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Effect of different diets on digestive enzyme activities, *in vitro* digestibility, and midgut gland structure in juvenile crayfish, *Cherax quadricarinatus*

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Abstract

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This study investigated the effects of food quality on digestive enzyme activities, *in vitro* protein digestibility and histological traits of the midgut gland in juvenile crayfish *Cherax quadricarinatus*. Animals of a wide weight range were fed different diets: two commercial diets with high or low lipid content (high lipid and low lipid, respectively) and were compared with a reference diet (RF) previously formulated for this species. Proteinase, lipase and amylase activities were significantly influenced by diet and weight. Specific trypsin activity was significantly higher for crayfish fed with the HL diet. Trypsin activity depended on diet and weight. Protein digestibility showed that HL was the most digestible diet and RF the least. The weight of the animals did not affect protein digestibility. Structural disorganization, hypertrophy of B-cells and presence of large vacuoles in R-cells were mainly observed in juveniles fed with HL, indicative of malnutrition. Thus, our data suggest that the HL diet would not be the most appropriate for *C. quadricarinatus*, while RF diet would be more convenient for culture of this species.

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Introduction

Aquaculture is a growing and important production sector for high-protein animal food. Feed cost represents a large portion (up to 70%) of the total operating cost in intensive aquaculture (Thompson *et al.* 2010). Adequate nutrition is essential for profitable aquaculture, and the effectiveness of the feeds administered depends on our knowledge of how organisms use the components of the diet (Carrillo-Farnés *et al.* 2006). The intake of sufficient amounts of nutrients by an organism depends on its ability to select and digest them and the capacity of its digestive enzymes (Bautista 1983). It is thus important to understand the digestive enzymes activities and the histological structure of the midgut gland, because they can be used as tools for developing feeds that cater specifically to each species metabolic requirements (Figueiredo *et al.* 2001).

The nutritive value of a feed ingredient is based on its chemical composition and on an animal's capacity to digest, absorb and utilize it (Saoud *et al.* 2012). In this sense, the study of digestibility, which refers to the quantity of a nutrient or energy in the ingested feedstuff that is not eliminated in the

faeces (NRC 1993; Lee and Lawrence 1997; Guillaume and Choubert 2001; Saoud *et al.* 2012) is also important.

The need for research on the mechanisms of food digestion and the impact of diet on growth and survival has long been recognized for crustacean's culture, such as penaeid shrimp (Guzman *et al.* 2001). Simon (2009), studying the effect of feeding and diet type on the digestive response of spiny lobsters *Jasus edwardsii*, demonstrated that this species display a natural enzymatic secretory response to formulated diet, which represents an important precondition for the development of successful diets for commercial culture. Additional studies on the relative ability of freshwater crayfish species to utilize different carbohydrate sources and assessment of their potential levels of inclusion in artificial diets can provide important data that will be required to formulate species-specific and cost-effective formulated feeds for farmed freshwater *Cherax* species (Dammannagoda *et al.* 2015).

The activity of digestive enzymes has been studied in many crustacean species, and it has been demonstrated to be affected by ontogeny, moulting, diet composition, circadian rhythms, photoperiod and quality of light, temperature, stage of larval development, vitellogenesis, feeding habits and even habitat (Saoud *et al.* 2013).

The midgut gland of crustaceans is the main organ for digestion, absorption and storage of nutrients. Histologically, the functional unit of the midgut gland is a blind tubule with a simple cylindrical epithelium (Sousa and Petriella 2006), consisting of different cell types, that is E-cells (embryonic), R-cells (resorptive), F-cells (fibrillar) and B-cells (blisterlike) (Gibson and Barker 1979; Al-Mohanna and Not 1987a).

The redclaw crayfish, *Cherax quadricarinatus*, is an omnivorous freshwater species native to the North of Queensland (Australia) and Southeast of Papua New Guinea. It has great aquaculture potential due to its high growth rate, easy management and high productivity. Nowadays, it is cultured intensively and semi-intensively in many countries throughout the world including Australia, United States, China, Ecuador and Mexico (reviewed by Ghanawi and Saoud 2012; Saoud *et al.* 2013).

Cherax quadricarinatus was introduced in Argentina in 1990, where it is cultured semi-intensively. The local trade of this species is primarily based on its consumption in restaurants and for ornamental purposes, while internationally, it is consumed in Europe (mainly Scandinavia, Italy, France and Germany), Asia and the United States (Luchini 2004).

Currently, diets for the commercial production of redclaw crayfish are based on formulations produced for other aquatic species, primarily penaeid shrimp (*Litopenaeus vannamei*) but sometimes prawn (*Farfantepenaeus paulensis*, *Litopenaeus monodon*, *Litopenaeus stylirostris*, *Macrobrachium rosenbergii*) and fish feeds (review Saoud *et al.* 2013). There are few commercial feeds specifically formulated and manufactured for redclaw crayfish. Progress in this area of commercial feeds has been made over the past decade, but there are still knowledge gaps inasmuch as nutrient requirements of the species. Some of the areas that require further research include essential amino acids, vitamin and mineral requirements, pelleting technology to produce a dry but malleable pellet, estimation of optimal feed regimes, broodstock nutrient requirements, and formulation of diets using low-cost regionally available ingredients (Saoud *et al.* 2013).

This study was aimed to evaluate and compare formulated and commercial feeds used for the culture of *C. quadricarinatus* juveniles in Argentina. For this purpose, we analysed digestive enzyme activity, *in vitro* protein digestibility and histological analysis of the midgut gland of juveniles of different masses fed with distinct diets: two commercial diets – one with a high lipid (HL) (Made in Argentina, Fundus®, crude protein 41.58%) and one with a low lipid (LL) content (Made in Germany, TetraColor®, Tropical Granules, crude protein 49.51%), and a reference diet specifically formulated for this species (RF) (Gutiérrez and Rodríguez 2010).

Materials and Methods

Animals

Juvenile redclaw were reared under laboratory conditions from a reproductive stock supplied by Centro Nacional de Desarrollo Acuícola (CENADAC), Corrientes, Argentina. Nine ovigerous females (mean wet body weight: 60.42 ± 4.53 g) were maintained in an individual glass aquarium (60 × 40 × 30 cm) containing 30 L of dechlorinated tap water under continuous aeration (dissolved oxygen: 5.4–8.0 mg/L). The temperature was held constant at 26–27 °C by ALTMAN (Guangdong, China) water heaters (100 W, precision 1 °C), and the photoperiod was maintained on a 14-h light: 10-h dark cycle. Each aquarium was supplied with a PVC tube (10 cm in diameter and 25 cm long) for shelter (Jones 1995). Females were fed daily *ad libitum* with *Elodea* sp. and dry commercial pellets (TetraColor® Tropical), according to previous studies (Bugnot and López Greco 2009; Sánchez de Bock and López Greco 2010). When juveniles became independent at stage 3 (Levi *et al.* 1999), they were separated from their mothers and were maintained under the same experimental conditions, as previously described until they reached the desired weight.

Feeding trials and experimental design

Three dietary treatments were used in this study. In the first treatment, crayfish were fed with a commercial feed (Fundus®) (Fideos Don Antonio SA, Junín, Buenos Aires, Argentina) usually used for culture of redclaw in some Argentinian farms, consisted largely of fishmeal as a protein source. This feed was previously assayed in our laboratory with good results in terms of growth and survival (Stumpf *et al.* 2011). The second diet, a commercial feed, which is the usual food used in our laboratory for *C. quadricarinatus* (Vazquez *et al.*

2008; Stumpf *et al.* 2010; Tropea *et al.* 2010, 2011), is composed of proteins from animal as well as plant origin (fish, shrimp, grains and yeast). The third diet adopted was the reference diet (RF), specially formulated for this species by Gutiérrez and Rodríguez (2010). The RF (Table 1) was prepared at first by mixing the dry ingredients with oil, and then adding water and prejellified starch until the mixture reached a crumbly dough consistency. The mixture was then placed in a mechanical mixer (Model S-1, Davis Mixer, Bonner Springs, KS) using a 3-mm-diameter die to produce 0.5-cm pellets which were dried at 60 °C for 24 h. Fundus® and TetraColor® are referred in our experiments inasmuch as their relative amount of lipids as high lipid (HL) and low lipid (LL) content, respectively. Crude protein, total lipids, and ash and moisture content of the diets were determined at the National Institute for Fisheries Research and Development (INIDEP, Mar del Plata, Argentina), according to AOAC (1990), and the proximal composition is detailed in Table 2.

