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In vivo and *In vitro* Protein Digestibility of Formulated Feeds for *Artemesia longinaris* (Crustacea, Penaeidae)

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ABSTRACT

This study was undertaken to determine the in vivo *crude protein apparent digestibility in the prawn* Artemesia longinaris, *using feeds with 0.25% of chromic oxide and animal (fish meal, meat and bone meal and squid protein concentrate) and plant (soybean meal) ingredients. Three replicate groups of prawn were fed and the feces were collected. The rate of protein hydrolysis was measured* in vitro *using midgut gland enzyme extract from the prawns fed the respective feeds and was compared with those found with enzyme extract of wild prawn. The* in vivo *apparent digestibility coefficients showed significant differences among the feeds ($P<0.05$). Fish meal feed presented the highest digestibility (92%); intermediate digestibility (83%) was found for meat and bone meal feed, and the less digestible feed (63%) was that containing soybean meal and squid proteins concentrate. No significant differences in the* in vitro *protein digestibility were found among the experimental feeds. The results indicated the limitation of* in vitro *enzyme assays and that it should be complemented by* in vivo *studies.*

Key words: Prawn; nutrition; protein sources; enzyme assays

INTRODUCTION

A formulated feed for shrimps can be well balanced and contain all dietary essential nutrients but still it does not produce adequate growth because the nutrients are not readily available. The digestibility of feedstuffs can be affected by the relative ratio of nutrients as well as the presence of inhibitory components in the ingredients (Lee and Lawrence, 1997). Digestibility data are important for the formulation of suitable feeds. Protein is the major and the most expensive ingredient in shrimp feeds and is also a growth limiting. The high amount of protein is also required in shrimp feeds increases the cost and makes the feed into a major expense in shrimp production. Therefore, it is very important to identify the low-cost protein rich ingredients in order to reduce the production costs of shrimp.

Variations in protein requirements in marine shrimp are attributed mainly to different sources of proteins used in the formulations. In recent years, the interest to identify and use alternative protein sources for use within aquafeeds has been increasing (Tacon et al., 1998). Alternate protein ingredients were used to supplement a formulated feed containing fish meal as the main protein source (Divakaran et al., 2000). Soybean meal is the most important source of plant protein, which is currently used to supplement the feeds for

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cultured shrimp to reduce the cost. However, it cannot be used as the only source of protein because it lacks certain essential amino acids and contains antinutritional factors such as lectins and proteinase inhibitors (Córdoba Murueta and García Carreño, 2002). Squid protein has been used as a protein source in the feeds and results suggest a favorable response of penaeids (Cruz Ricque et al., 1987; Díaz et al., 1999). This component may, thus, be of economic potential in those areas of the world in which squid can be obtained at low cost. Meat and bone meal is the main by-products of animal slaughterhouse, including meat scraps and trimmings. The quality of meat meal as a protein supplement depends on its production process as well as on the raw material used (Tacon and Akiyama, 1997).

Argentina is one of the main producers of meat and bone meal and soybean meal on a commercial scale. The squid *Illex argentinus* is an abundant resource in South West Atlantic ocean. The use of these protein sources as part of penaeoid shrimp diets has been shown to be important (Díaz et al., 1999; Díaz and Fenucci, 2002).

In aquaculture, feeding trials have been used to measure the nutritive value of feeds for growth performance; but they are too long and expensive, and the results may be affected by the environmental factors (Lan and Pan, 1993; Wasielesky Jr. et al., 2003). Studies focused to ascertain the nutrients digestibility coefficients usually consist of feeding a feed containing fixed concentrations of a nutrient and an indigestible marker to measure in the feces (Fenucci et al., 1980; Divakaran et al., 2002). Species specific *in vitro* digestibility methods have been developed using digestive enzymes from the species in question (Lan and Pan, 1993; Ezquerra et al., 1998; Ramos Díaz et al., 2001; Lemos et al., 2004). These methods are usually faster and less expensive than *in vivo* methods. The *in vitro* assay using shrimp digestive gland enzyme extract has potential as a promising tool in estimating the digestibility of alternative protein sources. However, digestibility *in vivo* data are needed to validate data obtained in the digestibility in vitro (Ezquerra et al., 1997). *In vivo* and *in vitro* assays must correlate well to predict protein quality (Ezquerra et al., 1997; 1998; Lazo et al., 1998).

