Effect of starvation and feeding on respiratory metabolism in *Leptomysis lingvura* (G.O. Sars, 1866)

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Abstract

The mysid, *Leptomysis lingvura*, is found along east coast of Gran Canaria (Spain) swimming in the plankton above sandy bottoms at depths between 5 and 15 meters. As with many mysids around the world, it is an important component in the food chain for many coastal fish and could be a potential live prey for use in aquaculture (Herrera et al., 2011; Jumars, 2007). We studied *L. Lingvura*'s survival and reproduction in captivity and determined its suitability for physiological and biochemical research in the laboratory. This mysid proved to adapt well to aquarium life and to be highly suitable for studying respiratory metabolism. This investigation documents the effect of feeding and starvation on the enzymology and physiology of respiration. The research strategy was to follow a simple time course of both the oxygen consumption rate of whole mysids and the activity of their respiratory electron transport system (ETS). Respiration (R) decreased logarithmically during starvation whereas the ETS activity remained constant. As a consequence, the ratio of R to ETS activity decreased along with the respiration. Superimposed on the declining respiration rate was an unforeseen diel rhythm that elevated R during the light and depressed it during the dark. The slope in

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the R-biomass log-log Kleiber plot in well fed mysids is close to 0.75 while for starved mysids it was lower than 0.75.
Keywords: mysids, respiration, starvation, feeding, ETS.

1. Introduction

Mysids are peracaridan crustaceans that inhabit many varied aquatic habitats. They are abundant in coastal regions, some benthic, others planktonic, but they also occur in the pelagic waters throughout the oceanic water column far from land. (Mauchline, 1980; Jumars, 2007). They are omnivorous filter feeders eating small planktonic organisms such as copepods, tinntinids, and diatoms as well as organic detritus (Tattersall and Tattersall, 1951; Mauchline, 1980; Murano, 1999). In cultures, cannibalism occurs if not enough food is provided (Lussier et al., 1988; Domingues et al., 1999). Some species exhibit daily feeding rhythms. Dauby (1995) has studied the behavior of Mediterranean *Leptomysis* species and finds that during the day they form swarms and rest just above the bottom while during the night they swim to feed on sediments and particulate organic matter (phytoplankton, seagrasses, macro and micro algae). Females carry the embryos in a marsupium where larval development occurs. Juvenile mysids emerge morphologically similar to adults.

Leptomysis lingvura inhabits the east coast of Gran Canaria (27°51 N; 15°23 W) and grows well in the laboratory. Not only does it survive in culture, but it can complete its life cycle in captivity. These characteristics enabled us to document its growth and respiration under controlled conditions (Herrera, 2009; Herrera et al., 2011).

Respiration is a good index of physiological activity and energy production in zooplankton (Gómez et al., 1996). The direct measurement of zooplankton respiration is difficult in practice, because it is difficult to simulate natural conditions in laboratory cultures. Differences between laboratory conditions and those of the natural environment include predation stress, food limitation, schooling, omnidirectional migration tendencies, and variability in temperature, light, and ocean currents. As a result a laboratory measurement of respiration is not equivalent to *in situ* respiration and proxies, models, or some combination of the two are needed to calculate in situ oceanic respiration accurately. Hence we investigate the biochemical basis of respiration. ETS (electron transport system) activity is the biochemical foundation of respiration and energy production (Lane, 2005). We use the term, electron transport system as a synonym for the electron transport chain. It is measured in plankton to estimate the "potential" respiration (ϕ) (Packard and Gómez, 2008). This technique uses the cytoplasmic reduction of an artificial electron acceptor: tetrazolium-salt (INT), to stoichiometrically measure the capacity of the cytoplasm to consume O_2. This can be done because the reduction of 2 moles of INT by the ETS is equivalent of the ETS-driven reduction of 2 atoms of oxygen (or 1 molecule of O_2). The relationship between the ETS activity and the respiration rate is complicated. Respiration is likely to be depressed during starvation and stimulated during feeding, mating, and avoiding predation (Thor, 2003; Kiorboe et al., 1985; Lampert, 1986; Bohrer and Lampert, 1988; Hernández-León and Gómez, 1996). ETS activity is likely to be relatively constant during these conditions. This study is an effort to test this hypothesis in the case of starvation.

The relationship between metabolic rates (R) and biomass (W), can be expressed by the equation:

$$R = W^b \tag{1}$$

or in logarithmic form :

$$R = log\ a + b\ log\ W \tag{2}$$

It is known as the Kleiber's law (Kleiber, 1961) and more recently has been used to develop a theory that combines the effect of two variables: temperature and body size. Reputed to be based on chemical, physical and biological principles (Gillooly et al., 2001, 2006; Brown et al., 2007), this theory is known as the Metabolic Theory of Ecology (MTE). This theory applies a single equation for metabolic rates of all organisms,

$$Y = i_0\ W^{3/4} e^{-E/k.T} \tag{3}$$

where i_0 is the normalization constant, W is the biomass, and $e^{-E/k.T}$ is the Boltzmann-Arrhenius factor, where E is the activation energy, k is the Boltzmann constant and T is the absolute temperature in degrees K (Gillooly et al., 2001). The regression coefficient b=3/4, as established by Kleiber's law, is a general average, but many authors have found variations in it (Glazier, 2005, 2006; Atanasov, 2010). Lane (2005) discusses the reliability of this exponent and concludes that based on detailed examination it is not a constant for all species and sizes of organisms as is claimed. In this study, we obtained the values of coefficient b for *L.lingvura* across a range of sizes and ages (1 day-adult) with three different feeding treatments, trying

to determine how this factor affects Kleiber's coefficient b. The present investigation of respiratory metabolism in *L.lingvura*, aims to examine:

1. How the periods of starvation affect respiration and ETS activity.

2. How different food conditions affect the respiration-biomass ratio and ETS-biomass relationship..

2. Materials and Methods

2.1. Collection and laboratory maintenance

Adults of *L. Lingvura* were captured in coastal waters off Risco Verde in Gran Canaria, at depths of between 5 and 15 m with SCUBA equipment and a hand net of 500 μm mesh size, cultured as described in Herrera et al. (2011), and identified microscopically following the keys of Tattersall and Tattersall (1951) and Wittmann (1986).

2.2. Effect of starvation on respiratory metabolism

After acclimation for 7 days, males of similar sizes were separated in individual containers to avoid cannibalism and subjected to different periods of starvation: 2, 6, 10, 22, 26, 30, 36, 46, 52 and 74 h. The experiment began at 10 am, the starvation period of 2 h occurred at 12 am, and the 74 h starvation period, 3 days later at 14 pm. At the end of each starvation period, five individuals were separated for measurements of *in vivo* respiration (μl O_2 per h) with an oxymeter (Strathkelvin 928 6-Channel oxygen system) in dark individual 50 ml chambers at 20.5℃. Afterwards, the mysids were

frozen at -196℃ in liquid nitrogen and then stored at -80℃ for ETS activity (Gómez et al., 1996) and for protein measurements according to Lowry method (Lowry et al., 1951), as modified by Rutter (1967).

2.3. Effect of feeding conditions on the respiratory metabolism-biomass relationship

For this experiment, 100 different sized mysids were put into each of 3 different tanks and fed 3 different treatments for a week:

Treatment A: twice-daily ration of 150 *Artemia* sp. (artemia) 48 hours- nauplii.

Treatment B: twice-daily ration of 75 artemia 48 hours- nauplii.

Treatment C: twice-daily ration of 10 artemia 48 hours- nauplii.

After one week 36 mysids from each of the different treatments were separated for measurements of in vivo respiration (μl O_2 h^{-1}) with an oximeter. Individual darkened cells of 50 ml were used. The mysids were subsequently photographed, sized, frozen at -196℃, and stored at -80℃ for ETS activity (Gómez et al., 1996) and for protein measurements.

2.4. ETS activity determination

Samples were homogenized by sonication for 45 s with an ultrasonic probe (Cole Parmer) in 1.5 ml of Milli-Q distilled water, then centrifuged for 10 minutes at 4000 rpm at 0℃. A 0.5 ml aliquot of the supernatant was added to 1.5 ml of a solution containing (0.2 (v / v) Triton X-100, 50 mM sodium phosphate (Sorensons) buffer pH 8, 0.133M disodium succinate, 0.835mM NADH, and 0.24mM NADPH) and 0.5 ml of 4mM INT (Sigma Lab). For each sample a blank was performed without ETS substrates. Samples were incubated

at 20.5℃ for 20 minutes after which the reaction was stopped with a quench solution consisting of 50% phosphoric acid 0.1M and 50% formaldehyde to 36%. Absorbance was read spectrophotometrically (Beckman DU 650, USA) at 490nm (INT-formazan) and 750nm (turbidity). Potential respiration was calculated from ETS activity according to Packard and Christensen (2004). Respiration rates (R) and potential respiration rates (ϕ) were normalized by biomass (protein) resulting in units of μl O_2 h^{-1}. mg $protein^{-1}$.

2.5. Statistical analysis

The starvation experiment data were analyzed using the program R Development Core Team 2010 (R Foundation for Statistical Computing, Vienna, Austria). To confirm normality, the respiration (R), ETS activity and R/ETS data were analyzed by the Shapiro Wilk test and the homoscedasticity of the residuals was assessed graphically. To study the correlation between respiration-biomass and ETS-biomass in different feeding conditions we use the program PASW Statistical Software version 18.0 to obtain the regression equations, using a confidence limits of 95% and the Pearson correlation coefficient.

3. Results

3.1. Effect of starvation on respiratory metabolism

The correlation in the relationship between *in vivo* R and starvation time (h) (figure 1) is represented by the equation:

$$R = 71.78 - 24.36\ log\ h \tag{4}$$

The Shapiro-Wilk normality test yields W=0.9551, p-value=0.07953; R^2=0.44, n=45, p< 0.001. Figure 1. depicts the decrease in R with starvation time in mysids.

Peaks in the respiration are observed at the beginning of the periods of darkness, if we take into account two variables: starvation time and time of day (t) (figure 1), the model that fits the data best is:

$$R = 44.49 - 9.98\ log\ h + 1.82\ t \tag{5}$$

The Shapiro-Wilk normality test yields W=0.9643, p-value=0.1778; R^2=0.65, n=45, p< 0.001.

