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Mobilization of energetic reserves during starvation in juveniles of different size of the redclaw crayfish *Cherax quadricarinatus*

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Abstract

Mobilization patterns of energy reserves during starvation are highly variable among crustaceans, and understanding this process is useful to satisfy the nutritional requirements of cultured organisms. The aim of this study was to elucidate the mobilization patterns on early and advanced juveniles—first free stage and one-gram juveniles—of redclaw crayfish (*Cherax quadricarinatus*). A biochemical approach was used to analyse the organic carbon, total proteins, lipids and glycogen levels of the entire animal, and a histochemical approach was adopted to identify location of metabolites in the hepatopancreas and abdominal muscle. While starvation did not affect early juvenile protein levels, it showed a significant decrease in advanced juveniles. Histochemical analysis showed that lipid storage of hepatopancreas R cells was depleted near point of no return (PNR) and recovered after feeding. Glycogen storage was localized in the F cells of the hepatopancreas, and among muscle fibres of the abdomen, where after feeding, a pronounced accumulation was observed. Early and advanced juveniles of redclaw crayfish showed different patterns of consumption mainly related to the protein level, which was the most abundant reserve in advanced juveniles and the most consumed during starvation. Hepatopancreas R cells were confirmed as the principal lipid storage, whereas the abdominal muscle was the main glycogen storage. In crustacean aquaculture, the understanding of resistance to temporary feed deprivation is relevant to achieve an adequate feeding management, thereby avoiding the overfeeding and diminishing operating costs.

KEYWORDS
crustacean aquaculture, glycogen, hepatopancreas, histochemistry, lipids, muscle, point of no return

1 | INTRODUCTION

Aquatic invertebrates frequently suffer periods of starvation due to seasonal variations and heterogeneous spatial distribution of feed (Espinoza, Guzmán, Bascur, & Urzúa, 2016; Gebauer, Paschke, & Anger, 2010). Accordingly, a variety of strategies have been reported such as reduction in metabolic energy expenditure (McCue, 2010), and behavioural changes including a reduction in locomotor activity or changes in feeding patterns (Calvo, Stumpf, Sacristán, & López Greco, 2013; Charron et al., 2015; Sacristán, Nolasco-Soria, & Lopez Greco, 2014). However, when nutritional conditions become favourable, most aquatic species can accumulate energy reserves in special storage organs, so that maintenance, growth and reproduction are ensured during subsequent periods of feed limitation (Castillo Díaz, Tropea, Stumpf, & López Greco, 2016; Espinoza et al., 2016).

Crustaceans alternate naturally feeding and non-feeding periods during moulting cycle (ecdysis) (Simon, 2015). This complex hormonally controlled process is accompanied by morphological, physiological and behavioural changes (Fanjul-Moles & Gonsebatt, 2012; Simon, 2015). The capacity of crustaceans to tolerate periods of starvation is believed to be essential for their survival in the wild, particularly in unstable habitats with variable feed availability (Calado & Leal, 2015; Oliphant, Ichino, & Thatje, 2014; Paschke, Gebauer, Buchholz, & Anger, 2004).

When feed is available, crustaceans can stock energetic reserves in specific organs such as muscle and hepatopancreas. The muscle is the main storage organ of protein and glycogen (Longo & Díaz, 2011; Pinoni, Iribarne, & López Mañanes, 2011; Simon, Fitzgibbon, Battison, Carter, & Battaglene, 2015), while hepatopancreas stores lipids and glycogen (Calvo et al., 2013; Ong & Johnston, 2006; Watts, McGill, Albalat, & Neil, 2014).

During starvation, most decapods deplete protein, glycogen and lipid reserves (Comoglio, Goldsmit, & Amin, 2008; Comoglio, Smolko, & Amín, 2005; Oliveira, Rossi, Kucharski, & Da Silva, 2004; Sánchez-Paz et al., 2006; Silva-Castiglioni, Valgas, Machado, Freitas, & Oliveira, 2016; Simon, Fitzgibbon, et al., 2015). Hence, starvation studies show the implications of the energy resources utilized by crustaceans under these conditions and provide clues to the biochemical pathways involved in these processes. The understanding of these biochemical processes may be useful to satisfy the energy requirements of crustacean in culture (Sánchez-Paz et al., 2006; Simon, Fitzgibbon, et al., 2015).

To experimentally quantify the nutritional vulnerability of a given species or developmental stage (Espinoza et al., 2016; Pantaleão et al., 2015; Stumpf, Calvo, Pietrokovsky, & López Greco, 2010), the point of no return (PNR) is estimated. Anger and Dawirs (1981) defined this critical point as the threshold time at which initially starved larvae lose their capability to recover from nutritional stress (even if they are fed *ad libitum* afterwards). It is estimated from the PNR_{25} or PNR_{50}, which represent the time when 25 or 50% of initially starved animals lose their capability to moult.

