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Prediction of culture performance of juvenile *Litopenaeus vannamei* by *in vitro* (pH-stat) degree of feed protein hydrolysis with species-specific enzymes

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Abstract

Rapid *in vitro* methods for measuring digestibility may be useful in analysing aqua feeds if the extent and limits of their application are clearly defined. The pH-stat protein digestibility routine with shrimp hepatopancreas enzymes was previously related to apparent protein digestibility with juvenile *Litopenaeus vannamei* fed diets containing different protein ingredients. The potential of the method to predict culture performance of shrimp fed six commercial feeds (T3, T4, T5, T6, T7 and T8) with 350 g kg^{-1} declared crude-protein content was assessed. The consistency of results obtained using hepatopancreas enzyme extracts from either pond or clear water-raised shrimp was further verified in terms of reproducibility and possible diet history effects upon *in vitro* outputs. Shrimps were previously acclimated and then maintained over 56 days (initial mean weight 3.28 g) on each diet in 500-L tanks at 114 ind m^{-2}, clear water closed system with continuous renewal and mechanical filtering (50 μm), with four replicates per treatment. Feeds were offered four times daily (six days a week) delivered in trays at feeding rates ranging from 4.0% to 7.0% of stocked shrimp biomass. Feed was accessible to shrimp 4 h daily for 1-h feeding period after which uneaten feed was recovered. Growth and survival were determined every 14 days from a sample of 16 individuals per tank. Water quality was monitored daily (pH, temperature and salinity) and managed by water back flushing filter cleaning every 7–10 days. Feeds were analysed for crude protein, gross energy, amino acids and pepsin digestibility. *In vitro* pH-stat degree of protein hydrolysis (DH%) was determined for each feed using hepatopancreas enzyme extracts from experimental (clear water) or pond-raised shrimp. Feeds resulted in significant differences in shrimp performance ($P < 0.05$) as seen by the differences in growth rates (0.56–0.98 g $week^{-1}$), final weight and feed conversion ratio (FCR). Shrimp performance and *in vitro* DH% with pond-raised shrimp enzymes showed significant correlation ($P < 0.05$) for yield ($R^2 = 0.72$), growth rates ($R^2 = 0.72$–0.80) and FCR ($R^2 = -0.67$). Other feed attributes (protein : energy ratio, amino acids, true protein, non-protein nitrogen contents and *in vitro* pepsin digestibility) showed none or limited correlation with shrimp culture performance. Additional correlations were found between growth rates and methionine ($R^2 = 0.73$), FCR and histidine ($R^2 = -0.60$), and DH% and methionine or methionine + cystine feed contents ($R^2 = 0.67$–0.92). pH-stat assays with shrimp enzymes generated reproducible DH% results with either pond (CV ≤ 6.5%) or clear water (CV ≤ 8.5%) hepatopancreas enzyme sources. Moreover, correlations between shrimp growth rates and feed DH% were significant regardless of the enzyme origin (pond or clear water-raised shrimp) and showed consistent R^2 values. Results suggest the feasibility of using standardized hepatopancreas enzyme extracts for *in vitro* protein digestibility.

KEY WORDS: digestibility, feed, *in vitro*, *Litopenaeus vannamei*, protein, shrimp

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Introduction

Over 2.4 million mt of penaeid shrimp are estimated to be produced annually by aquaculture (FAO, 2006). More than three million mt of feeds are required to sustain a sector valued at over $9 billion. Feeds are often among the largest variable costs in shrimp aquaculture. Accordingly, protein is a major limiting nutrient and a primary cost in compound

feeds, representing over 1 million mt into shrimp feeds yearly. In semi-intensive and intensive farming systems, dietary protein is generally the main source of indispensable amino acids for the deposition of new tissue protein into somatic growth. On the other hand, efficient dietary protein conversion into growth depends on the feed having adequate amino acid composition and availability.

Current methods to determine protein digestibility involve *in vivo* trials, and the collection and analysis of feeds and faeces (Forster *et al*. 2003; Smith & Tabrett 2004). Because of limitations in output (time-consuming and expensive) and the need for specialized facilities for animal rearing, rapid and practical *in vitro* methods are desirable as a responsive input to be considered in feed manufacturing (Tacon 1996; Lazo & Davis 2000). Most of the available *in vitro* tests employed in aqua feeds follow or adapt methodologies routinely used for warm-blooded terrestrial livestock including reactions with purified commercial enzymes (Dimes & Haard 1994). However, the non-specific nature of these enzymes has been a major limiting factor to consistent application into nutritional assessments for fish and shrimp species (Dimes *et al*.1994; Ezquerra *et al*. 1997; Lemos *et al*. 2004).

An *in vitro* pH-stat routine to determine protein digestibility using species-specific shrimp digestive enzymes is a promising alternative to assess the protein quality of ingredients and finished feeds (Lemos *et al*. 2000; Nates & Tacon 2005). Though capable to predict apparent protein digestibility in some feedstuffs for *Litopenaeus vannamei* (Ezquerra *et al*. 1998; Lemos & Nunes 2007), additional validation with shrimp performance data could check if digestible dietary protein *in vitro* would render better growth in culture trials (Sugiura 2000; Forster *et al*. 2003). The general objective of this study was to verify if effective feed protein hydrolysis by shrimp enzymes can be reflected into growth. Thus, the potential of the *in vitro* pH-stat method using shrimp hepatopancreas enzymes was assessed in the prediction of culture performance of shrimp-fed commercial feeds of similar protein content. The consistency of using hepatopancreas enzyme extracts obtained from either pond or clear water-raised shrimp was further determined in terms of reproducibility and possible diet history effects upon *in vitro* degree of protein hydrolysis.

