Change in Protein Digestion Capacity During Juvenile Fish Ontogeny: Approach on Spotted Rose Snapper (*Lutjanus guttatus*)

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Abstract

Aquaculture is facing a challenge in order to search new alternative nutritional sources to generate highly digestible and profitable diets for aquaculture species. In addition, the understanding of changes in digestive capacity in fish species with aquaculture potential is of relevance importance, as the capacity of assimilation of different nutrients may change during the juvenile development of the species. Numerous research has been focused on understanding the changes and adaptations of the development and capacities of the digestive system during the early ontogeny of fish, minimizing the importance of possible changes during juvenile ontogeny, as a trigger for the grow-out efficiency increase in fish culture.

Thus, few studies address the digestive changes during juvenile fish ontogeny and their implications in the ability to assimilate different nutritional sources, considering that there should be no changes during this stage, which in general represents the period of grow-out until commercial size, prior to their reproductive stages.

The present work deals with the importance to characterize changes the digestive capacity during grow-out on the spotted rose snapper (*Lutjanus guttatus*). Comparative studies of juvenile sizes of the species (20 to 400 grams) have shown existence of changes in the optimum alkaline protease activity, as well as a diversification and increase in the number of digestive enzymes of the alkaline phase in relation to juvenile ontogeny, resulting in changes of *in vitro* hydrolysis degree and total release of amino acids from different protein sources.

Keywords: Proteases, enzyme characterization, electrophoresis, *in vitro* digestibility, Lutjanidae.

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Introduction

Based on their eating habits and digestive morphology, fish are classified as detritivores, herbivores, omnivores or carnivores. Independent of the classification of dietary habits, fish are able to modify their digestive behavior and metabolism in response to changes in dietary sources as well as food availability (Rust, 2002; Pérez-Jiménez *et al*. 2009). Thus, growth and efficiency caused by the ingestion of a food in fish will depend on its physiological and biochemical ability to digest and transform nutrients, however many biotic and abiotic factors can influence the physiological state of the fish and therefore, the processes related to the digestion, absorption and transformation of these nutrients (Furnè *et al*. 2008).

Due to the rapid growth of the aquaculture sector, as well as the emergence of new species with aquaculture potential, there are currently a large number of researches related to development of formulated diets with the objective to search new ingredient sources of low cost and high digestibility. During ingredients searching, it is important to understand the digestive capacity of the species of interest, where the understanding of the number and type of digestive enzymes, their enzymatic activity, as well as the affinity that present to different nutritional sources, will be important for the design of new formulations that tend to generate a sustainable aquaculture industry.

From digestive enzymes, proteases play a key role in digestion, which translates into high growth and survival. The proteases found within the digestive organs of fish are responsible for catalyzing the hydrolysis of peptide bonds (Klomklao, 2008), which includes enzymes such as pepsin, gastricins, trypsins, chymotrypsins, collagenase, elastase, carboxypeptidases and carboxylesterases (Haard, 1994; Simpson, 2000), where trypsin, chymotrypsin and pepsin are considered as the most important digestive enzymes due to their abundance and high proteolytic activity according to studies in different fish species (Castillo-Yáñez *et al*. 2004, 2005, 2006; Klomklao *et al*. 2004, 2007).

In fish, a great effort has been made in understanding the changes in physiological digestive capacity during the early ontogeny of different fish species, which has promoted the development of zootechnics in larval and juvenile production of fish (Kolkovski, 2001; Zambonino-Infante and Cahu, 2001; Lazo *et al*. 2007; Rønnestad *et al*. 2007; Álvarez-

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González *et al*. 2008; Galaviz *et al*. 2012; Salze *et al*. 2012; Moguel-Hernández *et al*. 2013).

Digestibility of nutrient or diet depends on its chemical composition, type of ingredients and digestive capacity of the species to breakdown macronutrients to micronutrients to be absorbed (Lemos & Tacon 2011). The major criteria to determine the nutritional value of protein sources seems to be apparent digestibility coefficient (ADC) (Dimes *et al.* 1994) in which, total assimilation (digestion and absorption) of specific nutrients are obtain by feces collection and analyses. By the other side, pH Stat system is a practical tool to conduct *in vitro* measurement using the degree of hydrolysis (DH%) as criteria, providing multiple advantages such as: specific response by using standardized species enzymes, stable conditions, rapid, precise, test different ingredients in small amounts and appropriate for different ingredients sources, include marine-based, animal and plant ingredients (Lemos *et al*. 2009; Yasumaru & Lemos 2014). Actually, great interest and efforts exist for standardization of pH Stat method in fish species (Dimes *et al*. 1994; El-Mowafi *et al*. 2000; Tibbetts *et al*. 2011a, b; Yasumaru & Lemos 2014) and crustaceans (Ezquerra *et al*. 1997; Lemos *et al*. 2000; Lemos *et al*. 2009; Perera *et al*. 2010), because main limitation in pH-Stat assays seems to be the complete knowing of enzymes origin and activities, given that, variations in species, fish size/age and phenotype could generate poor reproducibility over *in vitro* digestion assays (Tibbetts *et al*. 2011a).

Few studies in aquatic organisms aboard enzyme changes or diversification during juvenile or adult ontogeny in a same species. Reports in species such as roach (*Rutilus rutilus* L.), cuban gambusia (*Gambusia punctata*), Japanese eel (*Anguilla japonica*) and tilapia (*Oreochromis niloticus* L.) showed that proteolytic activities and zymogens could differ during juveniles/adults stages in the same species (Chiu & Pan 2002; Kuz'mina 1996; Falcón-Hidalgo *et al*. 2011; Unajak *et al*. 2012).

