Time to (dia)pause

Clock gene expression patterns in the calanoid copepod *Calanus finmarchicus* during early and late diapause

MASTER-THESIS

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I declare that I have developed and written the enclosed master thesis entitled

Time to (dia)pause - clock gene expression patterns in the calanoid copepod *Calanus finmarchicus* during early and late diapause

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Zusammenfassung

Im Winter geht der calanoide Copepode *Calanus finmarchicus*, der in den gemäßigten/subpolaren Gebieten verbreitet ist, in eine jahreszeitliche Diapause, eine Art Winterruhe, um Zeiten mit geringer Nahrungsmittelverfügbarkeit zu überdauern. Tages- und Jahresrhythmen von Zooplankton könnten unter der Kontrolle einer endogenen zirkadianen Uhr sein, um eine optimale Synchronisation der physiologischen, biochemischen und verhaltensbezogenen Prozesse an lokal herrschende Umweltbedingungen zu gewährleisten. Hierbei soll als verlässlichster Zeitgeber für eine Synchronisation mit der Umwelt die Photoperiode (Tageslänge) gelten. Ein geringfügiges, zeitliches Ungleichgewicht zwischen biologischen Prozessen und der Umwelt, wie z.B. eine durch den Klimawandel verursachte zeitliche Verschiebung der Phytoplanktonblüte, könnte verheerende Folgen für das gesamte *Calanus*-basierte Ökosystem haben. Jedoch ist das Wissen über die Synchronisation mit der Umwelt von *C. finmarchicus* und marinen Organismen in polaren Gebieten im Allgemeinen begrenzt. Das Ziel dieser Studie war es, die Performance der Uhr zu unterschiedlichen Zeitpunkten während der Diapause zu untersuchen, um zu wissen, ob die Uhr eine Rolle in der saisonalen Diapause von *C. finmarchicus* spielt. Dafür wurden Genexpressionsmuster von Uhrgenen in *C. finmarchicus* aus früher (September 2014, 10 h L:14 h D) und später (Januar 2015, DD) Diapause über den Tagesverlauf untersucht. Copepoden wurden vor Ort in Kongsfjorden, Svalbard (78.6 ° N, 11.6 °E), über 24 Stunden gesammelt. Primer wurden für jüngst beschriebene potentielle Uhrgene (*cry1, cry2, clk, cyc, per1, tim, dbt2, vri*) in *C. finmarchicus* konzipiert. Expressionsmuster der Uhrgene wurden mittels quantitativer Echtzeit-PCR analysiert. Wir konnten nachweisen, dass die meisten Uhrgene eine tagesrhythmische Oszillation während der frühen Diapause (LD), viele Uhrgene der Copepoden aus später Diapause hingegen keine signifikante rhythmische Oszillation zeigen. Ein Vergleich der einzelnen Tageszeitpunkte zwischen früher und später Diapause zeigte signifikante Unterschiede. Copepoden, die während der frühen Diapause gefangen wurden, hatten einen insgesamt höheren relativen mRNA Level im Vergleich zu den Copepoden aus der späten Diapause. Diese Ergebnisse lassen eine tageszyklische Uhr in *C. finmarchicus* erkennen. Das könnten erste Anzeichen für eine zirkadiane Uhr in *C. fimarchicus* sein und eine mögliche Beteiligung der Uhr in der saisonalen Diapause darstellen. Weitere Studien müssen tagesabhängige Proteinlevel und uhrbezogene Gene untersuchen, um das Zusammenspiel von Uhrgenen, Photoperiode und der Diapause in *C. finmarchicus* besser verstehen zu können.

Abstract

During winter the temperate/subpolar calanoid copepod *Calanus finmarchicus* enters seasonal diapause, a type of dormancy, to overcome this period of low food availability. Daily and seasonal rhythms of zooplankton might be under the control of an endogenous circadian clock ensuring optimal synchronizatzion of physiological, biochemical and behavioral processes to prevailing local environmental conditions. Photoperiod (daylength) is supposed to be the most reliable entrainment cue of an animals seasonal cycle for synchronization with the environment. A small timing mismatch between biological processes and the environment such as temporal shifts of the onset of phytoplankton blooms caused by climate change could potentially have severe consequences for the entire *Calanus*-based ecosystem. Nevertheless, limited knowledge is available concerning the synchronization of *C. finmarchicus* and marine organisms inhabiting polar regions with their environment. This study aimed to investigate the performance of the clock at distinct times during diapause to gain knowledge concerning the role of the clock in seasonal diapause of *C. finmarchicus*. Thus, diurnal clock gene expression patterns in *C. finmarchicus* being in early (September 2014, 10 h L: 14 h D) and late (January 2015, DD) diapause. Copepods have been collected by 24 h *in situ* sampling from Kongsfjorden, Svalbard (78.6°N, 11.6°E). Primers were designed for recently described potential clock genes (*cry1, cry2, clk, cyc, per1, tim, dbt2, vri*) in *C. finmarchicus*. Clock gene expression patterns were analyzed with Real-Time quantitative PCR. We could show that most clock genes showed a diel rhythmic oscillation during early diapause (LD), whereas in late diapause (DD) a significant rhythmic oscillations was not detectable for most of the investigated genes. Comparison of early and late diapause between each diel time point revealed significant differences. Overall, copepods caught in early diapause had higher relative mRNA levels compared to copepods sampled in January. These findings indicate a diurnal clock in *C. finmarchicus*. This might be the first sign of a circadian clock in *C. fi•nmarchicus* and the potential involvement of the clock in seasonal diapause. Further studies need to investigate diurnal protein levels and clock-associated genes to get an understanding of the interplay of clock genes, photoperiod sensing and diapause in *C. finmarchicus*.

Keywords: Arctic, photoperiod, entrainment, Kongsfjorden, housekeeper

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List of Abbreviations

actb (ACTB)	*beta-actin* (BETA-ACTIN)
ArW	Arctic Water
AW	Atlantic Water
cDNA	complementary Desoxyribonucleic acid
CK2	casein kinase 2
clk (CLK)	*clock* (CLOCK)
cry (CRY)	*cryptochrome* (CRYPTOCHROME)
cry1 (CRY1)	*cryptochrome 1* (CRYPTOCHROME 1)
cry2 (CRY2)	*cryptochrome 2* (CRYPTOCHROME 2)
Ct	cycle threshold
cyc (CYC)	*cycle* (CYCLE)
dbt2 (DBT2)	*doubletime 2* (DOUBLETIME 2)
DD	Dark:Dark (24 h darkness)
ef1-α (EF1-α)	*elongation factor 1*-α (ELONGATION FACTOR 1-α)
ESC	East Spitsbergen Current
fwd	forward
gDNA	genomic Desoxyribonucleic acid
IQR	inter quartile range
LD	Light:Dark (alternating periods of light and darkness)
mRNA	messenger RNA
NT	no template
per1 (PER1)	*period 1* (PERIOD1)
PDP1	par domain protein 1
PP2A	protein phosphatase 2
RAIN	Rhythmicity analysis incooperating non-parametric methods
rev	reverse
RNA	ribonucleic acid
rnap	*RNA polymerase*
rpl32	*ribosomal protein L32*
rRNA	ribosomal RNA
RT	room temperature
+RT	with Reverse Transcriptase
-RT	without Reverse Transcriptase
RT-qPCR	real-time quantitative polymerase chain reaction
SEM	standard error of the mean
SGG	shaggy
tim (TIM)	*timeless* (TIMELESS)
vri (VRI)	*vrille* (VRILLE)
WSC	West Spitsbergen Current

CRY/PER/TIM	Capitalized terms indicate proteins
cry/per/tim	Low-case italic words indicate genes

1 Introduction

Within most of the North Atlantic and Arctic Ocean the pelagic calanoid subpolar/temperate *Calanus finmarchicus* is the biomass dominating zooplankton species representing an important trophic link in the pelagic lipid-based arctic food web (Hirche et al., 1997). Feeding on the annual phytoplankton bloom *C. finmarchicus* converts low-energy carbohydrates and proteins into high-energy wax esters (Lee et al., 2006; Falk-Petersen et al., 2009). Due to its high biomass and lipid storages, it represents the key prey species for other zooplankton species, pelagic fishes including polar cod (*Boreogadus saida*) and capelin (*Mallotus villosus*), and some seabird species such as the little auk (*Alle alle*) (Norderhaug, 1980; Lønne and Gulliksen, 1989; Hassel et al., 1991; Weslawski et al., 1999). Polar regions have experienced significant warming in the last decades (IPCC, 2013). The pelagic calanoid copepod *C. finmarchicus* has shifted its distribution northwards to up to $\sim$80° N at Svalbard, caused by climate change (Fromentin and Planque, 1996; Beaugrand et al., 2002). As many other polar and temperate species, *C. finmarchicus* has evolved rhythmic daily and seasonal physiological and behavioural functions which are synchronized with the cyclic changes of the environment (Hagen, 1999; Falk-Petersen et al., 2009; Daase et al., 2013). Daily rhythms include feeding and diel vertical migration (DVM), normally an ascent at dusk to shallow waters and a descent at dawn to greater depths, which is known to be the biggest synchronized movement in terms of biomass on earth (Hays et al., 1997; Fortier et al., 2001; Hays, 2003). Optimisation of feeding as well as predator avoidance are considered to be the ultimate factors behind DVM (Hays, 2003). As proximate trigger mostly day length (photoperiod) as an exogenous cue is mentioned (Fortier et al., 2001; Ringelberg and Van Gool, 2003; Berge et al., 2009, 2014) resulting in the occurrence of DVM during arctic seasons with a pronounced light:dark cycle (autumn and spring) (Cottier et al., 2006; Falk-Petersen et al., 2008; Wallace et al., 2010).

As part of a seasonal rhythm, *C. finmarchicus* enters seasonal diapause, a state of arrested development and reduced metabolic reduction, to overwinter at deeper waters in adaptation to the seasonal scarce food supply during winter (Hirche, 1996a). The calanoid copepod *C. finmarchicus* has a one year life cycle, which involves metamorphosis through six naupliar stages (NI-NVI) and five copepodid stages (CI-CV) prior to moulting to adults. During late summer/autumn mainly the fifth

and final juvenile stage of *C. finmarchicus* (CV copepodids), but also CIV copepodids, start initiating diapause, sink out of the surface waters and overwinter in deeper waters (Hill, 2009; Daase et al., 2013). During diapause copepods stop ingestion and, thus, CV copepodids accumulate large lipid amounts prior to entering diapause, which are utilized as nourishment during dormancy at deeper waters (Hirche, 1996a; Miller, 2000). After descending down to deeper waters in summer/autumn CV copepodids undergo a state of arrested development (Dahms, 1995; Hirche, 1996b), reduced metabolism and respiration, accompanied by suppressed reproduction (Hirche, 1983; Ingvarsdóttir et al., 1999). Arrested development is indicated by reduced transcriptional activity such as low RNA/DNA ratios (Wagner et al., 1998), low ecdysteroid levels (Johnson, 2004) as well as delayed moult progression (Miller et al., 1991). Heat shock protein expression patterns have also been shown to vary considerably over the seasonal cycle (Aruda et al., 2011). By comparing gene expression patterns of active and diapausing copepods, genes associated with lipid synthesis, transport and storage, including ELOV (elongation of very long chain fatty acids), FABP (fatty acid binding protein) and RDH (reductase/dehydrogenase), were downregulated in diapausing copepods (Tarrant et al., 2008). Furthermore, expression of *ferritin*, linked to protection of cells from oxidative stress, and *ecdysteriod reporter* (*EcR*), which is responsible for endocrine regulation of copepod development, was greater in diapausing copepods (Tarrant et al., 2008). *EcR* expression levels were also examined within diapausing copepods having lowest expression levels in December, when animals are assumed to be in diapause, whereas expression levels increased in January, when the animals were terminating diapause. Such seasonal rhythms are a response to a pronounced seasonality in environmental conditions like light, temperature, sea ice and food availability (Enright and Hamner, 1967; Hays, 2003; Berge et al., 2009; Hut et al., 2013). Distinct environmental cycles like the change of the seasons, the monthly cycle of the tides and the diel light/dark cycle are the result of the annual movement of the earth around the sun, the rotation of the earth itself once every 24 hours and the moon orbiting the earth (Berge et al., 2009; Søreide et al., 2010; Kronfeld-Schor et al., 2013; Shimmura and Yoshimura, 2013). Especially in the Arctic organisms have to cope with extreme light regimes, ranging from periods of constant light (midnight sun) to constant darkness (polar night) and only limited periods of pronounced light:dark cycles. Understanding possible endogenous and exogenous mechanisms driving life-cycle strategies such as diapause is important to predict the impact of climate-induced changes on the entire *Calanus*-based ecosystem of the Arctic/northern Atlantic (Kwasniewski et al., 2012; Ji et al., 2013). Temperature, food availability, saturation of lipid reserves

and photoperiod have been proposed as cues for seasonal entrainment initiating/terminating seasonal diapause (Hirche et al., 1997; Niehoff and Hirche, 2005; Johnson et al., 2008; Ji, 2011; Clark et al., 2012; Pierson et al., 2013). However, little is known about the triggers initiating and terminating diapause as well as internal processes and molecular underpinnings associated with these triggers.

