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Removal of Exogenous Stimuli Reveals a Canalization of Circadian Physiology in a Vertically Migrating Copepod

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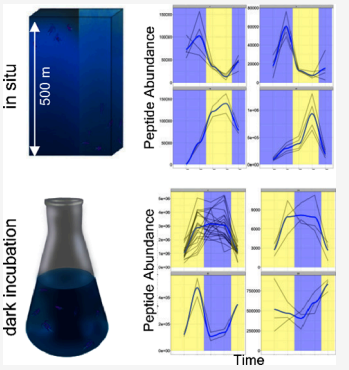
ABSTRACT: Diel rhythms are observed across taxa and are important for maintaining synchrony between the environment and organismal physiology. A striking example of this is the diel vertical migration undertaken by zooplankton, some of which, such as the 5 mm-long copepod *Pleuromamma xiphias* (*P. xiphias*), migrate hundreds of meters daily between the surface ocean and deeper waters. Some of the molecular pathways that underlie the expressed phenotype at different stages of this migration are entrained by environmental variables (e.g., day length and food availability), while others are regulated by internal clocks. We identified a series of proteomic biomarkers that vary across ocean DVM and applied them to copepods incubated in 24 h of darkness to assess circadian control. The dark-incubated copepods shared some proteomic similarities to the ocean-caught copepods (i.e., increased abundance of carbohydrate metabolism proteins at night). Shipboard-incubated copepods demonstrated a clearer distinction between night and day proteomic profiles, and more proteins were differentially abundant than in the *in situ* copepods, even in the absence of the photoperiod and other environmental cues. This pattern suggests that there is a canalization of rhythmic diel physiology in *P. xiphias* that reflects likely circadian clock control over diverse molecular pathways.



KEYWORDS: *proteomics, diel vertical migration, Pleuromamma, circadian clock, diel*

INTRODUCTION

Circadian cycles are recapitulated across diverse taxa.[1−3] Circadian cycles are endogenous 24 h rhythms that can be synchronized by environmental cycles. These rhythms are manifested in cyclical patterns of behavior and physiology that allow for periods of activity and rest (e.g., refs 4−6). In nature, environmental cues can entrain, or dictate, the period of these cycles so that physiology is appropriately aligned with the local environment. This alignment of physiology and environment allows for upregulation of metabolism when food resources are available,[4] emergence of adult life stages during certain times in the 24 h cycle,[7] and various other phenomena. Rhythms under circadian controls will continue even in the absence of the environmental cues alongside which they have evolved (e.g., refs 8 and 9).

The cosmopolitan copepod *Pleuromamma xiphias* (*P. xiphias*) is among the diverse zooplankton taxa that undergo diel vertical migration (DVM).[10] At dusk, *P. xiphias* migrate ∼500 m from depth to the surface ocean where it feeds with decreased threat from visual predators. Their migration pattern is closely aligned with the photoperiod. Because many other processes and behaviors, such as feeding, swimming, digestion, excretion, etc., are dependent on food availability and depth/time of the day, a wide range of physiological responses are also likely entrained in the DVM pattern. Zooplankton maintain the DVM pattern even in the absence of normal photoperiod cues,[4,9] which makes them an appropriate model for extending investigations of the relationship between physiology and the circadian clock into the marine habitat.

Disruption of typical circadian cues, such as incubations under constant darkness, uncovers the variety of physiological processes under circadian clock control (e.g., ref 8). Circadian clocks are molecular timekeepers that control rhythmic expression of molecules and phenotypes and are usually aligned with specific environmental cues. For at least a short period of time, circadian clock genes will continue to be expressed following the same pattern established in the natural environment (e.g., ref 4). As long as the clock genes are expressed following the *in situ* pattern, other physiological pathways under clock control will follow. These revealed pathways would be among the most essential for an organism's functional physiology.[8] Alternatively, there are biological rhythms expressed in the natural environment that follow environmental cues that are correlated to the photoperiod; even though these rhythms may appear to be circadian, if they

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are solely under environmental control, they are independent of clock control and may mask endogenous rhythms.

In this study, we explore proteomic biomarkers under putative circadian clock control in *P. xiphias* both *in situ* and in a fully dark incubation. We follow up on a whole proteome discovery (data-dependent acquisition) analysis,[11] applying these biomarkers to wild caught and dark-incubated copepods using targeted proteomics (selected reaction monitoring). These results provide foundational data for cross-taxa comparisons, a deeper understanding of a ubiquitous plankton's physiology, and discovery of novel biomarkers for future research.

EXPERIMENTAL PROCEDURES

Experimental Design

Adult female *Pleuromamma xiphias* were collected as described previously.[11] Briefly, copepod capture occurred aboard the R/V Atlantic Explorer from May 20−22, 2019 in the waters off of Bermuda (sampling site descriptions are described in Supporting Information 1). Two experimental approaches were employed: analysis of organisms collected rapidly after retrieval from their natural environment (the *in situ* samples) and those maintained in dark conditions after collection from the surface waters at night (the incubated samples). Time points for *in situ* collection were 22:00 on May 21st and 2:00, 9:00, 15:00, and 22:00 on May 22nd (local time; sunrise during this cruise was ∼6:00, and sunset was ∼22:00). Time points included in the "nighttime" samples in downstream analyses were both 22:00 time points and 2:00; "daytime" time points included 9:00 and 15:00. During the nighttime, when the animals migrated to the surface, organisms were captured using a 1 m^2 Reeve net[12] deployed to a 200 m depth, with a 150 μm mesh, a 20 L cod end, and a miniSTAR-ODDI pressure and depth sensor. During the daytime, after animals had migrated to their colder, deeper daytime depth, tows were conducted from a 400 to 600 m depth using a 1 m^2 MOCNESS with a 150 μm mesh and a custom-built thermally insulated closing cod end. Net tows were oblique with 200 or 400−600 m of wire out to the surface of the water.

For the incubation portion of the experiment, individual female copepods from the 22:00 Reeve tow on May 22nd were incubated in the dark at 20 °C and sampled at 6 h intervals for 36 h. Upon collection, the copepods spent not more than 1.25 h in the net at constant temperature. After they were pulled on deck, it took about 20 min to put them in the incubation jars. Individuals were maintained in 115 mL stoppered glass jars of filtered seawater with ∼30 mL of headspace. Water for the experiment was collected during midday CTD casts from the deep chlorophyll max (∼120 m). This water was filtered through a 0.2 GFF using a Georig and was stored for ∼12 h in a stand-up incubator to come to temperature. We set up 10 jars for each of the eight time points of the experiment (80 separate individuals). Jars had been DOC cleaned in the combustion furnace (450 °C for 5 h) prior to the cruise and were always handled with gloves. Jars were placed in cardboard boxes to prevent ambient-light interference and allocated randomly in the incubator, which had no lights or windows, to create total darkness. Every 6 h (starting at 6:00 May 23rd and ending at 12:00 May 24th), five actively swimming individuals were removed from jars with a plastic transfer pipet and were flash frozen for proteomics analysis (the other five sampled at each time point were used for a separate transcriptomics experiment). Time points will hereafter be referred to as 6:00, 12:00, 18:00, 24:00 (midnight), and 36:00. The 6:00 time point on May 24th (30:00) was excluded from the analysis due to issues with sample size.