Some consistent examples about advantages related to the presence of some digestive isoenzymes in aquatic organisms exist, where oyster (*Crassostrea gigas*) presents a genetic polymorphism in two alpha-amylase genes (AMYA and AMYB), that are related to growth (Prudence *et al*. 2006). Spiny lobster (*Panulirus argus*) presents genetic variation in digestive trypsin pattern (three phenotypes; A, B and C), that generate *in vitro* differences in digestion efficiency over different protein sources (Perera *et al*. 2010, 2015).

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In this sense, Atlantic salmon (*Salmo salar*) is the most studied fish, where fish possessing a certain trypsin phenotype (TRP-2*92), shows better growth rate and/or feed conversion efficiency, related with protein digestion capacity (Bassompierre *et al*. 1998; Rungruangsak-Torrissen *et al*. 1998; Torrissen *et al*. 1987; Torrissen 1991).

Hence, understanding of digestive physiological aspects that directly affects feed efficiency and growth in the target species is required. The species studied is the spotted rose snapper (*Lutjanus guttatus*), which is part of the Lutjanidae family, consisting of predatory fish with variable feeding habits, where all are carnivores, feeding mainly on fish and benthic crustaceans (Allen 1987; Vázquez *et al*. 2008). In the species, great efforts have already been made in the search for alternative protein sources for the manufacture of feeds in the juvenile stage, in order to reduce dependence on fishmeal, however studies focused on differences in capacity digestion during juvenile ontogeny are lack. Therefore, the objective of the present work was to characterize the digestive proteases in a comparative way during the juvenile ontogeny of the spotter rose snapper and determine the possible differences in protein digestive capacity using *in vitro* techniques.

Material and Methods

Experimental animals

Fish for this study were obtained from the Laboratory of Reproduction and Marine Finfish Hatchery (CIAD), Sinaloa, México, where all juvenile stages were obtained from single spawning batch, conducted as described by Álvarez-Lajonchère *et al*. (2012). After one batch larval culture, all juvenile fish continued under normal culture (nursery step) and fattening process, until were collected in different times from one cycle. According to their wet weight, fish where classified in three groups (all considered in the juvenile stage): early juvenile (EJ; 21.3±2.6 g; 3 months after hatchery, MAH), middle juvenile (MJ; 190±4.4 g; 7 MAH) and late juvenile (LJ; 400±11.5 g; 12 MAH). Fish were adapted to control diet reported by Silva-Carrillo *et al*. (2012). Fish were starved for 24 hours to ensure the emptiness of the gut, euthanized ethically by a single puncture in the head with scalpel and immediately dissected to extract the digestive tract.

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Dissection and extract preparation

The digestive tract of each fish was individually divided into five segments: stomach (ST), pyloric caeca (PC), and intestine in three sections (proximal (PI), middle (MI) and distal intestine (DI). All of the procedures were conducted at temperatures of 0-4 °C. All segments were frozen individually at -64 °C until the assay was conducted. Prior to analysis, segment was diluted in a ratio of 1:10 (wet weight: volume) in a physiological saline solution (NaCl 9g L^{-1}) and ice-cold-homogenized with an Ultra-Turrax homogenizer. Homogenates were centrifuged (8500 × *g*) at 4 °C for 15 min, and the supernatant was used to perform enzyme activity assays (Matus-de-la-Parra *et al*. 2007).

Enzyme activity assay

The pepsin-like or total acid protease activity was measured by a modified method of Sarath *et al*. (1989), with denatured hemoglobin (2 % pH 2) as substrate. Alkaline protease activity was estimated by method of Walter (1984) using casein as substrate. The trypsin activity was determined by modified method of Erlanger *et al*. (1961), using Nα-benzoyl-L-arginine-4-p-nitroanilide hydrochloride (BAPNA 1 mmol L^{-1}) as substrate. The protein content of the supernatant solution was determined by Bradford assay (1976) using bovine serum albumin as the standard. One unit (U) of enzymatic activity was defined as the amount of enzyme that produced 1 µg of product released per minute. Tyrosine amount liberated from haemoglobin and casein hydrolysis was determined at 280 nm, while amount of p-nitroaniline liberated from BAPNA was determined at 410 nm.

1) Total activity (Units ml^{-1}) = [Δabs*reaction final volume (ml)]/[MEC*time (min)*extract volume (ml)]

2) Specific activity (Units mg $prot^{-1}$) = Total activity/soluble protein (mg)

Δabs represents the increase in absorbance, and MEC represents the molar extinction coefficient of tyrosine or p-nitroaniline (0.005 and 0.008 mL/µg/cm, respectively).

Characterization of digestive enzymes

Pepsin-like, total alkaline protease and trypsin were characterized by determining the relative activity (%) as a function of pH and temperature. The temperature effect for pepsin-like was measured from 10 to 50 °C; alkaline protease and trypsin were measured

from 10 to 60 °C, with similar assay conditions as previously described. The pH effect on digestive activity was measured at 37 °C, and the buffers were in range from pH 1 to 10 using buffers as previous described by Matus-de-la-Parra *et al*. (2007).