Crayfish were divided into four groups according to their weight: group (A), animals weighing 0.53 ± 0.14 g (N = 90; density: 41.66 individuals/m^2); group (B), animals weighing 1.32 ± 0.29 g (N = 90; density: 41.66 individuals/m^2); group (C), animals weighing 7.41 ± 1.09 g (N = 45; density: 20.83 individuals/m^2); and group (D), animals weighing 14.08 ± 2.35 g (N = 36; density: 12.50 individuals/m^2). Within each group, animals were randomly assigned to the three diets described above as follows: 30 crayfish *per* diet, placed in three different plastic aquaria (10 animals *per* aquarium) for groups (A) and (B); 15 crayfish *per* diet, placed in three different plastic aquaria (five animals *per* aquarium) for group (C); and 12 crayfish *per* diet, placed in four different plastic aquaria (three animals *per* aquarium) for group (D). Each plastic aquarium (32 × 26 × 19 cm) was considered a replicate; all aquaria contained 10 L of dechlorinated tap water under continuous aeration (dissolved oxygen: 5.4–8.0 mg/L). Synthetic nets and PVC tubes were provided for shelter. Photoperiod and temperature were the same as those described above. Crayfish were acclimated to these experimental conditions for 1 week before the onset of the experiment.

Table 1 Reference diet formulation for *Cherax quadricarinatus* prepared according to Gutiérrez and Rodríguez (2010)

Ingredients	Percentage
Fish meal	28
Soybean meal	39
Pre-jellified starch	19
Soybean oil	6
Bentonite	6
Mineral premix[1]	1
Vitamin premix[2]	1

[1]Mineral premix (mg/kg): $ZnSO_4$, 50; $MgSO_4$, 35; $MnSO_4$, 15; $CoSO_4$, 2.5; $CuSO_4$, 3; KI, 3.

[2]Vitamin premix (mg/kg, unless otherwise noted): A (retinol), 3000 UI/kg; D, 600 UI/kg; E (alpha tocopheryl acetate), 60; K, 5; C (ascorbic acid), 150; B1 (thiamin), 10; B (riboflavin), 10; Vitamin B6 (piridoxin), 7; B12, 0.02; biotin, 0.4; pantothenic acid, 35; folic acid, 6; niacin, 80; choline, 500; inositol, 100.

Table 2 Proximal analyses of the three diets assayed in *Cherax quadricarinatus* juveniles

Nutrients	Fundus® (HL)	TetraColor® (LL)	Reference diet (RF)
Crude protein (%)	41.58 ± 1.76	49.51 ± 0.09	37.98 ± 0.94
Total lipids (%)	21.29 ± 0.29	4.60 ± 0.02	6.05 ± 0.08
Ash (%)	6.87 ± 0.08	8.78 ± 0.01	16.05 ± 0.11
Moisture (%)	6.10 ± 0.04	6.45 ± 0.10	4.03 ± 0.03

Juveniles were fed once a day in the morning (09:00 h) during 24 days with feed equivalent to 7% (groups A and B), 5% (group C) or 3% (group D) of the mean weight for each group. Moults and dead organisms were removed daily to avoid interference in the study. Growth performance was evaluated through the specific growth rate (SGR) based on the following equation: SGR (%/day) = 100 (Ln BWf – Ln BWi)/time (days) where BWf is the final body weight and BWi is the initial body weight.

Additionally, five crayfish for every combination of weight group and diet (N = 60) were maintained for histological analysis of the midgut gland, in individual glass containers 13.5 cm diameter × 13.5 cm high containing 1.5 L of dechlorinated tap water, under similar conditions (percentage of feed, experimental time, photoperiod and temperature).

Extraction of the midgut gland and enzymatic preparation

At the end of the experiment, animals were weighed (precision 0.1 mg) and then were anesthetized in cold water, and the midgut glands were dissected out, weighed and immediately frozen at −20 °C. All animals were anesthetized and sacrificed between 09:00 h and 12:00 h to avoid a possible effect of the circadian rhythm in the parameters to be analysed, according to Sacristán *et al.* (2013).

Midgut glands from three (groups A, B and C) or four (group D) crayfish were pooled together to obtain a sufficient sample for all determinations. Samples were lyophilized according to Fernández Gimenez *et al.* (2001, 2002) and stored at −20 °C until analysis. For enzymatic analysis, midgut glands were rehydrated to 70% of their original weight, homogenized in chilled distilled water and centrifuged at 10 000 × g for 30 min at 4 °C (Fernández Gimenez *et al.* 2009). The lipid layer was removed, and the supernatants were used as the enzyme extract.

Midgut gland somatic index was determined for wet weights using the following equation: midgut gland somatic index (%) = (midgut gland wet weight/whole body weight) × 100.

Enzymatic activity assays

Protein quantification. Total soluble protein was evaluated with the Coomassie blue dye method according to Bradford (1976) using serum bovine albumin as the standard.

Proteinase activity. Total proteinase activity was assayed using 1% azocasein as the substrate in 50 mM Tris–HCl, pH 7.5 (García-Carreño 1992). Reference tubes were prepared similarly, but the enzyme extract was added after the TCA solution. One proteinase unit was defined as the amount of enzyme required to cause an increase of 0.01 optical density (OD) units at 440 nm/min (López-López *et al.* 2005).

Trypsin activity. Trypsin activity was evaluated by the rate of hydrolysis of *Na*-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) (Sigma B4875, Saint Louis, MO, USA) as the specific substrate. BAPNA (1 mM) was dissolved in 1 mL of dimethylsulphoxide (DMSO) and brought to 100 mL with Tris–HCl, pH 7.5, containing 20 mM $CaCl_2$. The enzyme extract samples (5 μL) were added to 0.75 mL of substrate solution at 37 °C, and changes in absorbance were recorded at 410 nm for 10 min (Erlanger *et al.* 1961). The assay included blanks and commercial enzymes (1 mg/mL) as internal controls. Trypsin activity was expressed as change in absorbance/(minute*mg protein). Determinations were run in triplicates.

Lipase activity. Lipase activity of each enzyme extract was determined according to Versaw *et al.* (1989). The assay mixture consisted of sodium taurocholate 100 mM, buffer Tris–HCl 50 mm, pH 7.5 and the enzyme extract. After incubation (25 °C for 5 min), the substrate β-naphthyl caprylate (Goldbio N-100) dissolved in dimethyl sulfoxide (DMSO) was added to the mix. The mixture was incubated at 25 °C for an additional 30 min before adding 20 μL Fast Blue BB (100 mM in DMSO). The reaction was stopped with trichloroacetic acid (TCA) (0.72 N) and clarified with ethyl acetate: ethanol (1:1 V/V). Absorbance was recorded at 550 nm. One lipase unit was defined as the amount of enzyme required to cause an increase of 0.01 OD units at 550 nm/min (López-López *et al.* 2005).

Cellulase activity. Cellulase activity was determined by measuring the production of reducing sugars resulting from α-cellulose (Sigma C8002) hydrolysis as described by Linton and Greenaway (2004). Enzyme extract (50 μL) was mixed with 100 μL of substrate solution (2% α-cellulose in acetate buffer 0.1 M, pH 5.5), and the mixture was incubated at 30 °C for 60 min. The reaction was stopped by the addition of 25 μL of 0.3 M TCA, and excess acid was neutralized with 5 μL of 2.5 M K_2CO_3. Precipitated protein was pelleted by centrifugation at 10 000 × *g* for 10 min. A blank (100 μL acetate buffer + 50 μL homogenate) was prepared for each sample analysed. Immediately after incubation, the production of reducing sugars was determined by adding 500 μL distilled water and 500 μL dinitrosalicylic acid (DNS) reagent to the reaction mixture and boiling for 15 min, according to López-López *et al.* (2005). Volume was adjusted to 5 mL with distilled water, and the absorbance of the coloured solution was read at 550 nm. One cellulase unit was defined as the amount of enzyme required to cause an increase of 0.01 OD units at 550 nm/min (López-López *et al.* 2005).

Amylase activity. Total α-amylase activity of each crude extract was determined according to Vega-Villasante *et al.* (1993). 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). Following incubation at room temperature for 10 min, amylase activity was determined by measuring the production of reducing sugars resulting from starch hydrolysis by adding 200 μL sodium carbonate (2 N) and 1.5 mL DNS reagent to the reaction mixture and boiling for 15 min. Volume was adjusted to 10 mL with distilled water, and the coloured solution was read at 550 nm. Reference tubes were prepared similarly, but crude extract was added after DNS reagent. One amylase unit was defined as the amount of enzyme required to cause an increase of 0.01 OD units at 550 nm/min (López-López *et al.* 2005).

