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Daily patterns in parasite processes: diel variation in fish louse transcriptomes

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ABSTRACT

Parasites, similar to all other organisms, time themselves to environmental cues using a molecular clock to generate and maintain rhythms. Chronotherapeutic (timed treatment) techniques based on such rhythms offer great potential for improving control of chronic, problematic parasites. Fish lice are a key disease threat in aquaculture, with current control insufficient. Assessing the rhythmicity of fish lice transcriptomes offers not only insight into the viability of chronotherapy, but the opportunity to identify new drug targets. Here, for the first known time in any crustacean parasite, diel changes in gene transcription are examined, revealing that approximately half of the *Argulus foliaceus* annotated transcriptome displays significant daily rhythmicity. We identified rhythmically transcribed putative clock genes including core clock/cycle and period/timeless pairs, alongside rhythms in feeding-associated genes and processes involving immune response, as well as fish louse drug targets. A substantial number of gene pathways showed peak transcription in hours immediately preceding onset of light, potentially in anticipation of peak host anti-parasite responses or in preparation for increased feeding activity. Genes related to immune haemocyte activity and chitin development were more highly transcribed 4 h post light onset, although inflammatory gene transcription was highest during dark periods. Our study provides an important resource for application of chronotherapy in fish lice; timed application could increase efficacy and/or reduce dose requirement, improving the current landscape of drug resistance and fish health while reducing the economic cost of infection.

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1. Introduction

Temporal rhythms are a core element of life on earth, with most environmental cues cycling over tidal (12.4 h), daily (24 h), lunar (monthly) and/or seasonal scales (Helm and Stevenson, 2014; Neumann, 2014). Of these rhythmic cues, the most dominant is the daily change in light (Vitaterna et al., 2001). These highly predictable light cycles – which in turn drive diel variation in other factors such as temperature and food availability – have led to the evolution of endogenous rhythms, wherein environmental cues entrain feedback loops to regulate patterns in activity and biological processes (Vitaterna et al., 2001; Bell-Pedersen et al., 2005). Organisms often face entrainment from multiple oscillators, with symbionts experiencing an additional set of cues: the rhythms of their host (Rijo-Ferreira et al., 2017a). Despite the impact of parasites on human and animal health, parasite rhythms are severely understudied compared with free-living organisms with most investigations into parasite-host systems focusing on the host. Parasites and their associated hosts are intertwined in an arms race; to fully understand one you must consider the other, thus investigating parasite rhythms is key to improving global health (Martinez-Bakker and Helm, 2015).

Parasite rhythm studies focus on transmission and reproductive behaviours as these are key in infection dynamics (Martinez-Bakker and Helm, 2015). These include temporal peaks in malaria asexual reproduction (with the exact mechanism behind this still unconfirmed; Mideo et al., 2013), rhythmical discharge of infective stages including eggs, oocysts and cercariae, which typically synchronise with host behaviours to maximise transmission (Hawking, 1975; Bogéa et al., 1996; Lu et al., 2009; Martinez-Bakker and Helm, 2015) and timed behavioural manipulation of hosts to promote transmission to a secondary host (Trail, 1980; de Bekker et al., 2014). Genetic studies of parasite rhythms have primarily involved identification of putative ‘clock’ genes required to generate rhythms (Hevia et al., 2015; Sun et al., 2018; Rawlinson et al., 2021). More recently, whole transcriptome rhythmicity has been assessed in a few parasitic species (fungi, de Bekker et al.,...
2. Materials and methods

2.1. Animal husbandry

Rainbow trout (*Oncorhynchus mykiss*) were selected as hosts due to their economic importance and the susceptibility of trout in UK fisheries to *Argulus* spp. infections (*Fornshell, 2002; Taylor et al., 2006*). Juvenile triploid female rainbow trout were acquired from Bisbury Trout Farm in January 2019 and maintained at Cardiff University at 14 °C in a recirculating aquaculture system. Fish were cleared of external parasites, established by lightly anesthetising fish with 0.02% MS222 (tricaine methanesulfonate; Merck, Darmstadt, Germany) and examining them under a dissection microscope with fibre optic illumination (*Nikon, Amsterdam, Netherlands*). Throughout the study, fish were maintained under a 12:12 light–dark light cycle and fed daily with commercially available trout food (*Nutraparr, Skretting, UK*) ad libitum.