Material and methods

Shrimp, management and experimental design

Juvenile *L. vannamei* (2.30 ± 1.01 g, $n = 384$) obtained from a commercial farm were stocked into 24 indoor polypropylene tanks of 500-L volume (bottom area of 0.57 m^2) at 114 shrimp m^{-2} (65 shrimp $tank^{-1}$). The rearing system operated under clear water conditions with continuous water recirculation and with artificial aeration provided from two 2.0-hp mechanical blowers. Rearing water was partially discharged and replaced with new filtered seawater every 7–10 days through backflushing of sand filters. Water quality was monitored daily for pH, temperature and salinity. Over the rearing period, water quality was kept at 7.6 ± 2.5 pH, 29.5 ± 0.6 °C temperature and 33.0 ± 4.1 g L^{-1} salinity.

Shrimps were exposed to feed for 4 h daily with excess feed for 1 h at 0700, 1000, 1300 and 1500 h. All feed were delivered in feeding trays of 14.3 by 3.5 cm (diameter by height). No feeding took place on Sundays. Animals were fed to apparent satiation following feeding rates based on the maximum meal determined for *Farfantepenaeus subtilis* (Nunes & Parsons 2000). Every 2 weeks, 16 animals per tank were collected, weighted and returned to their respective tank. During shrimp sampling, survival was visually estimated and the stocked biomass determined for adjustment of the daily ration.

After stocking, shrimps were acclimated for 20 days with a crumbled feed containing 400 g kg^{-1} of crude-protein (CP). Animals started to be fed with treatment diets on the 21st day of culture, after reaching 3.28 g mean body weight. Shrimps were reared on treatment diets for an additional 56 days. Four tanks were randomly assigned for each treatment. At the end of rearing period, feed conversion ratio (FCR), final body weight, weekly growth and final survival were determined. FCR was calculated based on apparent feed intake (dry basis) estimated after recovery and weighting of feed remains from feeding trays after each meal. Apparent feed consumption values were corrected for moisture content, water absorption and leaching rates of each tested feed (Nunes *et al*. 2006).

Feeds and analysis

Treatment diets consisted of six different commercial feeds manufactured in Brazil for the grow-out culture of *L. vannamei*. Diets were manufactured either by pelleting or extrusion technology. Feed labels reported minimum guaranteed levels of 350 g kg^{-1} CP. In the laboratory, feeds were tagged from T3 through T8 and stored at under −20 °C until use. Feeds were analysed for total nitrogen using a Perkin-Elmer 2400 Ser. 2 C, H, N auto analyzer (Perkin-Elmer, Waltham, Massachusetts, USA) with acetanilide and benzoic acid as standards for assessment of crude-protein content (N × 6.25). Energy content was measured in ground samples of 8–12 mg by wet combustion when the amount of oxygen

expended in the combustion was converted to energy (Karzinkin & Tarkovskaya 1964). Amino acid profile was determined in prehydrolysed ground feed samples injected into a Dionex DX 3000 (Dionex, Bannockburn, IL, USA) ion chromatograph for amino acid separation through cation exchange column following postcolumn reaction with ninhydrin (Spackman *et al.* 1958). Standards were obtained from Sigma Chemical Co. (Sigma, St Louis, MO, USA) Tryptophan was separately analysed (Spies 1967). Levels of amino acids considered indispensable for penaeid shrimp were corrected for possible partial degradation during hydrolysis: methionine, lysine and cystine at 10%, threonine, valine, isoleucine, leucine, phenylalanine, histidine, tryptophan, arginine and tyrosine at 5%. Thus, data presented for these nutrients can be considered as maximum values. Amino acids expressed as per cent of crude protein were related to recommended levels for penaeid shrimp (Guillaume 1997). An essential amino acid index was calculated as the product of each amino acid by its respective recommended level $[=(aa_1/rec_1) \times (aa_2/rec_2) \times \cdots \times (aa_n/rec_n)]$. In contrast, an index for relative essential amino acid deficit corresponded to the sum of deficits with respect to recommended levels ($= def_1 + def_2 + \cdots def_n$, as % diet crude protein). True protein was denoted as the sum of total amino acid mass and the difference with crude-protein values resulted in the non-protein nitrogen fraction.

In vitro *determination of protein digestibility*

The degree of feed protein hydrolysis (DH%) was assessed using the *in vitro* pH-stat system with hepatopancreas enzymes from juvenile *L. vannamei* (Lemos *et al.* 2004). To test for prediction of shrimp culture performance and reproducibility, enzyme extracts were obtained from healthy shrimp from distinct sources; pond-raised (commercial farms in Northeastern and Southern Brazil, denoted as HPf_2 to HPf_4) and shrimp from the current laboratory trial (named as HPt_3 to HPt_8, according to each respective feed treatment), individual weight range 6–9 and 11–14 g, respectively. Shrimp hepatopancreas from the clear water laboratory trial were sorted and used for *in vitro* assays to verify possible diet effects upon protein hydrolysis (Divakaran *et al.* 2004). Enzyme extracts recovered from individuals fed a particular treatment were used to determine *in vitro* DH% for each one of the six feeds tested. After shrimps were sacrificed, the hepatopancreas were pooled into plastic vials under decreased temperature (4 °C) and immediately frozen in liquid nitrogen or dry ice. Enzyme extracts were recovered after homogenization with chilled distilled water and centrifugation at 10 000 × ***g*** for 30 min at 4 °C. After elimination of the lipid layer, pH of recovered supernatants was adjusted to 8.0 with 0.1 N NaOH, labelled according to origin and frozen at −20 °C prior to analysis. Shrimp hepatopancreas extracts were standardized according to the total alkaline proteinase activity. This was determined by the rate of hydrolysis of 1% azocasein in 50 mM Tris buffer, pH 7.5 (Garcia-Carreño 1992). Triplicate 10-μL enzyme extracts were mixed with 0.5-mL substrate solution at 25 °C. The reaction was stopped 10 min later by the addition of 0.5 mL 20% trichloroacetic acid and the mixture was centrifuged for 5 min at 6500 × ***g***. The supernatants were separated from the undigested substrate, and the absorbance for the released dye was recorded at 366 nm. The rate of absorbance change over time was calculated by the difference from reactions stopped at zero (blank controls) and 10 min. Total proteinase activity was expressed as units of change in absorbance per min per volume of the enzyme extract (ΔAbs min^{-1} μL^{-1}).

