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# GDH activity and ammonium excretion in the marine mysid, *Leptomysis lingvura*: effects of age and starvation


Researcher work accomplished by:

**Igor Fernández Urruzola**

Directed by:

**Theodore Train Packard**

**May Gómez Cabrera**


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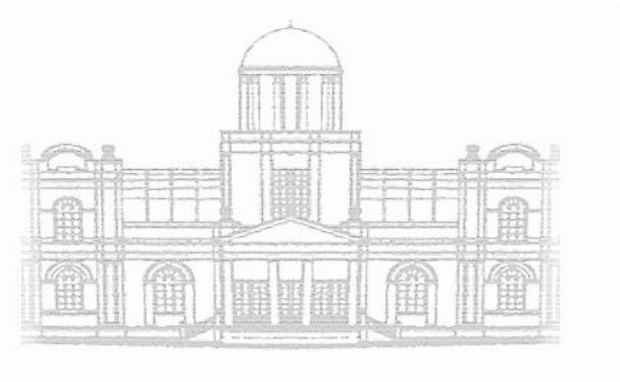


Laboratory of Biological Oceanography
Facultad de Ciencias del Mar
Campus de Tafira, 35017

Tel: 928 454 546
Fax: 928 452 922

E-mail: ifernandez@becarios.ulpgc.es
http://www.ulpgc.es

# GDH activity and ammonium excretion in the marine mysid, *Leptomysis lingvura*: effects of age and starvation

I. Fernández-Urruzola[1,*], M. Gómez[1], T. Packard[1]

[1] *Facultad de Ciencias del Mar, Universidad de las Palmas de Gran Canaria, Spain*

[*] Corresponding author. E – mail: ifernandez@becarios.ulpgc.es

**Running head:** GDH activity and $NH_4^+$ excretion in *L. lingvura*

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## Abstract

Ammonium ($NH_4^+$) release by heterotrophic organisms is the largest recycled nitrogen source in the euphotic zone. It determines the regenerated fraction of phytoplankton productivity, so the measurement of $NH_4^+$ in marine systems is necessary to characterize both the magnitude and the efficiency of the nitrogen cycle. The enzyme glutamate dehydrogenase (GDH) is the main enzyme responsible for $NH_4^+$ formation in animals and consequently, should be useful in estimating $NH_4^+$ excretion by marine zooplankton. Here, we study the rate of $NH_4^+$ excretion and the GDH activity in an important Canary Island mysid, *Leptomysis lingvura*. We address body size and starvation as sources of variability on the GDH to $NH_4^+$ excretion ratio (GDH/$R_{NH_4^+}$).

We found a strong correlation between the $R_{NH_4^+}$ and the GDH activity ($r^2$ =0.81, n = 48) during growth. Both variables were regressed against protein mass in order to obtain the allometric scaling exponent. Since GDH activity maintained a linear relation with biomass (b = 1.00) and $R_{NH_4^+}$ scaled exponentially (b = 0.59), in well fed mysids the GDH/$R_{NH_4^+}$ ratio trended to increase with size. However, the magnitude of its variation increased when adult mysids were starved. In this case, the GDH/$R_{NH_4^+}$ ratio ranged from 11.2 to 102.3, while apparent $K_m$s varied from 2.84 to 19.45 without a trend throughout the experiment.

**Keywords:** GDH activity, ammonium regeneration, *Leptomysis lingvura*, starvation, body size.

## Introduction

Nitrogen is essential for life. It is constituent to many biological structures and in all enzymatic reactions, but its availability is frequently limited in ocean ecosystems. As a result, nitrogen plays a critical role in biogeochemical cycles (Falkowski *et al.*, 1998). Despite its existence in multiple oxidation states and in many chemical compounds in oceans, the nitrogen which supports primary production occurs mainly in the forms of ammonium and nitrate (Bronk *et al.*, 1994; Yool *et al.*, 2007). The availability of these compounds determines the productivity of the ocean and thus, the capacity of this huge ecosystem to act as a carbon dioxide sink. Ammonium ($NH_4^+$) excretion from glutamate deamination in heterotrophic organisms constitutes the major recycled nitrogen source in the euphotic zone (Harrison, 1987), even though the nitrate remineralized in the near-surface mixed layer also sustains the regenerated production (Zehr and Ward, 2002; Beckmann and Hense, 2009). However, the nitrate produced in deep waters via nitrification, once it reaches the surface by vertical transport, is largely responsible for new production (Dugdale and Goering, 1967; Eppley and Peterson, 1979).

The relevance of the regenerated nitrogen to the phytoplanktonic growth rate and biomass has been widely addressed in the literature. The heterotrophic $NH_4^+$ release, on average, supplies around 80% of the primary producers' requirements (Harrison, 1992), which reflects the significance of this metabolic process at a global scale. Factors such as temperature (Ikeda, 1985), nutritional composition of ingested food (Glibert, 1993; Miller and Roman, 2008; Saba *et al.*, 2009) and the interaction between the different trophic levels (Glibert, 1998), among others, can modify locally this percentage. Therefore, ammonium recycling efficiencies range from 50% in coastal waters to about 95% in the less productive areas of tropical latitudes (Eppley and Peterson, 1979). Quantifying this physiological process in the oceans is then, necessary to characterize the efficiency of the nitrogen cycle and to understand the basis of an aquatic ecosystem's productivity.

In order to assess the $NH_4^+$ excretion in zooplankton, water bottle–incubations have been traditionally used by oceanographers. However, although direct, this delicate method is complicated by artifacts derived from organism manipulation, overcrowding or starvation that may occur during long incubation times (Bidigare, 1983). On a physiological scale such

measurements can be made, but at a low data acquisition rate. This is fine for physiology, but oceanography requires many measurements made over large time and space scales and so a high data acquisition rate is needed. In recognizing these requirements of oceanography, Bidigare and King (1981) introduced a biochemical approach by proposing the enzyme glutamate dehydrogenase (GDH) as an index for $NH_4^+$ formation in the marine systems. They chose GDH because it is found in high levels in planktonic crustaceans and because its role in amino acid catabolism argues for its control over a great proportion of $NH_4^+$ excretion. In these ammoniotelic organisms, proteins are broken down into amino acids and then, transaminated with α–Ketoglutarate to produce α–Ketoacids and glutamate. The glutamate is oxidized by NAD-dependent GDH (EC 1.4.1.3) into $NH_4^+$, NADH, α–Ketoglutarate, and a proton. Thus, the potential $NH_4^+$ excretion can be calculated from the rate of the GDH reaction (i.e., GDH activity).

The interest in GDH persists in spite of the problems associated with using enzyme assays to predict the physiology of the organisms. First of all, enzyme analyses are classically designed to measure the $V_{max}$ of an enzyme reaction. Accordingly, an enzyme assay requires the addition of externally added substrate, which insures that the assay determines the potential enzymatic activity ($V_{max}$) instead of some undefinable other level of activity. It would be desirable to measure the actual rate of activity in the sample, the *in vivo* rate, but the technology is just not available now. Thus, any enzyme, under unlimited substrates, operates at its maximum rate, and the product generated over a time period is simply a function of the amount of enzyme present. In addition, there is variability in the ratios of GDH activity to $NH_4^+$ excretion due to changes in specific composition, body size and trophic conditions. How these parameters impact the biochemistry and physiology of $NH_4^+$ excretion is part of this investigation. In spite of these uncertainties, at an operational level, the GDH analysis is a simple, fast and inexpensive proxy for heterotrophic $NH_4^+$ release. Strengthening our knowledge of the relationship between GDH activities and the $NH_4^+$ excretion rates ($R_{NH_4^+}$) under different conditions, would lead to more meaningful interpretations of the mesoscale variations in plankton $NH_4^+$ excretion.

