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# Diel metabolic patterns in a migratory oceanic copepod

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ABSTRACT

Diel vertical migration of zooplankton profoundly impacts the transport of nutrients and carbon through the water column. Despite the acknowledged importance of this active flux to ocean biogeochemistry, these contributions remain poorly constrained, in part because daily variations in metabolic rates are not considered or are modeled as simple functions of temperature. To address this uncertainty, we sampled the subtropical copepod *Pleuromamma xiphias* at 4- to 7-h intervals throughout the daily migration and measured rates of oxygen consumption, ammonium excretion, fecal pellet production and metabolic enzyme activity. No significant patterns were detected in rates of oxygen consumption or ammonium excretion for freshly caught animals over the diel cycle. Fecal pellet production was highest during mid-night, consistent with several hours of feeding near the surface. Surface feeding resulted in fecal pellet production at depth in the morning, providing direct evidence that active flux of particulate organic carbon occurs in this region. Electron transport system activity was highest during the afternoon, contrary to our prediction of reduced daytime metabolism. Activity of both glutamate dehydrogenase and citrate synthase increased during early night, reflecting higher capacity for excretion and aerobic respiration, respectively. Overall, these results show that activities of metabolic enzymes vary during diel vertical migration. The surprising observation of elevated afternoon enzyme activity coupled with daytime fecal pellet and ammonium production suggests that additional characterization of the daytime activity of migratory zooplankton is warranted.

## 1. Introduction

Diel vertical migration (DVM) of zooplankton is typified by the presence of migrators in the photic epipelagic during the night followed by their movement into deeper water during the day. These daily migrations, during which individuals of a few millimeters in length or less travel hundreds of meters in a few hours, are thought to be energetically expensive. Although costly, this typical pattern of migration is primarily driven by avoidance of visual predators at shallow depths during daytime and by the pursuit of prey (Antezana, 2009; Gliwicz, 1986; Hays, 2003; Pinti et al., 2019).

DVM is a key component of the biological pump (Siegel et al., 2016). Migrators release surface-derived carbon and nutrients as respiratory $CO_2$ and other excretory waste products (e.g., urea, ammonium, fecal matter, and dissolved organic compounds) below the thermocline (Longhurst et al., 1990; Longhurst and Harrison, 1988; Maas et al., 2020; Zhang and Dam, 1997). This process, known as active flux, has been estimated to account for 15–40% of the total global organic carbon export from the surface to the mesopelagic (Aumont et al., 2018; Bianchi et al., 2013; Steinberg et al., 2000). Through the excretion of nitrogenous compounds at depth, DVM also reduces the availability of this limiting nutrient to phytoplankton in surface waters, influencing the potential for new production (Longhurst and Harrison, 1988). In many cases, this daily shuttling of material meets or exceeds the vertical transport associated with passively sinking particles (Hernández-León et al., 2019a; Kobari et al., 2013; Steinberg et al., 2008). At depth, some zooplankton species also consume particles, aggregates, and one another, significantly modifying the availability and export of nutrients

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and carbon from the mixed layer (Robinson et al., 2010; Schnetzer and Steinberg, 2002a). The magnitude and relative importance of active transport varies regionally and seasonally, but the factors driving this variation are poorly understood (Burd et al., 2010; Steinberg and Landry, 2017) and have been identified as a priority for future research on the biological pump (Burd et al., 2016).

Estimates of active flux are typically made by measuring biomass of the migratory community then applying mass-specific and temperature scaling factors (i.e., $Q_{10}$ relationships) to experimentally calculated oxygen consumption rates, as well as organismal nitrogen and carbon excretion rates (e.g., Kiko et al., 2020; Le Borgne and Rodier, 1997). However, depth-dependent metabolic rates are driven not only by temperature differences, but also by differences in swimming activity and oxygen availability (Bianchi et al., 2013; Hernández-León et al., 2019b; Herrera et al., 2019). Daily cycles in feeding activity would also be expected to affect metabolic rates through specific dynamic action, the metabolic costs of assimilating nutrients and incorporating them into biomass (Kiørboe et al., 1985). In addition, a few studies in krill and copepods have identified circadian cycles in respiration rate, swimming behavior and the expression of metabolic genes (Häfker et al., 2017; Maas et al., 2018; Teschke et al., 2011). If these patterns are widespread and there are cycles in other major physiological processes, like fecal pellet production and ammonium excretion, failure to account for physiological cycles may lead to errors in the estimations of organismal contributions to biogeochemical flux during daytime at depth.

In addition to direct metabolic measurements of oxygen consumption, nitrogen excretion and fecal pellet production, aspects of active flux have been estimated by measuring the activity of key enzymes including the electron transport system (ETS) for respiration or glutamate dehydrogenase (GDH) for ammonium excretion (Bidigare, 1983; Fernández-Urruzola et al., 2011; Hernández-León et al., 2019b; Packard and Gómez, 2013). Such measurements have the advantage that they avoid artifacts associated with bottle incubations, but it is unclear over what time scale the fluctuations in enzymatic activity correspond to changes in organismal metabolic rates. Predictions of organismal metabolic rates from enzymatic activity measurements of field-collected zooplankton typically have large uncertainties, e.g., 31–38% for oxygen consumption and ETS (Packard and Gómez, 2013; Packard et al., 1988) and 42.5% for ammonium excretion and GDH (Fernández-Urruzola et al., 2016). Part of this uncertainty can be explained by variation in physiological activity and metabolic rates as a percentage of the maximum rate that could be supported by a given enzyme. For example, food availability and quality affect substrate availability, and contribute to decoupling between measurements of oxygen consumption and ETS activity (Hernández-León and Gómez, 1996; Osma et al., 2016).

For organisms that undergo DVM, daily cycles in food availability are somewhat predictable, so migrators might modulate their enzymatic capacity in anticipation of this variability. Daily physiological and behavioral cycles can be directly triggered by environmental conditions, and can also be regulated through endogenous circadian clocks. In nature, these two mechanisms are interrelated because circadian clocks are entrained by environmental cues, such as light, temperature and food availability. Conserved components of the circadian clock have been identified in a few planktonic crustaceans, including the euphausiids *Euphausia superba* (Teschke et al., 2011; De Pittà et al., 2013) and *Meganyctiphanes norvegica* (Blanco-Bercial and Maas, 2018), and the copepods *Calanus finmarchicus* (Christie et al. 2013, Häfker et al., 2017) and *Pleuromamma xiphias* (Maas et al., 2018). In *E. superba* and *C. finmarchicus,* circadian cycles in expression of circadian regulatory genes, metabolic enzymes (e.g., citrate synthase), and oxygen consumption have been described, and similar daily cycles have been detected in field populations (Teschke et al., 2011; De Pittà et al., 2013; Häfker et al., 2017). While external environmental cues have a large direct influence on DVM, laboratory experiments with krill (Gaten et al. 2008), copepods (Hüppe 2016) and nereid worm larvae (Tosches et al. 2014) suggest that circadian pathways can also contribute to this behavior. Regardless of the relative importance of circadian regulation versus direct responses to the environment, knowing when particular metabolic pathways are activated would allow prediction of when and where their end products are released into the water column, contributing to active flux transport and providing important nutrients to the midwater.