Feeds were ground so that over 80% of the total particles were smaller than 355 μm. Samples corresponding to 80 mg protein per assay were stirred with distilled water in the hydrolysis vessel for approximately 45 min and the pH of the mixture was continuously adjusted to 7.9 with 0.1 N NaOH. After the pH had stabilized, the protein hydrolysis by shrimp enzymes was determined with a pH-stat titration (Pedersen & Eggum 1983) using a 718 STAT TITRINO (Metrohm, Switzerland) with the adequate software for data logging (Metrodata Menu Program 718 STAT TITRINOPC). Prior to starting the reaction, the pH was automatically adjusted to 8.0. The hydrolysis was initiated by the addition of an enzyme extract volume corresponded to 4.0 U of enzyme activity. Nitrogen was bubbled into the hydrolysis vessel to avoid absorption of atmospheric CO_2 and its possible effect on the pH during hydrolysis. Assays were carried in triplicate and temperature was maintained at 25 ± 0.2 °C using a jacketed reaction vessel and a circulated bath. After a 35-min reaction, the degree of protein hydrolysis (DH%) was calculated as (Adler-Nissen 1986):

$$DH\% = (B \times Nb \times 1/\alpha \times 1/Mp \times 1/H_{tot}) \times 100$$

where B = mL of standard alkali (0.1 N NaOH) consumed to maintain the reaction mixture at pH 8.0; N_b = normality of the titrant; $1/\alpha$ = pK for amino groups at 25 °C pH 8.0; M_p = protein mass in sample (g); H_{tot} = total number of peptide bonds in the protein substrate (meqv g $protein^{-1}$).

To compare this test with a conventional protein quality test, feeds were further analysed for pepsin digestibility at 0.002% porcine pepsin (Sigma Chemical Co.) following A.O.A.C. (1984).

Statistical analysis

Mean results of feed treatments were compared by one-way ANOVA followed by Student–Newman–Keuls's multicomparison test after checking for normality and equal variance of data. In case data did not meet these prerequisites, Kruskall–Wallis' rank analysis was applied followed by Dunn's multiple comparison. Pearson's correlation analysis was applied to correlate feed compositional and culture performance attributes. Differences were considered significant at $P < 0.05$ (Zar 1984).

Results

Feed composition and in vitro *digestibility*

Compositional analysis indicated that tested feeds had crude-protein contents (CP) ranging from 348 g kg^{-1} to 371 g kg^{-1} (Table 1). Gross energy varied from 14.5 to 16.4 KJ g^{-1}. Resulting protein-energy ratios varied between 21.7 and 24.8 mg KJ^{-1}, which is in the range proposed for *L. vannamei* (Cuzon & Guillaume 1997). Levels of 18 amino acids analysed also varied among tested feeds. Some indispensable amino acids such as methionine (T3 to T8), lysine (T6) and the combinations of methionine + cystine (T3 to T8) and phenylalanine + tyrosine (T5 to T7) displayed levels below the recommendation for penaeid shrimp feed (Guillaume 1997; Table 2). Protein and amino acid features also showed variation among feeds (Table 3). The number of deficient individual amino acids ranged from 2 in T4 to 5 in T6 while essential amino acid index was lower in T3 (0.88) and T6 (1.05) compared to T5 (1.62), T4 (1.89), T7 (2.01) and T8 (2.51). Essential amino acid deficit (% CP) had the lowest value in T8 (1.21%) followed by T5 (1.71%), T7 (1.89%) and T4 (1.91%). Higher deficiencies were found with T3 (2.34%) and T6 (2.72%). True protein levels ranged from 31.7% to 34.9% in T6 and T8, respectively, while non-protein nitrogen was higher in T3 and T6 (10.4 and 10.7%, respectively) compared to other feeds.

In vitro degree of protein hydrolysis (DH%) by pond-raised shrimp hepatopancreas enzymes (HPf, mean values among different enzyme extracts) was significantly higher ($P < 0.05$) in T5 (4.73%) and T8 (4.49%) followed by T7 (4.29%) (Table 4). Significant lower values were observed for

	T3	T4	T5	T6	T7	T8
Crude protein (g kg^{-1})	371 (1.2)	348 (0.9)	361 (0.4)	350 (1.2)	356 (0.1)	359 (1.3)
Gross energy (kJ g^{-1})	16.4 (0.77)	16.0 (1.14)	15.2 (1.13)	16.1 (0.86)	15.8 (1.04)	14.5 (0.88)
Protein : energy (mg kJ^{-1})	22.7	21.7	23.7	21.8	22.5	24.8

Table 1 Protein and energy contents of tested commercial diets. Values expressed as mean (SD)

	T3	T4	T5	T6	T7	T8	Recommended level*
Arginine	6.92	7.20	6.71	6.31	6.65	7.37	5.8
Histidine	1.99	2.12	2.01	2.13	2.31	2.20	2.1
Isoleucine	3.66	3.84	3.56	3.70	3.90	3.93	3.4
Leucine	6.87	7.32	7.20	7.90	7.78	7.42	5.4
Lysine	5.45	6.07	5.94	5.14	6.09	6.10	5.3
Methionine	1.38	1.47	1.91	1.46	1.75	1.73	2.4
Methionine + cystine	2.46	2.62	2.83	2.44	2.68	3.07	3.6
Phenylalanine	4.43	4.75	4.35	4.72	4.73	4.84	4.0
Phenylalanine + tyrosine	7.04	7.54	6.74	6.67	6.77	7.78	7.1
Threonine	3.92	4.18	4.05	3.73	4.11	4.17	3.6
Tryptophan	0.91	0.97	0.99	1.05	0.89	0.79	0.8
Valine	4.14	4.36	4.14	3.97	4.32	4.40	4.0
Alanine	5.18	5.49	6.07	5.02	5.68	5.10	NI
Aspartic acid	9.27	10.12	8.89	8.85	9.33	9.59	NI
Cystine	1.08	1.15	0.92	0.98	0.94	1.33	NI
Glutamic acid	14.99	16.22	16.85	17.93	17.45	17.26	NI
Glycine	6.93	7.04	7.70	5.14	6.24	6.69	NI
Proline	5.82	5.89	6.10	5.80	5.70	6.05	NI
Serine	5.01	5.35	4.60	4.60	4.83	5.46	NI
Tyrosine	2.61	2.78	2.39	1.95	2.04	2.93	NI

