2014-04-20

Species specific in vitro protein digestion (pH-stat) for fish: method development and application for juvenile rainbow trout (Oncorhynchus mykiss), cobia (Rachycentron canadum), and Nile tilapia (Oreochromis niloticus).

Aquaculture Vs. 426–427, n. 20, p. 74–84, 2014
http://www.producao.usp.br/handle/BDPI/46436

Aquaculture 426–427 (2014) 74–84

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Species specific *in vitro* protein digestion (pH-stat) for fish: method development and application for juvenile rainbow trout (*Oncorhynchus mykiss*), cobia (*Rachycentron canadum*), and Nile tilapia (*Oreochromis niloticus*)

F. Yasumaru, D. Lemos *

Aquaculture Laboratory (LAM), Instituto Oceanográfico, University of São Paulo, Praça do Oceanográfico, 191, Butantã, São Paulo, SP 05508-120, Brazil

ARTICLE INFO

Article history:
Received 24 June 2013
Received in revised form 17 December 2013
Accepted 20 January 2014
Available online 25 January 2014

Keywords:
Compound diets
Digestion
Feedstuff
Formulation
Screening
Titration

ABSTRACT

Aqua feed manufacture requires flexible formulations and effective methods to screen suitable feed ingredients. *In vitro* digestion may assist in the characterization and quality control of protein in feedstuffs for fish species once standardized species-specific digestive enzyme extracts are available. This study aimed to develop a species-specific *in vitro* enzymatic method to assess protein digestion in fish under the pH-stat concept. Two carnivorous (rainbow trout, *Oncorhynchus mykiss*, and cobia, *Rachycentron canadum*) and one omnivorous (Nile tilapia, *Oreochromis niloticus*) fish species were used as models. Crude digestive enzyme extracts were recovered from stomach and pyloric caeca or intestine of individuals of different weight groups, feeding status, and farming systems. The hydrolytic capacity of the species-specific enzyme extracts was standardized on purified protein substrates and measured as degree of protein hydrolysis (DH) in the pH-stat assay. A group of twenty-four feed ingredients, including fish meals and by-products of plant and animal origin, was assessed for DH using the recovered enzymes from stomach and pyloric caeca/intestine. Ingredients were hydrolyzed with fish (i) stomach extract, (ii) pyloric caeca/intestine extract or (iii) stomach enzymes followed by pyloric caeca/intestine extract. Among plant by-products, cotton seed meal presented the highest DH with stomach plus pyloric caeca/intestine enzymes, followed by soy protein concentrate and soybean meals. Blood meals were the land animal by-product with higher DH outputs compared to poultry by-product meals and feather meals. No significant difference was observed among the DHs of fish meals. The significance of measuring the DH with stomach enzyme extract is still not well understood but, overall, the pre-hydrolysis of feedstuffs with stomach enzymes increased pyloric caeca/intestine DH value. For cage and pond farmed Nile tilapia, ingredient DHs followed the same trend, describing a significant correlation and a high determination coefficient regression. Routine use of the method may yet depend on the prompt availability of more practical sources of enzymes. The determination of the degree of protein hydrolysis by the *in vitro* pH-stat with species-specific enzymes has shown to be a precise method that may be a useful tool to rank feed ingredients, and also an accessory method in the quality control of feedstuffs.

1. Introduction

The aquaculture feed industry has been faced with the challenge to find nutritionally suitable and economically feasible protein ingredients to formulate compound diets. Aqua feed manufacture requires flexible formulations based on nutrient delivery from feed ingredients and additives to replace scarce raw materials like fish meals, as well as other sources subject to fluctuation in cost and availability (Tacon et al., 2011). In this context, searching for novel ingredients, monitoring potential nutritional variability and nutrient availability to the target species are required in the routine of the feed manufacturer. Consistent methods of nutritional evaluation are needed to assist in the characterization and quality control at the industrial level (Moughan, 1999). Nutrient digestibility and availability have been determined *in vivo* in research laboratories and accepted for feedstuff assessment (NRC, 2011). On the other hand, these trials may be laborious, complex, time consuming and expensive, and possibly not adequate for application at industrial level (Lemos and Tacon, 2011). This motivated the development of *in vitro* methods based on the digestion of small amounts of feedstuff samples. Reported *in vitro* protein digestion methods include the use of digestive enzymes that may be available from commercial sources (Lazo et al., 1998; Shipton and Britz, 2002; Tonheim et al., 2007) or recovered from the target species (Dimes and Haard, 1994; Ezquerra et al., 1998; Tibbetts et al., 2011a). Nevertheless, different results in the *in vitro* digestion have been found depending on the enzyme origin, suggesting a species-specific feature of *in vitro* protein digestion (Ezquerra et al., 1998; Lemos and Nunes, 2008; Moyano and Savoie, 2001).

* Corresponding author. Fax: +55 11 3091 6607.
E-mail address: dellemos@usp.br (D. Lemos).

0044-8486/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aquaculture.2014.01.012

The *in vitro* pH-stat determination of the degree of protein hydrolysis (DH) with species-specific enzymes has been investigated in some fish and crustacean species with significant potential to distinguish feedstuff quality and also to predict protein digestibility in the live animal (Córdoba-Murueta and García Carreño, 2002; Dimes et al., 1994; El-Mowafi et al., 2000; Ezquerra et al., 1998; Lemos and Yasumaru, 2010; Lemos et al., 2009; Tibbetts et al., 2011b). The principle of the pH-stat method consists in the potential shift in medium pH related to protein hydrolysis at certain pH levels. In the course of a reaction set for a constant pH, variation derived from hydrolysis may be automatically corrected by titration and, thus, the amount of peptide bonds cleaved is proportional to the volume of titrant consumed (Adler-Nissen, 1986). Some of the advantages of the pH-stat technique may be the non-use of buffer solutions while maintaining constant pH during reaction and the possibility to quickly calculate the degree of protein hydrolysis by the automatically plotted titration curve (Pedersen and Eggum, 1983). Besides being simple, rapid, precise, and safe, it does not require complex equipment, provides specific response (standardized species enzymes), stable reaction conditions, and tests different ingredients in small amounts (Grabner, 1985; Lemos et al., 2009).

The assessment of the species-specific degree of protein hydrolysis for distinct species would require the development of consistent analytical procedures considering the status of the donor individuals and standardization of the recovered digestive enzyme extracts. The present study describes a pH-stat method to determine the *in vitro* protein digestion for stomached fish species (rainbow trout, *Oncorhynchus mykiss*, cobia, *Rachycentron canadum*, and Nile tilapia, *Oreochromis niloticus*) with different characteristics in terms of domestication level, feeding habit and farming system. Digestive enzyme extracts of the species were recovered at different fish weight and feeding status, and standardized according to digestive potential as degree of hydrolysis of standard substrates. Standardized species-specific enzyme extracts were additionally used to determine the degree of protein hydrolysis of some practical ingredients commonly used in aqua feeds.

2. Materials and methods

2.1. Fish sampling

Individuals of the three species tested (rainbow trout, *Oncorhynchus mykiss*, cobia, *Rachycentron canadum* and Nile tilapia, *Oreochromis niloticus*) of different weight groups (three groups for rainbow trout and Nile tilapia and two for cobia), feeding status (fed commercial diet or unfed −15 h fasting for Nile tilapia or 24 h for rainbow trout and cobia), and commercial farming systems were sampled for the recovery of digestive enzyme extracts. Rainbow trout was farmed in freshwater raceways, Nile tilapia in fertilized ponds or in cages (in a freshwater reservoir), and cobia in circular concrete tanks with flow-through seawater. Ten healthy fish were sampled per species, weight group, feeding status and farming system. Fish were killed by rapid cephalic concussion then measurements were taken (body weight, body total length, individual digestive organ pH, weight and length). Mean individual body weight ± s.d. values of groups evaluated were: rainbow trout, 165.2 ± 38.9, 262.1 ± 22.5 and 393.1 ± 35.8 g; cobia, 550.5 ± 135.9 and 1052.6 ± 273.3 g; cage farmed Nile tilapia, 124.5 ± 23.2, 372.3 ± 68.1 and 598.3 ± 110.1 g; and pond farmed Nile tilapia, 116.1 ± 18.5, 332.3 ± 38.2, and 669.7 ± 82.6 g. Prior to organ excision, pH was measured in the stomach, pyloric caeca (absent in Nile tilapia) and intestine of each individual with a combined glass electrode connected to a pH meter (7 mm immersion depth and 3 mm electrode tip diameter — Biotrode 744 pH meter, Metrohm AG, Switzerland). After excision, the digestive tract of fasted fish was found empty, whereas of fed fish it had to be thoroughly cleansed with distilled water. Stomach, pyloric caeca and intestine were cleaned of visceral fatty tissue, dissected, measured (length, weight), pooled in plastic bags according to fish weight group and feeding status, and frozen at −20 °C. Samples were transported on dry ice to the laboratory and stored at −20 °C until further processing.

2.2. Crude digestive enzyme extract recovery

The frozen sampled organs were allowed to partially thaw at room temperature. For rainbow trout and cobia enzyme extracts from stomach and pyloric caeca were recovered. For fish possessing pyloric caeca it is considered as the most suitable enzyme source for protein hydrolysis assays in alkaline medium because the number of caeca or blind diverticula may account for ca. 70% of the total enzymatic digestion (Buddington and Diamond, 1986; Tibbetts et al., 2011a). The organs were chopped into small pieces with scissors as they were difficult to homogenize. For Nile tilapia, stomach and intestine enzyme extracts were used. Each pool of sampled organs was placed in a glass beaker on ice with cold distilled water at ratios of 1:3 (w/v) for stomach and 1:1 (w/v) for pyloric caeca and intestine. For pyloric caeca of cobia, the 1:3 (w/v) ratio was employed to allow proper homogenization of the harder tissue. This comparatively higher dilution was computed in the standardization of the enzyme extracts. Tissue was homogenized (T25 digital Ultra-Turrax®, 18G dispersing element, IKA WORKS, Inc., Wilmington, NC, USA) in several pulses of ca. 20 s to avoid engine overheating and possible damage to the sample. The mixture was centrifuged at 16,800 ×*g* for 30 min at 4 °C and the supernatant, which constituted the crude enzyme extract, was recovered and pooled in a glass beaker on ice. The pHs of the crude enzyme extracts were measured (pH Meter 744, resolution 0.01, 0.1 °C, 1 mV, Metrohm, Switzerland) and adjusted to 2.0 (stomach extract) by adding HCl 0.1 N and to pH 8.0 (pyloric caeca and intestine) by adding NaOH 0.1 N, under constant agitation on ice to keep low temperature (ca. 4 °C) to avoid enzyme activation. Enzyme extracts were aliquoted in 2-mL polypropylene cryogenic vials and stored at −20 °C until used. A schematic sequence of enzyme extract recovery is shown in Fig. 1.

