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The effect of starvation on respiration rates, gut
fluorescence and electron transfer system
activity in *Daphnia magna*.

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Abstract

Respiration rates, gut fluorescence and electron transfer system (ETS) activity were studied during starvation using the classical water bottle method in mixed epipelagic copepods and, more extensively in individuals of *Daphnia magna*. In organisms incubated immediately after capture for short periods of time, the respiration rates decreased exponentially reaching the standard metabolism only after 3.5 hours of incubation. The difference between the higher value at the beginning of the experiments and the standard metabolism was more than 4-fold for copepods, and 1.6-fold for *D. magna*. Gut fluorescence followed the same trend, showing a closer relationship to respiration rates than other classical indirect methods such as the ETS activity, suggesting that the gut content could be a suitable index of metabolic states in proper herbivorous mesozooplankton.

Key words: zooplankton, respiration, gut fluorescence, electron transfer system activity.

INTRODUCTION

The role of the ocean in the global carbon cycle is one of the most important problems of the current science; whether it is a sink or a source of carbon is still an open question (del Giorgio and Duarte, 2002). Respiration is the main way to remineralize the organic matter produced by phytoplankton and thus, the major part of carbon fixed as primary production remains at the upper layers. Only a small fraction of carbon reaches the ocean's sediments, and it is related to gravitational flux and vertical migrations. Thus, the knowledge of respiration is essential to understand the vertical transport of carbon in the ocean.

Heterotrophic plankton is responsible for the bulk of respiration in the ocean, particularly planktonic microbes, but the contribution of mesozooplankton could have been underestimated. Hernández-León and Ikeda (2005a), in a global assessment, found that mesozooplankton respiration is 3 to 8-fold higher than other values previously estimated. Thus, accurate measurement of mesozooplankton respiration is needed in order to improve our knowledge of the energy flow through the water column.

The methods used to estimate respiration are mainly based on the measurement of oxygen consumption. Omori and Ikeda (1984) developed what is known as the “water bottle method”. In this simple method, the respiration rate is measured as the difference between the oxygen content in a control bottle (without organisms) and in an experimental bottle (with organisms) filled completely in both cases with filtered sea water. The use of filtered water produces starvation and, in these conditions, the respiration of the zooplankters decreases to a standard metabolic rate in few hours of incubation (Thor, 2003; Lehette and Hernández-León, 2010). In natural conditions, the metabolic rate of organisms reaches somewhere in between active metabolism, when animals swim actively to get food, and standard metabolism, when animals are at the minimum expenses of energy, normally because of the lack of food. This point in between is what is called “routine metabolism” and is the best approximation to field conditions (Hernández-León and Ikeda, 2005b). In previous studies, after the catch of organisms and before the experiments, there was a time of acclimation (from 1-2 hours to a whole day) to prevent a stress condition in the animals at the start of the measurements (Ikeda, 1977a; Ikeda 1977c; Hernández-León and Gómez, 1996). It was thought that the high values of respiration during the first 2-3 hours after the capture

were due to stress conditions. But Ikeda and Skjoldal (1980) found that this decrease in the metabolic rate is due to the lack of food during the incubation rather than the stress after capture. A recent study (Lehette and Hernández-León, 2010) shows that in experiments carried out during a short period of time, the values of respiration rate during the first 2 hours of incubation (measured as CO₂ production) could be 11-fold compared to standard rates in starving conditions, and this was observed only after 4-6 h of incubation.

In order to avoid the hard routine and time consuming work of direct measurements of respiration rates, some indirect methods have been developed during the last decades. In 1971, Packard proposed the use of the enzymatic activity of the electron transfer system (ETS) as an index of respiration. The ETS is the last enzymatic complex which takes part in the production of ATP and uses Oxygen as the last electron acceptor. This is the reason why the ETS activity is, theoretically, a good index of respiration. As a method, the ETS activity is estimated in saturating conditions, and this is why ETS activity is considered an index of potential metabolism. Through the last few years, some works have attempted to find a global ratio between ETS activity and respiration rate (Hernández-León and Gómez, 1996). However, the great variability in the relationship between ETS activity and respiration rate (Hernández-León and Gómez, 1996) has prevented the commonly use of ETS as a standard method for estimating physiological rates.