Working with the marine mysid *Praunus flexuosus*, Bidigare and King (1981) established a high correlation ($r^2 = 0.92$, n= 7) between GDH activity and the production of the main nitrogenous waste of crustaceans, $NH_4^+$ (Regnault, 1987; Ikeda *et al*., 2000). These

results were confirmed in mixed communities of zooplankton by Park *et al.* (1986) ($r^2$ = 0.98, n = 10), with a small range of variation in their GDH to excretion ratio (18.18 ± 6.72). However, this ratio is expected to vary according to the body size and nutritional status since GDH is a regulatory enzyme which is modulated by the allosteric effectors adenosine diphosphate (ADP) and guanosine triphosphate (GTP). As a consequence, Park (1986) obtained substantial fluctuations in the GDH/$R_{NH_4^+}$ (36.92 ± 29.9) for two species of copepods, when food availability conditions were modified during the experimentation. Similar behavior on that relationship (values from 1.2 to 42.5, n = 59) was described by Hernández-León and Torres (1997) on mixed zooplankton from waters around Gran Canaria, where the so-called “late winter bloom” changes the trophic situation. These authors demonstrated a significant correlation between the GDH/$R_{NH_4^+}$ and respiration to excretion ($R_{O_2}/R_{NH_4^+}$) ratios, since the amount of $NH_4^+$ excreted is determined by the nitrogen content of the metabolized matter required for energy. On the other hand, Berges *et al.* (1993) pointed out the necessity of considering that enzyme activities scale allometrically with body mass and body protein in order to avoid erroneous interpretation of data when size structure of population is different. However, no attempt has been made to determine the influence of biomass scaling in both GDH and $NH_4^+$ excretion. If GDH is used as $NH_4^+$ excretion proxy, then it should follow equivalent size dependence.

Here, we address the issue of age and starvation as sources of variation in the relationship between GDH activity and $NH_4^+$ excretion in the mysid shrimp *Leptomysis lingvura*. We chose *L. lingvura* because of its widespread distribution in shallow marine waters around Canary Islands, which implies a significant role in the nitrogen cycle of the coastal ecosystem. Furthermore, our choice is strengthened by the arguments that mysids are critical in nutrient cycling and selective grazing in the near coastal environments (Linden and Kuosa, 2004). The main objective of this research is to provide better biochemical insight into *L. lingvura*’s nitrogen metabolism, which might be controlled under different physiological conditions by fluctuations in the glutamate pool and by allosteric regulation of GDH. Furthermore, we introduce spectrofluorometry as a technique for increasing the sensibility of the GDH assay and hence decreases the biomass needed for a successful analysis.

## Materials and methods

### Location and sampling

Marine mysids were sampled by diving in Risco Verde coast (27º51'26'' N, 15º23'11'' W), located in the south-east of Gran Canaria island. The zooplankters were collected over shallow sandy bottoms, between 8 – 12 m depth. They were patchily distributed, swimming in small swarms close to the sand layer and usually in the shade generated by rocks, which provides more protection from predators. Along with each sample, *in situ* temperature was recorded (19.5 ± 2 ºC). Scuba diving equipment, a 500 µm mesh size plankton net and plastic containers for storing the animals were used for sampling. Three species of mysids were identified: *Paramysis nouveli, Siriella armata* and *Leptomysis lingvura*. However, only *L. lingvura* was used for experimentation because its survival and fertility rates are high in culture (Herrera, 2009).

### Culture conditions

Once the mysids were captured, they were immediately transferred in 10 l buckets to a culture system installed in Las Palmas de Gran Canaria University (ULPGC). The installation consisted of six plastic trays (20 L each) suspended in a circulating water bath. Around 15% of the total water volume was replaced every day with new filtered seawater. The system was additionally stabilized by biological filtration, which oxidized the highly toxic ammonia to harmless nitrate via nitrification in colonies of *Nitrosomas sp*. Furthermore, mechanical filters installed in each tank removed excess food particles and other organic matter, which is produced during sloppy feeding and defecation. Physicochemical parameters were daily examined and maintained close to natural conditions. The ammonium, nitrite and nitrate never exceeded 0.1, 0.02 and 0.2 mg $L^{-1}$ respectively with pH and salinity being constant at 8.1 and 35 psu, respectively. Mysids were cultured under a 14:10 ligth:dark cycle and a thermostated temperature (21 ± 0.5 ºC) within the range registered in the sea (above). The tanks were gently bubbled to keep saturating oxygen conditions. Except for starvation experiments, mysids were fed twice a day with 48 h nauplii of *Artemia sp*., enriched with Easy-DHA Selco® (INVE, Belgium). Since other studies (Lussier et al. 1988; Domingues 1998) observed cannibalism of

the smallest sizes if food became limiting, we provided *ad libitum* conditions by offering 100 artemias per organism.

After an acclimation period of 24 h, the healthiest mature mysids were selected and separated in two new tanks in order to spawn. According to Domingues *et al.* (1999), optimal densities for maximizing reproduction ranged from 50 to 100 mysids $L^{-1}$. Populations that are too dense lead to a higher proportion of females with empty brood pouches (Luissier et. al, 1988), while concentrations below 50 mysids $L^{-1}$ would reduce the reproductive output. Consequently, 60 females and 10 males were placed together in a 1 L volume breeder box, while a total of 5 breeder boxes were arrayed in each reproductive tank. The hatchlings were collected and counted after one day. This cohort, which was composed of 420 hatchlings, was used to study the effect of age on physiological rates and GDH activity. For starvation experiments, however, male adults were required, but otherwise the procedures were the same.

**The influence of age on $NH_4^+$ excretion and GDH activity in *L. lingvura*.**

**(I) Excretory metabolism.** In the research reported here, we have assessed the impact of body mass on $NH_4^+$ excretion and GDH activity during the first thirty days of a mysid cohort, after which time these organisms become adults. In all the cases, animals were fed prior to experimentation. After feeding on artemia for an hour, mysids were placed for half an hour in Whatman GF/F filtered seawater in order to reduce stress during incubation. The experiments began once this acclimation period finished. Two to six mysids, depending on the biomass, were placed carefully in glass-capped bottles (60 ml each) filled with filtered seawater at 21 ºC and incubated in the dark. All the experiments included 1 control and 3 experimental flasks. After 30 minutes, the organisms were immediately transferred to new bottles and the water was siphoned for $NH_4^+$ determination. $NH_4^+$ excretion was measured spectrofluorometrically according to the Holmes method (1999), optimized for the $NH_4^+$ concentrations expected in this study. The mysid excretion rates were quantified by subtracting the $NH_4^+$ concentration in control flasks from the $NH_4^+$ concentration in experimental flask at the end of each incubation period. The procedure was accomplished with the same mysids for 2 hours, which allowed testing the linearity of these rates. Thus, there was no effect of incubation time (t) since the rates were constant over the 2 h incubation period. This short experimental time minimized

induction and repression of the mysid's enzymatic composition and also minimized potential effects of starvation.

**(II) GDH assay.** Once the incubation experiments ended, mysids were immediately frozen in N liquid and stored in the freezer (-80 ºC) for subsequent GDH analysis and protein determination. Later, the samples were thawed and kept on ice while awaiting analysis in order to prevent a decline in the protein activity. The mysids were placed in 2 ml of sonication medium composed of 100 mM Tris buffer, made up to pH 8.6 with acetic acid. Mysids were then sonicated for 50 seconds at 70% amplitude in a VXC 130 Sonics® device and centrifuged (0 – 4 ºC) for 10 min at 4000 rpm.

