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Influence of light and temperature cycles on the expression of circadian clock genes in the mussel *Mytilus edulis*

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ABSTRACT

Clock genes and environmental cues regulate essential biological rhythms. The blue mussel, *Mytilus edulis*, is an ecologically and economically important intertidal bivalve undergoing seasonal reproductive rhythms. We previously identified seasonal expression differences in *M. edulis* clock genes. Herein, the effects of light/dark cycles, constant darkness, and daily temperature cycles on the circadian expression patterns of such genes are characterised.

Clock genes *Clk*, *Cry1*, *ROR/HR3*, *Per* and *Rev-erb/NR1D1*, and *Timeout*-like, show significant mRNA expression variation, persisting in darkness indicating endogenous control. Rhythmic expression was apparent under diurnal temperature cycles in darkness for all except *Rev-erb*. Temperature cycles induced a significant expression difference in the non-circadian clock-associated gene *aaNAT*. Furthermore, Suppression Subtractive Hybridisation (SSH) was used to identify seasonal genes with potential links to molecular clock function and revealed numerous genes meriting further investigation. Understanding the relationship between environmental cues and molecular clocks is crucial in predicting the outcomes of environmental change on fundamental rhythmic processes.

1. Introduction

Intertidal habitats are under the influence of multiple fluctuating conditions, a number of which, such as diel day/night cycles, tidal immersion/exposure and annual seasonal progression, are cyclical in nature and can provide reliable environmental cues to inform biological timekeeping. Rhythmic biological processes, producing diverse physiological and behavioural outputs over a wide range of temporal and spatial scales, are essential to marine ecosystems (Tessmar-Raible et al., 2011). Common examples include rhythmic locomotor activity (Newcomb et al., 2014), oxygen consumption (Kim et al., 1999), feeding (Houki et al., 2015), reproduction (Wayne, 2001), cell renewal (Zaldibar et al., 2004), nerve impulses (Jacklet, 1969) and gene expression (Connor and Gracey, 2011).

Endogenous biological rhythms are underpinned by a circadian clock mechanism operating on a sub-cellular level. Clock gene expression, evolved to occur over a ~24 h period, is regulated by a negative feedback system in which clock proteins inhibit their own expression (Allada et al., 2001; Young and Kay, 2001). Though able to persist in the absence of environmental cues, synchronisation of these endogenous molecular rhythms by cycling environmental parameters like light and temperature is known as entrainment, and is an essential component of circadian rhythms (Golombek and Rosenstein, 2010; Dubruille and Emery, 2008; Rensing and Ruoff, 2002). Entrainment is considered adaptive and influences the timing of diverse physiological and behavioural processes via clock-output genes (Zhang et al., 2009).

Despite the importance of clock genes in regulating endogenous rhythmic processes in diverse phyla, the molecular mechanisms governing timekeeping ability are an understudied aspect of molluscan ecology. Recently emerging studies have characterised clock genes in gastropods (Cook et al., 2018; Duback et al., 2018; Schnytzer et al., 2018; Bao et al., 2017; Constance et al., 2002), cephalopods (Heath-Heckman et al., 2013), and bivalves (Perrigault and Tran, 2017; Sun et al., 2016; Pairett and Serb, 2013) including mussels (Chapman et al., 2017; Connor and Gracey, 2011). Blue mussels, *Mytilus edulis*, are an edible species of commercial importance (FAO, 2018), a biogenic keystone species (Gutiérrez et al., 2003) and are considered early indicators of the effects of climate change (Zippay and Helmuth, 2012).

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Important rhythmic processes documented in mussels include circadian valve activity (Gnyubkin, 2010), circatidal rhythms of epithelial cell renewal (Zaldibar et al., 2004), seasonal cycles of gametogenesis (Seed, 1969), and both circadian and tidal gene expression patterns with the former being more prevalent (Connor and Gracey, 2011). Among these rhythmic transcripts are the clock genes *Cry1* and *ROR*, which cycle on a circadian basis in the gills of *Mytilus californianus* (Connor and Gracey, 2011). In other bivalves, endogenous circadian expression of multiple clock genes occurs in the Pacific oyster *Crassostrea gigas* (Perrigault and Tran, 2017; Mat et al., 2016) whereas only weak/non-rhythmic clock gene oscillations were apparent in the limpet *Cellana rota* (Schnytzer et al., 2018). *Cry1* and *Cry2* are also rhythmically expressed in the head of the squid *Euprymna scolopes* (Heath-Heckman et al., 2013). The putative endogenous nature of the blue mussel molecular circadian clock is yet to be investigated.

In addition to their 24 h periodicity and endogenous nature, circadian rhythms are temperature-compensated, maintaining their periods over a range of constant temperatures (Ruoff, 2004; Rensing et al., 2001). However, both periodic and pulse temperature changes affect molecular clock function directly and indirectly in many organisms by impacting input pathways, molecular mechanisms, amplitude levels, phase shifts and entrainment (Rensing and Ruoff, 2002). Furthermore, temperature acts as an important cue (zeitgeber) for rhythm entrainment in both vertebrates and invertebrates (Waite et al., 2017; Glaser and Stanewsky, 2005; Lahiri et al., 2005) with daily temperature cycles of as little as 1–2 °C able to entrain ectotherms (Rensing and Ruoff, 2002). Though elevated temperature during exposure at low tide disrupts rhythmicity of genes with circadian and tidal periodicities in mussels (Connor and Gracey, 2011), the influence of temperature cycles on bivalve clock gene activity is unknown. Wild intertidal blue mussels experiencing natural seasonal photoperiod and temperature shifts, exhibit clock gene expression differences between seasons (Chapman et al., 2017), but the relative importance of light and temperature are yet to be elucidated. In addition, seasonal expression differences in genes involved in gametogenesis, metabolism, stress response and immunity have been documented in mussels (Banni et al., 2011; Ciocan et al., 2011), however there is further scope to investigate seasonally expressed genes with putative links to the molecular clock mechanism.

The primary purpose of this study was to assess whether *M. edulis* clock genes (1) exhibit a circadian pattern of mRNA expression under light/dark conditions, (2) have endogenous rhythmicity that persists in constant darkness and (3) are influenced by 24 h temperature cycles in the absence of light. To address these aims, we conducted a laboratory-based experiment to investigate the mRNA expression patterns of *M. edulis* clock genes (*Clock, Cry1, ROR/HR3, Period, Rev-erb/NR1D1*) as well as genes with closely associated clock functions (*ARNT/HIF-1β, Timeout*-like, *aaNAT*) under different 24 h light and temperature cycles. The secondary purpose of this study was to isolate and identify novel seasonal *M. edulis* genes with putative links to the molecular clock mechanism. A global transcriptomic approach was used to compare seasonal cDNA libraries from wild male and female *M. edulis* respectively, at standardised stages of gametogenesis. For the first time we reveal the endogenous nature of *M. edulis* clock gene expression in the mantle tissue and investigate the effect of 24 h thermocycles on a bivalve molecular clock mechanism. Characterising these underlying mechanisms of circadian regulation is an essential first step in predicting the effects of environmental change on the essential rhythmic processes of this important marine species.