On the other hand, the gut fluorescence method has been commonly used as a tool to assess mesozooplankton feeding. Gut fluorescence measures the amount of chlorophyll *a* and pigments derived from chlorophyll *a* in the gut of the organisms.

Moreover, in zooplankters (especially herbivorous organisms as *Daphnia magna*) gut fluorescence represents the source of metabolic substrates and thus, their metabolic state. In this sense, Schmoker and Hernandez-León (2003) found a correlation between gut fluorescence and respiration rate using a flow-through system. However, a comparison of respiration, gut fluorescence and ETS activity is lacking in the literature about the water bottle method. Thus, the aim of this study was: i) To gain knowledge about the respiration rate of mesozooplankton in the field and in a laboratory culture and ii) to compare the values of respiration rates, ETS activity and gut fluorescence during short-period incubations, simulating the development of the above parameters during the classical water bottle method.

MATERIAL AND METHODS

Field experiments.

Epipelagic copepods were collected to the north of Gran Canaria (Fig. 1) on board the R.V. Atlantic Explorer. The capture of the organisms was carried out in vertical hauls (0-100 m) using a WP-2 net equipped with a 100 μm mesh size. The cod-end content was transferred to a 2L beaker filled with filtered sea water (Whatman GF/F). The mesozooplankton 500-2000 μm size fraction was selected for experiments. Mesozooplankton was transferred subsequently to a two beakers of 500 ml and 50 ml, filled with filtered sea water, in order to dilute the unfiltered sea water from the cod-end and to concentrate the organisms as much as possible.

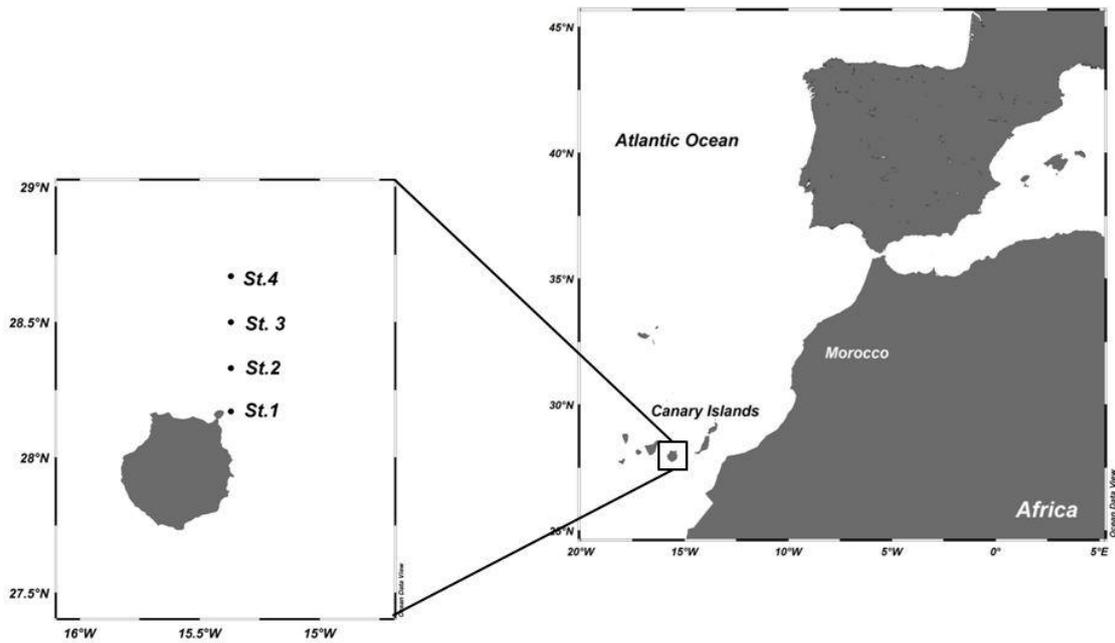


Figure 1. Location of the sampling stations during the R.V. Atlantic Explorer cruises.

The respiration rate was estimated by single measurements of oxygen consumption following the water bottle method proposed by Omori and Ikeda (1984). Nine 300 ml dark glass bottles were filled with aerated filtered sea-water. From 20 to 60 individuals were placed into the bottles. For each time interval (1 hour, 2 hours and 12 hours), one control and two experimental bottles were used. To measure oxygen content in the water we used polarographic electrodes connected to a Strathkelvin Instrument. The incubation took place in darkness at 20°C. The difference between initial and final oxygen concentration was taken as the respiration ratio.

