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Energetic reserves and digestive enzyme activities in juveniles of the red claw crayfish *Cherax quadricarinatus* nearby the point-of-no-return

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ABSTRACT

Cherax quadricarinatus displays biological attributes that make it suitable for commercial aquaculture and, as a freshwater species, it has high starvation resistance. Previous studies demonstrated that after 40 days of starvation only the 25% (PNR_{25}) and after 50 days the 50% (PNR_{50}) of one-gram juveniles died. The objective of this study is to characterize the pattern of use of energetic reserves through the analysis of digestive enzyme activities and the occurrence of lipids, proteins and glycogen in the hepatopancreas and pleon muscle, nearby PNR_{25}, PNR_{50} and after a feeding period. One-gram juveniles were randomly assigned to one of two feeding protocols: continuous feeding throughout 90-days (control) and starvation until day 50 and then feeding for the following 40 days (treatment). Juveniles from each feeding protocol were weighed, measured and sacrificed at day 40, 50 or 90. Total lipids, glycogen and proteins were determined on hepatopancreas and pleon; and lipase, amylase and protease activities were also estimated in the hepatopancreas. Growth stopped during starvation and resumed when food was supplied. Close to the point-of-no-return 50 pleon muscle began to degrade diminishing protein content and the lipid content decreases significantly in the hepatopancreas. However, after the feeding period both reserves were completely replenished. Although glycogen levels were not affected during starvation, a pronounced accumulation of this nutrient in the pleon was triggered when food was available. The lipase activity decreased during starvation suggesting that the lipase whose activity was measured may not be synthesized when food is not available. Although starvation had no significant effect on the protease and amylase activities, they tended to decrease around the point-of-no-return and to increase after the feeding period. In this context, the present research provides new and relevant biological information on physiological and biochemical responses of crustaceans nearby PNR_{25} and PNR_{50}.

1. Introduction

Crustacean growth is the result of sequential molting (ecdysis), a complex hormonally controlled process that might last for days or weeks and is accompanied by morphological, physiological and behavioral changes (Anger, 2001). During this process an alternation of feeding and non-feeding periods is evident. When food is available, crustaceans can accumulate energy reserves in special storage organs. Their hepatopancreas is not only the most important site of synthesis and secretion of digestive enzymes, but also the main organ for digestion, absorption and storage of nutrients (lipids and, to a lesser degree, glycogen), which can be mobilized during the non-feeding periods (Icely and Nott, 1992; Ong and Johnston, 2006).

Studies on metabolism have shown that the pattern of energy reserve mobilization, and in particular, the sequence in which reserves are used as energetic source during short or long starvation periods, is highly variable among crustaceans (reviewed by Sánchez-Paz et al., 2006). This may be the result of the vast diversity of environments inhabited by crustaceans and their long evolutionary histories (Sánchez-Paz et al., 2007). During starvation, most decapods reduce their metabolic rate and deplete protein, glycogen and lipid reserves (Comoglio et al., 2005, 2008; Oliveira et al., 2004; Vinagre and Da Silva, 1992). Hence, the understanding of these biochemical processes may be useful to satisfy the energy requirements of organisms and optimize crustacean pond rearing efforts (Sánchez-Paz et al., 2006).

Anger and Dawirs (1981) defined the threshold time at which initially starved larvae lose their capability to recover from nutritional stress (even if they are fed ad libitum afterward) as the "point-of-no-return" (PNR). This point has often been utilized as an index to quantify the 'nutritional vulnerability' or 'nutritional flexibility' (Sulkin, 1978; Sulkin and van Heukelem, 1980) of a given species or developmental stage (e.g. Figueiredo et al., 2008; Gebauer et al., 2010). It is estimated from the PNR_{25} or PNR_{50}, which represents the time when 25 or 50%

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of initially starved animals lose their capability to molt. Several studies demonstrated that decapod larvae developing in habitats where food availability may be unpredictable or low (e.g., freshwater, high latitudes) are highly tolerant to starvation (Anger et al., 2004; Bas et al., 2008; Paschke et al., 2004).

