The Open Access Israeli Journal of Aquaculture – Bamidgeh

As from **January 2010** The Israeli Journal of Aquaculture - Bamidgeh (IJA) will be published exclusively as **an on-line Open Access (OA)** quarterly accessible by all AquacultureHub (http://www.aquaculturehub.org) members and registered individuals and institutions. Please visit our website (http://siamb.org.il) for free registration form, further information and instructions.

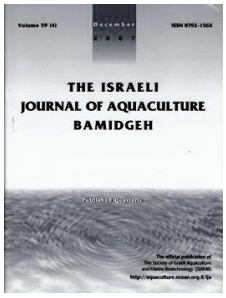
This transformation from a subscription printed version to an on-line OA journal, aims at supporting the concept that scientific peer-reviewed publications should be made available to all, including those with limited resources. The OA IJA does not enforce author or subscription fees and will endeavor to obtain alternative sources of income to support this policy for as long as possible.

Published under auspices of
The Society of Israeli Aquaculture and Marine Biotechnology (SIAMB),
University of Hawai'i at Mānoa Library
&
University of Hawai'i at Mānoa Aquaculture Program
in association with
AquacultureHub
http://www.aquaculturehub.org

ISSN 0792 - 156X

PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il



Testing Protein Digestibility in Red Grouper *Epinephelus Morio* using *In Vitro* and *In Vivo* Methods

Silva A.F.[1]*, Escalante K.[2], Alvarez-González C.A.[3], Guerrero-Olazarán, M.[4], Cuzon G. [5], Gaxiola G[2].

[1]Posgrado de Ciencias del Mary Limnologia, UNAM México
[2] UMDI Sisal, Fac. de Ciencias, UNAM México
[3]Lab. Acuic. Trop.-DACBIOL-UJAT, México
[4]Inst. Biotec., Fac. Cienc. Biol., UANL, México
[5]Ifremer-COP, Tahiti

(Received 1.1.2014, Accepted 27.1.2014)

Key words: ADC, in vitro digestibility, enzymes, fish, feedstuffs

Abstract

The digestibility of pre-selected ingredients was evaluated for *Epinephelus morio* using pH-stat and multi-enzyme extract (stomach, intestine, and ceca). The degree of hydrolysis (DH) was tested with acid and alkaline proteases using crustacean meals, squid meal, fish protein concentrate (FPC), fish meal, dried whey, as well as canola paste (w/ or w/o phytase), soybean meal (w/ or w/o phytase), soyprotein concentrate (SPC), and wheat gluten. SPC produced DH values between 3.6-4.1%, obtained from the three different parts of the digestive tract. Canola paste produced high DH in the stomach by adding phytase to 0.8 and 2% feed. This process was repeated under alkaline conditions for DH, with 0.6-2.6% in the pyloric ceca, and 1-2.2% in the intestine. The DH from stomach with crustacean meals (shrimp meal and crab meal) ranged from 32-87%, while values for dried whey from the ceca and intestine ranged from 6-7.4%. It did not differ from crab meal with values of 4.5 and 8.7% from pyloric ceca and intestine respectively. Based on the above results, 4 diets were developed using shrimp meal, dried whey, soy protein concentrate, and canola+phytase. The DH, FAA (fish amino acid) and SDS-PAGE (analysis of protein gels) for each diet were analysed. After an in vivo test, results on juveniles fed whey protein diet (WPd) and CPd+ ranked high (95% and 92% respectively) compared with other treatments. pH-Stat provided consistent results regarding digestibility of selected protein sources. Therefore, dried whey, shrimp meal, and canola+phytase ranked first to be included in *E.morio* feed formulation with a pool of 55% of these components, complemented with a minimum of fish meal, FPC, grains and wheat gluten.

* Corresponding author. Tel.: +52 9996 45 8208; e-mail: dricazoo@hotmail.com

Introduction

Red grouper *Epinephelus morio* is one of the main fishery resources in the state of Yucatan, Mexico. This is completely dependent on wild fishery catch; however decreases in natural stocks (Burgos et al., 2003) and seasonal variation in size of the catch have limited expansion of marketing. Therefore, appropriate conditions which include including formulation of a suitable diet are essential for the promotion of red grouper culture. In aquaculture, digestibility can be related to growth and other benefits, but is also relevant to environmental pollution, since the greatest source of nutrients in tank water is from food that has not been ingested or has been digested (Lemos, 2003). Fishmeal is the most widely used ingredient in the preparation of feed, however high cost and gradual decrease in worldwide production has triggered the search for, and evaluation of, new substitute ingredients (Viola et al 1994). In recent decades, there has been renewed interest in *in vitro* digestibility studies for a number of reasons which include teamwork, environmental pollution, the high cost of analysis, and concerns regarding animal welfare (Alarcón et al., 2002). *In vitro* tests can provide preliminary information on the suitability certain ingredients in the formulation of diets. From this perspective, the *in vitro* digestibility can be useful in rapidly and efficiently evaluating and comparing the nutritional quality of different sources of proteins (Frias-Quintana et al., 2010). But, *in vitro* tests do not completely replace *in vivo* tests.