In vitro *protein digestibility*

Methods used to measure *in vitro* digestibility of protein were similar to those described by Divakaran *et al.* (2004). The assay mixture consisted of 100 mg of each feed, 3.5 mL of triethanolamine buffer, pH 8.5 containing 0.2M $CaCl_2$ and 0.5 mL of the crude enzyme extract. Measurement of free amino groups released by enzyme hydrolysis was achieved by adding 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Fluka 92822) (Alder-Nissen 1979). Leucine was used as a standard and the results expressed as μM leucine liberated by 100 μg/mL protein in the enzyme extract from 100 mg of feed by minute, according to Divakaran *et al.* (2004). This value indicates the quantity of amino acids liberated and is a relative index of *in vitro* digestibility.

Histological analysis

After being cold-anaesthetized at −20 °C for 15 min, the carapace of each animal was removed and the midgut gland dissected and quickly fixed in Bouin's solution for 4 h at room temperature. The tissues were finally dehydrated and embedded in paraffin. Sections, 5–7 μm thick, were stained with haematoxylin–eosin (López Greco *et al.* 2007). At least three slides from each crayfish were inspected under light microscope.

Statistical analysis

One-way analyses of variance (ANOVAS) were used to compare the initial weights, specific growth rate, postorbital cephalothorax length, and the midgut gland somatic index between diets for each weight group. Two-way ANOVAS were used to analyse soluble protein, enzymatic activity assays and *in vitro*

digestibility, with the following fixed factors: diet (RF, HL and LL) and weight group (A, B, C and D). When the interaction between factors was significant, a test was applied to detect differences between groups. When the interaction was not significant, the Tukey's test was used to test for main effects of weight group and diet. ANOVA assumptions were verified and transformations applied to data if lack of normality and/or variance heterogeneity were detected (Sokal and Rohlf 1995). All statistical analyses were performed using the InfoStat® software. When the number of replicates *per* treatment is unequal, InfoStat applies the modification proposed by Tukey–Cramer (Miller 1981). The level of significance was 0.05 for all analyses.

Results

Feeding trials

Crayfish initial weight, SGR and midgut gland somatic index for each diet and weight group are shown in Table 3. In weight groups A and B, crayfish fed with HL presented a significantly higher midgut gland somatic index ($P < 0.05$) than those fed with RF and LL; no significant differences were found between RF and LL.

Enzymatic activity assays

All enzymes evaluated showed activity in the midgut glands pools of all crayfish fed with the different diets, although activities varied between treatments (Table 4). As detailed below, the digestive enzyme profiles of proteinase, lipase and amylase activities obtained from midgut gland extracts were significantly influenced by dietary formulation and animal weight (groups); cellulase activity showed no differences ($P > 0.05$).

The specific activity of total proteinase (Table 4) did not differ significantly between crayfish fed RF or LL; in both cases, enzymatic activity profiles were similar in all weight groups. For the HL diet, group B showed a significantly higher proteinase enzymatic activity ($P < 0.05$).

Lipase activity showed a significant interaction ($P < 0.05$) between diet and weight group (Table 4). The enzymatic activity for HL was significantly different and inversely proportional to the weight of the animals ($P < 0.05$). For LL, crayfish of group C exhibited the highest activity; when fed with the RF diet, crayfish in group A showed significantly higher activity levels ($P < 0.05$) than the other groups. No significant differences were observed in cellulase activity in midgut gland extracts for any treatment (Table 4); the activity values ranged from 0.94×10^{-3} to 6.47×10^{-3} U mg/protein. Amylase activities in midgut extracts ranged from 0.19 to 0.65 U mg/protein, except for crayfish in groups B and C fed with HL, which showed a higher activity than all others (1.27 and 1.01 U/mg protein, respectively) (Table 4).

Specific trypsin activity (STA) showed distinct activity for all treatments with a significant interaction ($P < 0.05$) between type of diet and animal weight. Values for the HL diet were significantly higher than for the other two diets, being highest for groups B and C (Fig. 1).

Table 3 Initial weight, SGR and midgut gland somatic index for each diet and weight group of *Cherax quadricarinatus* juveniles treated during 24 days

Diet	Group	Initial weight (g)	SGR (%/day)	Midgut gland index (%)
RF	A	0.55 ± 0.01^{a}	1.34 ± 0.30^{b}	7.31 ± 0.15^{a}
	B	1.33 ± 0.07^{b}	2.04 ± 0.13^{a}	7.33 ± 0.20^{a}
	C	7.28 ± 0.18^{c}	0.73 ± 0.04^{a}	6.75 ± 0.34^{a}
	D	14.10 ± 0.34^{d}	0.31 ± 0.17^{a}	6.66 ± 0.33^{a}
HL	A	0.54 ± 0.01^{a}	2.53 ± 0.32^{a}	9.10 ± 0.26^{b}
	B	1.37 ± 0.06^{b}	2.22 ± 0.14^{a}	9.29 ± 0.30^{b}
	C	6.87 ± 0.08^{c}	0.56 ± 0.04^{a}	7.34 ± 0.55^{a}
	D	13.88 ± 0.39^{d}	0.31 ± 0.17^{a}	6.69 ± 0.25^{a}
LL	A	0.48 ± 0.04^{a}	1.68 ± 0.15^{b}	7.88 ± 0.27^{a}
	B	1.22 ± 0.03^{b}	0.97 ± 0.38^{b}	7.69 ± 0.33^{a}
	C	8.08 ± 0.08^{c}	0.31 ± 0.12^{b}	6.57 ± 0.29^{a}
	D	14.25 ± 0.28^{d}	0.31 ± 0.17^{a}	6.69 ± 0.25^{a}

HL, high lipid content (Fundus®); LL, low lipid content (TetraColor®); RF, reference diet.

Values represent mean ± standard error. Different letters indicate significant differences ($P < 0.05$). Comparisons were made among diets for the same weight group. Weight groups A, B and C: three replicate aquaria (10, 10 and 15 animals *per* aquarium); and weight group D: four replicate aquaria (12 animals *per* aquarium).

In vitro *protein digestibility*

Protein digestibility revealed a similar pattern as trypsin activity (Fig. 1). Significant differences ($P < 0.05$) were barely observed between diets, HL was the most digestible diet, followed by LL and RF was the less digestible. HL was well digested by crayfish with values ranging from 0.8 to 1.3 μM leucine released/(mg protein*100 mg diet); LL showed lower values, and the lowest were obtained with RF from 0.3 to 0.4 μM leucine released/(mg protein*100 mg diet). The weight of the animals did not affect the digestibility of the diet ($P > 0.05$).

Histological analysis

The structure of the midgut gland of redclaw resembles that of other decapod crustaceans. It is composed of numerous blind end tubules with four main cell types, namely, E-, F-, B- and R-cells. E-cells are cuboidal and have a prominent nucleus occupying most of the basophilic cytoplasm; they are located at the distal part of the tubules. F-cells are cylindrical, with a central nucleus and basophilic cytoplasm; they are found in the medial and proximal parts of the tubules. B-cells are the largest cell type, with a large vacuole that displaces the nucleus basally; they are more abundant in the medial and

Table 4 Enzymatic activities in midgut gland extracts from *Cherax quadricarinatus* juveniles

Diet	Group	Proteinase (U/mg protein)	Lipase (U/mg protein)	Cellulase[1] (U/mg protein)	Amylase (U/mg protein)
RF	A	4.55 ± 0.05^{cd}	35.5 ± 1.83^{e}	2.37 ± 0.67^{a}	nd
	B	4.22 ± 0.30^{c}	17.07 ± 4.53^{ab}	4.35 ± 1.00^{a}	0.48 ± 0.23^{ab}
	C	2.02 ± 0.11^{a}	12.00 ± 0.82^{a}	1.67 ± 1.00^{a}	0.65 ± 0.41^{ab}
	D	4.36 ± 0.51^{cd}	15.69 ± 1.93^{a}	1.58 ± 1.00^{a}	0.28 ± 0.08^{a}
HL	A	4.09 ± 0.22^{bc}	49.58 ± 1.10^{f}	2.63 ± 1.00^{a}	0.19 ± 0.05
	B	8.83 ± 0.44^{f}	32.45 ± 3.85^{cde}	3.74 ± 1.00^{a}	1.27 ± 0.26^{c}
	C	4.39 ± 0.31^{cd}	26.30 ± 4.07^{cd}	6.47 ± 2.24^{a}	1.01 ± 0.18^{bc}
	D	5.65 ± 0.45^{de}	17.31 ± 0.38^{ab}	1.56 ± 1.00^{a}	0.36 ± 0.07^{ab}
LL	A	4.99 ± 0.49^{cde}	26.30 ± 3.22^{cd}	2.32 ± 0.65^{a}	0.37 ± 0.08^{ab}
	B	5.19 ± 0.41^{cde}	24.53 ± 4.17^{bc}	0.94 ± 0.10^{a}	0.43 ± 0.03^{ab}
	C	2.73 ± 0.08^{ab}	33.62 ± 2.04^{de}	2.19 ± 0.76^{a}	0.47 ± 0.03^{ab}
	D	6.11 ± 0.06^{e}	28.87 ± 0.74^{cde}	3.16 ± 2.23^{a}	0.51 ± 0.22^{ab}

HL, high lipid content diet (Fundus®); LL, low lipid content diet (TetraColor®); RF, reference diet.