The red claw crayfish, *Cherax quadricarinatus*, is a freshwater omnivorous species native to the North of Queensland (Australia) and the Southeast of Papua New Guinea. It displays a number of physical, biological, and commercial attributes that make it suitable for commercial aquaculture. In particular, crayfish are gregarious and non-aggressive, and they tolerate relatively high stocking densities. The species is also physiologically robust and can grow rapidly under a broad range of water quality conditions, such as hardness, alkalinity, oxygen concentration, temperature and salinity. Currently, the species is cultured intensively and semi-intensively in many countries including Australia, China, Ecuador, Costa Rica, México, Uruguay and Argentina. Many aspects about molting, reproductive biology, and hatchery management of the red claw crayfish have been recently reviewed by Ghanawi and Saoud (2012) and Saoud et al. (2013).

As a freshwater species, *C. quadricarinatus* has a strong starvation resistance. The first experimental laboratory studies on starvation resistance of juveniles were performed by Stumpf et al. (2010) and Calvo et al. (2012). They demonstrated that juveniles can tolerate intermittent feeding and long starvation periods, and that they are capable of undergoing compensatory growth following a feeding period. Also, they showed that 25% of one-gram juveniles died after 40 days of starvation (estimated $PNR_{25} = 41.2 \pm 0.1$ days) and 50% of them died after 50 days of starvation (estimated $PNR_{50} = 51.0 \pm 1.5$ days). The remaining animals considerably recovered after a feeding period of 40 days, based on the analysis of growth parameters and hepatopancreas structure (Calvo et al., 2012). More information about starvation capability and their energetic reserves may be useful to design new food or feeding protocols diminishing production costs and improving water quality.

The objective of this study is to characterize the pattern of use of energetic reserves through the analysis of digestive enzyme activities and the occurrence of lipids, proteins and glycogen in the hepatopancreas and pleon muscle, around the PNR_{25} and PNR_{50} and following a feeding period.

2. Material and methods

2.1. Conditions for broodstock maintenance and selection of juveniles

Juveniles were obtained under laboratory conditions from adult stocks supplied by Centro Nacional de Desarrollo Acuícola (CENADAC), Corrientes, Argentina. The reproductive stocks were placed on into 30-L glass aquaria (60 × 40 × 30 cm). Each aquarium containing 4 females and 1 male was checked weekly looking for ovigerous females. Four ovigerous females (mean wet body weight ± SD 59.8 ± 3.2 g) were placed individually into 30-L glass aquaria (60 × 40 × 30 cm) containing dechlorinated water (pH 7–8, hardness 70–100 mg $*$ L^{-1} as $CaCO_3$ equivalents) under continuous aeration to maintain a dissolved oxygen concentration of 5–8 mg $*$ L^{-1}, and a photoperiod of 14L:10D (Jones, 1997). Temperature was held constant at 27 ± 1 °C by electronic heaters (100 W). The females were fed daily ad libitum with frozen *Elodea* sp. and commercial balanced food for tropical fish, Tetracolor (Tetra holding (US) Inc., Blacksburg, Germany), containing 475 g $*$ kg^{-1} crude protein, 65 g $*$ kg^{-1} crude fat, 20 g $*$ kg^{-1} crude fiber, 60 g$*$$kg^{-1}$ moisture, 15 g $*$ kg^{-1} phosphorus, and 100 mg $*$ kg^{-1} ascorbic acid. This diet was previously found to be adequate for the studied species (Calvo et al., 2012; Stumpf et al., 2010; Vazquez et al., 2008). After reaching the free-living stage III, juveniles were separated from their mothers and maintained under the laboratory conditions described above until reaching about 0.5 g and then stocked individually. They were weighed after every molt and those of 1 ± 0.2 g were randomly assigned to the feeding treatments described below, according to Calvo et al. (2012).

2.2. Experimental conditions

The experimental design consisted of two feeding protocols based on previous results (Calvo et al., 2012). A total of 240 juveniles (mean initial weight ± SD: 0.94 ± 0.13 g) were sampled from 4 different mothers and randomly assigned to one of the two groups (control or treatment). The control juveniles were continuously fed throughout the 90-day experiment. The treated juveniles were not fed till day 50 and then were fed ad libitum by 40 days. From each group, 40 of the 120 juveniles were initially assigned to be sacrificed at day 40, 50 or 90 (Fig. 1). Forty and 50 days correspond to the PNR_{25} and PNR_{50} values previously estimated using the sigmoidal curve $M = 107 / (1 + \exp(-(x - 52.1) / 92.2))$, were M was the mortality (%) and x, the period of starvation (days) (Calvo et al., 2012).