Proteomics Sample Preparation

Individual copepods (5 per time point from the *in situ* and incubation collected animals) were processed for proteomics as described previously.[11] Briefly, each copepod was homogenized in 120 μL of ammonium bicarbonate with protease inhibitors with a plastic pestle in a 1.5 mL Eppendorf tube. Homogenized copepods were sonicated three times with a Fisher sonic dismembrator model 100 set to power of 2 (Thermo Fisher Scientific, Waltham, MA). The protein content was measured using a Pierce BCA assay kit (Thermo Fisher) following the manufacturer's guidelines for a limited sample volume in a microplate. Protein extraction/digestion was performed on 100 μg of the sample. To each sample, 0.5 μL of 250 unit/μL benzonase nuclease and 4 μL of 500 mM diothiothreitol were added followed by an incubation for 10 min at 95 °C. Then, 8 μL of 500 mM iodoacetamide was added followed by an incubation at room temperature in the dark for 30 min. Phosphoric acid (13.6 μL of 12%) and an S-trap binding buffer (700 μL) were added to each sample. Each copepod lysate was added to a single S-trap column (Protifi, Farmingdale, NY) and spun through followed by 3 additions of the S-trap binding buffer and 3 additions of 50/50 chloroform/methanol. After an additional wash with the S-trap binding buffer, peptides were on-column digested with 10 μg of trypsin (Promega, Madison, WI) suspended in 50 mM TEAB over 1 h at 47 °C. Peptides were eluted with 80 μL of 50 mM TEAB followed by 80 μL of 50% acetonitrile + 0.2% formic acid. Liquid was evaporated from the tubes using a speed vacuum at room temperature, and samples were resuspended in 100 μL of 5% acetonitrile + 0.1% formic acid.

Proteomics: Data-Dependent Acquisition (DDA)

For each time point in the incubation experiment (6:00, 12:00, 18:00, 24:00, and 36:00), 3 individual copepods were analyzed on a Q-Exactive Plus mass spectrometer (Thermo Fisher) in the data-dependent acquisition (DDA) mode as described previously[11] with additional details in Supporting Information 2. The DDA data set for the *in situ* copepods is described in detail elsewhere[11] and was used as a hypothesis generating data set for circadian biomarker identification for this study. The DDA experiment of the incubated copepods, as described here, was generated to compare whole proteome trends with the *in situ* copepods, but with a limited sample size. Raw mass spectrometry and supporting files for analysis were deposited to the ProteomeXchange Consortium via PRIDE with the data set identifier/accession PXD021156.

Acquired mass spectrometry spectra were searched against the *P. xiphias* transcriptome (NCBI accession GJVP01000000),[11] translated to predicted protein sequences using TransDecoder v. 2.0.1 (github.com/Transdecoder) and concatenated with standard laboratory contaminant sequences from the cRAPome.[13] Each raw file was searched against the predicted proteome using Comet v. 2019.01 rev. 4[14,15] (parameter file available on the GitHub repository: https://github.com/Nunn-Lab/Publication-Pleuromamma-targeted-proteomics). Probability scores for peptide and protein detection were assigned using PeptideProphet and ProteinProphet.[16,17] Consensus protein inferences for proteins with a

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false discovery rate of 0.01 or less were determined using Abacus.[18]

Input files were prepared for differential abundance (Qspec[19]) from the Abacus output file. Proteins with at least two unique peptides across all MS/MS experiments were used for downstream analysis. For comparison of day (time points 6:00, 12:00, and 36:00) versus night (18:00 and 24:00) protein abundance in QPROT, a file was created that contained spectral count data for each sample, as well as protein ID and protein length. Columns containing spectral count data were renamed 0 or 1, depending on if the sample was a nighttime or a daytime copepod, respectively.

GO term enrichment analysis was performed on significantly different proteins (proteins with a log fold change greater than |0.5| and with a *Z*-score greater than |2|) to find the overrepresented GO terms, with a *p*-value cutoff of 0.1 using a project-specific compGO:[20] https://meta.yeastrc.org/compgo_emma_sub_copepod/pages/goAnalysisForm.jsp.

To perform nonmetric multidimensional scaling (NMDS) and analysis of similarity (ANOSIM), adjusted normalized spectral abundance factor (NSAF) values (calculated in Abacus) were log(x + 1) transformed. NMDS was performed on a Bray−Curtis dissimilarity matrix in the vegan package.[21] Protein eigenvalues were exported for annotation and interpretation. For ANOSIM, a row standardization was performed before creating a Bray−Curtis dissimilarity matrix with a significance cutoff of 0.05.

Differentially abundant proteins were compared between the incubation DDA data set and the previously published DDA data set for the *in situ* collected copepods.[11] If a protein was not annotated by BLASTx against the UniProt trembl database, a secondary BLASTx against NCBI's nr database was performed to achieve an annotation (*e*-value cutoff of 1×10^{-10}).

Amino acid sequences for the significantly different proteins were analyzed using BlastKOALA (https://www.kegg.jp/blastkoala/). "Animals" was selected as the taxonomy group of the genome, and "family_eukaryotes" was selected for the database.

Proteomics: Data-Independent Acquisition (DIA)

To refine peptide detection for targeted proteomics, we generated a data-independent acquisition (DIA) chromatogram library on two pools of copepod peptides to verify detection of peptides to be used in the targeted proteomics experiment (see below). Two pools of samples from the *in situ* copepods were created to make DIA chromatogram libraries: "night" samples (both 22:00 time points and 2:00) and "day" samples (9:00 and 15:00). We used *in situ* copepods to create the libraries because the biomarkers tested in this experiment were chosen to represent physiological processes of importance to *in situ* copepods. Copepod peptides from each time point (5 individuals per time point, 2 μL each) were added to the sample pool. Sample pool peptides and a PRTC external standard were combined prior to MS/MS analysis; each 2 μL injection contained 1 μg of peptide and 25 fmol of PRTC. Additional mass spectrometry details are provided in Supporting Information 2.

