

SHORT COMMUNICATION

Tide-related biological rhythm in the oxygen consumption rate of ghost shrimp (*Neotrypaea uncinata*)

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ABSTRACT

The effects of tidal height (high and low), acclimation to laboratory conditions (days in captivity) and oxygen level (hypoxia and normoxia) were evaluated in the oxygen consumption rate (OCR) of the ghost shrimp *Neotrypaea uncinata*. We evaluated the hypothesis that *N. uncinata* reduces its OCR during low tide and increases it during high tide, regardless of oxygen level or acclimation. Additionally, the existence of an endogenous rhythm in OCR was explored, and we examined whether it synchronized with tidal, diurnal or semidiurnal cycles. Unexpectedly, high OCRs were observed at low tide, during normoxia, in non-acclimated animals. Results from a second, longer experiment under normoxic conditions suggested the presence of a tide-related metabolic rhythm, a response pattern not yet demonstrated for a burrowing decapod. Although rhythms persisted for only 2 days after capture, their period of 12.8 h closely matched the semidiurnal tidal cycle that ghost shrimp confront inside their burrows.

KEY WORDS: Biological clock, Synchrony, Metabolism, Tidal cycle, Acclimation, Hypoxia

INTRODUCTION

The intertidal zone is one of the harshest habitats on Earth, presenting unique features determined by interactions between terrestrial, aerial and marine environments. These interactions are driven by the earth's geophysical cycles, including the tidal cycle (12.4 h), moon day (24.8 h), solar day (24 h), fortnight (14 days), lunar month (29.5 days) and year duration (365.25 days) (Palmer, 1995). To a certain degree, most organisms synchronize their behavior and physiology with these cycles (Dunlap et al., 2004). Such synchronization is, in turn, driven by endogenous cycles that can oscillate autonomously and sustain specific physiological and behavioral rhythmic outputs. The molecular and physiological components of endogenous oscillators act as a kind of biological clock, which can be revealed experimentally when the environmental stimulus or 'Zeitgeber' disappears but the biological rhythm persists (de la Iglesia and Hsu, 2010).

Although synchrony and biological clocks are poorly understood, they help animals reorganize their physiology. For example, *Mytilus californianus* exhibits noticeable metabolic rhythms during simulated tidal cycles (Connor and Gracey, 2012). In other

invertebrates, such as decapod crustaceans, several reports show the existence of biological rhythms in locomotory activity and oxygen consumption (Naylor, 2010; Palmer, 1995). Nonetheless, no published studies have evaluated the existence of synchrony between the tidal cycle and physiological responses in burrowing crustaceans, inhabitants of the intertidal zone, where they face drastic but highly regular and predictable changes in temperature, salinity, partial pressure of carbon dioxide, sulfide, humidity and partial pressure of oxygen (P_{O_2}) (Atkinson and Taylor, 2005). P_{O_2} is probably one of the most relevant of these environmental factors, as the water in intertidal burrows may become hypoxic or even anoxic during low tide (Torres et al., 1977).

A recent study of the burrowing shrimp *Neotrypaea uncinata* (Milne Edwards 1837) showed that it lowered its metabolic rate under hypoxia (1 kPa). When returned to normoxic conditions (21 kPa), it recovered rapidly, showing normal concentrations of hemolympathic metabolites before the end of a tidal cycle (Leiva et al., 2015). In the present study, we evaluate the hypothesis that *N. uncinata* reduces its oxygen consumption rate (OCR) during low tide and increases it during high tide, regardless of oxygen level or acclimation. Additionally, we examined whether metabolic rhythms in *N. uncinata* were endogenous and whether they were synchronized with tidal, diurnal and semidiurnal cycles.

MATERIALS AND METHODS

Animal collection and maintenance

Intermoult adult males of *N. uncinata* were collected during low tide from the intertidal zone of Tenglo Island, near Puerto Montt, Chile. Individuals were captured and transported to the Laboratory of Crustacean Ecophysiology at the Universidad Austral de Chile, where they were acclimated following procedures similar to those described by Leiva et al. (2015). After 3 weeks, a new batch of animals was collected from the same site to compare their oxygen consumption with that of animals acclimated to the laboratory.

Experimental protocol

All experiments were conducted using pre-filtered (1 μ m) and UV-sterilized seawater pumped from the subtidal zone at 0.5 km distance off shore. Salinity (32 \pm 0.3) and seawater temperature (11.3 \pm 0.8°C) remained relatively stable during acclimation and experiments.