2. Materials and methods

2.1. Sample collection, acclimation and histology

For the photoperiod/thermocycle experiment, adult *M. edulis* (mean length ± SEM, 40.53 ± 0.25 mm), were collected from Filey beach, North Yorkshire, UK (54° 13′ longitude and 0° 16′ latitude) during low tide on 24.11.16. Mussels were transferred to the laboratory and divided across 9 aerated aquaria (n = 56 in each) containing 30L of 35 ppt artificial seawater (Tropic Marin, Germany). Mussels were acclimated for 10 days to constantly submerged conditions at 9.7 ± 0.4 °C (mean ± SD) with a photoperiod of 10 h light/14 h dark. Throughout both the acclimation period and the experiment, mussels were fed daily at irregular times with PhytoGreen-M Suspension (Brightwell Aquatics, UK) containing *Tetraselmis* sp. phytoplankton at a final concentration of ~0.43 million cells per mL and 50% water changes were conducted on alternate days at irregular times. Formalin-preserved mantle tissue from all samples were wax embedded, sectioned (10 μm), stained with haematoxylin and eosin, and viewed under the microscope to assess gender and gametogenesis stage according to Seed (1969).

For the seasonal field experiment, *M. edulis* were collected at low tide from Filey beach during winter 2014, spring 2015 and summer 2015 as described in detail in Chapman et al. (2017). Sexually developing females at gametogenesis stages βII to βIII (Seed, 1969) were randomly selected from the late morning winter and summer sampling time (n = 7 each) which differ in photoperiod by 10 h. Sexually developing males at gametogenesis stages βIII to βIV (Seed, 1969) were selected at random from late morning winter and spring time-points (n = 6 each), with a photoperiod difference of 4.5 h as insufficient βIII/βIV males were obtained in summer to allow a winter/summer comparison.

2.2. Photoperiod and thermocycle exposures

To investigate the effects of diel light and temperature cycles, laboratory-acclimated mussels (n = 168 per treatment) were exposed for 13 days to one of the following regimes: 10:14 h light/dark cycles at constant temperature (LD), constant darkness at constant temperature (DD), and constant darkness with thermocycles (DDTC) of 10:14 h thermophase:cryophase varying by 3.6 ± 0.2 °C (mean ± SD). This diel temperature range is environmentally relevant (Kawai and Wada, 2007) and the LD regime has previously been used to investigate daily and tidal activity and *Cry1* expression rhythms in the bivalve *C.gigas* (Mat et al., 2016). Each of the 3 aquaria used per treatment were independent and were each continually aerated by two air stones. Abiotic conditions were recorded throughout using a Hi 97500 Portable Luxmeter (Hanna Instruments Ltd, Leighton Buzzard, UK), "ama-digit ad 15th" Electronic Thermometer (Amarell Electronic, Kreuzwertheim, Germany), a V2 salinity refractometer (TMC, UK), and a 3510 pH Meter (Jenway, Bibby Scientific Limited, Stone, UK). Water parameters were maintained as follows (mean ± SD): water temperature in LD and DD, 9.7 ± 0.5 °C; water temperature at end of DDTC cryophase, 10.1 ± 0.4 °C; water temperature at end of DDTC thermophase, 13.7 ± 0.4 °C; salinity, 35.4 ± 0.6 ppt and pH, 7.8 ± 0.1. Thermocycles were controlled by adjusting ambient air temperature in a climate-controlled room to simulate gradual water temperature change.

Zeitgeber times 0 to 10 (ZT 0–10) correspond to the photophase and the thermophase in the LD and DDTC treatments respectively. At the end of the experiment, n = 28 mussels were sampled from each treatment at each of the 6 time points over the course of a day: ZT 23 (an hour before photophase/thermophase), ZT 1, ZT 5, ZT 9, ZT 11 and ZT 15 (Fig. S1). Mantle tissue was dissected and stored in RNAlater® Stabilisation Solution (Thermo Fisher Scientific, Loughborough, UK) at −80 °C for molecular analysis and in 10% formalin solution (Sigma Aldrich, Gillingham, UK) for histological examination.

2.3. Total RNA isolation and cDNA synthesis

For the photoperiod/thermocycle experiment, total RNA was extracted from a subset of randomly-selected male mussels from each time-point from each of the treatments (n = 7–9) using 10 mg of mantle tissue. Reagents from the High Pure RNA Tissue Kit (Roche, Burgess Hill, UK) were used according to manufacturer's instructions, which included DNase I treatment. RNA concentrations were measured using the Qubit

1.0 Fluorometer (Life Technologies, Paisley, UK) and 180 ng of RNA was used for cDNA synthesis using the Precision Nanoscript2 Reverse Transcription Kit with random nonamer (9 bp oligonucleotide) primers (PrimerDesign, Cambridge, UK).

2.4. Isolation of clock genes

Degenerate primers for *Per* were designed from a nucleotide alignment using sequences from the scallop *Mizuhopecten yessoensis* (XM_021519834.1) and the oyster *C. gigas* (JH816853.1) (Table S1). 0.5 μL of each 10 μM primer was used for PCR containing 2.5 μL Fisher BioReagent 10X Taq Buffer A (Fisher Scientific, UK), 0.5 μL dNTP mix (10 mM each), 0.25 μL Fisher BioReagents™ Taq DNA Polymerase (Fisher Scientific, UK), 0.5 μL $MgCl_2$ (25 mM), 1 μL cDNA and molecular-grade water (Fisher Scientific, UK) to a total volume of 25 μL. Thermal cycling conditions were 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 45 °C for 30 s and 72 °C for 30 s, with a final extension of 72 °C for 2 min. 5 μL of PCR product was subjected to a second PCR under the same conditions and the resulting band purified from the gel using the Macherey Nagel NucleoSpin Gel and PCR Clean-up Kit (Fisher Scientific, UK) and eluted in 20 μL 5 mM Tris/HCl buffer.

Rev-erb primers (Table S1) were designed from the *Mytilus galloprovincialis* sequence EF644354.2 (Raingeard et al., 2013) and were tested on *M. edulis* using a PCR with 5 μL 5X Herculase II Reaction Buffer (Agilent Technologies, UK), 0.5 μL dNTP mix (10 mM each), 0.25 μL Herculase II Fusion DNA Polymerase (Agilent Technologies), 0.5 μL of each primer (10 μM), 1 μL cDNA and molecular-grade water (Fisher Scientific, UK) to a total volume of 25 μL. Thermal cycling conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, with a final extension of 72 °C for 5 min.

All PCR products were run on 1% agarose TBE gels stained with Gel Red Nucleic Acid Gel Stain (Cambridge Bioscience, Cambridge, UK) and sequenced using the EZ Seq Sanger sequencing service (Macrogen Europe, Amsterdam, The Netherlands). Sequences were edited and aligned using BioEdit (Version 7.0.9.0) and identities were confirmed using blastn/blastx searches against the NCBI GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). *Clk*, *Cry1*, *ARNT*, *Timeout*-like, *ROR/HR3* and *aaNAT* have previously been isolated from blue mussels (Chapman et al., 2017).