The supernatant fluid was assayed for GDH activity following a slightly modified Bidigare and King (1981) methodology. This modification consisted of using fluorometry rather than spectrophotometry to detect the NADH produced in the GDH reaction (above). The assay was run on an aliquot of the centrifuged extract (200 µl) that was mixed with $NAD^+$ and ADP solutions (300 µl and 250 µl respectively). Each reagent was made fresh daily and added separately to the mixture, prior to acclimation to the assay temperature. The resultant solution was incubated for a few minutes in a 1 cm path-length quartz cuvette until no fluctuations in NADH fluorescence were detected. Then, following the addition of glutamate (500 µl), the increase of fluorescence was monitored during 2 minutes with a Horiba Jobin Ybon Fluoromax 4® spectrofluorometer, at 360 nm excitation and 460 nm emission wavelengths. The final volume of the reaction mixture was 1.25 ml; it contained 1.2 mM $NAD^+$, 2 mM ADP and 50 mM glutamate. Assay temperature was controlled to the *in situ* temperature (21 ºC) by a thermostated multi-cell holder attached to a refrigerated recirculator. The fluorescence units were converted to activities (µmol $NH_4^+ \cdot h^{-1}$) from the [NADH]-fluorescence standard curve, which was prepared from pure GDH (1.4.1.3) extracted from bovine liver (from Sigma-Aldrich) for each batch of work. This curve was determined over a range of $2.8 \cdot 10^{-2}$ – $2.1 \cdot 10^{-4}$ international units (U) of GDH activity, where one U equals the amount of enzyme that converts 1 µmol $NAD^+$ per minute.

**(III) Biomass and protein determination.** Biomass was estimated as protein in the samples using the Lowry method (Lowry *et al.*, 1951) modified by Rutter (1967). Calibration curves were made from standard solutions of bovine serum albumin (BSA), in which

absorbance was read at 750 nm in a Beckman DU 650 spectrophotometer. Each data point represented the mean of triplicate analyses.

**The impact of starvation on physiological rates and GDH activity in *L. lingvura*.**

A new experimental approach was designed to address the effect of starvation on the biochemistry and the physiology of $NH_4^+$ excretion in *L. lingvura*. After acclimating the mysids in the culture system for two days, 60 mature well-fed males were transferred to individual containers. This procedure prevented cannibalism during the experimental period. In the base of each container was a 1 mm mesh size net, which allowed fresh filtered seawater to enter and mysid fecal pellets to exit. During 4 days, successive incubations were carried out in triplicate, using three control bottles in each experiment. Ammonium excretion, GDH activity and protein were assayed as previously described, although apparent Michaelis constants ($K_m$) for glutamate were also calculated on each time. Furthermore, in order to obtain the oxygen consumed to ammonia excretion ($RO_2/RNH_4^+$) ratio, $O_2$ consumption rates were estimated in the same incubation experiments by the continuous measurements of dissolved $O_2$ concentrations through a 6 – channel Strathkelvin 928 Oxygen System® respirometer. Mysid respiration rates were calculated from $O_2$ time courses as the difference between the linear $O_2$–t slopes in experimental and control chambers.

## Results

GDH activities are linear over an order of magnitude of biomass (0.01 – 0.14 mg protein) for both spectrophotometry and spectrofluorometry (Fig. 1). The results show a high coherence between the activities analyzed by the two techniques, with a high correlation coefficient of 0.93 ($p<0.01$) obtained from the non-parametric Spearman's test in SPSS® statistics software. This fact facilitates the comparison of our data with the others found in the literature. Furthermore, spectrofluorometry increases the sensitivity because it detects activity in at least three more dilutions below 0.011 mg protein in the assay. GDH activity in these dilutions is undetectable by the spectrophotometer. The low standard deviations of the samples quantify the superiority of fluorometry over spectrophotometry in this range.

The dependence of the GDH reaction on the substrates in a well-fed adult *L. lingvura* is characterized in Fig. 2. Both glutamate and $NAD^+$ follow the classic Michaelian hyperbole, where the $V_{max}$ was 1.60 µmol $NH_4^+ \cdot h^{-1} \cdot$ mg $protein^{-1}$ and $K_m$ was 5.61 mM for glutamate, while for $NAD^+$ the $V_{max}$ and $K_m$ were 1.97 µmol $NH_4^+ \cdot h^{-1} \cdot$ mg $protein^{-1}$ and 0.44 mM, respectively (Fig. 2). Kinetic parameters were calculated by classic Lineweaver-Burk transformation plots.

**The influence of age on $NH_4^+$ excretion and GDH activity in *L. lingvura*.**

The increases with age of $NH_4^+$ excretion and GDH activity during the life cycle *of L. lingvura* (Fig. 3) are consistent with allometric principles. Both physiological rate ($r^2$ = 0.89, n= 48) and enzyme activity ($r^2$ = 0.93, n = 48) show a strong correlation with age. The relationship between the physiology and the biochemistry is presented in Fig. 4. Statistical analysis based on the Spearman's test exhibits a strong correlation of 0.81 (n = 48, $p < 0.01$) when they are compared per mysid. However, this relationship is obscured by protein normalization (Fig. 5), so that the correlation becomes insignificant. Thus, while the normalized $NH_4^+$ excretion rates decrease with age, GDH activities remain relatively constant. As a consequence, the GDH activity to ammonium excretion ratio tends to increase slightly ($r^2$ = 0.5) as mysids grow, with an overall value of 9.90 ± 4.39 (mean ± SD).

Both metabolic rate and enzymatic activity per whole animal were regressed against protein mass in Fig. 6. The slope of the regression of the log-transformed data for GDH shows that its activities scale to a global exponent of 1.00 for protein data, which is considerably greater than the scaling exponent registered for $NH_4^+$ excretion (0.59).

**The influence of starvation on physiological rates and GDH activity.**

The time courses of $NH_4^+$ excretion rates and the GDH activity during food deprivation are presented in Fig. 7. In contrast to the decrease in the specific $NH_4^+$ excretion after 10 h of starvation, the specific GDH activities did not change as the mysids became starved. The GDH activity held a constant value around 1.73 (± 0.29) µmol $NH_4^+ \cdot h^{-1} \cdot$ mg $protein^{-1}$ throughout the entire experiment, so that the GDH/$R_{NH_4^+}$ ratio, as one would expect, increase. From an initial value of 11.2, it increases almost ten-fold to 102.4 (see table I). The apparent $K_m$s seemed to

increase during the first 26 h of starvation and then decrease following two days, with a slight increase when the mysids were fed again.

In regard to the effect of starvation on $O_2$ consumption rate, the maximum respiration coincided with the highest value registered for $NH_4^+$ excretion at the level of 10 h after feeding. Then, there is a decrease of more than six-fold in 1.5 days. The $RO_2/RNH_4^+$ ratio remains fairly constant since the variability on the highest values overlaps the smaller ones.