Proteomics mass spectrometry in the data-independent acquisition (DIA) mode was performed on each pooled copepod sample on a Lumos mass spectrometer (Thermo Fisher). Reverse-phase high-performance liquid chromatography was performed inline using an EasyLC (Thermo Fisher) with the mass spectrometer using a 5 cm precolumn (150 μm i.d. fused silica) and a 30 cm, 75 μm i.d. fused silica analytical PicoTip column (New Objective), both packed in-house with Dr. Maisch 3 μm Reprosil-Pur C18 beads (20 A). Peptides were eluted from the column using an acidified (formic acid, 0.1% v/v) water−acetonitrile gradient (4−28% acetonitrile over 90 min). Mass spectrometry data collection has been previously described.[22]

The DIA data set was analyzed as previously described.[23] The Skyline document and raw files can be found on PanoramaPublic[24,25] https://panoramaweb.org/CshqGB.url, along with the biomarker fasta file, .dlib, and .elib files. The final transition list included 498 transitions (including transitions for the internal PRTC standard), which corresponded to 125 peptides and 63 proteins.

For each protein, we selected 2 peptides (4 transitions, or fragments, per peptide) to be in the SRM assay. Proteins included in the assay are listed in Supporting Information 3.

Proteomics: Selected Reaction Monitoring (SRM)

Peptide biomarkers of putative circadian-controlled processes for selected reaction monitoring (SRM) mass spectrometry were selected from a previous DDA analysis of *in situ* collected copepods[11] to test hypotheses of proteins under circadian control that have physiological relevance for *in situ P. xiphias*. Briefly, differentially abundant proteins were selected from the proteins detected in the DDA experiment. This list was narrowed down to 291 proteins for screening for suitability in SRM analysis.

Selected reaction monitoring (SRM) was applied to compare diel abundances of specific peptides hypothesized to be under circadian control in the *in situ* and incubation experiments. SRM mass spectrometry was performed on an Altis mass spectrometer (Thermo Fisher) coupled with an EasyLC (Thermo Fisher). Additional details are included in the Supporting Information 2.

Differential protein abundance for each experiment (*in situ* and incubation) was determined using MSStats[26] in R. Differential abundance between grouped time points for night and day was calculated. For the grouped time points, day for the incubation experiment included 6:00, 12:00, and 36:00; night included 18:00 and 24:00. For the *in situ* sampling, day included 9:00 and 15:00; night included 22:00 and 2:00. Data were normalized using equalized medians before differential abundance analysis. Proteins were considered differentially abundant if the adjusted *p*-value was <0.05.

Principal coordinate analysis (PCoA) was performed on the two SRM data sets (*in situ* and incubated) on a Euclidean distance matrix and visualized with ggplot2.[27] Hierarchical clustering analysis of SRM peptide abundances was performed to detect peptides that followed a similar trajectory of abundance across time points sampled. Peptide abundances were averaged across replicates for each time point. A Bray−Curtis dissimilarity matrix was calculated using the vegan package, and the average clustering method was used. For both the *in situ* and incubation experiments, the tree height was cut at 0.3. Peptide abundances over time for each cluster were visualized using ggplot2.

KEGG pathway maps that had high coverage across the proteomic data sets were visualized using KEGG Mapper Color (https://www.genome.jp/kegg/mapper/color.html). Pathways selected for this visualization included purine metabolism, cell cycle, the citrate acid cycle, and carbohydrate

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digestion and absorption. These figures situate differentially abundant proteins and proteins with distinctive abundance profiles into a larger context of cellular metabolic pathways.

All relevant .raw and skyline documents can be found on PanoramaPublic[24,25] at https://panoramaweb.org/CshqGB.url. All relevant R codes and input files are available on this project's GitHub page: https://github.com/Nunn-Lab/Publication-Pleuromamma-targeted-proteomics.

RESULTS

DDA

Copepod proteomes showed clear differentiation by time point in the DDA incubation data set (Figure 1 and Supporting Information 4). ANOSIM revealed a significant proteome distinction by time (ANOSIM R = 0.5763, p = 0.001). Axis 1 of the NMDS separated two of the daytime time points (6:00 and 36:00) from the three additional time points (12:00, 18:00, and 24:00). This first axis had strong eigenvector loading values (p < 0.05, loading value of >0.80) for 14 proteins, including the myosin light chain, myosin heavy chain, heat shock protein 70, collagen IV, and glucose-regulated protein 78 kDa. The second axis of the NMDS separated the nighttime points from the 6:00 and 12:00 time points and represented strong eigenvector loadings for 18 proteins, including the myosin heavy chain, NADH dehydrogenase, peroxiredoxin-4, heat shock protein 70, sodium/potassium-transporting ATPase, and cytochrome c-oxidase subunit 5B.

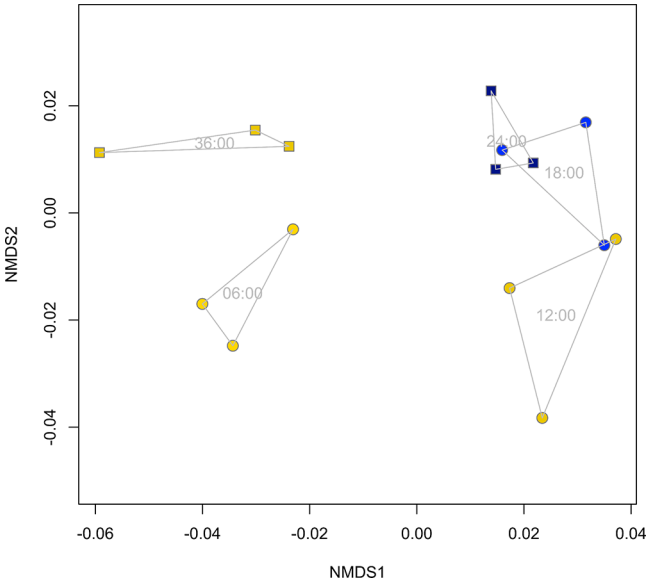

Figure 1. Nonmetric multidimensional scaling plot of the incubation DDA proteomics data set. Yellow indicates daytime samples, and blue indicates nighttime samples. Circles represent copepods sampled on May 23rd, and squares represent copepods sampled on May 24th.

The two noon time points (12:00 and 36:00) are distinct from each other, suggesting a change in proteome response with increased time in the incubation jars. Eighty-five proteins were significantly differentially abundant between 12:00 and 36:00; 28 were elevated at the 12:00 time point, and 57 were elevated at 36:00. Many of these proteins were myosin or troponin, but a few were more clearly linked to metabolism and cellular energy (e.g., peptidase, cytochrome c, and ATPase).

Differentially abundant proteins (LFC > |0.5| and Z-statistic > |2|) for night vs day in the incubation DDA data set included several proteins annotated as myosin and tropomyosin, cytochrome c, vitellogenin, peroxiredoxin, a ubiquitin-like-activating enzyme, multiple tubulins, and phosphoglycerate kinase (glycolysis), among others (Figure 2 and Supporting Information 4).

In the enrichment analysis of the differentially abundant DDA proteins, the GO term "lipid transporter activity" (GO:0005319, GO Aspect "Molecular Function", p-value = 0.06) was overrepresented. Five proteins were associated with this term, all of which were annotated as vitellogenin. In the statistically significant differentially abundant proteins, most vitellogenins were at elevated abundance in the day, but one was elevated at night (Figure 2).