2.5. Sequence alignments and phylogenetic analysis

Multiple species amino acid sequence alignments were created for PER and Rev-erb using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). For phylogenetic analysis MEGA7, Molecular Evolutionary Genetics Analysis version 7.0 (Kumar et al., 2016), was used for sequence alignment, manual editing and phylogeny construction using maximum likelihood analysis. Sequences selected included those from model species of vertebrates and insects in addition to available marine invertebrate sequences from molluscs and crustaceans. The Jones-Taylor-Thornton (JTT) model and the Nearest Neighbour Interchange (NNI) method for heuristic searches were used. The bootstrap method with 1000 replicates was applied to assess support for the tree with values displayed on nodes.

2.6. qPCR expression of clock and clock-associated genes

Real-time qPCR assays were optimised and performed for *Clk*, *Cry1*, *ROR/HR3*, *Per*, *Rev-erb*, *ARNT*, *Timeout*-like and *aaNAT*. Relative quantification was performed using *18S* and *EF1* as reference genes as their suitability for *M. edulis* qPCR experiments has previously been assessed (Chapman et al., 2017; Cubero-Leon et al., 2012; Ciocan et al., 2011) and their geometric mean expression was not significant between groups ($KW = 27.199_{17}$, $p = 0.0552$). qPCR primers gave reaction efficiencies of 90–110% (Table S2) in line with the MIQE guidelines (Bustin et al., 2009). qPCR reactions contained 10 μL PrecisionPLUS 2x qPCR MasterMix with SYBR Green for the ICycler (PrimerDesign, Southampton, UK), 7 μL molecular-grade water, 2 μL primer mix (see Table S2) and 1 μL ½ diluted cDNA. Reactions were performed in duplicate on a CFX96 Real Time PCR Detection System (Bio-Rad, Hemel Hempstead, UK) as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 1 min and 72 °C for 1 min. Melt peaks were generated at the end of each reaction to confirm primer specificity and template-negative reactions were included for each primer pair on all plates. The data were normalised to the geometric mean of the reference genes using the $2^{-\Delta Ct}$ version of the comparative Ct method (Schmittgen and Livak, 2008), and presented graphically using Microsoft Excel.

2.7. Suppression Subtractive Hybridisation (SSH)

SSH is a molecular technique allowing the identification of mRNAs that are differentially expressed between a pair of sample sets. The SSH approach was used to identify genes that were differentially expressed between (1) winter and summer females (βII/βIII) and (2) winter and spring males (βIII/βIV). Total RNA was extracted and quantified as previously described. RNA integrity was assessed on a 1% formaldehyde agarose denaturing gel stained with 0.15 μg/mL ethidium bromide (Invitrogen, UK). Four RNA pools were created (winter females, summer females, winter males, and spring males) with each sample represented at an equal concentration (female pools: 357.1 ng, $n = 7$; male pools: 416.7 ng, $n = 6$), totalling 2.5 μg RNA. cDNA was synthesised using SMARTer™ PCR cDNA Synthesis Kit (Clontech, Saint-Germain-en-Laye, France) reagents according to manufacturer's instructions. Forward and reverse-subtracted libraries were created using reagents from the PCR-Select cDNA Subtraction Kit (Clontech, France) and the Advantage 2 PCR Kit (Clontech, France) according to the manufacturer's protocols. The differential PCR products were cloned using the Original TA Cloning Kit with pCR 2.1 Vector (Life Technologies, UK) and chemically competent MAX Efficiency DH10B *E. coli* cells (Life Technologies, UK). Colonies were screened for inserts by PCR using vector-based primers and reagents from the Nucleospin Plasmid DNA purification Kit (Machery-Nagel, UK) were used to purify plasmids before sequencing with the EZ-seq DNA Sanger sequencing service (Macrogen Europe, The Netherlands). Sequences were edited using Bioedit (version 7.2.5) and identified using BLAST searches against the NCBI nucleotide and protein databases. Sequences were accepted when E-values, the number of hits expected to be obtained by chance, were $<10^{-5}$.

2.8. qPCR analysis of seasonal genes

Six mRNAs identified by SSH were selected for further investigation using real-time qPCR. qPCR reactions were performed as previously described with the exception that assays were prepared as follows: 10 μL FastStart Universal SYBR Green Master (Rox) (Roche, UK), 7 μL molecular-grade water (Fisher Scientific, UK), 1 μL of each primer (Table S3) and 1 μL of cDNA (derived from 190 ng RNA). qPCR was performed on the same samples used for SSH, supplemented with additional samples at the same gametogenesis stages to increase the sample number to $n = 8$–11 per group.

2.9. Statistical analysis

Statistical analyses were performed in GraphPad InStat v3 on the normalised expression values. For the photoperiod/thermocycle experiments, one-way Analysis of Variance (ANOVA) was performed for each treatment, followed by the post hoc Tukey-Kramer Multiple Comparisons Test when significance was detected. In instances where the Bartlett's test detected unequal variance, the non-parametric Kruskal-Wallis test was applied, followed by the Dunn's Multiple Comparisons Test when significance was detected. mRNA expression that significantly varied across the 6 time points was considered rhythmic expression. For the seasonal experiment, the data were analysed using unpaired t-tests

or the Mann-Whitney Test when appropriate. In all cases, statistical significance was accepted at the $p < 0.05$ level.

3. Results

3.1. Isolation of clock genes Per and Rev-erb from M. edulis

A 575 bp partial *Per* sequence (GenBank accession MH836580) was isolated from *M. edulis*. The conceptually translated amino acid sequence (blastx) shared 41% similarity with a period-like isoform from the oyster *Crassostrea virginica* (XP_022345656.1) and 38% with sea snail *B. gouldiana* period (AAK97374.1). *Per* was subsequently identified in *M. galloprovincialis* by comparing *M. edulis Per* against the transcriptome database generated by Moreira et al. (2015), where a 96% similarity match was obtained with a 3.3 Kb sequence (Unigene27326). A multiple-species amino acid alignment, showing a PAS domain and the Period C terminal region, indicates relatively low PER sequence homology even among bivalves (Fig. S2), as has previously been noted for other bivalve species (Pairett and Serb, 2013). The *M. galloprovincialis* PER sequence, as the longer of the two *Mytilus* sequences, was used for phylogenetic analysis. This sequence grouped with other mollusc sequences, particularly bivalves, and showed a greater degree of similarity with other invertebrate PER sequences than with vertebrate PERs (Fig. S3).