Cherax quadricarinatus juveniles have been characterized as highly resistant to prolonged periods of starvation when compared to other decapod crustaceans, due to the fact that juveniles accelerate their growth with high survival values when feed is supplied (Calvo, Tropea, Anger, & López Greco, 2012; Sacristán, Ansaldo, Franco-Tadic, Fernández Gimenez, & López Greco, 2016).

The redclaw crayfish, *Cherax quadricarinatus* (von Martens), is a freshwater omnivorous species native to the North of Queensland (Australia) and the south-east of Papua New Guinea. This species is 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 (García-Guerrero, Hernández-Sandoval, Orduña-Rojas, & Cortés-Jacinto, 2013; Nuñez-Amao, Villarreal, Naranjo-Paramo, & Hernandez-Llamas, 2016; Prymazok, Pasqualino, Viau, Rodríguez, & Medesani, 2016).

The aim of this study was to generate complementary information to understand the pattern of use of energetic reserves in different size specimens of juvenile redclaw crayfish *C. quadricarinatus*, through biochemical and histochemical approaches.

2 | MATERIALS AND METHODS

2.1 | Conditions for broodstock maintenance and selection of juveniles

Stage III (JIII) and 1-g juveniles (J1 g) were obtained under laboratory conditions from reproductive stocks supplied by Centro Nacional de Desarrollo Acuícola (CENADAC), Corrientes, Argentina. Nine 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 tap water (pH 7–8, hardness 70–100 mg/L as $CaCO_3$ equivalents) under continuous aeration to maintain a dissolved oxygen concentration of 5–8 mg/L, 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 *Elodea sp.* and commercial balanced feed for tropical fish Tetracolor® (Tetra holding (US) Inc., Blacksburg, Germany), containing 475 g/kg crude protein, 65 g/kg crude fat, 20 g/kg crude fibre, 60 g/kg moisture, 15 g/kg phosphorus and 100 mg/kg ascorbic acid. This diet was previously found to be adequate for the studied species (Castillo Díaz et al., 2016; Stumpf & López Greco, 2015). After reaching the first free-living stage III, the juveniles were separated from their mothers and randomly assigned to the feeding treatments described below. One-gram juveniles were obtained from the same broodstock as JIII. The former was maintained in 30-L glass aquaria (60 × 40 × 30 cm) until reaching about 1 ± 0.2 g and randomly assigned to the feeding treatments described below.

2.2 | Experimental conditions for biochemical analysis

The assay comprised two experiments: the former with stage III juveniles (JIII; cephalothorax length = 9.06 ± 1.19 mm) and the second with one-gram juveniles (J1 g; cephalothorax length = 26.1 ± 2.5 mm). Control juveniles were daily fed in both experiments, while the treated

ones were starved till the end of the experiment (9 and 50 days, respectively, based on a previous study (Calvo et al., 2012). Control juveniles were offered the nutritionally balance food Tetracolor® (see above), which was provided *ad libitum* once a day. Samples were taken from each aquarium at PNR_{25} and PNR_{50}. The sampled juveniles were weighed, sacrificed and lyophilized for posterior biochemical analyses (organic carbon, total proteins, lipids, glycogen).

During the experiments, juveniles were placed in plastic aquaria (33.5 × 25 × 19 cm) containing 8L of dechlorinated tap water with continuous aeration, and a piece of synthetic net provided as shelter (30 × 60 cm). The water was maintained at 27 ± 1°C by water heaters (Altman, Argentina) of 100W and accuracy of 1°C. For both experiments, water quality was monitored and water was changed weekly. Physicochemical parameters were within the optimal ranges recommended for *Cherax quadricarinatus* (Jones, 1997): dissolved oxygen 5.6–7.7 mg/L, pH 7.6–7.9, hardness 65–95 mg/L as $CaCO_3$ equivalents, nitrites <0.05 mg/L.

2.2.1 | JIII experiment

A total of 960 stage III juveniles (mean initial weight ± SD: 14.8 ± 1.8 mg) were sampled from four different mothers (240 *per* mother). At the beginning of the experiment, the first sample was taken, forty juveniles from each mother were sacrificed and stocked. The remaining 200 juveniles were randomly assigned to one of four aquaria (initial density, 6.25 juveniles/L), two aquaria from each mother were assigned to the fed group (control), and two to the starved group (treated); eight aquaria *per* group. During the experiment, two samples of 20 juveniles were taken from each aquarium, one at day 8 and another at day 9. These days correspond to the PNR_{25} and PNR_{50} values previously estimated using the sigmoidal curve $M = 100.1(1 + \exp(-(x-.74)^{-1}\ 8.65)^{-1}$ where M was the mortality (%), and x the period of starvation (days; Calvo et al., 2012). At any point in time that a sample was taken, juveniles were weighted (wet weight, WW) using analytical balance (Ohaus; accuracy 0.1 mg, Florham Park, NJ, USA), lyophilized and weighted again using the same balance (dry weight, DW). For biochemical analysis, the two samples obtained from different aquaria that corresponded to the same mother were joined. Finally, a total of four replicates of 40 individuals, one sample *per* mother, were taken at the beginning, at PNR_{25} and at PNR_{50}.