Although the data are scattered, ϕ shows no correlation with the diel periodicity. Furthermore, it does not decrease with increasing starvation-time in *L. lingvura* (R^2 =-0.02, p = 0.754) (figure 2) reflecting the constitutive nature of the ETS in the mysids mitochondria. Since ϕ is constant, it is the decreasing R that forces the R/ϕ ratio to decrease with starvation time as in figure 3. The regression equation is:

$$R/ETS = 2.03 - 0.29\ log\ h \tag{6}$$

The Shapiro-Wilk normality test yields W=0.9687, p-value=0.3116; R^2= 0.44, n=41, p< 0.001. If we consider both variables starvation time and time of day, the model that fits the data best is:

$$R/ETS = 1.16 - 0.27\ log\ h + 0.06\ t \tag{7}$$

The Shapiro-Wilk normality test yields W=0.9893, p-value=0.9614; R^2= 0.72, n=41, p< 0.001.

The biomass range of male mysids studied was between 0.129 and 0.319 mg of protein. The mean *in vivo* R and ϕ for each period of starvation are shown in (table 5).

3.2. Effect of feeding conditions on the respiratory metabolism-biomass relationship

The relationship between R and biomass expressed in logarithms for treatment A was:

$$log\ R = 2.18 + 0.84\ log\ W \tag{8}$$

(R^2=0.64, n=34) with a Pearson correlation coefficient =0.798, p< 0.01; for treatment B:

$$log\ R = 2.72 + 0.92\ log\ W \tag{9}$$

(R^2=0.69, n=36) with a Pearson correlation coefficient =0.829 p< 0.01; and treatment C:

$$log\ R = 1.87 + 0.71\ log\ W \tag{10}$$

R^2=0.81, n=35) with a Pearson correlation coefficient =0.902, p< 0.01 (figures 4, 5, 6).

ETS activity represents the ϕ , the maximum reaction rate of Complex I, the NADH dehydrogenase iron-sulfur protein flavin mononucleotide conglomerate, that controls the electron flux through the mitochondrial. The relationships between this activity and biomass was observed in the 3 treatments are as follows:

198 Treatment A:

$$log\ ETS = 2.73 + 0.72\ log\ W \tag{11}$$

199 (R^2=0.84, n=34) with a Pearson correlation coefficient =0.916, p< 0.01; for
200 B:

$$log\ ETS = 2.85 + 0.71\ log\ W \tag{12}$$

201 (R^2=0.77, n=36) with a Pearson correlation coefficient =0.879, p< 0.01; and
202 C:

$$log\ ETS = 2.44 + 0.54\ log\ W \tag{13}$$

203 (R^2=0.85, n=35) with a Pearson correlation coefficient =0.924, p< 0.01 (fig-
204 ures 4, 5, 6).
205 The exponent, b, in Kleiber plots is the coefficient in these log-log equations.
206 For both R and ϕ , b is lower in poorly fed mysids than in the well fed ones
207 (table 5).
208

209 **4. Discussion**

210 According to the results, when *L. lingvura* is starving its respiratory
211 rate decreases, other authors found similar results in the bathypelagic mysid
212 *Gnathophausia ingens* (Hiller-Adams and Childress, 1983). This is not the
213 case with ϕ, represented by the enzymatic activity of the ETS. Over a period
214 of 74 h it does not decrease. The enzymes of the ETS are responsible for
215 90% of all biological O_2 consumption (Nelson and Cox, 2005) and unless the

number of mitochondria or their size changes, their capacity (V_{max}) should not change, at least during short-term changes in physiological state. In *L. lingvura* other metabolic rates, such as the ammonia excretion rate also show similar behavior. It decreases logarithmically with the starvation time, while the activity of GDH, the enzyme that controls this process, is not affected (Fernández-Urruzola et al., 2011).

In our method for monitoring the ETS Vmax we saturate the enzyme assay with ETS substrates (NADH, NADPH and succinate) and so we insure that we are measuring the amount (the concentration) of the enzymes available to consume O_2 and to maintain the proton-motive force in the mitochondria. In this manner we also insure that the measurement of ETS activity is insulated from variability in the substrate supply systems which can be modulated by starvation and other forms of physiological stress. R in mitochondria can be impacted by the availability of ADP as a substrate for phosphorylation. Outside the mitochondria, when the speed of some cellular energy-requiring processes such as protein synthesis increases, there is an increased rate of ATP degradation to ADP. Transported back to the mitochondria, this increases the availability of ADP for oxidative phosphorylation which, by lowering the pH and emf gradient across the mitocondrial inner membrane, can stimulate the ETS and hence R (Chance and Williams, 1955; Nelson and Cox, 2005; Lane, 2005). R increases with feeding known as "specific dynamic action" (SDA) has been noted by Kiorboe et al. (1985) and Thor (2003) in *Acartia tonsa*. They found that the respiration rate in copepods during food-saturated conditions was 4 times greater than during conditions of starvation, and postulated that this increase is mainly related