The pH-Stat program directly measures the percentage of hydrolyzed peptide bonds during the reaction (Navarrete and García-Carreño, 2002). Another advantage of this technique is the use of digestive extracts from the target organism, which is more appropriate than using the exogenous multi-enzyme mixture. Hydrolysis caused by an exogenous mixture is the result of a reaction with three or four commercial enzymes (trypsin, chymotrypsin, aminopeptidase, and bacterial proteases), whereas hydrolysis of proteins using digestive extracts of the target organism is the result of native digestive enzymes, or for example, trypsin, chymotrypsin, aminopeptidase, pepsin, and carboxypeptidase (Alarcon et al. 2002).

Therefore, the aim of the present study is to determine the digestibility of protein in food sources with the specific *E. morio* enzyme in order to partially or totally substitute fish meal and formulate practical feeds based on its digestive physiology.

Materials and Methods

Five wild *E. morio* juveniles (100±1g) captured from the Yucatan coast near Sisal were given an overdose of clove oil (4-allyl-2-methoxyphenol>0.1mg/ml) in the laboratory and were then dissected on ice to remove the stomach, pyloric ceca, and intestine, and frozen at -40°C for further analysis. Enzyme extraction was performed subsequent to soaking the stomachs, intestines, and ceca in distilled water using an Ultra Turrax IKA T18 Basic tissue homogenizer (North Chase, Wilmington). Samples were centrifuged at 14,000 rpm for 30 min at 5°C; the aqueous part was recovered and each sample was divided into two parts, one adjusted with HCl to pH 3.5 (gastric protease activation) and the other with NaOH to pH 8 (alkaline protease activation) and stored at -40°C until use.

Enzyme activity was calculated in units of acid protease (pepsin) using hemoglobin (Hb) as the substrate, 1 ml of hemoglobin (0.5%) on a cotton pad 0.1 M glycine-HCl at pH 2.0 (Anson, 1938). The alkaline protease was produced using the method by Kunitz (1947) modified by Walter (1984), using casein as the substrate on a cotton pad with 50 mM Tris-HCl and10 mM CaCl2 at pH 9. Subsequently, 20 µl of enzyme extract was added to the mixtures and both solutions were incubated for 30 minutes at 37ºC. The reaction was stopped to add 0.5 ml trichloroacetic acid (TCA at 20%). The reaction mixture was left to rest (15 minutes) at 4ºC, and then centrifuged at 14,000 rpm for 5 min.

The amount of tyrosine released into the supernatant was measured at (ABS280nm) with a UV-visible spectrophotometer. One unit of activity was defined as the amount of enzyme that catalyses the formation of 1 mg of tyrosine per minute based on the molar extinction coefficient 0.005 (Alarcón et al., 2002). All assays were performed in triplicate.

In vitro degree of protein hydrolysis (DH) with fish enzymes: In vitro protein DH results for several animal and plant ingredients are reported in Table 1.

Table 1. Proximal analysis of selected ingredients.

Ingredient	*moisture (%)*	*Protein (%)*	*lipid (%)*
casein[a]	2	90	1.2
Hb[b]	2	90	<1
CPSP[70c]	7.5	70	2.5
squid meal[d]	5	75	7
crab meal[d]	4.9	30	1
Shrimp meal[d]	5	28	2
dried whey[d]	8	11	0.7
fish meal[e]	7.4	72	11.1
soybean concentrate[d]	7	65	0.7
soybean meal[d]	6	49	0.5
canola meal[d]	10	36	3.5
wheat gluten[d]	5	80	7

[a] Hammerstein quality Casein Res. Organics # Catalog 1082C,
[b] Bovine erythrocytes. USBiological # Catalogo H1850
[c]soluble fish protein concentrate,
[d]Proteínas marinas y agropecuarias S.A. de C.V., Guadalajara, Jalisco, Ferpac Internacional, S.A. de C.V.,Querétaro, México,
[e]APLIGEN.

Soybean meal and canola meal were pre-treated with an enzyme of microbial origin FTE II (543 U/ml activity) according to the method of Saunders et al. (1972) and modified by Dimes et al. (1994). The DH value of each ingredient was determined automatically using a pH-stat 718 Stat Titrino, Metrohm with an electrode Idrolyte (Num 6.0224.100) pH1-11/0-60ºC. A solution containing 40 mg of protein was adjusted to a final volume of 5 ml (8 mg of protein/ml distilled water). Acid digestibility (multi-enzyme extracts adjusted to pH 3.5) was evaluated for 15 minutes using 50 UAE/ml, and the consumption of HCL 0.1 N was recorded.

In contrast, 200 UAE/ml was used for alkaline digestibility and the reaction was evaluated for 45 min, adjusting the protein solution to pH 8 and recording the consumption of NaOH 0.1 N. Both sources of digestibility were kept at 37°C. Hb and casein were used as standard reference protein sources for acid and alkaline digestibility respectively. Based on the consumption of HCl (acid phase) and NaOH (alkaline phase) the degree of hydrolysis (DH) was determined. Similarly, the level of self-hydrolysis of the different ingredients in the absence of digestive proteases was estimated. In all cases, the DH was determined in the absence of the enzyme extract, which was replaced by an equal volume of distilled water. All the estimates were performed in triplicate (Frías-Quintana et al, 2010; Tibbetts et al, 2011).