2.2.2 | J1 g experiment

A total of 84 juveniles (mean initial weight ± SD: 0.96 ± 0.13 g) were sampled from a pool of five different mothers. At the beginning of the experiment, the first sample was taken; four juveniles were sacrificed and stocked. The remaining 80 juveniles were randomly assigned to one of 16 aquaria (initial density, 0.625 juveniles/L). Eight aquaria were assigned to the fed group (control), and eight to the starved group (treated). During the experiment, two samples of two juveniles were taken from each aquarium, one sample at day 40 and another at day 50. These days correspond to the PNR_{25} and PNR_{50} values previously estimated using the sigmoidal curve $M = 107(1 + \exp(-(x-52.1)^{-1}\ 9.2)^{-1}$, where M was the mortality (%), and x the period of starvation (days, Calvo et al., 2012). At any time that a sample was taken for biochemical analysis, juveniles were weighted (wet weight, WW) using analytical balance (Ohaus; accuracy 0.1 mg, Florham Park, NJ, USA), lyophilized and weighted again using the same balance (dry weight, DW). Finally, a total of four replicates were taken at the beginning of the experiment, eight at PNR_{25} and eight at PNR_{50}.

2.3 | Biochemical analyses

The organic carbon (OC g/kg) was quantified using the protocol described by Walkley and Black (1934) later modified by Jackson (1958). Total lipids, proteins and glycogen determinations were performed in triplicate using spectrophotometric methods using the UV/VIS spectrophotometer (JASCO, model 7850). They were quantified homogenizing the lyophilized juveniles in distilled water (1:1 m/V). Total lipids were extracted and determined using the Folch method (Folch, Lees, & Sloane Stanley, 1957) and Sulfophospho-vanillin method (Frings, Fendley, Dunn, & Queen, 1972), respectively. Finally, the samples were measured with a spectrophotometer at 530 nm. Glycogen levels were determined using the Antrona method described by Van Handel (1965) with a spectrophotometer at 630 nm. Total soluble protein was determined with the BCA method according to Fujimoto, Goeke, Olsom, and Klenk (1985) and measured with a spectrophotometer at 562 nm. All concentrations were expressed as mg/g of dry tissue.

2.4 | Experimental conditions for histochemical analysis

The assay consisted of an experiment with one-gram juveniles exposed to one of two feeding protocols: continuous feeding throughout 90 days (control) and starvation until day 50 (PNR_{50}), and feeding for the following 40 days (treatment). Control juveniles were offered feed Tetracolor® (see above), which was provided *ad libitum* once a day. 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 freshwater (0.1602 ppt) 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). Water quality was monitored, and water was changed at weekly intervals. Physicochemical parameters were within the optimal ranges recommended for *Cherax quadricarinatus* and detailed above.

Samples were taken at PNR_{25}, PNR_{50} and at the end of the experiment (day 40, 50 and 90 respectively). At each time, three juveniles were sacrificed from control and treated groups. Their hepatopancreas and abdominal muscle were removed and cut into two pieces. Half of them were fixed in Baker solution and the other half in Bouin liquid. The subsamples fixed in Baker solution were embedded in Crioplast. Ten-μm sections were cut with cryostat and stained by Sudan IV to detected lipids. The subsamples fixed in Bouin liquid were embedded in Paraplast. Six-μm sections were stained by periodic acid–Schiff (P.A.S.)

to detect neutral glycoconjugates and Best's Carmine to detect glycogen (Kiernan, 2008).

2.5 | Calculations and statistical analyses

For each group of juveniles, a factorial ANOVA, in a repeated-measures design using mixed models, was used to evaluate effect of feed regimen (fixed factor with two levels: fed and starved), the time (fixed factor with two levels: PNR_{25} and PNR_{50}), and experimental unit (random factor) on the following variables: wet and dry body mass, percentage of organic carbon, protein, lipids and glycogen concentration. When suitable, least squares difference (LSD) method was used for multiple comparisons (Zar, 2010). All values were expressed as mean ± standard deviation. Differences among means were considered significant at $p < .05$. The statistical analyses were performed using Infostat Software (Infostat version 2014, Grupo Infostat, FCA-UNC, Argentina).

3 | RESULTS

The wet weight (WW) of stage III juveniles was similar between groups during the experiment ($F = 0.36$; $df = 1,4$; $p = .58$), while their dry weight (DW) showed significant differences. Fed JIII presented higher values during the experiment than those starved ($F = 9.10$; $df = 1,4$; $p = .04$). The percentage of dry mass that corresponds to organic carbon (OC) was similar in both groups during the experiment ($F = 1.48$; $df = 1,6$; $p = .27$; Figure 1). Consequently, the protein concentration was also similar between groups. However, starved juveniles showed lower values not only for glycogen, but also for lipid concentrations ($F = 67.9$; $df = 1,6$; $p = .001$ and $F = 66.9$; $df = 1,6$; $p = .002$, respectively) (Figure 2).