2.2. Parasite culture

*Argulus foliaceus* eggs were collected by capturing adult females (morphologically identified according to *Fryer, 1982*) from Risca Canal (Newport, UK; grid reference: ST 24344 90686) by hand netting naturally infected three-spined stickleback, *Gasterosteus aculeatus*. Lice were removed from fish in the field by lifting the host fish out of water using a net for a 10 s period; upon re-submersion into a container of freshwater the parasite detached and was collected using a wide-bore pipette. Lice were transported to the laboratory off host in sealed containers of dechlorinated water. Once in the Cardiff Aquarium, UK, eggs laid by female parasites were collected and stored at <10 °C to prevent incubation (*Shafir and van As, 1986*). To hatch *A. foliaceus* metanauplii, eggs were warmed to 24 °C (over approximately 8 h by placing eggs at ambient temperature) and maintained at 24 °C for 20 days with daily monitoring until hatching. Due to the high mortality of lice during the first week of infection (*Hunt and Cable, 2020*) and to ensure lice would be large enough to harvest sufficient RNA, *A. foliaceus* were cultured on rainbow trout (average 10 cm standard length, (SL)) prior to experimentation. Rainbow trout were infected with 50 metanauplii each (within 24 h of hatching) by placing the fish into a small receptacle of water and introducing the parasite via a pipette, allowing for natural attachment of the parasite to the host. Fish were monitored daily for 1 week at 18 °C, after which all *A. foliaceus* were removed from hosts.

2.3. Sample collection

*Argulus*-naïve rainbow trout (SL = 60.1 ± 4.94 mm, range 47.5–69.0 mm, n = 49) were infected using the same method as above with seven individuals of *A. foliaceus* per fish (collected from the culture, average parasite length = 0.88 ± 0.10 mm, range = 0.73–1.04 mm). Fish were held individually in 4 L tanks at 14 °C, with cleaning every 48 h and feeding at 10:00 h every day. After 1 week, seven fish were sampled every 4 h over a 28 h period (starting at 07:00 h when lights turn on and finishing with a sample at 07:00 h the next day). Samples were labelled with zeitgeber time (ZT, standardised 24 h notation of time) as follows: ZT0 = 07:00 h, ZT4 = 11:00 h, ZT8 = 15:00 h, ZT12 = 19:00 h, ZT16 = 23:00 h, ZT20 = 03:00 h. Samples could not be taken over a longer time period (to capture multiple cycles) due to limitations regarding sequencing cost and animal use. It is preferable for rhythms to be examined over multiple cycles; thus here one timepoint was sampled twice to ensure capture of a full cycle (*Yúfera et al., 2017; Biscontin et al., 2019*). During sampling all *A. foliaceus* on an individual host were collected using sterilised fine tweezers, 2017; protists *Plasmodium* spp., *Smith et al., 2020* and trypanosomes, *Rijo-Ferreira et al., 2017b*). These studies reveal substantial portions of parasite gene repertoires may be expressed rhythmically over 24 h periods, even in endoparasites not directly exposed to light cycles. Similar to free-living organisms, parasites likely need to anticipate daily variations in risks and rewards such as host immune activity and nutrient availability (*Rouzine and McKenzie, 2003; O’Donnell et al., 2011; Prior et al., 2018*).

Research into rhythms of host immune systems and their pathogens has led to the development of chronotherapeutic control strategies – timed application of treatments to improve efficacy and/or reduce dosage (*Smolensky and Peppas, 2007*). Synchronisation to patient rhythms such as cell-cycle stage or blood pressure is at the forefront (*Hermida et al., 2003; Altnik et al., 2007*), with treatments timed to exploit parasite rhythms under investigation. Experimental trials with malaria show promise (*Cambi et al., 1991; Owolabi et al., 2021*) and the potential application of timed treatment has been documented for other parasite species (*Honorio-Francà et al., 2013; Davis et al., 2018*). Despite the rapidly developing chronotherapeutic revolution in human medicine, the potential for harnessing chronobiology to understand the dynamics of both captive and wild animal health remains largely unexplored (*Reinberg, 1983; Smolensky and D’Alonzo, 1993; McKenna et al., 2018*).