During the experiment, the juveniles were placed in individual plastic containers (500 cm^3) with a piece of synthetic net provided as shelter (3 × 3 cm), and 350 mL of dechlorinated water with continuous aeration. These containers were placed in aquaria of 53 × 40 × 12 cm with water maintained at 27 ± 1 °C by ALTMAN water heaters (100 W, accuracy of 1 °C) (Calvo et al., 2011, 2012). Following the feeding protocols the juveniles were offered the nutritionally balance food Tetracolor (see above), which was provided ad libitum once daily. The water quality was monitored weekly. Physicochemical parameters were within the optimal ranges recommended by Jones (1997) for *C. quadricarinatus*: dissolved oxygen 5.6–7.7 mg $*$ L^{-1}, pH 7.6–7.9, hardness 65–95 mg $*$ L^{-1} as $CaCO_3$ equivalents, and nitrites <0.05 mg $*$ L^{-1}.

At the corresponding day (40, 50 or 90) the sacrificed juveniles were dried with a paper towel, weighed to the nearest 0.01 g and measured. The post-orbital cephalothorax length (POL) of each juvenile was measured from the postorbital margin to the lateral posterior end of the cephalothorax. The hepatopancreas and pleon were removed and weighed separately. From the total of 40 replicates for each time, three were oven-dried for 48 h at 50 °C to determine hepatopancreas and pleon dry weight and the remaining samples were stocked frozen at −80 °C for biochemical analyses.

2.3. Biochemical analyses

Glycogen and total lipids were determined in homogenates of wet pleon muscle or hepatopancreas from 2/3 juveniles, while total proteins and enzymatic activities were determined in homogenates of tissues from 5 juveniles. All determinations were performed in triplicate using spectrophotometric methods.

Total lipids were extracted by homogenizing the tissue with a mixture of chloroform–methanol (2:1V $*$ V^{-1}) described by Folch et al. (1957). According to that, the homogenate was filtered through a funnel with a folded filter paper to recover the liquid phase. Samples were washed with NaCl solution (0.9%) to obtain two layers. Then, the determination was made using the sulfo-phospho-vanillin method described by Frings

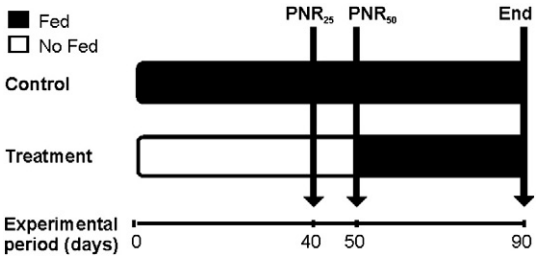

Fig. 1. General schedule of the protocol applied to one-gram juveniles of *Cherax quadricarinatus*. Black arrows indicate the times when juvenile samples were sacrificed.

and Dunn (1970). This method consists of oxidizing cellular lipids to small fragments after chemical digestion with hot concentrated sulfuric acid. After the addition of a solution of vanillin and phosphoric acid, a red complex is formed and is measured with a spectrophotometer at 530 nm.

Glycogen was extracted from tissues following the method described by Van Handel (1965), and glycogen levels were determined as glucose equivalent (glucose-oxidase method), after acidic hydrolysis (HCl) and neutralization (Na_2CO_3), according to the method described by Geary et al. (1981). Glucose was quantified using glycemia commercial kit (Wiener-Lab AA) and measured with a spectrophotometer at 505 nm.

Total soluble protein was determined with the Coomassie blue dye method, using serum bovine albumin as the standard, according to Bradford (1976), and measured with a spectrophotometer at 595 nm. All concentrations were expressed as $mg * g^{-1}$ of tissue.