From the BlastKOALA analysis of the differentially abundant DDA proteins, most were categorized under "Cellular Processes" or "Genetic Information Processing". In a decreasing order of representation, these proteins also represented the categories "environmental information processing", "energy metabolism", "protein families: genetic information process", "organismal systems", and "carbohydrate metabolism".

SRM

There was a clearer proteomic distinction between day and night proteomes for the incubation experiment than for the *in situ* experiment (Figure 3) in our targeted SRM analysis, which parallels patterns observed across the whole proteome (Figure 1 and Supporting Information 5).

Statistically differentially abundant proteins were detected across time points in both experiments (p < 0.05, Table 1). More proteins were differentially abundant in comparisons in the incubation experiment (n = 18) compared to in the *in situ* samples (n = 4). A summary of the differentially abundant proteins is in Table 1. Profile plots for these proteins over time, for the incubation and *in situ* samples, can be found in Supporting Information 6.

In the *in situ* copepods, six clusters showed a trend of peptide transitions with elevated abundance during night time points (Figure 4 and Supporting Information 7). These clusters included proteins dnaJ homologue subfamily C member 7, glycoside hydrolase, NADH dehydrogenase subunit 7, prosaposin, succinate dehydrogenase cytochrome b560 subunit, superoxide dismutase, troponin I, and venom dipeptidyl peptidase 4. Four clusters contained peptide transitions that were more abundant during the day. These peptides represented the proteins carbonic anhydrase and cuticle protein 6. None of these are significantly differentially abundant.

In the incubation experiment, nine clusters contained peptide transitions that show a trend of elevated abundance at night (Figure 4 and Supporting Information 8). These transitions were derived from the proteins listed in Table 2. These proteins represent a variety of metabolic enzymes, including lipid metabolism, carbohydrate metabolism, TCA enzymes, mitochondrial respiration, cell growth (cell cycle proteins), and antioxidant response. Proteins with peptide fragments elevated at night in both the *in situ* and incubation data sets included venom dipeptidyl peptidase 4, dnaj homologue, NADH dehydrogenase, glycogenin-1, and glycoside hydrolase.

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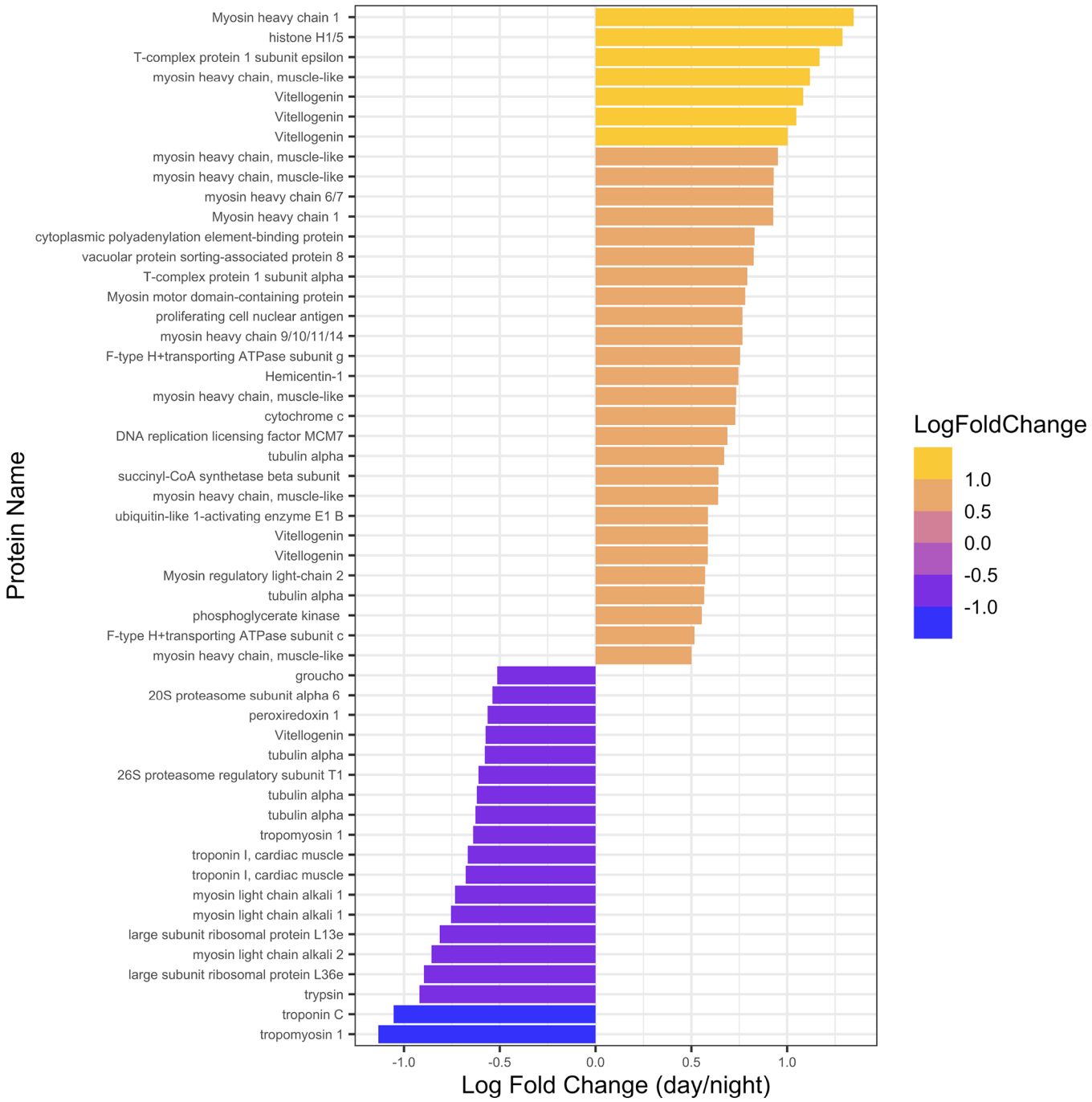

Figure 2. Log fold change (LFC) of differentially abundant DDA incubation proteins. Proteins with a positive LFC (yellow) were higher in the daytime proteome; proteins with a negative LFC (blue) were higher in the nighttime proteome.

Peptide transitions with a trend of higher abundance during the day in the incubation experiment were detected in 7 out of 26 clusters (Figure 4 and Supporting Information 8). These clusters included representatives of proteins 26S proteasome non-ATPase regulatory subunit 13 (significantly differentially abundant); allantoate amidinohydrolase; long-chain fatty acid-CoA ligase (significantly differentially abundant); ovoinhibitor (significantly differentially abundant); prosaposin (significantly differentially abundant); superoxide dismutase (different from SOD higher at night); troponin I (significantly differentially abundant); trypsin-7 (significantly differentially abundant); venom allergen 3-like (significantly differentially abundant); vitellogenin (different from Vg higher at night). Proteins with peptides elevated during day time points in both *in situ* and incubation data sets were trypsin and venom allergen 3-like.