Table 2 Amino acid composition of tested commercial diets (% of crude protein) and recommendation for essential amino acids in penaeid shrimp

NI, non-indispensable.
*According to Guillaume (1997).

Table 3 Protein and amino acid features of tested commercial diets. Further details in Material and methods

	T3	T4	T5	T6	T7	T8
Deficiency (no. of individual amino acids below recommendation level)	4	2	4	5	3	3
Essential amino acid index ($aa_1/rec_1 \times aa_2/rec_2 \times ... \times aa_n/rec_n$)	0.88	1.89	1.62	1.05	2.01	2.51
Essential amino acid deficit ($def_1 + def_2 + ... def_n$, % crude protein)	2.34	1.91	1.71	2.72	1.89	1.21
True protein (g kg^{-1})	336	335	341	317	337	349
Non-protein nitrogen (g kg^{-1})	104	38	59	107	56	27

Table 4 *In vitro* protein digestibility of tested commercial diets. DH: degree of protein (80 mg) hydrolysis with pond-raised shrimp (*Litopenaeus vannamei*) hepatopancreas enzyme extracts (HPf, mean values among different enzyme extracts); 4 units caseinolytic enzyme activity, 25 °C, 35 min. reaction time. Pepsin digestibility determined at 0.002% enzyme concentration. Results expressed as mean (SD). Values in the same row with common superscripts are not significantly different ($P < 0.05$)

	T3	T4	T5	T6	T7	T8
pH-stat DH, HPf (%)	3.82^{a} (0.22)	3.72^{a} (0.10)	4.73^{b} (0.26)	3.71^{a} (0.20)	4.29^{c} (0.28)	4.49^{bc} (0.18)
Pepsin (%)	78.2^{a} (0.81)	73.7^{b} (0.01)	72.7^{b} (0.20)	81.8^{c} (0.96)	82.1^{c} (0.30)	70.1^{d} (0.78)

T3 (3.82%), T4 (3.72%) and T6 (3.71%). Pepsin digestibility showed significant higher levels in T7 (82.1%) and T6 (81.8%) followed by T3 (78.2%). In contrast, decreased values were verified for T4 (73.7%), T5 (72.7%) and T8 (70.1%). Results between DH% and pepsin digestibility did not present any trend of correlation for the present feeds.

Shrimp culture performance

After 56 days of rearing with the commercial shrimp diets, *L. vannamei* individual mean body weight increased from 3.3 to up to 11.3 g. However, weight increase varied over time among feed treatments (data not shown). Better growth performance was achieved with T5, T7 and T8 which exhibited consistent biweekly weight increments, reaching higher final weights at harvest. On the other hand, decreased growth in T3, T4 and T6 after initial 28 days have resulted in lower final body weights at harvest. In spite of elevated stocking density, survival was relatively high (>90%) and showed no significant difference among treatments, except with T5 (81.9%, Table 5). Significant higher yield (kg m^{-2}) was achieved with T7 and T8 followed by T5 and T6, whilst T3 and T4 resulted in lower values. Individual growth rates of over 0.90 g $week^{-1}$ for T5, T7 and T8 were satisfactory for intensive clear water culture denoting adequate conditions for shrimp health and development. These growth rates contrasted significantly with those achieved with T3, T4 and T6 ($P < 0.05$) which ranged from 0.56 to 0.73 g $week^{-1}$. Growth rates could not be directly correlated to total apparent feed consumption over the experiment. Increased apparent feed consumption was verified with T8 followed by T6, T7 and T5 while T3 and T4 were marked by lower values. Higher shrimp biomass gain was correlated to increased growth rates observed in T8 and T7. Lower survival resulted in decreased biomass gain in T5 with values comparable to T6 followed by T3 and T4. Overall, FCRs were within the range expected for clear water rearing trials (Boyd 2005; Coutteau *et al*. 2007). Although not significantly different ($P > 0.05$), reduced FCR was found with T8, T7 and T5 which provided higher shrimp growth rates.

Table 5 Culture performance of juvenile *Litopenaeus vannamei* in clear water after 56 days of rearing fed six different commercial diets at 29.5 °C and 33.4 g L^{-1} salinity. Results expressed as mean (SD). Initial and final weights: 3.28 (0.31) and 9.65 (1.60) g, respectively, stocking density: 114 ind m^{-2}. Values in the same row with common superscripts are not significantly different ($P < 0.05$)

	T3	T4	T5	T6	T7	T8
Survival (%)	92.7^{a} (1.94)	91.5^{a} (5.10)	81.9^{b} (9.26)	93.8^{a} (2.18)	91.2^{a} (2.31)	90.8^{a} (3.32)
Yield (kg m^{-2})	0.50^{a} (0.12)	0.44^{a} (0.09)	0.61^{ab} (0.10)	0.60^{ab} (0.13)	0.77^{b} (0.11)	0.78^{b} (0.14)
Growth (g $week^{-1}$)	0.63^{a} (0.13)	0.56^{a} (0.10)	0.91^{b} (0.04)	0.73^{a} (0.14)	0.97^{b} (0.13)	0.98^{b} (0.14)
Feed consumed (g)	755.9^{a} (23.6)	691.9^{b} (55.9)	879.7^{c} (62.0)	915.4^{c} (32.7)	887.9^{c} (23.7)	977.9^{d} (31.6)
Biomass gain (g)	286.2^{a} (68.0)	252.2^{a} (50.0)	349.1^{ab} (58.7)	342.9^{ab} (71.5)	439.2^{b} (64.8)	444.1^{b} (81.3)
Feed conversion ratio (FCR)	2.75 (0.63)	2.80 (0.41)	2.56 (0.37)	2.75 (0.49)	2.05 (0.27)	2.26 (0.44)