In addition, the enzymatic activities of lipases, amylases and proteases were determined in the hepatopancreas. The lipase activity of each extract, expressed as units lipase $*$ mg protein^{-1} (one lipase unit is the amount of enzyme required to increase absorbance by 0.01 units at 540 nm per min), was determined according to Versaw et al. (1989). The assay mixture consisted of 100 μL sodium taurocholate (100 mM), 920 μL TRIS HCl (50 mM, pH 8) and 10 μL enzyme extract. After 10 μL β-naphthyl caprylate substrate [100 mM in dimethyl sulfoxide (DMSO)] was added, the mixture was incubated for 30 min at room temperature (25 °C ± 1). Then 10 μL Fast Blue BB (100 mM in DMSO) was added, and the mixture was incubated at the same temperature for 5 min. The reaction was stopped with 100 μL TCA (12%), and clarified with 1.35 mL ethyl acetate: ethanol (1:1V $*$ V^{-1}). Absorbance was recorded at 540 nm. The control tubes were prepared similarly, but crude extract was added after TCA solution. An additional negative control was made by replacing crude extract with TRIS HCl buffer.

Amylase activity of each crude extract was determined according to Vega-Villasante et al. (1993) with modifications. The assay mixture consisted of 500 μL TRIS HCl (50 mM, pH 7.5), 5 μL enzyme extract, and 500 μL starch solution (1% in TRIS HCl, 50 mM, pH 7.5). The mixture was incubated at room temperature (25°C ± 1) for 10 min. Enzyme activity was determined by measuring the production of reducing sugars resulting from starch hydrolysis, as follows: immediately after incubation, 200 μL sodium carbonate (2N) and 1.5 mL DNS reagent were added and the mixture was boiled for 15 min. Volume was adjusted to 7.3 mL with distilled water, and the colored solution was read at 550 nm. The control tubes were prepared similarly, but crude extract was added after DNS reagent. One amylase unit is defined as the amount of enzyme required to increase absorbance by 0.01 units at 550 nm $*$ min^{-1}.

Protease activity was assayed using 0.5% azocasein as the substrate in 50 mM TRIS HCl, pH 7.5 (García-Carreño, 1992). One proteinase unit is defined as the amount of enzyme required to increase absorbance by 0.01 units at 440 nm $*$ min^{-1}.

2.4. Calculations and statistical analyses

Growth was quantified in terms of a percentage growth increment (GI) calculated as follows: $GI = 100 * (W_t - W_0) * W_0^{-1}$ where W_t and W_0 were the juvenile wet weights when they were sacrificed (day 40, 50 or 90) and when the experiment started, respectively. Wet and dry hepatosomatic indexes were calculated according to Jones and Obst (2000): HSI_W (%) = 100 $*$ wet weight of hepatopancreas $*$ wet body weight^{-1} and HSI_D ($mg * g^{-1}$) = dry weight of hepatopancreas $*$ wet body weight^{-1}. Wet and dry pleon indexes were calculated as follows: PI_W (%) = 100 $*$ wet weight of the pleon $*$ wet body weight^{-1} and PI_D ($mg * g^{-1}$) = dry weight of pleon $*$ wet body weight^{-1}. The GI, HSI_W and PI_W were calculated for each sacrificed juvenile. The HSI_D and PI_D of treated juveniles sacrificed at day 50 could not be calculated due to insufficient samples. All variables were expressed as mean ± SD.

Parametric tests were applied when data met the model assumptions; otherwise, equivalent non-parametric tests were used. One-way ANOVA or the Kruskal–Wallis test (non-parametric) was used to test for differences in the GI, POL, HSI_W, HSI_D, PI_W, PI_D, lipid concentration, protein concentration, glycogen concentration, amylase activity, lipase activity and protease activity between treatments.

3. Results

The percentage of growth increment (GI), the post-orbital cephalothorax length (POL), and the hepatosomatic (HSI_W, HSI_D) and pleon (PI_W, PI_D) indexes was different between the experimental groups ($p < 0.05$) during the starvation period, with these variables being higher for the control animals than the treated ones at days 40 and 50. At the end of the experimental period, the treated juveniles showed increased values of the variables mentioned above, but only HSI_W, PI_W and PI_D were statistically similar ($p > 0.05$) to those of the control juveniles (Table 1).

3.1. Biochemical analyses

Total lipid concentration was significantly lower ($p < 0.05$) in the hepatopancreas and pleon muscle of treated juveniles at days 50 and 40, respectively, with respect to the control juveniles. This variable was similar ($p > 0.05$) to the control values afterwards (Fig. 2a and b).

