Starvation effects on physiological parameters and biochemical composition of the hepatopancreas of the southern king crab *Lithodes santolla* (Molina, 1782)

Efectos del ayuno sobre parámetros fisiológicos y composición bioquímica del hepatopancreas de la centolla *Lithodes santolla* (Molina, 1782)

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Resumen.- Este estudio evalúa los efectos del ayuno en la centolla *Lithodes santolla*. Machos adultos de la centolla fueron mantenidos en condiciones de ayuno durante 12 días. Cada 3 días (*i.e*., 0, 3, 6, 9 y 12 días) se evaluó en subgrupos el consumo de oxígeno, la excreción de amonio, la relación atómica O:N y la composición bioquímica del hepatopáncreas. El índice hepatosomático (calculado individualmente como el porcentaje del peso húmedo del hepatopáncreas en relación al peso húmedo total del individuo) fue menor a 12 días de ayuno, coincidiendo con el menor contenido de proteínas en el hepatopáncreas. No se detectaron diferencias significativas en la composición bioquímica porcentual (contenido de agua, materia orgánica y cenizas). El contenido lipídico no fue significativamente diferente durante el experimento aunque el mayor valor fue registrado a los 3 días de ayuno. Entre los 3 y 9 días de ayuno, la excreción de amonio y el contenido de proteínas en el hepatopáncreas fue mayor y la actividad enzimática de tripsina, menor. Esto podría indicar que el catabolismo de proteínas fue el más importante durante ese período. Este hecho también se confirma por los bajos valores de O:N detectados en el período mencionado. A los 9 días de ayuno se incrementó el consumo de oxígeno, acompañado con un alto valor de excreción de amonio, originados probablemente por una demanda extra de energía producida por el ayuno. Se concluye que *L. santolla* para estos rangos de tiempo experimental utiliza proteínas como fuente primaria de energía.

Palabras clave: Cangrejos, consumo de oxígeno, excreción de amonio, lípidos

Abstract.- This study evaluates the effects of starvation on the physiological aspects of the southern king crab, *Lithodes santolla.* Adult males of this crab species were maintained in starving conditions for 12 days. Every 3 days (*i.e.,* 0, 3, 6, 9 and 12), subgroups were taken to evaluate oxygen consumption, ammonia excretion, O:N atomic ratio and biochemical composition of the hepatopancreas. The hepatosomatic index (calculated individually as the percentage of hepatopancreas wet mass in relation to the total body wet weight) was lower at 12 days starving period, coincidentally with the minor amount of hepatopancreatic protein. No significant differences in the relative biochemical composition (percentage of water, organic matter and ash) were detected. The lipid content was not significantly different during the experiment; nevertheless the higher value was detected at 3 days of starvation. Between the 3 and 9 days of fasting period ammonia excretion and hepatopancreatic protein were higher and the trypsin activity, lower. These results could indicate that protein catabolism was the most important in these periods. Besides, the lower values of O:N detected for all periods confirm this effect. On the ninth day of starvation oxygen consumption increased together with a high value of ammonia excretion probably due to an extra energy demand produced by starvation. We conclude that *L. santolla* in the experimental time-range study uses protein as the primary source of energy.

Key words: Crabs, oxygen uptake, ammonia excretion, lipids

Introduction

The southern king crab *Lithodes santolla* (Molina) together with *Paralomis granulosa* (Jacquinot) are the most important shellfish in Tierra del Fuego, Argentina. *L. santolla* is distributed from Chiloe (South Pacific) to Tierra del Fuego, and from the Magellan Strait to Uruguay (South Atlantic) (Macpherson 1988).