Series 1

OCR measurements were obtained from a total of 24 individuals. Twelve of these were collected from the wild and measurements were taken on the same day [days *ex situ*=1; wet mass (WM)=13.98 \pm 2.85 g; cephalothorax length (CL)=19.80 \pm 1.14 mm, mean \pm s.d.]; the other 12 had been previously acclimated to laboratory conditions during a period of 3 weeks (days *ex situ*=21; WM=14.47 \pm 3.21 g; CL=20 \pm 1.28 mm). During acclimation, animals were fed with krill flakes and maintained in individual refuges (diameter=7.5 cm; height=20 cm) under a photoperiod of 12 h:12 h (light:dark) with

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List of symbols and abbreviations

CL	cephalothorax length
DM	dry mass
H	tide height
OCR	oxygen consumption rate
P_{O_2}	partial pressure of oxygen
RA	relative amplitude
t	a rational quantity representing time (0–24 h)
T	period
WM	wet mass
γ	time delay

the same conditions of salinity and temperature mentioned above. A subset of six individuals from each acclimation group was allocated to one out of two experimental P_{O_2} conditions: hypoxia or normoxia, with nominal values of 1 and 21 kPa, respectively. Hypoxic water was prepared by bubbling N_2 into a 60 liter seawater tank, then shutting off the N_2 bubbles and leaving it to equilibrate. Oxygen concentration in this reservoir was checked after 30 min using a needle-type oxygen optic fiber connected to a Microx TX3 AOT (PreSens, Germany) oxygen meter. Then, 1 liter closed respirometry chambers were filled with either hypoxic or normoxic water and placed in a temperature-controlled bath ($11.5 \pm 0.5^\circ\text{C}$). After measuring the actual O_2 concentration at each chamber, experimental individuals were transferred to each one and left to acclimate for approximately 1 h. After a full water replacement for each treatment, initial and final O_2 concentrations were measured again, 1 h before and 1 h after the time the highest tide level had been reached at the collection site. Then, both groups of individuals were maintained under normoxic conditions, in the same respirometry chambers, waiting for the time at which the lowest tide had been reached at the collection site. Approximately 2 h before this time, a second OCR measurement was taken, applying the same two experimental P_{O_2} conditions to the same animals that received them during the time of the highest tide and following the same general procedures described before.

Five chambers without animals (blanks) for each oxygen level were used to estimate and correct for potential bacterial OCR. Initial and final O_2 concentrations were measured using a needle-type oxygen optic fiber introduced through a hermetic septum, and connected to a Microx TX3 AOT (PreSens) oxygen meter. The optic fiber was calibrated prior to the start the experiments, using a two-point calibration in water (0% and 100% air saturation). OCR was normalized to 1 g of dry tissue mass (DM), computed from a published WM–DM curve (Leiva et al., 2015), and expressed as $\mu\text{mol } O_2 \text{ h}^{-1} \text{ g}^{-1}$.

Series 2

On the same day of collection, nine individuals of similar WM ($14.29 \pm 1.11 \text{ g}$) and CL ($19.93 \pm 0.44 \text{ mm}$) were selected and then used to obtain five repeated weekly OCR measurements on each individual, during five consecutive weeks. Each week, semi-continuous OCR measurements were obtained every 40 min under normoxic conditions (21 kPa), lasting for two tidal cycles ($\sim 26 \text{ h}$), and yielding a total of ~ 1000 OCR measurements after 5 weeks. Normoxic water was supplied by gravity from a 60 liter head tank and directed through silicone tubes to the chambers continuously supplied with pre-filtered ($1 \mu\text{m}$) and UV-sterilized seawater. Solenoid valves were fitted to each outlet tube and used to fill/renew the water in the respirometry chambers and/or to conduct incubated water to a Flow-Through Cell Housed Oxygen Microsensor connected to a Microx TX3 AOT (PreSens) oxygen meter. Oxygen sensors were calibrated periodically, prior to each weekly OCR measurement and under the same protocol used for Series 1. For initial measurements, each solenoid valve was opened for 15 s, just to flush and fill the oxygen sensor. New oxygen measurements were then taken every 40 min until the end of the experiment. For all subsequent measurements, solenoid valves were opened to re-establish normoxic conditions inside each respirometry chamber, at a flow rate of 30 ml s^{-1} . Weekly measurements normally started in the morning, and were conducted in a temperature (12°C)- and light-controlled room. Between weekly measurements, animals were maintained under conditions similar to those described above for Series 1.