To address these knowledge gaps, we investigated daily physiological changes in the copepod *P. xiphias* (Giesbrecht, 1889), which is abundant, occurs throughout the tropical and subtropical oceans, and exhibits strong DVM (Goetze, 2011 and references therein). The contributions of *P. xiphias* to nitrogen and carbon flux have previously been characterized using classical bottle sampling methods and abundance estimates (i.e. Steinberg et al., 2000; Steinberg et al., 2002; Teuber et al., 2013). At the Bermuda Atlantic Time-Series (BATS) site, near the sampling site of our study, seasonal measurements have indicated that *P. xiphias* is the most biogeochemically relevant of the *Pleuromamma* copepods in the region. Together these copepods and the euphausiid *Thysanopoda aequalis* make up 23% of the surface zooplankton biomass on average, suggesting that they are the most important contributors to active flux (range 4–70%; Fig. 3 within Steinberg et al., 2000). We have previously shown that *P. xiphias* exhibits a circadian pattern in oxygen consumption when held under constant laboratory conditions, with a peak during dawn and lowest levels during the evening (Maas et al., 2018). In the present study, diel metabolic variation of *P. xiphias* was examined in the natural context of its daily migration using organismal metabolic measurements, as well as enzymatic activity assays. We hypothesized that the combined influences of the circadian machinery and the environment would create emergent molecular and physiological cycles that cannot be accounted for solely by $Q_{10}$ relationships. Specifically, we expected that excretion rates would be elevated during the night and that oxygen consumption rates would be highest at dawn, as we had previously observed. Finally, we predicted that patterns in GDH activity would mirror ammonium excretion rates and that patterns in citrate synthase (CS) and ETS activity would reflect oxygen consumption rates.

## 2. Materials and methods

### 2.1. Sample collection

*P. xiphias* were collected offshore from the Bermuda Institute of Ocean Sciences (BIOS) during a cruise aboard the *R/V Atlantic Explorer* from May 20–22, 2019 (Fig. 1). All times are reported as solar times. On May 21, sunrise occurred at 5:02 and sunset at 18:59; solar noon corresponded to 13:15 Bermuda local time (UTC -3 during Daylight Saving Time). Net tows were conducted at 12 time points, spaced 4–7 h apart to target afternoon, early night, mid-night and morning (Table 1). The timing of morning and early night tows was selected to target recently arrived migrants based on empirical observations from previous tows and the first day of sampling. Nighttime tows (early- and mid-night) were conducted using a 1-$m^2$ Reeve net (Reeve, 1981) deployed to 200 m depth, with 150 μm mesh, a 20-L cod end, and a miniSTAR-ODDI pressure and depth sensor. Daytime tows (morning and afternoon) were conducted using a 1-$m^2$ MOCNESS with 150 μm mesh and a custom-built thermally-insulated closing cod end. The thermally-insulated cod end was used because copepods were also sampled for transcriptomic and proteomic analyses that will be presented elsewhere. Because the goal of the MOCNESS sampling was to collect copepods from a single depth stratum, only one closing net was used each time to sample from 400 to 600 m depth. To obtain temperature profiles, CTD rosette casts were conducted prior to six of the net tows using a Seabird 911 CTD equipped with additional oxygen, fluorescence, turbidity and backscatter sensors. After each tow, copepods were examined under a Leica M205 C stereomicroscope to identify adult *P. xiphias* and determine their sex. Copepods were either used immediately for respirometry and excretion measurements or flash-frozen for subsequent enzyme activity

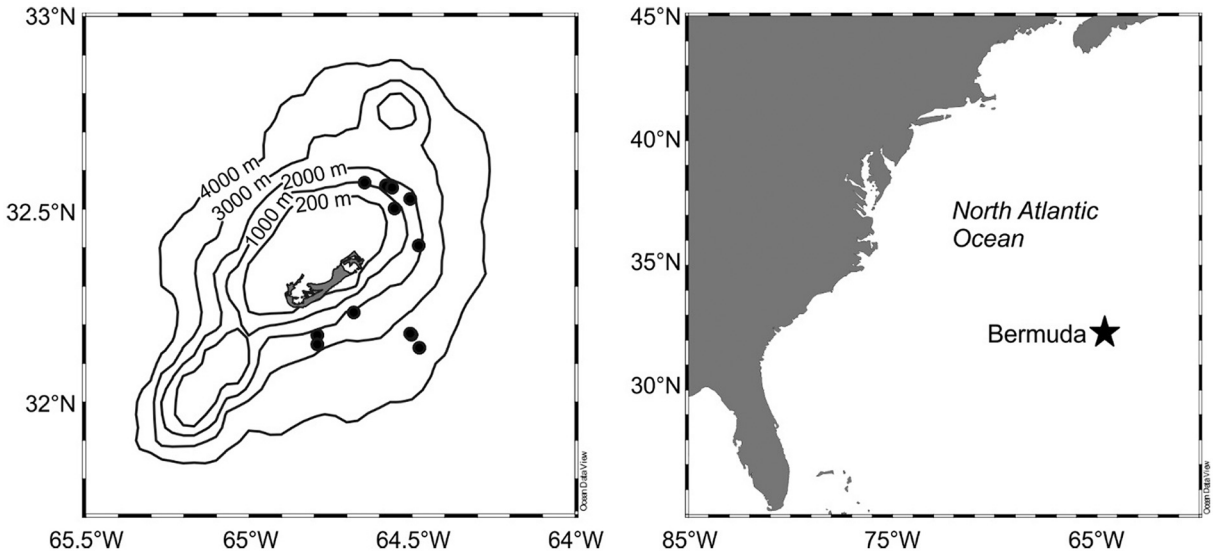


**Fig. 1.** (Left) Map showing bathymetry of the sampling region. Black circles mark tow locations. (Right) Regional map showing the position of Bermuda within the North Atlantic Ocean.