In addition, characterizations of acid and alkaline proteases were performed according to Guerrero-Zárate *et al*. (2014) using specific inhibitors. Pepstatin A (1 mmol L^{-1}) was used as an inhibitor of acid proteases from stomach and alkaline protease activity inhibition in pyloric caeca and intestine sections were performed using the following inhibitors: 250 mmol L^{-1} soybean trypsin inhibitor (SBT1), 10 mol L^{-1} N-tosyl-L-phenyl-chloromethyl ketone (TPCK), 100 mmol L^{-1} phenylmethylsulfonyl fluoride (PMSF), 10 mmol L^{-1} N_{α}-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), 10 mmol L^{-1} 1,10-Phenanthroline (Phen) and 250 mmol L^{-1} Type II-Turkey egg Ovomucoid (Ovo).

Chemical analysis

The moisture, protein, lipid and ash levels in the test ingredients were determined using standard methods AOAC (2000). The samples were homogenized and dried at 105 °C by 24 h prior to the chemical analyses. The level of crude protein was determined using micro-Kjeldahl method by Labcocnco System (Labconco, Kansas City, MO). The lipid content was analyzed using a micro Foss Soxtec Avanti 2050 Automatic System (Foss Soxtec, Hogan€as, Sweden) after extraction with petroleum ether and ash content was determined by calcination of the samples in a muffle furnace at 550 °C (Fisher Scientific International, Inc. Pittsburgh, PA, USA). NFE was determined by the difference between sums of all nutrients.

In vitro degree of hydrolysis (DH)

Digestibility of 13 different protein sources was evaluated by *in vitro* pH-Stat system Tritando Meltrohm 901, where list of ingredients used is summarized in Table 1. *In vitro* hydrolysis assays were performed with crude extracts from stomach (St) or pyloric caeca-intestine (PC-I) only in early and late juvenile stages. To determine protein degree of hydrolysis (DH), every single protein source was incorporated in a concentration of 8 mg ml^{-1} to be used as substrate solution, according to Saunders *et al*. (1972) and modified by Dimes & Haard (1994). For both juvenile stages, St extracts were adjusted to be added in

substrate solution at 193 U mL^{-1} and start acid hydrolysis at pH 3.0 in continuous agitations for 15 min (900 s) at 37 °C. Hydrochloric acid (HCl 0.1N) spent to maintain constant pH 3.0, was recorded every 100 seconds. Alkaline hydrolysis degree was performed adding PC-I pool extracts. As previously described, extracts from PC-I were adjusted to be added in the substrate solution at 23 U mL^{-1} and start alkaline hydrolysis at pH 8.0 in continuous agitation for 45 min (2700 s) at 37 °C. Sodium hydroxide (NaOH 0.1N) spent to maintain constant pH 8.0 was recorded every 250 seconds. All assays were performed by triplicated and procedure was performed for both juvenile stages under same parameters. The DH was calculated using the algorithm according to Adler-Nissen (1986).

During pH-Stat hydrolysis reaction, samples of mixture reactions (40 µl) were collected every 100s for acid hydrolysis reaction and every 250s for alkaline hydrolysis reaction to perform amino acid quantification analysis.

Table 1. Nutrient composition of protein sources used in assays

PROTEIN SOURCE	Abbreviation	%PROTEÍN	%LIPIDS	%ASH	%NFE
Casein [a]	Cas	90	1.2	----	----
Hemoglobin[b]	Hm	90	< 1	----	----
Fish meal [c]	FM	70.7	9.0	12.9	7.41
Tuna by products meal [d]	TM	59	14.9	22.4	3.61
Krill meal [e]	KM	56.7	19.6	9.6	14.1
Squid meal [e]	SM	68.5	2.6	11.6	17.3
Meat porcine meal [f]	MPM	59.7	10.7	12.8	16.8
Meat and bovine meal [f]	MBM	49	13.8	25.1	12.1
Poultry by products meal [f]	PM	61.6	15.3	10.4	12.7
Wheat gluten meal [g]	WGM	81.1	0.73	1.2	16.9
Corn gluten meal [g]	CGM	72.7	3.4	1.4	22.5
Soybean meal [f]	SBM	47.3	0.66	7.0	45.0
Canola meal [f]	CM	42.8	2.1	7.2	47.8
Control diet [h]	D-Control	45.5	10.5	9.9	34.1

[a]Hammarsten quality Casein, Research Organics # Catalog 1082C, [b]Bovine erythrocytes US Biological # Catalog H1850, [c]Premium grade fish meal was obtained from Selecta de Guaymas, S.A. de C.V. Guaymas, Sonora, México, [d]Maz Industrial, S.A de C.V. Mazatlán, Sinaloa, México, [e]PROAQUA, S.A. de C.V. Mazatlán, Sinaloa, México, [f]Proteínas marinas y agropecuarias S.A. de C.V., Guadalajara, Jalisco, [g]Droguería Cosmopolita, S.A. de C.V. México, D.F., México, [h]Diet manufactured in CIAD for snapper feeding as a reference diet.

Total amino acid release (TAAR)

Total amino acids (AA) released analysis was performed according to Church *et al*. (1893). An *o*-phtaldialdehyde (OPA) solution was prepared with 50 ml of sodium tetraborate 100 mmol l^{-1}, 5 ml of SDS at 20%, 80 mg of OPA diluted in 1ml of methanol and 0.2 ml of β-mercaptoethanol, solution was mixed and brings to 100 ml with distilled water. Briefly, 20 µl of the samples collected in digestion mixture reactions were fixed in 20 µl of 12% TCA and centrifuged at 14000 rpm during 15 min. Supernatant samples of 10 µl were added to 1 ml of OPA solution and absorbances were read at 340 nm. TAAR was calculated using standard curve made with decrees L-leucine concentrations.