A 427 bp partial *Rev-erb/NR1D1* sequence (MH748543) was also obtained. The conceptually translated sequence shared 96% amino acid similarity with both *M. galloprovincialis NR1D1* (ABU89807.2) and *NR1D2* (ABU89808.2) followed by a 72% match with *C. gigas NR1D* (AHV90297.1), and contained a DNA-binding domain of Rev-Erb receptor-like (Fig. S4). Phylogenetic analysis grouped the *Mytilus* sequence with other bivalves, closer to other invertebrates groups than vertebrates (Fig. S5).

3.2. Rhythmic clock mRNA expression under LD persists in DD

Expression patterns of clock and clock-associated genes were investigated in *M. edulis* exposed to 10:14 h photocycles (LD) and constant darkness (DD) in parallel. Significant variation in mRNA expression levels were observed under LD conditions for all five of the canonical clock genes investigated: *Clk*, *Cry1*, *ROR/HR3*, *Per* and *Rev-erb* (Figs. 1 and S6; Table S4). Generally, expression significantly increased in LD after lights on at zeitgeber time (ZT) 0 and then decreased by the end of the photophase at ZT 9 with some cases, *Cry1* in particular, showing a second peak in expression after the lights on/off transition at ZT11 (Figs. 1 and S6). For each of these genes, significant variation was also observed in the absence of environmental light cues under DD with two significant increases in expression, at the start of the subjective day and night respectively, apparent for *Clk*, *Cry1* and *ROR* (Figs. 1 and S6; Table S4). For the clock-associated genes, a non-significant trend was detected for *ARNT* under LD, which was significant under DD with increases in expression at the start of the subjective day and night respectively. *Timeout*-like exhibited significant variation under LD and DD, peaking at ZT16 during the LD scotophase, and showing peaks during both the subjective day and night under DD (Figs. 1 and S6; Table S4). Conversely, *aaNAT* exhibited constant expression under both LD and DD (Figs. 1 and S6; Table S4).

3.3. Effect of DDTC on rhythmic clock mRNA expression

The impacts of 3.6 ± 0.2 °C (mean ± SD) temperature cycles applied to mussels kept in constant darkness (DDTC) were also assessed in terms of clock and clock-associated gene mRNA expression. For four of the five clock genes investigated, *Clk*, *Cry1*, *ROR/HR3* and *Per*, the significant mRNA variation under LD and DD was also apparent under DDTC (Figs. 1 and S6; Table S4). However, thermocycles resulted in a loss of significant rhythmicity for *Rev-erb* (Table S4). For the clock-associated genes, significant daily expression differences were apparent for *ARNT* and *Timeout*-like with peak expression occurring in the cryophase in both cases (Figs. 1 and S6; Table S4). The constant expression of *aaNAT* exhibited under both LD and DD was significantly modulated by thermocycles (Table S4); mRNA expression peaked during the mid thermophase, and was lowest immediately after the transition to the cryophase (Figs. 1 and S6).

3.4. Suppression Subtractive Hybridisation analysis

A total of 40 clones containing successfully ligated plasmids were sequenced from the female SSH cDNA libraries and blastx and blastn searches against the NCBI database revealed the following matches: 37.5% with known identities and function were suggested to be either up- or downregulated in summer females compared to winter females (Table 1), 12.5% were unnamed sequences, 7.5% had no matches and 42.5% were duplicate sequences. For males, a total of 18 ligated plasmids were sequenced: 27.7% matched to sequences with known identities and function which were indicated to be either up- or downregulated in males from spring compared to males from winter (Table 2), 5.5% matched to unnamed sequences, 27.7% had no database matches and 38.8% were duplicate sequences. Of the successfully identified sequences, the majority of matches were to molluscs (Tables 1 and 2).

3.5. qPCR validation of SSH

Six target mRNAs isolated during the SSH experiment were selected for qPCR investigation. Transcripts with a range of different functions (Tables 1 and 2) were selected from across the 4 cDNA libraries as follows: *PDXK* (indicated by SSH to be upregulated in summer females), *GTPBP1-like*, *ABCE1* and *Neuroplastin-like* (downregulated in SSH summer females), *ND4* (upregulated in SSH spring males) and *eIF-4A-like* (downregulated in SSH spring mussels) (Fig. 2). *GTPBP1-like* expression was significantly lower in the summer compared to the winter (Mann-Whitney: $U = 23.0$, $p = 0.0465$), consistent with the SSH findings (Table 1). Significant differences were not detected between seasons for the other genes in this preliminary dataset.

4. Discussion

This study investigated the rhythmic characteristics of the blue mussel molecular circadian clock. The impacts of light and temperature cycles on clock mRNA expression patterns were investigated in a laboratory-based mesocosm experiment. For the first time in the species, we isolated *Per* and *Rev-erb* sequences and examined their diel expression alongside other clock (*Clk*, *Cry1*, *ROR/HR3*) and clock-associated (*ARNT*, *Timeout*-like, *aaNAT*) genes under 24 h light/dark cycles (LD), constant darkness (DD) and 24 h 3.6 °C temperature cycles in constant darkness (DDTC). Results indicated rhythmic endogenous expression of clock genes, revealed by persistence of expression variation in the absence of light/dark cues, and the ability of temperature cycles to modulate gene expression patterns in some instances.

4.1. Effect of photocycles and constant darkness on clock mRNA expression

Clock genes and their protein complexes interact via negative feedback loops, forming the molecular clock mechanism at the heart of biological timekeeping (Hardin, 2005; Young and Kay, 2001; Shearman et al., 2000). The single *Per* homolog present in molluscs (Sun et al., 2016; Constance et al., 2002) was isolated in *M. edulis* herein and grouped phylogenetically with other molluscan PER sequences (Fig. S3). Also isolated was *M. edulis Rev-erb* (*E75*), containing the characteristic DNA-binding and ligand-binding domains (Fig. S4), which also shared most sequence similarity with other mollusc sequences (Fig. S5).

Table 1

Table 1 mRNAs indicated by SSH to be potentially differentially expressed between summer and winter in the gonads of female *M. edulis*, at sexual development stages βII to βIII. Sequences shown gave NCBI database matches to sequences of known identities. * blastn nucleotide search, all other results are from a blastx search.