Values represent the mean of three replicates ± standard error. Different letters indicate significant differences ($P < 0.05$). Comparisons were made among weight groups fed the same diet and between diets for a given weight group. Weight groups A, B and C: three replicate aquaria (10, 10 and 15 animals *per* aquarium); and group D: 4 replicate aquaria (12 animals *per* aquarium). D: RF 0.5 g weight group not determined (nd).

[1]Cellulase activity $\times 10^{-3}$.

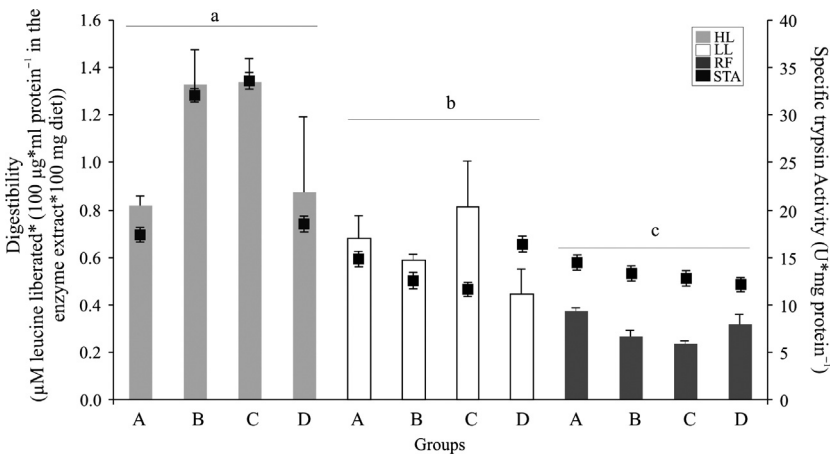

Fig. 1—*In vitro* protein digestibility (bars) and Specific Trypsin Activity (STA) (black squares) of midgut gland extract from juveniles of *C. quadricarinatus* fed the different diets (Each value represents the mean of three replicates ± SD). *In vitro* protein digestibility is expressed as µM leucine released/(mg protein*100 mg diet) and Specific Trypsin Activity is expressed as U mg/protein*minute. HL, High Lipid content diet (Fundus®): white gray bars; RF, Reference Diet: dark gray bars; LL, Low Lipid content diet (TetraColor®): white bars. Different letters indicate significant differences ($P < 0.05$) between diets for *in vitro* protein digestibility.

distal parts of the tubules. R-cells are the most numerous cell type; they are cylindrical, contain many small vacuoles and the nucleus is located centrally or basally; they are more abundant in the medial and proximal parts of the tubules (Fig. 2 A, B) (Al-Mohanna and Not 1987a; López Greco *et al.* 2011; Sacristán *et al.* 2011).

After 24 days of experiment, the main changes in the midgut gland of juveniles fed with HL were as follows: structural loss and disorganization of the tubules (Fig. 2 C); extensive areas with a notable reduction in epithelial height (Fig. 2D and detail, C); hypertrophy of B-cells with one or more large vacuoles, tending to coalesce into single larger ones (Fig. 2E, F); R-cells showing larger vacuoles (Fig. 2D, E,); scarce differentiation between B- and R-cells (Fig. 2D, F); loss of cell boundaries (Fig. 2E, F); reduction in the number of F-cells *per* tubule (Fig. 2G); and loss of the typical star-shape of the tubular lumen (Fig. 3H). There were no major structural differences for RF or LL (Fig. 2A, B). The structural changes observed were not related to the weight of juveniles.

Discussion

Juveniles of *C. quadricarinatus* showed similar digestive enzymatic activities and *in vitro* protein digestibility when they were fed with RF and LL diets. Additionally, RF has a lower content of crude protein and therefore lower cost. The information in this study reinforces the first results showed by Gutiérrez and Rodríguez (2010) about growth rates, survival and energetic reserves levels in early *C. quadricarinatus* juveniles (mean weight: 22 mg) fed with RF.

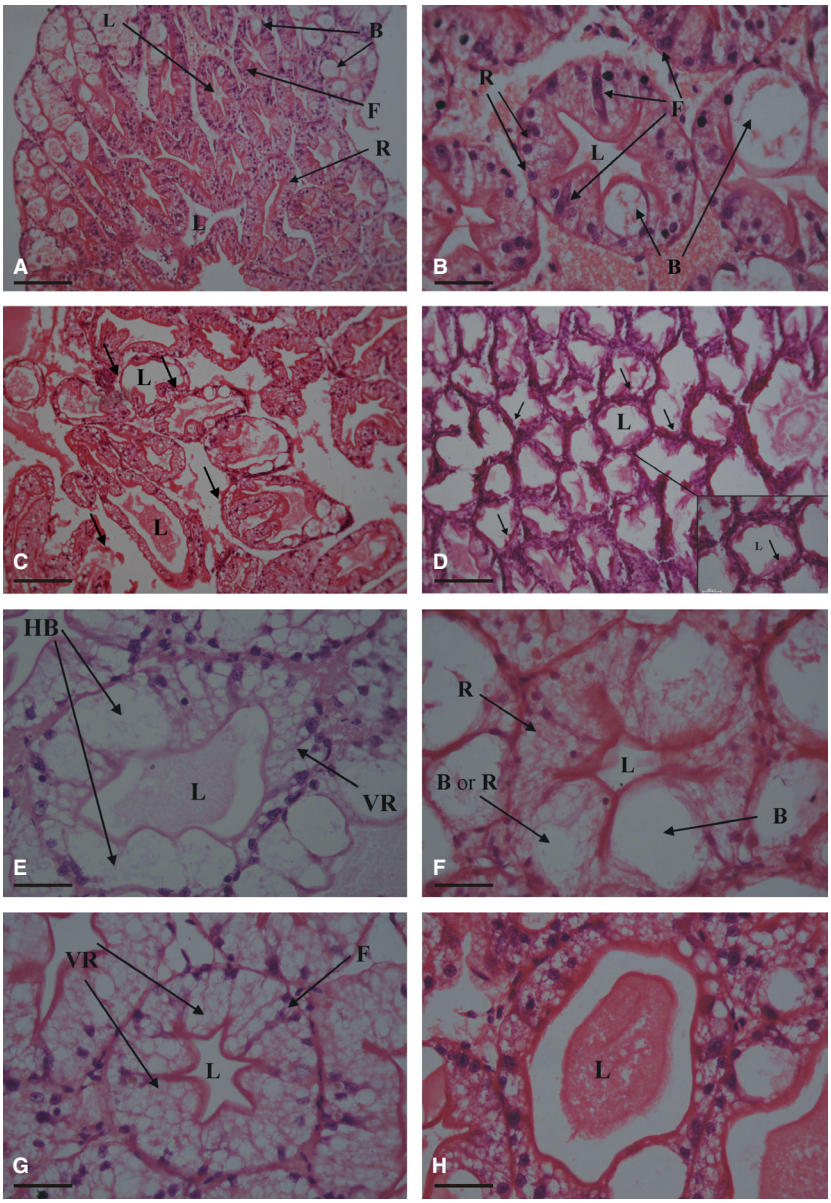

Fig. 2—Histological sections of the midgut gland from juveniles of *C. quadricarinatus* fed with different diets. —**A**. animal fed with the Reference Diet (RF); no structural changes were observed; B: animal fed with the Low Lipid content diet (LL); no structural changes were observed; **C-H**: crayfish fed with the High Lipid content diet (HL); **C:** structural loss and disorganization of the tubules (arrows); D and detail: extensive areas with a significant reduction in epithelial height (arrows); **E**: hypertrophy of B-cells with one or more larger vacuoles, tending to coalesce into a larger one; R-cells showing larger vacuoles; scarce differentiation between B- and R-cells; **F**: scarce differentiation between B and R cells and loss of cell boundaries; G: R-cells showing larger vacuoles; loss of cell boundaries; and reduction in the number of F cells *per* tubule; **H**: loss of the typical star-shaped tubular lumen. Scale bar: **A**, **B**, **C** = 200 μm; **D**, **D** detail, **E**, **F**, **G**, **H** = 50 μm. B: B-cell; F: F-cell; HB: hypertrophy of B-cells; L: tubular lumen; R: R-cell; VR: enlarged vacuoles from R-cells.