2.3. Crude enzyme extract standardization

The crude enzyme extracts recovered from rainbow trout, cobia and Nile tilapia were standardized according to their hydrolytic capacity using the *in vitro* pH-stat degree of protein hydrolysis (DH) to determine the volume of extract most suitable for the analysis. Briefly, in the pH-stat concept, the enzymatic hydrolysis of peptide bonds results in pH shift (increase or decrease, for acid and alkaline hydrolysis, respectively) which is automatically stabilized by the addition of the titrant (0.1 N HCl or 0.1 N NaOH). The volume of titrant added is equivalent to the DH by the digestive enzyme extract, i.e., at certain pH levels the relation between equivalent peptide bonds cleaved and equivalent titrant consumed is proportional (Adler-Nissen, 1986; Diermayr and Dehne, 1990).

Standard substrates for stomach and pyloric caeca or intestine assays were analytical grade hemoglobin from bovine blood (95% crude protein, H2625, Sigma-Aldrich, St. Louis, MO, USA) and casein from bovine milk (90% crude protein, C7078, Sigma-Aldrich, St. Louis, MO, USA), respectively. Hemoglobin was chosen because it is a rapidly digested reproducible substrate (Anson, 1938) and casein, a highly digestible purified and standardized product. Fish stomach enzyme extracts were standardized at pH 2.0. Rainbow trout and cobia pyloric caeca and Nile tilapia intestine extracts were standardized at pH 8.0.

The assays were carried out simultaneously in three automated titrators, i.e., one single and two double measuring interfaces with burettes (718 Stat Titrino, Titrando 836, Titrando 907 — Metrohm AG, Switzerland), connected to a single controlling and data logging software (Tiamo™ v. 2.2, Metrohm AG, Switzerland) operated by one person. Standard substrate samples corresponding to 80 mg protein were stirred in distilled water in the reaction tube (8.0 mL total suspension volume) and the pH was adjusted to 2.0 for the stomach or 8.0 for the pyloric caeca/intestine extract assays by the addition of HCl 0.1 N or

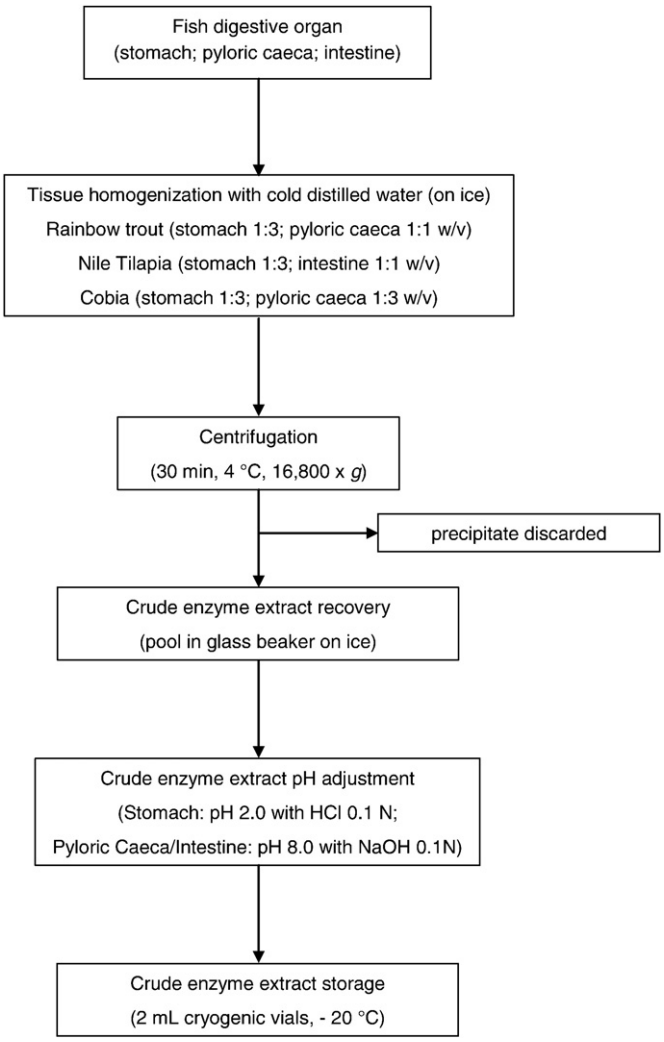

Fig. 1. Flow diagram of digestive enzyme extract recovery for the determination of the *in vitro* degree of protein hydrolysis (DH) in rainbow trout, *Oncorhynchus mykiss*, cobia, *Rachycentron canadum* and Nile tilapia, *Oreochromis niloticus*. After recovery, the hydrolytic capacity of crude enzyme extracts was standardized according to DH of specific protein substrates. Frozen stored enzyme extracts (−20 °C) showed to be stable for use after several months. Further details are in Materials and methods.

NaOH 0.1 N, respectively, and kept stable for 30 (hemoglobin) and 60 (casein) minutes. Minimum and maximum titrant addition rates were 5 μL and 0.8 mL min^{-1} (HCl) and 1.5 μL and 3.0 mL min^{-1} (NaOH). Suspension final volume was adjusted to 10 mL (considering the enzyme extract volume) by adding distilled water. If necessary, after the stabilization step the pH was automatically finely adjusted to 2.0 or 8.0. Protein hydrolysis assay started with the addition of the respective enzyme extract (from stomach or pyloric caeca/intestine) and carried out for 60 min. Titrant (HCl and NaOH) minimum and maximum addition rates were 3.0 μL and 5.0 mL min^{-1}, respectively. Reaction temperature was maintained at 25 ± 0.2 °C in jacketed water flow through reaction vessels connected to a heated/refrigerated constant temperature water bath (temperature uniformity ± 0.1 °C, RSWB 3222A Lindberg/BlueM, Thermo Electron Corp., MA, USA). The water was recirculated through plastic hoses from the water bath tank to the jacketed vessels with the aid of a submersible aquarium pump. During the assays, nitrogen gas was purged into the mixture and the reaction tube was covered with plastic film to avoid interference of atmospheric CO_2 in the reaction pH (Adler-Nissen, 1986). The hydrolysis assay was carried in triplicate.

The degree of protein hydrolysis (DH) with stomach extract was calculated based on the formula proposed by Diermayr and Dehne (1990):

$$DH = [(V \times N)/E] \times (1/P) \times F_{pH} \times 100\%.$$

Where:

V volume of acid consumed in the hydrolysis reaction (mL);
N normality of the acid;
E mass of substrate protein (g);
P number of peptide bonds cleaved (mol g $protein^{-1}$). For proteins which amino acid composition is not determined, P is generally suggested as 8.0.
F_{pH} 1.08 (correction factor for pH 2.0 at 25 °C).

The DH with pyloric caeca/intestine extract was calculated according to Adler-Nissen (1986):

$$DH = B \times Nb \times (1/a) \times (1/MP) \times (1/H_{tot}) \times 100\%.$$

Where:

B volume of alkali consumed (mL);
Nb normality of the alkali;
α average degree of dissociation of the α-NH groups (1/α=1.50 for pH 8.0 at 25 °C);
MP mass of substrate protein (g);
H_{tot} total number of peptide bonds in the protein substrate [7.6–9.2 meqv g $protein^{-1}$, according to the source of protein (Adler-Nissen, 1986)].

To assess the proper volume of each enzyme extract batch to be used in the DH applications, the study considered the minimum enzyme volume to produce high output. The standardization comprised the determination of the hydrolytic capacity of different volumes (50, 200, 600, 1000 μL) of stomach or pyloric caeca/intestine extracts on the same substrate amount (80 mg protein). DH values were plotted against the volumes of enzyme extracts used in the assay to check for hydrolysis performance and for the model that best describes these relationships. These regressions also served to compare the possible effects of fish weight and feeding status on the hydrolytic output of digestive enzyme extracts.

2.4. Test ingredients

The DHs of twenty-four feed ingredients of different types and sources were assessed for the three fish species. The ingredients were obtained from feed manufacturers, ingredient manufacturers and suppliers. The set included marine and land animal (fish meals, blood meals, feather meals, and poultry by-product meals) and plant (corn grain, corn gluten meal, cotton seed meal, rapeseed meal, soybean meals, soy protein concentrate, wheat bran, wheat flour, and wheat gluten) ingredients commonly used for aqua feed manufacture. Source details corresponded to samples of blood meals, poultry by-product meals (feed-grade and pet food grade), fish meals (anchovy, herring, mackerel, menhaden, salmon by-product, miscellaneous fish by-product), and soybean meals (full-fat and solvent extracted). The proximate composition (moisture, crude protein, crude fat, crude fiber, and ash) of the feed ingredients was determined (Silva and Queiroz, 2009). Moisture was determined by drying the sample at 103–105 °C; protein content was determined with the micro-Kjeldahl method; lipids were determined with the Soxhlet method; crude fiber was determined by acid and alkaline hydrolysis; and ash was determined by combustion at 550–500 °C. Nitrogen-free extract was calculated by difference [100 − (crude protein + crude fiber + crude fat + ash + moisture)]. Ingredients were processed as received from the suppliers ('as is') and sieved through a series of screens with

Table 1
Mean values (s.d., n = 10) of individual body weight (g) and total length (cm), digestive tract organs — weight (g), length (cm) and pH, determined in juvenile rainbow trout (*Oncorhynchus mykiss*), cobia (*Rachycentron canadum*), and Nile tilapia (*Oreochromis niloticus*) farmed in raceways, concrete tanks and cage or pond, respectively, and at two feeding status (fed or unfed).

