*T. californicus*	AFN54260.1	0.0	76%
fatty acid-binding protein	*fabp*	lipid anabolism	GAXK01168773.1	*C. clemensi*	ACO15363.1	1e-32	49%
elongation of very long chain fatty acids protein	*elov*		GAXK01161646.1	*M. rotundata*	XP_003700522.1	5e-79	51%

Table A2.1 (continued).

Calanus finmarchicus				NCBI top-hit		Comparison	
Gene	**Abbrev.**	**Function**	**Accession No°**	**Species**	**Accession No°**	**e-value**	**Identity**
2-hydroxyacyl-CoA lyase	*hacl*	lipid catabolism	GAXK01181632.1	*P. xylostella*	XP_011549888.1	0.0	54%
3-hydroxyacyl-CoA dehydrogenase	*hoad*		GAXK01110478.1	*C. gigas*	XP_011451193.1	1e-53	43%
ATP synthase γ subunit	*ATPsyn*	respiration chain	GAXK01169235.1	*L. salmonis*	ACO12344.1	3e-121	69%
arginine kinase	*argk*	anaerobic metabolism	GAXK01169213.1	*N. denticulata*	BAH56608.1	0.0	83%
eukaryotic translation initiation factor 4E	*if4E*	translation	GAXK01100448.1	*Z. nevadensis*	KDR11192.1	2e-72	55%
glutathione peroxidase	*gshpx*	stress response	GAXK01101248.1	*L. polyphemus*	XP_013772703.1	5e-52	52%
ferritin	*fer*		GAXK01170130.1	*P. annandalei*	AGT28487.1	4e-86	74%
ecdysteroid receptor	*ecr*	molting	GAXK01076616.1	*C. finmarchicus*	ABQ57403.1	2e-111	100%
cyclin B	*cyclB*	cell cycle	GAXK01175362.1	*C. carpio*	ABX89586.1	1e-84	42%
couch potato	*cpo*	diapause-associated	GAXK01052139.1	*C. finmarchicus*	DAA64514.1	5e-117	99%
chymotrypsin	*chtrp*	digestion	GAXK01169625.1	*D. pulex*	EFX79589.1	3e-75	49%
opsin	*ops*	light perception	GAXK01018191.1	*T. californicus*	ADZ45237.1	9e-158	62%
pigment-dispersing hormone receptor	*pdhr*		GAXK01203172.1	*A. aegypti*	XP_001653651.2	6e-73	38%
hemocyanin subunit	*hc*	blood O_2 transport	GAXK01053068.1	*N. inaurata*	CAD68057.1	2e-106	36%
elongation factor 1 α	*ef1α*	housekeeper	GAXK01169633.1	*M. demolitor*	XP_008547400.1	0.0	86%
ribosomal protein S13	*rps13*		GAXK01165987.1	*C. sinicus*	ALS04988.1	1e-103	100%
RNA polymerase II	*RNApoly*		GAXK01026612.1	*C. vicinus*	BAO20839.1	0.0	83%

Table A2.2: Changes of gene expression over the seasons and with depth. Related to Fig.3.7. For each gene, significant differences between seasonal time points identified via Kruskal-Wallis ANOVA on Ranks are indicated by different capital letters (A,B,…) with relative expression decreasing in alphabetical order. If no letters are shown, the Kruskal-Wallis ANOVA was insignificant. The seasonal time point with maximum expression is marked with an asterisk (*) at the respective capital letter. Significant differences between depth layers identified via Mann-Whitney U test are represented by arrows indicating higher expression in the shallow layer (↑), higher expression in the deep layer (↓) or no insignificant difference (–). max mRNA level indicates the specific maximum expression for the respective gene (minimum = 1.0). Per individual gene, month, and depth *n* = 36-40 replicates were measured. A detailed characterization of genes is given in Table A2.1.