Clone accession number	Length (bp)	Identity	Species match	Result accession number	e-value	Function	Reference(s)
Up-regulated in female summer solstice samples (compared to winter solstice)							
Signal transduction							
JZ970419	222	**Heat shock protein 90**(*HSP90-1*)	*M. galloprovincialis*	CAJ85741.1	2e-29	- Molecular chaperone - Signal transduction - Circadian clock interactions with BMAL1	Picard (2002) Zhao et al. (2011) Schneider et al. (2014)
JZ970427	274	**Calmodulin (CaM)**(*calmodulin*)	*Lymnaea stagnalis*	ABB85281.1	6e-11	- Calcium-activated signal transduction - Biomineralisation - Phototransduction	Van Eldik et al. (1998) Li et al. (2004) Hardie (2001)
Metabolic function							
JZ970420	776	**Pyridoxal kinase-like** (*PDXK)*	*Lottia gigantea*	XP_009054441.1	3e-117	- Transferase enzyme involved in vitamin B_6 metabolism	Schibler (2005)
Protein synthesis							
N/A	592	**16S (mitochondrial rRNA)***	*M. trossulus*	KU925349.1	0.0	- Mitochondrial ribosomal RNA involved in protein synthesis	Taanman (1999)
Down-regulated in female summer solstice samples (compared to winter solstice)							
Cell adhesion							
JZ970421	534	**Neuroplastin-like**	*C. gigas*	XP_011450189.1	9e-20	- Immunoglobulin superfamily (IgSF) transmembrane protein involved in cell adhesion and cell-cell recognition	Owczarek and Berezin (2012)
MH359088	609	**Hemicentin-like**	*Mizuhopecten yessoensis*	XP_021370012.1	1e-16	- Cellular adhesion - Mitotic cytokinesis - Retinal function	Xu et al. (2013)
Ribosome biogenesis							
JZ970422	545	**Nucleolar GTP-binding protein 1-like** (*GTPBP1-like*)	*Lingula anatina*	XP_013398687.1	5e-112	- Ribosome biogenesis - Linked to circadian rhythm regulation by enabling *aaNAT* degradation	Woo et al. (2011)
JZ970426	1004	**ATP-binding cassette sub-family E member 1** (*ABCE1*)	*C. gigas*	XP_011449756.1	0.0	- Initiation of translation - Ribosome biogenesis and recycling	Pisarev et al. (2010)
Energetic metabolism							
JZ970423	679	**ATP synthase lipid-binding protein, mitochondrial-like**	*Biomphalaria glabrata*	XP_013066447.1	2e-41	- Subunit of an enzyme catalysing adenosine triphosphate (ATP) synthesis (Electron transport chain/ energy metabolism)	De Grassi et al. (2006)
N/A	459	**Cytochrome *c* oxidase subunit II** (*COX2*)	*M. trossulus*	ADE05891.1	9e-53	- Subunit of an enzyme involved in the mitochondrial electron transport chain/energy metabolism	García-Horsman et al. (1994)
Cytoskeleton							
JZ970424	1033	**Alpha tubulin** (*alpha-tubulin*)	*B. floridae*	XP_002601443.1	0.0	- Microtubule formation: Cytoskeleton - Chromosome separation - Cilia/flagella structure	Keeling and Doolittle (1996)
JZ970428	540	**Centrosomal protein of 131 kDa-like (5-azacytidine induced protein 1)** (*CEP131*)	*C. gigas*	EKC38807.1	2e-16	- Cilia formation	Hall et al. (2013)
Protein cleavage							
JZ970425	338	**Mitochondrial-processing peptidase subunit beta-like (β-MPP)**	*Saimiri boliviensis boliviensis*	XP_010349997.1	4e-62	- Subunit of an enzyme that cleaves targeting signals from mitochondrial proteins	Gakh et al. (2002)

(*continued on next page*)

Table 1 (*continued*)

Clone accession number	Length (bp)	Identity	Species match	Result accession number	e-value	Function	Reference(s)
Metabolism							
JZ970429	421	**Chitinase/chitotriosidase-like**	*Mytilus coruscus*	AHC08445.2	3e-64	- Enzyme involved in chitin metabolism - Biomineralisation	Weiss and Schönitzer (2006) Banni et al. (2011)
Phosphorylation							
MH359089	724	**Dual specificity testis-specific protein kinase 1-like** (*TESK1*)	*Mizuhopecten yessoensis*	XP_021367571.1	3e-30	- Phosphorylation - Spermatogenesis role in males	Meng et al. (2014)

Table 2

Table 2 mRNAs indicated by SSH to be potentially differentially expressed between winter and spring in gonads of male *M. edulis*, at sexual development stages βIII to βIV. Sequences shown gave NCBI database matches to sequences of known identities. All are from blastx searches.

Clone accession number	Length (bp)	Identity	Species match	Result accession number	e-value	Function	Reference(s)
Up-regulated in male spring equinox samples (compared to winter solstice)							
Energetic metabolism							
N/A	1018	**NADH dehydrogenase subunit 4** (*ND4*)	*M. edulis*	AAV68419.1	4e-154	- Mitochondrial electron transport chain	Craft et al. (2010)
Cytoskeleton organisation							
JZ970430	328	**Beta tubulin** (*beta-tubulin*)	*Parascolymia vitiensis*	BAD11697.1	2e-75	- Microtubule formation: - Cytoskeleton - Chromosome separation - Cilia/flagella structure	Keeling and Doolittle (1996)
JZ970431	434	**Actin**	*Cyrenoida floridana*	AAS20336.1	1e-99	- Microfilament formation: - Cytoskeleton - Cell contraction - Cell motility	Mitchison and Cramer (1996)
Down-regulated in male spring equinox samples (compared to winter solstice)							
Protein synthesis							
JZ970432	979	**Eukaryotic Initiation Factor 4A-like** (*eIF-4A-like*)	*C. gigas*	XP_011421890.1	3e-142	- Helicase involved in protein synthesis by binding mRNA to ribosome	Andreou and Klostermeier (2013)
Metabolism							
JZ970433	915	**Chitinase/chitotriosidase-like**	*M. galloprovincialis*	AKS48199.1	8e-84	- Enzyme involved in chitin metabolism - Biomineralisation	Weiss and Schönitzer (2006) Banni et al. (2011)

The five clock genes investigated (*Clk*, *Cry1*, *ROR/HR3*, *Per* and *Rev-erb*), in addition to *Timeout*-like, showed significant oscillations in expression under LD cycles (Figs. 1 and S6; Table S4). In each case, significant variation in expression persisted under DD (Figs. 1 and S6; Table S4), indicative of endogenous circadian control. Expression differences in *M. edulis Clk*, *Cry1* and *ROR/HR3* were previously detected at comparable daily time-points between seasons (Chapman et al., 2017) but few studies to date have investigated clock gene expression patterns over 24 h in molluscs. *Cry1* and *ROR* exhibit circadian expression in the gills of the mussel *M. californianus* under LD and tidal conditions, whereas no rhythmicity was apparent for *Clk* or *Bmal* (Connor and Gracey, 2011). In the gills of the oyster *C. gigas*, expression of *Clk*, *Bmal*, *Per*, *Tim*, *Rev-Erb*, *Cry1*, *Cry2*, *pCry* and *6-4photolyase* varied under LD and was modulated by DD exposure (Perrigault and Tran, 2017). RNA interference of *C. gigas Clk* disrupts expression of other clock components including *Per*, *Bmal*, *Rev-erb*, and *Cry1* (Payton et al., 2017). *Cry1* in this same species also oscillated under LD but not DD in the adductor muscle, though oscillations under tidal entrainment persisted in constant darkness (Mat et al., 2016).