Nutritional deprivation is a natural part of the life cycle of many aquatic organisms (Sanchez Paz *et al.* 2006, Vinagre *et al.* 2007), which principally occurs during molt processes. Molting in crustaceans involves several stages with different feeding behavior and therefore, different kinds of energy from the available food. Artificially induced fasting and starvation may enlighten the metabolic routes used -in hierarchical order- during molt and may describe novel biochemical and physiological adaptation mechanisms (Barclay *et al.* 1983). Starvation induction of crustacean in

the intermolt stage is probably a good model to try to understand the molecular and enzymatic changes that occur naturally during their growing process, although the effect of hormones must not be forgotten (Sanchez Paz *et al.* 2006). Therefore, starvation studies may be useful predictors to determine energetic and metabolic requirements (Guderley *et al.* 2003).

As it has been pointed out by Wen *et al.* (2006), several studies of crustaceans´ metabolism have shown high variability of energy reserves mobilization as well as the sequence of substrates used for energy during starvation among crustacean species. The numerous possibilities must be the result of the vast diversity of environments that crustaceans inhabit and their long evolutionary histories (Sanchez Paz *et al.* 2007).

The hepatopancreas is involved in diverse metabolic activities, such as accumulation and cyclic mobilization of reserves, contribution of nutrients to the ovary during vitellogenesis, digestion and absorption (Sang & Fotedar 2004, Vazquez Boucard *et al.* 2004, Hasek & Felder 2005). In this sense, Sureshkumar & Kurup (1999) have established that the relative weight of the hepatopancreas reflects the provision of energy utilization for growth and metabolism. Particularly, the hepatopancreas is also considered the major storage organ in decapods crustaceans, mainly accumulating lipids and to a lesser degree, glycogen (see Sanchez Paz *et al.* 2007), as well as according to Rosas *et al.* (2000) who support that carbohydrates have low storage capacity and low capability of enzymatic processing.

On the other hand, levels of the digestive enzymes in decapod crustaceans do not remain constant during the developmental cycles (Van Wormhoudt 1974). The most common causes of the alterations of enzymatic values are seasons, circadian rhythm and amount and quality of food (Rodriguez *et al.* 1994, Fernandez *et al.* 1997), including the starving conditions (Cuzon *et al.* 1980, Leung *et al.* 1990, Comoglio *et al.* 2004). Among the digestive enzymes detected in crustaceans, trypsin is considered the most important in the digestion of proteins (Fernandez *et al.* 1997).

A strong relationship between food availability, metabolic rate and biochemical composition of the species considered have been pointed out by Mayzaud (1976). The O:N atomic ratio is considered a good indicator of used substrate for oxidative metabolism (Regnault 1981, Dall & Smith 1986, Chu & Ovsianico-Koulikowsky 1994, Rosas *et al.* 1995). Several theoretical minimum values of the O:N ratio have been proposed by different authors for strictly proteinic catabolism, depending on the basis of calculation. In this sense, to compute these theoretical ranges of values it is necessary to take into account the nature of the amino-acids actually entering the Krebs cycle and the catabolic pathway of fatty acids (Mayzaud & Conover 1988). According to Mayzaud & Conover (1988) theoretical values between 3 and 16 correspond to catabolism of pure proteins, whereas catabolism of equal quantities of proteins and lipids increases O:N ratio form 50 to 60. Greater values of O:N correspond to increase catabolism of lipids and carbohydrates.

Considering the importance to overcome starving periods in the life cycle, the aim of this study was to evaluate the effect of short starving periods on oxygen consumption, ammonia excretion, O:N ratio and biochemical composition of hepatopancreas in adult males of *Lithodes santolla* to describe the patterns of use of reserves.

Material and methods

Experimental conditions and design

Adults of *L. santolla* present different patterns of molt. While females have a molting-mating period during spring months (November-December), males molt during autumn months (March-April) (Vinuesa 1984). On the other hand, the fishery is locally regulated by a protective program which allows extracting only adult males being these under an important fishery pressure. For these reasons, only males were used in the present study in order to have an homogeneous group.

Adult males of *L. santolla* were provided by the local commercial fishery 'Pesquera del Beagle'. The organisms (N=25; average carapace length=73.7± 8.4 mm; average wet weight =292.5± 81.9) were transported to the laboratory and fed *ad libitum* with fresh mussels *Mytilus edulis* during two days of acclimation. This period allows the organisms to adapt adequately to the laboratory conditions, and minimize variations due to this factor. The experimental organisms were in intermolt period (Vinuesa 1984).