Correlating feed composition, in vitro *digestibility and culture performance of juvenile* L. vannamei

Protein : energy ratio, essential amino acid index, essential amino acid deficit, the number of deficient amino acids, true protein, non-protein nitrogen, pepsin digestibility and pH-stat *in vitro* degree of protein hydrolysis (DH%) with different enzyme extracts of feeds were correlated to *L. vannamei* performance. Feed consumption could not be correlated to any feed attribute. Determination coefficient (R^2) and P from correlations between performance and feed composition and digestibility showed wide variation (Table 6). The *in vitro* DH% showed overall best R^2 and was the main significant descriptor ($P < 0.05$) for shrimp performance. Hepatopancreas enzyme extracts used to DH% determination (HPf_3 and HPf_4) were able to predict yield significantly, growth rates and FCR (Table 6). Yield correlated positively with DH% with HPf_3 ($R^2 = 0.72$, $P = 0.033$) (Table 6, Fig. 1d). Growth rate was significantly predicted by DH% ($P < 0.05$) using enzyme extracts HPf_2, HPf_3 and HPf_4, $R^2 = 0.79$, 0.80 and 0.72, respectively (Table 6, Fig. 1a–c). In contrast, true protein content could not describe growth rates ($R^2 = 0.23$, Table 6). Biomass gain showed positively correlated ($P = 0.033$) with DH% HPf_3 (Fig. 2a). A significant negative correlation was found between FCR and DH% with HPf_3 and HPf_4 enzyme extracts, $R^2 = -0.65$ and -0.67, respectively (Table 6, Fig.2b,c). Moreover, DH% with HPf_4 was negatively correlated with essential amino acid deficit, $R^2 = 0.70$, $P = 0.038$ (Fig. 2d). As expected, the non-shrimp-specific pepsin digestibility test, regularly applied to discriminate among fish meal quality in terrestrial animal and fish feed industries, was not correlated with culture performance (Pedersen & Eggum 1983; Lemos 2003).

Significant correlation was also found between growth rates and feed methionine content, and between DH% HPf (mean) and methionine as well as methionine + cystine (Table 7). Despite not significant, elevated R^2 values were verified for correlations between FCR and histidine, and growth rate and methionine + cystine. Overall, feed quality ranking varied among tested feeds depending on the criterion employed (Table 8). The DH% showed better prediction of shrimp performance compared to compositional parameters, as it closely matched feed ranking according to growth rates.

Reproducibility and the effect of diet history on in vitro *DH% of feeds*

The variability in DH% output with enzymes from clear water or pond-raised shrimp hepatopancreas was verified (Table 9). DH% of feed protein with pond-raised enzymes was higher compared to their clear water counterparts. The difference varied between 16.6% and 34% for feeds T8 and T3, respectively. The effect of diet history upon the *in vitro* protein digestibility of feeds was assessed by the variability of DH% data (CV%). In general, a slightly higher CV of feeds assayed with clear water was observed compared to pond-raised enzyme extracts, with values ranging from 5.13% in T3 to 8.50% in T6. Pond-raised enzymes resulted in CVs from 2.66% to 6.51% in T4 and T7, respectively.

The capacity of DH% to predict *L. vannamei* performance (growth rates) was not affected by the source of enzyme extract as a significant correlation ($P < 0.05$) was found for either pond or clear water-raised shrimp enzyme extracts (Table 10). Determination coefficients (R^2) of regressions between growth rates and DH% were similar or even higher in clear water compared to pond-raised enzyme extracts, with values ranging from 0.80 in T6, T8 to 0.95 in T3, T5.

Discussion

Simple, rapid and precise *in vitro* methods such as the pH-stat hydrolysable protein using shrimp enzymes are required

Table 6 Pearson's correlation analysis between yield (kg m^{-2}), growth rate (g $week^{-1}$) and FCR of juvenile cultured *Litopenaeus vannamei* and tested commercial diets attributes. DH%: degree of protein hydrolysis, HPf: pond-raised shrimp (*L. vannamei*) hepatopancreas enzyme extract

	Yield (kg m^{-2})		Growth rate (g $week^{-1}$)		Feed conversion ratio (FCR)	
Descriptor	R^2	P	R^2	P	R^2	P
Protein : energy	0.37	0.203	0.50	0.115	−0.25	0.311
Essential amino acid index	0.37	0.210	0.35	0.218	−0.46	0.137
Essential amino acid deficiency	0.25	0.312	0.35	0.215	−0.34	0.226
Number of deficient amino acids	0.01	0.959	0.03	0.921	0.07	0.614
True protein	0.14	0.463	0.23	0.332	−0.26	0.301
Non-protein nitrogen	0.12	0.492	0.16	0.429	0.24	0.324
Pepsin digestibility 0.002%	0.00	0.981	0.02	0.132	0.00	0.946
pH-stat *in vitro* DH% HPf2	0.50	0.119	0.79	0.017	−0.45	0.147
pH-stat *in vitro* DH% HPf3	0.72	0.033	0.80	0.015	−0.65	0.051
pH-stat *in vitro* DH% HPf4	0.55	0.093	0.72	0.032	−0.67	0.045