Statistical analyses**Series 1**

Given the nature of our data, the effects of P_{O_2} , acclimation and tidal height upon OCR were assessed using a linear mixed-effects model approach (Pinheiro and Bates, 2000), aimed to account for lack of independence of repeated measures within individuals, which were considered as random effects. All fixed effects were treated as categorical variables, i.e. tide=high/low, P_{O_2} =normoxia/hypoxia, acclimation=yes/no. After fitting the model by maximum likelihood (Bates et al., 2012), hypothesis testing was performed using a three-way ANOVA, followed by a Tukey pairwise comparison among all treatment combinations.

Series 2

We also used a mixed-effects model approach, oriented to account for temporal autocorrelation, as well as lack of independence among repeated OCR measures within individuals. Thus, errors were assumed to follow an auto-regressive structure of order one, which

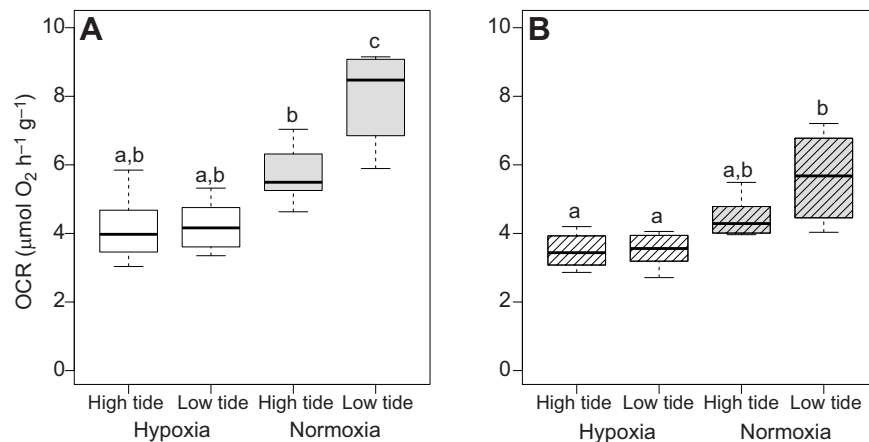


Fig. 1. Effect of the oxygen level, tidal height and acclimation days on the *Neotrypaea uncinata* oxygen consumption rate (OCR). Measurements of OCR obtained of animals exposed to hypoxia (1 kPa) and normoxia (21 kPa) during high/low tide events of the collection site after (A) 1 day and (B) 21 days *ex situ*. Boxes show median (horizontal line) ± 1.5 times the interquartile range (whiskers). Boxes with the same shading and pattern indicate that the same groups of animals were used in the treatments after exposure to high and subsequent low tide ($n=6$ per group). Different lowercase letters indicate significant differences in the mean values among all treatment combinations according to Tukey's test on linear mixed-effects models.

Table 1. Summary outputs of linear mixed-effects and nonlinear regression models for marginal data

Model	k	$\log(L)$	AIC
Null (M_0)	4	-2013	4035
Tidal cycle (M_1)	13	-2009	4045
Tidal cycle lagged (M_2)	12	-1582	3189
Discrete tidal cycle (M_3)	13	-1986	3998
Diurnal cycle (M_4)	13	-2005	4037
Semidiurnal cycle (M_5)	13	-2011	4049
Endogenous cycle (M_6)	12	-1581	3187

k , number of parameters; $\log(L)$, model log likelihood; AIC, Akaike's information criterion. Bold indicates models that were more informative than the null model.

yielded the most informative model [lowest Akaike's information criterion (AIC); Akaike, 1973] among several tested models. A null and six alternative hypotheses were considered and expressed as the following linear and non-linear mixed models, where α and β represent model parameters, H is the tide height (m), t is a rational quantity representing time (0–24 h) and γ represents a time delay in hours.

M_1 : linear, in-phase relationship between OCR and tide height (m) at the collection site:

$$\text{OCR}_t = \beta_0 + \beta_1 H_t, \quad (1)$$

M_2 : non-linear, lagged relationship between OCR and tide height (m) at the collection site:

$$\text{OCR}_t = \beta_0 + \beta_1 (H_{t-\gamma}), \quad (2)$$

M_3 : linear relationship between OCR and tide height category (low/high) at the collection site:

$$\text{OCR}_t = \beta_0 + \beta_1 (H_{t,0/1}), \quad (3)$$

M_4 : linear, in-phase relationship between OCR and diurnal cycle:

$$\text{OCR}_t = \beta_0 + \beta_1 \sin(2\pi t - \pi/2), \quad (4)$$

M_5 : linear, in-phase relationship between OCR and semidiurnal cycle:

$$\text{OCR}_t = \beta_0 + \beta_1 \sin(4\pi t - \pi/2), \quad (5)$$

M_6 : endogenous sinusoidal OCR cycle, defined as:

$$\text{OCR}_t = \alpha \sin(2\beta\pi t - \pi/2). \quad (6)$$

Normality and homogeneity of variance assumptions were tested and fulfilled by all tested models in both experimental series. Null hypotheses were rejected using a significance level of $P \leq 0.05$. Because no evidence of WM, temperature and salinity effects were found in preliminary ANCOVAs, these covariates were dropped for all analyses presented in this paper.