Integrated chronotherapeutic strategies could be key to improving parasite control. Ectoparasitic fish lice – large crustaceans that attach to fish skin and cause extensive mechanical damage (*Moller, 2011*) – have plagued farmers since the conception of aquaculture. Light appears to be an important external cue for both marine and freshwater species, influencing activity (*Novales Flamarique et al., 2000*; Bandilla et al., 2007; Yoshizawa and Nogami, 2008; Novales Flamarique et al., 2009; Hunt et al., 2021), transmission (* Mikhail et al., 1999* and reproduction (*Shimura and Eguada, 1980; Bai, 1981; Harrison et al., 2007*). Until recently, chemical treatments have been the main control method in fish farms (*Grave et al., 2004,*). However, rising drug resistance, stress to fish and environmental concern over their use means treatments need to be used more sparingly while remaining effective (*Aaen et al., 2015*). Considering the strong evidence of daily rhythmic phenotypes in fish lice, and the urgent need to improve louse control in aquaculture, profiling of fish lice at the transcriptional level over daily cycles is a vital first step towards a deeper chronobiological understanding of these economically devastating parasites and the development of novel mitigation strategies.

Here we examined transcriptome rhythmicity of *Argulus foliaceus*, the most common freshwater fish louse in the United Kingdom (UK; also found throughout temperate areas of Europe, central Asia and North America). *Argulus foliaceus* are a chronic problem in UK fisheries with treatment limited compared with marine systems due to environmental concerns. Despite taxonomic differences, *Argulus* spp. also act as a sea louse model due to their comparable functionality and impact on hosts. *Argulus* spp. transcriptomes have been generated previously to mine for specific targets including neuropetides, hemocyanin and feeding-associated proteins (*Sahoo et al., 2013; Christie, 2014; Pinnow et al., 2016*; Ambu Ali, A., Taggart, J.B., Bekkaert, M., Wehner, S., Monaghan, S.J., Bron, J.E., 2021). Identification of transcripts relating to host–parasite interactions and feeding-associated activities through de novo transcriptome sequencing of branchiuran fish lice, *Argulus foliaceus* (*Linnaeus, 1758*) and *Argulus coregoni* Thorell, 1865. Research Square. [https://doi.org/10.21203/rs.3.rs-285509/v1]{https://doi.org/10.21203/rs.3.rs-285509/v1}. We believe this study is the first to investigate diel changes in gene transcription and identify putative clock genes in a crustacean parasite, together with rhythmical key processes relating to infection and potential drug targets.
taking care to only touch the lice and not the fish skin. All lice from an individual fish were pooled together into RNAlater to form one sample; the number of *A. foliaceus* collected from each fish ranged from 4 to 7 lice.

### 2.4. Transcriptome sequencing

Total RNA was extracted from 49 individual samples using the RNeasy® Mini Kit (Qiagen, Manchester, UK) and quantified using Qubit High-Sensitivity RNA assays (Qubit® 3.0 Fluorometer, ThermoFisher Scientific, UK; RNA concentration = 245 ± 63 ng/µl, range 88–352 mm). RNA sequencing was performed in the School of Biosciences Genome Hub, Cardiff University, UK. Quality control of the RNA samples was confirmed by Tape Station (TapeStation Analysis Software 3.2, Agilent, Santa Clara, CA, USA) with a minimum RNA integrity number (RIN) score of 10.0. Following quality control, 48 individual samples were sequenced. Of these, *n* = 13 for ZT0 and *n* = 7 for ZT4, 8, 12, 16 and 20; ZT0 had more replicates as it was the start and end point of recording. Libraries were generated according to manufacturer's instructions (Illumina, San Diego, CA, USA) using an Illumina NeoPrep Library Workstation. Prior to equimolar pooling, library quality was quantified and assessed using Agilent Tapestation. The library pool was run on an Illumina NextSeq500 Sequencer (2 x 75 PE) to achieve a minimum of 16 million read pairs per sample. Raw reads are available at NCBI BioProject ID: PRJNA764202. Metadata for the raw RNA sequence reads is available in Supplementary Table S1 (available in Mendeley Data, see Section 2.8).