Zymogram analyses

Electrophoresis techniques were performed in Mini PROTEAN 3 Cell (Bio- Rad) with four plates vertical gels of 8x10x0.075 cm with 10 sample capacity per plate. For the analysis of acid proteases from stomach, electrophoresis was run under non-denaturing native conditions (Native-PAGE) composed by continuous acrylamide gel (10 %) in buffer Tris (25 mmol l^{-1}) and glycine (192 mmol l^{-1}, pH 8.3, 80 volts) according to Davis (1964). Plate was composed by stacking gel with 4% poly-acrylamide (PAA) and resolving gel with 10% PAA. Electrophoresis was run under denaturalizing conditions (SDS-PAGE), with SDS 0.1 % in buffer Tris (25 mmol l^{-1}) and glycine (192 mmol l^{-1}, pH 8.3, 100 volts), according to Laemmli (1970) and adapted by García-Carreño *et al*. (1993).

After Native-PAGE electrophoresis, the gels were treated to reveal proteases isoforms according to the procedure of Díaz-López *et al*. (1998). The gel was submerged for 90 min at 25 °C in solution containing 0.25% hemoglobin (0.1 mol l^{-1} Glycine-HCl buffer, pH 2.0). The gels were fixed in trichloroacetic acid (12%) solution by 15 minutes. After alkaline SDS-PAGE electrophoresis, the gels were washed and directly incubated for 30 min at 5 °C in 0.5% casein solution (Tris–HCl 0.1 mol l^{-1} buffer, pH 9). The gels were then incubated for 90 min in the same solution at 37 °C. Finally, the gels were washed and fixed as previously described. For acid and alkaline gels, after areas of enzyme activity had been developed, the gels were stained according to Weber and Osborn (1969), using 0.1% Coomassie brilliant blue R-250 solution. Electrophoretic techniques were complemented

with the use of specific inhibitors previous described. Molecular weight marker was applied to each SDS-PAGE. Molecular weight (MW) of each band in the SDS-zymograms (alkaline protease) was calculated by a linearly adjusted model between the Rf and the decimal logarithm of MW protein markers.

Statistical analysis

For comparison, the percent inhibition and percent of relative activity in enzyme characterization and pH-Stat degree of hydrolysis was arcsin ($x^{1/2}$) transformed. The data for each parameter were tested for normality and homoscedasticity. One- or two-way ANOVA analyses were run when required. When differences were found, Tukey's HSD test was used ($P \leq 0.05$). Total amino acids released (mg L-Leucine equivalent) was plotted describing relationship between cumulative amino acid release and time of digestion for different meals with linear adjustment ($y = a + bx$). Differences among rate of digestion (slopes) between protein sources in specific hydrolysis phase and juvenile stage were assessed with ANCOVA ($P \leq 0.05$) (Zar 1984). All of the statistical analyses were performed using Statistica 7.0 Software for Windows (StatSoft, USA).

Results

Enzyme activity assays

The acid and alkaline proteases activities of different digestive tract sections in three juvenile stages are presented in Table 2.

Table 2. Protease activity in the stomach (S), pyloric caeca (PC), proximal (PI), middle (MI) and distal intestine (DI) in three juvenile stages of spotted rose snapper *Lutjanus guttatus*.

	Specific Activity (U mg protein^{-1}) of crude extract				
Stage	ST	PC	PI	MI	DI
EJ	1754.4±307.8^{c}	17.4±5.9^{b}	15.0±1.1^{c}	15.6±2.9^{b}	15.8±3.2^{c}
MJ	3864.2±796.0^{b}	22.2±3.8^{b}	20.0±2.4^{b}	27.5±5.0^{a}	23.0±3.8^{b}
LJ	6210.1±657.6^{a}	32.3±4.2^{a}	28.2±3.0^{a}	29.1±6.4^{a}	34.0±6.2^{a}

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The stomach acid proteolytic activity showed significantly higher specific activities ($P \leq 0.001$) value with increasing life stage. No significant differences in specific activity of alkaline proteases were observed between pyloric caeca and intestine sections for all juvenile stages ($P \leq 0.001$). Meanwhile, significantly higher specific activities in the LJ stage ($P \leq 0.001$) were found between stages when individual sections were compared. The trypsin-like specific activity showed a significantly higher ($P \leq 0.001$) value in the EJ stage than MJ and LJ stages (Table 3).

Table 3. Trypsin-like activity in the pyloric caeca in three juvenile stages of spotted rose snapper *Lutjanus guttatus*. Different superscript within rows indicate significant differences ($P<0.05$).

Specific Activity (U mg protein^{-1})		
EJ	MJ	LJ
82.50 ± 2.24^{a}	23.18 ± 2.47^{b}	22.77 ± 9.66^{b}

Temperature and pH effect on acid and alkaline protease activity

The three juvenile stages presented optimum temperature of acid proteases at 45°C (Fig. 1A) ($P \leq 0.001$). The optimum temperature of total alkaline proteases was 55°C for EJ, 50°C for MJ and LJ (Fig. 1B) ($P \leq 0.001$).

The optimum activity of acid proteases was measured at pH 3 for EJ and LJ and at pH 2 for MJ, with 80 to 90% of remnant activity at pH 2 and 3, respectively (Fig. 1C) ($P \leq 0.001$). Alkaline protease activity showed high relative activity (%) over a wide pH range (5-10) and an optimum at pH 9.0 in the three juvenile stages (Fig. 1D) ($P \leq 0.001$). Differences were found in relative activity percent at pH 5 between LJ (80%) and EJ, MJ (50%) ($P \leq 0.001$).