The prawn *Artemesia longinaris* is distributed along the South American coast, from 23°S to 43°S (Boschi and Gavio, 2005) and is one of the most valuable species in the markets of Brazil, Uruguay and Argentina. Because of the yearly fluctuations of catches, it is important to establish the feasibility of culturing *A. longinaris* in order to maintain a continuous supply to the market.

Some studies have demonstrated good survival and growth under culture conditions (Fenucci et al., 1983; Petriella et al., 1984), determined the nutritional requirements (Fernandez Gimenez and Fenucci, 2002; Romanos Mangialardo and Fenucci, 2002) and characterized the digestive proteinases in relation to the molting cycle (Fernandez Gimenez et al., 2002). The present study aimed to evaluate the apparent digestibility of protein in formulated feeds for *A. longinaris*, compared the results with *in vitro* assays of formulated feeds and protein feed ingredients, and evaluated the possible adaptation of digestive enzymes according to the dietary protein sources.

MATERIAL AND METHODS

Feed and Feeding Trials

Artemesia longinaris prawns, weighing 1.3 ± 0.51 g, were obtained from a commercial fisherman in the coastal waters of Mar del Plata, Argentina (38°S).

The treatments consisted of three dry pelletized feeds with 0.25% chromic oxide as an inert marker. The composition of the formulated feeds were: one of the formulated feeds contained fish meal as main protein source (D1), and the others had meat and bone meal (D2) and soybean meal and squid protein concentrate (D3) in partial substitution of fish meal (Table 1). Feed ingredients were obtained from a local feed manufacturer. Formulations were made according to the chemical composition of the by-products in order to obtain isoproteic and isolipidic diets. The chemical composition of the formulated feeds was confirmed through the proximate analysis (Table 2) according to AOAC (1990). All ingredients were mixed and cold pelleted (<50°C) by extrusion (Fenucci and Zein-Eldin, 1976). The pellets were oven-dried at 50°C for 24 h.

Table 1 - Ingredient composition of formulated feeds.

Ingredient	Formulated feed (g/100g dry feed)		
	D1	D2	D3
Fish meal[a]	48	27	27
Meat and bone meal[b]	-	23	-
Soybean meal[c]	17	17	23
Squid protein concentrate[d]	-	-	10
Manioc starch	20	20	22
Wheat	9.25	7.25	12.25
Fish oil	2	2	2
Fish soluble	2	2	2
Lecithin	0.5	0.5	0.5
Cholesterol	0.5	0.5	0.5
Vitamin supplement[e]	0.5	0.5	0.5
Chromic oxide[f]	0.25	0.25	0.25
Proximate composition (% dry matter)			
Dry matter	99.55	99.47	99.60
Crude protein	39.30	39.80	37.20
Total lipid	8.30	7.80	7.00
Ash	6.23	5.94	5.20

[a] Agustinier S.A. Mar del Plata, Argentina.
[b] Oleochemicals Materia Hnos. S.A.C.I.F. Mar del Plata, Argentina.
[c] Melrico S.A. Argentina.
[d] Díaz et al. 1999.
[e] Vitamin premix (mg/kg of premix): cholecalciferol 35; thiamin 163; rivoflavin 156; pyridoxine 213; calcium pantothenate 250; biotin 250; niacin 500; folic acid 25; B_{12} HCL 20; ascorbic acid Rovimix STAY C 781; menadione 34; inositol 300; choline chloride 200; a-tocopherol acetate 1750; vitamin A acetate 180 (Laboquímica SRL, Argentina).
[f] Hopkin and Williams Ltd. England.

All the individuals were kept in 150 l glass aquaria with continuous aeration. Filtered seawater (5 μm) was exchanged at a rate of 50% per day. Prawns were exposed to an 11 h light-14 h dark photoperiod, temperature 18°C, pH 7, salinity 31ppt. The ammonium concentration never exceeded 0.2 mg/l. All the groups were fed *ad libitum* once a day (09:30 h). Formulated feeds were tested in triplicate groups of eight prawns randomly chosen.