Although various external factors might influence biological rhythms (Dalley, 1980; Wallace et al., 2010; Ji, 2011), many species display cycles that persist under constant conditions and, therefore, are considered as endogenous rhythms (Enright and Hamner, 1967; Cavallari et al., 2011; Teschke et al., 2011). On a daily scale, these genetically controlled rhythms are called circadian (Latin: circa = about and dies = day). A rhythm is classified as circadian, if the oscillation runs with a period of $\sim$24 h and persists under constant conditions (free-running). It is assumed that DVM of zooplankton slows down or ceases during winter and is only performed during arctic seasons with a pronounced light:dark cycle (Cottier et al., 2006; Falk-Petersen et al., 2008; Wallace et al., 2010). However, Enright and Hamner (1967) found that DVM of calanoid copepods was also performed during constant darkness (DD) in experimental studies. Furthermore, during polar night and day, where ambient light is generally assumed to be insufficient to cue zooplankton (Cottier et al., 2006), DVM was also performed by several zooplankton species (Berge et al., 2009, 2012) pointing towards the existence of a self-sustaining endogenous control mechanism, a so called circadian clock. Such a clock consists of transcriptional-translational autoregulatory feedback loops involving rhythmic clock gene expression patterns, which generate 24 h rhythms on a molecular level. Cyanobacteria as well numerous eukaryotes (Aréchiga, 1993; Bradshaw and Holzapfel, 2007; Axmann et al., 2014) have evolved such a circadian clock in order to anticipate environmental oscillations on a daily basis (Kuhlman et al., 2007) allowing activation of rhythmic outputs at the appropriate time of the day such as locomotory activity, DVM and metabolic functions (Marcus, 1985; Aréchiga, 1993; Strauss and Dircksen, 2010).

Although circadian rhythms can continue to oscillate with cycles of approximately 24 hours (Berge et al., 2009; Shimmura and Yoshimura, 2013) under constant environmental conditions (Enright and Hamner, 1967; Kuhlman et al., 2007; Cavallari et al., 2011; Teschke et al., 2011), a reliable environmental cue is needed to entrain endogenous rhythms to their $\sim$24 h cycle (Aschoff, 1965). Photoperiod is supposed to be the most reliable entrainment cue (*Zeitgeber*) for synchronization with the environment (Marcus, 1986; Kuhlman et al., 2007; Tosches et al., 2014). Nevertheless, there is also evidence that in the absence of photoperiod animals can display arrhythmicity or switch to

alternative time cues (Lu et al., 2010; Wallace et al., 2010). Besides photoperiod, temperature and food availability have been proposed as cues for seasonal entrainment triggering diapause (Hirche and Kwasniewski, 1997; Niehoff and Hirche, 2005; Cavallari et al., 2011; Ji, 2011; Clark et al., 2012; Pierson et al., 2013).

On a molecular level, the circadian clock consists of three key components. Further description of clock functions and components includes certain genes and their protein products. Genes are written as low-case italic words (e.g. *cry1*), whereas their proteins can be identified by capitalized terms (e.g. CRY1). First, a central oscillator independently keeps circadian time generated by temporal delays between activation and repression of clock genes mediated by negative transcription-translation feedback loops (Hardin, 2005; Mackey, 2007) resulting in a approx. 24 h oscillation of many clock gene transcripts (Dubruille and Emery, 2008; Zheng and Sehgal, 2008). Post-translational modifications including phosphorylation, localisation and degradation of clock proteins help to maintain a circadian rhythm (Zheng and Sehgal, 2008). The second component, the input pathway, synchronizes the clock to its environment. The *Drosophila*-like cryptochrome protein (CRY), analogous of the vertebrate-like CRY1, is light-sensitive and primarily function as clock-specific photoreceptors entraining the molecular clock to changing environmental cycles of light and dark. Cryptochrome proteins are able to absorb light and transmit the information directly to the oscillator allowing the period and phase of the clock to adjust to prevailing light:dark cycles (Ceriani et al., 1999; Emery et al., 2000). A null mutation in the *cryptochrome* gene, *cry* ^{b}, in the fruit fly *Drosophila melanogaster* showed a failure to synchronize to light:dark cycles indicating that cryptochrome's normal function involves circadian photoreception (Stanewsky et al., 1998; Helfrich-Förster et al., 2001). The output pathway, the third component, is responsible for transmitting the information to temporally organize behaviour and physiology like pupal eclosion, locomotor activity, neuronal function and hormonal secretion (Allada and Chung, 2010).

The endogenous control mechanism in *D. melanogaster* is one of the best studied clocks (Allada and Chung, 2010) functioning with two feedback loops, *period/timeless* and *clock* (Figure 1.1). The first feedback loop includes the activation and repression of *period (per)* and *timeless (tim)*. During late day the positive regulators CLOCK (CLK) and CYCLE (CYC) activate the transcription of the negative regulators *per* and *tim* leading to an accumulation of PER and TIM proteins in the cytoplasm. After dimerization of the two proteins PER and TIM during early night, they are translocated into the nucleus (midnight) binding to CLK/CYC.

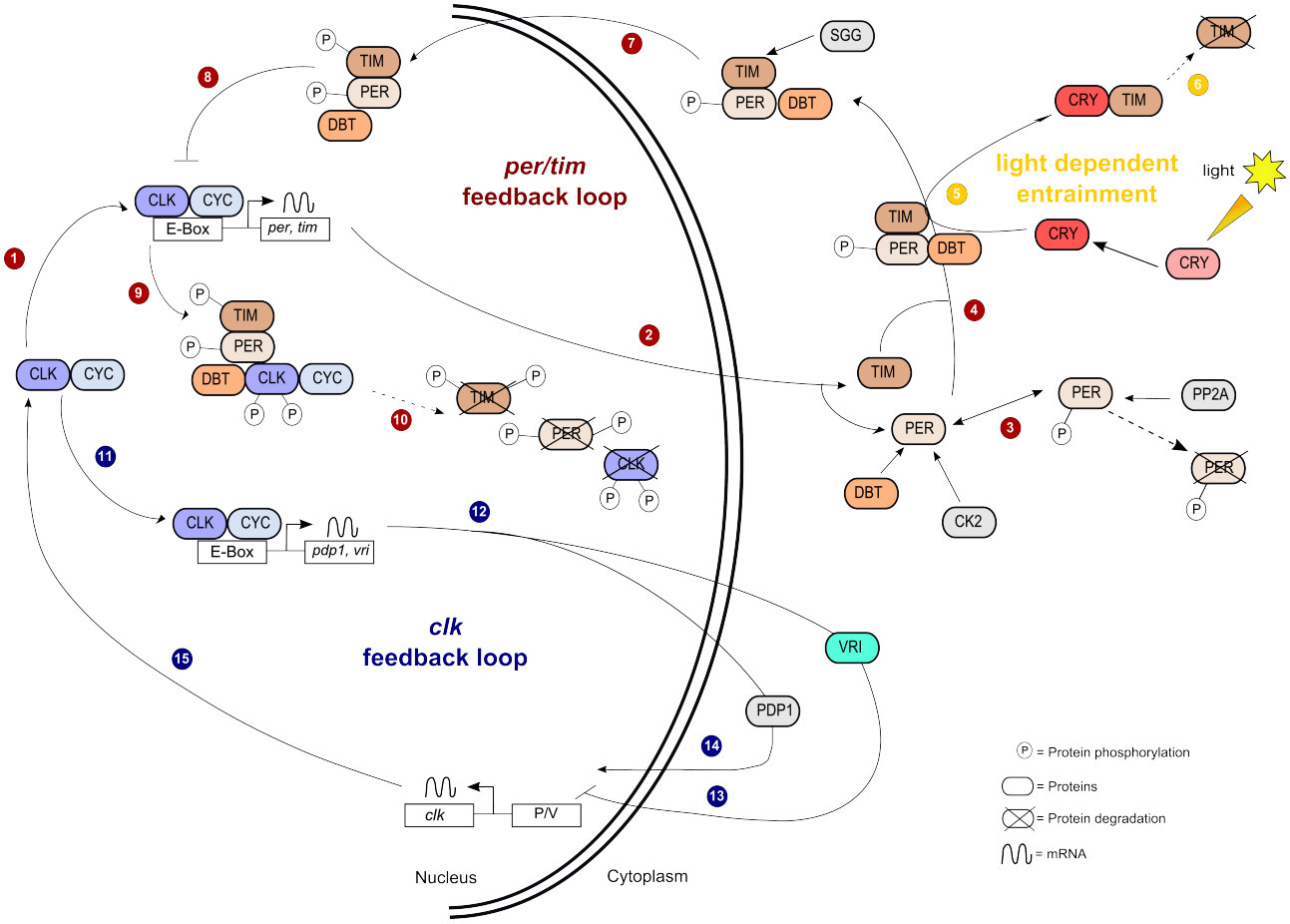

Figure 1.1: Circadian clock of *Drosophila melanogaster* - Circadian time is generated by two transcription-translation negative feedback loops (*per/tim* feedback loop and *clk* feedback loop) of clock genes and their protein products as well as a light dependent entrainment of the oscillator. Capitalized terms stand for proteins, low-case words indicate genes. ① CYC-CLK heterodimers activate transcription of *per* and *tim* (peaking during late day) by binding at the *per* and *tim* promoters containing a CLK/CYC target E-box sequence. ② Transcription factors PER an TIM are transported out of the nucleus into the cytoplasm. ③ PER is phosphorylated by DBT and CK2, which leads to its degradation. PER is also stabilized by PP2A which removes phosphates that were added to PER (late day/early night). ④ As stabilisation TIM binds to phosphorylated PER (early night), which remains bound to DBT. ⑤ During the day, light activates the photoreceptor CRY, which then binds to the TIM-PER-DBT complex removing TIM, ⑥ leading to its degradation (early morning). ⑦ In the absence of light TIM-PER-DBT complexes are further phosporylated by SGG promoting their transport into the nucleus (midnight). ⑧ PER and TIM transcription is inhibited by TIM-PER-DBT complexes binding to CLK-CYC. ⑨ The whole complex is removed from the E-box (late night). ⑩ TIM, PER and CLK are degraded (early day). Accumulation of non-phosphorylated CLK leads to heterodimerization with CYC and another cycle of PER an TIM transcription starts. ⑪ Further, CLK-CYC heterodimers bind to the E-box ⑫ activating the transcription of VRI and PDP1. ⑬ VRI binds to P/V boxes and inhibits *clk* transcription. ⑭ A delayed accumulation of PDP1 leads to a replacement of VRI from P/V (PDP1 and VRI) boxes and restimulates *clk* transcription. ⑮ Accumulation of non-phosphorylated CLK leads to heterdodimerization with CYC and another cycle of *vri* and *pdp1* transcription [adapted from Hardin (2005)]

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Thus, CLK and CYC are removed from the E-box resulting in inhibition of *tim* and *per* transcription (late night). Post transcriptional regulation and modification of clock components including phosphorylation via DOUBLETIME (DBT), CASEIN KINASE 2 (CK2), PROTEIN PHOSPHATASE 2 (PP2A) and SHAGGY (SGG) result in temporal delays between CLK/CYC transcriptional activation (late day) and PER/TIM repression (late night). Negative feedback on CLK/CYC activity is relieved at dawn when light activates CRY, a light-sensitive protein (Emery et al., 1998), promoting the degradation of TIM (Ceriani et al., 1999). Additionally, PER and CLK are degraded during early day. Within the second feedback loop, CLK/CYC heterodimers activate the transcription of VRILLE (VRI) and PAR DOMAIN PROTEIN 1 (PDP1). VRI represses *clk* transcription, whereas a delayed accumulation of PDP1 results in a replacement of VRI leading to a restimulation of *clk* transcription (Figure 1.1).

Some aspects regarding the control of the circadian clock are assumed to be common to all insects (Bradshaw and Holzapfel, 2010). This includes the *cyc* and *clk* transcription/translation feedback loop promoting transcription of *per* and *tim* as well as the involvement of cryptochromes. In *Drosophila* cryptochrome protein CRY, which is an analogue to *cry1* in the monarch butterfly *Danaus plexippus* (Figure 1.2) and *Calanus finmarchicus*, is known to function as photoreceptor entraining the clock to the prevailing photoperiod and transmitting that information directly to the clock by promoting the rapid degradation of the TIM protein (Emery et al., 1998; Stanewsky et al., 1998; Helfrich-Förster et al., 2001; Collins et al., 2006). CRY2, the vertebrate like cryptochrome, on the other hand, is light insensitive and might act as a negative-acting transcriptional regulator of CLK:CYC mediated transcription (Zhu et al., 2008).