**Table 1**
Tow sampling data. All times are reported relative to solar noon (12:00).

| Timepoint[a] | Type[b] | Date | Nominal time | Tow start | Tow stop | Incubation start[c] | Lat (N) | Long (W) |
|---|---|---|---|---|---|---|---|---|
| 1; CTD | Reeve | 05/20/19 | Early night | 16:38 | 17:31 | 21:25 | 32° 10.435′ | 64° 30.018′ |
| 2 | Reeve | 05/20/19 | Mid-night | 19:50 | 21:00 | 1:15* | 32° 08.434′ | 64° 28.581′ |
| 3; CTD | MOC | 05/21/19 | Morning | 5:10 | 6:25 | 7:35 | 32° 10.689′ | 64° 30.293′ |
| 4 | MOC | 05/21/19 | Afternoon | 11:50 | 13:06 | 14:30 | 32° 24.363′ | 64° 28.749′ |
| 5 | Reeve (2 tows) | 05/21/19 | Early night | 16:44 | 17:44 | 21:45 | 32° 33.650′ | 64° 34.754′ |
| | | | | 18:07 | 19:04 | | 32° 33.707′ | 64° 34.759′ |
| 6 | Reeve | 05/22/19 | Mid-night | 21:02 | 22:00 | 1:55* | 32° 33.387′ | 64° 33.609′ |
| 7 | MOC | 05/22/19 | Morning | 6:19 | 7:38 | 8:30 | 32° 31.592′ | 64° 30.307′ |
| 8; CTD | MOC | 05/22/19 | Afternoon | 12:09 | 13:27 | 14:55 | 32° 30.127′ | 64° 33.194′ |
| 9; CTD | Reeve | 05/22/19 | Early Night | 16:43 | 17:45 | 21:35 | 32° 34.141′ | 64° 38.701′ |
| 10 | Reeve | 05/23/19 | Mid-night | 21:53 | 22:55 | 2:45* | 32° 10.416′ | 64° 47.369′ |
| 11; CTD | MOC | 05/23/19 | Morning | 5:57 | 7:15 | 8:30 | 32° 08.950′ | 64° 47.354′ |
| 12; CTD | MOC | 05/23/19 | Afternoon | 11:50 | 12:52 | 13:50 | 32° 13.948′ | 64° 40.649′ |

[a] "CTD" indicates that a tow was directly preceded by CTD profiling.
[b] Reeve net tows had a maximum depth of 159 ± 12 m (mean ± SD). MOCNESS (MOC) tows sampled from 400 to 600 m depth.
[c] Asterisk (*) indicates that time corresponds to the day following the start of tow.

measurements.

### 2.2. Organismal metabolic measurements

Water for physiological experiments was obtained daily from 120 m depth using the rosette on the CTD. It was gravity-filtered past a 0.2 μm Supor filter in a Georig 142 mm filter holder and equilibrated to 20 °C in an upright incubator. Two types of experiments were conducted. In the first, rates of oxygen consumption, ammonium excretion and fecal pellet production were measured at four time points per day (discrete incubations). In the second, ammonium, urea and DOC excretion were measured from individual copepods over time (time-course measurements).

#### 2.2.1. Discrete incubations over a daily cycle

At each time point (four per day), up to six copepods were transferred into individual respiration chambers (i.e., one animal per chamber) that consisted of 50-mL glass syringes containing an optically sensitive oxygen sensor (OXFOIL: PyroScience, Aachen Germany) and 30 mL of 0.2 μm filtered seawater. A glass bead was placed at the bottom of each syringe to avoid trapping the copepod in the small region of the syringe outlet, and all air bubbles were purged from the chamber. To control for bacterial respiration, two chambers were filled with water but were left without a copepod. Chambers were placed upright (plunger facing upward) in a dark 20 °C incubator, and the oxygen concentration in each chamber was measured non-invasively and continuously (every 60 s) for approximately 3 h using two FireSting optical oxygen meters (PyroScience, Aachen Germany), initialized with a 2-point calibration procedure (100% air-saturated water and 0% oxygen-free water by sodium sulfite reaction) on May 19, 2020. At the end of the experiment, the chambers were visually inspected to ensure that the copepods were still swimming. A 15-mL subsample of water was filtered at a 30° upward angle (to avoid damaging copepods or fecal pellets) through 0.7 μm GFF filters into 15-mL conical vials that had been pre-treated with o-phthalaldehyde (OPA) working reagent (21 mM sodium tetraborate, 0.063 mM sodium sulfite, 50 mL $L^{-1}$ o-phthalaldehyde in ethanol). This filtered water was refrigerated (4 °C) for less than 24 h and then ammonium concentration was assayed at sea, as described below. The copepod and any fecal pellets from each chamber were rinsed into a petri dish. Fecal pellets were counted and photographed under a stereomicroscope. Copepods were rinsed once in deionized water and frozen at −80 °C. Frozen copepods were subsequently weighed on a Mettler-Toledo XPR microbalance, dried, and reweighed.

Individual respiration rates were corrected for bacterial respiration by plotting the oxygen concentration (μmol $O_2$ $L^{-1}$) in each chamber

over time then subtracting the mean slope (reduction in oxygen per hour) of the controls from those of each organismal chamber to calculate net copepod respiration rates. Rates were corrected for chamber volume and copepod dry mass (mmol $O_2$ $g_{DM}^{-1}$ $h^{-1}$).

Ammonium was measured using the OPA method (Holmes et al., 1999). Each day, a standard curve (0–3 μmol $L^{-1}$) was created in duplicate, and refrigerated samples were equilibrated to room temperature. Samples and standards were then spiked with the working reagent and were maintained in the dark for 3 h prior to analysis on a Turner fluorometer with the ammonium module (1 cm path length cuvette). Ammonium concentration was calculated based on the linear equation generated by the standards and corrected for background fluorescence. This value was adjusted by chamber volume, experimental duration, and copepod dry mass (mmol $NH_4^+$ $g_{DM}^{-1}$ $h^{-1}$).

Oxygen consumption rate, ammonium excretion rate, and dry mass were measured for 60 copepods ($n = 10$–18 per time point, 40 females, 19 males, and 1 juvenile CV female). Log-transformed rates of oxygen consumption and ammonium excretion were compared across timepoints (i.e., 4 times, pooled across days) using ANCOVA with log-transformed mass as a covariate (SPSS version 22). Equality of variances was confirmed with Levene's test. Differences in fecal pellet production between timepoints were assessed using the nonparametric independent-samples median test. Significance of all analyses was assessed at $p < 0.05$.

#### 2.2.2. Time-course excretion measurements

Two experiments were conducted to measure ammonium excretion by *P. xiphias* over time. For the first, copepods were collected from repeated Reeve tows from 19:45 to 22:45, and for the second copepods were collected from a single Reeve tow at 19:45. Copepods were placed into individual pre-filled ~115-mL pre-combusted glass jars at 23:15 for both experiments, which were incubated in the dark at 20 °C. Every six hours, five experimental jars and one control jar (with the same water but no copepod) were sampled using a positive pressure system past a 0.2 μm polytetrafluoroethylene (PTFE) filter. The 10-mL samples were stored in 15-mL conical vials that were pre-treated with OPA working reagent, and ammonium concentration was measured daily in the samples, as described in Section 2.2.1. Changes in ammonium concentrations and excretion rates were analyzed via linear regression.

### 2.3. Enzyme assays

For enzyme activity measurements, copepods were thawed on ice, blotted on a lint-free tissue (Kimwipe, Kimberly-Clark, Irving TX), and quickly weighed on a Cahn C-33 microbalance. Groups of 2–5 copepods were pooled into 300 μL of ice-cold enzyme-specific buffer in a 5-mL Potter-Elvehjem homogenizer. Copepod tissue was homogenized using a motorized PTFE pestle for two 30-s bursts with 30 s of ice cooling between bursts. Homogenates were centrifuged at 14,000 *g* for 20 min at 4 °C, and the supernatant was retained. Protein concentration was measured in the supernatant using the Bradford protocol (Bradford, 1976). Except where noted, enzyme activity measurements were made with 20 μL homogenate per well in triplicate wells of a 96-well plate. Measurements were made at 26 °C using a SpectraMax plate reader. The automix function was used prior to each set of measurements.