#### 2.5. De novo assembly

Reads were filtered and trimmed using Trimmomatic version 0.38 to perform the following: cut 15 bases from the start of the read and cut bases off the start/end if they were below Q3, cut adapter/other Illumina-specific sequences from the read, trim anywhere within each read where a 4 bp window dropped below Q15 and drop any trimmed reads below 36 bp long. Fastqc version 0.11.7 was used before and after trimming as part of quality assessment. Prior to assembly, putative contaminant host reads were removed using BBSplit (Bushnell, B., 2014). BBMap: a fast, accurate, splice-aware aligner (No. LBNL-7065E). Lawrence Berkeley National Lab. Berkeley, CA, United States) and the published rainbow trout genome (Berthelot et al., 2014). De novo assembly was performed on the filtered trimmed reads using Trinity version 2.6.6 and the default parameters. Assembly quality was assessed by examining the expression-dependant N50 (ExN50) value generated by the trinity report and applying benchmarking universal single-copy orthologs (BUSCO) using BUSCO version 4.1.4 with the eukaryota_odb10 (2020-09-10) BUSCO set (Simão et al., 2015). To further ensure no host or other contaminant sequences were included, all contigs were screened using double index alignment of next-generation sequencing data (DIAMOND; Buchfink et al., 2015) BLASTx searches against the NCBI non-redundant (nr) database, retaining only those with an arthropod as the top hit (51,755 contigs). Annotation including gene ontology (GO) assignment was performed using the Trinotate automated pipeline (Bryant et al., 2017).

#### 2.6. Gene transcription analyses

Filtered and trimmed reads were mapped against the de novo assembly using Trinity scripts (salmon abundance estimation method) to generate raw and normalised (trimmed mean of M-values, TMM) count matrices at the gene level. Raw counts were imported into R and differential transcription tests were performed using DESeq2 version 1.30.1 (Love et al., 2014) to compare transcription between all time points. Rhythmically transcribed genes were determined using empirical Jonckheere-Terpstra-Kendall (eJTK; Hutchison et al., 2015) analyses to examine normalised count data, with circacompare version 0.1.1 (Parsons et al., 2020) used to plot rhythm graphs. For both DESeq2 and eJTK, false discovery rate (FDR) corrected *P* values of less than 0.05 were considered significant for differential transcription and rhythmicity. GO functional enrichment tests were carried out using topGO version 2.42.0 (R) to detect significantly overrepresented biological processes of groups of rhythmically transcribed genes with ViSEAGO version 1.4.0 used to visualise GO enrichment at each time point. Putative clock genes were identified via manual searching with BLAST using model *Drosophila melanogaster* clock genes. The web-based tool Galaxy was also used to perform phylogenetically-informed annotation (PIA) via a tree-based approach to look for light-interacting genes (Blankenberg et al., 2010; Speiser et al., 2014). Genes were putatively assigned as feeding-related by selecting protein sequences listed in AmbuAli et al. (2020) and NCBI entries which matched to proteins listed in AmbuAli et al. (2021, Research Square, cited earlier) to manually search for hits against the transcriptome using BLAST.

#### 2.7. Ethics statement

All animal work was conducted under UK Home Office License PPL 303424. This work was approved by the Cardiff University Animal Ethics Committee, followed ARRIVE guidelines and conformed to UK legislation under the Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039).

#### 2.8. Data accessibility

Raw RNA sequence reads generated by this project are available in the NCBI Sequence Read Archive and can be found under BioProject PRJNA764202. Supplementary data and tables are available on Mendeley Data at https://doi.org/10.17632/vcsfshfdph.1.