In the laboratory, crabs were measured (carapace length, CL hereafter; ±0.1mm) and weighed (wet weight; ±0.01 g) previously drained. Subgroups of 5 crabs each, were maintained in 60-L glass aquaria for 0 (control group), 3, 6, 9 and 12 starvation days. All groups were maintained at a water salinity of 30 psu, a temperature of 8±0.5°C and at a 12:12h light/dark photoperiod cycle. Experimental water was completely renewed daily to avoid eventual consumption of faeces and increment of ammonia concentration.

Seawater was filtered through a 10 µm polypropylene cartridge filter and UV-sterilized to minimize the activity of microorganisms, and then kept with constant aeration in 250 L dark tanks until use.

Physiological measurements

After each starving period, organisms were placed individually in 5240 mL respirometer chambers under a flow-through system (flow = 200±10 mL min^{-1}). During the adaptation period and physiological measurements all crabs were maintained in a minimum locomotive activity (routine). To obtain comparable data, all measurements were done at the same time of the day. Crabs were acclimatized for 2 h to reduce stress and, afterwards, a water sample from each chamber was taken to determine the initial concentration of both oxygen and ammonia. Then, chambers were sealed during 15 min, after which new samples were taken to measure final concentrations. One chamber without crab was added as experimental control for each treatment. Duplicate measurements of each sample were performed. The flow and the sealed period of the chambers were previously adjusted to avoid a depletion of oxygen concentration higher than 0.5 mg L^{-1}.

Oxygen concentration was determined by a polarographic oxygen electrode (YSI® 5100). Ammonia was determined according to Strickland & Parsons (1972). Consumed oxygen and excreted ammonia were taken as the net difference between the start and end of the sealed period. The O:N atomic ratio was estimated according to Taboada *et al.* (1998) using the individual values of oxygen consumption and ammonia excretion transformed to µg At g^{-1} h^{-1}.

Biochemical analyses

After each physiological measurement, crabs were weighed, sacrificed and then the hepatopancreas dissected and weighed (±0.01 g). Subsamples of approximately 1g of hepatopancreas were taken to determine the water and ash content, drying until reaching constant weight at 60ºC and 550°C, respectively. For lipid and protein analysis other subsamples of hepatopancreas were frozen until analysis. Lipid content was determined gravimetrically by the method of Bligh & Dyer (1959). Protein measurements were carried out according to Markwell *et al.* (1978), reading the optical density at 750 nm in spectrophotometer. All biochemical measurements were done by duplicate. The hepatosomatic index (HI) was calculated individually as the percentage of hepatopancreas wet weight in relation to the total body wet weight.

Trypsin activity

Subsamples of approximately 0.5 g of hepatopancreas were homogenized in 0.1 M Tris buffer (pH 7.8 at 4°C) in 1:3 w/v ratio. Homogenates were centrifuged (at 1000 g for 10 min, 4°C) and the aqueous supernatant, diluted (1:10 v/v), and then immediately used for enzyme analysis. Trypsin activity was measured by the method of Erlanger *et al.* (1961) with N-á-benzoyl-DL-Arg-p-nitroanilide (BAPNA) as substrate at 25°C. The absorbance was measured at 405 nm and one unit of enzyme activity was defined as 1 µmol of p-nitroanilide liberated in 1 min at 25°C.

The soluble protein content was measured by the same method described previously. Duplicate assays for each sample were made.

Statistical analysis

To detect significant differences among starving periods, one-way analysis of variance (ANOVA) was employed after confirmation of normality and homogeneity of variance. For the data to be expressed as percentage, an arcsine transformation was performed. When ANOVA revealed statistically significant differences among groups, *a posteriori* comparisons (Least Significant Differences) were used to identify which groups differed from one another. When data were not normally distributed, Kruskal-Wallis test and multiple comparisons of Dunn´s test (Daniel, 1978) were used. For both tests, P was set at 0.05. All statistical analyses were performed using STATISTICA (Statsoft) and analyses details were obtained from Sokal & Rohlf (1981).