## Discussion

Most of the oceanographic research on GDH has been focused on the larger sizes of zooplankton despite the observation by Park *et al.* (1986) that nitrogen recycled by microzooplankton is four times greater, in terms of GDH activity, than the contribution of the macrozooplankton. The main problem with studying microheterotrophs' GDH lies in concentrating them in the field sufficiently to obtain a detectable signal in the spectrophotometer. As a consequence of this difficulty, King *et al.* (1987) could not evaluate accurately the potential $NH_4^+$ regeneration in the fraction of zooplankton below 153 µm. In this work we have approached the problem by applying of the advantages of spectrofluorometry (Segel, 1993) which increases at least six-fold the sensitivity of the assay by measuring NADH fluorescence. At low levels of activity, spectrophotometry has difficulty in discriminating between the true GDH signal and background artifacts. This explains the high variability in the spectrophotometric determinations when the enzyme concentrations in the analysis are too low (< 0.045 mg protein). On the other hand, high enzyme concentrations (> 0.075 mg protein) quench fluorescence and leads to underestimation of the true signal. This problem, however, can be solved by a simple dilution of the homogenate. Consequently, when available, fluorometry is superior to spectrophotometry and here we make the transition to this more sensitive methodology.

The Henri-Michaelis-Menten constant ($K_m$) for an enzyme is the most important biochemical property of the enzyme that one can measure (Friedman, 1981). It defines the chemical affinity that the enzyme has for its substrate, the potential control by the substrate over the enzyme reaction, and the approximate concentration of the substrate in the cell (its *in vivo*

concentration). Our $K_m$ estimation for glutamate on well-fed *L. lingvura* (5.61 mM) fall in the range of $K_m$ from other well fed marine zooplankters, i.e., from 2.6 mM (Bidigare and King, 1981) to 11.8 mM (Park, 1986). The dependence of GDH on $NAD^+$ has been much less addressed. Only Batrel and Regnault (1985) established a relationship with a first plateau at the level of 1.3 mM, but their measurements were irregular and scantily described. Our $K_m$ for $NAD^+$ (0.44 mM) confirms that the affinity of GDH from *L. lingvura* is higher for $NAD^+$ than for glutamate, and suggests that the role of $NAD^+$ in GDH control is more important than previously thought. This is consistent with the results described by Jeffries (1969), who did not find pronounced changes in zooplankton glutamate levels over a year. Their data imply that *in vivo* glutamate seems to stay constant through transamination. We conclude from our results that *in vivo* concentrations of glutamate and $NAD^+$ in a well fed *L. lingvura* are around 5.61 and 0.44 mM, respectively.

**The influence of age on $NH_4^+$ excretion and GDH activity in *L. lingvura*.**

The amount of $NH_4^+$ excreted by a well-fed adult *L. lingvura* over time (13.9 ± 1.93 µmol $NH_4^+ \cdot h^{-1} \cdot ind^{-1}$) accords with the rates of other mysids such as *Mysis relicta* (45 ± 3.2 µmol $NH_4^+ \cdot h^{-1} \cdot ind^{-1}$) (Linden and Kuosa, 2004), given the fact that these mysids are about three-times, or more, the size of *L. lingvura*. Furthermore, the values of GDH activity measured during mysid growth could account for the total $NH_4^+$ excretion registered at the same time. The high correlation between both parameters suggests an important role for GDH in the nitrogen metabolism, as was argued previously (Bidigare and King, 1981; Park *et al.*, 1986). On the other hand, Hernandez-Leon and Torres (1997) proposed that this strong correlation could be biased by the biomass unit used and by the small number of analyses given. However, in this work we have examined a similar number of samples (n =48) and find no evidence of bias. In addition, it is necessary to considerer that the $V_{max}$ obtained here represents a potential $NH_4^+$ excretion capacity of more than one order of magnitude greater than the directly measured $NH_4^+$ excretion rate. Since the substrate concentration required for the theoretical $V_{max}$ tends to infinity, the actual enzyme velocity (apparent $V_{max}$) measured, in function of the amount of substrate added (50 µM), was around the 90% of the true $V_{max}$, so the difference between the potential and the *in vivo* rate becomes even bigger.

So, why does the GDH activity exceed the $NH_4^+$ excretion rate by factors ranging from 6 to 18? If we assume that GDH activity sets the upper limit for the physiological rate of $NH_4^+$ excretion and that the $K_m$ is a proxy for the intracellular (*in vivo*) concentration of glutamate (Cleland, 1963), then other factors are limiting GDH activity to reduce it to the *in vivo* $NH_4^+$ excretion rate. In addition to substrate-based regulation, enzyme activity can be modulated by molecules serving as activators or repressors as ADP and GTP serve in the GDH reaction. Consequently, to understand the GDH/$R_{NH_4^+}$ ratio better, more research is needed involving the role of GTP and ADP as a regulatory molecules under different biological conditions.

The strong correlation between GDH activity and biomass ($r^2$ = 0.97) suggests that GDH is a constitutive enzyme and that GDH activity could serve as an index of zooplankton biomass in a mixed plankton sample. The potential constitutive nature of GDH would predict that its variability in face of environmental fluctuations should be more moderate than the variability of activity in enzymes that are known to be induced or repressed by environment changes. Assimilatory nitrate reductase, found in marine phytoplankton, is an example of such a sensitive enzyme. In the case of GDH, if it is a permanent component of cells, then accordingly, it will vary with carbon and nitrogen, i. e., biomass. However, as part of a cell's biomass, it would decompose with the cell death, and as a result, GDH would be a good index of living zooplankton biomass. This fact was previously noted with other mitochondrial enzymes, such as ETS (Martínez *et al.*, 2010).

It is well known that metabolic processes, including excretion, scale with body size in an allometric form defined by the equation $Y = a \cdot W^b$, where *Y* is the metabolic process, *W* is body weight and, *a* and *b* are constants. In that equality, *b* constitutes the scalar component which determines the relationship between metabolic rate and body mass. It is traditionally assumed that *b* is 0.75 when body mass is expressed as wet mass (Kleiber, 1961; Brown, 2007). However, in invertebrates, as in the case of the organism used here, body composition is highly variable with age (Mayzaud, 1986) so that the exponent must be reconsidered in terms of protein mass, which constitutes a relatively constant proportion of weight during growth. In this research we show how $NH_4^+$ excretion is affected by age with an exponent *b* of 0.59 ($r^2$ = 0.89), so that the smaller mysids hold higher metabolic rates per unit of protein than do the larger sizes. This value is slightly lower than the nitrogen-based slopes (b values) reported by Ikeda

and Skjoldal (1989), which ranged from 0.65 ($r^2$ = 0.83) in many species of Antarctic zooplankton to 0.8 ($r^2$ = 0.6) in zooplankters from Barents Sea. This dissimilarity can be explained partially by the different species of zooplankton studied, but also by the more rapid growth in *L. lingvura* due to its high metabolism. In contrast, GDH activity is linearly related to protein in the sample ($b$ = 1.003, $r^2$ = 93), which means that its specific activity is invariant over different body sizes. This finding is in agreement with the behavior of GDH ($b$ = 0.978, $r^2$ = 0.93) described by Berges *et al.* (1990) on different sizes of *Artemia franciscana*, but somewhat at odds with results by Mayzaud *et al.* (1994) on the copepod *Acartia clausi* ($b$ = 0.8, $r^2$ = 0.772). However, the latter presents a weaker correlation as a consequence of the use of a narrower range of sizes. Consequently, our resultant GDH/$R_{NH_4^+}$ ratio shows a small trend to increase as mysids become adults, especially in the first development stages. This fact suggests that the effect of size acts unevenly on physiological rate and enzymatic activity, since otherwise the slope would be close to zero. Nevertheless, the mean ratio obtained in this work is in the range of the other calculations in the literature (table II). The low value exhibited here is likely favored by the high post-feeding metabolism and by the relatively high excretion rates in the initial development stages.