### 3. Results

#### 3.1. De novo assembly

Raw reads generated per sample ranged from 7.8 to 10.7 million reads, with a total of 168,393 transcripts generated for whole tissue samples of *A. foliaceus*. Out of 51,755 total putative arthropod genes, 10,290 were annotated. The percent GC was 41.05 with an ExN50 of 2125. BUSCO results reported 98.4% completeness. Putative clock genes were identified via manual searching with AmbuAli et al. (2021, Research Square, cited earlier) to manually search for hits against the transcriptome using BLAST.

#### 3.2. Transcriptome rhythmicity

The eJTK analyses found 4906 genes significantly rhythmic out of a total of 10,290 annotated genes (47.7%; Supplementary Table S2). Rhythmicity totals varied by phase, with 14% of genes peaking at phase 0, 19% at 4, 18% at 8, 9% at 12, 15% at 16 and 25% at 20 (Fig. 1). The genes with highest significance in rhythmicity included the core clock gene timeless and various genes relating to molecular processes and feeding activity. Results from DESeq2 analyses did not show high numbers of significant genes when examining differences between two timepoints (Supplementary Data S1), however when examining all significant genes across all timepoint comparisons (raw *P* value with cut-off at <0.01)
96.6% of gene IDs matched to those found significant in eJTK analyses.

Functional profiles displayed distinct blocks of activity and changes over a 24 h period (Fig. 2, Supplementary Data S2). Metabolic processes and localisation displayed most activity at ZT20/03:00 h, with high enrichment at ZT4/11:00 h (1 h after host feeding) relating to amino sugar metabolic process (GO:0006040), glucosamine-containing compound metabolic process (GO:1901071) and chitin metabolic process (GO:0006030). Nucleobase-containing compound metabolic processes at ZT20/03:00 h were related to RNA processing. Development mostly at ZT20/03:00 h and ZT0/07:00 h involved reproductive development and neurogenesis, while cuticle development peaked at ZT4/11:00 h (GO:0040003 and GO:0042335). Immune system processes were particularly apparent at ZT4/11:00 h (e.g. haemocyte activation/degranulation: GO:0043303, GO:0045576), and included a Toll-like receptor (TRINITY_DN15356; TLR2-like) and lectins (e.g. TRINITY_DN3872, lectin-1-like). Response to stress was enriched at ZT12/19:00 h, including regulation of acute inflammatory response (GO:0002673 and GO:0002526). Transport function peaks occurred across the 24 h period, with regulation of transport focused at ZT20/03:00 h.

3.3. Clock genes

In total 10 rhythmic putative clock genes were identified in A. foliaceus (Table 1). Clock genes peaked in all phases except for ZT12. Cycle peaked from ZT20-ZT0 when lights turned on, with transcription lowest just after at ZT4-ZT8, the inverse of Clock which peaked from ZT4-ZT12 and was lowest from ZT20-ZT0 (Fig. 3). Both Period and Timeless followed the same pattern with transcription low during the light period and highest at ZT16 in the dark period. Timeless (TRINITY_DN37479_c0_g1) was also the second most significant rhythmic gene according to eJTK analyses.

3.4. Feeding-related genes

A total of 42 significantly rhythmic genes were found associated with feeding enzymes (Fig. 4, Table 2). Overall, the majority of these genes peaked at ZT20/03:00 h (4 h before lights on; 31%) with few genes peaking at ZT12/19:00 h (lights off; 4.8%). Anti-haemostatic proteins were highly transcribed at ZT4 and ZT20 (21.9% and 31.3%) as were digestion/degradation proteins (ZT4 = 28.6%, ZT20 = 35.7%), while anti-inflammatory proteins peaked mostly at ZT20 (37.5%) followed by ZT4 and ZT8 (both 25%). Other specific proteins of note include: hemocyanin which was highest at ZT16 and lowest at ZT4, ferritin which was also lowly transcribed at ZT4 but peaked at ZT12, and immune system protein adenosine deaminase, which displayed a linear decrease in transcription with highest at ZT0 and lowest at ZT20.