Calculation of DH of enzyme reaction:

$$DH = \frac{h}{htot} X100$$

where h is the number of hydrolysed peptide bonds,
h tot is the number of total peptide bonds of the protein substrate (meqv/g protein) where Htot was 7.8 (soy proteins), 8.3(wheat gluten proteins) and 8.35 (other proteins) (Adler-Nissen, 1986).

$$h = V_b N_b x \frac{1}{\alpha} \frac{1}{MP}$$

where h is the number of hydrolysed peptide bonds; Vb is consumption of the base in ml; Nb=normality of titrant (meqv/mL); α=average degree of dissociation of the α-NH group; 1/α=1.5 at 25°C and pH 8.0; Mp=total mass of protein (g) in the reaction mixture (e.g. protein contributed from test ingredient and added enzyme);

Preparation of diets: Once evaluated, the best protein ingredients were selected based on the highest DH values. Four practical diets were formulated: whey protein diet (WPd), canola paste treated with phytase (CPd+), shrimp powder (ShPd), and a soyprotein concentrate diet (SPCd) (Table 2).

Table 2. Diets prepared to evaluate *in vivo* digestibility for juvenile *E. morio*

	[1]*RD*	[2]*WPd*	[3]*ShPd*	[4]*CPd+d*	[5]*SPCd*
dried whey *		30			
shrimp meal			30		
canola meal (FTEII)**				30	
SPC***					30
dried whey	10	7	7	7	7
fish meal	40	28	28	28	28
soybean meal	15	10.5	10.5	10.5	10.5
wheat gluten	9	6.3	6.3	6.3	6.3
wheat flour	16	11.2	11.2	11.2	11.2
cod liver oil	2	1.4	1.4	1.4	1.4
soy lecitin	1	0.7	0.7	0.7	0.7
robimix C	2	1.4	1.4	1.4	1.4
vit Robimix	1.5	1.05	1.05	1.05	1.05
CMC****	2	1.4	1.4	1.4	1.4
Zeolite	1	1	1	1	1
[6]Proximal evaluation	43.68	30.25	43.05	42.36	47.41
fat	5.46	6.09	3.81	3.57	2.98
ash	6.61	8.18	9.83	5.87	5.45
fibre	-	-	0.03	0.89	0.04
NFE	19.79	30.29	22.86	19.2	18.98
[7]GE (kJ g/DM)	20.9	18.3	19.7	21.5	19.9

*dried whey used as test diet
Phytase FTEII (1.6 U/g Units of phytase for each g of meal)*soy protein concentrate.
****Carboxymethyl-cellulose.
[1]Reference diet, [2] dried wey diet, [3] shrimp diet, [4] canola+ phytase diet and [5] soy protein concentrate diet.
[6]Results are expressed on moist basis in % *Method AOAC (1980) for proximate analysis. Lab chemical analysis for feeds FMVZ-UNAM.
[7]Coefficients for GE were 4:9:4 with protein:lipid:carbohydrate respectively.

A ratio of 30:70 (experimental ingredient: reference diet for trout, Cho, 1992) was used, plus the addition of 1% of Zeolite as an inert marker. All the macro ingredients were sieved (<250 µm) and mixed with the micro-ingredients for 15 minutes, adding 20-30% water. The mixture was subsequently pelletized in a meat grinder. The pellets were cut manually and frozen at -20°C until use.

In vitro assessment of diets. Prior to the in vivo study, a sample of the four diets and trout feed (Cho, 1992) were prepared according to the techniques described above, in order to perform in vitro digestibility analysis. While the degree of hydrolysis was determined for the diets using the enzyme extracts from fish, samples of the hydrolyzed ingredients (40 µl) were collected as follows: 1) Acid digestion, adding extract to the stomach samples were taken at 0 (i) and 900 (f) seconds during acid hydrolysis. 2) Alkaline digestion, adding intestinal extract, samples were taken at 0, 100, 250, 500, 750, 1000, 1500 and 2700 seconds. From the sample taken for each reaction time during the acid and alkaline phases, 20 µl of protein solution was used to measure total free amino acids(FAA) and another 20 µl the enzyme extracts was used for the SDS-PAGE technique. The latter technique consisted of mixing 20 µl protein solution in equal parts with the sample buffer (0.125 M Tris-HCI, pH 6.8, 4% (w:v) SDS, 10% (w:v) 2-β mercaptoethanol, 20% (w:v) glycerol, and 0.04% (w:v) bromophenol blue. The mixture was allowed to cool to room temperature and was then stored at -40°C until analysis. SDS-PAGE was developed using 10% polyacrylamide gels run at 30 V for 1h and then increased to 80 V for 3h at 5°C. Gels were fixed for 30 min in a 12% TCA solution, stained for 2**-**3 h at room temperature in another solution 0.1% Coomassie brilliant blue (BBC R-250), and acetic acid methanol (0.25:10:50), then washed overnight with 10% methanol, 10% acetic acid and distilled water.