Fish species	Farming system	Feeding status	Body		Stomach			Pyloric caeca [a]			Intestine		
			Weight (g)	Length (cm)	Weight (g)	Length (cm)	pH	Weight (g)	Length (cm)	pH	Weight (g)	Length (cm)	pH
Rainbow trout (*Oncorhynchus mykiss*)	Raceway	Fed	165.2 (38.9)	22.2 (1.6)	2.7 (0.8)	5.0 (0.6)	3.40 (0.92)	2.9 (0.7)	3.1 (0.5)	7.40 (0.18)	1.8 (0.6)	9.2 (1.9)	7.80 (0.10)
			262.1 (22.5)	26.9 (0.9)	4.7 (1.3)	6.5 (1.2)	4.74 (0.39)	5.2 (1.5)	4.2 (0.4)	7.68 (0.27)	2.5 (0.6)	10.5 (1.7)	7.76 (0.14)
			393.1 (35.8)	30.7 (0.9)	8.2 (2.5)	7.3 (1.1)	3.86 (1.36)	8.1 (1.7)	4.2 (1.4)	7.75 (0.18)	5.2 (1.0)	14.1 (1.6)	8.01 (0.18)
		Unfed	165.2 (38.9)	22.2 (1.6)	2.8 (1.0)	5.8 (1.0)	2.29 (0.69)	2.6 (1.2)	3.5 (0.6)	7.58 (0.23)	1.8 (0.6)	9.5 (2.2)	7.68 (0.22)
			262.1 (22.5)	26.9 (0.9)	4.2 (1.9)	6.2 (1.3)	3.44 (0.64)	3.8 (1.2)	3.7 (1.1)	7.73 (0.14)	2.2 (0.7)	9.5 (1.4)	7.98 (0.27)
			393.1 (35.8)	30.7 (0.9)	8.7 (2.3)	7.0 (0.6)	2.23 (0.54)	5.8 (0.9)	4.4 (0.6)	7.64 (0.29)	3.9 (0.6)	13.1 (1.6)	7.91 (0.22)
Cobia (*Rachycentron canadum*)	Concrete tank	Fed	550.5 (135.9)	39.9 (2.5)	10.92 (3.6)	6.01 (0.9)	4.68 (0.80)	15.39 (6.0)	3.04 (0.7)	6.95 (0.22)	3.61 (1.2)	14.5 (2.8)	6.94 (0.28)
			1052.6 (273.3)	48.1 (4.5)	19.01 (4.2)	7.41 (1.5)	4.39 (0.23)	29.73 (10.6)	4.24 (1.4)	6.75 (0.26)	6.15 (1.9)	18.7 (3.1)	6.80 (0.20)
		Unfed	550.5 (135.8)	39.9 (2.5)	11.00 (1.1)	5.99 (0.8)	6.66 (0.41)	13.04 (1.8)	2.48 (0.5)	7.02 (0.25)	2.82 (0.2)	14.8 (2.0)	7.08 (0.19)
			1052.6 (273.3)	48.1 (4.5)	21.07 (5.6)	7.44 (1.4)	6.42 (1.38)	26.77 (7.0)	3.25 (0.5)	7.03 (0.19)	5.33 (1.5)	18.3 (3.8)	7.13 (0.25)
Nile tilapia (*Oreochromis niloticus*)	Cage	Fed	124.5 (23.2)	18.2 (1.1)	0.5 (0.2)	3.2 (0.7)	3.46 (0.75)				2.8 (0.4)	114.6 (16.8)	6.85 (0.18)
			372.3 (68.1)	24.8 (1.7)	1.5 (0.3)	6.1 (0.6)	3.34 (0.50)				8.5 (2.1)	191.1 (33.6)	6.44 (0.12)
			598.3 (110.1)	29.3 (1.7)	n.d.	7.1 (0.6)	3.97 (0.74)				n.d.	203.3 (26.0)	6.27 (0.18)
		Unfed	124.5 (23.2)	18.2 (1.1)	0.5 (0.2)	2.3 (0.4)	4.12 (1.69)				3.5 (1.0)	127.4 (19.9)	7.13 (0.32)
			372.3 (68.1)	24.8 (1.7)	n.d.	3.2 (0.5)	2.61 (0.57)				n.d.	149.5 (27.4)	6.81 (0.26)
			598.3 (110.1)	29.3 (1.7)	n.d.	3.9 (0.9)	5.08 (1.82)				n.d.	180.5 (30.0)	7.12 (0.12)
	Pond	Fed	116.1 (18.5)	18.1 (0.9)	0.71 (0.1)	2.5 (0.5)	2.55 (0.75)				5.89 (1.2)	123.0 (17.2)	6.64 (0.28)
			332.3 (38.2)	26.1 (1.0)	1.53 (0.2)	4.5 (0.8)	3.98 (0.95)				8.33 (1.0)	200.5 (36.0)	6.60 (0.34)
			669.7 (82.6)	32.3 (1.4)	2.80 (0.5)	4.74 (0.5)	3.89 (0.74)				16.16 (2.5)	242.6 (35.1)	6.92 (0.38)
		Unfed	116.1 (18.5)	18.1 (0.9)	0.54 (0.1)	2.04 (0.3)	3.01 (1.61)				3.55 (0.6)	104.2 (13.5)	7.46 (0.36)
			332.3 (38.2)	26.1 (1.0)	1.67 (0.2)	3.35 (0.5)	3.44 (0.73)				8.49 (1.5)	193.8 (30.2)	6.76 (0.37)
			669.7 (82.6)	32.3 (1.4)	2.97 (0.5)	3.93 (0.7)	2.91 (0.65)				13.62 (1.6)	202.8 (25.3)	6.86 (0.28)

[a] Not present in *Oreochromis niloticus*. n.d. = not determined.

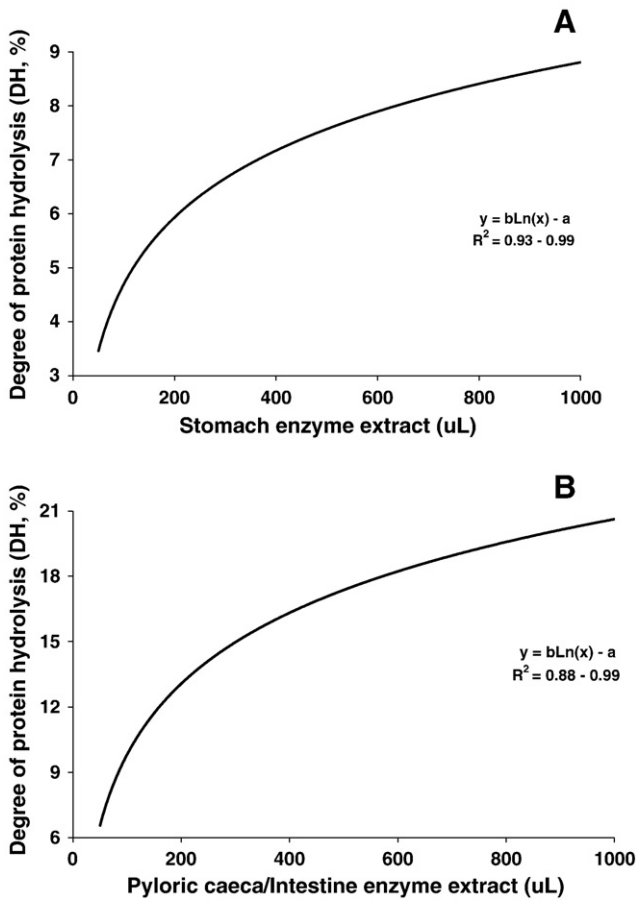

Fig. 2. Hydrolytic performance of the enzyme extracts recovered from (A) stomach and (B) pyloric caeca/intestine of juvenile rainbow trout, *Oncorhynchus mykiss*, cobia, *Rachycentron canadum* and Nile tilapia, *Oreochromis niloticus*. Analytical grade protein products (hemoglobin and casein for stomach and pyloric caeca/intestine enzymes, respectively) were used as substrates. Plotting *in vitro* pH-stat protein hydrolysis carried out for 60 min at 25 ± 0.2 °C, with 80 mg protein substrate (y) and 50, 200, 600 and 1000 μL of enzyme extracts (n = 12) (x) resulted in well adjusted logarithmic function [y = bLn(x) − a] with coefficient of determination R^2 = 0.88–0.99. Further details are in Materials and methods.

mesh sizes >1000 μm, 500–1000 μm, 250–500 μm, 125–250 μm, 63–125 μm, and <63 μm to describe particle size distribution (%), as grinding of raw materials is a costly process for the feed manufacturer and *in vitro* DH may depend on particle size.

2.5. Species specific in vitro pH-stat determination of protein hydrolysis of feed ingredients

Following digestive enzyme extract standardization, the *in vitro* degree of protein hydrolysis of the test ingredients was determined for rainbow trout, cobia and Nile tilapia. Ingredients were hydrolyzed in single assays with (i) stomach extract, (ii) pyloric caeca/intestine enzyme extract or (iii) stomach extract followed by pyloric caeca/intestine enzymes (two-stage hydrolysis). Assays were carried out with enzyme extracts recovered from fed fish at selected average individual weight: rainbow trout (393.1 ± 35.8 g), cobia (550.5 ± 135.9 g), and Nile tilapia (cage farmed: 598.3 ± 110.1 g or pond farmed: 669.7 ± 82.6 g). The enzyme extract volumes chosen were 250 μL from stomach and 200 μL from pyloric caeca (rainbow trout and cobia) or intestine (Nile tilapia). Four replicates of DH analysis were run per test ingredient. For the single assays with (i) stomach extract and (ii) pyloric caeca/intestine enzyme extract, samples were prepared and hydrolyzed as described in the enzyme extract standardization in Section 2.3 with a slight modification in the titrant addition rates, i.e., in the preparation step, HCl 0.1 N minimum and maximum rates were 5.0 μL min^{-1} and 0.3 mL min^{-1} and NaOH 0.1 N, minimum and maximum rates were 1.0 μL and 1.0 mL min^{-1}, and in the hydrolysis step, minimum was 1.0 μL min^{-1} and maximum was 0.3 mL min^{-1}. For the two-stage hydrolysis, immediately after hydrolysis with stomach extract sample was transferred to another measuring interface with an electrode and a burette, when pH was adjusted to 8.0 with the addition of NaOH 0.1 N for the hydrolysis with pyloric caeca/intestine extract. Each hydrolysis was carried out for 60 min at 25 ± 0.2 °C.