Both circadian and seasonal events rely on the ability to precisely measure time (Marcus, 1986; Kuhlman et al., 2007; Tosches et al., 2014) and might potentially involve the same genetically elements (Oster et al., 2002; Meuti and Denlinger, 2013). Evidence imposes that photoperiod and light intensity are essential for calibration of both events (Kuhlman et al., 2007). To determine the timing of seasonal events such as diapause it is assumed that organisms use and respond either to annual changes in day length or directly to the length of day/night (Meuti and Denlinger, 2013). Bünning (1936) first proposed the idea, that animals likely use their circadian clocks to measure photoperiod, and hence initiate photoperiodic responses, because the circadian clock already provides critical information on light:dark cycles. Evidences reveal that the timing of diapause could be related to photoperiod measurement as seasonal entrainment cue (Marcus, 1982; Stearns and Forward, 1984a,b; Cottier et al., 2006) involving a circadian clock (Davis, 2002; Oster et al., 2002; Schultz and Kay, 2003;

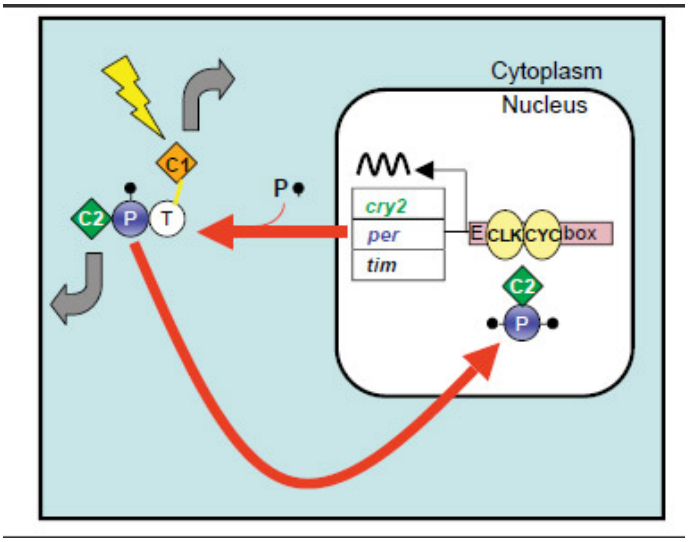


Figure 1.2: Proposed circadian systems of the monarch butterfly *Danaus plexippus* - Two CRYs (CRY1 and CRY2) are involved in this system. CRY1 functions as blue-light photoreceptor like the *Drosophila*-CRY, whereas CRY2, the vertebrate-like CRY, plays an important role as negative-acting transcriptional regulator. CLK and CYC heterodimers regulate transcription of *per*, *tim* and *cry2* by binding to their E-box. TIM, PER and CRY2 form a complex in the cytoplasm. PER is progressively phosphorylated and helps translocating CRY2 into the nucelus, where CRY2 inhibits CLK:CYC-mediated transcription. TIM degradation is caused by light exposure (lightning bolt). Grey arrows indicate output functions for CRY1 and CRY2. C=cryptochrome, P (blue circle)=period, T=timeless, CLK=clock, CYC=cycle, black P=Phosphorylation. Graphic adapted from Zhu et al. (2008).

Clark et al., 2013; Meuti and Denlinger, 2013). Several clock genes have already been shown to be involved in diapause (Tauber et al., 2007; Ikeno et al., 2010). Besides two identified crustacean clock gene sequences of *clock* (Yang et al., 2006) and *cryptochrome 2* (Mazzotta et al., 2010), Christie et al. (2013) could identify molecular components of a potential circadian clock in *C. finmarchicus* by using the *D. melanogaster* circadian system as reference for mining clock transcripts. This finding of potential clock genes in *C. finmarchicus* (Christie et al., 2013) as well as the persistence of DVM in calanoid copepods (laboratory experiments) and zooplankton species (field studies) under constant light conditions (Berge et al., 2009; Enright and Hamner, 1967) point to the existence of a potential circadian clock in this calanoid copepod. Nevertheless, limited knowledge is available concerning the molecular underpinnings of circadian and seasonal rhythms in pelagic calanoid copepods. Due to its importance as key species in the northern ecosystem, detailed knowledge concerning the physiology and biology of diapausing *C. finmarchicus* and the physical and biological factors controlling this behaviour is vital in order to understand the consequences of climate change on this species as well as on the entire food web.

The aim of this study was to detect potential rhythmic gene expression patterns of core clock genes (*clock, cycle, period, timeless, cryptochrome 2, doubletime, vrille, cryptochrome 1*) in the calanoid copepod *Calanus finmarchicus*. We suggest that the circadian clock might not only play a major role in DVM, but also entrains events of the seasonal cycle like diapause. Without the possibility to manipulate the clock and observe the impact on diapause, we, thus, rely on sampling diapausing copepods being in distinct phases and investigate variations within clock gene expression patterns such as amplitude and shifts in phase and period. This was realized for *C. finmarchicus* at two diapause phases (early and late diapause) for the first time in this study. Investigations of the performance of the clock during diapause allows us to get an understanding of the potential role of the circadian clock in diapause. As it is the major overwintering stage of *C. finmarchicus* CV copepodids were used, which were caught during 24 h *in situ* sampling in Kongsfjorden, Svalbard, in September 2014 (early diapause) and January 2015 (late diapause). We expect different clock gene expression patterns in *C. finmarchicus* being in early and late diapause. We assume that *C. finmarchicus* sampled in September (light:dark cycle (LD) 10 h L:14 h D) show a rhythmic expression of clock genes by sensing the diel light:dark cycle. Sampling in January took place during polar night. Thus, there is no entrainment of the circadian clock by sunlight, raising the question if the clock is then still ticking after a period of constant darkness lasting for almost seven months. We assume that rhythmic clock gene expression patterns might not be observable in *C. finmarchicus* in the absence of an external entrainment signal such as light during late diapause. Our findings indicate a diurnal clock in *C. finmarchicus*. This study might be the first evidence of a potential circadian clock in *C. finmarchicus* and the first step into the potential importance of a clock in seasonal diapause.

2 Material and methods

This study aimed to investigate the performance of the clock at distinct times during diapause to gain knowledge concerning the role of the clock in seasonal diapause of *C. finmarchicus*. Thus, we rely on sampling diapausing copepods being in distinct phases and investigate variations within clock gene expression patterns such as amplitude and shifts in phase and period.

2.1 Study site characteristics

The glacial fjord Kongsfjorden (78.55°N, 11.3°E) is located in the Svalbard archipelago on the north-west coast of Spitsbergen (Figure 2.1). Together with the adjacent Krossfjorden, it opens to the West Spitsbergen Shelf (WSS) (Svendsen et al., 2002). The two-armed fjord system is mainly influenced by the coastal East Spitsbergen Current (ESC) and West Spitsbergen Current (WSC), both flowing north along the WSS (Figure 2.1). Cold, fresh Arctic Water (ArW) is carried with the ESC and warm, saline Atlantic Water (AW) is transported by the WSC to the glacial fjord system (Saloranta and Svendsen, 2001). This shifts seasonal hydrography to states of Arctic as well as Atlantic dominance within an annual cycle (Cottier et al., 2005; Svendsen et al., 2002). During summer warm, saline Atlantic-derived waters are intruding into Kongsfjorden leading to a shift from Arctic to Atlantic Water dominated systems (Cottier et al., 2005). Limited or no sea ice formation occured in Kongsfjorden during the last decade caused by an increased influx of warm Atlantic waters (Svendsen et al., 2002; Cottier et al., 2005). In the fjord system upper water masses are mainly influenced by the tide, freshwater run-off as well as local wind forces affecting prevailing zooplankton community structure (Svendsen et al., 2002; Willis et al., 2006).

The zooplankton community in Kongsfjorden is comprised of co-occuring boreal and Arctic species and is mainly influenced by the advection of water masses (Willis et al., 2006). *Calanus finmarchicus* has its activity centre in the North Atlantic Ocean (Conover, 1988) and is additionally transported via the North Atlantic Current into subarctic and arctic seas. Thus, the high biomass of *C. finmarchicus* in Kongsfjorden is mainly contributed to advection of water masses.

However, overwintering of *C. finmarchcius* in these areas has also been reported, followed by reproduction in summer. Due to the balance between the Atlantic, Arctic and freshwater input, this fjord system is supposed to be very sensitive to climate change (Cottier et al., 2005). Thus, it is a site of great interest concerning investigations of anthropogenic climate change on the hydrophysical and biological fjord-system (Cottier et al., 2005).

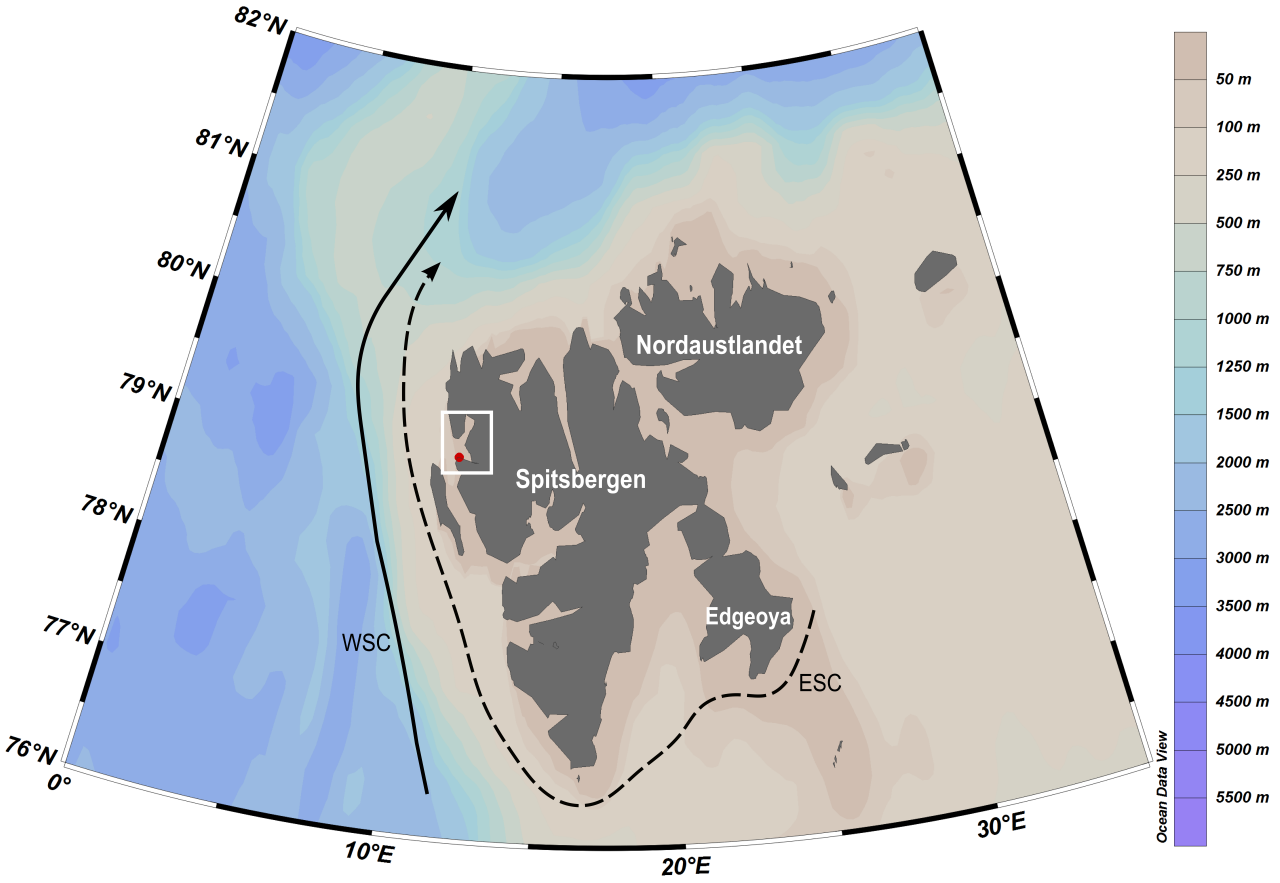


Figure 2.1: Svalbard archipelago - The Kongsfjorden–Krossfjorden system (white box) is located on the northwest coast of Spitsbergen. The fjord-system is mainly influenced by the coastal East Spitsbergen Current (ESC) carrying cold, fresh Artcic Water and the West Spitsbergen Current (WSC) transporting warmer Atlantic Water (Willis et al., 2006). Red point indicates sampling station (78.6°N, 11.6°E) in September 2014 and January 2015 during a research cruise with *R/V Helmer Hanssen*. The map was created with the programme Ocean Data View (Schlitzer, 2012).

2.2 Experimental organisms

For clock gene sequence analysis *Calanus finmarchicus* CV copepodids were used obtained from 24 hour *in situ* sampling at Kongsfjorden (78.6°N, 11.6°E), Svalbard on the 26th of September 2014 and the 13th of January 2015. Due to the sampling times, copepods were in their early and late diapause, respectively (Figure 2.2). Copepods were collected by vertical plankton net hauls (mesh size 200 µm) in depth intervals from around 345 m (~10 m above the bottom) to 220 m depth during the research cruise with *R/V Helmer Hanssen* (sampling conducted by Sören Häfker and Lukas Hüppe). Light conditions at Kongsfjorden during sampling were as follows: light:dark cycles (LD) 10 h:14 h on the 26th of September 2014 and constant darkness (DD) on 13th of January 2015. Photoperiod data were obtained from the following website http://pveducation.org/pvcdrom/properties-of-sunlight/calculation-of-solar-insolation. Information can be gained about daily solar irradiance, the solar insolation and the hours of sunrise and sunset.