In non-bivalve molluscs, *Per* expression in the sea snail *Bulla gouldiana* was rhythmic in the eye under LD, but constant under DD (Constance et al., 2002). Significant differences in the expression of *Clk*, *Per* and *Cry* genes, showing night-time expression peaks, occur under LD conditions in the brains of the nudibranch *Melibe leonina* (Duback et al., 2018) whereas clock gene expression rhythms in the limpet *Cellana rota* did not uphold significance (Schnytzer et al., 2018). Finally, *Cry1* and *Cry2* have a daily expression pattern in the head of the squid *E. scolopes* (Heath-Heckman et al., 2013). An increase in clock gene expression at the lights on transition (ZT0) was observed during LD for a number of *M. edulis* clock genes herein (Figs. 1 and S6), consistent with findings in *C. gigas* gills (Perrigault and Tran, 2017). However, second peaks in expression apparent during the subjective night for a number of *M. edulis* genes under LD (*Cry1* and *Rev-erb*) and DD (*Cry1*, *Clk* and *ROR*) suggest a possible ultradian (<24 h) rhythm. Hypothetical models of bivalve molecular clocks have been proposed to incorporate elements of both mammalian and insect core interactions (Perrigault and Tran, 2017; Sun et al., 2016), though functional studies are required to further clarify bivalve clock organisation. The molecular mechanisms by which ultradian rhythms, such 12 h tidal rhythms, are regulated are also yet to be elucidated.

Clock-associated gene *ARNT* encodes a bHLH-PAS protein closely related to CYC/BMAL1; both dimerise with melatonin-activated NPAS4 to activate *Cry1* expression (West et al., 2013). A non-significant trend was apparent in *M. edulis ARNT* expression under LD which showed significant variation under DD (Figs. 1 and S6; Table S4). Significant variation in *Timeout*-like expression occurred under LD and DD (Figs. 1

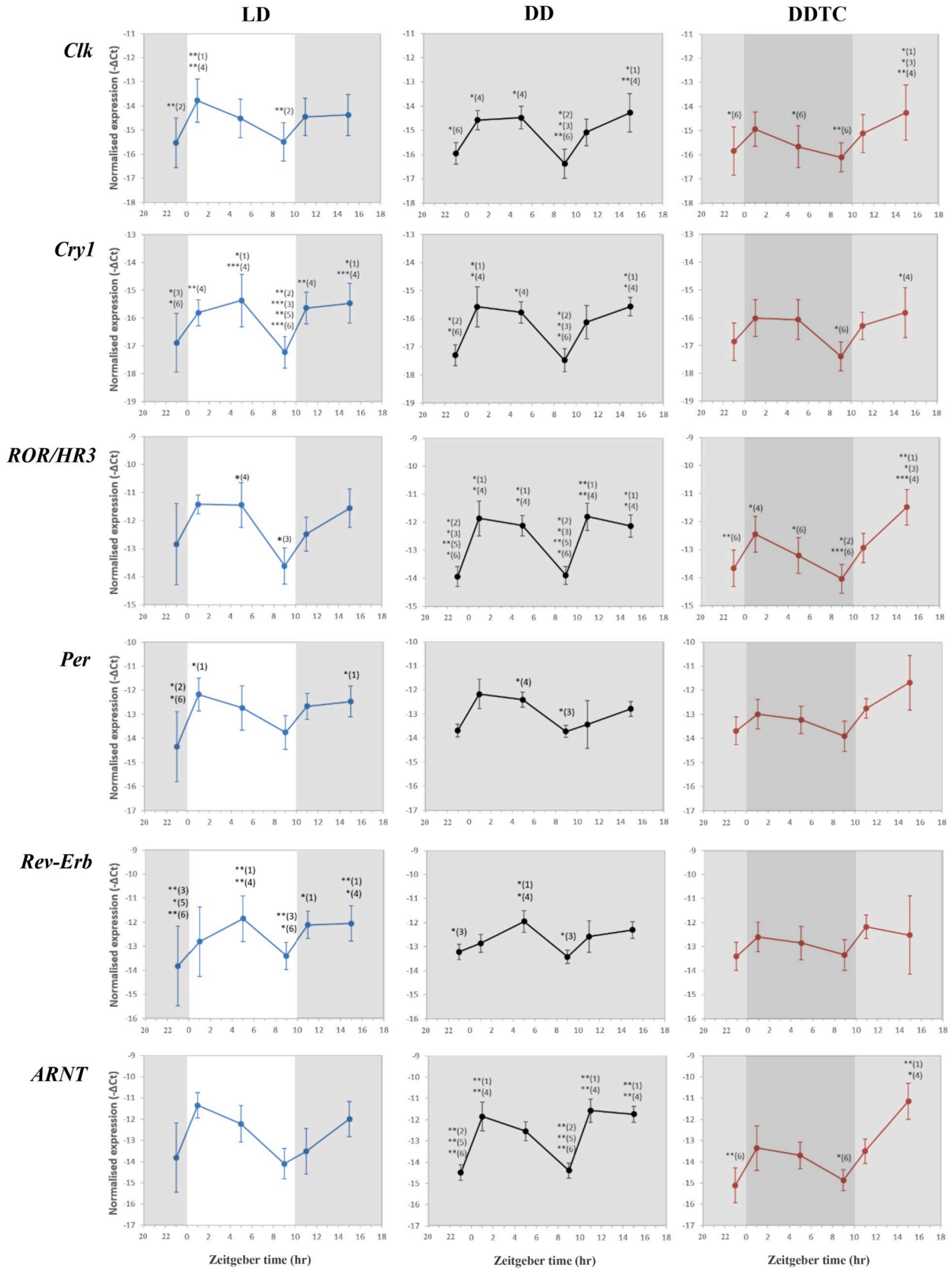

Fig. 1. Influence of photocycles and thermocycles on the daily variation of mRNA expression of clock and clock-associated genes in *M. edulis* male mantle tissue. Mean expression data, normalised to reference genes *18S* and *EF1*, are plotted ±SEM; $n = 5$–9. Significance denoted by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ with adjacent numbers in parentheses referring to significance from the time-points as follows: (1) ZT 23, (2) ZT 1, (3) ZT 5, (4) ZT 9, (5) ZT 11, (6) ZT 15. Abbreviations: LD, light/dark; DD, dark/dark; DDTC, dark/dark with ~3.5 °C thermocycles. Unshaded areas represent photophase, light shading represents darkness and heavy shading represents a thermophase during darkness.