DISCUSSION

Organisms spanning diverse taxa demonstrate patterns of circadian-type rhythms, which include patterns that are ancient and evolutionarily conserved, as well as taxonomically specific (e.g., refs 4, 28, and 29). Here, we provide evidence of specific metabolic pathways under putative circadian clock control in the diel vertically migrating copepod *Pleuromamma xiphias* from the Sargasso Sea. In both a full proteome and a targeted proteomics data set, *P. xiphias* proteomes showed signs of canalization of a rhythmic response when exogenous food and

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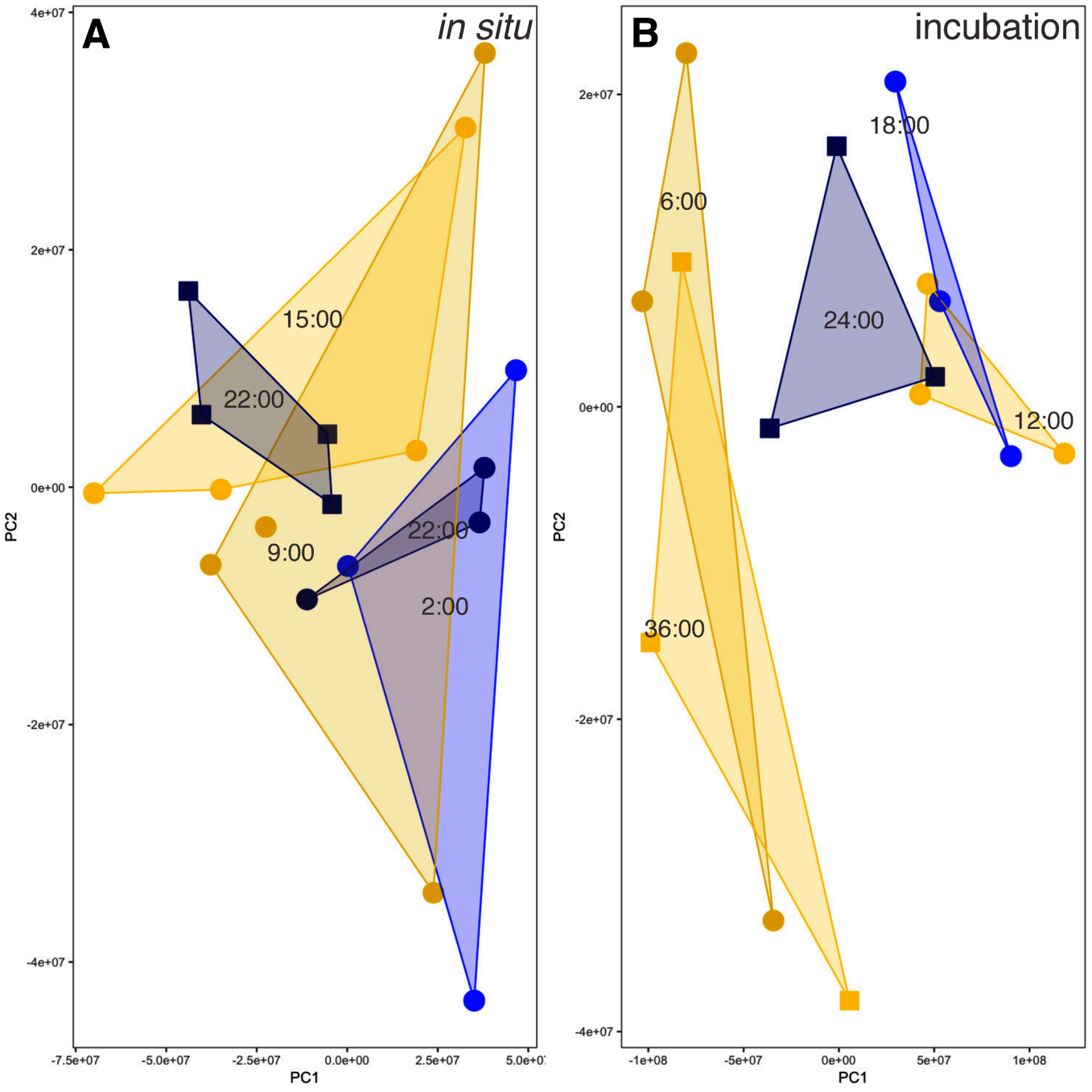

Figure 3. PCoA plots of the SRM proteomics data for *in situ* collected copepods (A) and copepods from the bottle incubations (B). Both data sets span 2 days with circles representing time points on the first day and squares time points on the second day. Yellows represent daytime time points, and blues represent nighttime. Trends of abundances over time are presented in Figure 4.

photoperiod cues were removed. Canalization can refer to the evolved specificity, or a lack of plasticity, of molecular or phenotypic environmental response and has been observed in a variety of contexts across taxa (e.g., refs 30−33). The observed maintenance of rhythms in protein abundances suggests an evolutionarily maintained pattern specific to the environment and lifestyle of *P. xiphias*. If specific proteins follow a diel pattern of abundance changes across *in situ* and incubated copepods, then those proteins may be under clock control. Alternatively, if general patterns of protein abundance fluctuate with the diel cycle and the proteins that change in abundance are specific to the given environment (entrained by environmental cues), then there is likely more general circadian control over mechanisms such as protein translation rates,[34] rather than control of a specific protein. There is evidence of some *P. xiphias* proteins that show direct circadian control. Metabolic proteins, for example, responsible for carbohydrate and fatty acid degradation, continued to follow a pattern of day-to-night abundance in the absence of environmental cues (Figure 4 and Supporting Information 6 and 10), suggesting a circadian metabolic response that aligns with the copepod's daily migration pattern. Targeted analysis of some of these proteins during an incubation experiment can help to resolve

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Table 1. Differentially Abundant Proteins from the SRM Experiment[a]

experiment	protein	LFC
incubation	venom dipeptidyl peptidase 4	−1.6
incubation	ovoinhibitor	0.8
incubation	troponin I	Inf
incubation	alpha-amylase	−1.0
incubation	esterase D	−0.6
incubation	superoxide dismutase	Inf
incubation	histidine ammonia-lyase	0.6
incubation	2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline decarboxylase	0.7
incubation	dolichyl-diphosphooligosaccharide—protein glycotransferase	0.5
incubation	low-density lipoprotein receptor	0.6
incubation	26S proteasome non-ATPase regulatory subunit 13	0.5
incubation	2,4-dienoyl-CoA reductase	0.4
incubation	long-chain fatty acid-CoA ligase 3-like	−0.6
incubation	trypsin-7	1.1
incubation	prosaposin	1.3
incubation	aminopeptidase	0.7
incubation	dnaJ homologue subfamily C member 7	−1.0
incubation	solute carrier family 2, facilitated glucose transporter member 1	0.5
in situ	tropomyosin	0.8
in situ	ATP-dependent 6-phosphofructokinase	0.8
in situ	tumor suppressor candidate 3	0.6
in situ	protein obstructor-E	0.5

[a]The first column contains the experiment type, the second the protein name, and the third the log fold change (LFC). A positive LFC indicates a higher abundance in the combined daytime times, and a negative LFC indicates a higher abundance at night. An LFC value of "Inf" translates to detection only in daytime time points.

ambiguity surrounding the responsiveness of these proteins to environmental drivers versus circadian control.