Laboratory experiments.

In the laboratory, individuals of *Daphnia magna* were grown in saturating food concentration in a large container. This culture was grown in old tap water, between 23°C and 26°C and feeding on *Spirulina sp.*

The experiments were made in two steps. The first step was the feeding on green algae in saturating conditions. Three hundred individuals of *D. magna* were taken from the general culture and incubated in a 1L beaker filled with 300 ml of filtered (0.22 µm Whatman GF/F filter) and aerated old tap water from a common container. In this case, the supply of green algae came from a general culture of *Ankistrodesmus*. The final concentration was between 1.8×10^5 and 3×10^5 cells per ml, enough to get saturating conditions for *Daphnia* according to Simčič and Brancelj (1997) and Yebra (2001). The incubation period of the experiment 1 was 48 hours. For the experiments 2, 4 and 5 the incubation time was 24 hours. The temperature for all cases was maintained between 22°C and 23.2°C. The experimental conditions are shown in Table 1.

| Experiment | Date | t ^a of acclimation | Density of <i>Ankistrodesmus</i> (cell mL ⁻¹) | Density of <i>Daphnia</i> (ind mL ⁻¹) | t ^a of resp. rates measurements |
|---------------|----------|-------------------------------|---|---|--|
| Experimento 1 | 20/07/10 | 22.5°C | 2.75×10^5 | 0.55 | 25.1°C |
| Experimento 2 | 26/07/10 | 22°C | 2.15×10^5 | 0.7 | 25.4°C |
| Experimento 4 | 16/08/10 | 23.2°C | 1.8×10^5 | 0.6 | 23.4°C |
| Experimento 5 | 06/09/10 | 22.8°C | 3×10^5 | 1.04 | 24.4°C |

Table 1. Experimental conditions, including date, temperature during feeding and during the incubation for metabolism, and density of food (*Ankistrodesmus*) and individuals of *D. magna* during the feeding period.

After the feeding period, the second step involved the measurement of three variables. A half of individuals were transferred to a beaker filled with 500 ml filtered (0.22 µm Whatman GF/F filter) and aerated old tap water for the measurement of gut fluorescence, ETS activity and protein content at different times. At the same time, the other half of individuals were moved to three dark glass bottles (300 ml) for respiration measurements. The incubation period for this second step was 12 hours.

The respiration rates were estimated by the water bottle method described by Omori and Ikeda (1984). Four dark glass bottles (300 ml) were filled with filtered and aerated water from the common container. One was the control bottle and in the other three, 40 to 50 individuals were transferred from the feeding incubation beaker for the experimental measurements. An electrode from a Strathkelvin Instrument (SI) was fitted to each bottle. Oxygen values were recorded every second during 12 hours of incubation. The first measurements were taken after 1.5 hours in order to allow the electrode to stabilize. The temperature of incubation varied from 23.4°C to 25.4°C depending on the experiment. After this time, the individuals from the bottles were recovered and immediately frozen for protein determination.

The organisms used for gut fluorescence, ETS, and protein measurements were incubated in parallel to oxygen consumption measurements, in the same conditions of temperature and light. For each measurement, 7 to 10 individuals were picked-up, transferred to an eppendorf tube and immediately frozen in liquid nitrogen. All tubes were kept later at -80°C. The samples were homogenized in a Teflon pestle during 1 minute at 0-4°C using a phosphate buffer (0,05M, pH 8) containing Triton X-100. To measure gut fluorescence an aliquot of the homogenate was placed in a test tube with 10 ml of 90% acetone and kept at -20°C during 24 hours. For the measurement of fluorescence, a Turner Design fluorometer previously calibrated with pure chlorophyll (Yentsch and Menzel, 1963) was used. Pigments were calculated as chlorophyll equivalents using the measurements of fluorescence before and after acidification with 10% HCl by the equations given by Strickland and Parson (1972) slightly modified to:

$$\text{Chlorophyll } a = k \cdot (F_0 - F_a) \cdot \text{Individual}^{-1}$$

$$\text{Pheopigments} = k \cdot (R \cdot F_a - F_0) \cdot \text{Individual}^{-1}$$

Where k is the instrument calibration constant, F_0 and F_a are the fluorescence readings before and after acidification respectively and R is the acidification coefficient. The overall concentration of pigments refers to both chlorophyll a and pheopigments, expressed as $\text{ng pigments} \cdot \text{ind}^{-1}$.