Results

Survival, weight and hepatosomatic index (HI%)

During the experiment no mortalities were recorded. Besides, no differences were registered in the weight of crabs related with the initial individual weight (Table 1). The HI was lower at 12 days starving period (3.04± 0.24%) than at 9 days of starvation (5.14±0.15%). Particularly the average HI at 3 days of starvation was the highest but due to the register of a great deviation no significant differences with the other groups were detected (Table 2, Kruskal-Wallis, $P< 0.05$).

Oxygen consumption, ammonia excretion and O:N ratio

Oxygen consumption did not differ significantly till 6 days of starving conditions (mean value 25± 9 µg $h^{-1}g^{-1}$). At 9 days of starvation treatment, oxygen consumption increased significantly (56±14 µg $h^{-1}g^{-1}$) and at 12 days of treatment the consumption diminished again (37±2 µg $h^{-1}g^{-1}$) (ANOVA, $P<0.05$) (Fig. 1).

There were significant differences for ammonia excreted during the experiment. The highest values were detected in 3 and 9 starving groups (7.38±3.30 and 7.33±

Table 1

Differences in wet mass (mean± standard deviation) of *Lithodes santolla* after each starving period related with the initial weigh expressed as percentage

Diferencias en el peso húmedo (promedio± desviación estándar) de *Lithodes santolla* después de cada período de ayuno en relación al peso inicial, expresado como porcentaje

Days of starvation	Wet mass (average in g)	Difference in mass related to day 0 (%)
0	292.5±81.9	-------
3	288.9±91.3	0.1±0.9
6	271.6±98.9	0.0±1.0
9	267.5±122.1	-0.3±2.3
12	363.2±82.1	-0.3±1.4

2.46 µg N-NH_3 $h^{-1}g^{-1}$ respectively). The lowest values corresponded to the control and 12 days starvation groups (2.45±0.51 and 3.38±0.78 µg N-NH_3 $h^{-1}g^{-1}$ respectively) (ANOVA, P<0.05) (Fig. 2).

The O:N atomic ratio was significantly lower in the organisms starved for 3 and 6 days (4.30±1.05 and 2.21±0.86 respectively). At 9 and 12 days treatments the values (7.26±3.00 and 8.44±1.19 respectively) were similar to the control group (9.58±2.39) (ANOVA, P<0.05) (Fig. 3).

Biochemical composition of hepatopancreas

Along starving periods, there were no significant differences in the relative biochemical composition (mean values: water 73.92± 4.94%, organic matter 23.58±4.84% and ash 2.50±0.62%). The lipid content (in %) did not show significant differences among treatments either, but the highest value and deviation was detected in 3 days starvation treatment (6.40±2.76%) and the lowest value at 9 days starving condition (2.89±0.86%). Protein content (in %) was significantly different among treatments being high at 6 and 9 days starvation treatments (9.87± 0.33% and 9.15± 0.40% respectively). Considering the net amount of lipid in the hepatopancreas, there was no significant differences among treatments, but at 3 days of

Table 2

Hepatosomatic index (HI%) and relative biochemical composition of the hepatopancreas of *Lithodes santolla* after each starving period. All values are in average ± standard deviation

Índice hepatosomático (HI%) y composición bioquímica proximal del hepatopancreas de *Lithodes santolla* posterior a cada período de ayuno