2.6. Statistical analysis

Data were analyzed using a SigmaPlot version 11.0 statistical software (Systat Software Inc., Chicago, IL, USA). Protein DH percentage data from enzyme extract standardization were arcsine- and log10 + 1-transformed to statistically compare the effect of fish weight, feeding status and farming system on the hydrolytic performance of enzyme extracts (Gotelli and Ellison, 2004; Zar, 1999). Plotting DH against the enzyme extract volumes after transformation resulted in a linear adjustment (y = a + bx). Slope (b) and intercept (a) values from the linear regressions of fish weight groups in the same feeding status were submitted to ANOVA and between feeding status of fish of the same weight group were submitted to *t*-test and significant differences between values were detected with the post-hoc Tukey test. Since the same data were compared twice (fed vs. unfed; fish weight groups), *P*-value was divided by two and difference was considered significant at $P < 0.025$ (Zar, 1999). Ingredient DH data from hydrolysis with stomach, pyloric caeca/intestine extracts and two-stage hydrolysis were submitted to one-way ANOVA. Ingredient DH data of the two Nile tilapia farming systems were compared using *t*-test. Difference between means was detected with Tukey test at $P < 0.05$.

3. Results

Mean (s.d.) values of fish body weight and total length, digestive organs weight, length and pH, are presented according to fish species, farming system and feeding status (Table 1). The relation between intestinal length and body length for rainbow trout and cobia was 0.4 (0.06), whereas for Nile tilapia it was 6.6 (1.4). Stomach pH ranged from 2.23 (0.54) to 6.66 (0.41) with lower values in unfed rainbow trout and higher values in unfed cobia. Rainbow trout and cobia pyloric caeca pH varied from 6.75 (0.26) to 7.75 (0.18). Nile tilapia intestine pH ranged from 6.27 (0.18) to 7.46 (0.36).

The recovered enzyme extracts showed consistent hydrolytic responses upon analytical grade hemoglobin (stomach enzymes) and casein (pyloric caeca or intestine enzymes). The degree of protein hydrolysis (DH) of the substrate increased significantly ($P < 0.05$) with the stomach and pyloric caeca/intestine enzyme extract volumes employed (50, 200, 600, 1000 μL), following a well adjusted logarithmic function (R^2 = 0.88–0.99, n = 12, Fig. 2). As a log shaped model, higher DH increments were verified between 50 and 600 μL. The coefficients of variation (n = 3) of DH under different enzyme extract volumes were 4.24 ± 2.82% for stomach and 4.48 ± 2.12% for pyloric caeca/intestine enzyme hydrolysis. The recovered species-specific crude enzyme extract batches could be standardized according to the hydrolytic capacity upon purified substrates. The intercept (a) and slope (b) values from the linearized regressions (y = a + bx) between DH and enzyme extract volumes for stomach and pyloric caeca/intestine, according to fish species, were obtained as additional comparison criteria, with the respective regression coefficients of determination (R^2) (Table 2). Accordingly, the slope value indicates the rate at which DH increases with the enzyme extract volumes assayed, and intercept the overall DH level. With rainbow trout stomach enzyme extract, the rate of DH increase (b) was not statistically different ($P > 0.025$) among fish weight or between feeding status, whereas with pyloric caeca extract

Table 2
Standardization of crude digestive enzyme extracts using the *in vitro* pH-stat degree of protein hydrolysis (DH) in rainbow trout (*Oncorhynchus mykiss*), cobia (*Rachycentron canadum*), and cage and pond farmed Nile tilapia (*Oreochromis niloticus*). The hydrolytic capacity was standardized according to enzyme extracts from fish at different weight and feeding status. Standardization used hemoglobin and casein (80 mg protein per sample) as substrates for stomach and pyloric caeca/intestine assays, respectively. Enzyme extract volumes tested were 50, 200, 600 and 1000 μL, n = 12. Percentage DH data were arcsine- and log10 + 1-transformed for a linear adjustment (y = a + bx) with enzyme extract volume (x), and equation values are given as mean (s.d.). a = intercept or constant; b = slope; R^2 = coefficient of determination for the regression. Different superscript upper-case letters indicate significant difference between fish of different feeding status but same weight and different superscript lower-case letters indicate significant difference between the different fish sizes at the same feeding status ($P < 0.025$). Comparisons were made within the same fish species.

Fish species	Feeding status	Enzyme extract (organ)	Fish size (g)	a	b	R^2
Rainbow trout (*Oncorhynchus mykiss*)	Fed	Stomach	165.2 (38.9)	0.335 (0.00789)$^{a, B}$	0.174 (0.00927)$^{a, A}$	0.97
			262.1 (22.5)	0.304 (0.00783)$^{c, B}$	0.179 (0.00920)$^{a, A}$	0.97
			393.1 (35.8)	0.316 (0.00739)$^{b, A}$	0.183 (0.00869)$^{a, A}$	0.98
	Unfed		165.2 (38.9)	0.360 (0.00877)$^{a, A}$	0.153 (0.01030)$^{b, B}$	0.96
			262.1 (22.5)	0.315 (0.01310)$^{b, A}$	0.171 (0.01540)$^{a, A}$	0.92
			393.1 (35.8)	0.299 (0.00761)$^{c, B}$	0.182 (0.00894)$^{a, A}$	0.98
	Fed	Pyloric caeca	165.2 (38.9)	0.359 (0.01700)$^{a, B}$	0.288 (0.02000)$^{b, A}$	0.95
			262.1 (22.5)	0.317 (0.01950)$^{b, B}$	0.337 (0.02290)$^{a, A}$	0.96
			393.1 (35.8)	0.366 (0.02080)$^{a, B}$	0.286 (0.02440)$^{b, A}$	0.93
	Unfed		165.2 (38.9)	0.449 (0.01140)$^{a, A}$	0.244 (0.01330)$^{a, B}$	0.97
			262.1 (22.5)	0.453 (0.02110)$^{a, A}$	0.237 (0.02480)$^{a, B}$	0.90
			393.1 (35.8)	0.422 (0.02030)$^{b, A}$	0.245 (0.02380)$^{a, B}$	0.91
Cobia (*Rachycentron canadum*)	Fed	Stomach	550.5 (135.9)	0.299 (0.00956)$^{a, B}$	0.193 (0.01120)$^{a, A}$	0.97
			1052.6 (273.3)	0.282 (0.00991)$^{b, B}$	0.198 (0.01160)$^{a, A}$	0.97
	Unfed		550.5 (135.9)	0.326 (0.01250)$^{a, A}$	0.177 (0.01470)$^{a, B}$	0.93
			1052.6 (273.3)	0.335 (0.01050)$^{a, A}$	0.164 (0.01230)$^{b, B}$	0.95
	Fed	Pyloric caeca	550.5 (135.9)	0.278 (0.01990)$^{b, A}$	0.322 (0.02330)$^{a, A}$	0.95
			1052.6 (273.3)	0.311 (0.01490)$^{a, A}$	0.321 (0.01750)$^{a, A}$	0.97
	Unfed		550.5 (135.9)	0.260 (0.02180)$^{a, B}$	0.222 (0.02560)$^{b, B}$	0.88
			1052.6 (273.3)	0.258 (0.03070)$^{a, B}$	0.326 (0.03610)$^{a, A}$	0.89
Nile tilapia (*Oreochromis niloticus*) cage farmed	Fed	Stomach	124.5 (23.2)	−0.0385 (0.0327)$^{a, A}$	0.429 (0.03840)$^{b, A}$	0.93
			372.3 (68.1)	−0.0450 (0.0254)$^{a, B}$	0.428 (0.02990)$^{b, A}$	0.95
			598.3 (110.1)	−0.0979 (0.0255)$^{b, B}$	0.446 (0.02990)$^{a, A}$	0.96
	Unfed		124.5 (23.2)	−0.0300 (0.0405)$^{b, A}$	0.434 (0.04760)$^{a, A}$	0.89
			372.3 (68.1)	0.0196 (0.0278)$^{a, A}$	0.390 (0.03260)$^{b, B}$	0.93
			598.3 (110.1)	−0.0181 (0.0295)$^{b, A}$	0.411 (0.03470)$^{b, B}$	0.93
	Fed	Intestine	124.5 (23.2)	0.280 (0.02170)$^{c, A}$	0.368 (0.02550)$^{a, A}$	0.95
			372.3 (68.1)	0.325 (0.02940)$^{b, A}$	0.339 (0.03450)$^{b, A}$	0.91
			598.3 (110.1)	0.418 (0.01650)$^{a, A}$	0.248 (0.01930)$^{c, B}$	0.94
	Unfed		124.5 (23.2)	0.261 (0.01870)$^{c, B}$	0.379 (0.02200)$^{a, A}$	0.97
			372.3 (68.1)	0.306 (0.02730)$^{b, A}$	0.358 (0.03210)$^{a, A}$	0.93
			598.3 (110.1)	0.352 (0.02700)$^{a, B}$	0.292 (0.03180)$^{b, A}$	0.89
Nile tilapia (*Oreochromis niloticus*) pond farmed	Fed	Stomach	116.1 (18.5)	0.110 (0.02720)$^{a, A}$	0.324 (0.03200)$^{b, A}$	0.91
			332.3 (38.2)	0.0429 (0.0193)$^{c, A}$	0.365 (0.02270)$^{a, A}$	0.96
			669.7 (82.6)	0.0871 (0.0176)$^{b, A}$	0.293 (0.02070)$^{c, B}$	0.95
	Unfed		116.1 (18.5)	0.123 (0.02670)$^{a, A}$	0.299 (0.03130)$^{c, A}$	0.90
			332.3 (38.2)	0.0408 (0.0263)$^{b, A}$	0.360 (0.03090)$^{b, A}$	0.93
			669.7 (82.6)	−0.150 (0.03150)$^{c, B}$	0.484 (0.03710)$^{a, A}$	0.94
	Fed	Intestine	116.1 (18.5)	0.431 (0.02380)$^{a, B}$	0.216 (0.02790)$^{c, A}$	0.86
			332.3 (38.2)	0.231 (0.02170)$^{c, B}$	0.412 (0.02550)$^{a, A}$	0.96
			669.7 (82.6)	0.259 (0.01010)$^{b, A}$	0.344 (0.01190)$^{b, B}$	0.99
	Unfed		116.1 (18.5)	0.463 (0.01680)$^{a, A}$	0.201 (0.01970)$^{c, A}$	0.91
			332.3 (38.2)	0.272 (0.02050)$^{b, A}$	0.378 (0.02410)$^{b, B}$	0.96
			669.7 (82.6)	0.220 (0.03650)$^{c, B}$	0.413 (0.04280)$^{a, A}$	0.90

it was significantly higher ($P < 0.025$) in fed than unfed fish. With cobia stomach enzyme extract, fed fish showed a significantly higher regression slope than unfed fish, regardless of fish weight, and with pyloric caeca extract, DH slope did not differ between fed or unfed fish. With stomach enzyme extracts of cage farmed Nile tilapia, the rate of DH increment was significantly higher in fed fish but with intestine extract, it was not affected by feeding status. In pond farmed Nile tilapia, no difference in DH increasing rate with stomach extract was observed between fed and unfed fish and with intestine enzyme extract, no clear trend of feeding status effect could be observed. Cage farmed Nile tilapia presented higher DH increasing rate (b) for stomach enzyme extract than pond farmed Nile tilapia, whereas the opposite was observed for intestine enzyme extract. On average, no clear trend of effect of fish weight, feeding status or farming system on DH increment rate could be determined for the three fish species.