		Statistical analyses (α = 0.0001)						**max mRNA level**
Gene	**Depth**	**May**	**Jun**	**Aug**	**Nov**	**Jan**	**Mar**	
clk	shallow	AB*	AB	BC	A	CD	D	3.91
		↑	–	↓	–	↓	↓	
	deep	CD	AB	A	A*	BC	D	3.42
cyc	shallow	A*	A	A	AB	B	B	6.90
		↑	–	↑	–	–	–	
	deep	A	A*	AB	ABC	BC	C	4.12
per1	shallow	B	B	B	A*	B	C	4.38
		–	↓	↓	↓	↓	↓	
	deep	BC	A*	A	A	B	C	7.46
per2	shallow	A*	AB	B	AB	C	C	8.41
		–	–	–	–	↓	↓	
	deep	A	A*	A	A	B	B	7.22
tim	shallow	B	B	B	A*	B	C	5.06
		–	↓	↓	↓	↓	↓	
	deep	C	AB*	AB	A	B	C	9.59
cry2	shallow	B	B	B	A*	AB	C	6.98
		–	↓	↓	↓	↓	↓	
	deep	C	AB	AB	A*	B	C	13.09
cwo	shallow	AB	AB*	AB	A	BC	C	3.79
		–	–	–	–	↓	–	
	deep	BC	A*	A	B	B	C	4.10
vri	shallow	AB	A*	AB	BC	BC	C	2.75
		–	↓	↓	↓	–	–	
	deep	CD	A*	AB	BC	CD	D	5.22
cry1	shallow	B	AB	AB	B	AB	A*	1.60
		–	–	–	–	–	–	
	deep	B	B	Ab	AB	AB	A*	1.76
dbt2	shallow	A	A*	AB	BC	BC	C	3.19
		↑	–	–	–	–	–	
	deep	B	A*	AB	B	BC	C	3.81
ck2α	shallow	A	A	A	B	A	A*	1.63
		–	↑	↑	–	–	–	
	deep	AB*	BC	C	C	BC	A	1.55
tws	shallow	AB	A*	AB	C	B	A	4.40
		–	↑	↑	–	↑	↑	
	deep	A*	BC	CD	D	CD	AB	3.71

Table A2.2 (continued).

Gene	Depth	Statistical analyses (α = 0.0001) May	Jun	Aug	Nov	Jan	Mar	max mRNA level
wbt1	shallow	–	–*	–	–	–	–	1.12
		–	↓	↓	↓	–	–	
	deep	BCD	A*	ABC	AB	CD	D	1.64
sgg	shallow	AB	AB	B	A*	B	C	2.77
		–	↓	↓	↓	↓	↓	
	deep	CD	A	AB	A*	BC	D	4.16
pfk	shallow	A*	Ab	AB	BC	C	C	12.94
		↑	↑	↑	↑	–	–	
	deep	A*	AB	B	C	C	C	7.51
pepck	shallow	–	–*	–	–	–	–	2.87
		–	–	–	↓	–	–	
	deep	B	A*	B	B	C	BC	4.28
cs	shallow	A*	A	AB	B	AB	A	1.56
		–	–	–	–	–	–	
	deep	AB	BC	BC	ABC	C	A*	1.59
mdh	shallow	A	A	A	B	A	A*	3.23
		–	↑	↑	–	↑	–	
	deep	A*	B	B	B	B	A	3.14
gdh	shallow	A*	B	B	AB	C	C	10.78
		↑	↓	↓	↓	↓	↓	
	deep	BC	A*	AB	A	C	D	12.24
fabp	shallow	A*	AB	AB	BC	CD	D	16.44
		↑	↑	↑	↑	↓	↓	
	deep	A*	A	A	A	A	B	9.96
elov	shallow	A*	A	AB	BC	CD	D	5477.14
		↑	↑	↑	↑	↓	↓	
	deep	A*	B	BC	B	CD	D	2692.01
hacl	shallow	A*	AB	AB	B	C	C	9.93
		–	↑	↑	–	–	–	
	deep	A*	AB	BC	BC	C	C	7.11
hoad	shallow	A*	AB	BC	AB	CD	D	4.62
		↑	–	↓	↓	↓	↓	
	deep	C	A*	AB	A	BC	D	5.39
ATPsyn	shallow	A*	AB	B	C	B	B	2.11
		–	↑	↑	–	↑	–	
	deep	A*	B	B	B	B	A	2.33
argk	shallow	BC	C	C	AB*	C	D	3.95
		–	↓	↓	↓	↓	↓	
	deep	BC	A	A	A*	AB	C	5.80
if4E	shallow	B	AB	AB	C	AB	A*	2.89
		–	↑	↑	–	↑	–	
	deep	AB	D	CD	D	BC	A*	2.85
gshpx	shallow	A	A	A	A*	B	B	9.47
		↑	–	–	–	↓	↓	
	deep	B	A*	A	Á	BC	C	12.31
fer	shallow	D	BC	BC	A*	AB	CD	11.05
		↓	↓	↓	↓	↓	↓	
	deep	C	A*	A	A	B	BC	31.23