The assay for GDH is based on the rate of oxidation of NADH (Willett and Burton, 2003). Copepods were homogenized in buffer (100 mM Tris pH 8, 50 mM $NH_4Cl$, 10 mM EDTA, 0.0025% Tween-80), as described above. Then 180 μL of GDH assay buffer (200 μM ADP, 100 μM NADH in GDH homogenization buffer, made fresh daily) was added to the homogenate. Baseline absorbance was monitored for 8 min at 340 nm to ensure depletion of endogenous substrates. To measure enzymatic activity, 20 μL of substrate (5 mM α-ketoglutarate) was added to each well, and the change in absorbance at 340 nm was recorded over 8 min.

CS activity was measured modifying the protocol of Hawkins et al. (2016). Copepods were homogenized in buffer (25 mM Tris, pH 7.8, 1 mM EDTA, 10% glycerol). 170 μL of CS assay buffer (0.11% Triton X-100, 294 μM 5,5′-dithiobis-[2-nitrobenzoic acid] [DTNB], 588 μM acetyl-coenzyme A in CS homogenization buffer, made fresh daily) was added to the homogenate. After taking baseline absorbance measurements for 3 min at 405 nm, 10 μL of 10 mM oxaloacetate was added to each well, and the change in absorbance at 405 nm was recorded over 3 min.

ETS activity was measured modifying the protocol of Owens and King (1975). Aliquots of the same homogenates from the CS assay were diluted 1:3 in ETS phosphate buffer (0.1 M, pH 8.5, $Na_2HPO_4$, $KH_2PO_4$, Triton X-100). 40 μL of each diluted sample was added in triplicate along with a fourth aliquot used as a no-substrate control, followed by 120 μL of ETS assay buffer (1.25 mM NADH and 0.22 mM NADPH in phosphate buffer, made fresh daily) to the samples or 120 μL of phosphate buffer alone to the control wells. After taking baseline absorbance measurements for 8 min at 490 nm, 40 μL of 0.2% 3-(4-iodophenyl)-2-(4-nitrophenyl)-5-phenyl-2H-tetrazol-3-ium chloride (INT), pH 8.5 was added to each well, and the change in absorbance at 490 nm was recorded over 8 min.

CS activity was determined by comparing measurements of copepod homogenates with a standard curve derived from a dilution series of a pure enzyme standard (citrate synthase from porcine heart, Sigma-Aldrich). Other enzymatic activities were calculated using the Beer-Lambert Law with extinction coefficients of 6.22 $mM^{-1}$ $cm^{-1}$ for NADH (GDH assay, reported by Sigma-Aldrich, the supplier) and 15.9 $mM^{-1}$ $cm^{-1}$ for INT (ETS assay, as in Owens and King, 1975). The enzyme activity measurements were not conducted on the same copepods used in individual measurements of oxygen consumption and ammonium excretion, so the values do not represent calibrated activity rates.

Enzyme activity measurements were separately normalized to dry mass and to protein. Dry mass was calculated as 0.0513 times wet mass, based on the average ratio from measurements in the respirometry experiments ($n = 61$). Physiological and enzymatic measurements were log10-transformed prior to analysis. Differences in enzymatic activity of pooled copepod samples were analyzed using a one-way ANOVA (using the oneway.test function in R, which does not assume equal variances) with a Games-Howell post-hoc test to identify significantly different groups ($p < 0.05$).

## 3. Results

### 3.1. Site characteristics and sample description

Throughout the sampling period, a deep chlorophyll maximum (DCM) consistently occurred around 130–160 m depth; thus, nighttime sampling (to 200 m depth) would have included copepods feeding within this region (Fig. 2). Temperature was 23–24 °C at the surface, 20 °C at the DCM, and 17–18 °C at 500 m depth. Oxygen levels decreased with depth, but were always above 160 μmol $kg^{-1}$ at the depths sampled. The exact timing of the tows and incubations varied from day to day (Table 1). Timepoints were clustered into four groups sampled at the same depth range and similar times of tow recovery (R) and incubation start (I): morning (R: 6:25–7:38; I: 7:35–8:30), afternoon (R: 12:52–13:27; I: 13:50–14:55), early night (R 17:31–19:04; I: 21:25–21:45), mid-night (21:00–22:55; I: 1:15–2:45). The delay between each tow recovery and the start of the corresponding incubation primarily represented the time required to identify sufficient adult females for all study objectives.

### 3.2. Organismal metabolic measurements

There was no significant difference among time points in the mass-normalized rates of oxygen consumption or ammonium excretion (Fig. 3A-B, oxygen consumption $F(55,3) = 0.242$, $p = 0.867$; ammonium excretion $F(55,3) = 0.545$, $p = 0.653$). Rates did not vary by sex, and the

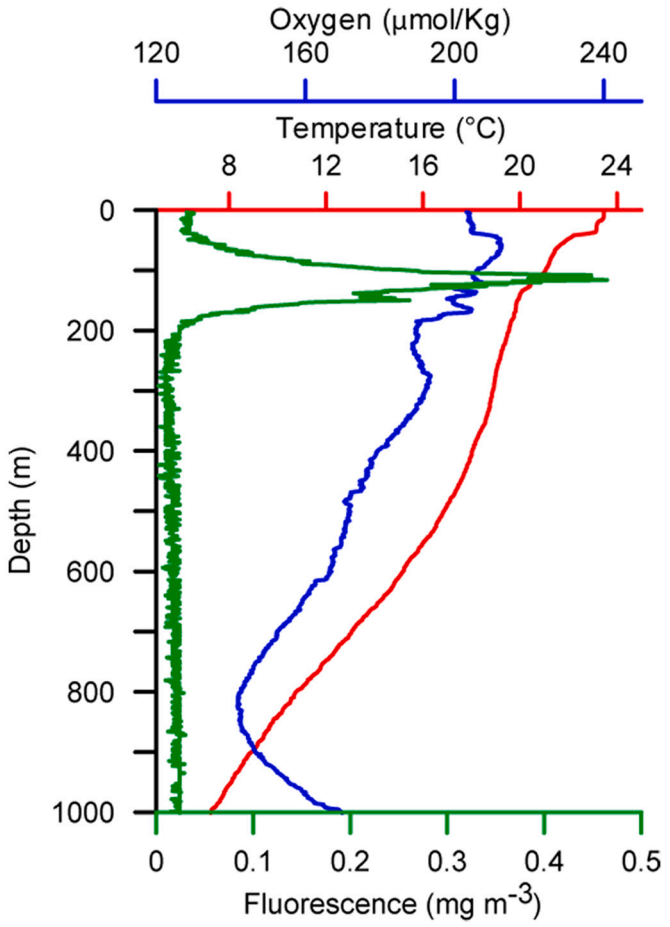


**Fig. 2.** Plots of hydrographic conditions on 20 May 2021 at 32° 10.435 N, 64° 30.018 W (i.e., prior to Tow 1, Table 1). Additional hydrographic data are available at https://www.bco-dmo.org/project/764114.

interpretation was not changed by limiting the analysis to adult females (not shown). Fecal pellet production varied significantly over the course of the day with maximum values at mid-night that were significantly higher than afternoon or early night (Fig. 3C, $X^2(3, N = 64) = 20.426$, $p < 0.001$). The fecal pellets produced at mid-night appeared densely packed and were brown or green in color. Some of the fecal pellets produced during daytime were similar in appearance; however, others were light-colored and loosely packed (Fig. 4).