The proximate composition of the set of 24 test ingredients of different sources and origins (Table 3) was within the ranges reported for standard raw materials (NRC, 2011; Rostagno, 2011; Tacon et al., 2009). For the ingredient DH assays, the criteria adopted to choose the volume of stomach and pyloric caeca/intestine enzyme extract were the minimum volume resulting in maximum DH and also volumes that could fit in the test tube conditions. Additionally, selection of the enzyme extract batches was based on the extract standardization results, i.e., fed fish of harvest weight. For rainbow trout (393.1 ± 35.8 g) DH values with stomach and pyloric caeca extracts on hemoglobin and casein were 8.22 and 14.03%, respectively. For cobia (550.5 ± 135.9 g), DH values were 7.87 and 10.93%, respectively. For cage farmed Nile tilapia (598.3 ± 110.1 g), DH values with stomach and intestine extracts were 3.63 and 15.35% and for pond farmed fish (669.7 ± 82.6 g), DH values were 4.51 and 10.82%, respectively.

In general, ingredient sample hydrolysis with stomach followed by pyloric caeca/intestine (two-stage DH) tended to be higher compared to results from hydrolysis with only stomach or pyloric caeca/intestine for rainbow trout and Nile tilapia (cage and pond farmed) (Figs. 3, 5). For rainbow trout, wheat gluten, soybean meal (USA), soy protein concentrate, cotton seed meal, blood meals, poultry by-product meals and feather meals presented the highest two-stage DH values (4.63–10.37%) and no significant difference ($P > 0.05$) was observed for DH among fish meals (average 6.90 ± 0.43%). With cage farmed

Table 3
Proximate composition (g 100 g^{-1}) of the 24 tested ingredients (as-fed basis). Ingredient information in brackets refers to ingredient type and/or origin.

Test ingredient	IFN[a]	Moisture	Crude protein[b]	Crude fiber	Crude fat	NFE[c]	Ash
Blood meal (Brazil)	5-00-380	5.8	90.6	0.0	0.3	1.0	2.2
Blood meal (USA)	5-00-380	7.2	89.3	0.0	0.2	0.6	2.7
Corn gluten meal (USA)	5-28-242	6.3	68.1	0.2	1.6	21.0	2.7
Corn grain (Brazil)	4-26-023	12.5	9.8	1.7	3.9	70.3	1.9
Cotton seed meal (Brazil)	5-01-621	8.0	41.1	14.8	3.1	27.2	5.8
Feather meal (Brazil)	5-03-795	6.5	82.9	0.0	7.1	0.3	3.2
Feather meal (European Union)	5-03-795	4.6	86.0	0.0	7.3	0.2	1.9
Fish meal (anchovy, Peru)	5-01-985	6.6	71.7	0.0	7.8	0.1	13.8
Fish meal (herring)	5-02-000	4.3	74.5	0.0	8.6	0.0	12.6
Fish meal (mackerel, Chile)	5-01-985	6.3	70.7	0.0	5.0	0.0	17.9
Fish meal (menhaden, USA)	5-02-009	5.7	66.3	0.0	7.7	0.8	19.5
Fish meal (by-product, Brazil)		6.8	61.1	0.1	5.2	4.0	22.8
Fish meal (salmon, Chile)	5-02-012	6.6	75.4	0.0	6.2	0.6	11.2
Poultry by-product meal (feed grade, Brazil)	5-03-798	12.3	61.0	0.2	12.5	0.8	13.1
Poultry by-product meal (feed grade, USA)	5-03-798	4.4	61.2	0.1	17.7	2.6	14.0
Poultry by-product meal (pet-food grade, USA)		3.7	67.4	0.3	13.2	1.5	13.8
Rapeseed meal (European Union)	5-06-145	19.3	28.5	9.8	3.5	33.0	5.9
Soybean meal (solvent extracted, Brazil)	5-20-637	11.3	47.4	6.0	0.5	28.5	6.3
Soybean meal (full fat, USA)	5-04-597	5.9	40.2	4.3	18.4	25.5	5.6
Soybean meal (solvent extracted, USA)	5-04-612	7.0	47.7	2.7	1.0	34.1	7.4
Soy protein concentrate (Brazil)	5-32-183	9.0	59.9	4.3	0.5	20.2	6.1
Wheat bran (Brazil)	4-05-190	9.0	16.7	7.2	3.3	58.9	4.8
Wheat flour (Brazil)	4-05-199	9.2	16.7	0.2	2.1	71.2	1.6
Wheat gluten (USA)	5-05-220	6.4	79.4	0.0	1.3	12.2	0.7

[a] International feed number.
[b] N × 6.25.
[c] Nitrogen-free extract = 100 − (crude protein + crude fiber + crude fat + ash + moisture).

Nile tilapia, soybean meal (USA), soy protein concentrate, cotton seed meal and blood meal (Brazil) presented the highest two-stage DH values (5.92–8.86%). For pond farmed Nile tilapia, the highest two-stage DH was determined with soy protein concentrate, cotton seed meal, blood meal (Brazil), poultry by-product meal (feed grade) and poultry by-product meal (pet food grade) (4.67–6.67%). For cobia, such trend could be observed in half of the tested ingredients, especially with fish meals (Fig. 4). High ingredient DH with stomach extract did not result in increased two-stage DH for soybean meal full-fat (rainbow trout), poultry by-product meals (cage farmed Nile tilapia), poultry by-product meal (pet food grade) (pond farmed Nile tilapia) and soybean meals, wheat gluten, blood meal (Brazil), poultry by-product meal (feed grade), poultry by-product meal (pet food grade), and fish meal (menhaden) (cobia). Additionally, two-stage DH did not differ from hydrolysis with pyloric caeca extract for corn gluten meal, rapeseed meal, poultry by-product meal (feed grade) (rainbow trout), soybean meals (cobia), soybean meal full-fat, corn gluten meal, wheat flour (cage farmed Nile tilapia), soybean meal full-fat, soybean meal (Brazil), feather meals, poultry by-product meal (feed grade) and fish meals (pond farmed Nile tilapia). In general, ingredient two-stage DH from cage and pond farmed Nile tilapia followed a similar hydrolysis pattern but the output values were comparatively higher in cage farmed fish (Fig. 5A, B).

Ingredient particle size distribution indicated that wheat flour and wheat gluten were the ingredients with the finest particle size (<250 μm), followed by blood meals, whereas soy protein concentrate and poultry by-product meal (pet food grade) were coarser with more than 20% of particles >1000 μm. Fish meals particle size was mostly <500 μm. In general, ingredients particle size was <1000 μm but no clear relation between ingredient particle size distribution and DH could be detected in this study.

4. Discussion

The use of species-specific crude enzyme extracts for *in vitro* digestion methods may be important since catalytic output may differ significantly among species (Dimes et al., 1994; Hamdan et al., 2009; Lemos et al., 2004; Márquez et al., 2013) and the donor organism (weight, age, feeding status, farming system) and habitat (water salinity, natural productivity) (Dimes and Haard, 1994; Lemos and Nunes, 2008). The consistency of *in vitro* techniques employing species-specific digestive enzymes depends on the standardization of the hydrolytic capacity of the recovered extract. Most commonly, extracts have been standardized to the activities of trypsin and/or chymotrypsin (Chong et al., 2002; Dimes and Haard, 1994; Grabner, 1985; Tibbetts et al., 2011a), or total alkaline proteinase (El-Sayed et al., 2000; Hamdan et al., 2009; Lemos et al., 2009; Márquez et al., 2013). The present study introduces the degree of protein hydrolysis (DH) as a standardization method using analytical grade proteinaceous substrates under the same conditions (pH-stat) as the *in vitro* digestion assays. Advantages of this approach would be the non-inclusion of buffers or other extra chemicals, stable pH during hydrolysis, and precision (average c.v. 4%). The adjusted function of DH output at fixed substrate protein quantity *versus* fish enzyme extract volume followed a similar trend (log function), either for stomach or pyloric caeca/intestine enzymes, regardless of the species or condition of the donor individuals (weight, feeding status, or farming system) (Fig. 2; Table 2). From such models, the volume of enzyme extract from different batches or species that may result in the same hydrolytic capacity (DH) may be obtained and then used for comparable determination of *in vitro* digestion of a given feedstuff. These methods of standardization according to digestive capacity may be more adequate than considering e.g. a fixed volume of enzyme extract to be used in the assays (Divakaran et al., 2004; Lan and Pan, 1993).