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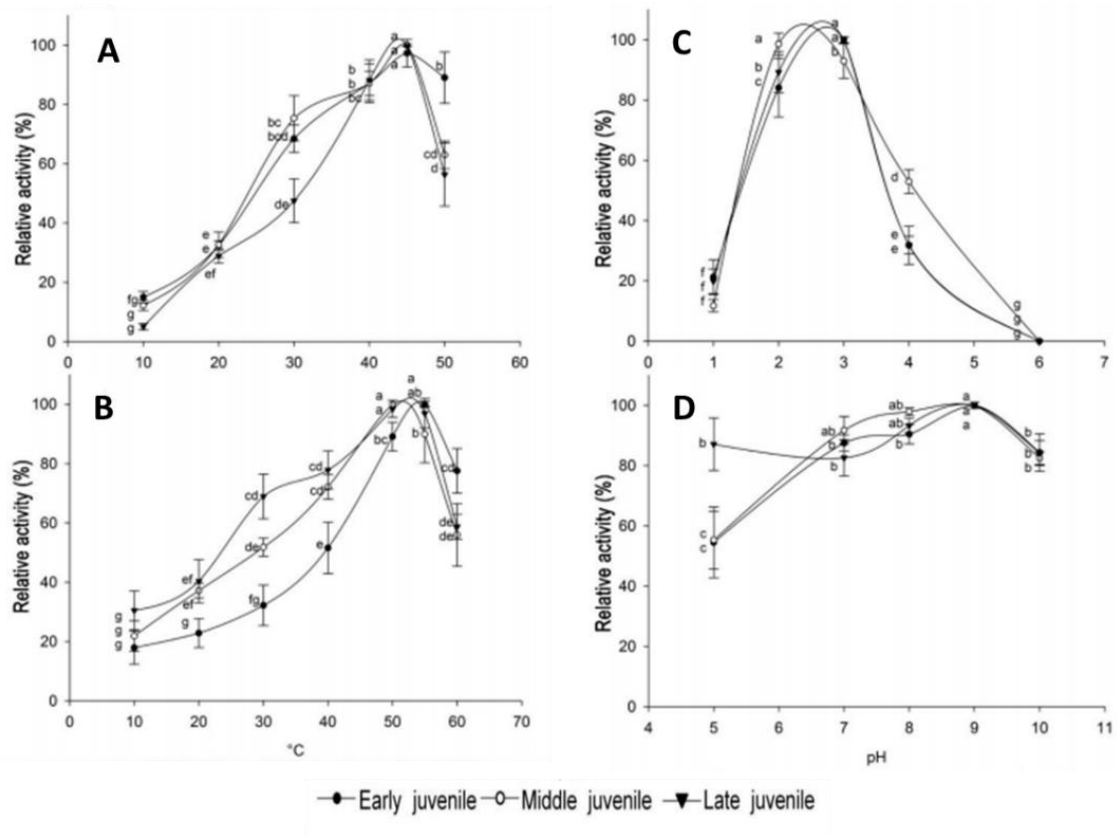

Figure 1. Temperature effects (°C) on the relative activity of acid (a) and alkaline proteases (b) and pH effects on the relative activity of acid (c) and alkaline proteases (d) in three juvenile stages of *Lutjanus guttatus*.

Temperature and pH effect on trypsin activity

The optimum temperature of trypsin was 50 °C for MJ and LJ, while EJ presented an optimum at 60 °C. Differences were found in relative activity (%) between almost all temperatures tested ($P \leq 0.001$) (Fig. 2B). Trypsin activity showed optimum activity at pH 9 for all juvenile stages (Fig. 2B).

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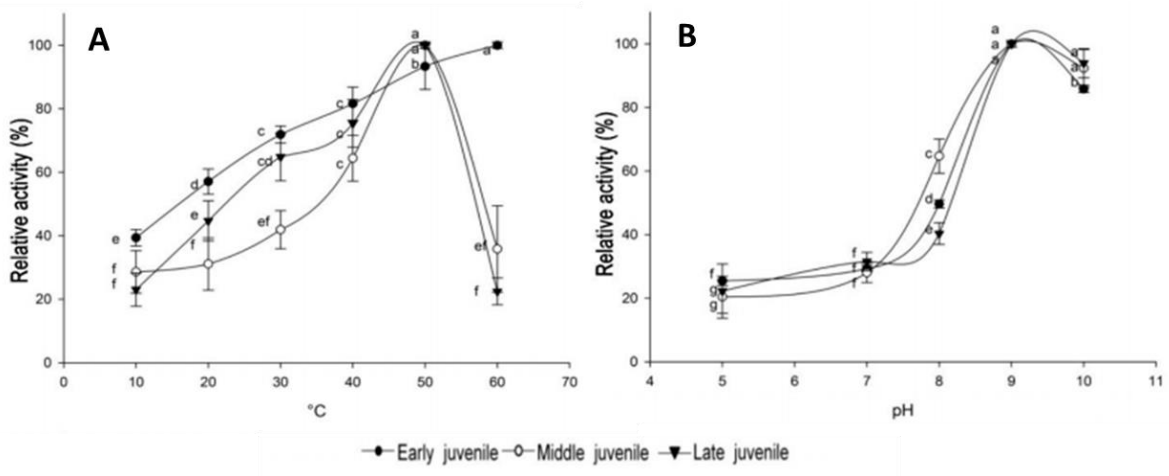

Figure 2. Temperature and pH effects on the relative trypsin-like activities in three juvenile stages of *Lutjanus guttatus*.