Pleuromamma xiphias Demonstrates Putative Clock Control over Diverse Processes

The preservation of a biological rhythm in the absence of exogenous cues is suggestive of circadian clock control on select physiological processes. Clear patterns of rhythmic gene expression are preserved in aquatic crustaceans during lab incubations.[8] In this study, the preservation, and intensification, of a biological rhythm of proteins was detected in copepods incubated in the dark, with no food source (Figures 3 and 4). Even though our sample size was small (n = 5) due to experimental constraints, these proteomic diel rhythms were still detectable. The absence of exogenous stimuli may create an apparent intensification of the circadian response since physiological response/compensation for the various external influences (depth, temperature, light, food, and inter- and intraspecific interactions) is no longer influencing physiology (e.g., ref 35). Even though DVM may effectively decrease variability in light exposure throughout a 24 h period (e.g., ref 36), the other environmental cycles and changes mentioned could still vary considerably in the natural environment. For example, even though *P. xiphias* does most of its feeding in the food-rich upper ocean layers, there may still be opportunistic feeding at lower depths. The acquisition and digestion of food at depth could dampen the detection of a potential circadian rhythm in metabolic enzymes. In another copepod species, some genes demonstrated clearer rhythmicity in lab-incubated organisms than in those caught in the field.[4] In both the *P. xiphias* targeted and DDA (whole proteome) data sets, there were stronger signals of day-to-night differences in incubated samples than the *in situ* collected samples[11] (Supporting

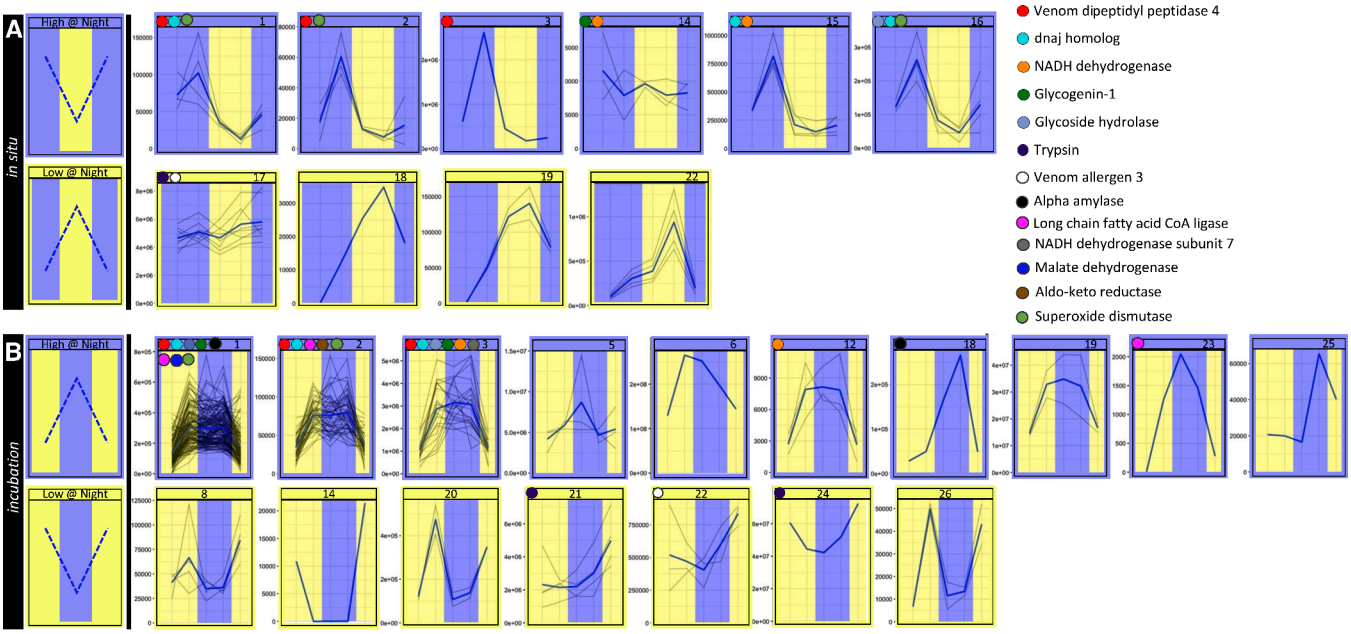

Figure 4. Dominant trends of protein abundances over time from the *in situ* (A) and incubation (B) copepods. Plots in the first column indicate if proteins identified peak during the night (blue) or the day (yellow). Clusters containing proteins that show similar abundance trends across experiments have a colored dot indicating a specific protein: red for venom dipeptidyl peptidase 4, aqua for dnaj homologue, orange for NADH dehydrogenase, green for glycogenin-1, light blue for glycoside hydrolase, purple for trypsin, white for venom allergen, black for alpha-amylase, magenta for long-chain fatty acid-CoA ligase, gray for NADH dehydrogenase subunit 7, bright blue for malate dehydrogenase, brown for aldo-keto reductase, and light green for superoxide dismutase. Venom dipeptidyl peptidase, dnaj homologue, trypsin, alpha-amylase, long-chain fatty acid-CoA ligase, and superoxide dismutase are also significantly differentially abundant between night and day in the incubation copepods (Table 1).