In contrast to JIII, J1 g showed differences between groups in wet weight (WW; $F = 9.10$; $df = 1,4$; $p = .04$) and in dry weight (DW; $F = 10.1$; $df = 1,1$; $p = .007$). The percentage of dry mass that corresponds to organic carbon was also different between groups (OC; $F = 31.7$; $df = 1,1$; $p < .001$). The fed J1 g presented higher values during the experiment than those starved. Accordingly, fed J1 g showed higher values in protein, glycogen and lipid concentrations than those starved ($F = 7.80$; $df = 1,14$; $p = .014$ $F = 1.6$; $F = 28.3$; $df = 1,14$; $p < .001$ and $F = 6.86$; $df = 1,14$; $p = .02$, respectively).

3.1 | Histochemical analysis

The histological observation revealed differences between control and treated J1 g hepatopancreas (Figure 3). At day 50, which corresponds to PNR_{50}, the P.A.S. technique showed small reserves of neutral glycoconjugates only in F cells of control J1 g (Figure 3c). These reserves were not seen in treated J1 g at 40, 50 or 90 days (Figure 3d,e). The abdominal muscle showed deposits of neutral glycoconjugates between fibres in both control and treated J1 g, however in the first were more frequent (Figure 4a,b). At the end of the experiment, the muscle of treated J1 g presented a considerable amount of deposits of neutral glycoconjugates after the feeding period, even more than the control J1 g (Figure 4c,d). The Best's Carmine stain confirmed that these reserves in hepatopancreas organ and deposits in the muscle tissue contained glycogen (Figure 5). The Sudan IV technique showed lipid reserves in vacuoles of R cell of the hepatopancreas in all J1 g. These vacuoles were larger in control J1 g than in treated ones during the experiment (Figure 6). This technique did not detect lipids in the muscle of any J1 g.

4 | DISCUSSION

Different sizes individuals of redclaw juveniles were affected differently by starvation. Stage III juveniles (JIII) were affected only in dry

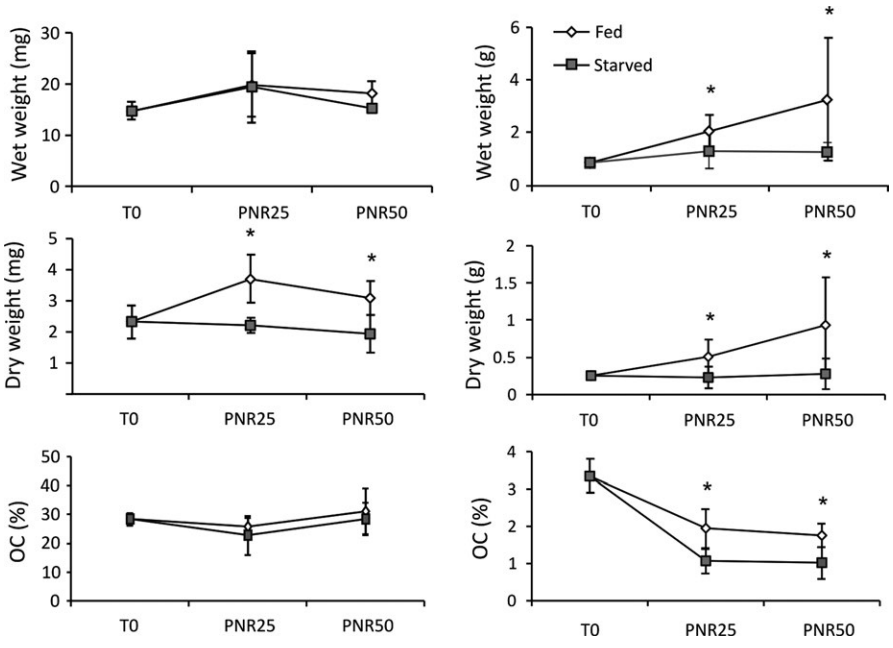

FIGURE 1 Wet and dry weight, and organic carbon percentage (OC) (mean ± SD) of stage III (left) and one-gram juveniles (right) of *Cherax quadricarinatus* after being exposed to feeding or starvation periods which correspond to points of no return 25 and 50 (PNR_{25} and PNR_{50}). Asterisks mean significant differences