to the biosynthesis and transport. They argue that gut activity, amino acid oxidation and urea excretion contributes less to the SDA. In any case, SDA is another zooplankton process likely to disturb the ADP/ATP ratio via the demand for ATP throughout the organism. Most physiological mechanisms that disturb the ADP/ATP ratio will lead to considerable variability in R. Ingestion, for example, such as studied in *Euphausia superba* by Ikeda and Dixon (1984) will also fall in this group. In this study, R in well fed mysids is three times higher than R in mysids starved for 46 h or more. In mysids that have an active metabolism, but a low nutrient reserve, the lack of substrates is rapidly reflected in R. The variation in the ratios R/ϕ is a direct consequence of all of the above processes. Hernández-León and Gómez (1996) studied the causes of the high variability of the R/ϕ ratios obtained in other zooplankton studies for different oceanic areas and oceanographic conditions and showed that the factors affecting this relationship are: chlorophyll, primary production, temperature and size of organisms. The variability of the R/ϕ in relation to chlorophyll and primary production suggests that these indices of the quantity or quality of food impact R, but not ETS (Hernández-León and Gómez, 1996). In this study, R and R/ϕ in fed mysids was three times higher than in mysids starved for 46 h or more. In addition, in figure 1 (R vs starvation period) there are two peaks in R that coincide with the start of the dark period at 20:00 h. These are likely maxima in the circadian R rhythm. Many mysids in the hyperbenthos have endogenous rhythms of activity. Mysids of the genus, *Gastrossacus*, rest on the sediment during the day and ascend swimming at night, this behavior persists even under experimental conditions of darkness for several days (Mauchline, 1980).

Mediterranean species of *Leptomysis* also have this type of feeding behavior (Dauby, 1995). Hecq et al. (1984) have conducted studies on the influence of experimental and environmental conditions in the consumption of O_2 in *L. lingvura*. They show that during the day R ranges between 20 and 24 mg O_2 h^{-1}.mg $protein^{-1}$. It increases progressively during the night reaching a maximum value at the end of the night (48.2 mg O_2 h^{-1}.mg $protein^{-1}$). Amylase activity increases in parallel.

It is not purpose of this paper to investigate how circadian rhythms affect respiration and respiration/ETS ratio, but when we observe the behavior of respiratory metabolism during the dark period we have seen that the model that includes two variables: starvation time and time of day have a better fit than the one that only includes the starvation time (figures 1 and 3), but this model is rather descriptive and missing data in both periods (light and dark) in the last days of the experiment to assess how influence both variables in the respiration. Experiments are needed for this purpose. In organisms that have internal daily rhythms as mysids in applying models to estimate metabolic rates is necessary to consider this variable and the feeding conditions because that directly affect these processes.

Regarding the relationship between respiratory metabolism and biomass, other authors (Martínez, 2007; Gómez et al., 2008; Packard and Gómez, 2008; Herrera, 2009) have found similar correlations in different groups of zooplankton (table 5). In these studies the slope b is also ranges between 0.5 and 1. This variability has lead others to question Kleiber's law and the MTE to describe the oxygen consumption in a small range of sizes, short time scale or in different physiological states (Dodds et al., 2001; Lane, 2005; Packard and

Gómez, 2008; Kolokotrones et al., 2010). The enzyme-kinetic-model (EKM) developed by (Packard et al., 1996, 2004) and Roy and Packard (2001) proposed that R is the product of ϕ and substrate availability that regulates it. These are the fundamental bases for regulating R. Biomass is indirectly related to R because it packages the mitochondria and the ETS enzymes (Martínez et al., 2010), but by itself biomass is an irrelevant factor. Here, the ETS-biomass relationship in the three treatments showed a better fit than the R-biomass ratio (figures 4, 5, 6, table 5). ETS activity is determined by the concentration of Complex 1-NADH dehydrogenase in the mitochondria, and this concentration varies with the number of cells in the mysid and hence the biomass. ETS, being a constituent part of the mitochondria, the cells, and the mysid, should not change rapidly with environmental conditions or the amount of metabolizable substrate, as does respiration. However, with prolonged acclimation to different conditions (as with forced activity) the ETS activity could change. If we analyze the data of the table 5, and assume that organisms in the regions of upwelling, coastal and eddies are well-fed (as in treatments A and B), the higher values of b ($\geq$0.75) become understandable. Likewise, assuming that zooplankton in oceanic regions are less-well fed (as in treatment C), the lower values of b (<0.75) become understandable.

5. Conclusions

1 - *L. lingvura* respiratory activity shows a variability related to feeding conditions and circadian rhythms. Since the activity of enzymes of the ETS is not altered in the short term, this variability of respiratory rate forces

parallel variability in the R/ϕ ratio, this ratio can be three times higher in feeding mysids than in starved ones. When performing *in vivo* experiments of respiratory metabolism in zooplankton it is necessary to take into account the physiological conditions and endogenous daily rhythms because they directly impact the respiratory activity.

2 - In *L. lingvura* the Kleiber coefficient, b, of the regression equation: $R=a\,W^b$, varies with food conditions, it is lower than 0.75 when the organisms are exposed to minimum conditions of food for long periods of time. Further testing of this hypothesis will require studies of respiration and ETS activity in other zooplanktonic organisms exposed to different feeding conditions.