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Table 2. Nighttime-Specific Processes as Indicated by Proteins Represented by Peptide Abundances that Peaked at Night in the Cluster Analysis of the Targeted Proteomics Incubation Data Set (Supporting Information 8)[a]

	function	protein	cluster assignment
Metabolism	metabolism of polyunsaturated fatty enoyl-CoA esters	2,4-dienoyl-CoA reductase*	2
	carbohydrate metabolism	aldo-keto reductase family 1; alpha-amylase*; ATP-dependent 6-phosphofructokinase; glycogenin-1; glycoside hydrolase; phosphotransferase	2; 18; 3; 5; 6
	multiple metabolic functions	esterase D*	12
	amino acid metabolism	histidine ammonia-lyase*	2
	luciferase/fatty acid metabolism	AMP-binding domain-containing protein; long-chain fatty acid-CoA ligase*; short-chain acyl-CoA dehydrogenase	2; 3; 23
	electron transport chain	complex I−B14.5a; cytochrome *b*-c1 complex subunit Rieske; NADH dehyrogenase [ubiquinone]	3; 2; 12
	tricarboxylic acid cycle	oxoglutarate dehydrogenase; pyruvate dehydrogenase; succinate dehydrogenase cytochrome b560 subunit	2; 3; 5
Protein Modification/ Turnover	ubiquitination/protein degradation	26S proteasome non-ATPase regulatory subunit 13*; cathepsin C; ubiquitin carboxyl-terminal hydrolase; ubiquitin-conjugating enzyme	2; 12
	aminopeptidase activity	aminopeptidase*	2
	protein glycosylation	dolichyl-diphosphooligosaccharide−protein glycosyltransferase*; tumor suppressor candidate 3	3; 2
	protein folding	dnaJ homologue*; protein disulfide-isomerase	2; 3
	protein translation	methionine−tRNA ligase	19
Macromolecule Transport	carbohydrate transport	facilitated glucose transporter	2
	cholesterol receptor/transport	low-density lipoprotein receptor; SCP2 domain-containing protein	2; 5; 3
	lipid storage/transport	seipin; vitellogenin	25; 3
Excretion	excretion	allantoate amidinohydrolase; Rh-related protein	3; 2
Cell/Tissue Growth and Maintenance	cell cycle	cell division control protein 2; spartin-like	19; 2
	structural constituent of cuticle/ chitin binding	cuticle protein 6; protein obstructor-E; putative LOC100871525	3; 2
	muscle	tropomyosin	2
ROS Response	oxidative stress response	superoxide dismutase*	2
Venom	venom	venom didpetidyl peptidase 4*	2; 3
pH Balance	carbonate dehydratase activity	carbonic anhydrase	2

[a]An asterisk indicates a protein that is significantly differentially abundant between night and day.

Information 5 and Figures 1 and 3). This pattern is likely due to the removal of the physiological noise of living in a dynamic environment, which may blunt signals of the circadian rhythm.

At night, *P. xiphias* migrate ∼500 m for active feeding at the productive ocean surface. While the behavior of DVM and some of the molecular effectors of metabolism may be under clock control, feeding activity is likely triggered by decreased light, either directly or by light acting on an endogenous control,[37] which coincides with migration to the food-rich surface waters. Both changes in light and food availability have been shown to entrain circadian rhythms and are likely the root of the observed rhythmicity in metabolic peptides.[1,37] This daily rhythm is reflected in the proteome with evidence of elevated nighttime levels of a range of proteins underlying carbohydrate and lipid metabolism, as well as proteins involved in processes "downstream" of metabolism (response to reactive oxygen species; cell/tissue growth). Proteins that are significantly elevated in the nighttime proteome in incubated copepods include alpha-amylase (carbohydrate metabolism), glycoside hydrolase (carbohydrate metabolism), and long-chain fatty acid-CoAligase 3 (fatty acid metabolism). Others demonstrate a consistent trend of nonsignificant higher abundances across SRM peptide targets: NADH dehydrogenase subunit 7 (electron transport), malate dehydrogenase (TCA), glycogenin-1 (glycogen biosynthesis), and aldo-keto reductase (carbohydrate metabolism) (Figure 4). Glycogenin-1, NADH dehydrogenase, and glycoside hydrolase all demonstrated peaks in abundance in both the *in situ* and incubation targeted data sets, suggesting an essential role of these proteins during the diel cycle. NADH dehydrogenase subunit 7 is among the four proteins representing mitochondrial respiration that were measured with SRM (29 were detected in the DDA data set, Supporting Information 9). Regulation of mitochondrial respiration, and specifically of the rate-limiting step at cytochrome *c*-oxidase, is complex and can be environmentally dependent.[38,39] Our low coverage of the entire pathway in the SRM data set does not yield definitive answers, but in the four proteins measured, all demonstrate trends toward increased abundance at night (Supporting Information 6), as does superoxide dismutase, a scavenger of reactive oxygen species generated during mitochondrial respiration. Cellular metabolism is known to be mechanistically linked to the circadian clock,[40] and metabolism likely entrains the circadian clock.[41] Across planktonic crustaceans, genes and enzymes associated with cellular metabolic processes observe clear diel or circadian cycles in expression.[8,35,42] In krill, many of these genes did not maintain a night/day expression pattern during 24 h of darkness[8] in contrast with the current study, suggesting either a taxonomic difference in biology or a reflection of the functional difference between genes and proteins. Food anticipation appears to be at least in part under circadian clock control in some taxa,[37,43] and the upregulation of metabolic enzymes could be part of that food anticipatory behavior. Upregulation of metabolism necessarily means that increased levels of reactive oxygen species and signals of that specific physiological response are found in the increased

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abundances of peroxiredoxin (DDA/whole proteome, Figure 2) and superoxide dismutase (targeted SRM data set, nonsignificant; Figure 4 and Supporting Information 6) at night. In the planktonic crustacean *Daphnia pulex*, increases in ROS scavenger expression were observed around the feeding time,[44] suggesting a direct link between food acquisition, ROS production, and antioxidant expression. The ROS system and peroxiredoxins in particular have proven to be entrained in circadian rhythms across all domains of life, highlighting their importance in mitigating cellular damage that occurs during daily metabolic cycles.[29] Even in the absence of their physical migratory pattern and food, *P. xiphias* continue to exhibit a metabolic proteomic signature strongly linked to diel vertical migration.

In the daytime proteome, there are fewer metabolic proteins with elevated abundance, suggesting that circadian control favors decreased abundance of metabolic enzymes at depth. It is likely that *P. xiphias* feed at depth during the daytime,[11] but this feeding may be more opportunistic and not as physiologically ingrained as the feeding that occurs at night. In the targeted analysis, both incubated and *in situ* collected copepods revealed peaks in abundance for the protein trypsin, which suggests a fixed pattern of digestion during the day even in the absence of food (Figure 4 and Supporting Information 6). One strong signal in the data set from the incubation daytime proteome that was not detected in the nighttime proteome is the increased abundance of proteins involved in cytokinesis and DNA replication (Figure 2 and Supporting Information 10). In a whole proteome analysis of the *in situ* copepods, we detected similar signals of increased abundance of proteins involved in digestion and tissue repair and maintenance.[11] The cellular morphology of the copepod digestive tract changes throughout the cycle of feeding to nonfeeding,[45] suggesting a period of repair and growth during nonfeeding periods. Similarly, in other crustaceans, hematopoiesis and neurogenesis are under circadian control, leading to cyclical patterns in cell production.[5,46] Time away from the surface during the day may be time spent on cellular growth and repair as the food acquired at night is digested and taken up by cells.