RESULTS AND DISCUSSION

For Series 1, we found significant main effects of tidal height (ANOVA, $\chi^2=11.31$, $P<0.001$), P_{O_2} ($\chi^2=64.40$, $P<0.001$) and acclimation ($\chi^2=22.49$, $P<0.001$), as well as significant effects of interactions between tidal height and P_{O_2} ($\chi^2=10.33$, $P<0.001$), and between P_{O_2} and acclimation ($\chi^2=4.35$, $P<0.05$), on *N. uncinata* OCR (Fig. 1A,B). We found a strong effect of P_{O_2} on *N. uncinata* OCR. Under hypoxia, OCR reached its lowest values within Series 1, with a mean of $3.84 \pm 0.40 \mu\text{mol O}_2 \text{ h}^{-1} \text{ g}^{-1}$ and no evident effects of tide height or acclimation (Fig. 1A,B). Much higher OCR values were found under normoxia, where a further increase during low tide was

observed, which became significant in non-acclimated animals. Here, OCR averaged $6.89 \pm 1.67 \mu\text{mol O}_2 \text{ h}^{-1} \text{ g}^{-1}$ (Fig. 1A).

For Series 2, the most informative model was the 'endogenous sinusoidal model' (M_6), followed by the 'lagged tidal-cycle model' (M_2) and the 'discrete tidal-cycle model' (M_3) (Table 1). Within endogenous sinusoidal models, we found that a model that estimated different parameters for each weekly measurement was more informative than another that estimated a single set of parameters. Hence, the predicted endogenous cycle tended to decrease with time in terms of both its period (T) and its relative amplitude (RA) (Fig. 2). Cycle T went down from 12.8 h at the first weekly series (days *ex situ*=1) to approximately 6 h in all