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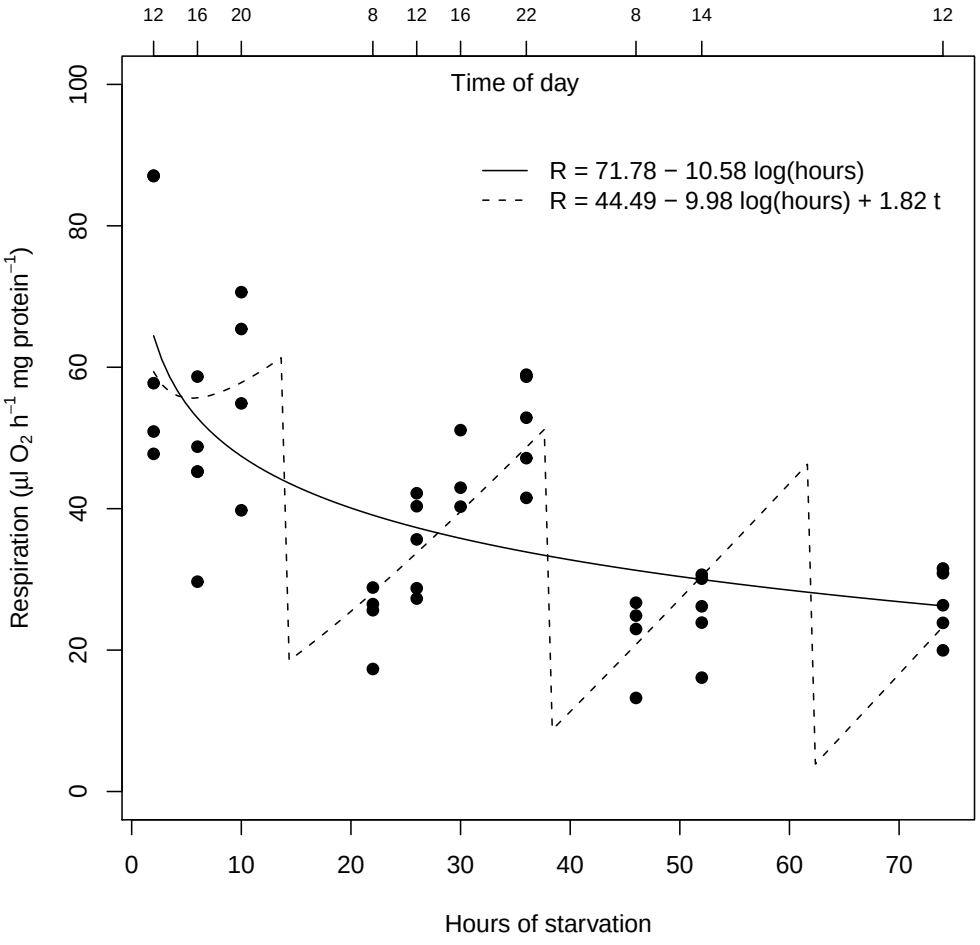

Figure 1: Relationship between R (μl O_2 h^{-1} mg prot.$^{-1}$) and starvation period (h), R^2=0.44, n=45 (solid line); and relationship between R, starvation period and time of day (t), R^2=0.64, n=45 (dotted line). The dark period started at 20:00 hs.

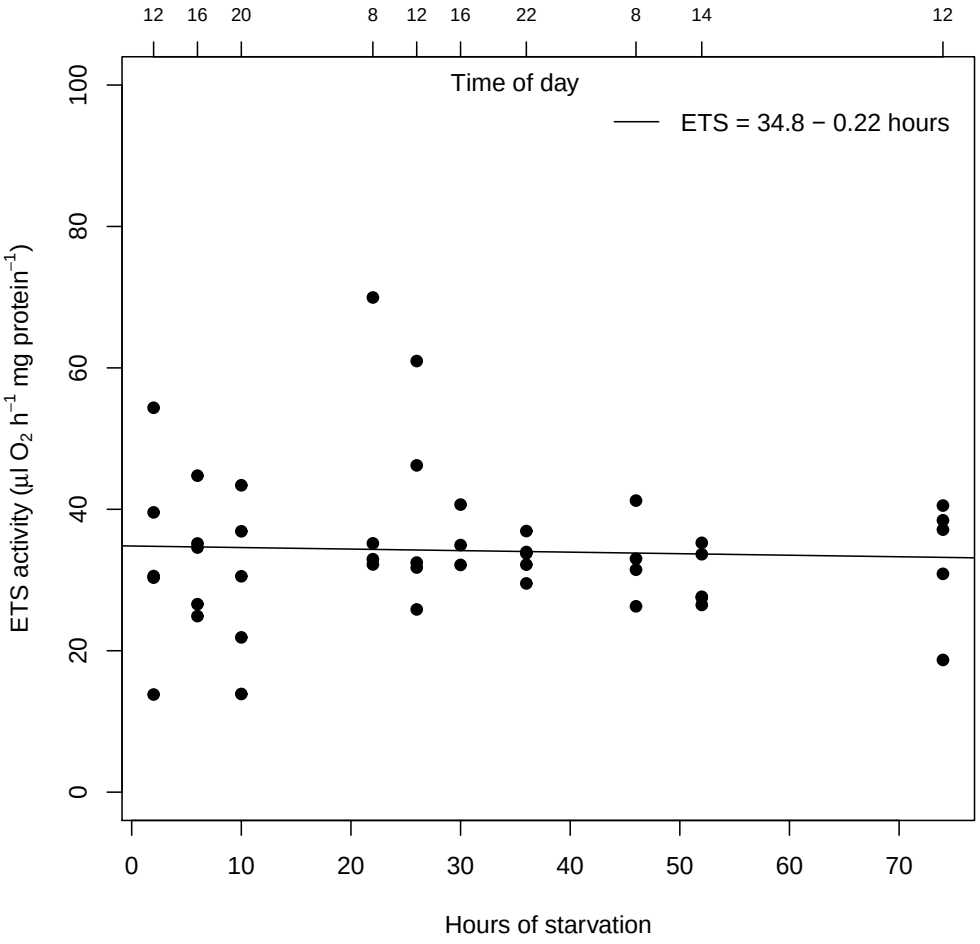

Figure 2: Relationship between ϕ (μl O_2 h^{-1} mg prot.$^{-1}$) and starvation period (h), R^2=0.021, n=45.