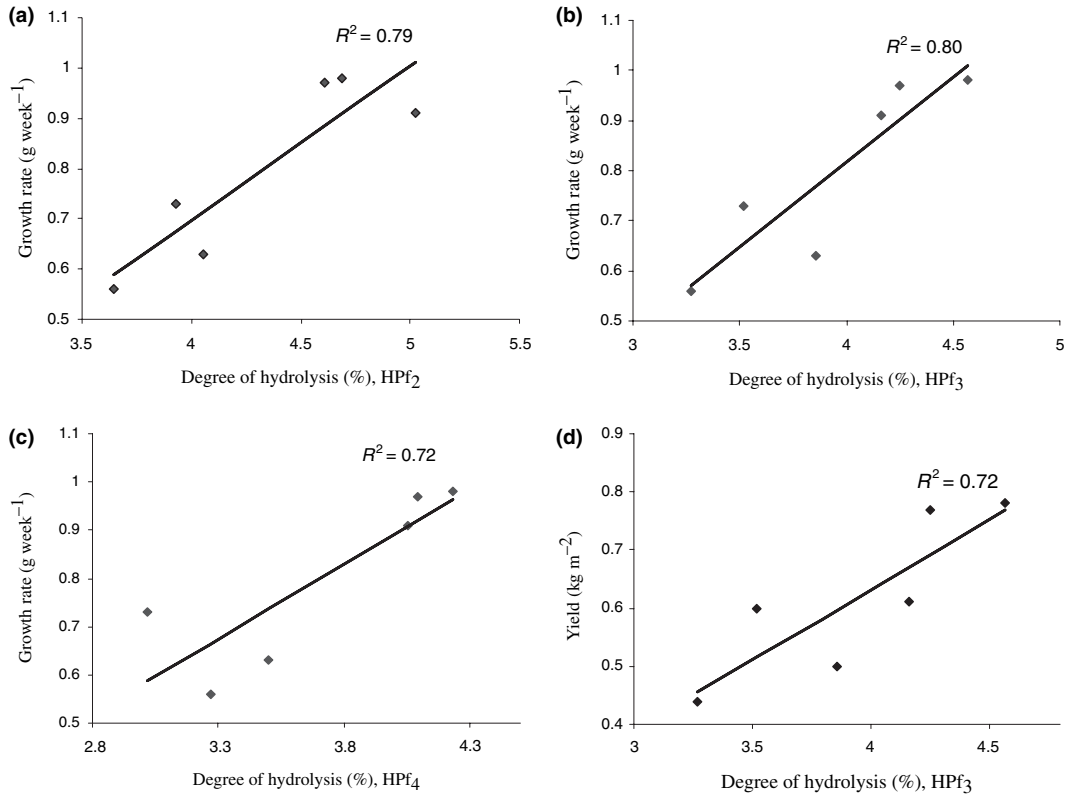

Figure 1 Regressions between growth rates (a–c) and yield (d) of juvenile *Litopenaeus vannamei* with *in vitro* degree of protein hydrolysis (%) of tested commercial diets with different pond-raised shrimp (*L. vannamei*) hepatopancreas enzyme extracts (HPf).

to assess the nutrient quality (including digestibility) of feedstuffs (Tacon 1996; Dong & Hardy 2000). Though feasible and reproducible *in vivo* apparent digestibility trials are expensive, time-consuming and require specialized facilities for animal rearing (Lazo & Davis 2000), and possibly do not fit for quality control in the feed industry (Castillo *et al.* 2002). However, digestion represents the first step of dietary protein incorporation into somatic growth, and apparent digestible protein content of diets may not be necessarily related to growth responses in culture trials (Sugiura 2000; Córdova-Murueta & Garcia-Carreño 2002; Forster *et al.* 2003). Though *in vitro* pH-stat routine with shrimp enzymes (DH%) was previously correlated with *in vivo* apparent crude-protein digestibility in some feed ingredients (Ezquerra *et al.* 1997; Lemos & Nunes 2007), the present validation of DH% to predict shrimp performance suggests a close relationship between effective protein polypeptide enzymatic breakage and growth, providing support for increased evaluation and applications.

Dietary protein is important as the main source of indispensable amino acids to produce new tissue protein and maintain previously synthesized tissue (Mente 2003), while protein growth occurs when protein synthesis exceeds protein degradation with a low protein turnover (Houlihan *et al.* 1995). Optimization of protein synthesis in tissue cells is directly related to the timely supply of indispensable and dispensable amino acids (Liebert 2005). In the present experiment using commercial feeds with similar crude-protein content, effective *in vitro* peptide bond breakage by hepatopancreas digestive proteases was found proportional to the capacity of somatic growth and feed efficiency in juvenile *L. vannamei*. A positive relationship between growth and DH% (R^2 = 0.67) in *L. vannamei* has been previously reported with experimental diets (Ezquerra *et al.* 1998) and with present results, the *in vitro* prediction could be further expanded for commercial diets in terms of yield (kg m^{-2}), growth rates (g week^{-1}) and FCR. The increased growth rates for treatments T5, T7 and T8 (0.91–0.98 g week^{-1}) may have resulted from the combination of proper supply and availability of indispensable amino acids despite of apparent methionine and methionine + cystine deficiencies (Guillaume 1997). The determination of nutrient digestibility combined with compositional analysis in quality assessment of feedstuffs is emphasized, as true protein content and amino acid

