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Research Article

Comparative characterization of protease activity in cultured spotted rose snapper juveniles (*Lutjanus guttatus*)

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ABSTRACT. Partial characterizations of digestive proteases were studied in three life stages of spotted rose snapper: early (EJ), middle (MJ) and late juvenile (LJ) with corresponding average weights of 21.3 ± 2.6 g (3 months after hatching, MAH), 190 ± 4.4 g (7 MAH), and 400 ± 11.5 g (12 MAH). At sampling points, the digestive tract was dissected into the stomach (St), pyloric caeca (PC), and the intestine in three sections (proximal (PI), middle (MI) and distal intestine (DI)). The effect of pH and temperature and specific inhibitors were evaluated for acid and alkaline proteases. Total acid and alkaline protease activity showed a tendency to increase with juvenile life stage of fish while trypsin activity decreased. Differences were found in acid and alkaline protease activities at different pH and temperatures during juvenile stages. Pepstatin A inhibited total activity in the stomach extract in all juvenile stages. Activity in total alkaline protease inhibition was significantly higher in EJ using TLCK, PMSF, SBTI, Phen and Ovo than in MJ and LJ, while no significant differences were found with TPCK inhibition. Therefore increases in protease activities with fish growth through juvenile stages in which a substitution or diversification in the type of alkaline enzymes exist. These results lead a better comprehension of changes in digestive potential of Lutjanidae fish.

Keywords: *Lutjanus guttatus*, spotted rose snapper, digestive enzymes, pepsin, trypsin, protease inhibitors.

Caracterización comparativa de la actividad de la proteasa en juveniles cultivados de pargo flamenco (*Lutjanus guttatus*)

RESUMEN. Se caracterizaron parcialmente las proteasas ácidas y alcalinas en tres estadios juveniles del pargo flamenco: temprano (EJ), medio (MJ) y juvenil tardío (LJ) con pesos promedios correspondientes a 21,3 ± 2,6 g (3 meses post-cultivo larvario, MAH), 190 ± 4,4 g (7 MAH) y 400 ± 11,5 g (12 MAH). El tracto digestivo fue seccionado en estómago (St), ciegos pilóricos (PC) e intestino en tres secciones (proximal (PI), medio (MI) e intestino distal (DI)). El efecto de la temperatura, pH e inhibidores específicos sobre proteasas ácidas y alcalinas fue evaluado en los tres estadios juveniles. Los resultados indican una tendencia de aumento en la actividad de proteasas ácidas y alcalinas totales con el aumento de edad, mientras que la actividad de tripsina disminuye con la edad. Se encontraron diferencias en actividad de proteasas ácidas y alcalinas a diferentes temperaturas y pH entre los tres estadios juveniles. Pepstatin A inhibió la actividad total de proteasas ácidas en los tres estadíos juveniles. La inhibición de la actividad de proteasas alcalinas con los inhibidores TLCK, PMSF, SBTI, Phen y Ovo fue significativamente mayor en el estadio EJ en comparación a MJ y LJ, mientras que no se encontraron diferencias en inhibición con TPCK. El pargo flamenco presenta un incremento en actividad total de proteasas ácidas y alcalinas en conjunto con su desarrollo juvenil, aunado a una sustitución o diversificación en el tipo de proteasas alcalinas. Estos resultados permiten una mejor comprensión de los cambios en el potencial digestivo de lutjánidos.

Palabras clave: *Lutjanus guttatus*, pargo flamenco, enzimas digestivas, pepsina, tripsina, inhibidores de proteasas.