The muscle and hepatopancreas glycogen concentration was similar ($p < 0.05$) in both experimental groups during the starvation period (days 40 and 50). However, after the feeding period (day 90) glycogen values were much higher ($p < 0.05$) in the pleon muscle of treated juveniles than in control juveniles (Fig. 2c and d).

The protein content in the hepatopancreas of the treated juveniles was significantly lower ($p < 0.05$) than that of the control group at day 40, and tended to reach control values ($p > 0.05$) at days 50 and 90 (Fig. 2e). The pleon muscle showed a lower ($p < 0.05$) protein

Table 1
Juvenile *Cherax quadricarinatus* exposed to two different feeding protocols. Mortality, growth increment (GI), post orbital length (POL), wet hepatosomatic index (HSI_W), dry hepatosomatic index (HSI_D) and relative pleon weight based on wet and dry weights (PI_W, PI_D) calculated at days 40, 50 and 90.

	Days			
Mortality (%)	40	5	0	
	50	10	15	
	90	30	20	
GI (%)	40	50.0 ± 21.7	−1.8 ± 6.7	*
	50	65.5 ± 37.8	3.4 ± 15.3	*
	90	149.8 ± 48.6	98.4 ± 29.7	*
POL (mm)	40	13.6 ± 0.7	12.1 ± 0.5	*
	50	14.0 ± 0.8	12.1 ± 0.6	*
	90	16.0 ± 0.8	15.3 ± 0.7	*
HSI_w (%)	40	7.2 ± 1.1	4.2 ± 1.1	*
	50	7.5 ± 1.5	4.2 ± 0.7	*
	90	7.8 ± 1.8	7.9 ± 0.9	
HSI_D ($mg * g^{-1}$)	40	2.0 ± 0.7	0.4 ± 0.1	*
	50	2.2 ± 0.6	nd	
	90	3.6 ± 0.6	3.3 ± 0.6	*
PI_W (%)	40	29.7 ± 1.7	25.3 ± 7.2	*
	50	29.2 ± 4.8	25.7 ± 6.1	*
	90	29.1 ± 5.4	29.5 ± 1.1	
PI_D ($mg * g^{-1}$)	40	66 ± 2.3	25.8 ± 2.4	*
	50	75 ± 8.0	nd	
	90	63 ± 14.8	71.6 ± 5.6	

All variables are expressed as mean ± SD; nd means that no data is available. Asterisks indicate significant differences ($p < 0.05$) between treatments at each time (40, 50 and 90 days).