In the time-course studies, ammonium concentrations increased over time, indicating some continued excretion over the 18-h incubation (Fig. 5A). There was not a statistically significant change in the integrated rate of excretion measured at each sampling point (Fig. 5B), but mean ammonium excretion rates decreased with each sampling interval (Fig. 5C).

### 3.3. Enzyme activity measurements

Patterns of enzyme activity were broadly similar whether the data were normalized to dry mass (Fig. 6) or protein concentration (Supplemental Fig. 1); however, the statistical significance varied with the method of normalization (Table 2). ETS and CS both exhibited significant differences in activity over time when normalized to mass, though the patterns were different (Fig. 6A and B, respectively). Within the deep water, ETS activity was higher during the afternoon than during the morning; this was contrary to our hypothesis that rates would be highest during morning. CS was highest at night, with significant differences between early night and both the daytime points. GDH activity also tended to be higher at night, with a significant increase between afternoon and early night when normalized to protein (Table 2, Supplemental Fig. 1). Mean GDH activity at night was approximately twice the afternoon activity at depth, and this difference would be magnified in the field due to differences in temperature with depth.

## 4. Discussion

Over a three-day period, *P. xiphias* copepods were sampled from depths that corresponded to their typical DVM. Fecal pellet production and ammonium excretion during daytime at depth were consistent with both active transport and some degree of midwater feeding. Daily patterns in enzymatic activity suggest that the copepods respond to or anticipate differences in food availability, temperature and/or other environmental conditions over the course of the migratory cycle.

We did not observe significant variation in oxygen consumption rate among discrete measurements of respiration. The failure to detect a diel rhythm in oxygen consumption in wild-caught animals was initially surprising, given previous reports of circadian rhythms in copepod respiration (Häfker et al., 2017; Maas et al., 2018); however, the present study was very different in design and goals than the previous circadian studies. The previous circadian studies consisted of continuous longitudinal measurements of oxygen utilization, whereas the present ecophysiological study consisted of independent samples collected directly from the field. Thus, rates measured in the current study reflect the combined influences of circadian patterns in physiology, as well as the variable feeding and swimming history of the individuals over their vertical migration.

Unexpectedly high inter-individual variability among samples in the

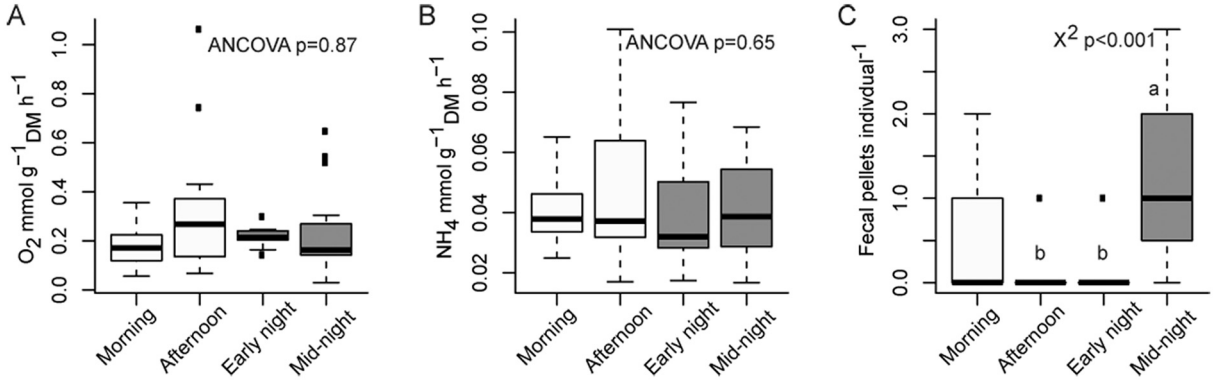


**Fig. 3.** *Pleuromamma xiphias* physiological rates. Distinct letters indicate statistically distinct time points. Open boxes denote samples collected from depth (400–600 m) during daytime, and solid boxes indicate samples collected from surface waters (<200 m) during night. Oxygen and ammonium rates per gram dry mass (DM) per hour. (A) Oxygen consumption, $N = 10–19$; (B) Ammonium excretion, N = 10–18; (C) Fecal pellet production (per 3-h incubation period), $N = 13–19$.