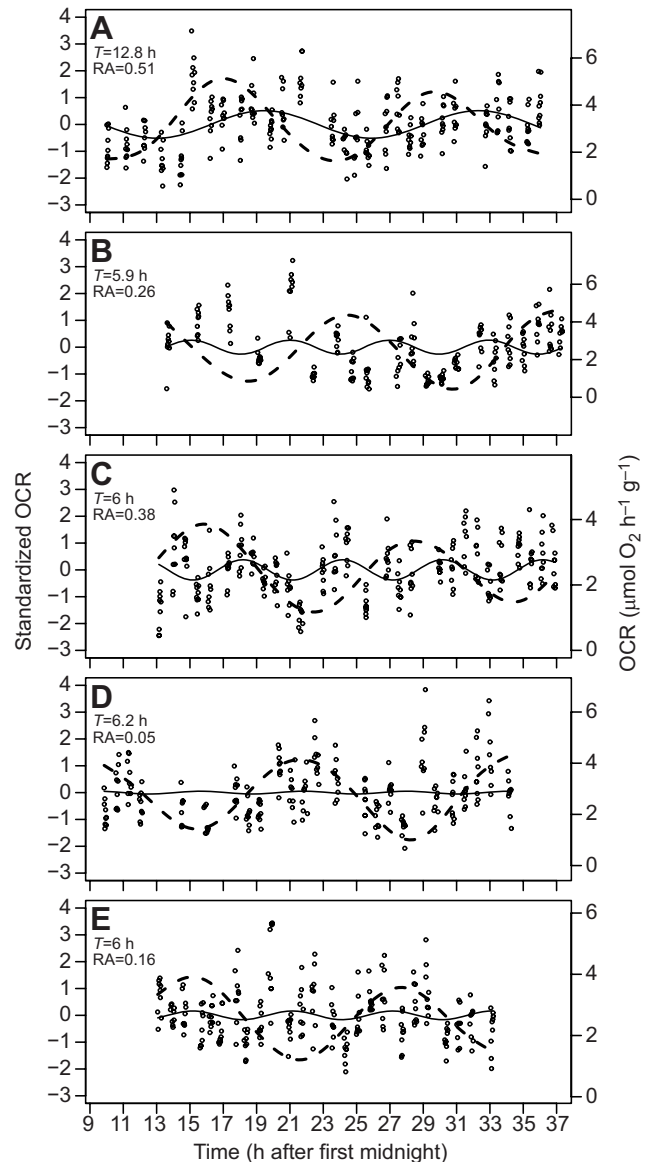


Fig. 2. Tidal cycle and OCR in *Neotrypaea uncinata*. Left and right y-axes represent standardized (i.e. subtracting the mean and dividing by the standard deviation) and non-standardized OCR values, respectively. Circles correspond to semi-continuous OCR measurements taken from nine individuals after (A) 1, (B) 8, (C) 15, (D) 22 and (E) 29 days *ex situ*. Segmented lines represent tidal height at the collection site, at the time of each measurement series. Solid lines represent predictions from the most informative model (endogenous cycle) fitted to OCR data. T and RA correspond to the period (hours) and relative amplitude of each endogenous cycle, respectively.

subsequent weeks. The estimated RA of the endogenous cycle decreased from 0.5 at the first week (days *ex situ*=1) to 0.05–0.38 in subsequent weekly measurements (Fig. 2). Given the ‘lagged tidal-cycle model’ (M_2), computed for the first weekly measurement, the maximum OCR occurred 4.6 ± 2.13 h after the highest tide or 1.6 ± 2.13 h before the lowest tide level.

Overall, OCR values found in both experimental series were in agreement with previous reports for the same species, under comparable oxygen availability and acclimation conditions (Leiva et al., 2015). The lowering of metabolic rates observed under hypoxia (1 kPa) in *N. uncinata* matched what is considered the most common and efficient physiological response to face hypoxia in the animal kingdom (Hochachka and Lutz, 2001). Thus, OCR was limited primarily by hypoxia and there was no evidence of any endogenous cycle being expressed under hypoxia.

A higher OCR was observed in Series 1 during the low tide period, under normoxia, for both acclimated and non-acclimated individuals (Fig. 1). These results contradict our hypothesis that OCR would be reduced towards the low tide period, matching lower oxygen availability in the burrows. One explanation, which is supported by results from Series 2, is that there is an anticipated increase in OCR, reaching its maximum before the low tide and its minimum before the high tide. The significant increase in OCR that we saw in the present study suggests that metabolism is increased prior to the low tide, as an anticipated response to the adverse hypoxic conditions occurring during this period in the burrows. The adaptive significance of such a biological rhythm has been discussed for other taxa (MacDougall-Shackleton et al., 2015), and it has been related to preparatory adjustments in physiology, as proposed in the Pacific oyster (*Crassostrea gigas*) prior to hypoxia exposure (Sussarellu et al., 2010). An alternative explanation might lead the endogenous cycle to be in phase with the actual dynamics of oxygen availability in the burrow’s water, which could be renewed much later than the low tide, depending on its relative position along the intertidal zone.

Both series showed evidence that the amplitude of the endogenous cycle found in *N. uncinata* decreased with time as the animals became acclimated to the laboratory conditions. Moreover, results from Series 2 showed that the period of the OCR cycle tended to decrease after the first week *ex situ*. Such a limited persistence (*ex situ*) is much shorter than has been found in other crustaceans, such as fiddler crabs from the genus *Uca*, which were found to keep their OCR rhythms up to 15 days (Naylor, 2010). The rapid loss of synchrony we found in *N. uncinata* suggests a strong dependence on environmental cues, such as oxygen availability or water column pressure, which might be related to the life cycle of this and other burrowing decapods that spend most of their lifespan inside burrows. Although environmental conditions, here, may reach extreme values, such variability fluctuates on a predictable, gradual and regular basis, which is reflected in several tide-related environmental cues that may act as exogenous triggers for the observed metabolic synchrony.

In summary, our results suggest the existence of a tide-related endogenous cycle in *N. uncinata* metabolism, a feature not yet described for any burrowing shrimp. This evidence shows lagged synchrony between tidal and metabolic cycles, indicating that this ghost shrimp may anticipate stresses associated with low tide. The metabolic patterns tended to be lost over time as animals were

acclimated to laboratory conditions. The results obtained when comparing OCR at discrete tide, oxygen and acclimation levels highlight the importance of including natural variability in controlled laboratory experiments. Although metabolic rate tends to decrease after acclimation to static conditions, higher metabolic rates would be expected under more realistic (variable) environmental conditions that provide multiple triggering signals and force animals to handle several environmental stressors at once. This might be particularly relevant to future research that seeks to properly evaluate the physiological consequences of ongoing environmental changes.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

F.P.L. devised the idea, collected the data and drafted the manuscript. E.J.N. realized most statistical analysis and, together with K.P., P.G. and M.A.U., contributed to, revised and edited the manuscript. All authors read and approved the final manuscript.

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