For most of the ingredients tested incubation with stomach enzyme extracts prior to hydrolysis with pyloric caeca/intestine extracts (two-stage) resulted in higher *in vitro* DH values when compared to single stage hydrolysis (only stomach or pyloric caeca/intestine extract) (Figs. 3, 4 and 5). *In vivo*, the role of the stomach in protein digestion is to initiate breaking down the food, partially hydrolyzing proteins into peptides and/or mechanical disruption via muscular contraction in preparation for further hydrolysis in the intestine (Clements and Raubenheimer, 2006; Hamdan et al., 2009). Gastric digestion could also increase the speed of intestinal hydrolysis, leading to a significant shift from soluble polypeptides to oligo- and dipeptides (Grabner and Hofer, 1989) and potentially increasing availability of soluble protein

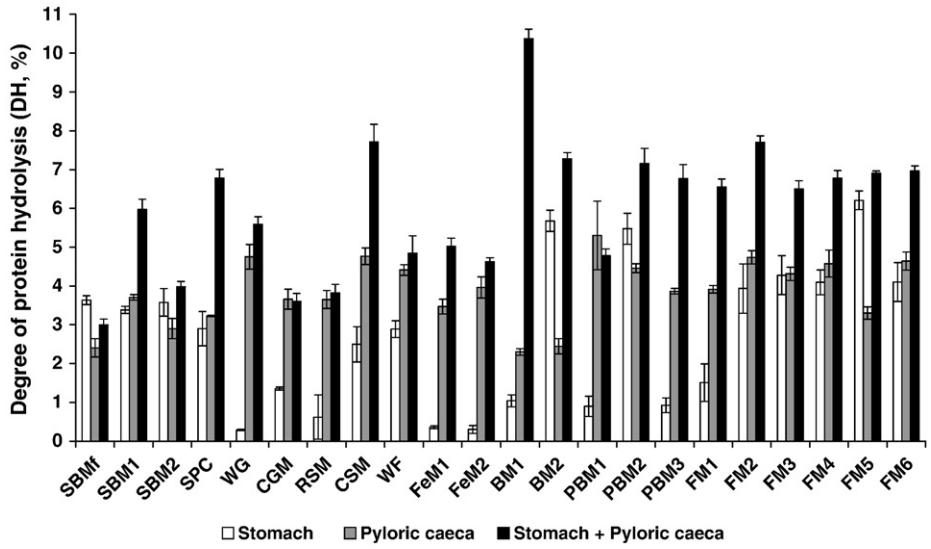

Fig. 3. *In vitro* pH-stat degree of protein hydrolysis (DH) of feed ingredients using digestive enzyme extracts from fed 400.5 ± 37.9 g rainbow trout, *Oncorhynchus mykiss*. Ingredients (80 mg crude protein per assay) were hydrolyzed for 60 min with (i) stomach, or (ii) pyloric caeca or 120 min with (iii) stomach (60 min) plus pyloric caeca (60 min) extracts at 25 ± 0.2 °C. SBMf: soybean meal full-fat; SBM1: soybean meal, USA; SBM2: soybean meal, Brazil; SPC: soy protein concentrate, Brazil; WG: wheat gluten, USA; CGM: corn gluten meal, USA; RSM: rapeseed meal, European Union; CSM: cotton seed meal, Brazil; WF: wheat flour, Brazil; FeM1: feather meal, Brazil; FeM2: feather meal, European Union; BM1: blood meal, Brazil; BM2: blood meal, USA; PBM1: poultry by-product meal (feed grade), Brazil; PBM2: poultry by-product meal (pet food grade), USA; PBM3: poultry by-product meal (feed grade), USA; FM1: fish meal (by-product), Brazil; FM2: fish meal (anchovy), Peru; FM3: fish meal (herring); FM4: fish meal (mackerel), Chile; FM5: fish meal (menhaden), USA; and FM6: fish meal (salmon), Chile. Results shown as mean (vertical bars) and s.d. (error bars), n = 4.

by the inactivation of protease inhibitors by low pH and/or pepsin activity (Hamdan et al., 2009; Pedersen and Eggum, 1983). This may be the case of some soybean meals tested that showed higher two-stage DH values (Figs. 3, 4 and 5). However, the meaning of measuring quantitatively the *in vitro* DH value of practical ingredients with fish stomach enzymes seems not well understood. For some of the ingredients, e.g., rapeseed meal and feather meal (European Union) for cobia and rainbow trout and soy protein concentrate, wheat bran, feather meals, and fish meal (mackerel) for pond farmed Nile tilapia, the coefficient of variation in DH with stomach extracts was very high (>50%), which may be due to the fact that they were comparatively coarser and less soluble than the substrate used for standardization (hemoglobin), a purified, highly soluble and digestible product (Díaz-López et al., 1998). Thus, present fish stomach enzymes seem to be sensitive to variation in the purity of protein substrate but further studies are necessary to understand the significance of measuring the DH value with stomach enzyme extract on the protein hydrolysis of a feedstuff. The DH of tested ingredients with digestive enzymes from cage and pond farmed Nile tilapia followed the same trend, describing a significant correlation and a high determination coefficient regression, as verified in shrimp (*Litopenaeus vannamei*) under clear water *versus* pond conditions (Lemos and Nunes, 2008).

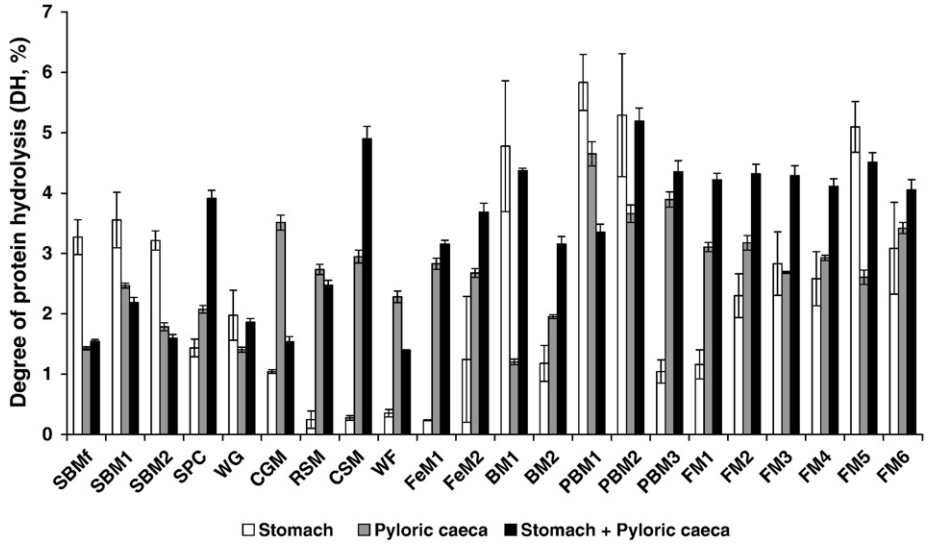

Fig. 4. *In vitro* pH-stat degree of protein hydrolysis (DH) of feed ingredients using digestive enzyme extracts from fed 542.80 ± 182.1 g cobia, *Rachycentron canadum*. Ingredients (80 mg crude protein per assay) were hydrolyzed for 60 min with (i) stomach, or (ii) pyloric caeca or 120 min with (iii) stomach (60 min) and pyloric caeca (60 min) extracts at 25 ± 0.2 °C. SBMf: soybean meal full-fat; SBM1: soybean meal, USA; SBM2: soybean meal, Brazil; SPC: soy protein concentrate, Brazil; WG: wheat gluten, USA; CGM: corn gluten meal, USA; RSM: rapeseed meal, European Union; CSM: cotton seed meal, Brazil; WF: wheat flour, Brazil; FeM1: feather meal, Brazil; FeM2: feather meal, European Union; BM1: blood meal, Brazil; BM2: blood meal, USA; PBM1: poultry by-product meal (feed grade), Brazil; PBM2: poultry by-product meal (pet food grade), USA; PBM3: poultry by-product meal (feed grade), USA; FM1: fish meal (by-product), Brazil; FM2: fish meal (anchovy), Peru; FM3: fish meal (herring); FM4: fish meal (mackerel), Chile; FM5: fish meal (menhaden), USA; and FM6: fish meal (salmon), Chile. Results shown as mean (vertical bars) and s.d. (error bars), n = 4.

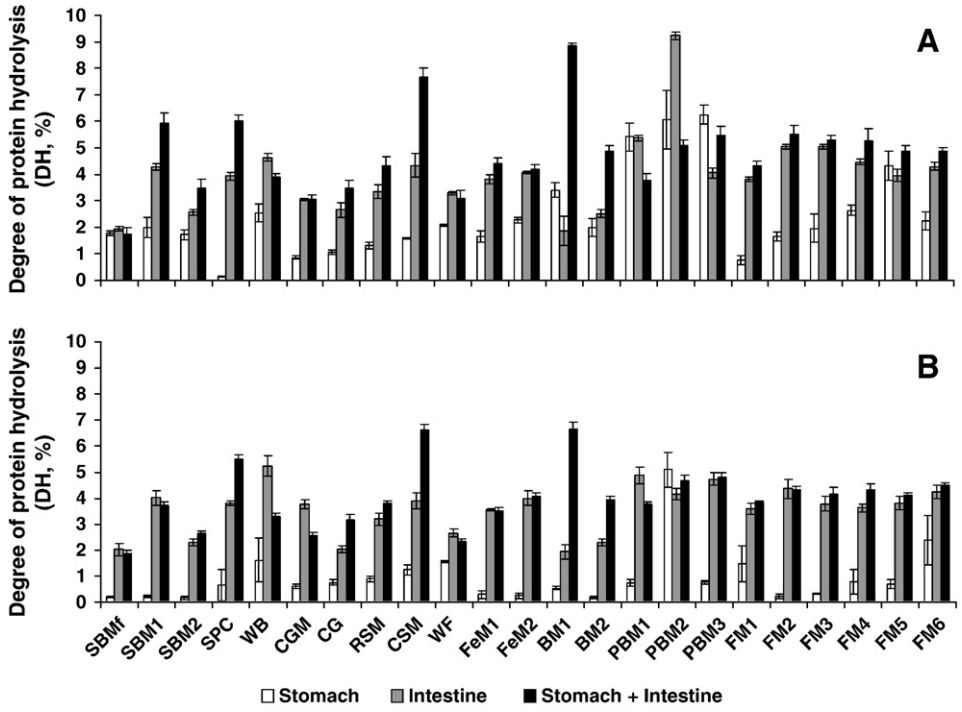

Fig. 5. *In vitro* pH-stat degree of protein hydrolysis (DH) of feed ingredients using digestive enzyme extracts from fed (A) cage farmed 573.0 ± 89.5 g and (B) pond farmed 696.7 ± 85.1 g Nile tilapia, *Oreochromis niloticus*. Ingredients (80 mg crude protein per assay) were hydrolyzed for 60 min with (i) stomach, or (ii) intestine or 120 min with (iii) stomach (60 min) plus intestine (60 min) extracts at 25 ± 0.2 °C. SBMf: soybean meal full-fat, USA; SBM1: soybean meal, USA; SBM2: soybean meal, Brazil; SPC: soy protein concentrate, Brazil; CG: corn grain, Brazil; CGM: corn gluten meal, USA; RSM: rapeseed meal, European Union; CSM: cotton seed meal, Brazil; WB: wheat bran, Brazil; WF: wheat flour, Brazil; FeM1: feather meal, Brazil; FeM2: feather meal, European Union; BM1: blood meal, Brazil; BM2: blood meal, USA; PBM1: poultry by-product meal (feed grade), Brazil; PBM2: poultry by-product meal (pet food grade), USA; PBM3: poultry by-product meal (feed grade), USA; FM1: fish meal (by-product), Brazil; FM2: fish meal (anchovy), Peru; FM3: fish meal (herring); FM4: fish meal (mackerel), Chile; FM5: fish meal (menhaden), USA; and FM6: fish meal (salmon), Chile. Results shown as mean (vertical bars) and s.d. (error bars), n = 4.