The percentage of crude protein of the RF diet is within the range of 31 to 43% which yielded the best results in terms of growth, biomass and survival in the study by Cortés-Jacinto *et al.* (2003) in juveniles of *C. quadricarinatus* (mean weight 1 g). Pavasovic (2008) proposed that redclaw tolerate varying quantities of lipid in their diets (7, 9 or 11%), values similar to the lipid content of the RF and LL diets, when included as part of a diet containing either low (19%) or high (32%) dietary protein levels.

The presence of a variety of enzymes in midgut glands of juvenile redclaw has been linked to the ability of this species to digest a wide range of dietary components (Xue *et al.* 1999; Figueiredo *et al.* 2001; López-López *et al.* 2005; Saoud *et al.* 2013). In the present study, the enzymatic activities of proteinase, lipase and amylase were dependent on the type of diet and the animal's weight. Our results are in agreement with Lee *et al.* (1984) and Gamboa-Delgado *et al.* (2003), who observed that digestive enzymes activity in *Litopenaeus* vannamei varies in relation to the protein level in the diet and body weight. The results of Dammannagoda *et al.* (2015) are in disagreement with our results because they observed that *C. quadricarinatus* adults showed significantly higher protease and amylase activities when fed the reference diet. The proteinase activity in animals of D group fed with RF showed relative high levels than those reported by Dammannagoda *et al.* (2015) in the same species. In addition, Andres *et al.* (2010)

found that proteinase and amylase activities depended on the larval stage in *Maja brachydactyla*, and Saborowski *et al.* (2006) demonstrated changes in the activity of digestive enzymes (trypsin and chymotrypsin) between different ontogenetic stages (eggs, zoea I, zoea II, zoea III/megalopae and crab I) in *Lithodes santolla*.

As argued by López-López *et al.* (2005), when the protein quantity in a given diet diminishes, a progressive increase in the use of carbohydrate–lipids may cause a decrease in the use of protein as an energy source. Therefore, the high lipid levels in the HL diet (21.3%) may have affected proteinase activity, even though the protein levels were well above the faint levels proposed by Pavasovic (2008) to be needed for proteinase activity to decrease (19%). This author also stated that changes in proteinase activity coupled to an increase in dietary lipid content may be associated with protein sparing. Serine proteinase activity may reflect this lower protein turnover and mobilization in the presence of higher lipid levels.

Serine proteinases, such as trypsin and chymotrypsin, are proposed to be the most important crustacean digestive enzymes, believed to be responsible for 40–60% of total protein digestion in penaeids (Hernández-Cortés *et al.* 1999a). Teschke and Saborowski (2005) demonstrated that cysteine proteinases were the predominant enzymes in the midgut gland of *Crangon crangon* and *C. allmani*, but their activity was the lowest in *Euphausia superba*. In agreement with this result, when Coccia *et al.* (2011) characterized the alkaline proteases of *C. albidus*, serine proteases (trypsinlike and chymotrypsin-like enzymes) were the most active enzymes along the digestive tract, a common feature with other decapods (Celis-Guerrero *et al.* 2004; Navarrete Del Toro *et al.* 2006; Linton *et al.* 2009). A recent study (Rojo *et al.* 2010) suggests that in crustaceans, some groups have a higher proportion of serine proteinases, while others have a preponderance of cysteine (cathepsin L and B) or aspartic proteinases (cathepsin D). The effect of *C. quadricarinatus* juveniles, fed with the different diets, on the activity of other types of proteinases could not be evaluated, as the present study did not focus specifically on these enzymes, and only trypsin was measured.

Trypsin and chymotrypsin-like enzyme activity has been reported in other crustaceans when characterizing the proteinase classes present (García-Carreño *et al.* 1993) as a result of the ontogenetic development (Lemos *et al.* 1999a; Luo *et al.* 2008; Buarque *et al.* 2009) and moulting cycle (Fernández Gimenez *et al.* 2001, 2002). Luo *et al.* (2008) determined the activities of trypsin and chymotrypsin in different stages of embryonic development for *C. quadricarinatus*; the present study confirmed that the midgut gland of *C. quadricarinatus* juveniles of a wide range of body weights contains serine proteases as well.

In terms of digestibility, the HL diet was the most digestible, followed by LL and RF. Allan *et al.* (2000), analysing the replacement of fish meal in diets for Australian silver perch *Bidyanus bidyanus*, explained that meat and bone meals have low dry matter digestibility due to their high ash content. In the study by Pavasovic *et al.* (2007), low digestibility of a diet made from meat and bone meal was also attributed to high levels of ash (20.4%). In agreement with these results, the RF diet in the present study, which showed high ash content (16%), also showed low *in vitro* protein digestibility. Lemos *et al.* (1999b) demonstrated low digestibility of soya bean meal in shrimp *Farfantepenaeus paulensis*, so poor digestibility of RF diet due to high soya bean meal contained (39%). In spiny lobster, *Jasus edwardsii*, Simon and Jeffes (2008) demonstrated that this species showed low dry matter digestibility of the formulated diet (61.2%) compared to natural diet (fresh mussel; 89.2%), and they explained that is not the consequence of a shortcoming in enzymatic secretion (Simon 2009). Moreover, given the participation of proteinases in digestion process, it is believed that to enhance protein assimilation of feed, proteinases have been used as feed supplements (Dabrowska *et al.* 1979; Maugle *et al.* 1983; Divakaran *et al.* 2004). The feed supplementation with proteinases or mixed enzymes might have positive effects on the development of animals (González-Zamorano *et al.* 2013). Therefore, more research is needed in that area to improve feed industry.

Our results showed that animals fed a diet with a high percentage of protein (HL and LL, 41.58% and 49.51, respectively) had more trypsin activity than animals fed with a lower percentage of protein in the diet (RF, 37.98%). This is related to the fact that carnivorous organisms (such as some penaeid shrimp) feed mostly on zooplankton or benthos, and thus produce more proteolytic enzymes and are capable of digesting animal proteins more efficiently than herbivores or omnivores (García-Carreño *et al.* 1994). However, in the present work, the activity of trypsin in crayfish fed the HL diet was higher than in those fed LL, which could be indicating that the quality of dietary protein also influences enzyme activity. The protein quality of both commercial diets used was not known, so this effect in our experiment is yet to be confirmed. Le Vay *et al.* (2001) and Fernández Gimenez *et al.* (2009) demonstrated that digestive enzyme activity can increase in crustaceans when fed with low nutritional quality feed.

The redclaw, *C. quadricarinatus*, is an omnivorous species that inhabits rivers where food sources are mostly vegetal; however, the species is also an opportunistic carnivore, able to efficiently consume vegetal and/or animal ingredients from the diet, as has been reported by Villarreal and Hutchings (1996) and Campaña-Torres *et al.* (2005).

The activity of some carbohydrases is age dependent (Figueiredo and Anderson 2003), and it changes with developmental stage in the redclaw. For example, amylase and laminarinase activities are significantly higher in large *C. quadricarinatus* than at other stages, whereas protease activities decrease as the species grows (Saoud *et al.* 2013). The presence of these carbohydrases would suggest that redclaw should be able to obtain a substantial amount of their metabolic energy needs from carbohydrates, even though research suggests that only a relatively small portion of their energetic needs are indeed obtained from carbohydrates (Pavasovic

et al. 2006; Saoud *et al.* 2013). Cellulase activity might also be contributed by microorganisms of the digestive system, as it is unclear whether endogenous cellulase enzymes in the redclaw represent secretion products from the cells of the digestive gland or if they originate from symbiotic bacteria residing in the lobes of the foregut.

Crawford *et al.* (2005), when comparing cellulase activity between freshwater crayfish (Genus *Cherax*) and marine prawns (Genus *Penaeus*), proposed that the higher levels of enzyme activity observed in crayfish are to be expected given that freshwater crustaceans tend to consume a greater proportion of plant material in their diets compared to marine species (Tacon and Akiyama 1997). Additionally, the observed greater cellulase activity in *C. tenuimanus* may indicate a specific genetic adaptation to a diet high in plant material, although it is important to note that digestive enzyme activity could be influenced to a degree by the diet. Adaptive changes were observed in α-amylase activity in lobster *J. edwardsii*; therefore, to improve the digestibility and utilization of the carbohydrate fraction for growth, it could change the formulated diet (Simon 2009). Previous studies have shown that cellulase activity in crustaceans can increase in response to higher levels of dietary polysaccharides, such as cellulose or starch (Gonzalez-Pena *et al.* 2002; Pavasovic *et al.* 2004). Increased availability of such substrates in the form of naturally occurring pond biota may also raise cellulase activity levels (Moss *et al.* 2001).