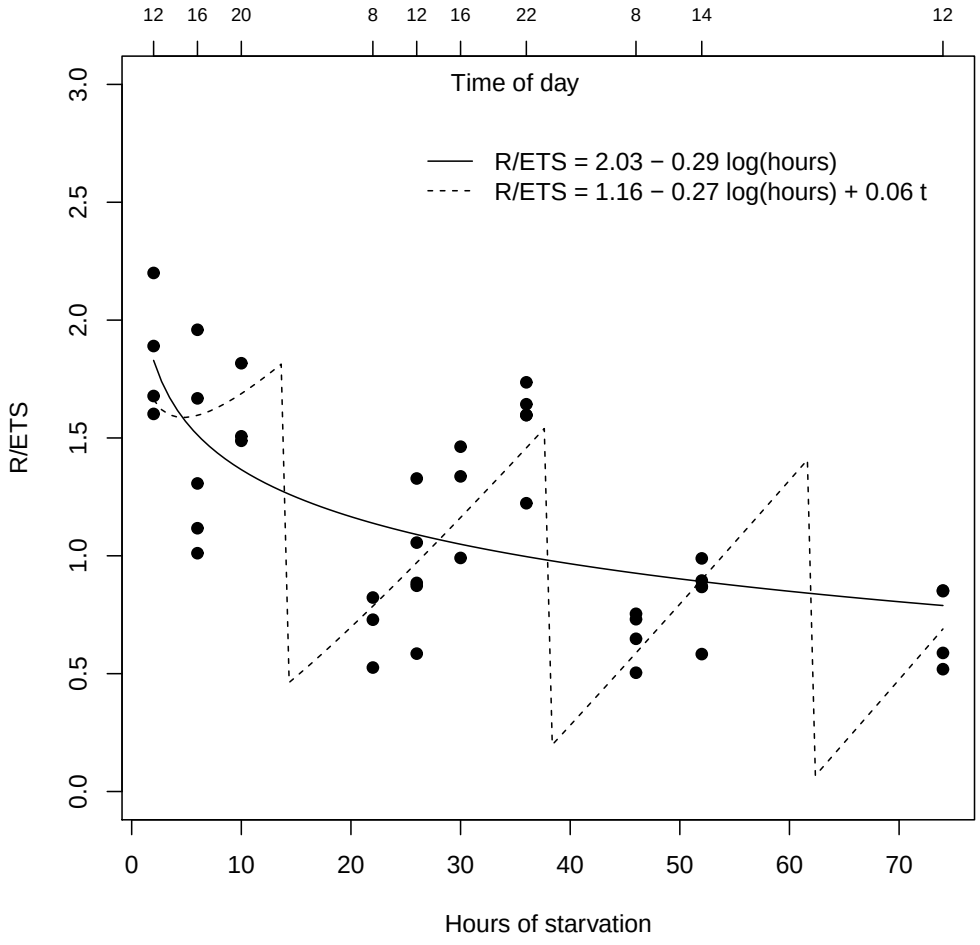

Figure 3: Relationship between R/ϕ ratio and starvation period (h), R^2=0.44, n=41 (solid line); and relationship between R/ETS, starvation period and time of day (t), R^2=0.72, n=41 (dotted line). The dark period started at 20:00 hs.