4. Discussion

Daily rhythms are ubiquitous to life and diel variation in wide-ranging physiological processes in both hosts and their parasites may be pivotal to infection outcomes. Investigation of parasite rhythmicity at the transcriptional level provides an opportunity to examine and understand which processes are temporally coordinated. Here, almost half of annotated genes in the fish louse A. foliaceus had significant diel variation under light–dark cycles, indicating a high level of transcriptome rhythmicity. These included genes related to key processes such as feeding enzyme activity, cuticle development and immune responses. Our study highlights the magnitude of rhythmicity that can be encountered in parasite transcriptomes and the potential for chronotherapy applications considering the patterns seen in key processes and drug targets.

Few other studies have examined parasite transcriptional rhythmicity, and for those that have, the results vary significantly across taxa. For example, trypanosomes and parasitic fungi appear
to express a relatively small proportion of their gene repertoire rhythmically \((Trypanosoma brucei \sim \sim 15\% \text{ of genes, } Rijo-Ferreira \text{ et al., 2017b}; Ophiocordyceps kimflemingiae \sim \sim 5\%, \text{ de Bekker et al., 2017})\). In contrast, the blood stage of \(Plasmodium falciparum\) exhibits rhythmicity in up to 93% of the transcriptome \(\text{Smith et al., 2020}\). For ectoparasites, previous studies have used transcriptomics to examine biological processes and drug targets \(\text{Copepoda – Chávez-Mardones et al., 2016}; \text{ Branchiura – AmbuAli et al. (2021, Research Square, cited earlier); Monogenea – Vorel et al., 2021})\) but not across daily timescales. We believe the current study is the first to examine diel gene variation in a parasitic crustacean despite their importance in aquaculture and fisheries as primary infections.

Fig. 2. Clustering heatmap for comparison of enriched gene ontology term functional profiles across time, generated from the \(Argulus foliaceus\) transcriptome. Dendrogram is based on Wang's semantic similarity distance and ward.D2 aggregation criterion, heatmap displays \(-\log_{10}(P \text{ value})\) from functional enrichment tests for each timepoint. Zeitgeber time \(\text{ZT0 = 07:00 h, lights turn on; ZT12 = 19:00 h, lights turn off.}\)

Table 1
Summary of rhythmic clock gene hits from the \(Argulus foliaceus\) transcriptome using Galaxy phylogenetically-informed annotation. Phase and rhythmicity \(P\) values are from empirical Jonckheere-Terpstra-Kendall (eJTK) analyses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Trinity ID</th>
<th>Hits (isoforms)</th>
<th>Phase (h)</th>
<th>Rhythmicity ((P \text{ value}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryptochrome 2</td>
<td>TRINITY_DN10110_c0_g2</td>
<td>1</td>
<td>0</td>
<td>0.015803</td>
</tr>
<tr>
<td>Pigment Dispersing Hormone</td>
<td>TRINITY_DN5965_c0_g1</td>
<td>2</td>
<td>0</td>
<td>0.027781</td>
</tr>
<tr>
<td>Clock</td>
<td>TRINITY_DN3505_c0_g1</td>
<td>3</td>
<td>4</td>
<td>0.026201</td>
</tr>
<tr>
<td>Slowpoke</td>
<td>TRINITY_DN31_c0_g1</td>
<td>3</td>
<td>4</td>
<td>0.006378</td>
</tr>
<tr>
<td>Vrille</td>
<td>TRINITY_DN6730_c0_g1</td>
<td>2</td>
<td>8</td>
<td>0.031003</td>
</tr>
<tr>
<td>Period</td>
<td>TRINITY_DN7846_c0_g1</td>
<td>2</td>
<td>16</td>
<td>0.008084</td>
</tr>
<tr>
<td>Timeless</td>
<td>TRINITY_DN37479_c0_g1</td>
<td>3</td>
<td>16</td>
<td>7.89E-05</td>
</tr>
<tr>
<td>Cycle</td>
<td>TRINITY_DN1637_c0_g1</td>
<td>5</td>
<td>20</td>
<td>0.017545</td>
</tr>
<tr>
<td>Lark</td>
<td>TRINITY_DN8177_c0_g1</td>
<td>4</td>
<td>20</td>
<td>0.014336</td>
</tr>
<tr>
<td>Tango</td>
<td>TRINITY_DN1637_c0_g1</td>
<td>4</td>
<td>20</td>
<td>0.017545</td>
</tr>
</tbody>
</table>
and vectors of secondary diseases. In non-parasitic crustaceans, however, 68.2% of copepod *Calanus finmarchicus* genes were rhythmic while in Antarctic krill *Euphausia superba* only 27% of genes were deemed rhythmic (of which, 2.7% were clock controlled; Biscontin et al., 2019). Considering the importance of light in *Argulus* spp. off host behaviour and host searching (Yoshizawa and Nogami, 2008; Mikheev et al., 1999; Hunt et al., 2021), it is perhaps unsurprising that almost half of the annotated transcriptome displayed significant light-based rhythmicity.