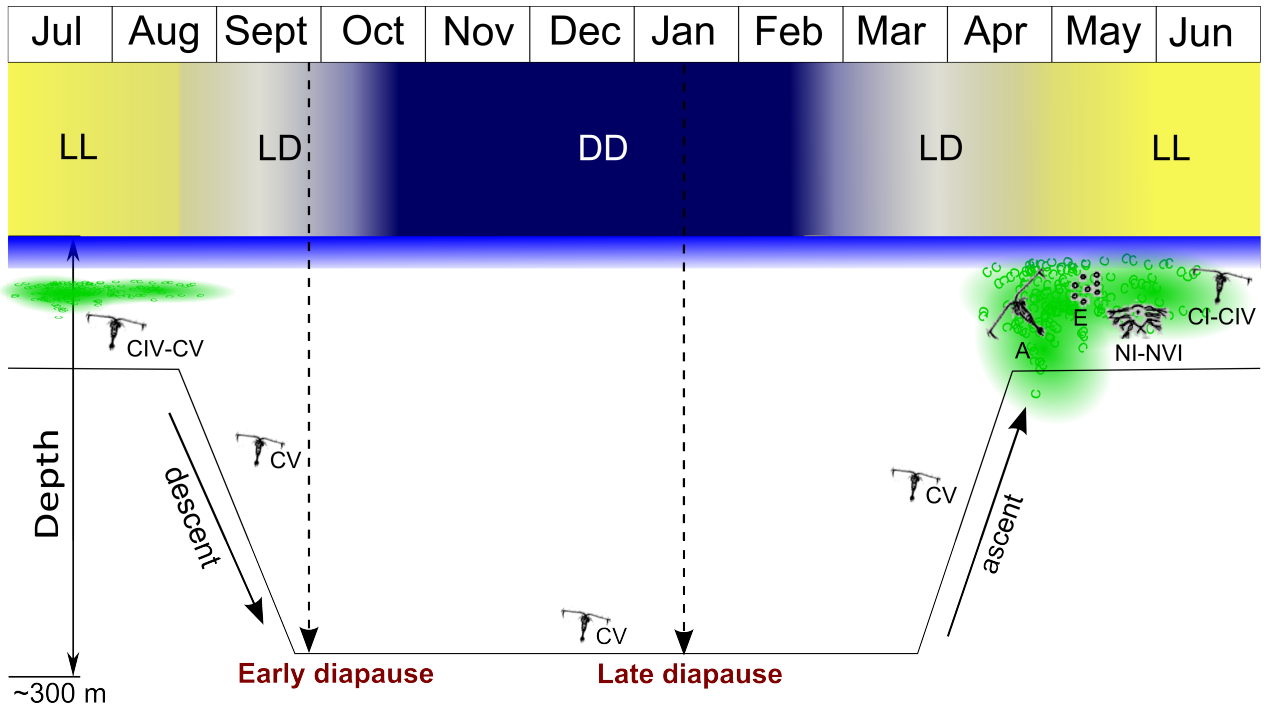


Figure 2.2: Life cycle of *Calanus finmarchicus* in Kongsfjorden, Svalbard - CV copepodids are descending down to overwinter at deeper depths (diapause). After ascending back to surface waters during March/April copepods feed on the annual phytoplankton bloom (green) followed by reproduction (E = eggs, NI-NVI = naupliar stages, CI-CV = copepodid stages, A = adult copepods). Sampling points of early (26th September 2014) and late (13th of January 2015) diapause are indicated by black dashed arrows. LL = continuous light, LD = light:dark cycles, DD = continuous darkness.

For copepods caught during late diapause in January, 7 samplings in 4 hour intervals (0h, 4h, 8h, 12h, 16h, 20h, 24h) could be conducted, whereas in September only 6 samplings (4h, 8h, 12h, 16h, 20h, 24h) were performed due to technical issues. For both September and January, sampling started and ended at midnight (0h = 24h). Copepods were immediately transferred in a cold and dark store. Copepods sampled in January 2015 were sorted directly after sampling on board and were stored in RNALater ® (Ambion, USA). Copepod samples from September 2014 were transferred directly into RNALater ® solution for postponed sorting in the home laboratory at the Alfred-Wegener-Institute in Bremerhaven. Copepods were sorted by species and life cycle stage (Kwasniewski et al., 2003) with a binocular (Leica MZ125) and cooling chambers for petridishes. For each diel sampling point 5 replicates were obtained by pooling 15 CV copepodids for each replicate. All sampled organisms were stored in Cryo vials (Fisher Scientfic) at -20℃ in RNALater ® for further analysis.

2.3 RNA extraction and quality control

Previous laboratory experiments concerning copepod RNA extraction (conducted by Sören Häfker) were highly time consuming. Thus, at the beginning of this study further investigations needed to be conducted to gain a reliable RNA extraction protocol. Furthermore, due to extremely low RNA quantities obtained with a single CV copepodid, RNA quality and quantity checks had to be performed with distinct amounts of pooled CV copepodids. The disadvantage of pooling individuals is the inability to identify individual gene expression patterns. Copepods caught during early diapause had generally higher gene expression patterns compared to early diapausing copepods.

Copepod RNA was extracted using the RNeasy ® Mini Kit (Qiagen, Germany) with some improvements for the RNA isolation of *Calanus*. Per each diel time point 15 pooled *C. finmarchicus* were transferred into 2 ml Precellys ® tubes containing 1.4 mm and 2.8 mm beads filled with 1000 µl of Buffer RLT (10 µl ß-mercaptoethanol per 1 ml Buffer RLT). Copepods were immediately homogenized with a Precellys ® 24 homogenizer (bertin Technologies, France) for 16 s and 6500 rpm at room temperature (RT). Homogenated *C. finmarchicus* were rest for 60 min at RT. Precellys ® tubes were centrifuged for 10 min at max. speed (20817 *g*) and RT. The supernatant was mixed with one sample volume of 70% molecular biology grade ethanol (AppliChem, Germany) and 700 µl were transferred to RNeasy spin column placed in a 2 ml collection tube.

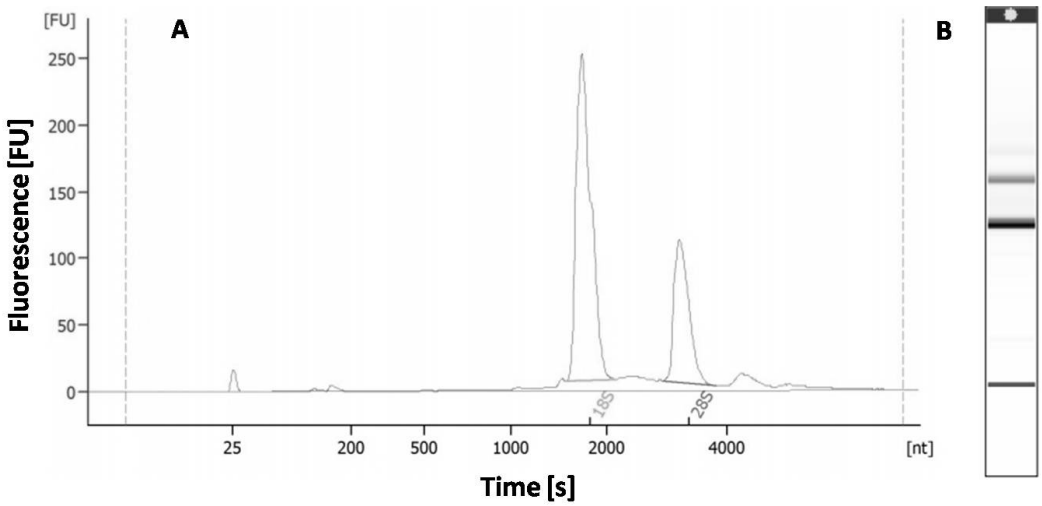


Figure 2.3: RNA quality control - Electropherogram **(A)** and gel-like image **(B)** of extracted RNA from 15 pooled *C. finmarchicus* produced by microfluidic electrophoresis in an Agilent 2100 Bioanalyzer using a RNA 6000 Nano Kit System. Time of ribsosomal RNA peak appearance (size related) is plotted against fluorescence of the peak (concentration related).

After centrifugation (30 s at 16000 *g*), flow-through was discarded and the remainig sample volume was added on the RNeasy spin column and centrifuged again. A volume of 700 µl Buffer RW1 were transferred into the spin column and samples were incubated for 2 min at RT. After inverting and rolling the tubes to remove possible RLT residues, samples were centrifuged for 30 s at 16000 *g* and flow through was discarded. Same procedure was conducted once, but instead of 700 µl Buffer RW1, 500 µl RPE was added into the spin column. An additional washing step with 500 µl Buffer RPE was conducted with a centrifugation of 1 min at 16000 *g*. The spin columns were transferred into 2 ml collection tubes and centrifuged for another 1.5 min at max. speed *g* to dry the membrane. After placing the spin column in a new collection tube, 60 µl RNase free water were added directly onto the column membrane and incubated at RT for 5 min. To elute the RNA, samples were centrifuged for 1 min at 10000 *g*. This step was conducted once more, but instead of adding new water the flow through was used for eluting RNA. Samples were stored on ice. The RNA concentration and purity were determined using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The 260/280 ratios ranged from 2.12 - 2.16 indicating reliable RNA quality for all RNA samples. Furthermore, the RNA quality was checked with the Agilent Bioanalyzer 2100 and the RNA 6000 Nano Kit (Agilent Technology) according to suppliers instruction (Figure 2.3).

2.4 cDNA synthesis

Exactly 2 µg RNA were reversely transcribed to cDNA. Total reaction volume of 50 µl for one reaction included 28.25 µl copepod RNA (end concentration: 2 µg; dilution with RNase free water) as well as 21.75 µl Master mix (for one reaction: 10 µl 5 x Buffer, 1 µl dNTPs 10 mM, 0.5 RNase inhibitor 40 U/µl, 5µl RNase free water, 5 µl Pentadecamere 500 µM, 0.25 µl Reverse Transcriptase; Thermo Fisher Scientific Molecular Biology). After mixing RNA was reversely transcribed to cDNA with the T100^{TM} Thermal Cycler (Biorad). Additionally, no template (NT) controls were run to observe possible contamination of the cDNA reagents. The -RT (without Reverse Transcricptase) controls, which were run for all samples, did include RNase free water instead of RNA to check for DNA contamination within each RNA sample. Settings were as follows: 25℃ at 10 min, 37℃ at 50 min, 70℃ at 15 min, 4℃ at ∞. To avoid decay cDNA was stored at -20℃.

2.5 Primer design

With the aid of the data from Christie et al. (2013) primers were designed to investigate the 8 potential clock genes including *clock* (*clk*), *cycle* (*cyc*), *period1* (*per1*), *timeless* (*tim*), *cryptochrome 2* (*cry2*), *doubletime 2* (*dbt2*),*vrille* (*vri*) and *cryptochrome 1* (*cry1*) Table 2.1). The usage of the online software Primer3Plus Version 2.3.6 (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) for obtained clock gene sequences resulted in primers of $\sim$20 bp for a query length of the targeted sequence of 70-150 bp. Received primers were checked for the occurrence of hetero dimers, self dimers and possible hairpin structures (Oligo Analyzer 3.1, http://eu.idtdna.com /analyzer/Applications/OligoAnalyzer/). With the DINAMelt web server including the UNAfold software package (similar to mfold) the possibility of folding and hybridization of two primers was analyzed as well as the melting profiles of the nucleic acids (http://mfold.rna.albany.edu/?q =DINAMelt/Hybrid2). Primer specifity was ensured by using BlastN and Primer-BLAST (http://www.ncbi.nlm. nih.gov/tools/ primer-blast/).

Six housekeeping genes (Table 2.1) were checked for stable gene expression. *Ribsosomal protein L32 (rpl32)* and *elongation factor 1-α (ef1-α)* have been identified as most reliable housekeepers under different photoperiodic treatments (Fu et al., 2013). *Beta-actin (actb)*, *RNA polymerase* (*rnap*), *16S* and *18S rRNA* were chosen due to their common use as housekeepers in many species (Teschke et al., 2007; Tarrant et al., 2008; De Pittà et al., 2013). Due to limited sequence availability in the NCBI database for specific potential housekeeping genes of *Calanus finmarchicus*, primers were

designed by using Local BlastN (version: ncbi-blast-2.2.30+). An own database was created with the transcriptome of *C. finmarchicus* (Lenz et al., 2014). Targeted housekeeping gene sequences of crustaceans within the NCBI database were used to identify the appropriate regions within the *Calanus*-transcriptome. Output sequences were aligned with NCBI BlastN and similar sequences were eliminated. Remaining alignments were checked with NCBI BlastX. The best sequence hit was used for designing primers as described above. Besides ensuring primer specificity with Primer-BLAST, specifity within the *Calanus*-transcriptome was additionally assured with local BlastN.