The process for the evaluation of the ETS activity was based on the method of Packard (1971) and Owens and King (1975) and the modification introduced by Kenner and Ahmed (1975) and Gomez et al. (1996). The homogenate was centrifuged at 4000 rpm and 0°C during 10 minutes. An aliquot of the homogenate was directly used for the incubation at nearest *in situ* temperature (24°C) and darkness, in the presence of NADH, NADPH, succinate and a tetrazolium salt (INT) as an artificial electron acceptor. After 20 minutes, the incubation was stopped by a quench solution. The ETS activity was estimated spectrophotometrically at 490 nm with a turbidity base-line of 750 nm. For the activity of *in situ* temperature, the Arrhenius equation and an activation energy of $15 \text{ kcal} \cdot \text{mole}^{-1}$ was used (Packard, 1975)

Protein content was measured using the method of Lowry et al. (1951) and the modifications made by Rutter (1967), and using Bovine Serum Albumin (BSA) as standard. The conversion factor used for the relationship mg protein/mg DW , was 0.192 given by Postel et al. (2000). To convert into carbon units, we assumed that it is 40% of dry weight (Omori and Ikeda, 1984; Båmstedt, 1986)

RESULTS

The oxygen consumption rates of marine copepods showed a significant decrease after the first hours of incubation. Despite the few respiration measurements, it showed high values during the first hours of the experiment (Fig. 2). The higher value was reached one hour after the capture and averaged $1.29 \pm 0.51 \mu\text{O}_2 \text{ ind}^{-1} \text{ h}^{-1}$. This is more than 4-fold higher than the standard rate obtained after 12 hours of incubation ($0.29 \pm 0.11 \mu\text{O}_2 \text{ ind}^{-1} \text{ h}^{-1}$). Respiration rates estimated for *Daphnia magna* (Fig. 2), showed an exponential decrease. In these experiments, the metabolic rate reached the standard metabolism 3.5 hours after the beginning of the experiments and averaged $0.41 \pm 0.07 \mu\text{O}_2 \text{ ind}^{-1} \text{ h}^{-1}$ from 3.5 to 12 hours of incubation. After 2 hours of incubation we took the first respiration rate measurement, which was the higher value during the whole incubation period. The average for this interval was $0.66 \pm 0.13 \mu\text{O}_2 \text{ ind}^{-1} \text{ h}^{-1}$, 1.6-fold higher than the standard rate.

Analysis of the gut content by the gut fluorescence method showed a range of total pigments between 5.12 and 78.9 ng ind⁻¹ (taking into account all experiments). As shown in figure 3, the gut content decreased in an exponential way, following the same model as respiration rate. After 4 h of incubation, the gut content was 37% of the initial food content (from 66.51 to 24.4 ng ind⁻¹). Afterwards, the gut content reached the lowest value (12.2 ng ind⁻¹).