Once bleached and hydrated in distilled water it was digitalized in GEL Doc model XR+ from Bio-Rad. Analysis of FAA came from the other 20µl aqueous solution fixed with 20 ml TCA 12% (Church et al., 1983). This was is based on binding the amino terminal; sample mixed in 20 ml OPA reagent, stirring vigorously and read at 340 nm. A standard reference diet curve was prepared with L**-**leucine (0.5mg/ml) from 0 to 20 µg/ml. These assays were performed in triplicate.

Apparent digestibility coefficient (ADC): For this assay, 150 juvenile *E. morio* (average weight of 141.4 ±6.3g) were caught, and distributed in 15 tanks (500l) operating with a recirculation system. For fifteen days before the experiment, the fish were fed a maintenance diet (Table 3)

Table 3. *In vitro* protein digestibility of ingredients (from a marine or terrestrial origin) using *E. morio* enzyme extracts. DH% results are expressed as mean±SD.

	%DH[1]		
	Acid	*Alkaline*	
Ingredients	*stomach*	*Pyloric ceca*	*Intestine*
Hb*/casein**	2.3 ± 0.7^{c}	3.2 ± 0.7^{ab}	3.1 ± 0.7^{b}
crab meal[40]	86.8 ± 8.4^{a}	4.5 ± 2.1^{a}	8.7 ± 2.6^{a}
shrimp meal[50]	32.1 ± 4.2^{ab}	1.4 ± 0.2^{b}	5.2 ± 1.0^{b}
dried whey[11]	4.5 ± 0.6^{c}	6 ± 1.5^{a}	7.4 ± 1.8^{a}
squid meal[72]	2.7 ± 0.2^{c}	1.4 ± 0.2^{b}	4.6 ± 0.8^{b}
CPSP[70***]	3.6 ± 1.9^{c}	2.1 ± 0.8^{b}	3 ± 0.1^{b}
fishmeal[62]	4.1 ± 1.0^{c}	2.6 ± 0.8^{b}	2.8 ± 0.7^{b}
soy protein concentrate[60]	3.6 ± 1.5^{c}	4 ± 0.4^{b}	4.1 ± 0.3^{b}
soybean meal[44]	1.2 ± 0.3^{d}	1 ± 0.2^{c}	0.9 ± 0.1^{d}
soybean meal+phytase	1.5 ± 0.4^{d}	1.6 ± 0.5^{b}	0.9 ± 0.2^{d}
canola paste[32]	0.8 ± 0.2^{d}	0.6 ± 0.3^{c}	1 ± 0.2^{d}
canola paste+phytase	2.0 ± 0.3^{cd}	2.6 ± 0.2^{b}	2.2 ± 0.3^{c}
wheat gluten[80]	1.6 ± 0.2^{d}	2.1 ± 0.7^{b}	1.7 ± 0.8^{c}

*Analytical standard reference diet for animal ingredients
** Analytical standard reference diet for plants ingredients
*** soluble fish protein concentrate,
[1]Methods are described in the text, (n=3 fish).
Different superscripts in the same column indicate significant differences.

The fish were not fed for 48 hours prior to feeding them with the experimental diets in order to completely empty their digestive systems and then they were given the experimental diet. The fish were offered food to satiety, twice a day. The feed which was not consumed was removed from tanks before feeding; waste was collected by siphoning after each feeding (to avoid leaching as a result of over-exposure in water). Feces was collected in a 400mm mesh, hourly for eight hours. The collected feces was subsequently washed with distilled water to remove salts, dried at 60°C until a constant weight was attained and stored until further analysis. Collection took place for 20 days.

The formulae used to determine (ADC) protein and dry matter were:

%PC=100x(1-(% zeolite in the feed/% zeolite in feces)(% protein in feces/% protein in feed)

%DM=100x(1- (% zeolite in the diet/% zeolite in feces)

To determine the apparent digestibility for each ingredient, the following formula was used:

ADC ingredient = (ADC diet-0.7*ADC ref. diet)/0.3

Statistical procedures. To determine acid DH, 3 replicates per ingredient and 3 measurements were performed for each enzymatic extract. A Kruskal-Wallis test was run since no variance homogeneity or differences between values were detected by the Nemenyi test. For alkaline DH of pyloric ceca and intestines a one way ANOVA and Tukey range test were used. A p-value < 0.05 was considered significant. DH values, and *in vivo* digestibility data, for protein and dry matter were analyzed using ANOVA and a posteriori Tukey test. Pearson correlation coefficients were also calculated between in *vivo* and *in vitro* digestibility values (Zar, 1999).

Results

The ingredients tested showed significant differences in protein hydrolysis using multi-enzyme extracts. DH values under acidic conditions for crab meal and shrimp meal were 86.6 ± 8.4% and 32.1±4.2% respectively ($p<0.01$; $H=83$). Dried whey values did not differ from other animal ingredients ($p>0.05$, Table 3). Soybean meal (1.2±0.3), soybean meal^{+} (1.5±0.4), and canola meal (0.8±0.2) had low DH% ($p<0.01$; $H=77$) in acidic conditions. Alkaline DH in the pyloric ceca and intestine produced the highest values in dried whey ($p<0.05$, Table 3). DH values from canola meal+phytase and soya

protein concentrate were significantly higher than other plant protein ingredients but similar to fishmeal. The lowest alkaline DH values were observed in the pyloric ceca and intestine of individuals fed plant pastes (Soy and canola) without phytase treatment, ($p<0.05$, Table 3). Shrimp meal and dried whey were selected as the best animal sources to formulate diets, although DH was higher for crab meal. Plant sources such as soy protein concentrate and canola meal+phytase were selected for their high DH value.
Total DH values and *in vitro* digestibility of the experimental diets are presented in Table 4.