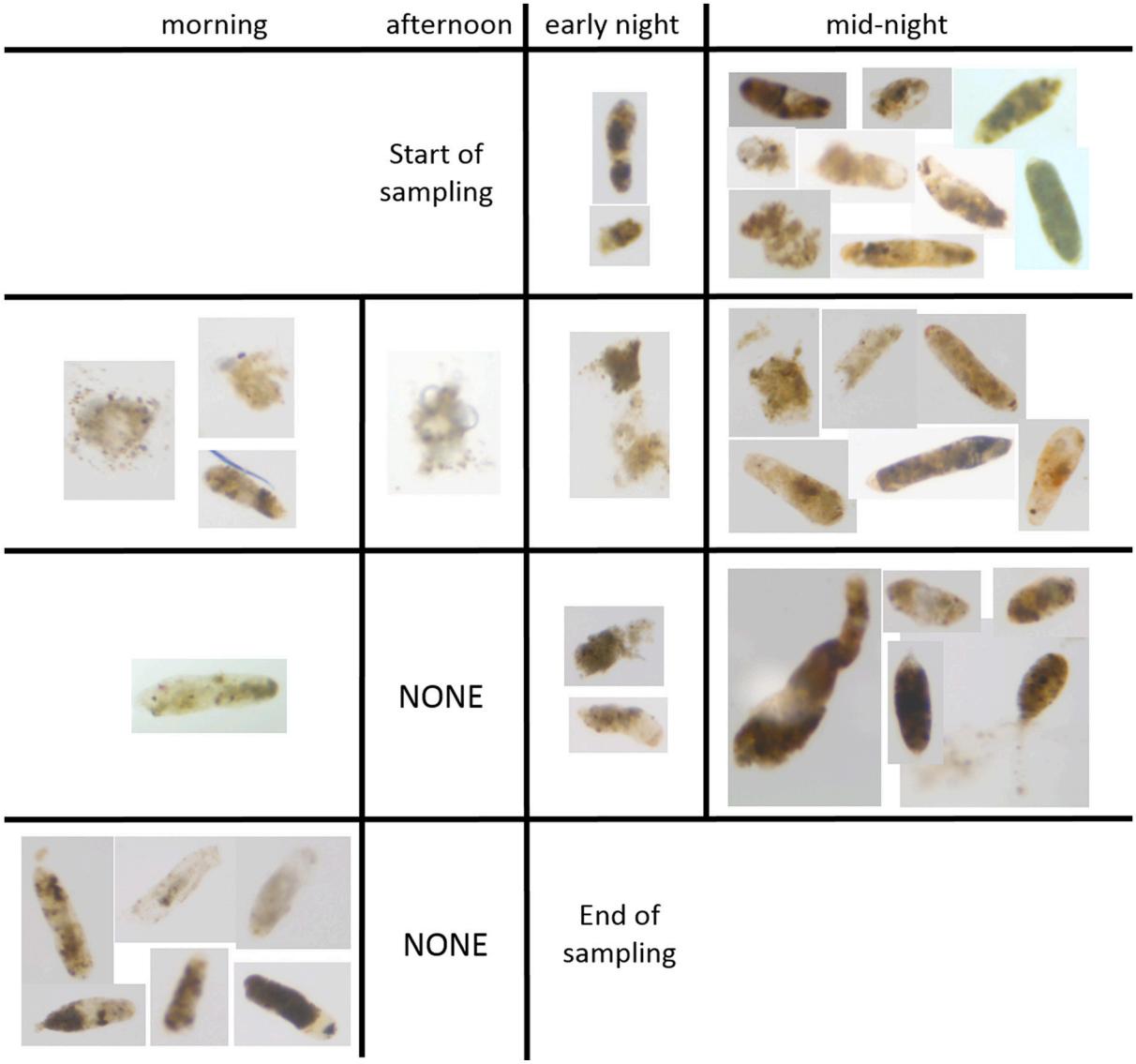


**Fig. 4.** Examples of fecal pellets produced by *Pleuromamma xiphias* during three-hour incubations over a three-day period, with sequential sampling points beginning in the upper left and proceeding to the right and down over time.

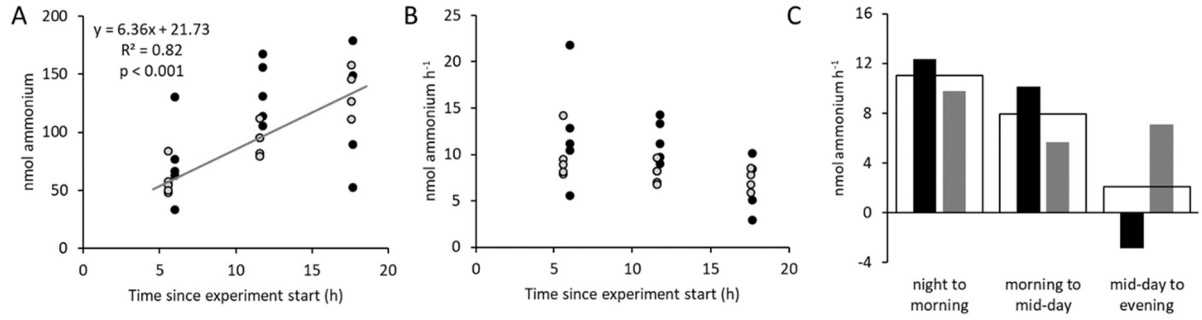


**Fig. 5.** Time series of *Pleuromamma xiphias* ammonium production from individual copepods that had been captured between 19:45 and 22:45 on May 20th (solid symbols) or between 19:45 and 20:45 on May 22nd (open symbols). Both excretion experiments were set up at ~23:15 and results are reported as time since the start of the experiment (x-axis) with (A) total production; (B) production integrated over time on the y-axis; (C) production per 6-h sampling window calculated by excluding the average production of the prior time points to demonstrate the excretion rate over the duration of the experiment in relation to solar time. White bars show the average production per time point.

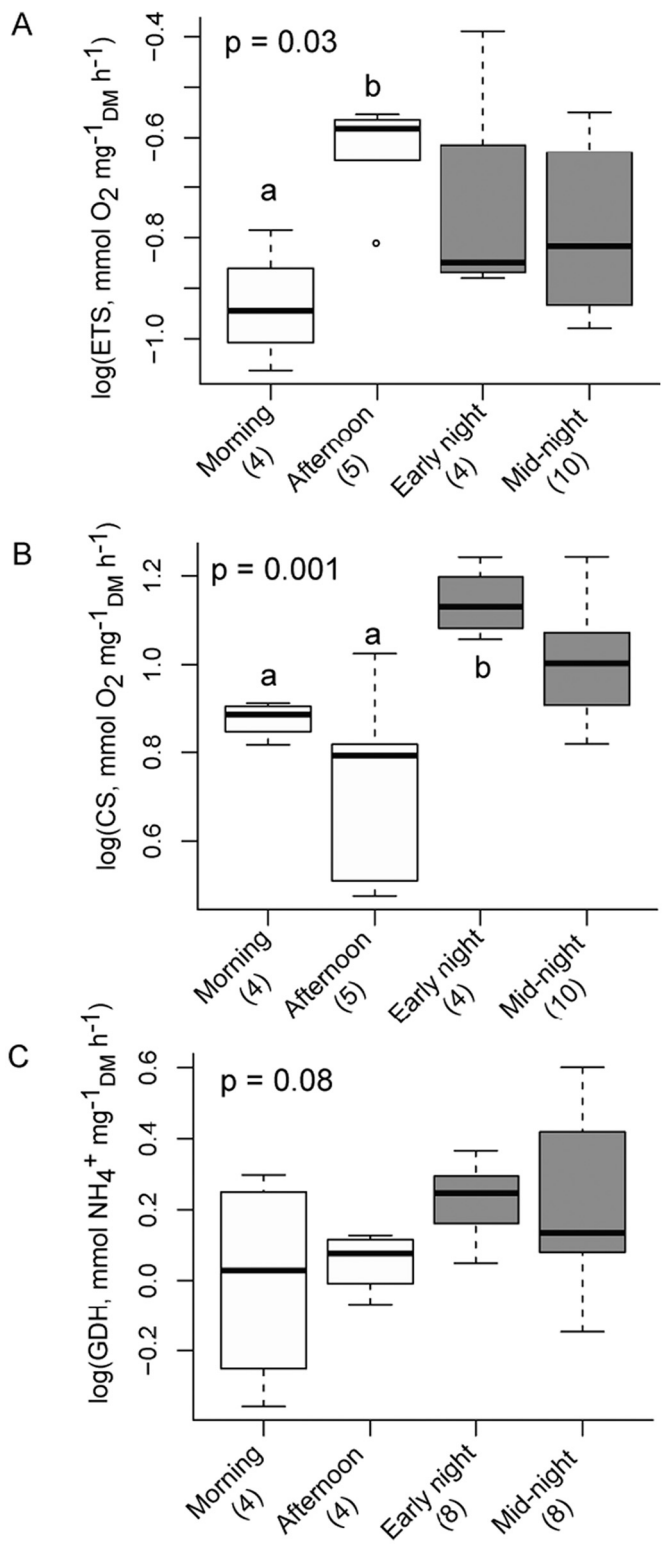


**Fig. 6.** Log-transformed enzymatic activity of electron transport system (ETS; A), citrate synthase (CS, B) and glutamate dehydrogenase (GDH, C). Activity normalized to dry mass (DM). Time of sampling and sample size indicated on the x-axis. Open boxes indicate samples collected from depth (400–600 m) during daytime, and shaded boxes indicate samples collected from surface waters (<200 m) during night. Letters indicate statistically distinct time points.