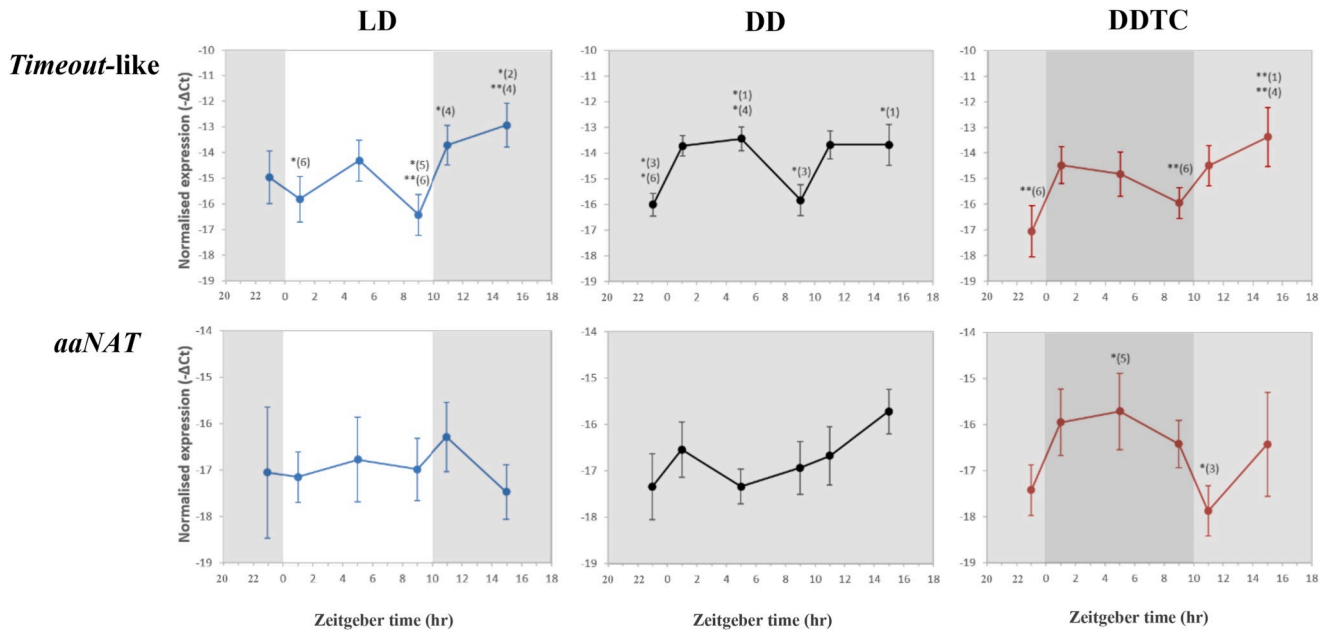

Fig. 1. (*continued*).

and S6; Table S4), indicating clock-control. *Timeout* is the ortholog of mammalian-type *Timeless* which, in addition to non-circadian roles, has been linked to mammalian clock function (Gotter, 2006; Barnes et al., 2003) and *Drosophila* light entrainment (Benna et al., 2010). *M. edulis Timeout*-like peaked during the LD scotophase (ZT15), as is the case in the sea anemone *Nematostella vectensis* (Reitzel et al., 2010). Finally, *aaNAT,* which encodes an enzyme involved in rhythmic melatonin production in mammals (Klein, 2007) and some invertebrates (Peres et al., 2014), did not oscillate under LD or DD and does not appear to be under circadian control.

4.2. Effect of thermocycles on clock mRNA expression

Acute temperature stress induces and represses a suite of genes across *Mytilus* species (Lockwood et al., 2015) and can disrupt expression patterns of rhythmic transcripts (Connor and Gracey, 2011). The effect of diurnal temperature cycles on the expression of bivalve clock genes has not previously been investigated. Though temperature-compensation ensures that the period of clock rhythmicity remains relatively constant over a range of temperatures, the phase of the clock can be reset by temperature cycles and changes (Sweeney and Hastings, 1960). This includes temperature entrainment of clock gene expression in vertebrates and invertebrates in both terrestrial and aquatic habitats (Glaser and Stanewsky, 2005; Lahiri et al., 2005; Rensing and Ruoff, 2002).

Diurnal temperature cycles modulated the expression of two genes herein. Firstly, the significant variation in *Rev-erb* expression under both LD and DD was no longer apparent under DDTC (Figs. 1 and S6; Table S4) indicating disruption. The endogenous nature of *Rev-erb* found herein is consistent with that of *C. gigas*; as is the case for vertebrates, *Rev-erb* is hypothesised to be a core bivalve clock component (Perrigault and Tran, 2017). Disruption of circadian *Rev-erb* expression has implications for core clock dynamics as well as its other functions which include lipid metabolism regulation in mice (Cho et al., 2012) and roles in reproduction, moulting and metamorphosis in arthropods (Cruz et al., 2012; Hannas et al., 2010). *Rev-Erbα* also provides a link between circadian and themogeneic processes in mice (Gerhart-Hines et al., 2013). Secondly, *aaNAT* expression varied over 24 h under DDTC, peaking during the thermophase (Fig. 1; Table S4), but expression was constant under both LD and DD indicating a clock-independent response to temperature. This is consistent with previous findings that this non-vertebrate version of the gene is involved in more ancestral functions such as detoxification (Pavlicek et al., 2010), contrasting with the rhythmically-expressed vertebrate form essential to melatonin synthesis (Klein, 2007). Rhythmic melatonin production does occur in molluscs (Muñoz et al., 2011; Abran et al., 1994) but any links to biological timekeeping require further investigation.

The mussel clock genes that exhibited variable expression under both LD and DD also showed significant variation in expression under DDTC (Figs. 1 and S6; Table S4) indicating that the oscillations were not disrupted by thermocycles. Variation in *ARNT* expression also occurred under both DD and DDTC (Figs. 1 and S6; Table S4). In the sea snail *Haliotis diversicolor*, *ARNT* (*HIF-1β*) expression in the gills and hemocytes was not affected by 3 °C heat shock, though the expression of HIF-1α, encoding the second subunit of the heterdimeric complex, was significantly elevated (Cai et al., 2014). HIF-1α links the circadian clock with the hypoxic signalling pathway in vertebrates via core clock genes (Peek et al., 2017; Egg et al., 2013). In other species, temperature affects further aspects of circadian regulation. Sensitivity of the *Drosophila* clock to temperature results from alternative 3' splicing of *Per* and *Tim* mRNAs in the untranslated regions, resulting in phase advancement of the clock at low temperatures (Helfrich-Förster et al., 2020; Dubruille and Emery, 2008). Temperature input pathways are therefore able to affect multiple levels of molecular clock organisation from transcription and translation to epigenetic modifications and protein degradation (Stevenson, 2018; Rensing and Ruoff, 2002). Differences between species in sensitivity of the molecular clock to temperature are considered a factor in allowing radiations into different climates (Helfrich-Förster et al., 2020; Rivas et al., 2018). Further investigation is required to determine the potentially synergistic effects of combined environmental stressors on the circadian functioning of *M. edulis* and other bivalves and functional studies will be key in determining associated changes in cellular function.