Table 4. Degree of hydrolysis (% DH total) and % digestibility of diets; reference diet (Rd); shrimp powder diet (ShPd) , canola paste+phytase diet (CPd+) , whey protein diet (WPd) and soy protein concentrate diet (SPCd), using multi-enzymatic extracts of juveniles *E. morio.*

diets	*DH[1]% total*	*digestibility%*
Rd*	17.2 ± 3.6^{a}	100^{a}
ShPd	16.6 ± 3.3^{a}	96.5 ± 1.9^{a}
WPd	15.8 ± 3.3^{ab}	91.9 ± 3.5^{ab}
CPd**	14.4 ± 2.2^{ab}	83.7 ± 2.8^{ab}
SPCd	13.1 ± 2.0^{c}	76.2 ± 3.3^{c}

* Control diet; **FTEII (1.6 U/g Unit of phytase for each g of meal), n=3 fish. Mean ± SD. [1]Methods are described in the text. Different superscripts in the same column indicate significant differences.

The highest DH values were recorded for ShPd, WPd and CPd+, with significantly higher values than for the SPCd. Comparison of TFAA concentrations at the start of acid digestion showed a significant difference ($p<0.05$) between the treatments. The WPd presented higher values for TFAA (1699 ± 0.9 and 2708 ± 21 ml, acid and alkaline respectively) released from the start to the end of hydrolysis; the other diets (ShPD, SPCd and CPd+varied in TFAA values throughout hydrolysis (Table 5).

Table 5. Free amino acids (FAA) concentration (µg/ml) released from acid and alkaline hydrolysis using multi-enzymatic extracts of juvenile *E. morio* on protein ingredients (mean±SD, n=3). Reference diet (Rd); shrimp powder diet (ShPd) , canola paste+phytase diet (CPd+) , whey protein diet (WPd) and soy protein concentrate diet (SPCd). Different superscripts in the same column indicate significant differences ($p<0.05$).

	acid phase		*alkaline phase*						
`time (s)	*0*	*900*	*0*	*100*	*250*	*500*	*1000*	*1500*	*2700*
Rd*	975±22^{c}	1107±17^{d}	1028±1^{c}	1064±1^{d}	1100±3^{d}	1130±5^{d}	1143±1^{d}	1159±18^{d}	1171±6^{e}
ShPd	1446±25^{a}	1557±14 ^{b}	1120±1^{b}	1245±2^{b}	1350±8^{b}	1366±1^{b}	1490±1^{b}	1582±19^{b}	1778±7^{b}
CPd+**	1426±23^{a}	1533±6^{c}	658±9^{e}	1105±1^{c}	1266±5^{c}	1319±9^{c}	1465±4^{c}	1477±18^{c}	1482±13^{c}
WPd	1261±12^{b}	1699±09^{a}	1370±1^{a}	1518±3^{a}	1704±1^{a}	2096±3^{a}	2226±3^{a}	2388±32^{a}	2708±21^{a}
SPCd	607±11^{d}	766±15^{e}	607±22^{d}	909±19^{e}	1015±5^{e}	1056±1^{e}	1125±9^{e}	1132±17^{e}	1341±9^{d}

*Control diet; **FTEII (1.6 U/g Unit of phytase for each g of meal)

The electrophoretic analysis of the diets (WPd, CPd+ and SPCd), Fig.1 (A, C and D), demonstrates how the proteins divide into their corresponding sub-units; CPd+ presented greater digestibility in the alkaline phase with fragments of 15 to 225 kDa. However, by increasing the hydrolysis time there is a partial degradation of the proteins, which can be seen as a decrease in the number of bands at 2700 seconds, with only the section of protein found in the highest concentration persisting (more intense bands). In the CPd+ (Figure 1-C), there was also partial degradation of the protein with increasing hydrolysis time.