**The influence of starvation on physiological rates and GDH activity.**

A common characteristic in the physiology of zooplankton is the rapid fall of the metabolic rates after depletion of the food source. Since Mayzaud (1976) described a dramatic decrease in nitrogen release after 12 h of starvation, the same trend has been widely reported in subsequent works (Ikeda and Skjoldal, 1980). In this study, $NH_4^+$ excretion diminishes almost three-fold in the first 20 h to a basal metabolism. Later, after 70 h of starvation, when a new pulse of food was offered to the mysids, the $R_{NH_4^+}$ increases.

With regard to the GDH activity and its apparent $K_m$, few studies have attempted to evaluate their variability under different trophic conditions. The first study was made by Park (1986) on two species of copepods, but on a larger time-scale and less resolution. Here, GDH seemed to be constant with external changes in food availability, although a small increase in its activity was observed as mysids starved. This might be explained by a reduction in substrate catabolism during the Tricarboxylic Acid (TCA) Cycle leading to decreased formation of GTP,

the main inhibitor of GDH. A more pronounced increase in GDH activity per mg of protein under food deprivation was found by Park (1986), who suggested a conversion of GTP into ATP due to the depletion of the high energy forms. However, the range of our results overlaps the variation he measured. This behavior of GDH explains occurrence of the highest value of the ratio GDH/$R_{NH_4^+}$ at the end of the starvation time. The variability in the kinetic parameters implies internal adjustments of amino acid catabolism as food becomes limiting. Healthy states result in a low apparent $K_m$ (4.7 mM) as a result of rapid protein consumption during growth. However, once the ingested food has been metabolized and no other fuel is available, mysids begin to use their own reservoirs as sources of energy and GDH reduces its affinity for glutamate during the first 24 h in order to prevent its depletion. Then, the basal metabolism seems to fall to its minimum level and the weak GTP generation via TCA restores a high apparent $V_{max}$. However, the *in vivo* activity is likely much lower due to the absence of substrates at this time.

The changes in excretion rate with starvation are dependent on the body reserves that animal metabolize for their energy expenditures. The atomic $R_{O_2}/R_{NH_4^+}$ ratio is used as an indicator of the fuel required for energy. Thus, it shows that the substrates oxidized are nearly constant, which is consistent with Kiørboe's (1985) finding in copepods. Values under 13 indicates a protein-based metabolism (Mayzaud, 1988), which largely occurs during the experimental period.

## Conclusions

**(I)** The use of fluorometry promises to improve the sensibility of the GDH assay at least six fold. This improvement could reduce substantially the amount of biomass required for the assay.

**(II)** GDH activity in *L. lingvura* can account the total physiological $NH_4^+$ excretion. The disparity between the potential and direct measurements, suggests a regulation of GDH by a regulatory mechanism stronger than a substrate control (allosteric control?).

**(III)** GDH activity trends with biomass and thus may serve as an index of zooplankton biomass in mixed plankton samples.

**(IV)** Body size affects the $NH_4^+$ excretion and GDH activity unevenly. This causes an increases in the variability of the GDH/$R_{NH_4^+}$ ratio.

**(V)** Starvation causes $R_{NH_4^+}$ and GDH activity to diverge as it does in $R_{O_2}$ and ETS activity.

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## Figures legends

**Figure 1.** (A) Effect of enzyme concentration (mg protein) on GDH activities measured spectrophotometrically and (B) spectrofluorometrically. Each point represents the mean of triplicates, with the standard deviations calculated for both techniques. (C) The correspondence between the activities (µmol $NH_4^+ \cdot h^{-1}$) obtained from the spectrophotometry (X axis) and from the spectrofluorometry (Y axis).

**Figure 2.** Enzyme kinetics of GDH exhibited in a well-fed mysid for the substrates of the reaction, glutamate (left) and $NAD^+$ (right). Top: the typical hyperbolic Michaelis–Menten curves. Bottom: Kinetic parameters extracted from the double-reciprocal transformations. Each *x* intersect is equal to $1/V_{max}$, and the regression slopes are defined by $K_m/V_{max}$.

**Figure 3.** (A) Protein mass, (B) $NH_4^+$ excretion rates and (C) GDH activities per mysid over the month of experimentation. Each data point represents the mean of triplicate analyses, where the standard deviation characterizes its variability.

**Figure 4.** Linear regression between GDH activity and physiological ammonium excretion per mysid.

**Figure 5.** Protein-normalized data of (A) $NH_4^+$ excretion rates and (B) GDH activities in function of age. (C) The time-course of the resultant $GDH/R_{NH4+}$.

**Figure 6.** Log-transformed $NH_4^+$ excretion rates (filled circles) and GDH activities (open circles) *versus* log-transformed protein mass. Linear regression equations are Log $NH_4^+$ excretion = 1.497 + 0.585 Log protein mass ($r^2$ = 0.89, n=16), and Log GDH activity = -0.072 + 1.000 Log protein mass ($r^2$ = 0.93, n = 16).

**Figure 7.** Impact of food deprivation on: (A) $NH_4^+$ excretion rates and (B) GDH activities of *L. lingvura*. The vertical broken line at 70 h represents the time in which one pulse of food was offered to the mysids again. Thus, filled circles represents the starved mysids, while open circles refers to the experimental organisms which were fed once more. Each data point was averaged from triplicate measurements except for the point at 68 h, where only one mysid was analyzed for being the only active swimmer after 2 h incubation. In both cases, only the data of starvation experiments were considered in calculating the curves.