Starvation days	HI (%)	Water (%)	Organic (%)	Ash (%)	Lipid (%) Net lipid (mg)	Protein (%) Net protein (mg)
0 (control)	4.41±0.22 $^{a\,b}$	76.30±0.73	21.24±0.80	2.46±0.11	3.03±0.60 *(362.63)*	7.33±0.48 $^{a\,b}$ *(866.07)*
3	5.51±2.63 $^{a\,b}$	71.35±7.37	26.47±7.75	2.18±0.34	6.40±2.76 *(1567.45)*	8.58±0.57 $^{b\,c}$ *(1459.42)*
6	4.39±1.03 $^{a\,b}$	74.80±4.49	22.61±4.78	2.59±0.31	4.40±1.17 *(411.36)*	9.87±0.33 ^{c} *(897.39)*
9	5.14±0.15 ^{a}	71.88±6.37	25.24±5.08	2.88±1.32	2.89±0.86 *(319.47)*	9.15±0.40 ^{c} *(835.74)*
12	3.04±0.24 ^{b}	75.25±3.66	22.32±3.68	2.40±0.20	4.43±1.21 *(426.89)*	6.89±0.29 ^{a} *(687.82)*

Values in the same column sharing different letter indicate significant differences (P <0.05) between starving periods. Values are expressed as percentage of mass of the total wet mass of the hepatopancreas. Values in parentheses indicate the average of total hepatopancreatic content in mg.

Los valores en una misma columna con diferente letra indican diferencias significativas (P <0.05) entre los períodos de ayuno. Los valores se expresan como porcentaje de masa del total de peso fresco del hepatopancreas. Valores en paréntesis indican el promedio del contenido total en hepatopáncreas en mg.

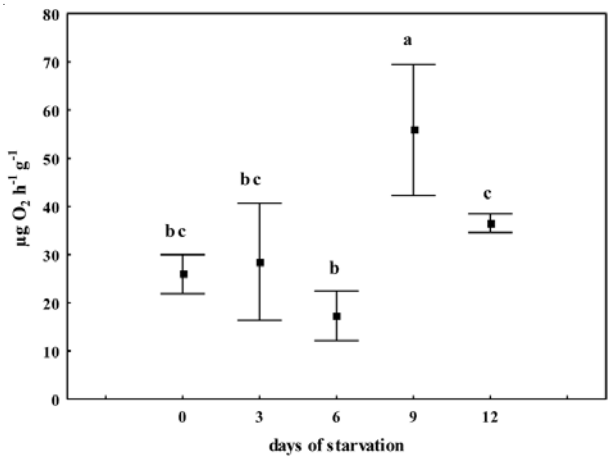

Figure 1

Routine oxygen consumption of *Lithodes santolla* after each starvation period. Dark points indicate averages and vertical lines indicate standard deviations. Different letters denote significant differences among treatments (*P* <0.05)

Consumo de oxígeno de *Lithodes santolla* a diferentes períodos de ayuno. Puntos negros indican el promedio y las líneas verticales indican las desviaciones estándares. Letras diferentes indican diferencias significativas entre los tratamientos (*P* <0.05)

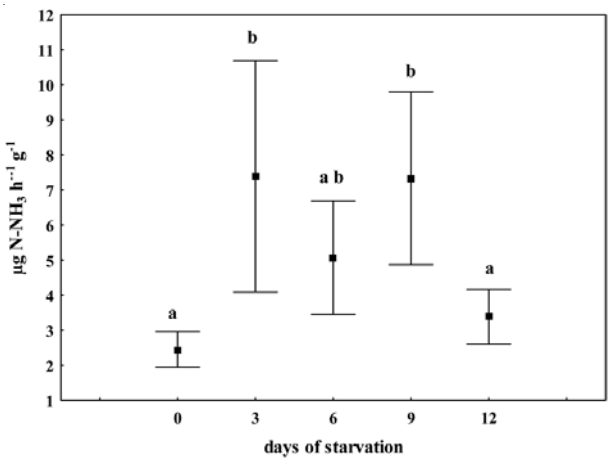

Figure 2

Routine nitrogen excretion of *L. santolla* after each starving period. Dark points indicate averages and vertical lines indicate standard deviations. Different letters denote significant differences among treatments (*P* <0.05)

Excreción nitrogenada de *L. santolla* a diferentes períodos de ayuno. Puntos negros indican el promedio y las líneas verticales indican las desviaciones estándares. Letras diferentes indican diferencias significativas entre los tratamientos (*P* <0.05)