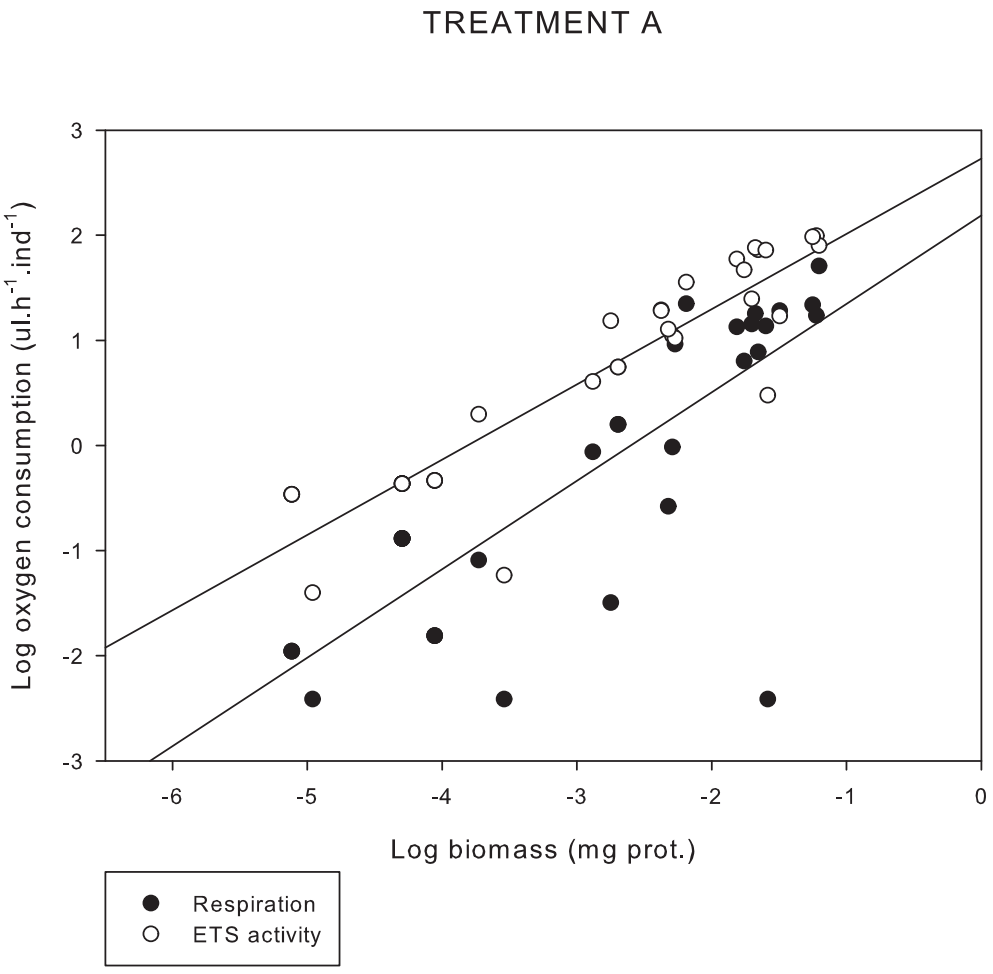

Figure 4: Relationship between R-biomass; and ϕ-biomass for mysids with treatment A.

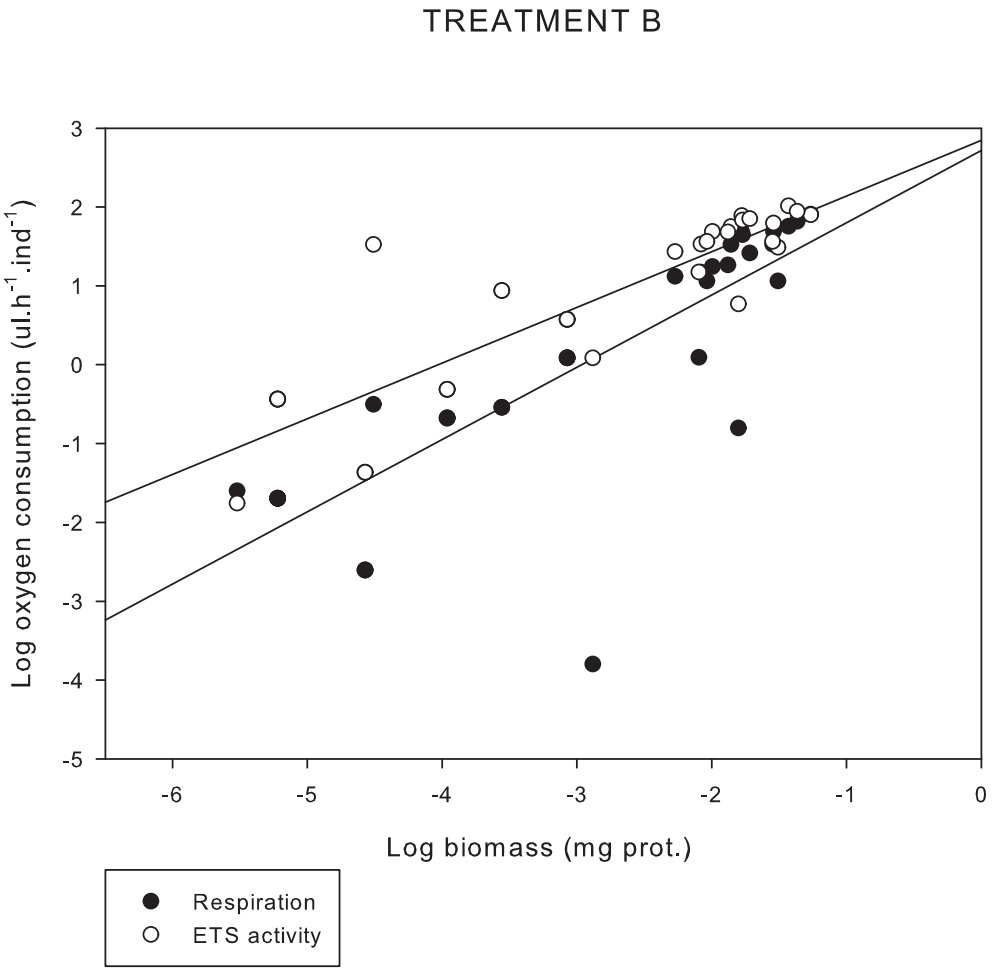

Figure 5: Relationship between R-biomass; and ϕ-biomass for mysids with treatment B.