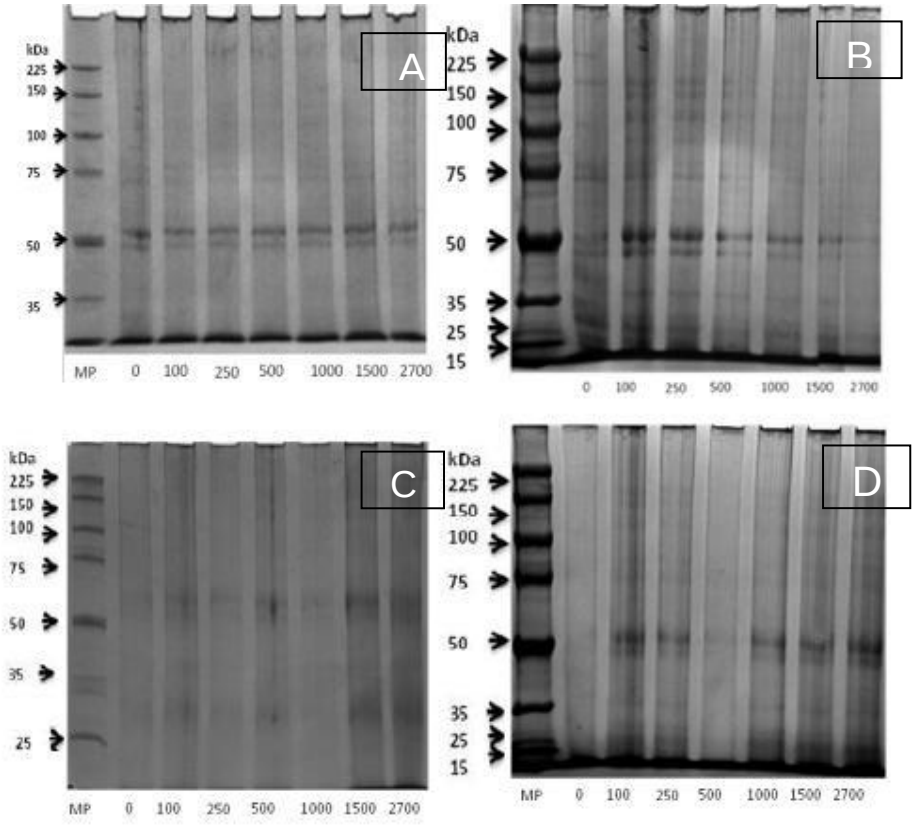


Fig 1. Protein fractions resulting from alcaline digestion (pyloric cecas and intestine) and by action enzyme extracts from juveniles red grouper of dried whey (A:upper left), shrimp meal (B: upper right) canola meal+phytase (C: bottom left) and soy protein concentrate (D: bottom right). The numbers below the figures indicate time in seconds.

In *vivo* digestibility results of experimental diets are displayed in Table 6. ADC protein values were significantly higher for the WPd (95.0±0.8%) and CPd+ (92.0±1.5%), and differed significantly from the ShPd (88.0±4.3), ($p<0.05$). The lowest ADC value for protein was displayed with SPCd (76.0±7.4)

Table 6. ADC dry matter and crude protein of experimental diets. Reference diet (Rd); shrimp powder diet (ShPd), canola paste+diet (CPd+), whey protein diet (WPd) and soy protein concentrate diet (SPCd) in fed *E. morio*. Mean±SD, n=3.

	ShPd	*CPd+*	*WPd*	*SPCd*
Dry matter	67.1±8.8^{b}	67.0±1.6^{b}	81.8±9^{a}	57.6±3.4^{c}
Crude protein	88.0±3.3^{b}	92.0±1.5^{ab}	95.0±0.8^{a}	76.0±2.4^{c}

*Phytase FTEII (1.6 U/g phytase Unit for each g of meal). Different letters in superscripts in the same line indicate significant difference ($p<0.05$).

As shown in Table 7, a negative correlation was observed between *in vivo* and *in vitro* digestibility for CPd+ (r2-0.59). However, there was a positive correlation between *in vivo* and *in vitro* digestibility for the SPCd (r2 0.98), WPd (r2 0.95) and ShPd (r2 0.51) respectively.

Table 7. Correlation between *in vivo* and *in vitro* digestibility (%) in compounded diets; Reference diet (Rd); shrimp powder diet (ShPd); canola paste+diet (CPd+); whey protein diet (WPd) and soy protein concentrate diet (SPCd).

diets	*in vitro*	*ADC*	r^2
shrimp meal	96.5±1.9	88±3	0.51
canola meal+phytased**	83.7±2.8	95±1	-0.59
dried whey	91.9±3.5	92±2	0.96
soy protein concentrate	76.2±3.3	76±2	0.98

Discussion

In vitro digestibility has been a useful complementary tool for predicting the digestibility of protein from fish egg powder, as compared to other ingredients, in rats (Pedersen and Eggum, 1983; Alarcón et al. 2002; Lemos et al., 2000). Acid hydrolysis from crab and

shrimp meal showed highest DH% values, confirming that red groupers feed on crustaceans (Brulé and Rodriguez-Canché, 1993). Similar values were seen for digestive enzymes when studying DH in *Atractoterus tropicus* demonstrating its high preference for food of marine origin (Frias-Quintana et al. 2010). However, crab meal contains ash and chitin therefore decreasing ADC above 15% (Tibbetts et al., 2011). Chitin is tested in a powdered form that could limit digestibility compared to a fresh cast from a molted animal. In this context, shrimp meal would be suitable for juvenile red grouper even though the DH value was not particularly high in acidic or alkaline conditions (4.1±1.0 and 5.3 ± 0.5% respectively). $FPC^{70,}$ showed significantly higher DH% especially in alkaline conditions.