Specific inhibitors effects

Pepstatin A inhibited the total activities in stomach extracts in all juvenile stages. The percent of alkaline protease inhibition are summarized in Table 4. In general, the inhibited percent of activity in total alkaline proteases was significantly higher ($P \leq 0.001$) in EJ using TLCK, PMSF, SBTI, Phen and Ovo compared to MJ and LJ, while no significant differences were found between inhibition percent with TPCK ($P=0.2402$).

Table 4. The percent of activity inhibition in pyloric caeca after incubation with enzyme specific inhibitors in three juvenile stages of spotted rose snapper *Lutjanus guttatus.*

	Percentage of activity inhibition					
Inhibitor type	TPCK	TLCK	PMFS	SBTI	Phen	Ovo
EJ	11.7±4.8 ^{a}	14.2±1.3^{a}	15.7±2.5^{a}	54.9±6.6^{a}	32.7±2.0^{a}	18.5±1.2^{a}
MJ	9.9±2.6 ^{a}	6.1±0.6^{b}	13.6±0.6^{a}	25.8±5.4^{b}	28.8±1.3^{b}	7.3±0.5^{b}
LJ	6.6±2.1 ^{a}	7.9±1.3^{b}	5.4±1.9^{b}	16.1±3.9^{c}	23.3±1.1^{c}	6.3±1.0^{b}

Different superscript within columns indicate significant differences ($P<0.05$).

In vitro degree of hydrolysis (DH)

Hemoglobin presented the highest DH among all ingredients in acid digestion for both juvenile stages ($P \leq 0.05$). Higher DH values differ in protein source between juvenile stages, where SBM (soybean meal), CM (canola meal) and D-control (control diet) showed

the higher DH values in LJ acid digestion, while TM (tuna by products meal), SBM and D-control showed the highest DH values in EJ acid digestion. By the other side, DH values of TM, SM (squid meal) and CM showed differences between EJ and LJ stages in acid hydrolysis ($P \leq 0.05$) (Fig. 3A).

Alkaline hydrolysis showed that FM (fishmeal) presented the higher degree of hydrolysis among all ingredients in LJ stage, while MBM (meat and bovine meal), WGM (wheat gluten meal), CGM (corn gluten meal) and D-control presented the highest DH among all ingredients in EJ stage ($P \leq 0.05$). Eight of the ingredients tested, showed differences in DH between EJ and LJ stages in alkaline hydrolysis, including animal protein sources (FM, MPM (meat porcine meal), MBM, PM (poultry by products meal)) and vegetable protein sources (WGM, CGM, CM) and D-control, as protein mix from balance diet ($P \leq 0.05$) (Fig. 3B).

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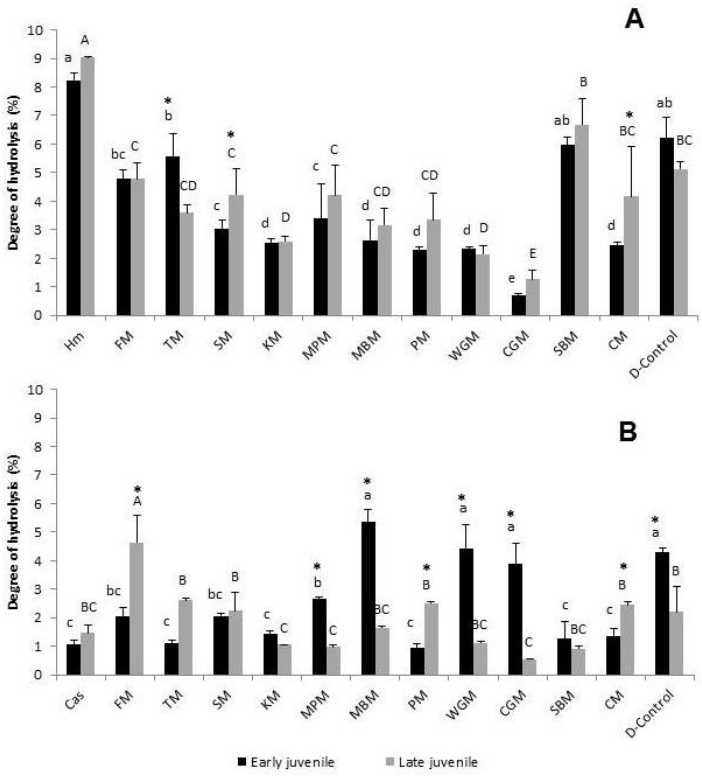

Figure 3. *In vitro* pH-stat degree of protein hydrolysis (DH) of feed ingredients using digestive enzyme extracts from *L. guttatus* early (20 g) and late juveniles (400g) from A) stomach and B) pyloric caeca. Lower-case show differences in EJ stage, upper-case show differences in LJ stage and asterisk show differences between juvenile stages ($P<0.05$). Values shown are means (n=3) ± standard deviation (error bars).

Total amino acid release (TAAR)

The kinetics of TAAR was assessed by analyzing the cumulative production of amino acid through time of digestion. These relationships were best described by linear regressions, all of them with high determination coefficients (R^2=0.90 to 0.98). The rate of amino acid liberation were compared by ANCOVA and showed significant differences between ingredients in acid and alkaline hydrolysis in both juvenile stages ($P\leq0.05$).

For both juvenile stages, hemoglobin presented the highest TAAR. Nevertheless, higher TAAR with stomach extracts in EJ stage was obtained by SM, followed by CM, SBM and KM (krill meal), while TM showed the lowest TAAR ($P\leq0.05$) (Fig. 4A).