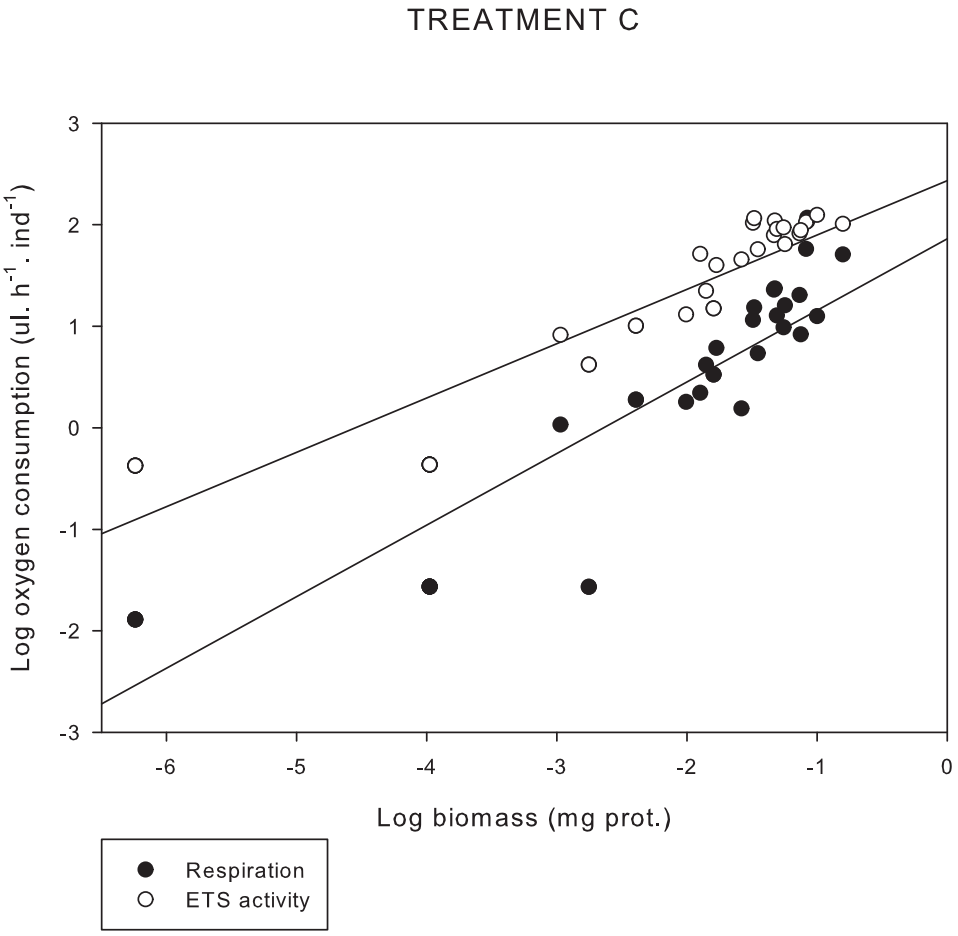

Figure 6: Relationship between R-biomass; and ϕ-biomass for mysids with treatment C.

Table 1: Values of respiration rate (R) and potential respiration rate (ϕ) (μl O_2 h^{-1}. mg $protein^{-1}$) (mean ± standard deviations) for different periods of starvation, n=number of samples. Ratio R/ϕ (mean ± standard deviations) for each period of starvation.

Hours of starvation	R ± SD	n	ϕ ± SD	n	R/ϕ ± SD
2	66.1±19.5	5	33.7±14.8	5	2.17±0.76
6	45.5±10.4	5	33.2±7.9	5	1.41± 0.40
10	57.7±13.6	4	29.3±11.7	4	1.60±0.18
22	24.6±5.0	4	42.6±18.3	3	0.69±0.15
26	34.8±6.7	5	39.5±14.2	5	0.94±0.27
30	44.8±5.6	3	35.9±4.4	3	1.26±0.24
36	51.8±7.5	5	33.3±2.7	5	1.56±0.20
46	22.8±6.0	4	33.0±6.2	4	0.66±0.11
52	25.4±6.9	5	30.1±4.0	5	0.84±0.15
74	26.5±4.9	5	33.1±8.8	5	0.70±0.17

Table 2: Regression constants of the relationship between potential respiration and biomass represented by the equation: log ETS= a logW^b. Mean scaling exponent ± 95% confidence limits (C.L.). *Significantly different from 0.75.

Organism	Food conditions or colect conditions	a	b ± 95% C.L.	n	R^2	Reference
Artemia salina	5000 *Nanoclorosis* sp. ind $^{-1}$	-0.05	0.59±0.39	10	0.60	Martínez et al. (2010)
Artemia salina	1000 *Dunaliella* sp. ind $^{-1}$	-4.40	0.50 ±0.18*	14	0.73	Martínez et al. (2010)
Zooplankton mix	upwelling areas	0.14	0.89 ±0.11*	248	0.53	Gómez et al. (2008)
Zooplankton mix	eddies areas	-0.12	0.98±0.40	30	0.48	Gómez et al. (2008)
Zooplankton mix	oceanic areas	-0.03	0.64±0.11	220	0.38	Gómez et al. (2008)
Zooplankton mix	coastal areas	0.08	0.79±0.15	64	0.64	Gómez et al. (2008)
Zooplankton mix	incubated for 1 day	0.52	0.79±0.12	14	0.94	Packard and Gómez (2008)
L.lingvura	200 *Artemia* nauplii.d^{-1}.ind^{-1}	4.15	0.92±0.07*	32	0.96	Herrera (2009)
L.lingvura	300 *Artemia* nauplii.d^{-1}.ind^{-1}	2.73	0.72±0.11	34	0.84	present work
L.lingvura	150 *Artemia* nauplii.d^{-1}.ind^{-1}	2.85	0.71±0.13	36	0.77	present work
L.lingvura	20 *Artemia* nauplii.d^{-1}.ind^{-1}	2.44	0.54±0.08*	35	0.85	present work