Table 2.1: Primer design - Primer sequences of target and housekeeping genes used for RT-qPCR

Target genes		**Primer sequence (5'-3')**	**Product (bp)**	**Sequence source**
clock (*clk*)	fwd	ACTCGGATTGGCTTTGATGG	122	Christie et al. (2013)
	rev	TTCTCAGGTGCAACGTTTCC		(comp76772 c1 seq1)
cycle (*cyc*)	fwd	CAGAGCAGGAAGGATAATGAGC	110	Christie et al. (2013)
	rev	TGTAAGCATTGGCACTCAGC		(comp160482 c0 seq1)
period 1 (*per1*)	fwd	ACATTGTCACAAGCCCTTGG	143	Christie et al. (2013)
	rev	ACAGATGCTCCTTGTGATGC		(comp171214 c0 seq1)
timeless (*tim*)	fwd	CCTAACCTGTTACCGTTGACC	121	Christie et al. (2013)
	rev	ATCGCTCACCAATGACTTCC		(comp88114 c0 seq1)
cryptochrome 1 (*cry1*)	fwd	GGGTTTCAACTGGCTTTGG	86	Christie et al. (2013)
	rev	CCTCTCACTTACCAGAAGATGC		(comp37700 c0 seq1)
cryptochrome 2 (*cry2*)	fwd	AGCAACCACCGAATATGACC	108	Christie et al. (2013)
	rev	AACTGACCTTGTGGCATTCC		(comp181328 c0 seq1)
doubletime 2 (*dbt2*)	fwd	ATGTGTCAGATGCAGCAAGC	74	Christie et al. (2013)
	rev	TAGTTTGGCCAGCTTGTTGG		(comp126103 c3 seq2)
vrille (*vri*)	fwd	TGCAGCCTCACAACATTACC	108	Christie et al. (2013)
	rev	AAACACGCAGGGATTTCACG		(comp71844 c0 seq1)
Housekeeping genes		**Primer sequence (5'-3')**	**Product (bp)**	**Sequence source**
ribsosomal protein L32	fwd	GTCCTGATCCACAACATCAAGG	95	Lenz et al. (2014)
(*rpl32*)	rev	CTGTTCTTTGCGGAGACTCC		(comp30 c2 seq1)
rna polymerase (*rnap*)	fwd	TCAATGACGAGGTTCTCAGG	79	Lenz et al. (2014)
	rev	ATCAACTGTTGCCACTCTCG		(comp19535 c1 seq1)
elongation factor 1-α	fwd	AGTTGCTGGCTTGTTCTTGG	142	Lenz et al. (2014)
(*ef1-*α)	rev	GGTTAAGTCCGTGGAGATGC		(comp8 c1 seq1)
beta actin (*actb*)	fwd	GCATCATCTCCAGCGAAACC	91	Lenz et al. (2014)
	rev	CAAACCCAAAGATGTGTGACG		(comp25 c4 seq1)
16S rRNA (*16S*)	fwd	CCGCGTTAGTGTTAAGGTAGC	143	Lenz et al. (2014)
	rev	CTTCTCGTCCTAGTACAACTGC		(comp2 c0 seq1)
18S rRNA (*18S*)	fwd	AAGCTCGTAGTTGGATCTCG	131	Lenz et al. (2014)
	rev	AAGTAAACCTGCCAGCATCC		(comp92 c0 seq1)

2.6 qPCR methodology

Transcription levels of target genes were determined using quantitative Real-Time PCR (RT-qPCR) with a SYBR-Green single gene assay. Therefore, cDNA was diluted 1:10 with RNase free water. Each RT-qPCR reaction was performed in a total volume of 20 µl containing 8 µl properly diluted cDNA of the sample, 10 µl of SYBR Green Master mix (FIRMA) and 2 µl of primer mix (forward and reverse, 3000 nM respectively). The qPCR was run with the software Vii-A7 (Applied Biosystems). Reaction conditions were as follows: 1 cycle of stage 1 with 50℃ for 2 min and 95℃ for 10 min, 40 cycles of stage 2 with 95℃ for 15 s and 60℃ for 1 min, ending with 1 cycle of 95℃ for 15 s and 60℃ for 1 min. Threshold values for all qPCR runs were set to a value of 0.1 to guarantee the comparability between data obtained from different genes and different runs. Blank controls with no template (NT) or no reverse transcriptase (-RT) were performed for each run. The -RT controls should indicate genomic DNA contamination of the RNA samples. These controls were lacking the enzyme reverse transcriptase during the cDNA synthesis. In case of contamination occurring Ct (cycle threshold) values would be similar to the Ct values of the samples. NT controls (RT-qPCR) were performed by substituting the RNA for sterile RNase free water to elucidate contamination of the used RT-qPCR Master mix. Further NT controls during the cDNA synthesis were conducted to control for contamination of the used chemicals. The results from the NT controls should be negative or have higher Ct values which might occur due to primer dimers, otherwise they are not reliable. PCR amplifications were always run in technical duplicates, except the -RT controls for January samples due to low sample volume caused by low RNA concentrations. The transcript levels of all target genes were normalized to a BestKeeper consisting of the three best housekeeping genes by using the $2^{-\Delta\Delta Ct}$ method, a convenient algorithm to analyze relative changes in genes expressions, calculated with Microsoft Excel 2007 software (Livak and Schmittgen, 2001).

Efficiencies of primers were generated with serial dilutions of *C. finmarchicus* cDNA (1:10, 1:20, 1:40, 1:80, 1:160, 1:320). Calculation of the efficiency of the PCR amplification was conducted with standard curves provided within the software Vii-A7 (Applied Biosystems). Efficiency is calculated using the slope of the regression line in the standard curve. A slope close to -3.32 indicates optimal, 100% PCR amplification efficiency.

2.6.1 Genomic DNA contamination

In general, more than five cycles difference between the -RT (without reverse transcriptase) and +RT (with reverse transcriptase) Ct values allow the assumption that no DNA contamination occured. Comparing -RT and +RT Ct values for all target as well as housekeeping genes revealed a genomic DNA contamination of our samples. This was indicated by a Ct difference between -RT and +RT of generally less than five cycles. Normally, a subequent DNase treatment of extracted sample RNA is a common procedure in molecular biology. Nevertheless, DNase digestion is known to affect RNA quantity leading to an additional RNA loss. Furthermore, the working group of Bettina Meyer had good experience with RNA extraction of larval and adult krill (*Euphausia superba*) without DNase digestion leading to reliable RT-qPCR results. During the establishment of the RNA extraction protocol we decided against a DNase digestion due to general low RNA quantity, especially of copepods caught in January 2015. Preliminary investigations of RNA extraction of *Calanus finmarchicus* had been considered promising without DNase digestion. Results of RNA quality and quantity with the Nanodrop and Bioanalyzer did not indicate a high genomic DNA contamination. Thus, we decided to extract the RNA of all the samples using the RNeasy ® Mini Kit (Qiagen, Germany) with some improvements for the RNA isolation of *Calanus*.

For further investigations concerning time series analysis of *C. finmarchicus*, RNA extraction followed by a DNase digestion or the usage of the RNeasy ® Plus Mini Kit (Qiagen, Germany), which already includes a gDNA Eliminator spin column for removing genomic DNA, should be considered to avoid DNA contamination and to reveal high reliable results. Due to the fact that the genomic DNA contamination occured in all samples, results are still reliable and can be interpreted.

2.7 Housekeeping gene validation

Normalizing the transcript levels of target genes to a stably expressed gene measured simultaneously in the same biological material is a useful and established method to gain reliable gene expression results. Such reference or housekeeping genes are used to correct inequalities in DNA concentrations which result from natural variations. Reference genes are supposed not to be influenced by biological or experimental conditions (Boda et al., 2009). Different methods can be applied to substantiate the suitability of these genes as endogenous controls. NormFinder (Andersen et al., 2004) , BestKeeper (Pfaffl et al., 2004) , geNorm Vandesompele et al. (2002) and delta CT method

(Livak and Schmittgen, 2001) are the most used tools for housekeeper validation. The web-based tool RefFinder (http://www.leonxie.com/referencegene.php?type = reference) combines these four straightforward software programs to compare and rank the tested candidate reference genes. Based on the rankings from each program, RefFinder assigns an appropriate weight to an individual gene and calculates the geometric mean of their weights for the overall final ranking. Anaylsis of our tested potential housekeeping genes revealed 16S as the most stable one, followed by EF1-α and RNA polymerase.

2.8 Statistics

After normalization of the RNA samples with a previously identified BestKeeper (RNA polymerase, EF1-α, 16S) possible outliers were detected by using four outlier tests (Thompson Tau, z-score, Nalimov and IQR). Data points have been removed, if three out of four methods indicated an outlier. Expression levels of mRNA were calculated relative to the minimal expression level for each gene and represent the mean ± SEM (n = 4-5) of used replicates per treatment (early and late diapause). Normal distribution of the data were tested with normal quantile-quantile plots and the Shapiro-Wilk-Test for each gene and the two sampling times (early and late diapause) using the statistical software R (Version 3.1.2, http://cran.r-project.org/bin/windows/base/). Where the criteria for normal distribution and equal variances were met, significance between the two sampling times (early and late diapause) was investigated with a two-sided t-test conducted with R. Furthermore, the software package RAIN (= rhythmicity analysis incooperating non-parametric methods) for R/Bioconductor was applied to detect possible rhythmic behaviour in our time series data (Thaben and Westermark, 2014). The significance level for all analyses was set at $p<0.05$.

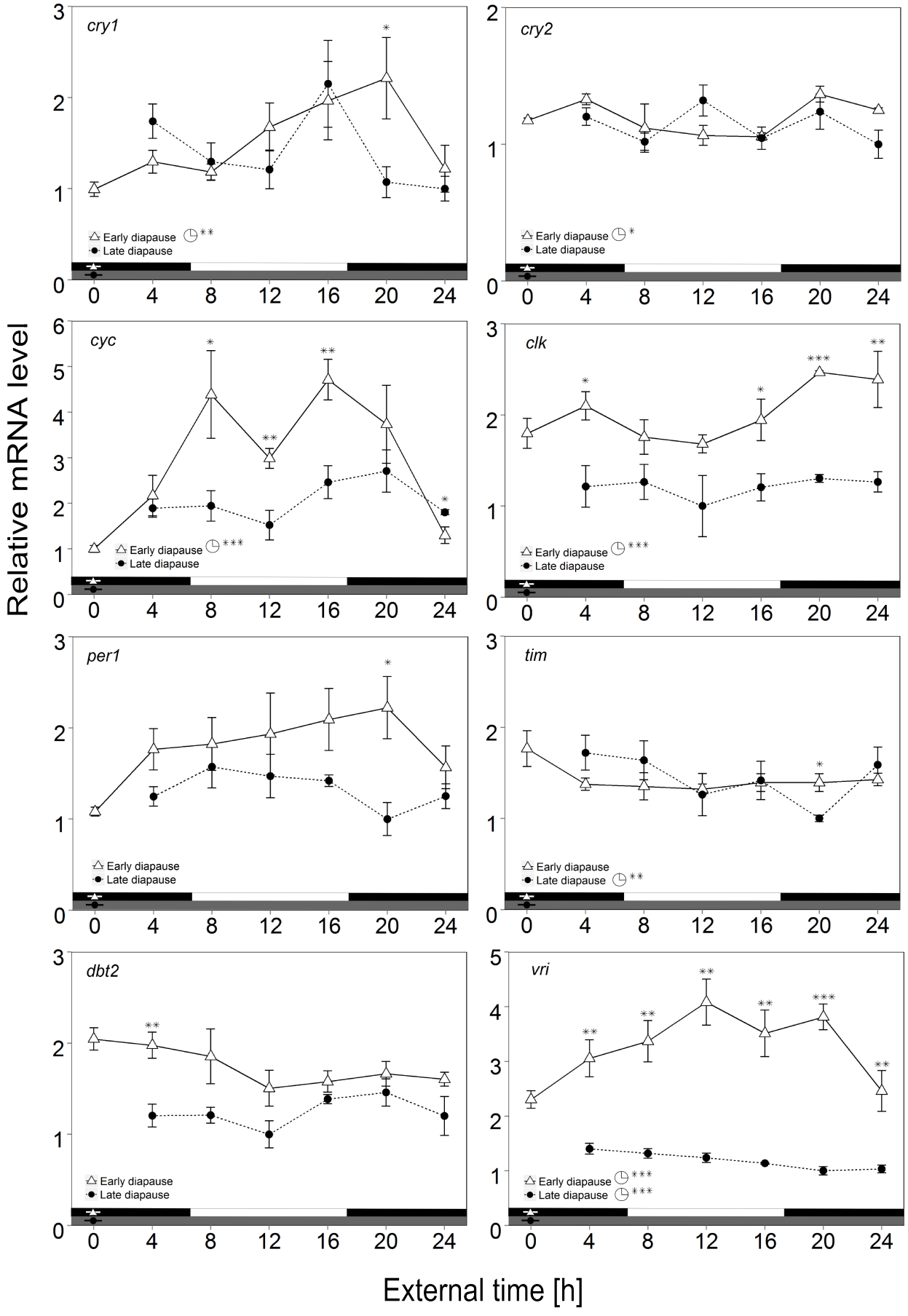
3 Results

Gene expression levels of eight potential core clock genes (*clock, cycle, period, timeless, cryptochrome 2, doubletime, vrille, cryptochrome 1*) were investigated of CV copepodids being in early (September 2014, 10 h light:14 h dark) and late diapause (January 2015, constant darkness DD). Copepods being in early diapause (LD) exhibited generally higher relative mRNA levels compared to copepods in late diapause (Figure 3.1). During early diapause half of the investigated genes (*cry1, cyc, per1, vri*) reached their maximum expression levels during the day or early night, whereas others (*cry2, clk, tim, dbt2*) showed lowest expression levels during the day. For copepods in late diapause a generalized pattern such as rising or falling levels linked to day/night phase was not observed. For most clock genes a diel rhythmic oscillation with a period of 24 h could be detected for copepods in early diapause (RAIN: $p<0.05$), while mRNA levels of copepods in late diapause showed no rhythmic behaviour over 24 h (RAIN: $p>0.05$) except for two genes (*tim, vri*).