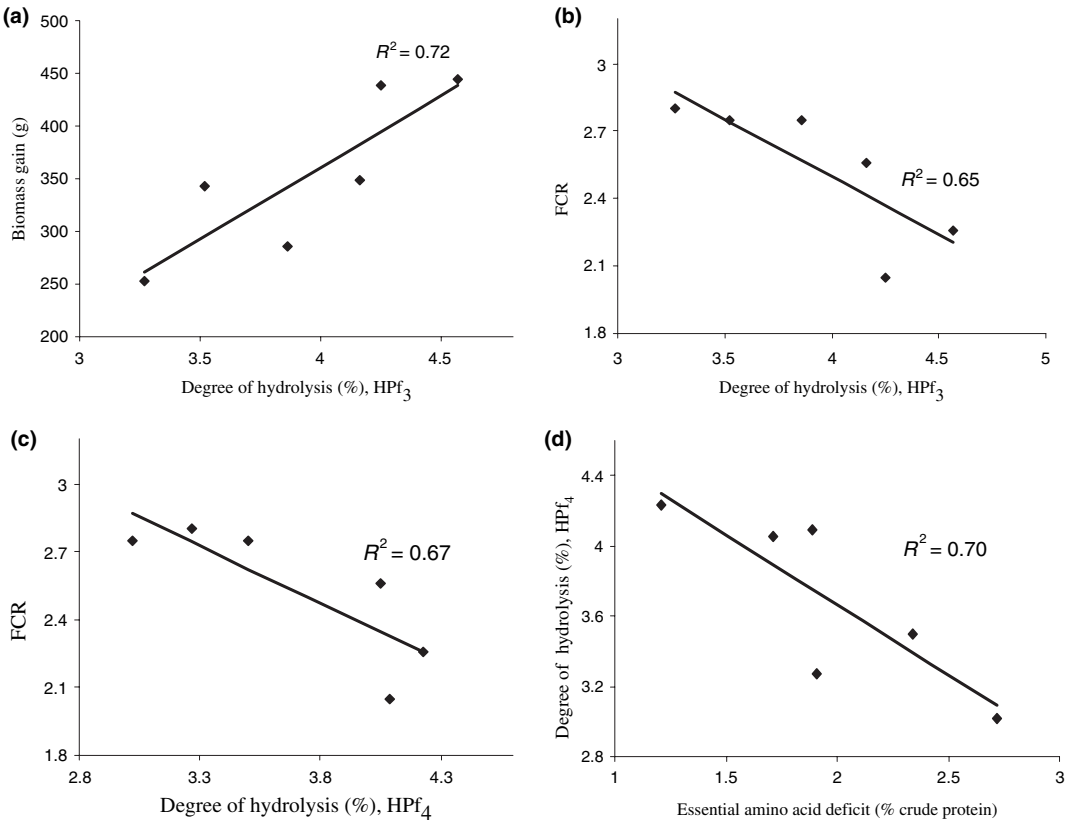

Figure 2 Regressions between biomass gain (a) and feed conversion ratio (b,c) of juvenile *Litopenaeus vannamei*, and essential amino acid deficit of tested commercial diets (d) with *in vitro* degree of protein hydrolysis (%) of tested commercial diets with different pond-raised shrimp *(L. vannamei)* hepatopancreas enzyme extracts (HPf).

Table 7 Pearson's correlation analysis between growth rates (g week^{-1}), FCR of juvenile *Litopenaeus vannamei*, pH-stat *in vitro* degree of protein hydrolysis (DH%) with shrimp enzymes and essential amino acids (% crude protein) in tested commercial diets. HPf: mean values of pond-raised shrimp (*L. vannamei*) hepatopancreas enzyme extracts

	R^2	*P*
Growth rate *versus* methionine	0.73	0.031
pH-stat *in vitro* DH% HPf versus methionine	0.92	0.003
pH-stat *in vitro* DH% HPf versus methionine + cystine	0.67	0.047
Feed conversion ratio *versus* histidine	−0.60	0.070
Growth rate *versus* methionine + cystine	0.52	0.108

Table 8 Quality ranking based on protein features of tested commercial diets (T3–T8) for juvenile *Litopenaeus vannamei*. DH%: degree of protein hydrolysis, HPf: pond-raised shrimp (*L. vannamei*) hepatopancreas enzyme extract

Criterion	Feed quality ranking
Number of deficient amino acids	T4 > T8 = T7 > T5 = T3 > T6
Non-protein nitrogen (%)	T8 > T4 > T7 > T5 > T3 > T6
Essential amino acid index	T8 > T7 > T4 > T5 > T6 > T3
Essential amino acid deficit (% crude protein)	T8 > T5 > T7 > T4 > T3 > T6
pH-stat *in vitro* DH% (pond-raised HP extract)	T5 ≥ T8 ≥ T7 > T3 ≥ T4 ≥ T6
Growth rate (g week^{-1})	T8 ≥ T7 ≥ T5 > T6 ≥ T3 ≥ T4

profile (individually analysed after forced acid hydrolysis) have not proved effective in predicting shrimp culture performance (Anderson *et al*. 1993; Lan & Pan 1993). On the other hand, some of dietary true protein may include free amino acids with high-leaching potential that may not be ingested or contribute to body protein synthesis. If amino acid requirements of *L. vannamei* are lower compared to suggested values for *P. monodon* (Millamena *et al*. 1996), different performance of shrimp-fed treatments T3, T4 and T6 could also be related to reduced feed protein DH%.

Levels of certain amino acids in diets correlated with some parameters of *L. vannamei* culture performance. Though not significant, FCR was negatively related to histidine content (R^2 = −0.60). A similar trend was observed in juvenile

Table 9 Mean and variation of pH-stat *in vitro* degree of protein hydrolysis (DH%) of tested commercial diets with shrimp (*Litopenaeus vannamei*) hepatopancreas enzyme extracts from different sources. Further details in Material and methods

Hepatopancreas source/diet	T3	T4	T5	T6	T7	T8
Clear water raised (HPt_3 to HPt_8)						
DH% (mean)	2.85	2.78	3.76	3.00	3.56	3.85
SD	0.15	0.19	0.23	0.25	0.26	0.29
CV (%)	5.13	6.82	6.10	8.50	7.37	7.66
Pond raised (HPf_2 to HPf_4)						
DH% (mean)	3.82	3.72	4.73	3.71	4.29	4.49
SD	0.22	0.10	0.26	0.20	0.28	0.18
CV (%)	5.72	2.66	5.58	5.38	6.51	4.06