**Table 2**
One-way Welch's ANOVA and significant post-hoc (Games-Howell) results from enzyme activity assays.

| Assay | Dry mass-normalized activity | Protein-normalized activity |
|---|---|---|
| ETS | $F(3, 7.94) = 4.83$; $p = 0.03$<br>Morning vs. Afternoon $p = 0.02$ | $F(3, 6.88) = 2.75$; $p = 0.12$ |
| CS | $F(3,8.59) = 12.09$; $p < 0.01$<br>Morning vs. Early Night $p = 0.01$<br>Afternoon vs. Early Night $p = 0.04$ | $F(3,7.80) = 3.36$; $p = 0.08$ |
| GDH | $F(3,8.35) = 3.10$; $p = 0.09$ | $F(3, 4.35) = 9.19$; $p = 0.02$<br>Afternoon vs. Early Night $p < 0.01$ |

current study may have prevented detection of a diel cycle in oxygen consumption rate. To provide context, the amplitude of the circadian cycle we previously reported in *P. xiphias* oxygen consumption rates (initially 173 μmol $g^{-1}_{DM}$ $h^{-1}$ with rapid dampening; Maas et al., 2018) is similar in magnitude to the difference (non-significant) in means between morning and afternoon observed in the present study (128 μmol $g^{-1}_{DM}$ $h^{-1}$). To detect a difference of this magnitude (128–173 μmol $g^{-1}_{DM}$ $h^{-1}$) given the observed variability of the field-collected samples (mean standard deviation of time points 140 μmol $g^{-1}_{DM}$ $h^{-1}$) we approximate a required sample size of 21–29 ($\alpha = 0.05$, $\beta = 0.80$; based on ANOVA of mass-normalized samples with 6 pairwise comparisons, Chow et al., 2007), a substantial increase from our actual sample size of 10–18 per time point. Our results also contrast previous observations by Pavlova (1994), who conducted endpoint measurements that were similar to those used in the present study, and who observed greatly increased respiration rates by *P. xiphias* around dawn and dusk. However, the oxygen consumption rates measured at dawn and dusk by Pavlova (1994) are an order of magnitude greater than any respiration rates observed for *P. xiphias* adults in several subsequent studies (Maas et al., 2018; Steinberg et al., 2000; Teuber et al., 2013). While we cannot provide a definitive explanation for this discrepancy, possible explanations could be unique physiology of the populations sampled by Pavlova (e.g., sampling was done from individuals captured at 5 m depth in the Indian Ocean, where there is a strong oxygen minimum zone), a failure of the present study to make measurements during ephemeral periods of peak respiration, or methodological artifacts (e.g., differences in handling stress).

Fecal pellet production rates were highest during mid-night, but there was also some evidence of daytime feeding with occasional pellets even in the afternoon. Our results suggest a 50% decrease in fecal pellet production rate when comparing mid-night (0–200 m depth) and early morning (400–600 m depth). This is consistent with the estimates made by Schnetzer and Steinberg (2002b) who estimated 57% production of surface-derived fecal pellets by *P. xiphias* at 300 m depth using gut evacuation rate experiments and migration speed. *P. xiphias* has been demonstrated to commonly have >50% of their gut contents consisting of material suspected to be of detrital origin (Schnetzer and Steinberg, 2002a), which might imply that substantial feeding activity could occur continuously throughout the water column and over the full diel cycle. However, the observed reduction in fecal pellet production at depth during the day suggests that midwater feeding is substantially less than surface nighttime consumption. Although validation of the origin of these pellets would require isotopic, microscopic or molecular analysis of their content, the similarity in the morphology of many of the mid-night (surface) and early morning (deep) fecal pellets suggests instead that at least some of these pellets are derived from surface feeding. These would then contribute to particulate organic carbon active flux, as predicted by Schnetzer and Steinberg (2002b).

Ammonium excretion rates dropped over an eighteen-hour incubation (time-course measurements) but exhibited no variation over the course of the day (discrete incubations). The decreasing rate in the time-course measurements likely reflects reduced excretion as food in the gut is cleared, suggesting that feeding only at the surface during night would not be sufficient to sustain continued excretion over the full daily cycle.

Thus, when taken in conjunction with the time series results, the lack in diel variation is intriguing and would be consistent with continued feeding at depth to support continued ammonium excretion in the field-caught samples.

While we did not detect diel changes in organismal-level measurements of oxygen consumption and ammonium excretion rates, there were diel changes in the activity levels of the three metabolic enzymes measured. This discrepancy could reflect the interplay between enzyme activity measurements and substrate availability that resulted in consistent metabolism despite variations in enzymatic capacity. Alternatively, the enzymatic measurements, which were made on rapidly flash-frozen individuals, may have been less impacted by the effects of handling or captivity that were associated with the experimental incubations needed to measure organismal rates.

ETS activity is generally considered to reflect the value of oxygen consumption if all enzymes in the electron transport chain were functioning at maximum activity, whereas in vivo respiration rate may be constrained by substrate limitation or the presence of inhibitors. While oxygen consumption rates and ETS activity are often concordant (e.g., Bidigare, 1983; Maldonado et al., 2012; Packard, 1985), they can respond differently to changes in food availability, temperature and other factors (Hernández-León and Gómez, 1996; Osma et al., 2016). Unlike respiration rate, which was consistent throughout the diel cycle, ETS activity in our study was significantly higher in the afternoon than in the morning. The increase in ETS activity between morning and afternoon is puzzling, as our previous work with this species demonstrated a circadian peak in oxygen consumption rates in the morning (6–12 h) and the lowest respiration rate in the early evening (18–24 h) under constant conditions in the laboratory. Multiple additional environmental factors could be influencing the in situ ETS activity, but the patterns cannot be clearly explained by predicted changes in swimming activity or metabolic changes due to specific dynamic action associated with food processing. Experimental studies with the copepod *Acartia tonsa* demonstrated that copepods maintained high rates of metabolism for about 8 h after feeding (Kiørboe et al., 1985). While this timing may be expected to vary among copepod species, a metabolic pattern driven solely by feeding activity and postprandial metabolic processes would be expected to have the lowest rates during the afternoon sampling period. Alternatively, the higher ETS capacity may be a strategy used to offset the $Q_{10}$ temperature effect, allowing for sustained aerobic metabolism despite lower midwater temperatures. While such a compensatory effect is consistent with elevated ETS activity observed during afternoon, it does not explain the low expression in the morning period. A third possibility is that the copepods are upregulating their metabolic capacity in preparation for their nighttime ascent. Analogous anticipatory rhythms have been characterized in model organisms, such as mammalian food anticipatory behavior (increased actiivity 1–3 h before meal time; reviewed by Silver et al., 2011) and anticipatory upregulation of catabolic liver enzymes (Díaz-Muñoz et al., 2000).