Although this species was able to tolerate the high lipid levels assayed in the HL diet (21.29%), juveniles showed several signs of malnutrition as well as structural loss and disorganization of the midgut gland tubules, along with hypertrophied B-cells, R-cells with larger vacuoles, scarce differentiation between B- and R-cells, loss of cell boundaries, reduction in the number of F-cells and loss of the typical 'star-shaped' tubular lumen. Most of these alterations have also been recently observed in juveniles under different starvation periods (Calvo *et al.* 2011, 2012), implying that they are not specifically triggered by the diet but rather demonstrating the capability of the midgut gland to undergo a 'quick' structural response to diet composition. The R-cells are the most abundant cells in the decapod midgut gland and appear along the length of the tubule, and they are resorptive or absorptive cells which are filled with vacuoles, lipid and glycogen. Al-Mohanna and Not (1987b) proposed that the rates at which energy reserves are accumulated by the R-cells could be used to monitor dietary requirements and even the B-cells have been found to reflect differences in diet. There is reason to believe that, in the present study, the hypertrophied B-cells and R-cells of crayfish possibly are due to lipid accumulation in these cells, but this suggestion needs confirmation. In other species, such as *Pleoticus muelleri*, suboptimal levels of vitamin A in the diet caused some histopathological changes in the hepatopancreas, such as cellular hyperplasia and hypertrophy, disorganized tissue and shrinkage of cells (Fernández Gimenez *et al.* 2008). Juveniles of *J. edwardsii* fed diets with different ratios of carbohydrate/lipid showed decreasing vacuolization in R-cells as the carbohydrate/lipid relation decreased (Johnston *et al.* 2003); juveniles of *L. vannamei* exposed to different concentrations of Cd and Zn showed reduction in the number of R- and B-cells and atrophy and sloughing of these cells, as well as infiltration of hemocytes (Frias-Espericueta *et al.* 2008). To our knowledge, the present study is the first to show that diet composition directly affects midgut gland structure in this species, and reinforces the concept of the digestive gland of crustaceans as a dynamic structure functioning as a 'mirror' in response to diet. Therefore, identification of the main changes in the structure of the midgut gland may be useful to evaluate optimal and suboptimal nutritional requirements in the culture of the species.

From all the results obtained and discussed in this study, HL diet would not be the most appropriate for *C. quadricarinatus* culture. We suggest that the RF diet would be more convenient, if ash content is reduced to increase protein digestibility. More research for this diet is necessary regarding binders, water stability, attractant incorporation and feeding protocols. The activity of digestive enzymes, *in vitro* protein digestibility and histological analysis of the midgut gland could be used as integrative tools to evaluate the effect of different diets in *C. quadricarinatus* juveniles or other cultured species in addition to the usual studies on growth performance.

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References

Alder-Nissen, J. 1979. Determination of degree of hydrolysis of food proteins hydrolysates by trinitrobenzenesulfonic acid. – *Journal of Agriculture Food Chemistry* **27**: 1256–1266.

Allan, G. L., Parkinson, S., Booth, M. A., Stone, D. A. J., Rowland, S. J., Frances, J. and Warner-Smith, R. 2000. Replacement of fish meal in diets for Australian silver perch, *Bidyanus bidyanus*: I. Digestibility of alternative ingredients. – *Aquaculture* **186**: 293–310.

Al-Mohanna, S. Y. and Not, J. D. 1987a. M-"midget" cells and the moult cycle in *Penaeus semisulcatus* (Crustacea: Decapoda). – *Journal of the Marine Biological Association UK* **67**: 803–813.

Al-Mohanna, S. Y. and Not, J. D. 1987b. R-cells and the digestive cycle in *Penaeus semisulcatus* (Crustacea: Decapoda). – *Marine Biology* **95**: 129–137.

Andres, M., Gisbert, E., Díaz, M., Moyano, F. J., Estévez, A. and Rotllant, G. 2010. Ontogenetic changes in digestive enzymatic capacities of the spider crab, *Maja brachydactyla* (Decapoda: Majidae). – *Journal of Experimental Marine Biology and Ecology* **389**: 75–84.

AOAC (Association of Official Analytical Chemist) 1990. Official Methods of Analysis of the Association of Official Analytical Chemist, Inc, AOAC (Association of Official Analytical Chemist), Arlington.

Bautista, M. 1983. The response of *Penaeus monodon* juvenile to varying protein/energy rations in test diets. – *Aquaculture* **53**: 229–242.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. –*Analytical Biochemistry* **72**: 248–253.

Buarque, D. S., Fernandes Castro, P., Silva Santos, F. M., Lemos, D., Carvalho Júnior, L. B. and Bezerra, R. S. 2009. Digestive peptidases and proteinases in the midgut gland of the pink shrimp *Farfantepenaeus paulensis* (Crustacea, Decapoda, Penaeidae). – *Aquaculture Research* **40**: 861–870.

Bugnot, A. and López Greco, L. 2009. Sperm production in the freshwater crayfish *Cherax quadricarinatus* (Decapoda, Parastacidae). – *Aquaculture* **295**: 292–299.

Calvo, N., Stumpf, L., Pietrokovsky, S. and López Greco, L. 2011. Early and late effects of feed restriction on survival, growth and hepatopancreas structure in juveniles of the red claw crayfish *Cherax quadricarinatus*. – *Aquaculture* **319**: 355–362.

Calvo, N., Tropea, C., Anger, K. and López Greco, L. 2012. Starvation resistance in juvenile freshwater crayfish. – *Aquatic Biology* **16**: 287–297.

Campaña-Torres, A., Martinez-Cordova, L. R., Villarreal-Colmenares, H. and Civera-Cerecedo, R. 2005. *In vivo* dry matter and protein digestibility of three-plant derived and four animal-derived feedstuffs and diets for juvenile Australian redclaw, *Cherax quadricarinatus*. – *Aquaculture* **250**: 748–754.

Carrillo-Farnés, O., Forrellat-Barrios, A., Guerrero-Galván, S. and Vega-Villasante, F. 2006. A review of digestive enzyme activity in Penaeid shrimps. – *Crustaceana* **80**: 257–275.

Celis-Guerrero, L. E., García-Carreño, F. L. and Navarrete Del Toro, M. A. 2004. Characterization of proteases in the digestive system of spiny lobster (*Panulirus interruptus*). – *Marine Biotechnology* **6**: 262–269.

Coccia, E., Varricchio, E. and Paolucci, M. 2011. Digestive enzymes in the crayfish *Cherax albidus*: Polymorphism and partial characterization. – *International Journal of Zoology* **2011**: 310371.

Cortés-Jacinto, E., Villarreal-Colmenares, H., Civera-Cerecedo, R. and Martínez-Córdova, L. 2003. Effect of dietary protein level on growth and survival of juvenile freshwater crayfish *Cherax quadricarinatus* (Decapoda: Parastacidae). – *Aquaculture Nutrition* **9**: 207–213.

Crawford, C. A., Richardson, N. R. and Mather, P. B. 2005. A comparative study of cellulase and xylanase activity in freshwater crayfish and marine prawns. – *Aquaculture Research* **36**: 586–592.

Dabrowska, H., Grudniewski, C. and Dabrowski, K. 1979. Artificial diets for common carp: effect of the addition of enzyme extracts. – *Progressive Fish-Culturist* **41**: 196–200.

Dammannagoda, L. K., Pavasovic, A., Hurdwood, D. A. and Mather, P. B. 2015. Effects of soluble dietary cellulose on specific growth rate, survival and digestive enzyme activities in three freshwater crayfish (*Cherax*) species. – *Aquaculture Research* **46**: 626–636.

Divakaran, S. and Velasco, M. 1999. Effect of proteolytic enzyme addition to a practical feed on growth of the Pacific white shrimp, Litopenaeus vannamei (Boone). – *Aquacultrue Research* **30**: 335–339.

Divakaran, S., Forster, I. A. and Velasco, M. 2004. Limitations on the use of shrimp *Litopenaeus vannamei* midgut gland extract for the measurement of *in vitro* protein digestibility. – *Aquaculture* **239**: 323–329.

Erlanger, B. F., Kokowsky, N. and Cohen, W. 1961. The preparation and properties of two new chromogenic substrates of trypsin. *Archives of Biochemistry and Biophysics* – **95**: 271–279.

Fernández Gimenez, A. V., García-Carreño, F. L., Navarrete del Toro, M. A. and Fenucci, J. L. 2002. Digestive proteinases of *Artemesia longinaris* (Decapoda, Penaeidae): Partial characterization and relationship with molting. – *Comparative Biochemistry and Physiology B* **132**: 593–598.