3.1 Clock gene expression patterns during early diapause

During early diapause expression patterns of the investigated genes could be divided in three patterns: upregulation during light phase (*cry1, cyc, per1, vri*), downregulation during light phase (*cry2* and *clk*) and a quite stable expression over 24 h (*tim, dbt2*). A diel rhythmicity for *cry1* during early diapause with a period of 24 h could be detected (RAIN: $p<0.01$) (Figure 3.1). *Cry1* seemed to be upregulated at the beginning of the light phase (8 h), having its highest expression levels at 20 h, fol-

Figure 3.1 ***(following page)*****: Clock gene expression patterns** - Eight potential clock genes *cry1, cry2, per1, tim, dbt2, vri, clk, cyc* of *C. finmarchicus* being in early (September 2014) and late (January 2015) diapause were analyzed. After normalization to a BestKeeper (consisting of RNA polymerase, EF1-α, 16S), data were expressed as relative mRNA levels. Each point represents the mean $\pm$ SEM (n=4-5). Bars beneath the graph indicate the photoperiod during sampling. Light conditions were as follows: LD 10 h:14 h in September and constant darkness in January (black/grey bars = darkness, white bars = light). Rhythmic oscillation with a period of 24 h was detected with RAIN (Thaben and Westermark, 2014) and is indicated by clocks and stars. Significant differences between each diel time point of early and late diapause were calculated with with a two-sided t-test (indicated by stars). Significance levels were as follows: '$***$' 0.001, '$**$' 0.01, '$*$' 0.05.


cry1
Early diapause **
Late diapause
cry2
Early diapause *
Late diapause
cyc
Early diapause ***
Late diapause
clk
Early diapause ***
Late diapause
per1
Early diapause
Late diapause
tim
Early diapause
Late diapause **
dbt2
Early diapause
Late diapause
vri
Early diapause ***
Late diapause ***
Relative mRNA level
External time [h]

lowed by a steep decrease until 24 h. An earlier increase in gene expressions starting at 0 h until 20 h was observed for *cyc, vri* and *per1*, whereas *cyc* and *vri* showed a drop in expression levels at 12 h and 16 h, respectively. Except for *per1*, a diel rhythmic oscillation with period of 24 h for *cyc* and *vri* could be detected (RAIN: $p<0.001$). Overall, *cyc* and *vri* had the highest expression levels reaching relative mRNA levels over 4. *Clk* and *cry2* showed a similar gene expression pattern characterized by a downregulation during light phase. For both genes, a rhythmic behaviour oscillating with a period of 24 h was detected by RAIN (*cry2* $p<0.05$, *clk* $p<0.001$), whereas *clk* had higher expression levels (range from 1 to 2.468) compared to *cry2*. Except for a higher relative abundance at 0 h, *tim* was constitutively expressed with low levels.

3.2 Clock gene expression patterns during late diapause

A division into defined expression patterns like for early diapause was not possible for late diapause mRNA levels. Most genes lacked a rhythmic oscillation with a period of 24 h during late diapause (RAIN: $p>0.05$), except for *tim* and *vri* (Figure 3.1). *Vri* mRNA levels almost stayed constant within the 24 h period (range from 1 to 1.4), whereas *tim* mRNA levels showed no clear pattern over time. *Cyc, clk* and *dbt2* all showed similar expression patterns reaching lowest mRNA levels at 12 h during light phase. *Per 1*, on the other hand, reached its lowest mRNA level at 20 h. Except for a peak at 16 h, *cry1* showed a decline over the 24 h period. Relative mRNA levels of *cry2* alternate between rising and falling parts every 4 h.

3.3 Early diapause vs. late diapause

A comparison of each diel time point between early and late diapause (two-sided t-test) was used to distinguish significant differences ranging from zero (*cry2*) up to six (*vri*) diel time points differences. Early and late diapausing relative mRNA levels are within the same range for *cry1*, *cry2* and *tim* by comparing both sampling times. For all other clock genes (*cyc*, *clk*, *per1*, *dbt2*, *vri*) expression levels were generally higher during early diapause over the investigated 24 h period. No significant difference between early and late diapause could be detected for *cry2* (two-sided t-test: $p<0.05$). *Cry2* mRNA levels of early and late diapause were within the same range and besides a higher expression at 12 h, gene expression patterns were quite similar (Figure 3.1). Only one significant difference between early and late diapause could be detected for the genes *cry1* ($p<0.01$ for 20 h),

per1 ($p<0.05$ for 20 h), *tim* ($p<0.05$ for 20 h) and *dbt2* ($p<0.01$ for 4 h). *Dbt2* mRNA levels showed a similar pattern in early as well as late diapause reaching a minimum at 12 h. For *cyc* and *clk* four diel time points were significantly different between early and late diapause. Significant differences for *clk* between early and late diapause could be detected for time points 4 h, 16 h, 20 h and 24 h (two-sided t-test; $p<0.05$, $p<0.05$, $p<0.001$, $p<0.01$, respectively). For *cyc* significant differences could be observed for 8 h ($p<0.05$), 12 h ($p<0.01$), 16 h ($p<0.01$) as well as 24 h ($p<0.05$) by comparing early and late diapause. Comparison between early and late diapause expression patterns of *vri* revealed a significant difference for all time points (two-sided t-test; 4 h-16 h and 24 h: $p<0.01$, 20 h: $p<0.001$).

4 Discussion

A previous study of Christie et al. (2013) already revealed the existence of several clock genes in the calanoid copepod *Calanus finmarchicus*. The present study aimed to investigate eight potential circadian clock gene expression patterns (*clock, cycle, period, timeless, cryptochrome 2, double-time, vrille, cryptochrome 1*) in CV copepodids of *C. finmarchcius*. Most clock genes showed a diel rhythmic oscillation during early diapause (LD), whereas in late diapause (DD) a significant rhythmic oscillation was not detectable for most of the investigated genes. Comparison of early and late diapause revealed significant differences between expression levels. Overall, copepods caught in early diapause had higher relative mRNA levels compared to copepods sampled in January. Investigations of clock gene expression patterns in copepods being in distinct diapause phases allows us to get an understanding of the potential role of the circadian clock in regulating seasonal diapause. This study provides first observations of the performance of the clock in diapausing *C. finmarchicus* at two times of this seasonal event, early (September) and late diapause (January).

One key component of this study was to sample diapausing copepods. Timing of *C. finmarchicus* entering and terminating diapause as well as the duration of overwintering varies among locations (Hind et al., 2000). Relatively little information is available concerning seasonal changes of zooplankton communities and their timing of life cycle strategies in high Arctic fjords like Kongsfjorden in Spitsbergen. In the Gulf of Maine *C. finmarchicus* CVs emerge in late December (Durbin et al., 1997), whereas in the Scotian shelf they return to surface waters in February (McLaren et al., 2001). The latest documented emerging period, March-April, is documented from copepods inhabiting the St. Lawrences Estuary (Plourde et al., 2001). At the Norwegian coast, CV copepodids overwinter from July to February and emerge to surface waters in February-March (Marshall and Orr, 1955; Tande, 1982; Falkenhaug et al., 1997). In Kongsfjorden CV copepodids have been reported as the most dominant stage in 65 m depths at the end of August, followed by extremely low surface abundances at the beginning of September (Willis et al., 2006) indicating that the majority of CV copepodids migrated down to deeper waters to overwinter. During diapause, *C. finmarchicus* reduces its metabolism and suppresses development and reproduction (Hirche, 1996a). Biochemical measurements would valid the diapausing state of *Calanus finmarchicus*. Activities of metabolic enzymes like citrate synthase

and malate dehydrogenase are known to serve as indicators for overall metabolic activity due to their role as key enzymes within the citric acid cycle (Meyer et al., 2002; Cullen et al., 2003; Donnelly et al., 2004). Due to time limitations within this study, such measurements could not be conducted, but respective samples were taken and await analysis. Abundance data collected during September revealed that *C. finmarchicus* CV copepodids were still present in the water column between 0-200 m (Häfker pers. comm.). However, the highest proportion of *Calanus finmarchicus* CVs was located deeper in the water column in depth of 200-320 m. Thus, we suggest that collected CV copepodids have been in diapause during September 2014 and January 2015, forming the basis of our research.

4.1 A functional clock in *Calanus finmarchicus*

Christie et al. (2013) could identify molecular components of a circadian clock in *C. finmarchicus* by using the fruit fly *Drosophila melanogaster* circadian system as reference for mining clock transcripts. Nevertheless, reverse blasting of the *Calanus* sequences revealed a closer correlation to non-*Drosophila*-isoforms rather than to *Drosophila* itself (Christie et al., 2013). Together with the existence of both cryptochromes, *cry1* and *cry2*, the potential circadian copepod clock might be an ancestral one as found in the monarch butterfly *Danaus plexippus* (Zhu et al., 2008). Such a clockwork mechanism with two distinct expressed functional *cry* genes has not been fully described yet (Zhu et al., 2008). Furthermore, instead of generally one *per* gene involved in the circadian clock system of insects (e.g. *D. melanogaster*, *D. plexippus*), Christie et al. (2013) could identify three *per* genes (*per1, per2, per3*) in *C. finmarchiucs* as found in mice (Tei et al., 1997; Takumi et al., 1998). All three mice *per* genes are the molecular relative of insect *per* (Tei et al., 1997). Due to their potentially similar role within the clock and time limitations within this study, we only investigated *per1*. Knowledge about circadian clocks in crustaceans with respect to distribution, oscillatory activity and chronobiological functions is scarce (Strauss and Dircksen, 2010) and information about the functioning of circadian clocks in polar marine crustaceans is missing. Thus, for further discussion of the potential role of each investigated clock gene, we compare our clock gene expression patterns for *C. finmarchicus* with one of the best studied clocks of *D. melanogaster* and known clock mechanisms of *D. plexippus* due to its closer correlation.

Drosophila CRY, analogue to CRY1 in *Calanus finmarchicus*, is activated during light exposure and binds to TIM promoting its rapid degradation through a proteasome dependent pathway (Ceriani

et al., 1999; Naidoo et al., 1999) to reset the clock to its 24 h cycle. *Per* and *tim* expression are both controlled by CLK:CYC heterodimers. Thus, in many insects *tim* and *per* transcriptional rhythms are robust and cycle almost in synchrony with rising mRNA levels during early night (Sehgal et al., 1995). In *C. finmarchicus* both genes showed distinct gene expression patterns. *Tim* was constantly expressed on a low level during the investigated 24 h period, whereas *per1* increased at the end of the night over the day reaching its peak at the beginning of the following night. If we expect that TIM has a similar function as in the monarch butterfly and *Drosophila*, TIM proteins could accumulate at the beginning of the night, when TIM degradation by light is not possible. For *Drosophila* it has been shown, that the circadian loop will continue to cycle as long as there is a delay generated by PER/TIM association and a suppression of PER accumulation in the absence of TIM (Dunlap, 1999). However, a rhythmic oscillation of *per1* and *tim* (early diapause) could not be detected with RAIN in this study, but other investigated genes still showed a rhythmic oscillation with a period of 24 h. Thus, TIM could also be modified by post-transcriptional mechanisms. Shaggy, for example, plays an important role within the clock. It is responsible for the timing of the nuclear transfer of the PER/TIM complex accomplished by promoting phosphorylation of TIM (Young and Kay, 2001) and should be considered as potential post transcriptional modification of TIM to ensure a delay in the feedback loop. In *Drosophila* DBT, analogue of DBT2 in *C. finmarchicus*, physically associates with PER and PER/TIM complexes. Furthermore, it promotes phosphorylation and degradation of single PER in the cytoplasm and nucleus leading to a delay within the feedback loop (Kloss et al., 1998). *Drosophila dbt* is constitutively expressed like *dbt2* in *C. finmarchicus* in our study.