When comparing studies of diel metabolism of migratory zooplankton, there is a consistent disconnect between observed patterns in respiratory peaks, which are often coincident with sunrise and sunset (Häfker et al., 2017; Maas et al., 2018; Pavlova, 1994), and the period of highest ETS expression. Although the precise peak in ETS activity differs among the migrating species that have been examined, it is consistently during the daytime portion of the diel cycle. For example, a study of euphausiid physiology in an area with a pronounced oxygen minimum zone found peak ETS activity levels during morning in animals caught at depth (400 m depth; Herrera et al., 2019). A pattern more similar to that detected in our study was observed in a laboratory-based study of krill by Biscontin et al. (2019), who report peak late-afternoon expression of genes associated with the electron transport chain and Krebs cycle. The discordance between ETS and respiration measurements and the variation in peak timing observed in studies conducted in different species and ecosystems together suggest a need for greater coordinated study, particularly since ETS measurements are used as a proxy for respiration in biogeochemical studies (Belcher et al., 2020; Hernández-León et al., 2019a; Hernández-León et al., 2019c; Packard and Gómez, 2013).

Alternate enzymatic proxies for respiration include individual enzymes within the citric acid cycle, the reactions which provide high-energy electrons to the ETS. Of these, citrate synthase (CS) is the first enzyme of the citric acid cycle that performs the irreversible condensation of acetyl-CoA with oxaloacetic acid to create citrate. Unlike the ETS machinery, the activity of CS in our study was elevated during nighttime, when *P. xiphias* is expected to be feeding most actively. High rates of nighttime feeding are consistent with observed increases in fecal pellet production during the mid-night period. CS has been used to indicate aerobic metabolic potential but does not always correlate well with oxygen consumption rates in invertebrates (Thuesen et al., 1998 and references therein). In copepods, previous studies have correlated CS activity with food availability on multiday timescales (e.g., 2–3 day lab incubations, Clarke and Walsh, 1993; pre−/post-bloom Geiger et al., 2001). Daily patterns in CS (i.e., cycles *within* days) have not been previously described in copepods, but are well-documented in mammalian tissues through measurements of both transcript expression and enzymatic activity (Crumbley et al., 2012; Glatz et al., 1984). Among other zooplankton, both circadian and ultradian patterns of CS expression have been observed in krill (Biscontin et al., 2019; De Pittà et al., 2013; Teschke et al., 2011). Meyer et al. (2010) suggested that malate dehydrogenase (MDH), another enzyme in the citric acid cycle that additionally shunts electrons between cytosolic and mitochondrial compartments, might better correspond to oxygen consumption rates. Diel patterns in copepod MDH expression have not yet been investigated and would be useful to include in future studies; however, seasonal studies in both copepods and euphausiids have indicated a general correspondence of both CS and MDH activity with oxygen consumption rates (Freese et al., 2017; Meyer et al., 2010).

GDH, which mediates the production of ammonium waste during amino acid catabolism and is associated with the urea cycle, exhibited peak activity at night. This contrasts with direct measurements of ammonium excretion, which showed no pattern. A possible explanation for the discrepancy between ammonium and GDH measurements could be artifacts in the ammonium measurements due to stress of capture, handling, captivity (small chamber size) and acclimation to starved conditions (Ikeda et al., 2000; Kodama et al., 2015). In addition, while GDH activity is used as a proxy for potential excretion rate, actual excretion rate may be limited by substrate availability (Fernández-Urruzola et al., 2016). We are not aware of any other studies tracking GDH activity during DVM. Bidigare (1983) measured depth-stratified GDH activity of bulk zooplankton communities within the upper 200 m depth in the Gulf of Mexico. The highest activities occurred within the mixed layer and were largely driven by zooplankton abundance. Protein-specific activity was only reported during the daytime and was highest in the upper 100 m. Within the first 24-h of laboratory incubations with starved mysids, Fernández-Urruzola et al. (2011) found an initial increase in ammonium excretion, followed by a sharp decrease; however, they observed high variability in GDH activity within time points and no consistent temporal patterns. The design of the mysid study was quite different from the present study in that a single cohort of animals was brought into the laboratory and sampled over time. Despite these differences, it supports the idea that zooplankton excretion rates could change on a daily scale with feeding activity.

To date, the handful of previous studies that have characterized aspects of circadian metabolism in zooplankton have been conducted using a limited range of taxa, primarily copepods (Häfker et al., 2017; Maas et al., 2018; Pavlova, 1994) and krill (Biscontin et al., 2019; De Pittà et al., 2013; Teschke et al., 2011). Further, most of this work has been conducted in polar or sub-polar environments. Consequently, assessing diel rhythms in multiple species across a range of environments will be important as we seek to better understand the cycling of nutrients in the euphotic and twilight zone. The studies cited above used

a combination of methods including organismal physiology (e.g., oxygen consumption, ammonium excretion), transcriptomics, proteomics, and enzyme activity assays. Each of these approaches can provide insight into the physiological ecology of zooplankton and zooplankton contributions to biogeochemical cycling; however, these types of measurements are subject to different sources of error and may indicate variability over different time scales. Moving forward, disentangling which factors these measurements are responding to (e.g., light, temperature, oxygen concentration, food availability, endogenous circadian rhythms) will help us to better apply these tools to quantify zooplankton contributions to biogeochemical cycles.

## 5. Conclusions

This study demonstrates that the copepod *P. xiphias* exhibits variation in fecal pellet production and activity of metabolic enzymes as it undergoes DVM, supporting our hypothesis that circadian rhythms and other environmental factors beyond temperature create emergent patterns in zooplankton physiology. Observed production of fecal pellets in deep water during morning indicates that surface feeding by migratory copepods contributes to active flux of particulate organic matter. Occasional late afternoon fecal pellets and sustained levels of ammonium excretion by copepods sampled during daytime suggests continued, although reduced, levels of midwater feeding. Despite the diel patterns in enzyme activity, there were no statistically significant variations in oxygen consumption or ammonium excretion over the diel cycle. Studying diel rhythmicity in physiological rates (respiration, ammonium excretion) remains difficult in field-caught organisms, yet further studies are needed both in *P. xiphias* and in other migratory species. Enzyme activity assays can complement direct physiological measurements, and the observed daily variation in metabolic enzymes indicates that copepods adjust their metabolic capacity in response to or perhaps in anticipation of variation in environmental conditions and metabolic demands. However, measurements of enzymatic activity indicate peaks in metabolic potential that may lead or lag actual cycles in physiological rates; this decoupling can contribute to uncertainty in applying enzymatic measurements to estimate zooplankton contributions to respiration and ammonium production.

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## Data archiving

CTD profiles and physiological data are available through the Biological and Chemical Oceanography Data Management Office (BCO-DMO); https://www.bco-dmo.org/project/764114.

## 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.

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