## Figures and Tables

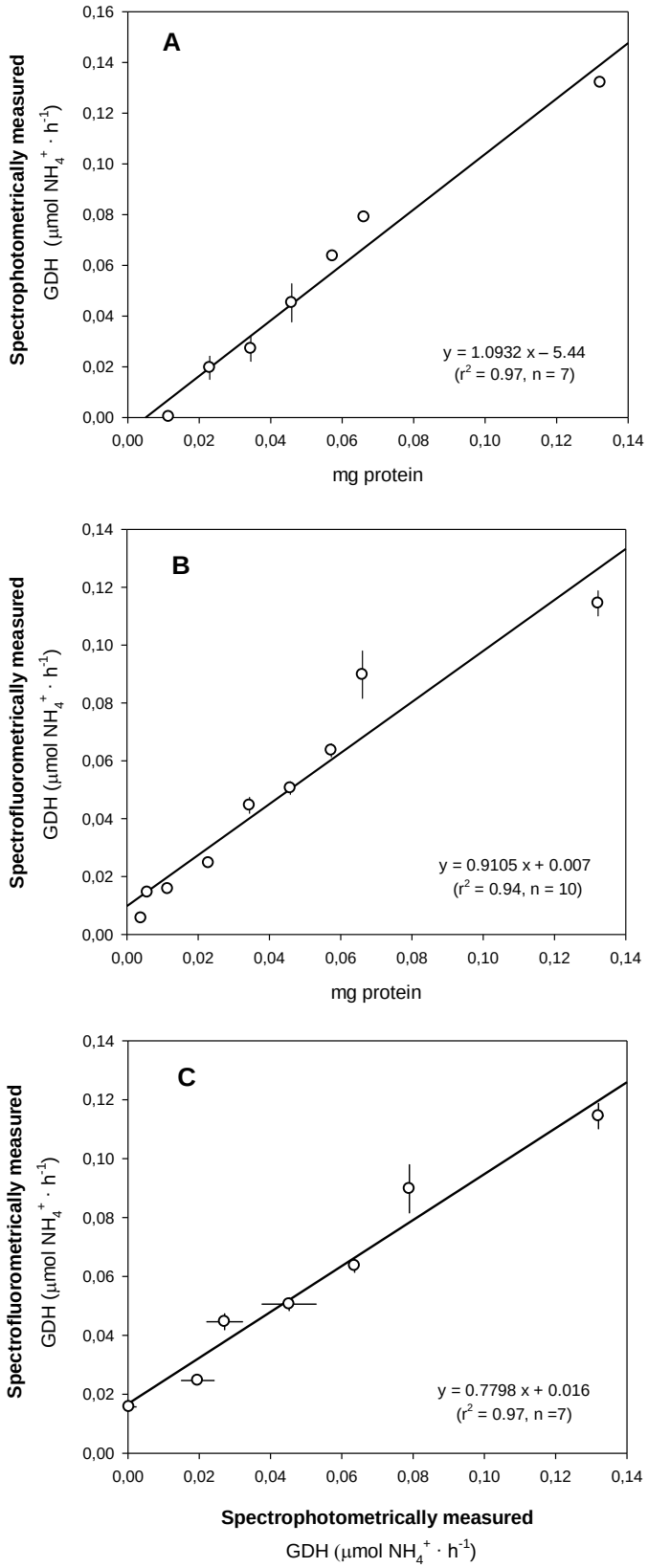



Figure 1

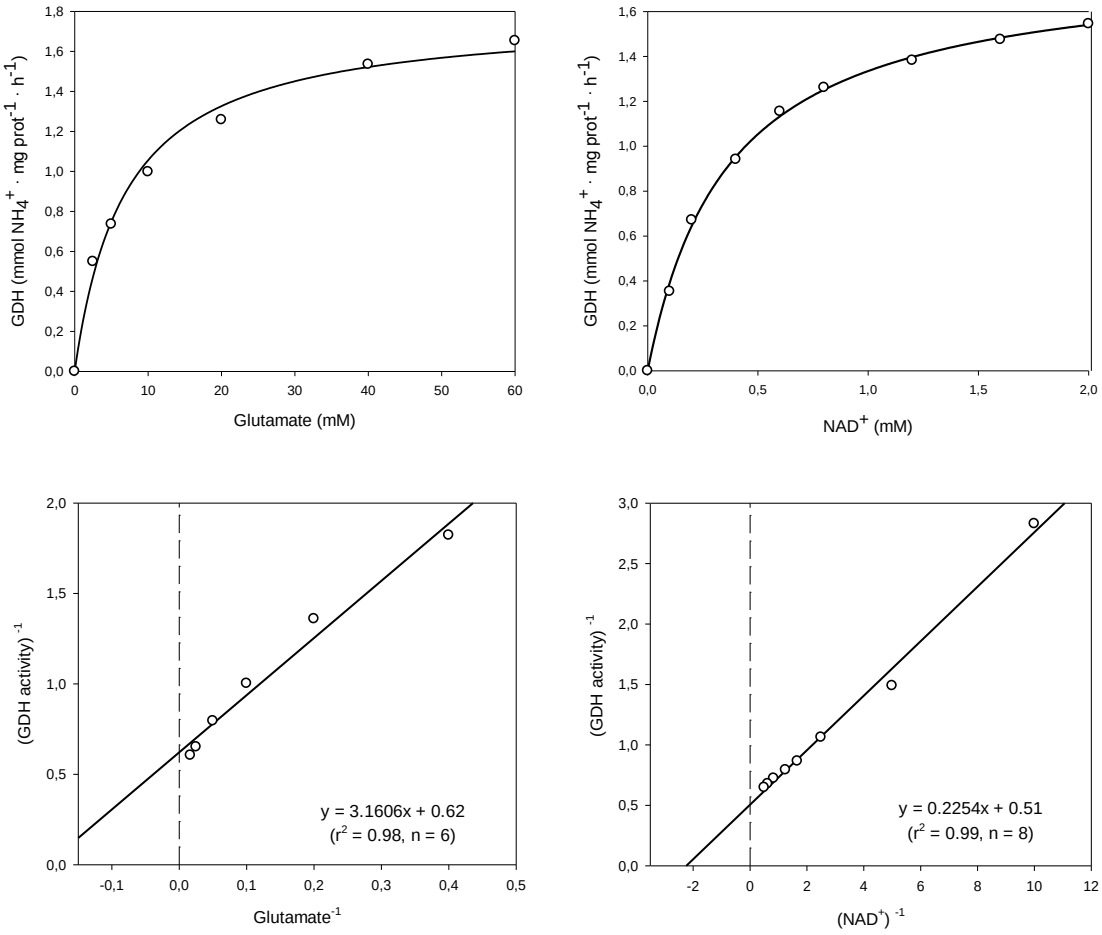

GDH (mmol $NH_4^+$ · mg $prot^{-1}$ · $h^{-1}$)
Glutamate (mM)
GDH (mmol $NH_4^+$ · mg $prot^{-1}$ · $h^{-1}$)
$NAD^+$ (mM)
(GDH activity) $^{-1}$
$Glutamate^{-1}$
y = 3.1606x + 0.62
($r^2$ = 0.98, n = 6)
(GDH activity) $^{-1}$
$(NAD^+)^{-1}$
y = 0.2254x + 0.51
($r^2$ = 0.99, n = 8)