In *Drosophila*, PER acts as the main negative regulator of the clock function by associating with TIM for translocation into the nucleus (Konopka and Benzer, 1971). However, in insects expressing *cry2*, PER's function seems to have been replaced by CRY2 (Sandrelli et al., 2008). Thus, *cry2* should have the same expression patterns as *tim* as well as *per* as it still associates with both proteins TIM and CRY2 (Zhu et al., 2008). Nevertheless, in our study *cry2* showed a daily rhythmic expression pattern with lowest expression levels during the day, whereas *tim* levels were constitutively expressed. Within the endogenous circadian timing system in the Antarctic krill *Euphausia superba*, *cry2* expression patterns were highly rhythmic in a light:dark cycle (LD 16:8) with an upregulation until the middle of the day (Teschke et al., 2011). Furthermore, expression levels of mRNA were 2-3 folds higher in *E. superba* compared to our findings.

Vrille is an essential component for embryonic development oscillating with a circadian rhythm as *per* and *tim* regulated by the transcription factors CLK and CYC (Blau and Young, 1999). Cycling *vri* is required for a functional *Drosophila* clock (Cyran et al., 2003). Accumulation of *per* and *tim* mRNA was repressed by a high constitutive expression of *vri*, what could explain the absence of cycling *per1* and *tim* in our study. *Clk* RNA levels cycle with a PER/TIM dependent manner, but with a different phase as compared to *per* and *tim* mRNA oscillations (Bae et al., 1998). However, in our study with a downregulation during the day *clk* showed a completely different pattern by comparing the oscillation with *per* and *tim* mRNA levels. PER and TIM proteins dimerize and enter the nucleus, where PER inhibits CLK/CYC activity to complete the negative feedback loop. In *Drosophila cyc* is constitutively expressed, whereas in several other insects *cyc* mRNA levels vary (Meireles-Filho and Kyriacou, 2013) as found in *Calanus finmarchicus cyc* mRNA levels.

Despite distinct clock gene expression patterns to other arthropod species, diurnal clock gene expression patterns in early diapause point to the existence of a functional clock in the calanoid copepod *Calanus finmarchicus*. By comparing clock gene expression patterns of *C. finmarchicus* with other arthropod expression patterns (*D. melanogaster*, *D. plexippus*, *E. superba*), detected differences might indicate distinct roles of clock genes within the potential circadian clock in *C. finmarchcius*, if we assume that copepods might still have detected photoperiod in sampling depths of $\sim$300 m. On the other hand, if we exclude the possibility of entrainment by photoperiod at these depths, these differences might also have occurred due to the fact, that sampled *C. finmarchicus* were already in diapause in September.

Photoperiod is generally regarded as the most reliable entrainment cue controlling the movement of migrators during DVM and seasonal diapause (Marcus, 1985; Kuhlman et al., 2007; Bartok et al., 2013). We assumed that *C. finmarchicus* sampled in September (light:dark cycle (LD) 10 h L:14 h D) might show a rhythmic expression of clock genes entrained by the light:dark cycle. It seems that *C. finmarchicus* sampled in September (LD) might be synchronized to prevailing light:dark cycles in Kongsfjorden as indicated by rhythmic oscillations of at least several investigated clock genes. However, also a sensor attached to a CTD (conductivity-temperature-depth instrument) could not detect any light in sampling depths of $\sim$300 m, whereby one should keep in mind that the light detecting sensor was not the most sensitive one. There is no literature available concerning light regime patterns in the water column down to 300 m in the high Arctic Kongsfjorden during autumn. However, it is known that sunlight intensity decreases exponentially with depth. Within the twilight or disphotic

zone (200-1000 m) light is not sufficient for photosynthetic processes, but faint sunlight is still detectable. Light availability of individuals depends on the spectral sensitivity of an organisms as visual systems are not sensitive to all wavelengths equally (Cohen and Forward, 2002). Only a few studies have investigated the phototactic response of copepods related to the rate of change and absolute intensity threshold as well as spectral sensitivity (Stearns and Forward, 1984b; Cohen and Forward, 2002; Cohen and Forward Jr, 2005). Buskey and Swift (1985) investigated the spectral sensitivity of *C. finmarchicus* by simulating bioluminescent flashes of different wavelengths and found the greatest responses with blue-green wavebands, matching ambient twilight at the time of migration. Further literature concerning the spectral sensitivity of *C. finmarchicus* in particular is not available. However, a recent study revealed the lowest irradiance (10^{-8} - 10^{-6} µmol photons m^{-2} s^{-1}) eliciting a significant phototactic response in *Calanus* spp. (Båtnes et al., 2013). In deep-sea plankton DVM was performed precisely to prevailing local sunrise and sunset between 500 and 650 m (van Haren and Compton, 2013). Together with the knowledge that zooplankton can sense even the smallest light quantities (Stearns and Forward, 1984b), photoperiodic entrainment of *C. finmarchicus* clock in these depths might still be possible. When we exclude the possibility of photoperiodic entrainment in samplings depths of $\sim$300 m, observed clock gene expression patterns might already indicate the existence of a circadian clock. *Calanus finmarchicus* conducts DVM within a day, down to depths ranging from 200-1000 m depending on the location. Thus, descending down to deeper waters to overwinter at 300 m could be performed within one day. We do not know, how long animals have been at these depths before sampling. Several studies revealed that clock gene expression patterns continued to oscillate in constant darkness for a few days with a lower amplitude (Emery et al., 1998; Blau and Young, 1999; Young and Kay, 2001; Teschke et al., 2007). These studies rely on several days in LD cycles followed by several days in DD. In our study one indication for an already free-running period could be detected rhythmic mRNA levels of the clock gene *cry1*. During light exposure CRY1 is activated leading to degradation of TIM and setting the clock to its 24 h cycle. Thus, expression levels of *cry1* are shown to have a close correlation to light exposure with increased mRNA levels during the day followed by a decline during dark phase (Emery et al., 1998; Zhu et al., 2008). In our study a slightly shifted pattern was observed for *cry1*, where mRNA levels increased during the day until beginning of the night. The increase until the dark phase could be explained by a delayed response due to the possible absence of photoperiod as entrainment cue at sampling depths.

4.2 Clock gene expression levels in late diapause

Inhabiting polar regions is accompanied with a strong variability in annual day length (Hut et al., 2013). Knowledge about the adaptions used by polar organisms to entrain their circadian clocks to prevailing extreme light conditions is scarce. Investigations of the circadian clock in the Arctic reindeer *Rangifer tarandus* indicate an absent or a reduced circadian rhythmic oscillation under constant light conditions. A circadian rhythmic of locomotory activity occurred only under pronounced LD cycles (autumn/spring) (van Oort et al., 2005; Lu et al., 2010). Some other Arctic species such as birds, squirrels and porcupines maintained their circadian activity cycles indicating an intact circadian clock during constant light conditions (Folk et al., 2006; Silverin et al., 2009). Thus, due to a possible wide plasticity of the circadian clock, polar organisms are able to cope with prolonged periods in the absence of a strong *Zeitgeber*. They can either display arrhythmicity or switch to alternative time cues such as spectral composition (Pohl, 1999; Lu et al., 2010; Wallace et al., 2010).

We assumed that rhythmic clock gene expression patterns might not be observable in *Calanus finmarchicus* in the absence of an external entrainment signal such as photoperiod during late diapause. In our study no significant rhythmic oscillation could be detected for most of the investigated clock genes in *Calanus finmarchicus* except for *tim* and *vri*. For *vri* the difference in gene expression relative levels range from 1-1.4 with extremely low standard errors, assuming that RAIN thus might have detected a rhythmic oscillation. Furthermore, for each diel time point 15 CV copepodids were pooled for analysis. It is known that there are differences of circadian clock expression patterns among species as well as among individuals of one population. Thus, pooling of organisms might also have influenced mRNA results of *tim* and *vri*. Furthermore, behavioural rhythmicity is controlled by a master clock located in the central brain and besides the coordination of rhythms in peripheral tissues by a master clock, autonomous clocks within peripheral tissues could be detected in some organisms (Hege et al., 1997; Plautz et al., 1997). Although similar rhythmic oscillations of central clocks and peripheral clocks could be observed (Hege et al., 1997), peripheral clocks play an important role sometimes independently of brain clocks (Allada and Chung, 2010).

By comparing gene expression patterns of early and late diapause in our study, genes lost or damped their diurnal gene expression patterns observed in September. Gene expressions were for almost all genes lower compared to early diapause. Several studies revealed that clock gene expression patterns (*cry1, cry2, tim, vri*) continued to oscillate in constant darkness for a few days with a lower amplitude (Emery et al., 1998; Blau and Young, 1999; Young and Kay, 2001; Teschke et al.,

2007). These studies rely on organisms held for several days in LD cycles followed by several days in DD, whereas copepods caught in January have lived for several months in constant darkness. After a defined time span under constant light conditions, rhythmicity of circadian clock gene expressions were damped in other studies (Aréchiga, 1993).

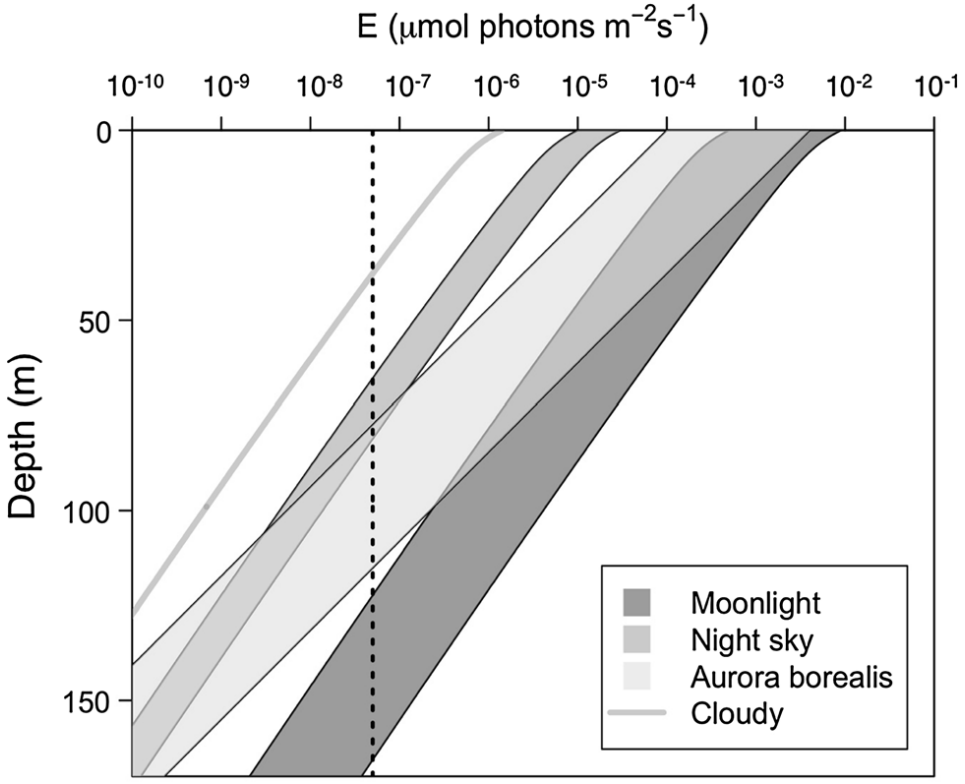


Figure 4.1: Irradiance levels during polar night - Irradiance from moon, night, sky and aurora borealis with depth, the shaded areas representing and approximate range of irradiance from each source. The vertical dotted line (0.05 x 10 $^{-6}$ µmol photons m^{-2} s^{-1}) represents the lowest irradiance value for photoperiodic response in *Calanus* spp. (Båtnes et al., 2013).

Sampling in January took place during polar night, excluding a possible entrainment by pronounced light:dark cycles. On the other hand, it is known, that zooplankton can perceive even the smallest light quantities (Stearns and Forward, 1984b; Båtnes et al., 2013) and might not only be entrained by solar illumination, but also by lunar illumination during polar night with intensities far below the threshold not detectable for human eyes (Berge et al., 2009; Cohen and Forward Jr, 2005). Båtnes et al. (2013) investigated the spectral sensitivity of field-collected *Calanus* spp. sampled during polar night. Irradiance (E) of moon, night sky and aurora borealis were linked to depth and the lowest irradiance value for photoperiodic response in *Calanus* spp., which corresponds to 0.0005-0.5 % of the polar night surface irradiance (Figure 4.1). Modelling results revealed a response of *Calanus*

spp. down to 70-80 m depth to clear night sky E, 120-170 m to moonlight and 80-120 m to aurora borealis (Båtnes et al., 2013). Furthermore, a recent study reported the ambient light regime in the high Arctic during polar night (Cohen et al., 2015). Several Arctic zooplankton species were able to detect and use ambient light down to 20-30 m depth during polar night. However, our copepods were sampled in 220-345 m depths and due to the absence or damped diurnal gene expression patterns we, thus, exclude a possible entrainment by photoperiod during late diapause.