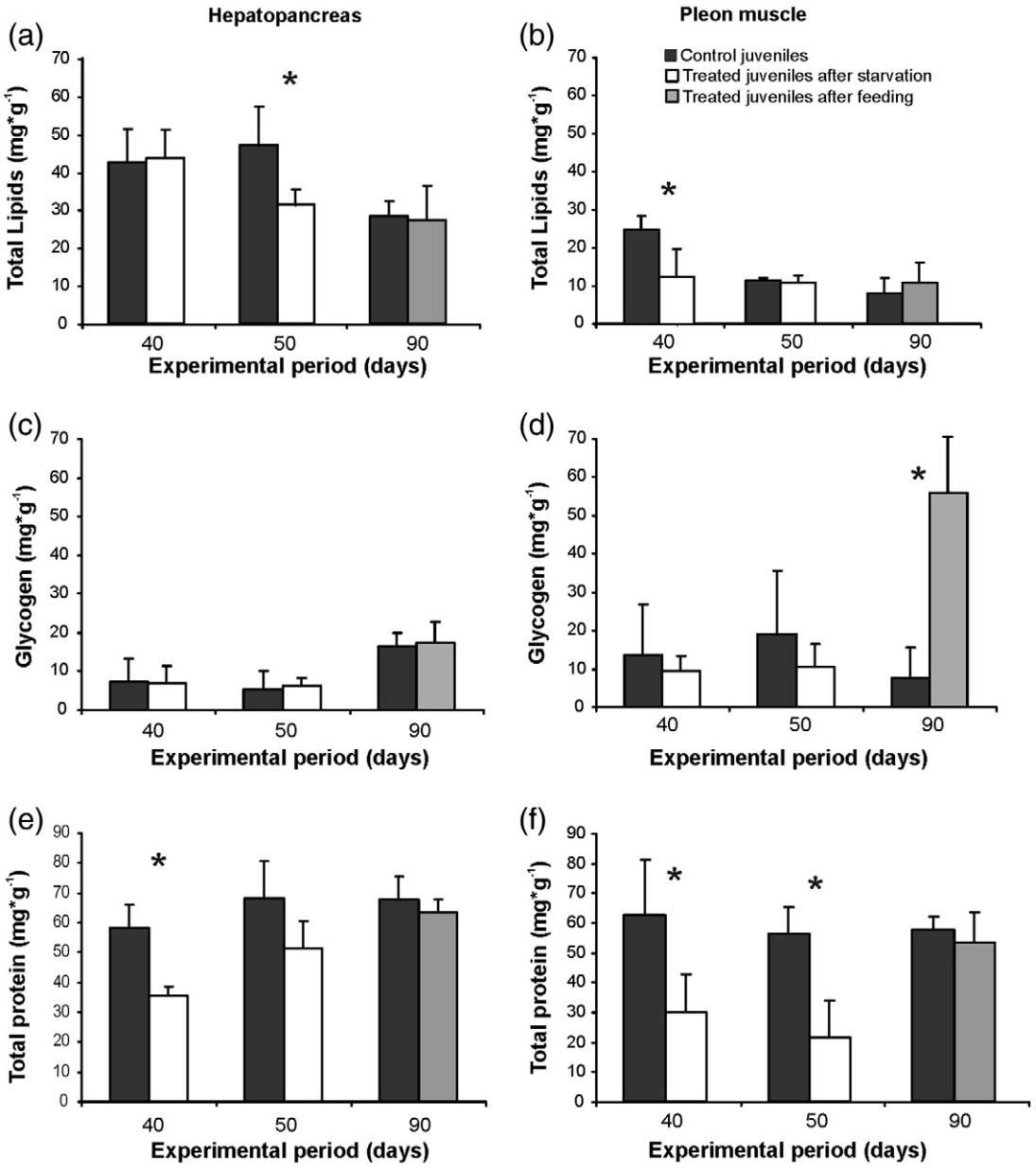

Fig. 2. Total lipid, glycogen and protein concentrations during the starvation period (50 days) and subsequent feeding until the end of the experiment (day 90) for *Cherax quadricarinatus* juveniles; error bars: mean ± SD. black bars: control group; white bars: treated group during starvation period; gray bars: treated group during feeding period; (*) indicates significant differences between groups ($p < 0.05$).

concentration during starvation period (days 40 and 50) in treated juveniles with respect to control juveniles, with this variable being similar ($p > 0.05$) in both experimental groups by the end of the experiment (Fig. 2f).

The lipase activity of treated juveniles was lower ($p < 0.05$) than that of control juveniles during the starvation period (days 40 and 50), but it was similar ($p > 0.05$) between both groups after the feeding period (day 90) (Fig. 3a). The protease and amylase activities were similar ($p > 0.05$) between the experimental groups throughout the experiment (Fig. 3b, c).

4. Discussion

C. quadricarinatus juveniles have been characterized as highly resistant to prolonged periods of starvation when compared to other decapod crustaceans (Calvo et al., 2012). Present results showed that growth (evaluated as GI and POL) stopped during a long starvation period. Moreover, the locomotion activity of treated juvenile decreased and their cuticle was duller than that of control juveniles after 30 days of food deprivation (personal observation). However, juveniles resumed their growth with high survival values when food was supplied. The fact that their weight increased much more than that of control juveniles after 40 days of feeding (98% and 50%, respectively) reflects their recovery capacity (see Table 1). Comoglio et al. (2004) and Pascual et al. (2006) studied the physiological response to starvation in other crustacean species and concluded that saving energy from exuvia, including the energy challenged to mobilize reserves, chitin digestion, and exuviations, is an adaptation to tolerate starvation. According to this, *C. quadricarinatus* may save energy from exuvia during starvation until food is available.