4.3. Identification of potential seasonal candidate genes

The success rate for cDNA sequence identification from the SSH approach was 31.7% for the female experiment and 27.7% for males, consistent with the 22–53% success rate in similar bivalve experiments (de Cerio et al., 2013; Ciocan et al., 2011, 2012; Yang et al., 2012). As

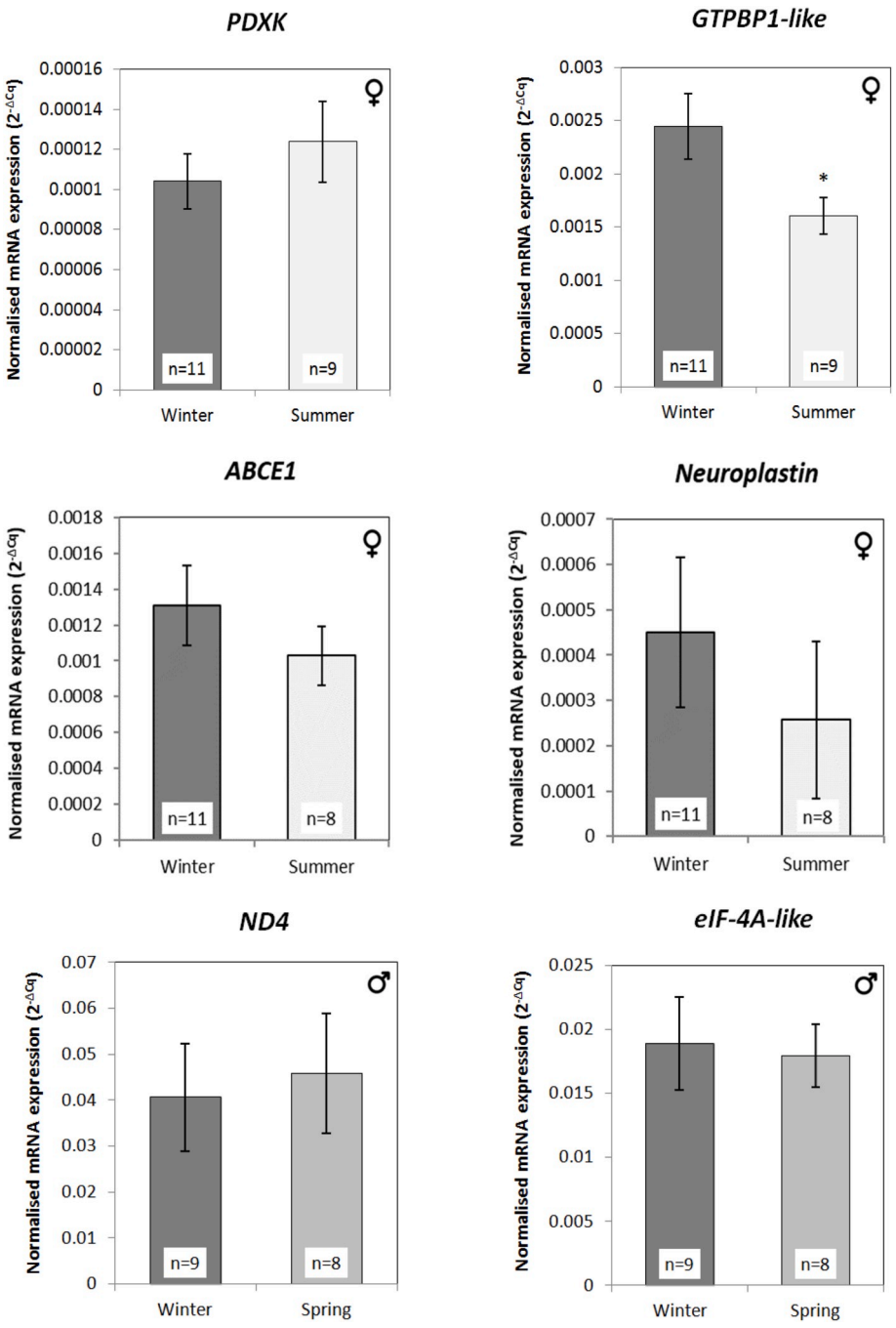

Fig. 2. mRNA expression of a subset of transcripts identified by SSH as potentially differentially expressed between seasons in female (*PDXK*, *GTPBP1-like*, *ABCE1*, *Neuroplastin*) and male (*ND4*, *eIF-4A-like*) *M. edulis* gonads. Mean data ±SEM. Units are arbitrary. *denotes significance at $p < 0.05$ level.

qPCR only validated one of the six genes tested (Fig. 2), further evidence is required to establish seasonality in the isolated transcripts. However, this preliminary dataset enabled the identification of a number of genes which may have potential as candidate genes in future investigations into seasonal expression differences in female and male mussels (Tables 1 and 2).

Genes indicated by SSH to be potentially differentially expressed in females between winter and summer at the same time of day are involved in multiple processes including signal transduction (*HSP90-1*, *calmodulin*), metabolic enzymatic processes (*PDXK*), cell adhesion (*Neuroplastin-like, hemicentin*), ribosome biogenesis (*GTPBP1-like*, *ABCE1*), energy metabolism (*ATP synthase lipid-binding protein, mitochondrial-like*; *MT-CO2*), cilia formation (*CEP131*), protein cleavage (*β-MPP*), phosphorylation (*TESK1*) and metabolism (*Chitinase/chitotriosidase-like*) (Table 1). Some encode proteins that interact with the molecular clock mechanism; HSP90 regulates mammalian BMAL1 protein levels (Schneider et al., 2014), calmodulin is a multifunctional messenger protein involved in phototransduction in *Drosophila* (Hardie, 2001), and *GTPBP1* is involved in regulating mRNA degradation, including that of *aaNAT* which regulates circadian melatonin production in mammals (Woo et al., 2011). The mRNA transcripts indicated by SSH to be potentially differentially expressed in males between winter and spring were involved in cytoskeleton processes (*beta-tubulin*, *actin*), the electron transport chain (*ND4*) protein synthesis (*eIF-4A-like*) and chitin metabolism (*chitinase/chitotriosidase-like*) (Table 2). Chitinases, which are involved in digestion and shell-remodelling processes, show

seasonal expression differences in *M. galloprovincialis* (Banni et al., 2011).

4.4. Conclusion

This study investigated the effect of 24 h light and temperature cycles on *M. edulis* clock gene expression patterns to increase understanding of how abiotic factors inform biological timekeeping in bivalves. We conclude that the canonical clock genes investigated exhibited endogenous expression in the mantle tissue of blue mussels, with significant variability in mRNA expression persisting under diurnal temperature cycles for all but one (Figs. 1 and S6; Table S4). Thermocycles modulated the variable expression of clock gene *Rev-erb* to constant levels and conversely induced significant variation in the otherwise constantly expressed gene *aaNAT* (Figs. 1 and S6; Table S4). Though further investigations are required to clarify the interactions comprising the mussel molecular clock mechanism, it is clear that clock genes play a vital role in regulating the timing of essential rhythmic processes in marine organisms and investigating their responses to environmental inputs is highly relevant to dynamic intertidal habitats in a changing marine environment.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Emma C. Chapman: Conceptualization, Investigation, Formal analysis, Data curation, Visualization. **Brodie J. Bonsor:** Investigation. **Daniel R. Parsons:** Data curation, Visualization. **Jeanette M. Rotchell:** Data curation, Funding acquisition, Conceptualization, Supervision, Visualization.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2020.104960.

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