Previous studies on this protein source resulted in high feed intake, good survival, and weight gain, and enhanced skeleton formation at the larval stage, (Cahu et al. 1999). Squid meal DH scored higher than the control under acidic or alkaline conditions (2.7±0.2 and 7.3±1.1% respectively) and substitution of 20-30% total dietary protein was proposed. Glycine, betaine, and other unidentified compounds stimulated feed intake (Cordova-Murueta and Garcia-Carreno et al. 1997). Under acidic or alkaline conditions, SPC ranked high for DH% and can influence digestibility, as found in catfish (Brown and Robinson, 1989) or *E. malabaricus* (Chen and Tsai, 1994), provided it did not exceed 20% inclusion. However, soy, canola, and local wheat gluten have been found to be less suitable for inclusion in diets due to low DH and other undesirable factors. Low DH% is linked to phytate present as an enzyme inhibitor (García-Carreño et al. 1997; Lemos et al. 2000), however when treated by phytase, it displayed good ADC. Dried whey contrasted in a DH% both from acidic and alkaline conditions (5 and 18% respectively) ($p<0.05$) but regular dried whey had limited digestibility due to a 50-70% lactose and low protein content. Ingredients classified from protein hydrolysis were then screened as potential feedstuffs to improve diet formulation for groupers (Lupatsch and Kissil, 2005). However, *in vivo* values are needed to produce data on energy coefficients (Cho, 1992).

ADC of an ingredient or a diet is critical in taking into account amino acid availability (Kaushik and Oliva-Telles, 1996). Sometimes weight gain remains poor due to a lack of palatability and therefore fish cannot reach their potential (Smith and Tabrett, 2004). Further studies on red grouper should evaluate energy partitioning to verify the effect of high nutrient density diets on energy metabolism. DP/DE must be optimized (Tsai, 1991) and weight gain, FCR, and body lipid content need to be controlled. Furthermore, the protein requirements in g of protein per kJ DE or g of protein per 100g biomass (absolute requirement) need to be critically reviewed (Shearer, 2000). The pH-stat provided initial information on the protein fraction of feedstuff for digestibility; however DH% values need to be correlated with *in vivo* data. Therefore, *in vitro* digestibility for a new candidate species produces only preliminary, but rapid information on feedstuff quality with no control over regulation of enzyme secretion, absorption, or rate of transport. The pH-stat provided initial information on ingredients, specifically regarding the protein fraction and its level of digestibility by fish, however DH values need to be correlated to *in vivo* studies.

The results of ADC and in vitro digestibility of diets prepared with selected sources showed a positive correlation, except for CPd+ and WPd which also showed high digestibility values both *in vitro* (91.9±3.5%) and *in vivo* (92±2%). SPCd demonstrated the lowest in vitro and in vivo digestibility values, while CPd+ presented contrast between low DH% in vivo condition even though in vitro hydrolysis was recommended (Fenerci and Sener, 2005).

Electrophoretic analysis and amino acid release revealed high molecular weight from shrimp meal protein, which was partially hydrolyzed in the intestine, indicating a high affinity of digestive enzymes for this protein. Results have shown that possible alternative feed ingredients for grouper and the approach used here regarding protein digestibility could help develop cost effective feeds by *in vitro* assays which are easier and less expensive to perform (Tonheim et al 2004).

Conclusion

The results show the possibility of using alternative sources to replace fish meal as a source of protein for grouper diets. Various approaches for assessing protein digestibility have been tested in order to develop cost effective and reliable feeds however *in vitro* assays, which are easier and less expensive to perform, should be confirmed by *in vivo* tests (Tonheim, et al. 2004).

Acknowledgements

Financial support was provided by UNAM (IN 220410 and 219713-3). Dr. A.F. Silva would like to thank CONACYT for the scholarship concession. The authors thanks Jaime Suárez and A. Sanchez for their assistance during the experimental aspect of the work.

References

AOAC (1980) Official methods of analysis 13rd. ed. Washington, DC.USA.

Adler-Nissen, J., Eriksen, S. and H.S. Olsen, (1983), Improvement of the functionality of vegetable proteins by controlled enzymatic hydrolysis. *Qual. Plant Foods Hum. Nutr*. (32) 411-423.

Alarcón, F.J., Moyano, F.J. and M. Diaz, (2002). Evaluation of different protein sources for aquafeeds by an optimised pH-stat system. *J. Sci. Food Agri.* (82) 697:704.

Anson, M.L., (1938). The estimation of pepsin, trypsin, papaïn and cathepsin with Hb. *Gen*. *Physiol***.** (22)79-89.

Brown, P.B., and E.H, Robinson, (1989). Comparison of practical catfish feeds contain 26 or 30% protein. *Progr. Fish-Cult*. (51) 149-151.

Brulé, T., and L.G.Rodríguez-Canché, (1993). Foods habits of juvenile red grouper *Epinephelus morio* (Valenciennes 1828) from Campeche Bank, Yucatán, Mexico. *Bull. Mar Sci*. (52)772-779.

Burgos, R., Moreno, V. and E. Giménez, (2003). Evaluación de la población y propuestas de para la pesquería del mero (*Epinephelus morio*) en el Banco de Campeche. Informe de Investigaciones conjuntas Mexico-Cuba sobre el mero (*Epinephelus morio* Valenciennes, 1828) en el Banco de Campeche. Convenio de pesca México-Cuba. *SAGARPA*, México, MIP, 25pp.