The relative wet weight of the hepatopancreas and pleon (IHS_W and PI_W) decreased during the starvation period and reached control values

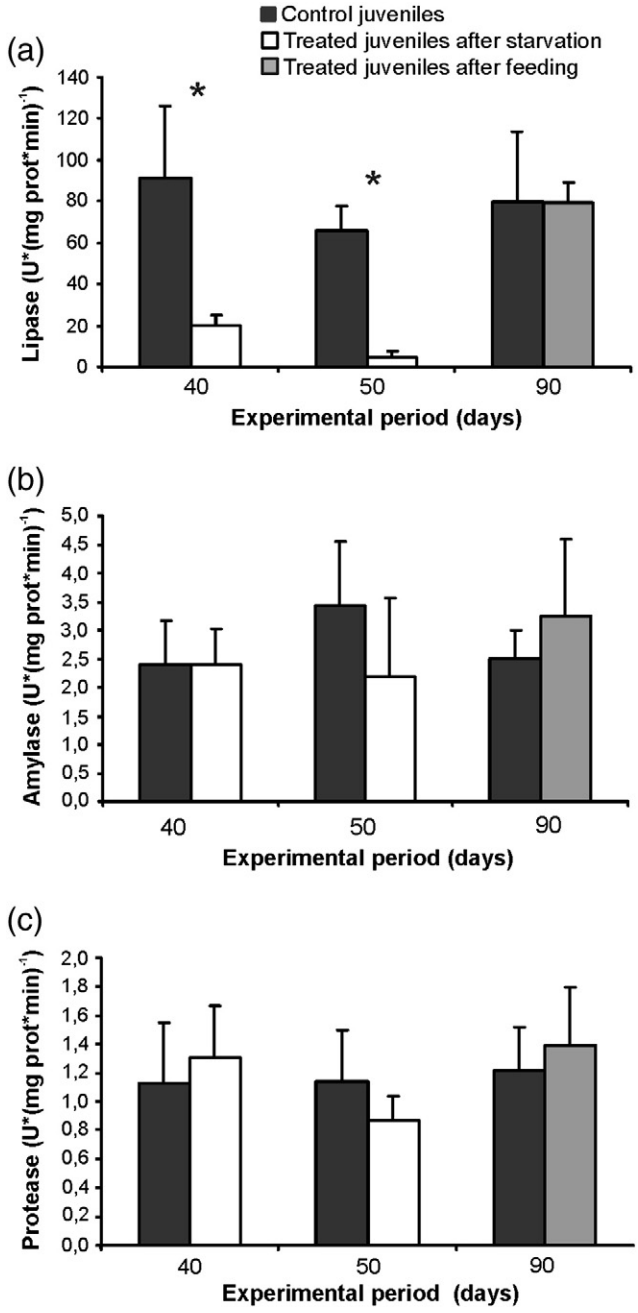

Fig. 3. Lipase, amylase and protease activities measured on hepatopancreas of *Cherax quadricarinatus* juveniles during the starvation period (50 days) and subsequent feeding until the end of the experiment (day 90); error bars: mean ± SD. black bars: control group; white bars: treated group during starvation period; gray filled bars: treated group during feeding period; (*) indicates significant differences between groups ($p < 0.05$).

after the feeding period. However, results concerning the relative dry weight of both organs in treated juveniles demonstrate that while matter content was recovered in the pleon after the feeding period (PI_D), the hepatopancreas weight reached the control value mainly by increasing its water content (IHS_D). Water uptake, which was also observed in other crustaceans such as shrimps and lobsters, could compensate for the lost of weight resulting from organic matter used under starvation conditions (Dall, 1974; Jones and Obst, 2000; Wilcox and Jeffries, 1976).

On the other hand, the energetic reserves analyzed in the present study were affected by starvation in different ways: while glycogen concentration in hepatopancreas and pleon muscle did not vary during the starvation period, lipid and protein content decreased in both organs compared to the control. The protein level was mainly affected in the pleon muscle, which is considered the most important protein reserve in crustaceans (Barclay et al., 1983; Dall and Smith, 1986). Taking into account the previously reported value of PNR_{50} (Calvo et al., 2012) and present results, it seemed that when half of *C. quadricarinatus* juveniles were close to the point-of-no-return some protein-rich organs, such as pleon muscle, began to degrade. Anger (1984) has previously observed at this critical point signs of epidermal atrophy, disintegration of tissues and changes of the muscle surfaces in *Hyas araneus* larvae. Other authors proposed that crustaceans may have the ability to use protein as an energy source for growth due to their high protein requirements and limited capacity for lipid and carbohydrate storage (Dall and Smith, 1986; Rosas et al., 2000; Sánchez-Paz et al., 2006).