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Results in TAAR by LJ stomach extract show higher values for SBM and CM, followed by MBM and WGM, while CGM showed the lowest TAAR (P≤0.05) (Fig. 4B).

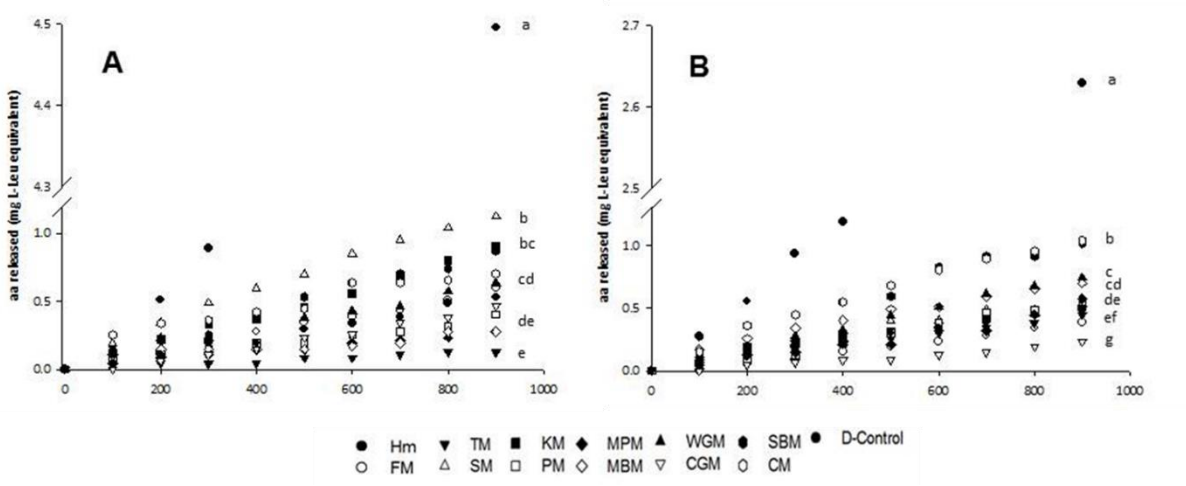

Figure 4. Kinetic of free amino acid released from ingredients using stomach enzyme extracts from *L. guttatus* a) early (20 g) and b) late juveniles (400g). Data points and regression lines of cumulative values against time for each meal are represented with the same symbol. Letters to the right of regression lines indicate differences (P≤0.05) among slopes.

Alkaline hydrolysis in EJ stage showed higher TAAR by PM, followed by Cas (casein), MPM and TM, while SBM and CGM showed the lowest TAAR (P≤0.05) (Fig. 5A). Alkaline hydrolysis in LJ stage showed higher TAAR in MPM, followed by FM, while Cas and SBM showed the lowest TAAR values (P≤0.05) (Fig. 5B).

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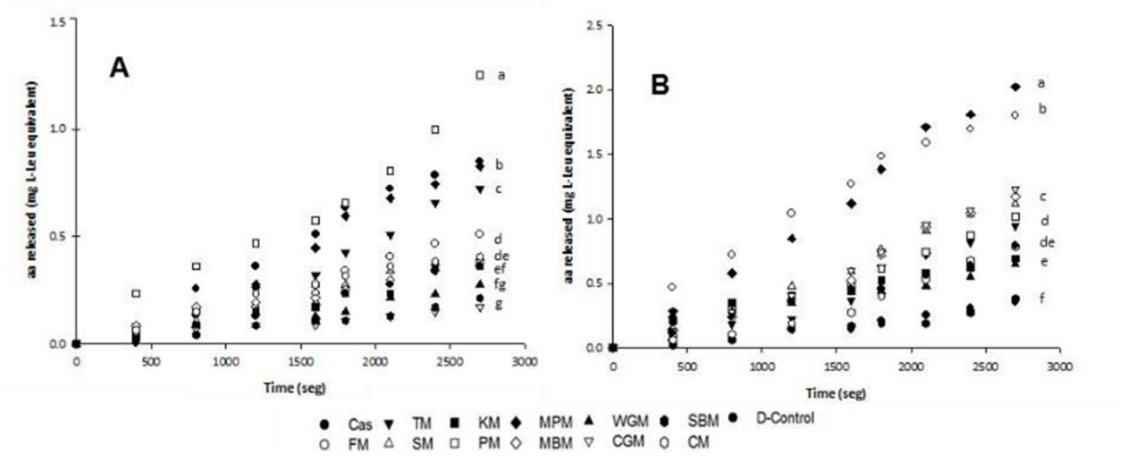

Figure 5. Kinetic of free amino acid released from ingredients using pyloric caeca-intestine enzyme extracts from *L. guttatus* a) early (20 g) and b) late juveniles (400g). Data points and regression lines of cumulative values against time for each meal are represented with the same symbol. Letters to the right of regression lines indicate differences ($P \leq 0.05$) among slopes.

Zymogram analyses

Electrophoresis under Native-PAGE conditions, reveal two bands with acid protease activity in both juvenile stages of SRS: one with an Rf of 0.72 and the other with an Rf of 0.77, where both bands were completely inhibited by pepstatin A (Fig. 6).