Generation and regulation of rhythms is reliant on transcription-translation feedback loops of “clock” genes. These molecular clocks are found from bacteria to mammals, although the constitution of core clock genes varies across taxa (Rijo-Ferreira et al., 2017a; Rijo-Ferreira and Takahashi, 2019). Previously, for crustaceans up to 15 rhythmic clock genes had been identified (Bernatowicz et al., 2016; Biscontin et al., 2019). Here, 10 putative clock genes were identified as having diel cycles in the *A. foliaceus* transcriptome: cycle, pigment dispersing hormone, timeless, lark, period, slowpoke, clock, tango, vrille and cryptochrome 2 (cry2). The presence of cry2 but lack of cry1 suggests *A. foliaceus* clock genes resemble those of sand hopper *Talitrus saltator* and isopod *Eurydice pulchra* (see Zhang et al., 2013; O’Grady et al., 2016), rather than *Drosophila melanogaster* or copepod *Calanus finmarchicus*, which possess only cry1 (Yuan et al., 2007), or the cladoceran *Daphnia pulex*, which possess both (Tilden et al., 2011). Clock genes are responsible for prolonged occurrence of rhythmic activity in the absence of zeitgeber cues (i.e. 24 h light or darkness), known as endogenous rhythms. While specific endogenous activity cannot be identified in this study, the presence of a suite of clock genes indicates the possibility. Endogenous rhythms in gene transcription have not yet been assessed in any parasitic crustaceans, although their presence has been confirmed in other parasites (monogeneans, parasitoid wasps and trypanosomes; Kearn, 1973; Bertossa et al., 2010; Rijo-Ferreira et al., 2017b).

Rhythmic transcriptomes produce rhythmic behaviours, the occurrence of which can potentially be predicted from transcriptome diel variation. Adult *Argulus* blood feed and as such express proteins to facilitate this process (Gresty et al., 1993; Walker et al., 2011; AmbuAli et al., 2020), although it is currently unknown if feeding occurs at specific times. As *Argulus* spp. feeding causes extensive external damage to their host, knowing when feeding occurs is important for understanding host vulnerability. Despite the *A. foliaceus* used in this study being too young to blood feed, genes were identified relating to blood feeding activity, indicating transcription of these proteins is not adult restricted and could facilitate juvenile feeding on mucous and skin cells. The cells identified here included trypsins, proteases and protease inhibitors, which have been found previously in *A. foliaceus* and other blood feeding parasites (Francischetti et al., 2009; Robinson et al., 2009; Tirloni et al., 2015; Xavier et al., 2019; AmbuAli et al., 2020). Further confirmation of these proteins through functional assays could aid development of compounds to mitigate parasite feeding impact or improve host defence against feeding. Numerous putative feeding genes also had significant rhythms, suggesting temporal patterns to feeding, with their transcription generally highest at ZT20/4h before lights on. This may be due to *A. foliaceus* feeding at this time, or in preparation for the host protective immune response (inflammation) which triggers at onset of the light period (Montero et al., 2019; Ellison et al., 2021).