Cahu, CL., Zambonino-Infante, JL., Quazuguel, P. and MM. Le Gall, (1999). Protein hydrolysate vs. fishmeal in compound diets for 10-day old sea bass *Dicentrarchus* labrax larvae. *Aquaculture* (171) 109-119.

Chen, H.Y. and J.C. Tsai, (1994). Optimal dietary protein level for the growth of juvenile grouper, *Epinephelus malabaricus*, fed semi-purified diets. *Aquaculture* (119)265-271.

Cho, C.Y, (1992).Feeding systems for RT and others salmonides with reference to current estimates of energy and protein requirements. *Aquaculture* (10)107-123.

Church, FC., Swaisgood, H., Porter, D. and G. Catignani, (1983). Spetrophotometric assay using o-phthaldehyde for determination of proteolysisin milk proteins. *J. Dairy Sci*. (66) 1219-1227.

Dimes, LE., Haar, NF., Dong, FM., Rasco, BA., Forster, IP., Fairgrieve, WT., Arndt, R., H, RW., Barrows, FT. and DA. Higgs, (1994).Estimation of digestibility. II. *In vivo* assay of protein in salmonid feeds. *Comp. Biochem. Physiol.* (108A) 363-370.

Fenerci, S.and E. Sener, (2005). *In vivo* and *in vitro* digestibility of rainbow trout(*Onchorhynchus mykiss* Walbaum, 1972) fed steam pressured or extruded feeds. *Turkisk. J. Aquac. Fish. Aquac. Sci.* (5) 17-22.

Frías-Quintana, CA Álvarez-González CA. and G. Márquez-Couturier, (2010) .Design of microdiets for the larviculture of tropical gar *Atractosteus tropicus*, Gill (1863). *Univ. y Ciencia* (26) 265:282.

Garcia-Carreno, F L., Navarrete del Toro, A. and M. Ezquerra, (1997). Digestive shrimp proteases for evaluation of protein digestibility in vitro. 1:Effect of protease inhibitors in protein ingredients, *J. Mar. Biotech*. (5) 36-40.

Kaushik, S.J.and A. Oliva-teles, (1986). Effect of digestible energy on nitrogen and energy balance in rainbow trout. *Aquaculture (*50)89-101.

Lemos, D., Ezquerra, J M. and F L. Garcia-Carreno, (2000). Protein digestion in penaeid shrimp:digestive proteinases, proteinase inhibitors and feed digestibility. *Aquaculture* (186) 89-105.
Lupatsch, I.and G.W. Kissil, (2005). Feed formulations based on energy and protein demands in white grouper (*Epinephelus aeneus*). *Aquaculture* (248) 83-95.
Kunitz, M. 1947. Crystalline soybean trypsin inhibitor II. General properties.
J. Gen. Physiol. 30, 291-310.
Pedersen, B.Y., and B.O. Eggum, (1983). Prediction of protein digestibility by an *in vitro* enzymatic ph-stat procedure. *J. Ann. Physiol.* (49) 265-277.
Saunders, R.M., Conner, M.A., Booth, A.N., Bickoff, E.M. and G.O. Kohler,(1972). Measurement of digestibility of alfalfa concentrates by *in vivo* and *in vitro* methods. *J. Nutr.* (103), 530-535.
Shearer, K.D., (2000). Experimental design, statistic analysis and modeling of dietary nutrient requirement studies for fish: a critical review. *Aquac. Nut*. (6) 91-102.
Smith, D.M. and S.J. Tabrett, (2004). Accurate measurement of in vivo digestibility of shrimp feeds. *Aquaculture* (**232)** 563-580.
Tibbetts, S.M., Milley, J.E., Ross, N.W., Verreth, J.A.J. and S.P. Lall, (2011). *In vitro* pH-Stat protein hydrolysis of feed ingredients for Atlantic cod, *Gadus morhua*. 1.Development of the method. *Aquaculture* (319)398-406.
Tsai, J.C. (1991).Protein and energy requirements of grouper, *Epinephelus malabaricus*. Tesis, National Sun Yat-Sen University, Fac. Sci., Kaoshiung, Taiwan. 70 pp.
Tonheim, S K., Espe, M., Raae, AJ., Darias, MJ. and Ronnestad, I. (2004) In vivo incorporation of [U]-^{14}C-amino acids: an alternative protein labelling procedure for use in examining larval digestive physiology *Aquaculture* (235)553-567.
Viola, S.; Angeoni, H.; Lahav, E. Present limits of protein sparing by amino acid supplementation of practical carp and tilapia feeds. *J. Aquac.,* v.46, p.203-211, 1994.
Walter, HE. (1984). Proteinases: methods with haemoglobin, casein and azocoll as substrates, In *Methods of enzymatic analysis*, (*ed*.), Bergmeyer, HJ., Verlag Chemie, Weinheim, pp 270-277.
v, J.H. (1999) *Biostatistical analysis* 4th ed. Prentice Hall, Upper Southern River, NJ. USA, 660pp.