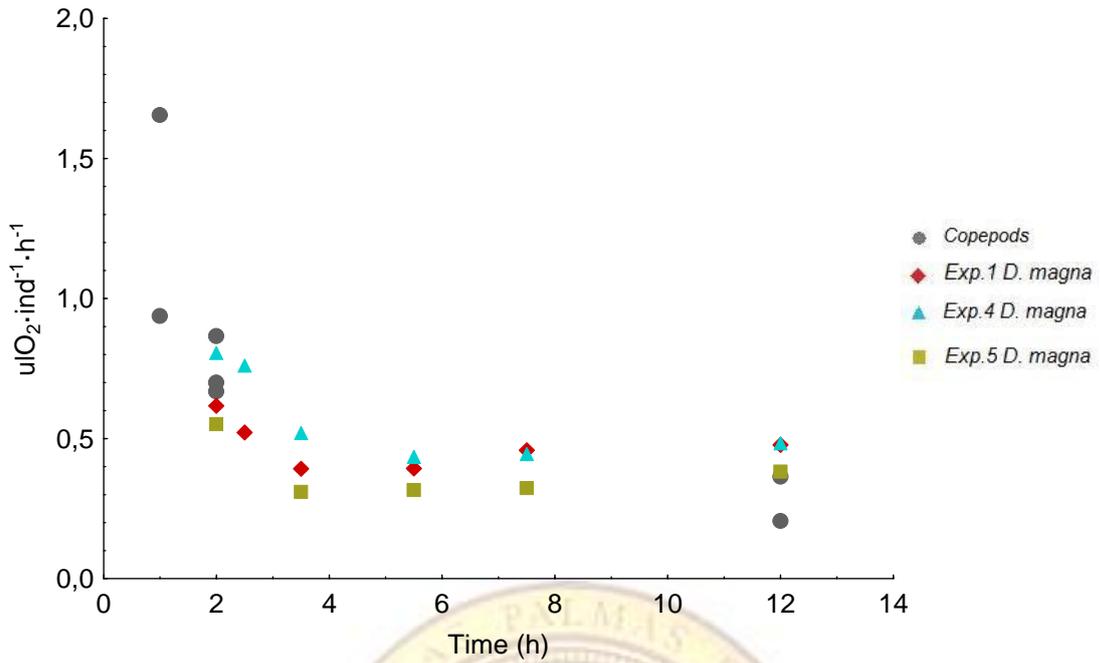


Figure 2. Respiration rates ($\mu\text{O}_2 \text{ ind}^{-1} \text{ h}^{-1}$) of epipelagic copepods (field conditions) and three experiments of *Daphnia magna* (laboratory conditions) versus time using the water bottle method.

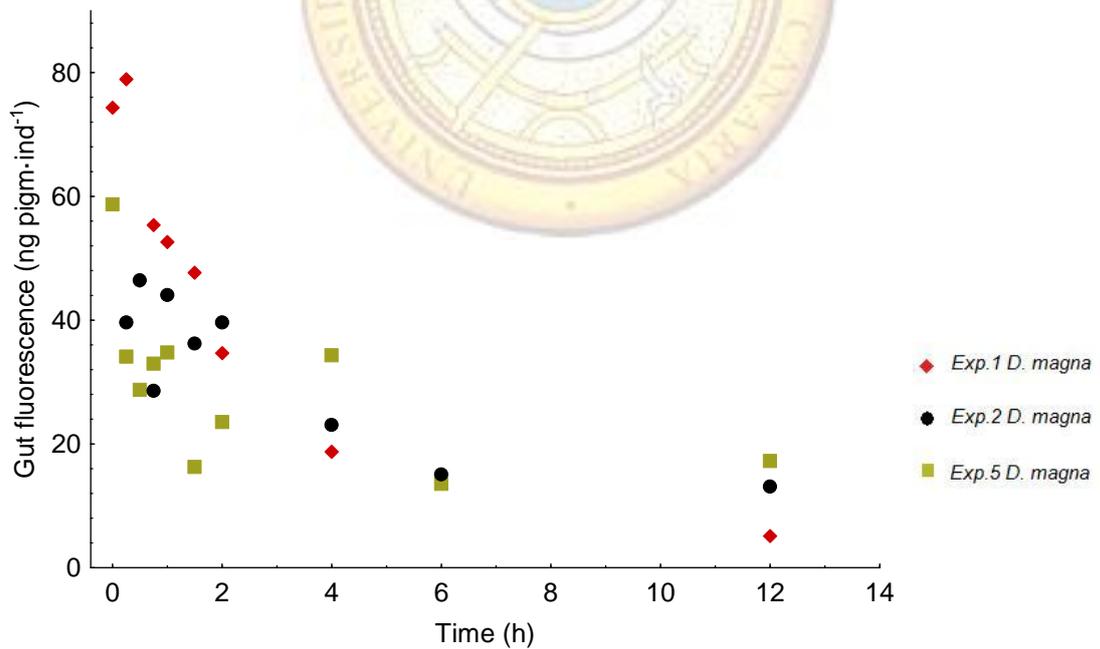


Figure 3. Gut fluorescence (ng pigment ind^{-1}) versus time in three experiments of *Daphnia magna*.