The *in vitro* pH-stat protein digestion assays of the present study were standardized adopting conditions that may not exactly simulate digestion in some fish species. Ingredient incubation time, temperature, and enzyme:substrate ratio were chosen considering previous studies (Lemos et al., 2009; Morales and Moyano, 2010), laboratory convenience and output rates. Since assay temperature and salinity may not be natural for rainbow trout and cobia, respectively, ingredient DHs should not be compared between these species. On the other hand, the quantitative meaning of DH enables the differentiation of ingredients according to their potential to be digested and also the comparison between feedstuff digestion by different species. Further aspects regarding assay conditions should be considered for the consistency of the *in vitro* determination. Sample preparation step to stabilize pH ensures that variation in pH during hydrolysis is due only to the enzyme activity and not to any possible sample instability (Dimes and Haard, 1994; Lemos et al., 2009; Pedersen and Eggum, 1983). Accordingly, ingredient particle size plays a significant role in aqua feed manufacturing (Tacon, 1988) and may affect feed digestion. Unfortunately, the influence of the ingredient particle size distribution on DH could not be detected in the present study, as individual particle size assays were not performed.

The pH-stat application to determine *in vitro* digestion with species-specific enzymes has shown to be a fast and precise method with reported use for different aquatic species such as fish (Dimes et al., 1994; Tibbetts et al., 2011a), shrimp (Ezquerra et al., 1998; Lemos et al., 2009), and mollusks (Aguilar et al., 2012). Significant relationship between *in vivo* apparent protein digestibility and *in vitro* DH has been reported for some feedstuffs using enzyme extracts from digestive organs of the target species (Table 4), indicating DH as capable of predicting digestibility in the live animal. Nevertheless, the correlation between *in vivo* apparent protein digestibility and *in vitro* DH digestion may be essentially dependent on the consistency and experimental details of feeding trials involved in the determination of apparent digestibility (Fuller, 1991; Tacon, 1996). This includes mainly (a) the formulation of experimental diets, types and inclusion levels of test ingredients, (b) the feeding regime, including feeding method and strategies for feces collection, to minimize nutrient leaching, and (c) the applied research and development focused on specific practical raw materials, manufacturing and farming conditions. Apart from correlation and method validation, the pH-stat *in vitro* DH technique may be important to qualify or distinguish raw materials, indicating how much it could be potentially hydrolyzed in the fish digestive tract and, to a certain extent, the availability for absorption. The complex nature of food digestion in the live fish may not be easily replicated and it may also not be strictly required for consistent screening of feed protein quality, as demonstrated for farmed land and aquatic species (Boisen and Eggum, 1991; Boisen and Fernández, 1995; Dimes et al., 1994; Pedersen and Eggum, 1983; Shipton and Britz, 2002; Tibbetts et al., 2011b). The DH has emerged with potential as an accessory method in quality control of raw materials in the aqua feed manufacturing industry (De Muylder et al., 2008; Lemos and Tacon, 2011). For feed manufacturers, the DH could assist ranking protein from different ingredients or a certain ingredient from different suppliers, as an additional criterion for the quality control of aqua feeds. It could be helpful also in the preliminary assessment of the effectiveness of feed additives (e.g. exogenous proteases) on the nutritional value of raw materials.

The regular use of DH by the industry as a standard method may still be restrained by the source of the enzyme extract, which is obtained from individuals of the target species. More practical sources and analytical routines should also be developed, e.g., easy-to-handle freeze-dried crude extracts and products to be supplied for prompt use in the feed industry. Additional studies should include the determination of a possible relationship between *in vitro* pH-stat DH and individual amino acid availability. Protein digestibility may not be the only factor that affects feed quality, but an objective assessment of feedstuffs is a

Table 4
Prediction of *in vivo* apparent protein digestibility (APD) of feed ingredients by *in vitro* pH-stat degree of protein hydrolysis (DH) using digestive enzyme extract from fish (rainbow trout, *Oncorhynchus mykiss*, haddock, *Melanogrammus aeglefinus*, Atlantic cod, *Gadus morhua*) and whiteleg shrimp (*Litopenaeus vannamei*). R^2 = coefficient of determination between *in vivo* APD and *in vitro* DH. n = number of feed ingredients tested.

Species	R^2	n	Ingredient[a]	Reference
Rainbow trout	0.87	5	FM	Dimes et al. (1994)
	0.94	8	FM, MBM, FeM, PBM, BM	El-Mowafi et al. (2000)
	0.81	8	FM	Lemos and Yasumaru (2010)
Haddock	0.80	7	FM, CrM, ShM, SBM, CM, CGM, CAS	Tibbetts et al. (2002)
Atlantic cod	0.61–0.99	21	FM, PBM, FeM, SBM, SPC, SPI, KM, CrM, SM, CM, CPC, FSM, CGM, WG, PPC, WLM	Tibbetts et al. (2011b)
Whiteleg shrimp	0.81	7	FM, SBM, LM	Ezquerra et al. (1998)
	0.69	6	KH, FH, SM	Córdoba-Murueta and García Carreño (2002)
	0.86	26	BM, CAS, CGM, CrM, DDGS, FeM, FM, GEL, KM, KF, PBM, SBM, SPI, SM, WG	Lemos et al. (2009)

[a] BM: blood meal, CAS: casein, CGM: corn gluten meal, CM: canola meal, CPC: canola protein concentrate, CrM: crab meal, DDGS: distiller's dried grains with solubles, FeM: feather meal, FH: fish hydrolysate, FM: fish meal, FSM: flaxseed meal, GEL: gelatin, KF: krill flour, KH: krill hydrolysate, KM: krill meal, LM: langostilla meal, MBM: meat and bone meal, PBM: poultry by-product meal, PPC: pea protein concentrate, SBM: soybean meal, ShM: shrimp meal, SM: squid meal, SPC: soy protein concentrate, SPI: soy protein isolate, WG: wheat gluten, and WLM: white lupin meal.

valuable input for aquaculture nutritionists in using different raw materials, achieving adequate, cost-effective, and sustainable species-specific feed solutions (Glencross et al., 2007).

5. Conclusions

The *in vitro* pH-stat species-specific method to determine the degree of protein hydrolysis (DH) has shown to be a useful tool to distinguish the raw materials available for feed production. The hydrolytic capacity of fish enzyme extracts could be standardized by the DH in the same conditions (pH-stat) as the *in vitro* digestion assays. The significance of quantitatively measuring the DH with stomach enzyme extract is still not well understood but, overall, the pre-hydrolysis of feedstuffs with stomach enzymes contributed to elevate pyloric caeca/intestine DH. Ingredient DH presented distinct patterns and may be compared among different species when enzyme extracts are originated from individuals reared under practical conditions. Assay conditions e.g. temperature, medium salinity, incubation time, and enzyme:substrate ratios according to the fish's physiological status should be taken into account for the consistency of the *in vitro* determination. Routine use of the method may yet be dependent on the prompt availability of more practical sources of enzyme extracts.

Acknowledgments

The authors are indebted to Dr. Vicente Gomes, Ms. Maria José de Arruda Campos Rocha Passos and Mr. Felipe Braga for their diligent effort in obtaining the digestive organs and extracts from cobia and for their laboratory assistance. Thanks are also given to Dr. Yara Tabata, Piscicultura Ipaussu and Aqualider for providing the fish; to Drs. Renata Guimarães and Vicente Gomes for their valuable comments on an earlier version of the manuscript; and to the two anonymous reviewers for their critical review and suggestions. The financial support from the Project MCT/CNPq/CT-Agronegócio/MPA 036/2009 and the CAPES/Ministry of Education for the scholarship of M. Sc. F. Yasumaru is acknowledged. D. Lemos appreciates the support from the MPA and CNPq (304895/2012-1; 400713/2012-8; 406472/2012-2), the FAPESP (2010/10756-7) and the USP grants.