Chronotherapies – timed treatments based on host-parasite immune rhythms and targeting/disruption of key processes – hold great potential for parasite control. Here *A. foliaceus* rhythmic immune transcription (including functional enrichment related to haemocyte degranulation, and lectin and Toll-like receptor genes; Theopold et al., 2004; Viswambari Devi et al., 2010;
Watthanasurorot et al., 2011; Lin and Söderhäll, 2011; Grigorian and Hartenstein, 2013) was predominantly highest at ZT4 (4 h after lights on). Mounting an immune response requires a high energetic cost; as such it is beneficial for organisms to time expression with when it is most required (Demas et al., 1997). The response to stress peaked at ZT12 when lights turned off, potentially in preparation for the host adaptive responses and wound repair which increase in activity during dark periods (Ellison et al., 2021). Intriguingly, genes related to crustacean parasite drug targets also showed daily rhythmicity. For example, acetylcholinesterase genes (a target of organophosphate treatments for sea lice, *Argulus* spp. and other parasites) peak mainly at/around ZT0 (lights turn on). Timed treatment could therefore improve efficacy, especially considering rising resistance concerns (Aaen et al., 2015; Agusti-Ridaura et al., 2018). This has been proven previously for glyphosate treatment of weeds where application time had a higher impact than dosage on control (Mulugeta and Boerboom, 2000). Chitinases are the only drug group currently not facing resistance (Aaen et al., 2015; Macken et al., 2015); they control fish lice by targeting development (Eichner et al., 2015). Chitinases are ineffective against adult sea lice as moulting stops upon reaching adulthood; conversely *Argulus* spp. continue to moult even as adults, making them ideal targets. Here *A. foliaceus* chitin development genes were highly rhythmic in their transcription with activity at ZT4 (4 h post lights on), comparable with chitin synthesis in krill which occurred 2–9 h post lights on (Biscontin et al., 2019).

**Fig. 4.** Rhythmic plots of *Argulus foliaceus* gene transcription associated with feeding proteins. Venom serine protease, Cysteine protease and Trypsin produce anti-coagulant activity. Apyrase is an anti-coagulant and anti-inflammatory. Protein SpAN-like is related to immunity and Hemocyanin is involved in respiration and protein storage. Error bars represent standard error, dotted line indicating rhythmic pattern predicted by circacompare package in R, white background = light period, grey background = dark period. TMM, trimmed mean of M-values.
Future testing of chronotherapeutic application of organophosphates (e.g. azamethiphos) and chitin inhibitors (e.g. benzoyl ureas, diflubenzuron and teflubenzuron) could, for example, be conducted by applying treatments at peaks/troughs in transcription of their targets (ZT0/12 for organophosphates, ZT4/16 for chitin inhibitors) and comparing efficacies.

Parasites are often side-lined with regard to examining rhythmic processes in favour of their hosts, despite the potential in exploiting parasite rhythms to improve control. Here, gene transcription related to numerous key processes including immune responses, chitin development and feeding were found to be significantly rhythmic in their transcription over day-night cycles. While further functional assays to confirm gene function are required, this work provides a baseline for rhythmic patterns within louse gene transcription and showcases the potential for chronotherapy. Going forward this will aid the reduction of drug use in aquaculture without compromising impact, improving fish welfare and mitigating economic loss.

**Acknowledgements**

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### Table 2

Significantly rhythmic genes associated with feeding proteins identified from *Argulus foliaceus*. Protein selection for transcriptome searches and functional profiles were obtained from AmbuAli et al., 2020, 2021 (Research Square, cited earlier). Phase and rhythmicity *P* values are from empirical Jonckheere-Terpstra-Kendall (eJTK) analyses.

<table>
<thead>
<tr>
<th>Protein class/domain</th>
<th>Protein function(s)</th>
<th>Protein hit(s)</th>
<th>Trinity ID</th>
<th>Phase (h)</th>
<th>Rhythmicity (P value)</th>
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<td><strong>Trypsin/Serine protease</strong></td>
<td>Digestion and anti-haemostatic</td>
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<td>Diphosphorydrolase</td>
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<td>Immunity including antifungal activity, food digestion, host penetration and immune evasion or activation</td>
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References