ETS activity, however, showed a quite different pattern (Fig. 4). Although experiments 2, 4 and 5 were carried out under the same conditions of temperature, light and body size, and similar conditions of food concentration, the results were rather variable. Organisms in experiment 1 were incubated for 48 hours at a food saturating level, but the ETS values were in the same range as in experiments 4 and 5, in which *D. magna* was feeding during 24h. The ETS activity in these experiments averaged $0.52 \pm 0.15 \mu\text{O}_2 \text{ ind}^{-1} \text{ h}^{-1}$ for the whole period of incubation. Experiment 2, however, showed an average ETS activity nearly 2 times higher ($1.1 \pm 0.28 \mu\text{O}_2 \text{ ind}^{-1} \text{ h}^{-1}$), despite organisms were incubated for 24h as in experiments 4 and 5.

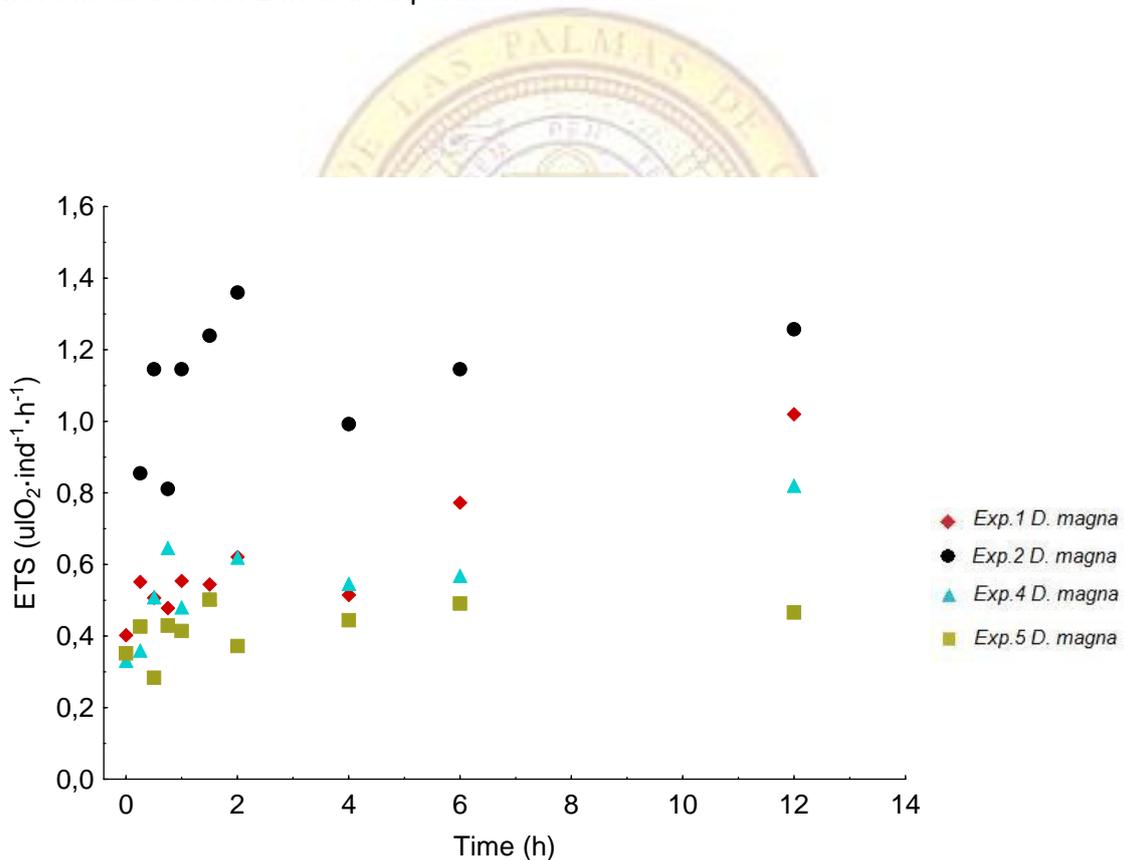


Figure 4. ETS activity ($\mu\text{O}_2 \text{ ind}^{-1} \text{ h}^{-1}$) versus time in four experiments of *D. magna* (laboratory conditions)