Apparent Digestibility

A seven day period of adjustment to the new conditions and diets was implemented before the beginning of fecal collection. To determine the apparent digestibility for crude protein, before each feeding, feces were collected during 16 days by siphoning and separated from uneaten diet by an appropriate mesh screen. Fecal samples were rinsed with distilled water to eliminate the excess of salts and frozen (-20ºC).

The diets and fecal samples were oven-dried at 50ºC for 24 h. Proximate analyses of diets (Table 1) and fecal samples were carried out using AOAC methods (1990). The chromic oxide levels in diets and feces were analyzed using a modified colorimetric method (Fenucci, 1981). The levels were measured with a spectrophotometer (540 nm) (Shimadzu UV-2102 PC, UV-visible Scanning Spectrophotometer) after perchloric acid oxidation and forming a colored complex with diphenylcarbazide (DPC).

The apparent digestibility coefficient (ADC) was estimated according to Fenucci et al. (1980):

$$ADC\ (\%\ protein\ digestibility) = 100 - (I_a/I_b \,.\, II_b/II_a \,.\, 100)$$

Where $I_{a=}$ $\%Cr_2O_3$ feed; $I_{b=}\%$ Cr_2O_3 feces; $II_{a=}\%$ protein food; $II_{b=}\%$ protein feces

In vitro Digestibility

To determine if the animals had acclimated to the formulated feed, the changes in protein digestion from the *in vitro* assay between the midgut gland of wild prawn (control) and of prawn from each treatment were compared at the end of the feeding trial (feeds D1, D2, and D3).

In vitro assays were conducted at the end of the experiment with each formulated feeds (D1, D2 and D3) to determine the protein digestibility in

the midgut gland of *A. longinaris*. Specimens were placed on ice and midgut gland were dissected. Samples were immediately stored at –20ºC. Samples of individuals from the same treatment were pooled. Frozen midgut gland were homogenized in chilled distilled water and centrifuged for 30 min (10,000 *g* at 4ºC). The lipid layer was removed and total soluble protein was evaluated in the supernatants (Bradford, 1976), with albumin from chicken egg white (Sigma) as standard. Total proteinase activity was assayed using 1% azocasein in 50 mM Tris-HCl buffer, pH 7.5 (García Carreño, 1992) (Table 2).

Table 2 - Protein content and specific activity in enzyme extracts of *A. longinaris.*

Treatment	Protein content (mg/ml)	Specific activity (Abs/min/mg protein)
D1	6.80[a]	0.33[a]
D2	5.03[a]	0.40[a]
D3	7.23[a]	0.20[a]
Wild	6.01[a]	0.44[a]

Values are means of three replicate analyses. Different superscript letters in the same column show statistical differences (P<0.05).

Formulated feeds were finely ground with a mortar and pestle to pass through at 80 mesh screen. The amount of each feed needed to provide 10 mg/l of crude protein, determined from the proximate analysis (Table 1), was mixed with 50 mM Tris-HCl buffer (pH 7.5). A sample of 6 ml supernatant was mixed with a volume of each crude enzyme extract with equivalent units of enzyme activity (Table 2) with vibration at 30ºC for 0.5, 1, 1.5, and 3 h. One mililitre of the mixture was taken for proteolysis, and the process was stopped by the addition of 1 ml of 20% trichloroacetic acid. The material was filtered and the absorbance (280 nm) was measured (Lan and Pan, 1993). Three replicates of the same treatment corresponding to the crude enzyme extract were used to measure the enzyme hydrolysis.

Statistical Analysis

Regression analyses were performed using Origin (4.1) on the actual and predicted chromic oxide values. A correlation coefficient was used to describe the fit of the data on the regression line. ANCOVA was used to test differences among the regression lines. Arc sine transformation was applied to percentages. ANOVA and Student's *t*-test were used to find the differences among the apparent digestibility coefficients and the hydrolysis values among the treatments (P<0.05) (Sokal and Rohlf, 1995).