Fernández Gimenez, A. V., Diaz, A. C., Velurtas, S. M., Petriella, A. M. and Fenucci, J. L. 2008. Effects of different dietary vitamin A levels in the red shrimp *Pleoticus muelleri* (Bate 1888) (Decapoda, Solenoceridae). – *Revista de Biología Marrina y Oceanografía* **43**: 483–490.

Fernández Gimenez, A. V., Díaz, A. C., Velurtas, S. M. and Fenucci, J. L. 2009. Partial substitution of fishmeal by meat and bone meal, soybean meal, and squid concentrate in feeds for the prawn, *Artemesia longinaris*: Effect on digestive proteinases. – *Israeli Journal of Aquaculture Bamidgeh* **61**: 48–56.

Figueiredo, M. S. R. B. and Anderson, A. J. 2003. Ontogenetic changes in digestive proteases and carbohydrases from the Australian freshwater crayfish, redclaw *Cherax quadricarinatus* (Crustacea, Decapoda, Parastacidae). – *Aquaculture Research* **34**: 1235–1239.

Figueiredo, M. S. R. B., Kricker, J. A. and Anderson, A. J. 2001. Digestive enzyme activities in the alimentary tract of redclaw crayfish, *Cherax quadricarinatus* (Decapoda: Parastacidae). – *Journal of Crustacean Biology* **21**: 334–344.

Frias-Espericueta, M. G., Abad-Rosales, S., Nevárez-Velázquez, A. C., Osuna-López, I., Páez-Osuna, F., Lozano-Olvera, F. and Voltolina, D. 2008. Histological effects of a combination of heavy metals on Pacific white shrimp *Litopenaeus vannamei* juveniles. – *Aquatic Toxicology* **89**: 152–157.

Gamboa-Delgado, J., Molina-Poveda, C. and Chau, C. 2003. Digestive enzyme activity and food ingesta in juvenile shrimp *Litopenaeus vannamei* (Boone 1931) as a function of body weight. – *Aquaculture Research* **34**: 1403–1411.

García-Carreño, F. L. 1992. The digestive proteases of langostilla (*Pleuroncodes planipes*, Deapoda): Their partial characterization, and the effect of fed on their composition. – *Comparative Biochemistry and Physiology B* **103**: 575–578.

García-Carreño, F. L., Dimes, N. and Haard, N. 1993. Substrate gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. – *Analytical Biochemistry* **214**: 65–69.

García-Carreño, F. L., Hernández Cortes, M. P. and Haard, N. 1994. Enzymes with peptidase and proteinase activity from the digestive system of a freshwater and a marine decapod. – *Journal of Agricultural and Food Chemistry* **42**: 145–146.

Ghanawi, J. and Saoud, P. 2012. Molting, reproductive biology, and hatchery management of redclaw crayfish *Cherax quadricarinatus* (von Martens 1868). – *Aquaculture* **358–359**: 83–195.

Gibson, O. and Barker, P. L. 1979. The decapod hepatopancreas. – *Oceanography and Marine Biology* **17**: 285–346.

Gonzalez-Pena, M., Anderson, A. J., Smith, D. M. and Moreira, G. S. 2002. Effect of dietary cellulose on digestion in the prawn *Macrobrachium rosenbergii*. – *Aquaculture* **211**: 291–303.

González-Zamorano, M., Navarrete del Toro, M.A. and García-Carreño, F. 2013. Exogenous proteinases as feed supplement for shrimp: in vitro evaluation.. – *Aquaculture Nutrition* **19**: 731–740.

Guillaume, J. and Choubert, G. 2001. Digestive physiology and nutrient digestibility in fishes. In Guillaume, J., Kaushik, P., Bergot, P. and Metailler, R. (Eds): Nutrition and Feeding of Fish and Crustaceans, pp 27–56. Springer-Praxis Publishing, Chichester.

Gutiérrez, M. L. and Rodríguez, E. M. 2010. Effect of protein source on growth of early juvenile redclaw crayfish *Cherax quadricarinatus* (Decapoda, Parastacidae). – *Freshwater Crayfish* **17**: 23–29.

Guzman, C., Gaxiola, G., Rosa, C. and Torre-Blanco, A. 2001. The effect of dietary protein and total energy content on digestive enzyme activities, growth and survival of *Litopenaeus setiferus* (Linnaeus 1767) postlarvae. – *Aquaculture Nutrition* **7**: 113–122.

Hernández-Cortés, M. P., Quadros, W., Navarrete, A., Portillo, G., Colado, G. and García-Carreño, F. L. 1999. Rate of ingestion and proteolytic activity in digestive system during continuous feeding of juveniles shrimps. – *Journal of Applied Aquaculture* **9**: 35–45.

Johnston, D. J., Calvert, K. A., Crear, C. G. and Carter, C. G. 2003. Dietary carbohydrate/lipid ratios and nutritional condition in juvenile southern rock lobster *Jasus edwardsii*. – *Aquaculture* **220**: 667–682.

Jones, C. M. 1995. Production of juvenile redclaw crayfish, *Cherax quadricarinatus* (von Martens) (Decapoda, Parastacidae) I. Development of hatchery and nursery procedures. – *Aquaculture* **138**: 221–238.

Lee, P. G. and Lawrence, A. L. 1997. Digestibility. In:D'Abramo, L. R., Conklin, D. E. and Akiyama, D. M. (Eds): Crustacean Nutrition, vol. 6, pp. 194–260. Advances in World Aquaculture, World Aquaculture Society, Baton Rouge, LA.

Lee, P. G., Smith, L. L. and Lawrence, A. L. 1984. Digestive proteases of *Penaeus vannamei* Boone: Relationship between enzyme activity, size and diet. – *Aquaculture* **42**: 225–239.

Lemos, D., Hernández-Cortés, M. P., Navarrete, A., García-Carreño, F. L. and Phan, V. N. 1999a. Ontogenetic variation in digestive proteinase activity of larvae and postlarvae of the pink shrimp *Farfantapenaeus*. *paulensis* (Crustacea: Decapoda: Penaeidae). – *Marine Biology* **135**: 653–662.

Lemos, D., Navarrete del Toro, A., Córdoba-Murueta, J. H. and Garcia-Carreño, F. 1999b. Testing feeds and feed ingredients for juvenile pink shrimp Farfantapenaeus paulensis: in vitro determination of protein digestibility and proteinase inhibition.. – *Aquaculture* **239**: 307–321.

Levi, T., Barki, A., Hulata, G. and Karplus, I. 1999. Mother–offspring relationships in the redclaw crayfish *Cherax quadricarinatus*. – *Journal of Crustacean Biology* **19**: 477–484.

Le Vay, L., Jones, D.A., Puello-Cruz,, A.C., Sangha, R.S. and Ngamphongsai, C. 2001. Digestion in relation to feeding strategies exhibited by crustacean larvae.. – *Comparative Biochemistry and Physiology* **128**: 621–628.

Linton, S. M. and Greenaway, P. 2004. Presence and properties of cellulase and hemicellulase enzymes of the gecarcinid land crabs *Gecarcoidea natalis* and *Discoplax hirtipes*. – *Journal of Experimental Biology* **207**: 4095–4104.

Linton, S. M., Allardyce, B. J., Hagen, W., Wencke, P. and Saborowski, R. 2009. Food utilisation and digestive ability of aquatic and semi-terrestrial crayfishes, *Cherax destructor* and *Engaeus sericatus* (Astacidae, Parastacidae). – *Journal of Comparative Physiology B* **179**: 493–507.

López Greco, L., Vazquez, F. and Rodríguez, E. M. 2007. Morphology of the male reproductive system and spermatophore formation in the freshwater 'redclaw' crayfish *Cherax quadricarinatus* (von Martens, 1898) (Decapoda, Parastacidae). – *Acta Zoologica* **88**: 223–229.

López Greco, L., Tropea, C., Sacristán, H. J., Calvo, N. S., Stumpf, L., Franco Tadic, L. M., Castillo Díaz, F., Arias, M. and Tomas, A. L. 2011. Estructura del hepatopáncreas de la langosta de agua dulce *Cherax quadricarinatus* (DECAPODA, PARASTACIDAE). Proceedings of Primeras Jornadas de Ciencias Morfológicas de las Sierras de Tandil, Argentina.

López-López, S., Nolasco, H., Villarreal-Colmenares, H. and Civera Cerecedo, R. 2005. Digestive enzyme response to supplemental ingredients in practical diets for juvenile freshwater crayfish *Cherax quadricarinatus*. – *Aquaculture Nutrition* **11**: 79–85.