DISCUSSION

Commonly, a decrease in mesozooplankton respiration rates during starvation has been reported (Ikeda, 1977c; Skjoldal, 1984; Thor, 2003; Lehette and Hernández-León, 2010). The estimation of respiration rate during an incubation period usually involved the use of an acclimation time previous to the measurements in order to prevent stress conditions at the beginning of the experiments (Table 2). However, some authors (Ikeda, 1977c; Ikeda and Skjoldal 1980) found that the decay in the metabolic rate is due to the shortage of food rather than a stress condition produced by the handling of organisms. Normally, the nutritional state of zooplankters before the measurements affects the metabolic activity during starvation (Corner and Cowey, 1968; Porter et al., 1982; Richman and Dodson, 1983). This fact refers to the feeding history of individuals and the storage of food in their guts. Schmoker and Hernández-León (2003) developed an experiment to evaluate the effect of food supply in respiration rate of *Daphnia magna*. They found that after the introduction of food, the respiration rate increased 4-fold after feeding. This scope for respiration rate during starvation was also found by Lehette and Hernández-León (2010), measuring the amount of CO₂ produced by copepods caught from the field. The maximum rates were obtained at 0.5 hour of incubation and were 11-fold higher than the standard metabolism (reached after only 4 hours of incubation). Our values for respiration rates were within the range obtained by other authors (Table 2). In our experiments with copepods (field conditions) the maximum value, reached one hour after the capture, was more than four times the standard rate; with *Daphnia magna* (laboratory conditions) the difference between maximum value (after 2 hours of incubation) and

standard values was 1.6. From these results we suggest that to estimate respiration rates, at the laboratory or in the field, the results obtained during the first hours of incubation seems to be the more realistic approach.

Since Packard (1971) proposed the use of the ETS activity as a simple indirect method to estimate respiration rates, many advances have been made in this area. However there is not a global ratio between respiration rate and ETS activity yet (Hernández-León and Gómez, 1996). These authors studied the feasibility of the ETS activity as an index of respiration rate. They estimated the variability of the R/ETS ratio in different conditions of food quantity and quality, body size of the organisms and temperature. In general, they found a high variability in the R/ETS ratio. In our experiments, we found that ETS activity and respiration rate did not show any correlation. Even when the experimental conditions were similar, the ETS activity was different.

| References | Organisms | Respiration rates | Units | Temperature | Acclimation time |
|------------------------------------|-------------------------------------|-------------------|--|-------------|------------------|
| Ikeda (1976) | Subtropical copepods | 0.46-2.12 | $\mu\text{LO}_2 \text{ ind}^{-1} \text{ h}^{-1}$ | 22°C | 36 hours |
| Ikeda (1977b) | <i>Acartia tonsa</i> | 0.089-0.181 | $\mu\text{LO}_2 \text{ ind}^{-1} \text{ h}^{-1}$ | 22°C | None |
| Schmoker and Hernández-León (2003) | Cladoceran (<i>Daphnia magna</i>) | 0.5-4 | $\mu\text{LO}_2 \text{ ind}^{-1} \text{ h}^{-1}$ | 19°C | 24 hours |
| Li et al. (2004) | zooplankton | 0.64-0.84 | $\mu\text{LO}_2 \text{ ind}^{-1} \text{ h}^{-1}$ | 18-27°C | None |
| Present study | Copepods <i>D. magna</i> | 0.66-0.38 | $\mu\text{LO}_2 \text{ ind}^{-1} \text{ h}^{-1}$ | 25°C | None |

Table 2. The range of respiration rates estimated in this study and the respiration rates obtained by other authors for comparison.

On the other hand, in the present work we found a good agreement between the gut fluorescence and respiration rates and this suggests a coupling between the food supply and metabolism. This agreement was previously noticed by Schmoker and

Hernández-León (2003). However, the limitations of the gut fluorescence method, due to pigment destruction or the omnivorous diet in field organisms, make us consider this method as a metabolic rate estimator carefully. Nevertheless, the limitations are lower in herbivorous organisms such as *Daphnia magna*. Despite the weakness of this method, it seems to correlate with respiration rate closer than other classical methods as ETS activity.

In conclusion, both respiration rates and gut fluorescence showed a similar trend and different from that showed by the ETS activity. As suggested by Schmoker and Hernández-León (2003) incubating *D. magna* using a flow-through system, the current work also showed a relationship between respiration rate and gut fluorescence in short incubation periods using the classical water bottle method. However, more research in this area should be made in order to compare the above results to those of e.g. other mesozooplankton species.

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