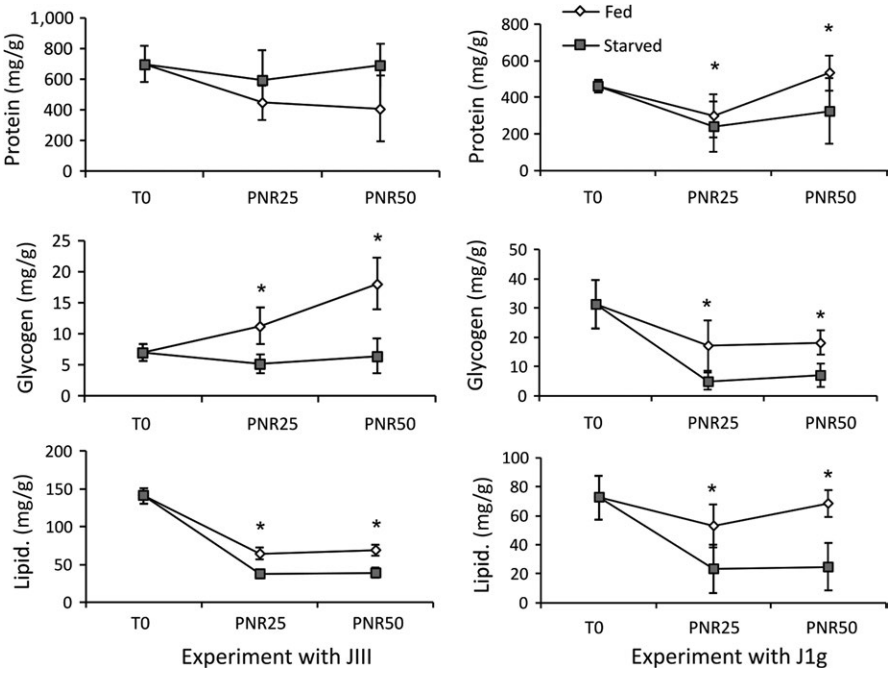

FIGURE 2 Protein, glycogen and lipid concentration of dry mass (mean ± SD) of stage III (left) and one-gram juveniles (right) of *Cherax quadricarinatus* after being exposed to feeding or starvation period which correspond to points of no return 25 and 50 (PNR_{25} and PNR_{50}). Asterisks mean significant differences