A significant decrease in the hepatopancreas lipid content was observed in this study around the PNR_{50}. Anger et al. (1985) suggested that the PNR represents an irreversible loss of the animal ability to restore lipid reserves depleted during initial starvation. Later, this author recognized three distinct phases of biomass degradation during starvation in crustaceans: initially, the main reserves mobilized are the lipid ones, which is the typical response to short-term food deprivation. When much of the accessible lipid pool has been depleted (long-term food deprivation), proteins are increasingly utilized. A significant part of the lipid pool is bound in crucial cell structures such as membranes, and hence is normally unavailable for energy metabolism. This phase of predominantly protein catabolism reflects the degradation of structures such as muscle and nervous tissue. In the final phase of starvation *prior* to death, structural lipids may also be degraded; in this condition, the individuals have passed their point-of-no-return and do not recover after refeeding (Anger, 2001). In the present study, lipid and protein reserves were completely replenished after a feeding period of 40 days. This suggests that at days 40 and 50 *C. quadricarinatus* juveniles were still on the second phase of the starvation period.

Although glycogen levels were not affected during starvation, a pronounced accumulation of this nutrient in the pleon was triggered when food was available. Due to the spatial and temporal patchiness of food in nature, a large glycogen reserve may allow juveniles to move looking for another food patch. A similar response was observed by Mendez and Wieser (1993) in *Rutilus rutilus* (Cyprinidae) juveniles. In this fish species the concentration of glycogen decreased during food deprivation and increased dramatically after refeeding, exceeding control values. This was proposed as a strategy to rapidly store food energy, which may be used later for the synthesis of body materials.

In general, the decline in reserves is associated with increased enzymatic activity: high protease activity indicates protein catabolism, lipase activity is indicative of lipid use, and amylase activity suggests carbohydrate mobilization (Johnston et al., 2003; Kamarudin et al., 1994; Rodríguez et al., 1994; Zhang et al., 2010). However, in the present study both lipid reserves and lipase activity decreased during starvation. Similarly, the activity of digestive enzymes decreased after a starvation period in two species of the *Penaeus* genus (Cuzon et al., 1980; Muhlia-Almazan and García- Carreño, 2002). Moreover, Yudkovski et al. (2007) found that the lipase transcripts decrease in the hepatopancreas of *C. quadricarinatus* during non-feeding stages. Hence, they suggested that the lipase enzyme is secreted to function as a digestive lipase. Rivera-Pérez et al. (2011) distinguished in the shrimp *Penaeus vannamei* two types of lipase with alternate presence. They observed that during starvation digestive lipase transcripts decrease while the intracellular lipase transcripts increase, suggesting that the intracellular lipase is responsible for catabolism. Based on these previous studies, we conclude that the lipase whose activity was measured in the present research may not be synthesized when food is not available, since it may not be responsible for lipid catabolism.

Although starvation had no effect on the protease and amylase activities, they tended to decrease around the point-of-no-return (day 50) and to increase after the feeding period (day 90). The amylase activity measured in this study was different to that reported by Zhang et al.

(2010) and Comoglio et al. (2004), while the protease activity pattern was similar to that observed by Zhang et al. (2010). However, it is important to be cautious when comparing results because the studies mentioned above were done on marine shrimps; unfortunately, no data is available concerning digestive enzyme activity in freshwater species.

Sánchez-Paz et al. (2006) explained that the understanding of the metabolic necessities of crustaceans would allow researchers to focus on more complicated issues, including the contributions of some other nutrients (such as proline, as in flying insects) to satisfy the energy requirements of organisms in undernourishment and how genes involved in energy metabolism respond to survival periods of starvation. They suggested that this would eventually provide basis to propose strategies that could lead to a marked improvement in health and productivity of cultured crustaceans.

In this context, the present research provides new and relevant biological information on physiological and biochemical responses of crustaceans around PNR_{25} and PNR_{50}. This information reinforces the idea of high resistance in *C. quadricarinatus* and contributes to the understanding of the energetic reserve use in this important species for worldwide aquaculture.

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