RESULTS AND DISCUSSION

Results showed that *in vivo* apparent digestibility coefficients of protein varied from 63 to 92% in *A. longinaris*, revealing significant differences among the feeds (P<0.05) (Table 3). The present *in vivo* digestibility results provided a ranking of protein quality for the feeds. The feed with fish meal as main protein source (D1) was more digestible than the meat and bone meal (D2), and soybean meal plus squid protein concentrate (D3) diets.

Table 3 - *In vivo* apparent protein digestibility coefficients for formulated feed consumed by *A. longinaris*.

Treatment	*In vivo* (%)
D1	92.15 ± 0.435[a]
D2	83.83 ± 3.482[b]
D3	63.13 ± 3.574[c]

Values are means ± standard error of triplicates.
Percentages with similar superscripts are not significantly different (P<0.05).

Akiyama et al. (1989) and Ezquerra et al. (1997) reported that chromic oxide seemed to be an appropriate digestibility indicator for penaeid shrimp using a chromic oxide level of 1%. In the present study, its use at a concentration of 0.25% was adequate because of the amount of feces collected, high digestibility rate, low standard deviation among replicates, and homogeneous mixing of chromic oxide into the formulated feed.

A previous study tried to determine the suitability of various protein sources and optimum protein levels for supporting the growth and survival of the shrimp *Pleoticus muelleri* (Díaz and Fenucci, 2002). In this work, four formulated feeds with 0, 20, 30, and 50% of meat and bone meal to replace fish meal were tested. No significant differences were detected in the final weight gain or survival among the treatments. In *A. longinaris*, a higher digestibility (83.83%) was observed with a formulated feed containing 50% of meat and bone meal in substitution of fish meal (D2). Forster et al. (2003) examined three meat and bone meals and reported similar values of digestibility coefficients for the crude protein in *Litopenaeus vannamei* (81.04 to 87.84%). The feeding experiments suggested that meat and bone meal could be utilized as a suitable replacement for fish meal in a formulated diet for penaeoid species.

In the present study, apparent digestibility for the formulated feed with soybean meal (D3) were lower to those reported by other researchers for *L. vannamei* (Ezquerra et al., 1997; Divakaran et al., 2000). Lee et al. (1984) and Smith et al. (1985) showed that an adaptative change occurred in the digestive enzymes of *L. vannamei* when shrimp exceeded 10 g, which determined a lower capacity to utilize the animal proteins than smaller shrimps. While shrimp grows up, the protein source is less important and it assimilates similarly both animal and plant proteins. *A. longinaris* reaches 160 mm total length (Vinuesa, 2005), the size used in this study corresponded to juvenile prawns (35 mm). Till now, studies have not been done yet about the aspects related to the ontogenic development of the digestive tract and its relation to feeding habits, and about the digestive physiology that allows determining the biochemical mechanisms used to metabolize the different feeds. The identification of the digestive enzymes and the knowledge of the differences in the digestive potential among the life stages are necessary to formulate the feeds adapted to the physiology of the studied species.

Lemos et al. (2004) found that in *Farfantepenaeus paulensis*, soybean meal exhibited reduced digestibility and a high degree of protease inhibition. The occurrence of trypsin inhibitor can be attributed to insufficient heat during processing (Swick, 2000, Genovese et al., 2006). Since the ingredients tested were obtained from the feed manufacturers, the poor digestibility observed for soybean meal in *A. longinaris* would be a reason to increase the quality control of this feedstuff prior to use in shrimp feeds.

The *in vitro* methods have been applied to the study of digestibility in crustacean (Lan and Pan, 1993; Ezquerra et al., 1997; Lazo et al., 1998). Such methods have potential as screening devices for determining the suitability of feed ingredients for shrimp feeds. *In vitro* methods based on the pH monitoring tend to overestimate protein source from plant origin with regards to proteins from animal origin (Haard, 1993). Lan and Pan method (1993) used in *Penaeus monodon* was chosen, because it confirmed the general pattern described for the apparent digestibilities of typical crustacean feedstuffs for shrimp: casein >wheat gluten >fish meal >soybean meal (Lee and Lawrence, 1997).

Fig. 1 shows the extent of feed protein hydrolysis with extracts from the midgut gland of wild and experimental prawns. No significant differences in the *in vitro* protein digestibility were found among the formulated feeds as indicated by rate of hydrolysis. At 30ºC and pH 7.5, all the feeds hydrolysate reached maximal A_{280nm} within 1.5 h.