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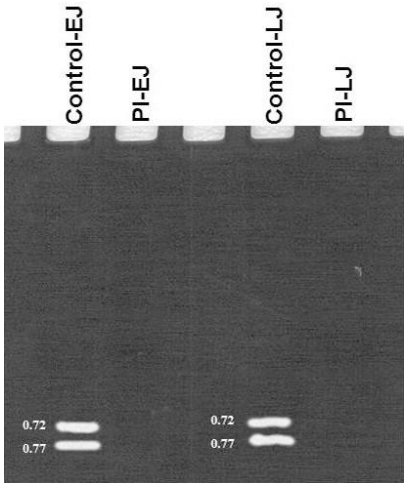

Figure 6. Zymogram of acid proteases from the multienzymatic stomach extracts of early juvenile (EJ; 20g) and late juvenile (LJ; 400g) stages of *L guttatus*, with the action of pepstatin A inhibitor (PI) on the isoforms.

Electrophoresis under SDS-PAGE conditions showed same band pattern in pyloric caeca and intestine sections, therefore results corresponds to all alkaline phase digestive tract in *L. guttatus* in a given juvenile stage. Total of nine bands in PC-I extracts were observed between EJ and LJ stages bands (Fig. 7A and Fig. 7B , respectively; 98.1, 90.2, 87.3, 71.4, 53.1, 40.6, 26.1, 19.8 and 16.7 kDa), referenced as first to ninth bands.

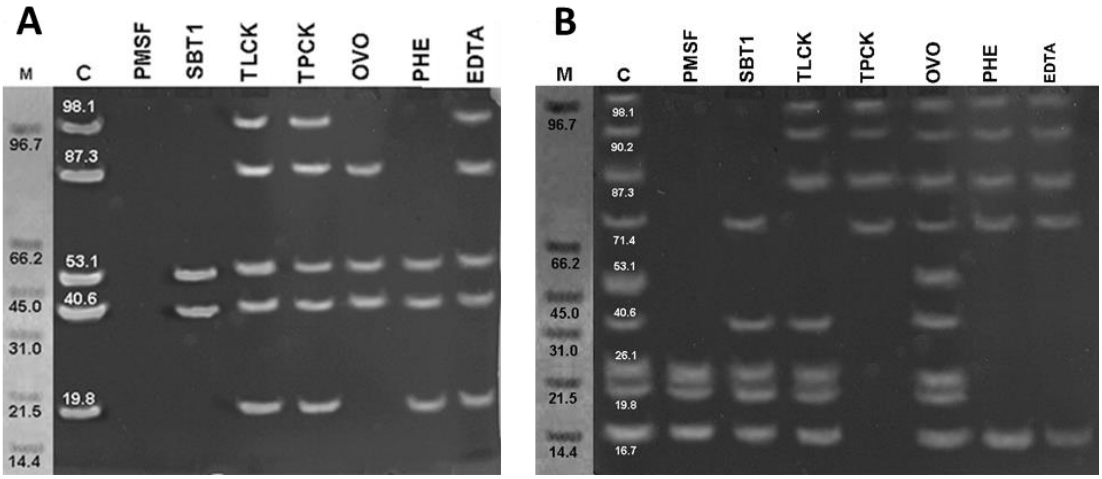

Figure 7. Zymograms of alkaline proteases from the multienzymatic pyloric caeca and intestine extracts of A) Early juvenile of *L. guttatus* (20 g) and B) Late juvenile of *L.*

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guttatus (400 g), with the action of the respective inhibitors on the isoforms. M: molecular weight marker (kDa).

In the case of alkaline enzyme pattern in EJ stage, five bands were observed in the control (98.1, 87.3, 53.1, 40.6 and 19.8 kDa, representing, first, third, fifth, sixth and eight bands) (Fig. 7A). Four additional bands were observed in LJ stage PC-I extracts, with MW of 90.2, 71.4, 26.1 and 16.7 (representing the second, fourth, seventh and ninth bands) with a total of nine bands (Fig. 7B).

Discussion

Although there are many investigations in characterization of digestive enzymes in different fish species, much of this research has focused on early ontogeny and / or characterization in a juvenile size of this species. During the early ontogeny of many fish species, there are successive changes in the activity and / or expression of different enzymes together with a rapid development of the digestive system and auxiliary organs (Zambonino-Infante & Cahu, 2001), that is helpful to close cultivation cycles of species with aquaculture potential.

On the other hand, the characterization of digestive enzymes that have been carried out during juvenile stages, take for granted that the number, type and / or activities of digestive enzymes do not change throughout the juvenile stage, where some studies have approached this subject in some species (Unajak *et al.* 2012, Yasumaru & Lemos, 2014). The presence of changes in digestive capacity during juvenile stages of different species is of great importance, since these stages of life correspond in the majority of the fish aquaculture species to the phase of culture before harvest. Therefore, understanding of digestive changes and / or adaptations will serve as a basis for the formulation of specific diets for grow-out juvenile stages, in order to increase the productive yield.

Thus, the present investigation serves as a basis for the development of research in different species with culture potential, where it is clear the existence of modifications in the digestive capacity of proteins in a same species during juvenile development.

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Conclusion

In conclusion, the digestive system of spotted rose snapper is highly efficient in the breakdown of protein. The high pepsin activities and the presence of two pepsin isoforms suggest the potential for hydrolysis of a wide range of protein sources joined to final alkaline digestion. This potential increases with fish growth through juvenile stages in which a diversification in the type of alkaline enzymes exists, affecting the degree of hydrolysis of different protein sources and the rate and degree of absorption of total free amino acids. Higher DH and TAAR values were documented in constituents such as fish and squid meal, animal porcine meal and poultry meal produced from recycled by-products and soybean meal and canola meal as vegetable products that provide better protein sources for use in the development of practical diets.

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