Figure 2

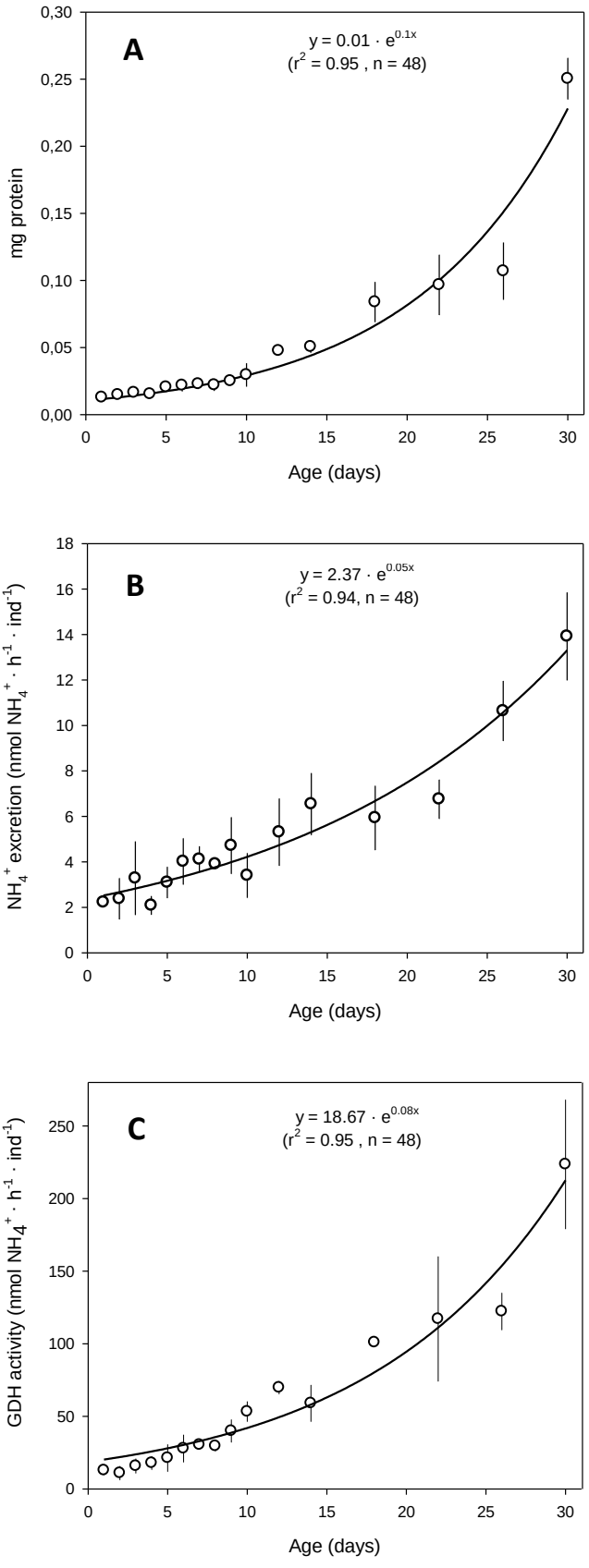

A
y = 0.01 · e^0.1x
(r² = 0.95 , n = 48)
mg protein
Age (days)
B
y = 2.37 · e^0.05x
(r² = 0.94, n = 48)
NH4+ excretion (nmol NH4+ · h-1 · ind-1)
Age (days)
C
y = 18.67 · e^0.08x
(r² = 0.95 , n = 48)
GDH activity (nmol NH4+ · h-1 · ind-1)
Age (days)

Figure 3

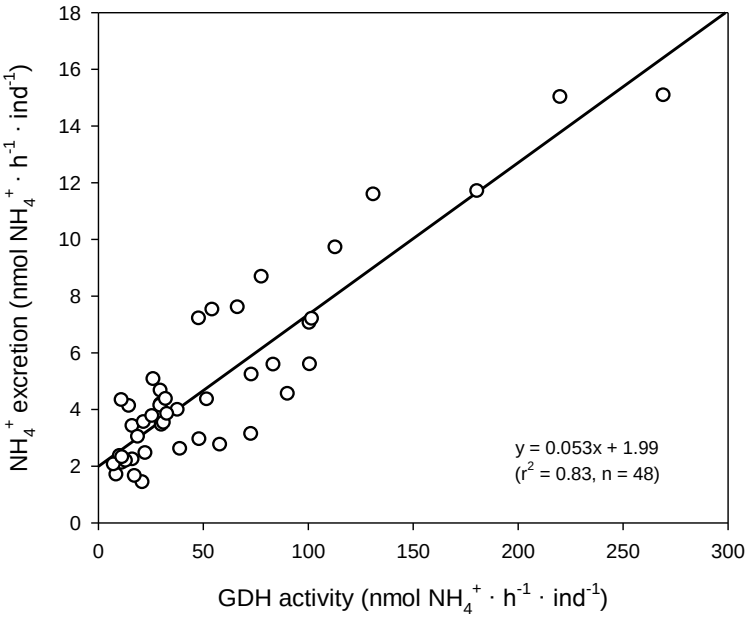

18
16
14
12
10
8
6
4
2
0
0
50
100
150
200
250
300
$NH_4^+$ excretion (nmol $NH_4^+ \cdot h^{-1} \cdot ind^{-1}$)
GDH activity (nmol $NH_4^+ \cdot h^{-1} \cdot ind^{-1}$)
y = 0.053x + 1.99
($r^2$ = 0.83, n = 48)

Figure 4

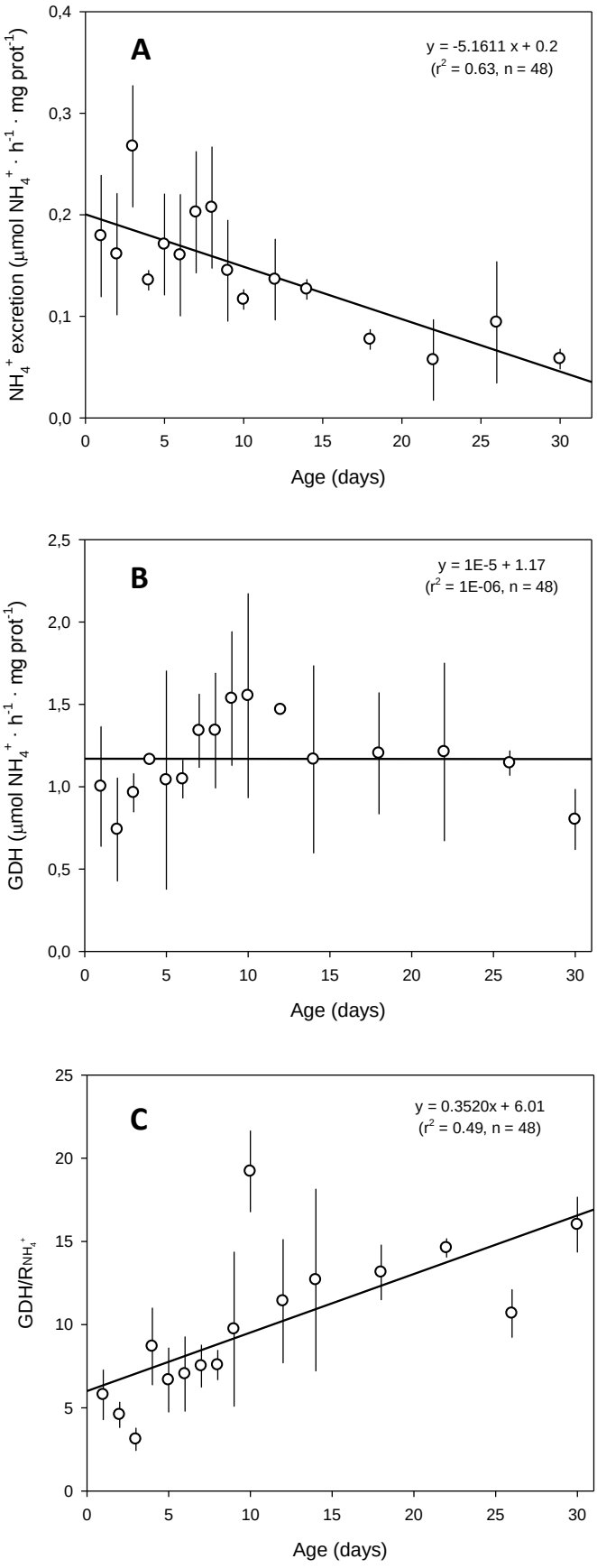

A
y = -5.1611 x + 0.2
(r2 = 0.63, n = 48)
NH4+ excretion (µmol NH4+ · h-1 · mg prot-1)
Age (days)
B
y = 1E-5 + 1.17
(r2 = 1E-06, n = 48)
GDH (µmol NH4+ · h-1 · mg prot-1)
Age (days)
C
y = 0.3520x + 6.01
(r2 = 0.49, n = 48)
GDH/RNH4+
Age (days)