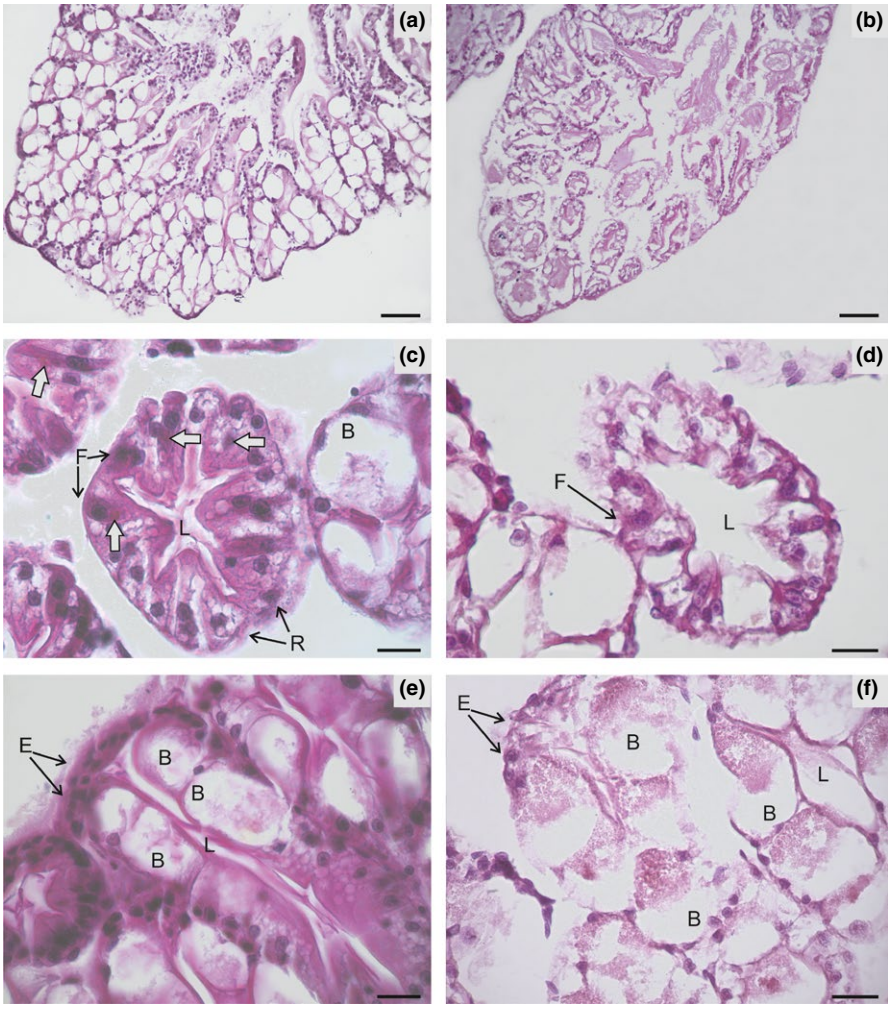

FIGURE 3 Histological sections of hepatopancreas of one-gram *Cherax quadricarinatus* juveniles stained by periodic acid–Schiff technique (P.A.S.); (a), (c), (e) control juveniles (b), (d), (f) treated juveniles; (a), (b), general view at day 50 of the experiment (PNR_{50}); (e), (f) longitudinal section of a tubule at the end of the experiment (day 90). Scale bars: (a), (b): 100 μm; (c), (d), (e), (f): 20 μm; B: B cell B; E: E cell; F: F cell; L: lumen of the tubule; R: R cell; grey arrows: vacuoles with deposits of neutral glycoconjugates

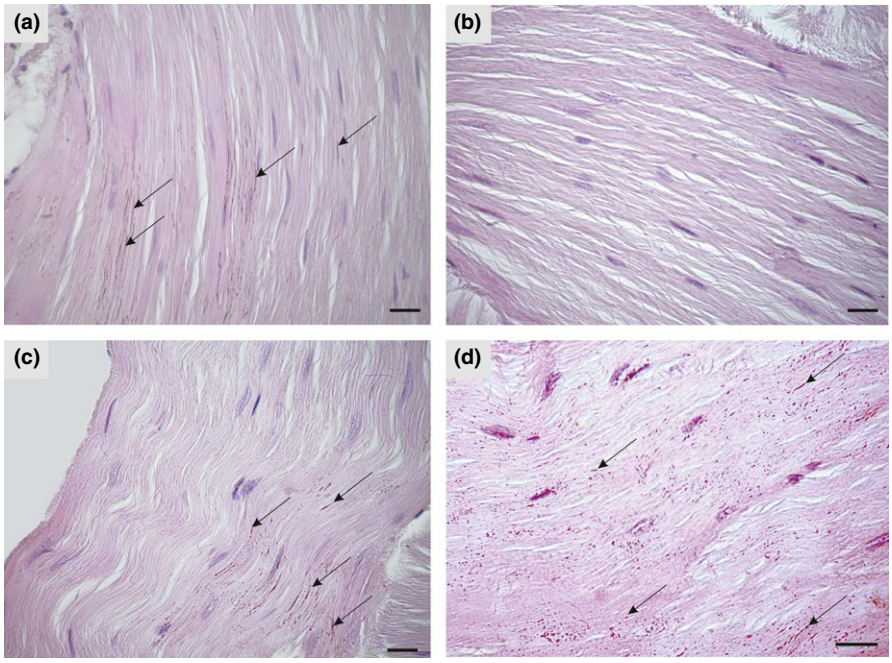

FIGURE 4 Histological sections of abdominal muscle of one-gram *Cherax quadricarinatus* juveniles stained by periodic acid–Schiff technique (P.A.S.); (a), (c), control juveniles (b), (d), treated juveniles; (a), (b), at day 50 of the experiment (PNR_{50}); (c), (d) at the end of the experiment (day 90); scale bars: 20 μm; arrows: vacuoles with deposits of neutral glycoconjugates

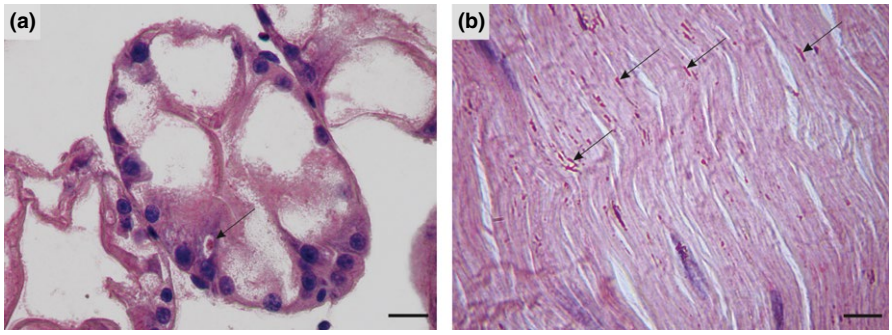

FIGURE 5 Histological sections of hepatopancreas (a) and abdominal muscle (b) of one-gram *Cherax quadricarinatus* juveniles stained by Carmine's Best technique; scale bars: 20 μm; arrows: vacuoles with deposits of glycogen

weight after the starvation period near PNR_{25} and PNR_{50}, while one-gram juveniles (J1 g) were affected not just in wet and dry weight, but also in the percentage of organic carbon after the starvation periods.