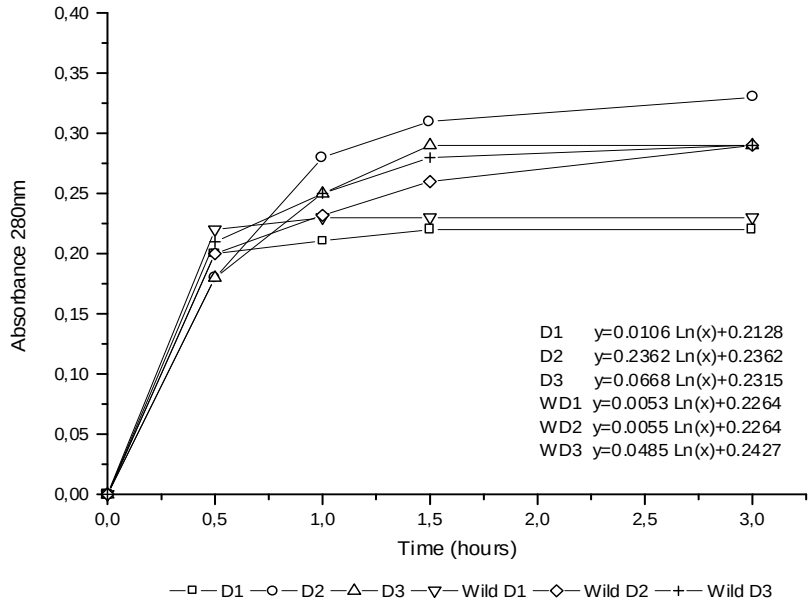

Figure 1 - Protein hydrolysis of formulated feeds (D1, D2 and D3) at pH 7.5 and 30ºC with crude extract from midgut gland from prawns fed each respective diet and wild *A. longinaris*.

In the present work, there were significant differences (P<0.05) among the feeds in the apparent protein digestibility of *A. longinaris.* However, there were no differences with *in vitro* assays. In *in vitro* methods, the reactions are more drastic than those that occur during the natural digestion of crustaceans and will free nutrients otherwise unavailable (Anderson et al., 1993). Similarly, there is a tendency to overestimate the digestibility of leguminous plants by *in vitro* methods compared with *in vivo* methods (Marletta et al., 1992). On the other hand, the results of *in vivo* methods may be affected by the environmental conditions (temperature, salinity, pH) (Ezquerra et al., 1997).

The present results showed that *A. longinaris* had a good digestibility for all the formulated feeds tested. The study of digestive enzymes in aquatic animals is a very important preliminary step to obtain valuable information that may be further applied to the development of *in vitro* digestibility assays. The knowledge of the specific pattern of protein digestion for this prawn could lead to the adequate formulation of feeds.

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RESUMO

O objetivo do presente trabalho foi determinar a digestibilidade aparente *in vivo* da proteína bruta de ingredientes de origem animal (farinhas de peixe, osso e carne e concentrado de proteína de lula) e ingredientes vegetais (farinha de soja) em camarões *Artemesia longinaris* utilizando rações contendo 0,25% de óxido de cromo. Três grupos de camarões, utilizados como replicatas, foram alimentados e as fezes coletadas. A velocidade de hidrólise da proteína de cada ração foi medida *in vitro* utilizando extrato enzimático da glândula do intestino médio dos camarões alimentados com a ração correspondente e foi comparado com

aqueles obtidos com o extrato enzimático de camarões selvagens. Os coeficientes de digestibilidade aparente *in vivo* mostraram diferenças significativas entre as rações testadas (P<0,05). A farinha de peixe apresentou a maior digestibilidade (92%), enquanto valores intermediários de digestibilidade (83%) foram encontrados para a farinha de carne e ossos. A ração contendo farinha de soja e concentrado de proteína de lula resultou em menor digestibilidade (63%). Não houve diferença significativa entre os valores de digestibilidade *in vitro* para as rações testadas. Estes resultados indicam a limitação inerente dos ensaios enzimáticos *in vitro*, os quais poderiam ser complementados com estudos *in vivo*.

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