Luchini, L. 2004. Algo más sobre el cultivo de la redclaw (*Cherax quadricarinatus*). Ed. Secretaría de Agricultura, Ganadería, Pesca y Alimentos (SAGPyA), Subsecretaría de Pesca y Acuicultura, Dirección de Acuicultura. Buenos Aires. 15 p. http://www.sagpya.mecon.gov.ar.

Luo, W., Zhao, Y., Zhou, Z., An, C. and Ma, Q. 2008. Digestive enzyme activity and mRNA level of trypsin in embryonic redclaw crayfish, *Cherax quadricarinatus*. – *Chinese Journal of Oceanology and Limnology* **26**: 62–68.

Maugle, P.D., Deshimaru, O., Katayama, T., Nagatani, T. and Simpson, K.L. 1983. Effect of microencapsulated amylase and bovine trypsin dietary supplements on growth and metabolism of shrimp. – *Bulletin of the Japanese Society for the Science of Fish* **49**: 1421–1427.

Miller, R. G. 1981. Simultaneous Statistical Inference, New York, Springer-Verlag.

Moss, S. M., Divakaran, S. and Kim, B. G. 2001. Stimulating effects of pond water on digestive enzyme activity in the Pacific white shrimp, *Litopenaeus vannamei* (Boone). – *Aquaculture Research* **32**: 125–131.

Navarrete Del Toro, M. D. L. A., García-Carreño, . F., López, M. D., Celis-Guerrero, L. and Saborowski, R. 2006. Aspartic proteinases in the digestive tract of marine decapod crustaceans. – *Journal of Experimental Zoology A* **305**: 645–654.

NRC (National Research Council, Subcommittee on Fish Nutrition) 1993. Nutrient Requirements of Fish. The National Academies Press, Washington, DC.

Pavasovic, A. 2008. Evaluation of the Nutritional requirements of redclaw crayfish, *Cherax quadricarinatus*. PhD Dissertation, Queensland University of Technology.

Pavasovic, M., Richardson, N. A., Anderson, A. J., Mann, D. and Mather, P. B. 2004. Effect of pH, temperature and diet on digestive enzyme profiles in the mud crab, *Scylla serrata*. – *Aquaculture* **242**: 641–654.

Pavasovic, A., Richardson, N. A. and Anderson, A. J. 2006. Influence of insoluble dietary cellulose on digestive enzyme activity, feed digestibility and survival in the red claw crayfish, *Cherax quadricarinatus* (von Martens). – *Aquaculture Research* **37**: 25–32.

Pavasovic, A., Anderson, A. J., Mather, P. B. and Richardson, N. A. 2007. Effect of variety of animal, plant and single-cell- based feed ingredients on diet digestibility and digestive enzyme activity in redclaw crayfish, *Cherax quadricarinatus* (von Martens 1868). – *Aquaculture* **272**: 564–572.

Rojo, L., Muhlia-Almazan, A., Saborowski, R. and García-Carreño, F. 2010. Aspartic cathepsin d endopeptidase contributes to extracellular digestion in clawed lobsters *Homarus americanus* and *Homarus gammarus*. – *Marine Biotechnology* **12**: 696–707.

Saborowski, R., Thatje, S., Calcagno, J. A., Lovrich, G. A. and Anger, K. 2006. Digestive enzymes in the ontogenetic stages of

the southern king crab, *Lithodes santolla*. – *Marine Biology* **149**: 865–873.

Sacristán, H. J., Franco Tadic, L. M. and López Greco, L. 2011. Efecto de la dieta sobre la estructura del hepatopancreas en la langosta *Cherax quadricarinatus* (Decapoda). Proceedings of Primeras Jornadas de Ciencias Morfológicas de las Sierras de Tandil, Argentina.

Sacristán, H. J., Franco Tadic, L. M. and López Greco, L. 2013. Influencia de la alimentación sobre el ritmo circadiano de las enzimas digestivas en el cultivo de juveniles de la langosta de agua dulce *Cherax quadricarinatus* (Parastacidae). – *Latin American Journal of Aquatic Research* **416–417**: 85–91.

Sánchez de Bock, M. F. and López Greco, L. 2010. Sex reversal and growth performance in juvenile females of the freshwater crayfish "red claw" *Cherax quadricarinatus* (Parastacidae) by effect of temperature and androgenic gland included on food. – *Aquaculture International* **18**: 231–243.

Saoud, I. P., Garza De Yta, A. and Ghanawi, I. J. 2012. A review of nutritional biology and dietary requirements of redclaw crayfish *Cherax quadricarinatus* (von Martens 1868). – *Aquaculture Nutrition* **18**: 349–368.

Saoud, I. P., Ghanaw, I. J., Thompson, K. R. and Webster, C. D. 2013. A review of the culture and diseases of redclaw crayfish *Cherax quadricarinatus* (von Martens 1868). – *Journal of the World Aquaculture* **44**: 1–29.

Simon, C. J. 2009. Digestive enzyme response to natural and formulated diets in cultured juvenile spiny lobster, *Jasus edwardsii*. – *Aquaculture* **294**: 271–281.

Simon, C.J. and Jeffs, A. 2008. Feeding and gut evacuation of cultured juvenile spiny lobsters, Jasus edwardsii.. – *Aquaculture* **280**: 211–219.

Sokal, R. R. and Rohlf, F. J. 1995. Biometry: The Principles and Practice of Statistics in Biological Research, 3rd edn. W. H. Freeman and Co., New York.

Sousa, L. G. and Petriella, A. M. 2006. Morphology and histology of *Paleomonetes argentinus* (Crustacea, Decapoda, Caridea) digestive tract. – *Biocell* **30**: 287–294.

Stumpf, L., Calvo, N., Pietrokovsky, S. and López Greco, L. 2010. Nutritional vulnerability and compensatory growth in early juveniles of the "redclaw" crayfish *Cherax quadricarinatus*. – *Aquaculture* **304**: 34–41.

Stumpf, L., Calvo, N., Castillo Díaz, F., Valenti, W. C. and López Greco, L. 2011. Effect of intermittent feeding on growth in early juveniles of the crayfish *Cherax quadricarinatus*. – *Aquaculture* **319**: 98–104.

Tacon, A. G. J. and Akiyama, D. M. 1997. Feed ingredients. In D'Abramo, L. R., Conklin, D. E. and Akiyama, D. M. (Eds): Crustacean Nutrition, vol. 6, pp. 411–472. Advances in World Aquaculture, World Aquaculture Society, Baton Rouge, LA.

Teschke, M. and Saborowski, R. 2005. Cysteine proteinases substitute for serine proteinases in the midgut glands of *Crangon crangon* and *Crangon allmani* (Decapoda: Caridea). – *Journal of Experimental Marine Biology and Ecology* **316**: 213–229.

Thompson, K. R., Bailey, T. J., Metts, L. S., Brady, Y. J. and Webster, C. D. 2010. Growth response and fatty acid composition of juvenile red claw crayfish (*Cherax quadricarinatus*) fed different sources of dietary lipid. – *Aquaculture Nutrition* **16**: 604–615.

Tropea, C., Piazza, Y. and López Greco, L. 2010. Effect of long-term exposure to high temperature on survival, growth and reproductive parameters of the "redclaw" crayfish *Cherax quadricarinatus*. – *Aquaculture* **302**: 49–56.

Tropea, C., Hermida, G. N. and López Greco, L. 2011. Effects of androgenic gland ablation on growth and reproductive parameters of *Cherax quadricarinatus* males (Parastacidae, Decapoda). – *General and Comparative Endocrinology* **174**: 211–218.

Vazquez, F., Tropea, C. and López Greco, L. 2008. Development of the female reproductive system in the freshwater crayfish *Cherax quadricarinatus* (Decapoda, Parastacidae). – *Invertebrate Biology* **127**: 433–443.

Vega-Villasante, F., Nolasco, H. and Civera, R. 1993. The digestive enzymes of the Pacific brown shrimp *Penaeus californiensis*. I- Properties of amylase activity in the digestive tract. – *Comparative Biochemistry and Physiology B* **106**: 547–550.

Versaw, K. W., Cupper, L. S., Winters, D. D. and Williams, E. L. 1989. An improved colorimetric assay for bacterial lipase in nonfat dry milk. – *Journal of Food Science* **54**: 1557–1568.

Villarreal, H. and Hutchings, R. W. 1996. Biología y Cultivo de la langosta de agua dulce *Cherax quadricarinatus*. Manual de Producción. Navimar, S.A., Guayaquil, Ecuador p 500.

Xue, X. M., Anderson, A. J., Richardson, N. A., Anderson, A. J. and Xue, G. P. 1999. Characterisation of cellulase activity in the digestive system of the redclaw crayfish (*Cherax quadricarinatus*). – *Aquaculture* **180**: 373–386.