Table 10 Pearson's correlation analysis between growth rates (g $week^{-1}$) of juvenile *Litopenaeus vannamei* and pH-stat *in vitro* degree of protein hydrolysis (DH%) of tested commercial diets with shrimp (*Litopenaeus vannamei*) hepatopancreas enzyme extracts from different sources. HPt: clear water-raised shrimp hepatopancreas fed indicated diet number. HPf: pond-raised shrimp hepatopancreas from different sampling locations

Descriptor (hepatopancreas source)	R^2	*P*
Clear water		
HPt_3	0.95	0.001
HPt_4	0.92	0.003
HPt_5	0.95	0.001
HPt_6	0.80	0.016
HPt_7	0.81	0.014
HPt_8	0.80	0.017
Pond raised		
HPf_2	0.79	0.017
HPf_3	0.80	0.015
HPf_4	0.72	0.032

Penaeus monodon under comparable histidine levels (1.52–2.70% crude protein, $R^2 = 0.60$) (Millamena *et al*. 1999). On the other hand, the positive correlation between growth rate and methionine content may be related to limiting amounts in tested feeds that might include availability, and indicate its relative importance for growth in *L. vannamei*. Under comparable ranges, growth has also increased linearly with methionine content in diets for *P. monodon* (Millamena *et al*. 1996). Lysine, arginine and methionine are reported as the most limiting indispensable amino acids in commercial formulations (Fox *et al*. 1995). In the present study, as recommendation for lysine (except for T6) and arginine were met, methionine levels could be limiting though elevated growth rates observed with T5, T7 and T8. Possibly, the requirement for some essential amino acids is lower than presently recommended for *L. vannamei* whilst for others as methionine, low levels appear to have a more pronounced growth-limiting effect (Millamena *et al*. 1999). This could be further corroborated by the positive correlation between DH% and methionine or methionine + cystine diet contents. Efficient enzymatic digestion has showed proportional to levels of these key nutrients though the nature of feed protein (e.g. native or processed sources) could not be assessed. A correlation between *in vitro* protein digestibility with shrimp enzymes and dietary levels of certain individual amino acids such as lysine and arginine has been previously reported in *P. monodon*, $R^2 = 0.98$ (Lan & Pan 1993). The consistency of these findings may further depend on the accuracy of methods for the determination of critical amino acids as methionine, cystine and histidine.

Protein digestion involves hydrolysis by several different enzymes, with specific action on different parts of the polypeptide (Gauthier *et al*. 1982). Thus, *in vitro* determination of protein digestibility should be preferably carried with a complete mixture of digestive enzymes occurring in the target species instead of purified proteolytic enzymes (Grabner 1985; Dimes *et al*. 1994; Lemos *et al*. 2004). In the pH-stat routine, more uniform activities are ensured to proteolytic enzymes in different samples during protein hydrolysis (Pedersen & Eggum 1983). However, the use of shrimp hepatopancreas extracts has been debated because of possible diet effects affecting the efficiency and predictive capacity of *in vitro* protein hydrolysis (Divakaran *et al*. 2004). These authors could not find a correlation between apparent crude-protein digestibility and *in vitro* digestibility determined by the release of free amino acids after buffered incubation of volume standardized hepatopancreas extracts of *L. vannamei*. In the present study, the hypothesis of diet effects upon *in vitro* response was tested by determining feed DH% with hepatopancreas extracts from different sources, clear water or pond-raised shrimp. The pH-stat assays with shrimp enzymes produced reproducible DH% results for either pond (CV ≤ 6.5%) or clear water (CV ≤ 8.5%) raised shrimp enzymes (Table 9). Increased DH% with pond compared to clear water enzyme sources (up to 34%) was detected and may be attributed to possible effects of natural food, organic and/or inorganic co-factors available in the pond environment (Moss *et al*. 2001). The hydrolysis of feed protein by pond enzyme extracts sampled in different farms (HPf_{2-4}) resulted in low variability in the estimates of DH%. Moreover, correlations between shrimp growth rates and DH% of

tested feeds were maintained significant regardless the enzyme source (Table 10). These results suggest the need of proper standardization of hepatopancreas enzyme extracts to be used for *in vitro* analysis according to total proteinase activity (Garcia-Carreño 1992; Lemos 2004). Effects of diet quantity and quality (Le Moullac *et al*. 1996; Lemos & Rodríguez 1998; Brito *et al*. 2001; Gamboa-Delgado *et al*. 2003) but also circadian rhythms (Cuzon *et al*. 1982) and molting stage (Muhlia-Almazán & García-Carreño 2002) upon digestive enzyme activities of penaeid species are rather well reported. Though the mechanisms of modifying the efficiency of enzymatic hydrolysis are unknown, diet effects upon *in vitro* pH-stat DH% with activity standardized enzyme extracts were not verified either in trout (Dimes & Haard 1994) or shrimp *L. vannamei* (Ezquerra & Garcia-Carreño 1997; Córdova-Murueta & Garcia-Carreño 2002; present study). Improved assay conditions such as temperature, reaction time and enzyme amount should optimize the predictive capacity of the *in vitro* pH-stat method with shrimp hepatopancreas enzymes in the development and quality control of biologically, economically and environmentally proper shrimp feeds.

Conclusions

Protein is one of the primary costs in compound shrimp feeds. As successful shrimp farming is currently associated to reduction in production cost and maximization of economic benefit, the formulation of nutritional, cost and environmentally effective feeds requires strict quality control of ingredients and finished feeds. A simple, safe, rapid, precise and species-specific *in vitro* method for protein digestibility using hepatopancreas enzymes has been shown to be capable of predicting culture performance of *L. vannamei*. The technique was reproducible and the enzyme extracts can be standardized for consistent output. The concept of *in vitro* species-specific degree of protein hydrolysis (DH%) combined with the amino acid profile may provide a suitable estimate of protein nutritional quality in shrimp feeds.

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