Proteins of unknown function can also be revealed by a circadian analysis, highlighting the complexity of the circadian response and providing additional information for future functional characterization. The abundances of two different venom proteins changed with the diel cycle in both groups of copepods (Figure 4). The ability to produce and use venom has evolved across a diverse range of crustaceans, including known venomous copepods (heterorhabdids) and some ectoparasites.[47] The copepod adaptations required for venomous feeding require both the evolution of venom and the accompanying morphological adaptations that allow injection and release of venom, as seen in some heterorhabdids.[48] However, none of the copepod taxa with known venom-producing abilities are closely related to *Pleuromamma*, and *P. xiphias* does not possess the specialized feeding morphology for releasing venom into prey.[49] *Pleuromamma xiphias* are known omnivores in the Sargasso Sea, but they likely accomplish their omnivory exclusively through particle feeding.[49] It is possible that these proteins are nonfunctional in terms of venom production yet could have other physiological roles. The importance of expression of these venom-related proteins and their change in abundance following a diel cycle in *P. xiphias* remain unknown.

Whole Proteome Analysis Reveals Common Processes between *In Situ* and Incubation Copepods

There was considerable overlap in classes of proteins that followed a night−day pattern between the whole proteome (DDA) data sets for the incubation copepods (this study) and the *in situ* copepods.[11] Across both data sets, there is evidence of ubiquitous expression of vitellogenin and myosin. We found evidence of changes in abundance of proteins involved in protein degradation, vitellogenesis, muscle function, amino acid biosynthesis, carbohydrate metabolism, and oxidative stress response in both data sets, but there was little direct overlap of specific differentially abundant proteins[11] (Figure 2). In both whole proteome data sets, some types of proteins (vitellogenin and myosin) were elevated at both night and day.[11] These seemingly contradictory patterns may suggest differing roles for proteins within the same functional group; for example, in the incubation data set, most of the myosins elevated in the day were heavy chains, while most of the myosins elevated at night were light chains. The similar patterns between data sets could suggest that none of these proteins are under direct circadian control, but rather, a more general regulatory mechanism is under clock control (e.g., ref 34). Alternatively, or additionally, some of these specific proteins that seem circadian-responsive in the *in situ* data set, but not in the incubation data set, may be more directly responsive to specific environmental changes, which are strongly correlated with the diel cycle. In the daytime incubation proteome, similar to the *in situ* data set, we also saw evidence of a changing abundance of proteins involved in cellular metabolism and repair (e.g., phosphoglycerate kinase, arginine kinase, cuticle protein, and DNA replication licensing factor MCM7) (Figure 2). These similar patterns in classes of proteins, from ocean-collected to lab-incubated copepods, suggest a canalization of the diel physiological cycle at the protein level.

The distinctive proteomic separation between the two noon time points (12:00 and 36:00) in the whole proteome data set may provide insight into acclimation to the jar environment. There were no distinctive signals of starvation in the copepods from the 36:00 time point, which would have been in the jars 24 h longer than the 12:00 time point copepods. However, many of the differentially abundant proteins between 12:00 and 36:00 were annotated as myosin, perhaps revealing a change in muscle proteins when the physical process of DVM is no longer possible. Out of 12 muscle-related proteins (myosin, tropomyosin, and troponin), 3 were elevated at 12:00, and 9 were elevated at 36:00. This lab incubation effect on the molecular level warrants further investigation.

Biomarkers under Circadian Control

In a comparison of differentially abundant proteins across the incubation and *in situ* data sets, we have compiled a list of proposed highly informative biomarkers of the circadian rhythm for *P. xiphias* (Table 3). The response of these protein biomarkers has been characterized in our study[11] across natural and full-darkness incubation environments and can thus serve as a robust reference for further studies in *P. xiphias*. They could also be applied for comparative studies across taxa and environments to better understand the universality of the mechanisms of circadian rhythms.

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Table 3. Proposed Highly Informative Biomarkers of the Circadian Rhythm in *P. xiphias*

protein	function	day or night elevated abundance
venom dipeptidyl peptidase 4	unknown	night
myosin heavy chain	muscle	day
myosin light chain	muscle	night
alpha-amylase	metabolism	night
superoxide dismutase	oxidative stress response	day
trypsin-7	digestion	day
dnaJ homologue	protein folding	night
long-chain fatty acid ligase	metabolism	night
prosaposin	glycolipid metabolism	day
troponin I	muscle	day
2,4-dienoyl-CoA reductase	metabolism	night
esterase D	metabolism	night
26S proteasome non-ATPase regulatory subunit 13	ubiquitination	night
aminopeptidase	aminopeptidase activity	night

CONCLUSIONS

Through proteomics analysis, we have uncovered a diversity of *P. xiphias* physiological processes that are under putative circadian clock control and developed biomarkers that can be applied to future circadian studies in *P. xiphias*, or even in cross-taxa comparisons. These results underline the importance of metabolic regulation during the copepod DVM cycle. This metabolic control continues in the lab, even in the absence of the photoperiod and other exogenous cues. From the data collected, it is impossible to determine if these important players in the circadian rhythm of *P. xiphias* are under direct (transcription and translation) circadian clock control, or if they are under some other, indirect control. If under indirect clock control, the translation of proteins in the circadian cycle may be a direct result of circadian control of cellular Mg^{2+} levels, which control protein translation rates,[34] rather than direct circadian control over specific genes and proteins, a hypothesis that was not directly explored in this study. There is evidence in this data set both for directed control of specific proteins (i.e., glycogenin-1, NADH dehydrogenase, and glycoside hydrolase) and for general control of physiology that may not be linked to a specific protein or pathway.

ASSOCIATED CONTENT

Data Availability Statement

Data-dependent acquisition raw and supporting files are available on the ProteomeXchange PRIDE repository under accession PXD021156. Data-independent acquisition and targeted proteomics raw files and Skyline projects are available on Panorama and PRIDE: https://panoramaweb.org/CshqGB.url. All R files for creating the figures in this paper are available at https://github.com/Nunn-Lab/Publication-Pleuromamma-targeted-proteomics.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.4c00086.

Supporting Information 1: Details for each sampling time point (TXT)
Supporting Information 2: Supplementary methods for mass spectrometry proteomics (PDF)
Supporting Information 3: Targeted proteomics peptide transition abundances (TXT)
Supporting Information 4: Data-dependent acquisition protein abundance for incubation data sets (TXT)
Supporting Information 5: Nonmetric multidimensional scaling plot for the *in situ* DDA data set (PDF)
Supporting Information 6: Peptide transition profiles across time, grouped by protein (PDF)
Supporting Information 7: Hierarchical cluster plot for the *in situ* data set (PDF)
Supporting Information 8: Hierachical cluster plot for the incubation data set (PDF)
Supporting Information 9: Protein abundance plots of mitochondrial respiration proteins identified in the DDA data set (PDF)
Supporting Information 10: KEGG pathway figures (PDF)

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Notes

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