Stage III juveniles showed a decrease in dry mass while no change was detected in wet weight, suggesting an increase in water content possibly for the replacement of organic mass. This observation is in accordance with other studies which showed that body weight reduction is not necessarily the response to starvation (Comoglio, Gaxiola, Roque, Cuzon, & Amín, 2004; Hu, Wang, Tsang, Cheung, & Shin, 2011; Watts et al., 2014; Wu, Zeng, & Southgate, 2017). McCue (2010) proposed that the growth in tissue water content results from the rising osmotic pressure in tissues, which stems from increasing metabolite levels, and cells somehow replace lost organic matter with water to maintain their size, during starvation (Hu et al., 2011). The difference observed in dry weight of JIII between fed and starved juveniles, together with the similar percentage of organic content found, suggests that starvation in the first stage affected both organic (protein, lipids and glycogen) and inorganic matter in a similar way. While starved JIII were expected to stop their growth, fed JIII would rise their organic content faster than inorganic matter. Assuming that most inorganic matter is concentrated in the cuticle, the body surface would show a proportional increase in this inorganic while the organic content would increase along with the body volume in fed animals. However, the proportional increase in both suggests that their cuticles become more calcified in the stages evaluated. The same pattern was registered in a study with *Hyas araneus* (Linnaeus) which increases its calcification in the terminal larval stage and equally compensates this increase in inorganic matter with tissue production (Anger, 2001). In contrast, J1 g showed strong effects of starvation on organic carbon; moreover, both dry weight and wet weight presented their consequences. These strong effects were mainly related to the reserve depletion by starved juveniles.

The biochemical analysis showed again differences between early and advanced redclaw juveniles. These differences were mainly related to protein consumption. While starvation did not affect the protein *per* gram of dry mass in JIII, a significant drop was observed on the protein *per* gram of dry mass in J1 g at PNR_{25} and PNR_{50}. Dry mass of starved juveniles, early and advanced, showed a significant decrease, and the content of all reserves decreased likewise. However, starved JIII, in comparison with fed JIII, consumed more lipid and glycogen than protein. In contrast, studies on spiny lobster (*Jasus edwardsii*) showed that during feed deprivation lipid catabolism was more important for later larval stages (Ritar, Dunstan, Crear, & Brown, 2003),

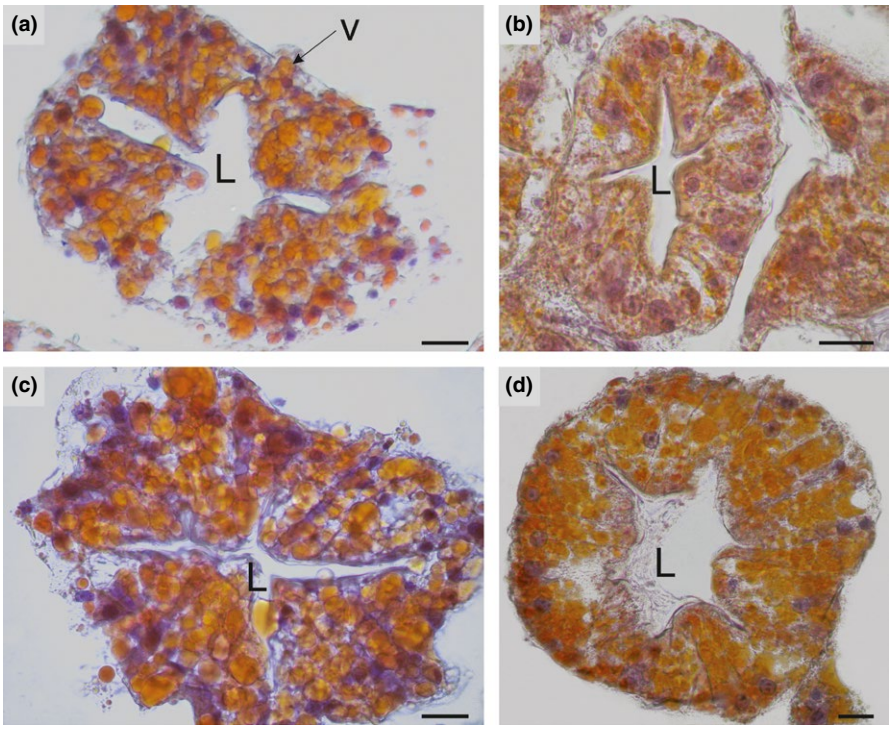

FIGURE 6 Histological sections of hepatopancreas of one-gram *Cherax quadricarinatus* juveniles stained by Sudán IV technique; (a), (c) control juveniles (b), (d) treated juveniles; (a), (b), cross section of tubule at day 50 of the experiment (PNR_{50}); (c), (d) cross section of tubule at the end of the experiment (day 90). Scale bars: 20 μm; L: lumen of the tubule; V: vacuoles with deposits of lipids

whereas protein catabolism was more important in the first larval stage (Smith, Thompson, Ritar, & Dunstan, 2003). The relative importance of metabolic reserves and their order of utilization vary among species (Sánchez-Paz et al., 2006).