Table A2.2 (continued).

Gene	Depth	Statistical analyses (α = 0.0001) May	Jun	Aug	Nov	Jan	Mar	max mRNA level
ecr	shallow	B	AB	B	C	B	A*	4.04
		–	↑	↑	–	–	–	
	deep	AB	C	C	BC	BC	A*	3.57
cyclB	shallow	B	B	B	C	AB	A*	20.11
		–	↑	↑	–	↑	–	
	deep	AB	C	C	C	B	A*	17.45
cpo	shallow	B	B	AB	A*	B	C	3.34
		–	↓	↓	–	↓	↓	
	deep	CD	AB	A	A*	BC	D	4.25
chtrp	shallow	A*	AB	B	B	C	C	68.08
		–	↑	↑	↑	↑	↑	
	deep	A*	CD	BC	D	BCD	AB	47.92
ops	shallow	C	C	BC	A*	AB	D	5.21
		–	↓	↓	–	↓	↓	
	deep	B	A	A	A*	A	B	8.81
pdhr	shallow	A	A	A	A*	B	B	4.12
		–	↓	↓	–	–	↑	
	deep	BC	A*	AB	B	CD	D	6.88
hc	shallow	D	BC	BC	A*	AB	CD	21.81
		↓	↓	↓	–	↓	↓	
	deep	B	A	A	A*	A	B	24.41

A3 Supplemental material to Publication III

Table A3.1: Gene expression in different life phases of *C. finmarchicus* (stage CV). Related to Fig.4.4. For each gene, the abbreviation (Abbrev.), the physiological function, as well as the mean expression ± SD in the three life phases are shown. Genes were analyzed individually and compared via Kruskal-Wallis ANOVA on Ranks and Dunn's post-hoc test. Significant differences are indicated by different numbers of asterisks (∗). In cases where two life phases differ significantly while the third is equal to both of them (e.g. *per1*), the asterisk is shown in brackets.