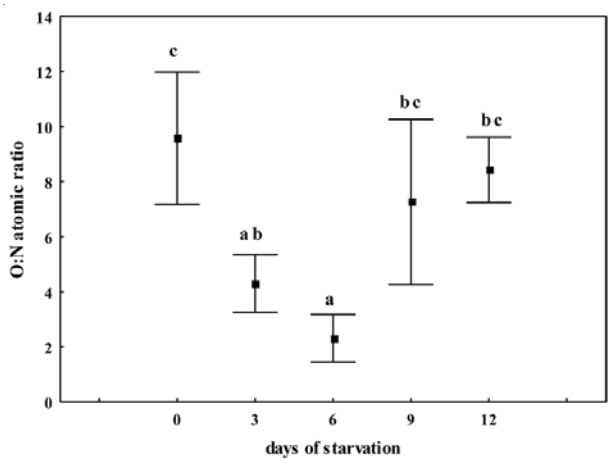

Figure 3

O:N atomic ratio of *L. santolla* after each starving period. Dark points indicate averages and vertical lines indicate standard deviations. Different letters denote significant differences among treatments (*P* <0.05)

Relación atómica O:N de *L. santolla* a diferentes períodos de ayuno. Puntos negros indican el promedio y las líneas verticales indican las desviaciones estándares. Letras diferentes indican diferencias significativas entre los tratamientos (*P* <0.05)

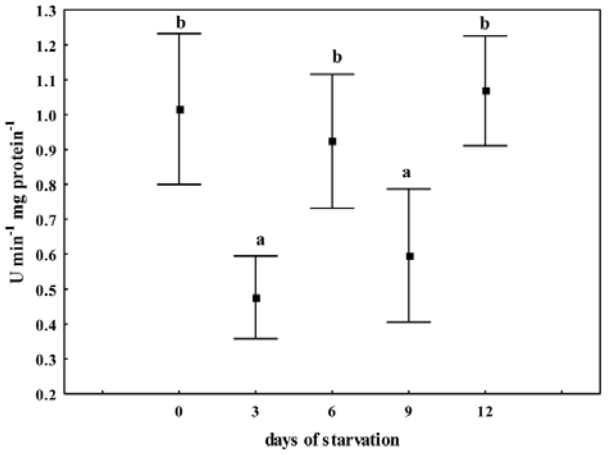

Figure 4

Trypsin activity of *L. santolla* after each starving period. Dark points indicate averages and vertical lines indicate standard deviations. Different letters denote significant differences among treatments (*P* <0.05)

Actividad enzimática de tripsina de *L. santolla* a diferentes períodos de ayuno. Puntos negros indican el promedio y las líneas verticales indican las desviaciones estándares. Letras diferentes indican diferencias significativas entre los tratamientos (*P* <0.05)

starvation the average lipid content was the highest (mean value 1567.45 mg). Respect to protein content the same tendency was observed at 3 days of starved conditions (mean value 1459.42 mg) and at 12 days treatment a significant decrease was registered (mean value 687.82 mg) (ANOVA, $P<0.05$) (Table 2).

Trypsin activity

The activity of trypsin was lower at 3 and 9 days starving periods (0.48±0.12 and 0.60±0.19 U min^{-1} mg $protein^{-1}$ respectively) (Fig. 4). The other treatments did not show significant differences with the control group (mean value 1.00± 0.19 U min^{-1} mg $protein^{-1}$) (ANOVA, $P< 0.05$).

Discussion

Due to the spatial and temporal food patchiness in the environment, nutritional deprivation is a natural part of the life cycle of many aquatic organisms (Mehner & Wieser 1994). The 100% survival of *L. santolla* in the present study showed the capacity of tolerance to starving conditions for 12 days in coincidence with what has been observed by Comoglio *et al.* (2004, 2005) for *Litopenaeus vannamei* and *Paralomis granulosa* respectively, exposed to starvation for similar periods.