Figure 5

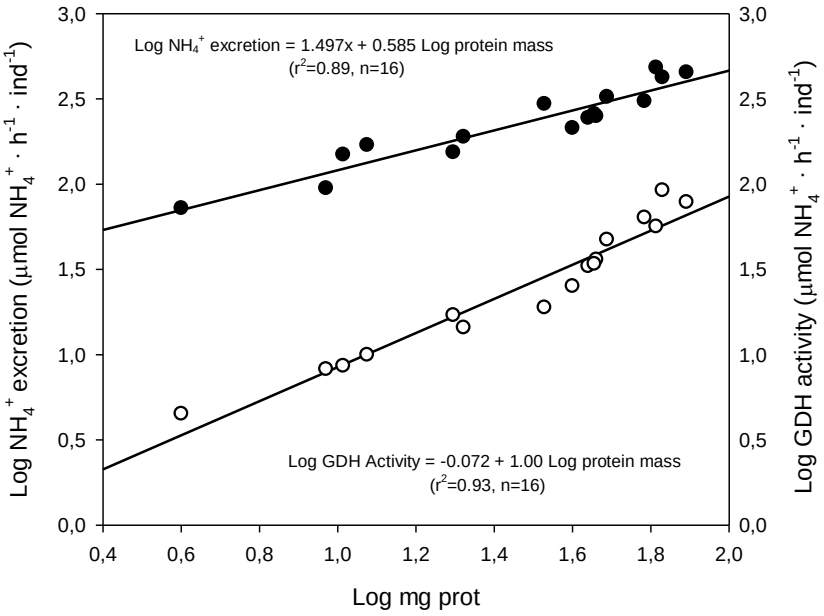
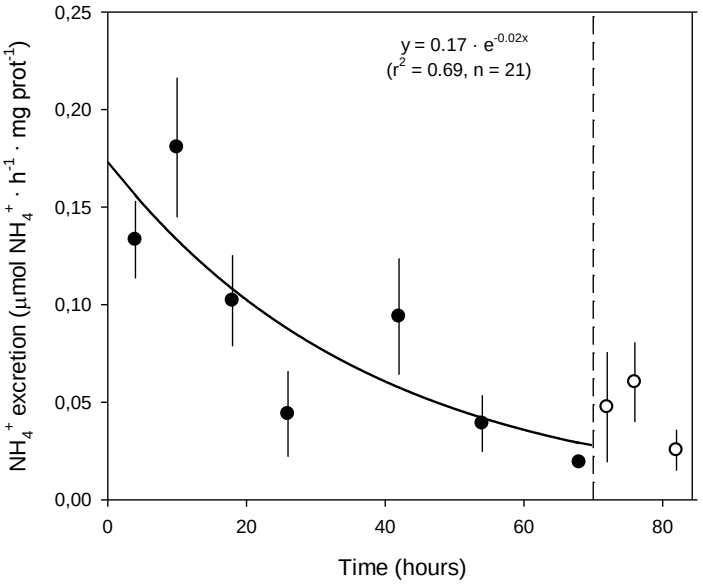

Log NH4+ excretion = 1.497x + 0.585 Log protein mass
(r2=0.89, n=16)
Log GDH Activity = -0.072 + 1.00 Log protein mass
(r2=0.93, n=16)
Log NH4+ excretion (µmol NH4+ · h-1 · ind-1)
Log GDH activity (µmol NH4+ · h-1 · ind-1)
Log mg prot
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0,4
0,6
0,8
1,0
1,2
1,4
1,6
1,8
2,0

Figure 6


y = 0.17 · e^-0.02x
(r² = 0.69, n = 21)
NH4+ excretion (µmol NH4+ · h-1 · mg prot-1)
0,25
0,20
0,15
0,10
0,05
0,00
0
20
40
60
80
Time (hours)

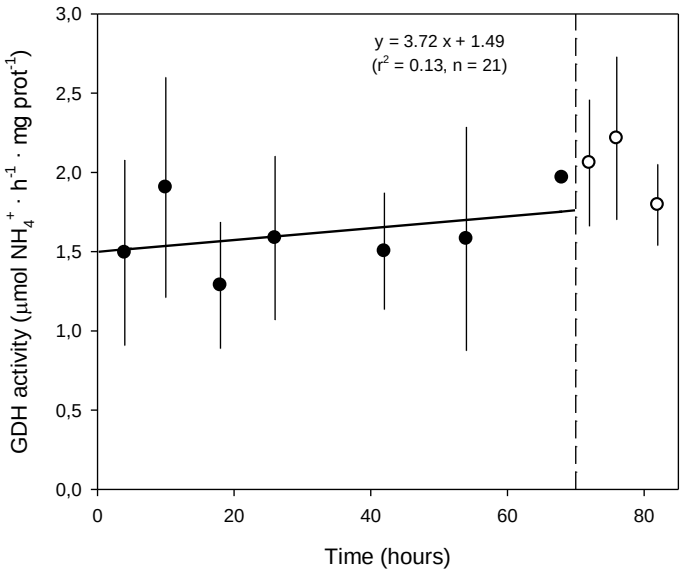

y = 3.72 x + 1.49
(r² = 0.13, n = 21)
GDH activity (µmol NH4+ · h-1 · mg prot-1)
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
20
40
60
80
Time (hours)

Figure 7

**Table I.** Effect of starvation on the Michaelis constants, and on the GDH/$R_{NH4}$+ and $R_{O_2}/R_{NH_4^+}$ ratios.

| **Starvation (h)** | **Apparent Km** (mM) | **GDH/$R_{NH4}$+** | **$O_2$ Consumption** (µmol $O_2 \cdot h^{-1} \cdot$ mg protein$^{-1}$) | **$R_{O_2}/R_{NH_4^+}$** |
|---|---|---|---|---|
| **4** | 4.69 ± 0.69 | 11.20 | 0.61 ± 0.09 | 6 ± 1.90 |
| **10** | 12.1 ± 5.9 | 10.54 | 1.29 ± 0.32 | 7.32 ± 1.65 |
| **18** | 8.55 ± 4.04 | 12.61 | 0.5 ± 0.15 | 4.94 ± 2.47 |
| **26** | 19.44 ± 10.8 | 37.78 | 0.6 ± 0.11 | 9.44 ± 1.73 |
| **42** | 4.67 ± 0.79 | 15.99 | 0.2 ± 0.18 | 3.73 ± 0.29 |
| **54** | 5.04 ± 0.27 | 40.42 | 0.22 ± 0.22 | 11.08 ± 3.25 |
| **68** | 2.84 | 102.37 | 0.24 | 12.9 |
| **2** | 4.89 ± 3.45 | 43.37 | 0.55 ± 0.05 | 15.23 ± 8.6 |
| **6** | 5.95 ± 2.48 | 36.71 | 0.74 ± 0.43 | 13.43 ± 2.96 |
| **10** | 6.25 ± 2.51 | 70.59 | 0.59 ± 0.36 | 21.16 ± 15.5 |

**Table II.** Values of the GDH activity to ammonium excretion ratios reported in the literature from zooplankters of different regions. Notice that the value of this work comes from the mean of the values measured in the well fed mysids.

| Sample | Location | GDH / $RNH_4^+$ | Reference |
|---|---|---|---|
| ***Leptomysis lingvura sp.* (n = 48)** | **Canary Islands** | **9.90 ± 4.39** | **Present work** |
| *Neocalanus plumchrus* | Strait of Georgia | 15.3 ± 4.3 | Campbell *et al*.. 2004 |
| Mixed mesozooplankton | Canary Islands | 17.86 ± 10.3 | Hernández-León and Torres., 1997 |
| Mixed zooplankton (n = 8) | Gulf of Maine | 23.4 ± 4.0 | King *et al*.. 1987 |
| Mixed macrozooplankton (n = 10) | Great South Bay | 18.18 ± 6.72 | Y. C Park *et al*.. 1986 |
| Mixed zooplankton (n = 5) | Gulf of Mexico | 18.7 ± 4.3 | Bidigare and King. 1982 |
| *Calanus finmarchicus* (n = 10) | Gulf of Maine | 16.8 ± 2.6 | Bidigare and King. 1981 |
| Mixed zooplankton (n = 5) | Antarctic | 18.7 ± 8.5 | Bidigare. 1981 |