The histochemical analyses of the storage organs were in accord with the biochemical results as they showed structural effects, such as disorganized tubular structures, and hypertrophy of B cells of hepatopancreas which are responsible for digestion. When *C. quadricarinatus* juveniles were close to the point of no return, some protein-rich organs—such as pleon muscle—began to degrade. These effects were previously registered after feeding restriction (Calvo, Stumpf, Pietrokovsky, & López Greco, 2011) and coincided with previous studies which observed decreases in protein levels in hepatopancreas and pleon of juveniles after starvation (Calvo et al., 2013; Sacristán et al., 2016; Simon, Fitzgibbon, et al., 2015).

In terms of biomass, the proteins were the most abundant and the most consumed during starvation in late juveniles. At the PNR_{50}, the difference between mean values of fed and starved animals was about 200 mg/g of dry mass. Other authors proposed that crustaceans could use protein as energy source to grow (Simon, Fitzgibbon, et al., 2015), due to their high protein requirement, and their limited ability to reserve lipids and carbohydrates (Comoglio et al., 2004; Sánchez-Paz et al., 2006).

Lipid levels were affected significantly after starvation near PNR_{50} in JIII as well as in J1 g. Anger (2001) stated that the PNR represents an irreversible loss of the animal ability to restore lipid reserves depleted during initial starvation. Biochemical results agreed with previous studies which reported significant decrease in lipid levels, mainly in hepatopancreas (Espinoza et al., 2016; Sacristán et al., 2016; Watts et al., 2014; Wu et al., 2017). The histochemical analysis confirmed that R cells of hepatopancreas are responsible for lipid storage whereas the abdominal muscle did not show lipid reserve. After refeeding, the juveniles showed a remarkable recovery of lipid deposits in the hepatopancreas. Previous biochemical analysis in the same species showed that the protein level was mainly affected in the pleon muscle during a starvation period around the PNR_{50}, and the lipid level significantly decreased in the hepatopancreas (Calvo et al., 2013). Moreover, the locomotion activity of starved individuals decreased, and their cuticle became duller than those of fed ones (Calvo et al., 2012; Charron et al., 2015). This information suggests that near to $PNR_{50,}$ juveniles do not merely use the reserves from the storage organs, but also the proteins, lipids, and glycogen from structural tissues.

The study of the nutritional vulnerability of the first free-living juveniles that start to eat exogenous feed is interesting, because it is regularly related to a bottle neck survival feature. Usually, these analyses are only possible with pooled samples comprising numerous individuals of larvae or early juveniles with homogeneous characteristics, as a consequence of the minimal sample size required or the technical limitations (Anger & Hayd, 2009; Silva-Castiglioni et al., 2016). Thus, comparative studies which include individuals of different sizes can help to understand the physiology of first stages, regarding storage and mobilization of energetic reserves under starvation conditions.

Both sizes of juveniles, JIII and J1 g showed significant effects on glycogen levels. These results were similar to those obtained by Sacristán et al. (2016), and contrast with previous results of redclaw juveniles of the same size which showed no effect of starvation on glycogen concentration (Calvo et al., 2013). A possible explanation for this difference could be that the extractions in the present work were carried out on a dry mass while the previous extractions were performed

on a wet mass. As water content of marine crustaceans ranges between 64 and 92 g/kg, because of moult or development stage, starvation or water quality (Anger, 2001; Simon, 2015), the methods used on a dry mass tend to be more sensitive to detect small variations (about 10 mg/g of dry mass) in the limited storage of glycogen.

Similarly to the biochemical analysis, the histochemical analysis showed that both hepatopancreas and muscle were positive for Best carmine. The former showed glycogen content in a central vacuole of F cells which syntheses and secretes proteins, presumably digestive enzymes (Gallardo et al., 2012). As energy is necessary for this process, a glycogen stock in these cells could be essential to ensure its function during starvation. The muscle presented the main storage of glycogen. The histochemical analysis showed the effect of starvation on this storage, and when feed was available, a pronounced accumulation of this nutrient was triggered into the abdominal muscle. This result agreed with a previous study in which biochemical analysis showed the same reaction after refeeding, thereby suggesting that a large glycogen reserve might allow juveniles to move around looking for food, probably another food patch (Calvo et al., 2013). This effect has also been described in juveniles of *Rutilus rutilus* (Linnaeus) (Vega-Villasante, Cortes-Jacinto, & Garcia-Gerrero, 2007).

Physiological evidence such as that reviewed by Sánchez-Paz et al. (2006) showed that differences in energy source usage may be species-specific, but an intricate issue when analysing the available information is that in several cases, not all the three energy sources (carbohydrates, proteins and lipids) were evaluated in crustacean under the same conditions. Starvation time, moulting or developmental stage, as well as type of protocol of extraction used, can cause important effects. Therefore, studies involving different approaches as well as developmental stages should be undertaken to achieve a deeper understanding of this complex process of crustacean nutrition.

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