The energy metabolism of crustaceans is characterized by high intra- and inter-specific variability, which makes it difficult to determine a standard metabolic profile (Oliveira *et al.* 2003). Most species reduce their metabolic rate and deplete protein, glycogen and lipid reserves during nutritional stress (Vinagre & Da Silva 1992, Oliveira *et al.* 2004, Comoglio *et al.* 2005). The relative importance of these reserves and their order of utilization vary according to the species, recent feeding history, diet composition and length of fast (Clifford & Brick 1983, Vinagre & Da Silva 2002, Vinagre *et al.* 2007). Some authors have mentioned that the metabolism in crustaceans is primarily based in glycogen and fatty acids (Welsh 1975, Wen *et al.* 2006), but in contrast decreased levels of protein have been noted during fasting in other marine decapods (Barclay *et al.* 1983, Dall & Smith 1986).

Starvation affects metabolic activities and during this period essential processes are maintained at the expense of accumulated endogenous energy reserves, which sometimes results in a loss of weight (Steffens 1989). However, some authors have detected that some crustaceans such as shrimps and lobsters compensate the weight of organic matter that they use in starving conditions with water uptake so that no loss of weight is detected (Dall 1974, Wilcox & Jeffries 1976). In the present study no changes in wet weight were registered, but we can not confirm that this corresponds to water incorporation; further research is needed to enlighten this effect.

On the other hand, lipids play an important role during the development of decapod crustaceans, not only as energy sources, but also as essential nutrients (Kanazawa *et al.* 1985). In the present study the decrease in hepatosomatic index observed in crabs starved for 12 days showed to be associated with the protein content, which was lower for this period, and not with the lipid content that remained constant along starving periods.

Between the 3 and 9 days of fasting period the increment in protein content of hepatopancreas, the increase of ammonia excretion and the low values of O:N could indicate, as mentioned by Mayzaud & Conover (1988), that the catabolism of protein was predominant. These authors have established that values between 3 and 16 would be possible for protein-dominated catabolism; this theoretical minimum was calculated for zooplankton species so that some variations could appear depending on the species considered.

In general terms, digestive enzymes follow the presence or absence of food. Samain *et al.* (1983) and Cuzon *et al.* (1980) have found that digestive enzymes increase in case of food deprivation with a peak, and then the enzyme production decreases as an adaptation to low nutrition status and to save energy. So these lower values (at 3 and 9 days of starving conditions) could be related to the mobilization of their own reserves, corresponding with the highest excretion of ammonia and higher values in hepatopancreas that indicated a dominating protein metabolism.

Taking into account that 1g of protein requires 0.94 L of oxygen in oxidative metabolism and that 1g of lipid requires 2.04 L (Mayzaud & Conover 1988), the increase in oxygen consumption detected at 9 days of the experiment accompanied with high value of ammonia excretion may be indicating an extra energy demand was produced by the starving and not by the use of lipids. Comparing the present results with the ones obtained previously for *P. granulosa* (Comoglio *et al.* 2005), we could conclude that both species, that co-inhabit the Beagle Channel, have different patterns of response to starvation, denoting in *L. santolla* the priority use of proteins in contrast with the use of lipids for *P. granulosa* for the same experimental time-range study.

The ability to withstand and recover from periods of nutritional stress is an important adaptation for survival of any organism that must sporadically endure periods of limited food supply (Stuck *et al.* 1996). For *L. santolla* although there were no significant differences detected

in the relative quantity of food consumed between seasons among mature crabs, during spring months the percentage of empty stomachs was higher and differences in the occurrence of different types of food was detected (Comoglio & Amin 1996). In general terms, for crab species feeding takes place throughout the year, except during a few weeks of the molting-mating period, when feeding ceases or is at a minimum (Jewett & Feder 1982). In this sense, the results of the present study give new and relevant biological information about the physiological and biochemical responses during starving condition about an important commercial species that inhabits the Beagle Channel.

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