References

Adler-Nissen, J., 1986. Enzymic Hydrolysis of Food Proteins. Elsevier, New York, NY (427 pp.).
Aguilar, F.D.M., Overa-Novoa, M., Rosas, C., 2012. In vitro digestibility: standardizing methods using gastric juice of Octopus maya in the way of improve foods for octopus culture. Book of Abstracts, Cephalopod International Advisory Council Symposium 2012, 27 Oct-02 Nov, 2012, 129. Florianópolis, Santa Catarina, Brazil.
Anson, M.L., 1938. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. J. Gen. Physiol. 22, 79–89.
Boisen, S., Eggum, B.O., 1991. Critical evaluation of *in vitro* methods for estimating digestibility in simple-stomached animals. Nutr. Res. Rev. 4, 141–162.
Boisen, S., Fernández, J.A., 1995. Prediction of the apparent ileal digestibility of protein and amino acids in feedstuffs and feed mixtures for pigs by *in vitro* analyses. Anim. Feed Sci. Technol. 69, 277–286.
Buddington, R.K., Diamond, J.M., 1986. Aristotle revisited: the function of pyloric caeca in fish. Proc. Natl. Acad. Sci. 83, 8012–8014.
Chong, A.S.C., Hashim, R., Ali, A.B., 2002. Assessment of dry matter and protein digestibilities of selected raw ingredients by discus fish (*Symphysodon aequifasciata*) using *in vivo* and *in vitro* methods. Aquac. Nutr. 8, 229–238.
Clements, K.D., Raubenheimer, D., 2006. Feeding and nutrition, In: Evans, D.H., Claiborne, J.B. (Eds.), The Physiology of Fishes, 3rd ed. CRC Press, pp. 47–82.
Córdoba-Murueta, J.H., García Carreño, F.L., 2002. Nutritive value of squid and hydrolyzed protein supplement in shrimp feed. Aquaculture 210, 371–384.
De Muylder, E., Lemos, D., van den Velden, G., 2008. Protein hydrolysis of PAP shows the nutritive value for shrimp feeds. Aquacult. Asia Pac. 4, 18–19 (Sep/Oct).
Díaz-López, M., Moyano-López, F.J., Alarcón-López, F.J., García-Carreño, F.L., Navarrete del Toro, M.A., 1998. Characterization of fish acid proteases by substrate–gel electrophoresis. Comp. Biochem. Physiol. 121B, 369–377.
Diermayr, P., Dehne, L., 1990. Controlled enzymatic hydrolysis of proteins at low pH values: 1. Experiments with BSA. Z. Lebensm. Unters. Forsch. 190, 516–520.
Dimes, L.E., Haard, N.F., 1994. Estimation of protein digestibility — I. Development of an *in vitro* method for estimating protein digestibility in salmonids (*Salmo gairdneri*). Comp. Biochem. Physiol. 108A, 349–362.
Dimes, L.E., Haard, N.F., Dong, F.M., Rasco, B.A., Forster, I.P., Fairgrieve, W.T., Arndt, R., Hardy, R.W., Barrows, F.T., Higgs, D.A., 1994. Estimation of protein digestibility — II. *In vitro* assay of protein in salmonid feeds. Comp. Biochem. Physiol. 108A, 363–370.
Divakaran, S., Forster, I.P., Velasco, M., 2004. Limitations on the use of shrimp *Litopenaeus vannamei* midgut gland extract for the measurement of *in vitro* protein digestibility. Aquaculture 239, 323–329.
El-Mowafi, A.F., Dorrell, H., Bureau, D.P., 2000. Potential of a pH-stat method to estimate apparent digestibility of protein in salmonids. IX International Symposium on Nutrition and Feeding of Fish, 21–25 May 2000, Miyazaki, Japan.
El-Sayed, A.F.M., Martínez, I.N., Moyano, F.J., 2000. Assessment of the effect of plant inhibitors on digestive proteases of Nile tilapia using *in vitro* assays. Aquac. Int. 8, 403–415.
Ezquerra, J.M., García-Carreño, F.L., Carrillo, O., 1998. *In vitro* digestibility of protein sources for white shrimp *Penaeus vannamei*. Aquaculture 163, 123–136.
Fuller, M.F., 1991. *In Vitro* Digestion for Pigs and Poultry. CAB International, Wallington, Oxon (209 pp.).
Glencross, B.D., Booth, M., Allan, G.L., 2007. A feed is only as good as its ingredients — a review of ingredient evaluation strategies for aquaculture feeds. Aquac. Nutr. 13, 17–34.
Gotelli, N.J., Ellison, A.M., 2004. A Primer of Ecological Statistics. Sinauer Associates Publishers, Sunderland, Mass.(510 pp.).
Grabner, M., 1985. An *in vitro* method for measuring protein digestibility of fish feed components. Aquaculture 48, 97–110.
Grabner, M., Hofer, R., 1989. Stomach digestion and its effect upon protein hydrolysis in the intestine of rainbow trout (*Salmo gairdneri* Richardson). Comp. Biochem. Physiol. 92A, 81–83.
Hamdan, M., Moyano, F.J., Schuhardt, D., 2009. Optimization of a gastrointestinal model applicable to the evaluation of bioaccessibility in fish feeds. J. Food Sci. Agric. 89, 1195–1201.
Lan, C.C., Pan, B.S., 1993. *In vitro* digestibility simulating the proteolysis of feed protein in the midgut gland of grass shrimp (*Penaeus monodon*). Aquaculture 109, 59–70.
Lazo, J.P., Romaire, R.P., Reigh, R.C., 1998. Evaluation of three *in vitro* enzyme assays for estimating protein digestibility in the Pacific white shrimp *Penaeus vannamei*. J. World Aquacult. Soc. 29, 441–450.
Lemos, D., Nunes, A.J.P., 2008. Prediction of culture performance of juvenile *Litopenaeus vannamei* by *in vitro* (pH-stat) degree of protein hydrolysis with species-specific enzymes. Aquac. Nutr. 14, 181–191.
Lemos, D., Tacon, A.G.J., 2011. A rapid low-cost laboratory method for measuring the *in vitro* protein digestibility of feed ingredients and feeds for shrimp. Aquaculture Asia Pacific Magazine 18–21 (Mar/Apr).

Lemos, D., Yasumaru, F., 2010. *In vitro* pH-stat determination of protein digestibility in fish species: recovery and standardization of enzyme extracts for acid and alkaline hydrolysis assays in juvenile rainbow trout and Nile tilapia. XIV International Symposium on Fish Nutrition and Feeding, May 31–June 4, 2010, Qindao, China.

Lemos, D., Navarrette del Toro, A., Córdova-Murueta, J.H., Garcia-Carreño, F., 2004. Testing feeds and feed ingredients for juvenile pink shrimp *Farfantepenaeus paulensis*: *in vitro* determination of protein digestibility and proteinase inhibition. Aquaculture 239, 307–321.

Lemos, D., Lawrence, A.L., Siccardi III, A.J., 2009. Prediction of apparent protein digestibility of ingredients and diets by *in vitro* pH-stat degree of protein hydrolysis with species-specific enzymes for juvenile Pacific white shrimp *Litopenaeus vannamei*. Aquaculture 295, 89–98.

Márquez, L., Overland, M., Martínez-Llorens, S., Morken, T., Moyano, F.J., 2013. Use of a gastrointestinal model to assess potential amino acid bioavailability in diets for rainbow trout (*Oncorhynchus mykiss*). Aquaculture 384–387, 46–55.

Morales, G.A., Moyano, F.J., 2010. Application of an *in vitro* gastrointestinal model to evaluate nitrogen and phosphorus bioaccessibility and bioavailability in fish feed ingredients. Aquaculture 306, 244–251.

Moughan, P.J., 1999. *In vitro* techniques for the assessment of the nutritive value of feed grains for pigs: a review. Aust. J. Agric. Res. 50, 871–879.

Moyano, F.J., Savoie, L., 2001. Comparison of *in vitro* systems of protein digestion using either mammal or fish proteolytic enzymes. Comp. Biochem. Physiol. 128A, 359–368.

NRC — National Research Council, 2011. Nutrient Requirements of Fish and Shrimp. The National Academies Press, Washington, DC (376 pp.).

Pedersen, B., Eggum, B.O., 1983. Prediction of protein digestibility by an *in vitro* enzymatic pH-stat procedure. J. Anim. Physiol. Anim. Nutr. 49, 265–277.

Rostagno, H.S. (Ed.), 2011. Tabelas Brasileiras para Aves e Suínos: Composição de Alimentos e Exigências Nutricionais, 3rd ed. UFV, DZO, Viçosa, MG (252 pp.).

Shipton, T.A., Britz, P.J., 2002. Evaluation of an *in vitro* digestibility technique for the prediction of protein digestibility in the South African abalone, *Haliotis midae* L. Aquac. Nutr. 8, 15–21.

Silva, D.J., Queiroz, A.C., 2009. Análises de Alimentos (Métodos Químicos e Biológicos), 3ª ed. Universidade Federal de Viçosa, Viçosa, MG (235 pp.).

Tacon, A.G.J., 1988. The Nutrition and Feeding of Farmed Fish and Shrimp — A Training Manual. 3. Feeding Methods (Available at: http://www.fao.org/docrep/field/003/AB467E/AB467E00.htm).

Tacon, A.G.J., 1996. Nutritional studies in crustaceans and the problems of applying research findings to practical farming systems. Aquac. Nutr. 1, 165–174.

Tacon, A.G.J., Metian, M., Hasan, M.R., 2009. Feed ingredients and fertilizers for farmed aquatic animals: sources and composition. FAO Fisheries and Aquaculture Technical Paper 540. FAO (Rome, 209 pp.).

Tacon, A.G.J., Hasan, M.R., Metian, M., 2011. Demand and supply of feed ingredients for farmed fish and crustaceans: trends and prospects. Rome, Italy, FAO Fisheries and Aquaculture Technical Paper 564 (87 pp.).

Tibbetts, S.M., Lall, S.P., Milley, J.E., 2002. *In vivo* and *in vitro* digestibility of common feed ingredients by haddock, *Melanogrammus aeglefinus*, and Atlantic halibut, *Hippoglossus hippoglossus*. Aquaculture Canada 2002, 19th Annual Meeting of the Aquaculture Association Canada, Sep 17–20, 2002, Charlottetown, Prince Edward Island.

Tibbetts, S.M., Milley, J.E., Ross, N.W., Verreth, J.A.J., Lall, S.P., 2011a. *In vitro* pH-stat protein hydrolysis of feed ingredients for Atlantic cod, *Gadus morhua*. 1. Development of the method. Aquaculture 319, 398–406.

Tibbetts, S.M., Verreth, J.A.J., Lall, S.P., 2011b. *In vitro* pH-stat protein hydrolysis of feed ingredients for Atlantic cod, *Gadus morhua*. 2. *In vitro* protein digestibility of common and alternative feed ingredients. Aquaculture 319, 407–416.

Tonheim, S.K., Nordgreen, A., Hogoy, I., Hamre, K., Ronnestad, I., 2007. *In vitro* digestibility of water-soluble and water-insoluble protein fractions of some common fish larval feeds and feed ingredients. Aquaculture 262, 426–435.

Zar, J.H., 1999. Biostatistical Analysis, 4th ed. Prentice Hall, Upper Saddle River, N.J. (663 pp.).