4.3 Time to (dia)pause - how to tell time

Investigations of diapausing *C. finmarchicus* under laboratory conditions is accompanied with difficulties, including realization of overwintering depth of 300 m. We cannot substantiate a functional causality of the circadian clock in regulation of diapause in this study without the possibility to manipulate the clock and observe the impact on diapause. We rely on sampling diapausing copepods being in distinct phases and investigate variations within clock gene expression patterns such as amplitude and shifts in phase and period. No external factor or combination of factors could yet be identified as directly responsible for the initiation or termination of diapause in calanoid copepods (Miller et al., 1991; Johnson et al., 2008; Ji, 2011). Some of the most convincing evidence that the circadian clock is involved in diapause initiation includes recent molecular studies on the clock genes themselves. In *D. melanogaster* mutant strains of *per*, *tim* and *clk* led to a disruption of a diapause imitating narcosis like state (Pegoraro et al., 2014). Ikeno et al. (2010) used RNA interference (RNAi) to knock-out core circadian clock genes in the bean bug *Riptortus pedestris* and assessed whether the bugs are able to enter diapause. The RNAi technique uses RNA molecules to inhibit gene expression by destruction of specific mRNA molecules. (Ikeno et al., 2010) suggest that the circadian clock as a functional unit, rather than individual genes regulates diapause initiation in *R. pedestris*. *Cycle* and *per* function as opposing regulators and have been shown to play an important role in diapause of *R. pedestris* (Ikeno et al., 2010). Inhibition of either *cycle* or *period* expression affected ovarian diapause. While *cyc* RNAi suppressed ovarian diapause even under long-day conditions, *per* RNAi led to an induction of ovarian development under short-day conditions (Ikeno et al., 2010). However, in *Drosophila* no association of *cyc* with diapause occurred, whereas *tim* and *cry* seemed to have significant, but independent effects on diapause (Yamada and Yamamoto, 2011). Yamada and Yamamoto (2011) even suggested that the occurrence of diapause might not be based on the circadian clock function

as a unit, but independently relies on individual clock genes. For example, Tauber et al. (2007) assumed that adaptation to seasonal conditions is enhanced by a natural mutant allele in *Drosophila melanogaster*. They discovered that natural and artifical alleles of the *timeless* gene affected the incidence of diapause in response to changes in light and temperature (Tauber et al., 2007). Besides different assumptions of the involvement of the whole clock as a unit or just several clock genes in diapause, it seems also to depend on the species, which clock genes might influence diapause. With our findings we can see, that during early diapause most clock genes still showed a rhythmic oscillation. However, for *tim, per* and *dbt2* a rhythmic oscillation was not detectable, what could indicate, that those three genes might influence the initiation of diapause. Further studies should include clock gene expression patterns of active *Calanus finmarchicus*, right before entering diapause (August) as well as in the middle of the midnight sun (e.g. June), to have the possibility to compare gene expression patterns of diapausing copepods with active copepods.

The circadian clock is tracking daily changes of light and other environmental factors and has been studied well at the molecular level, at least for several organisms including insects, mice, humans and plants (Aschoff, 1965; Allada and Chung, 2010; Foster and Helfrich-Förster, 2001). The circadian system plays an important role in photoperiodic measurements in almost all species studied to date (Kuhlman et al., 2007). When we assume that photoperiod might act as a entrainment cue for the circadian clock due to its reliability, *Calanus finmarchicus* must discriminate between long and short days. In most cases short days (long nights) result in a high occurrence, whereas long days (short nights) elicit a low occurrence of diapause (Tagaya et al., 2010). During sampling in September, the night was already 4 hours longer than the day (10 h L: 14 h D). Thus, *C. finmarchicus* might already have reached its critical photoperiod (CPP) in August at Kongsfjorden. The CPP of an organism is defined as the point at which the incidence of diapause is 50 % of its maximal level (Meuti and Denlinger, 2013). The CPP increases with latitude enabling populations at higher latitudes to adjust to the earlier onset of winter by entering diapause at an earlier date (Bradshaw and Holzapfel, 1975; Jordan and Bradshaw, 1978). Thus, the short days with 10 h and/or the long night with 14 h could have triggered the initiation of diapause in *C. finmarchicus*. There are controversial hypothesis concerning how animals might respond to changes in photoperiod concerning seasonal events.

In 1936 Bünning first proposed the idea, that the circadian clock regulating daily activities is also involved in a seasonal photoperiodic timing measurement system by measuring the length of day and night initiating photoperiodic responses such as diapause (Bünning, 1936). Bünning's hy-

pothesis, later named external coincidence model, states, that the relative size of the light and dark-requiring phases encodes the critical photoperiod that induces the seasonal response. This model suggests the existence of an endogenous rhythms of 24 h, which is composed of two 12 h cycles, the subjective day (photophil, photosensitive) and the subjective night (scotophil, photoinsensitive). In this model light has a dual effect. It entrains the endogenous circadian rhythm of subjective day and subjective night and it stimulates photoperiodic responses if it coincides with the photosensitive phase (subjective night). With longer days in spring light starts penetrating into the scotophil (photosensitive) phase triggering a physiological or behavioural response. Due to a longer shift of light into the scotophil phase during summer, animals recognize these days as long days and enter a non-diapause phenotype. Short day effects like diapause are seen in autumn, when light is restricted to the photophil phase (Saunders, 1978; Goto and Numata, 2009; Tagaya et al., 2010). Since Bünnings hypothesis, this assumption has been verified for various organisms (Pittendrigh, 1981; Saunders et al., 2004). The internal coincidence model, first described by Pittendrigh and Minis (1964), suggests two oscillators being entrained by dawn and dusk and does not require the dual role of light as described in the external coincidence model. The critical photoperiod is encoded by unique phase relationships between two internal oscillators. Light is only necessary for the synchronization of the circadian system. Changing photoperiods will alter the internal phase relationship of the two oscillators resulting in states of permission and inhibition of multiple circadian rhythms.

Excluding the involvement of a circadian clock in photoperiodic measurement, the hourglass model, the non-circadian timer, relies on the total number of hours of light per day (Lee, 1950). The hourglass model assumes the gradual accumulation of a physiological agent in the organism during one part of the light:dark cycle, whereas during the other part the agent is degraded. A threshold is reached, if the light or dark phase had been long enough to lead to an accumulation of this physiological reagent without a previous degradation. Thus, after reaching a certain amount of this agent a physiological response is triggered (Lee, 1950). With our findings, we cannot tell which model might be applicable for the initiation/termination of diapause in *Calanus finmarchicus*.

Copepods in late diapause (DD) showed a weak till no oscillation of most clock genes. The absence of rhythmic oscillations during late diapause also point to the fact, that the clock was not entrained by other environmental cues. In Kongsfjorden, *C. finmarchicus* is terminating diapause probably at the beginning of March. During this time a pronounced light:dark cycle already exists again. The polar night begins mid of October, lasts for 129 days and ends mid February. The

question remains, if clock genes start to show a rhythmic oscillation with recurring photoperiod or if other external factors trigger the termination of diapause.

Nevertheless, there is also evidence that in the absence of photoperiod animals can display arrhythmicity or switch to alternative time cues (Lu et al., 2010; Wallace et al., 2010). Sampling in January was conducted during polar night. Thus, photoperiod as entrainment cue during this time was not possible. Temperature and food availability have been proposed as cues for seasonal entrainment triggering diapause (Hirche and Kwasniewski, 1997; Niehoff and Hirche, 2005; Cavallari et al., 2011; Ji, 2011; Clark et al., 2012; Pierson et al., 2013). The descent of *Calanus glacialis* females in Norway to lower depths and the arrest of their reproductive activity where apparently related to a temperature increase in the surface layer, similar to earlier observations in the White Sea (Niehoff and Hirche, 2005). However, many circadian clocks are temperature compensated meaning that the period of oscillations is remarkably stable over a wide temperature compensation, whereas changes in the apparent phase can occur (Aréchiga, 1993; Pittendrigh, 1954; Bartok et al., 2013). Furthermore, different entrainment pathways of the circadian clock such as pressure in *Carcinus maenas* (Taylor and Naylor, 1977) or food in *Procambarus clarkii* for locomotory activity (Page and Larimer, 1972) were found. Hind et al. (2000) proposed that diapause might me cued directly by a decrease in food supply rather than photoperiod. Thus, it seems to depend also on the species, which environmental cue might be applicable for entrainment of the circadian clock.

A further assumption is the involvement of lipids, which could play a major role in initiating and terminating diapause. This so called lipid accumulation window hypothesis point to a lipid-modulated endogenous timer controlling dormancy duration (Johnson et al., 2008). Individuals can only enter dormancy if their lipid storage reached a defined accumulation threshold to endure overwintering, moulting and gonad maturation. Individuals that did not reach this threshold would remain at the surface. The length of dormancy might be controlled by the quantity of lipid reserve built up before entering dormancy. After depletion of lipid reserves to a certain level copepods might become active ascending back to surface waters (Irigoien, 2004; Hassett, 2006; Johnson et al., 2008). Prior to entering diapause, *C. finmarchicus* accumulates large amounts of lipids. However, not all individuals of *C. finmarchicus* CV copepodids enter a diapause state. This could be an indication for the lipid accumulation windows hypothesis. After migrating to surface waters in late winter, low lipid levels of *C. finmarchicus* indicate that feeding on the spring bloom is required prior to development and

reproduction (Willis et al., 2006), whereas some adult females might start reproducing prior to the bloom (Niehoff et al., 1999).

Overall, successful reproduction of *C. finmarchicus* strongly depends on the timing of the annual spring bloom, whose time of occurrence can be strongly affected by seasonal ice coverage and water temperatures (Sakshaug and Slagstad, 1991; Niehoff et al., 2000; Søreide et al., 2010; Hodal et al., 2012; Weydmann et al., 2013). Climate-mediated changes in ice dynamics and stratification of nutrient-rich water masses lead to short and intense Arctic phytoplankton blooms and impose temporal asynchronies between energy requirements and food availability for organisms inhabiting polar regions (Falk-Petersen et al., 2007). Thus, temporal shifts of occurring phytoplankton blooms can lead to timing mismatches between herbiviorous copepods, including *C. finmarchicus* and primary producers having also a negative impact on higher trophic levels (Søreide et al., 2010; Leu et al., 2011; Ji et al., 2013). It is from great importance to understand the exogenous and endogenous mechanisms controlling diapause to predict future scenarios caused by climate change.

4.4 Conclusion

Little is known about the molecular underpinnings of circadian and seasonal rhythms in pelagic calanoid copepods and generally in marine crustaceans (Miller et al., 1991; Johnson et al., 2008; Marcus and Scheef, 2010; Ji, 2011). The cue triggering diapause is still under discussion (Johnson et al., 2008; Clark et al., 2012, 2013). Due to its importance as key species in the northern ecosystem, physical and biological factors triggering and controlling the initiation/termination of diapause in *Calanus finmarchicus* needs to be investigated to understand the consequences of climate change on this species as well as on the entire arctic food web. We assume that a circadian clock might be involved in seasonal diapause. A closer correlation to non-*Drosophila*-isoforms (Christie et al., 2013) and the existence of both cryptochromes, *cry1* and *cry2* as in the monarch butterfly *D. plexippus* (Zhu et al., 2008) point to an ancestral circadian clock in the calanoid copepod *C. finmarchicus*. To gain knowledge about the potential involvement of a circadian clock, we need to investigate the performance of the clock at distinct diapause phases. This study provides the first investigations of clock gene expression patterns of diapausing *C. finmarchicus* at two distinct diapause phases, early (September) and late (January) diapause. The detection of rhythmic oscillations with a period of 24 h in *C. finmarchicus* being in early diapause (10 h:14 h LD) point towards the existence of a diurnal or even circadian clock in *C. finmarchicus*. Copepods sampled during late diapause showed no or at least a weak rhythmic oscillation of clock genes, indicating that the clock was not ticking in January during polar night with the absence of photoperiod as possible entrainment cue. Future studies need to focus on circadian clock gene expression patterns throughout the season (active copepods) and must include more time points of diapausing copepods to gain further knowledge about the performance of the clock and possible entrainment cues initiating/terminating diapause.

5 Outlook

To clearly prove the existence of a circadian clock in *Calanus finmarchicus* laboratory experiments with several days in light:dark cycles followed by several days in constant darkness (DD) should be conducted to valid the continuity of significant rhythmic oscillations with a period of 24 h in constant light conditions. Furthermore, comparison of clock gene expression patterns of active and diapausing *C. finmarchicus* shall give an opportunity to investigate circadian characteristics (e.g. amplitude, period, phase, relation) at different stages throughout the season. The maintenance of the feedback loop to a $\sim$24 h rhythm is accomplished by cyclic activation and inhibition of gene expression as well as post-transcriptional modifications such as phosphorylation, relocalisation and degradation of proteins (Mackey, 2007). Thus, to get an understanding of the whole clock machinery, it is necessary to investigate protein levels and their role within the clock. Knock-out experiments with the RNAi technique could clarify the role of investigated clock genes within the feedback loop. Investigation of photobehavioural sensitivity of *C. finmarchicus* will clarify the possibility of detecting light at the overwintering depths of $\sim$300 m. The open question remains what physiological/behavioural patterns are controlled by a circadian system of *C. finmarchicus*. Further investigations need to focus on the role of the circadian clock in seasonal time-keeping and on seasonal patterns of clock controlled target genes to understand regulatory processes in the phenology including the initiation and termination of diapause.

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