Gene	Abbrev.	Function	Active phase	Early diapause	Late diapause
clock	*clk*	clock gene	∗ 3.46 ± 1.37	1.23 ± 0.34	1.00 ± 0.35
cycle	*cyc*		∗ 3.22 ± 1.96	1.00 ± 0.40	1.31 ± 0.57
period1	*per1*		∗ 2.22 ± 0.91	(∗) 1.49 ± 0.46	1.00 ± 0.37
period2	*per2*		∗∗ 5.54 ± 1.44	∗ 2.51 ± 0.68	1.00 ± 0.37
timeless	*tim*		∗ 2.69 ± 0.90	1.49 ± 0.29	1.00 ± 0.40
cryptochrome2	*cry2*		∗ 2.02 ± 0.42	1.38 ± 0.27	1.00 ± 0.32
clockwork orange	*cwo*		∗ 3.76 ± 1.79	1.00 ± 0.27	1.29 ± 0.54
vrille	*vri*		1.21 ± 0.32	∗ 1.71 ± 0.46	1.00 ± 0.26
cryptochrome1	*cry1*	clock light input	∗ 2.58 ± 1.18	1.00 ± 0.36	1.22 ± 0.53
doubletime2	*dbt2*	clock-associated	∗ 2.94 ± 0.78	1.23 ± 0.32	1.00 ± 0.34
casein kinase II α	*ck2α*		∗ 1.52 ± 0.23	1.00 ± 0.19	∗ 1.49 ± 0.35
PP2A subunit twins	*tws*		∗ 1.69 ± 0.27	1.00 ± 0.25	∗ 2.21 ± 0.84
PP2A subunit widerborst	*wbt1*		∗ 1.61 ± 0.21	1.10 ± 0.14	1.00 ± 0.14
shaggy	*sgg*		∗ 2.54 ± 0.50	1.05 ± 0.25	1.00 ± 0.26
phosphofructokinase	*pfk*	glycolysis	∗ 6.71 ± 2.23	1.00 ± 0.25	1.21 ± 0.46
phosphoenolpyruvate carboxykinase	*pepck*	gluconeogenesis	1.28 ± 0.50	∗ 1.91 ± 0.54	1.00 ± 0.26
citrate synthase	*cs*	citric acid cycle	1.25 ± 0.24	1.15 ± 0.22	1.00 ± 0.35
glutamate dehydrogenase	*gdh*	nitrogen metabolism	∗∗ 3.53 ± 0.58	∗ 1.50 ± 0.18	1.00 ± 0.28
fatty-acid binding protein	*fabp*	lipid anabolism	∗∗ 11.37 ± 3.88	∗ 2.02 ± 0.49	1.00 ± 0.29
elongation of very long fatty acids protein	*elov*		∗ 39.82 ± 12.73	1.25 ± 1.40	1.00 ± 0.40
2-hydroxyacyl-CoA lyase	*hacl*	lipid catabolism	∗ 4.26 ± 1.58	1.28 ± 0.69	1.00 ± 0.45
3-hydroxyacyl-Coa dehydrogenase	*hoad*		∗ 2.58 ± 0.53	1.47 ± 0.34	1.00 ± 0.31

Table A3.1 (continued).

Gene	Abbrev.	Function	Active phase	Early diapause	Late diapause
ATP synthase γ subunit	*ATPsyn*	respiration chain	∗ 1.96 ± 0.34	1.00 ± 0.17	∗ 1.48 ± 0.21
arginine kinase	*argk*	anaerobic metabolism	∗∗ 4.43 ± 0.90	∗ 2.80 ± 0.58	1.00 ± 0.38
eukaryotic translation initiation factor 4E	*if4E*	translation	(∗) 1.24 ± 0.33	1.00 ± 0.34	∗ 1.53 ± 0.51
glutathione peroxidase	*gshpx*	stress response	∗∗ 9.79 ± 3.20	∗ 2.22 ± 0.66	1.00 ± 0.38
ferritin	*fer*		∗ 1.59 ± 0.44	∗ 4.17 ± 0.93	1.00 ± 0.31
ecdysteroid receptor	*ecr*	molting	1.00 ± 0.85	∗ 4.22 ± 1.57	∗ 10.89 ± 7.83
cyclin B	*cyclB*	cell cycle	1.00 ± 2.44	∗ 1.19 ± 0.41	∗ 7.43 ± 6.41
couch potato	*cpo*	diapause-associated	∗ 1.49 ± 0.28	(∗) 1.34 ± 0.19	1.00 ± 0.33
chymotrypsin	*chtrp*	digestion	∗ 64.34 ± 17.51	1.47 ± 3.35	1.00 ± 0.32
opsin	*ops*	light perception	1.05 ± 0.21	∗ 1.44 ± 0.27	1.00 ± 0.46
pigment-dispersing hormone receptor	*pdhr*		1.00 ± 0.25	∗ 2.36 ± 0.70	∗ 2.71 ± 1.22
hemocyanin subunit	*hc*	blood O_2 transport	1.00 ± 0.68	∗ 2.31 ± 1.09	∗ 2.00 ± 0.99

Supplemental references

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