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INTRODUCTION

The spotted rose snapper (*Lutjanus guttatus*) has a high potential for intensive culture in Latin American countries (Davis *et al*., 2000). In Mexico and Costa Rica, fish farmers capture wild juveniles and stock them in floating sea cages where they are fed until they reach the appropriate market size (450 g) (Herrera-Ulloa *et al.*, 2010). Reproduction techniques for juvenile mass production in hatcheries have been developed on a pilot scale for this species in Mexico (Ibarra-Castro & Alvarez-Lajonchère, 2011). The spotted rose snapper, similar to other members of the Lutjanidae family, are carnivorous marine fish distributed in tropical zones. They primarily feed on demersal organisms, such as crustaceans and fish (Allen, 1995). Under culture conditions, they require a high protein diet containing between 45 and 50% (Silva-Carrillo *et al*., 2012). This species has a well-defined stomach, with five to six blind sacs in a pyloric caeca, and a very short intestine. Little information is available regarding the digestive physiology Lutjanids and more knowledge in this area is required to develop appropriate feeds for rearing to market size.

Some studies describe the early ontogeny development of the digestive system in spotted rose snapper, presenting same pattern of digestive enzyme activity as previously reported for other species, in which pancreatic and intestinal enzymatic activities are present at hatching (Moguel-Hernández *et al*., 2013), and maturation of digestive function occurs around 20-25 days after hatching with pepsin secreted by functional stomach, described by Galaviz *et al*. (2012). Studies in others Lutjanidae species (Alarcón *et al.*, 2001) described the effect of plant regional protease inhibitors on digestive proteases of yellow snapper (*L. argentiventris*) and Pacific dog snapper (*L. novemfasciatus*). Additionally, Khantaphant & Benjakul (2008, 2010) reported the skin gelatin hydrolyzation capability in brown stripe red snapper (*L. vitta*) with proteases from pyloric caeca and performed a trypsin characterization for this species. Therefore, early development of digestive enzymes in *L. guttatus* has been described, but similar research on the juvenile or adult stage has not been performed.

Some authors have indicated that independent of feeding habits, fish digestive system responses closely correlate with diet and age (Pérez-Jiménez *et al.*, 2009; Falcon-Hidalgo *et al*., 2011). Differences in proteolytic enzyme activities and zymogens in fish at different ages have been reported, but the changes have been attributed to feeding habitats or diet changes and not solely influenced by age (Falcon-Hidalgo *et al*., 2011; Unajak *et al*., 2012). Other report presents the existence of variations of genetically trypsin-like isozymes correlated with fish size in *Salmo salar* fry (Torrissen, 1987), and these variations are related and could affect growth rate and/or feed conversion efficiency (Torrissen & Sharer, 1992). Hence, determine possible changes in proteases potential over juvenile ontogeny that represents culture time period is important, which could be useful to develop efficient specific size diets to optimize growth of *L. guttatus*.

Therefore, protease activity could change during juvenile stages of *L. gutattus* with different digestive potential and possible variations in protease enzymes or isozymes. Therefore, the objective of this study was to compare the partial characterization of acid and alkaline digestive proteases in the digestive tract of three spotted rose snapper juvenile stages using biochemical techniques to understand the protein digestive potential variations during the culture period of spotted rose snapper.

MATERIALS 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 Alvarez-Lajonchère *et al*. (2012). After one batch larval culture, all juvenile fish continued under normal culture (nursery step) and fattening process. Fish were collected in different times from one cycle. When given fish stage was required, fish were place in tanks and fed the same feed for 20 days. 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 month after hatchery, MAH), middle juvenile (MJ; 190 ± 4.4 g; 7 MAH) and late juvenile (LJ; 400 ± 11.5 g; 12 MAH). Diet adaptation was performed in fiberglass tanks (4000 L) with a constant water flow and the fish were fed twice at day (9:00 and 16:00 h) with a diet containing fishmeal as a main protein source (Table 1). Fish were starved for 24 h 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. The parameters and biometric indices of fish used in the assays are summarized in Table 2.

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 proce-

Table 1. Composition and proximate analyses of diet for spotted rose snapper *L. guttatus*. [1]Premium grade fish meal was obtained from Selecta de Guaymas, S.A. de C.V. Guaymas, Sonora, México. [2]Marine Protein and Agricultural, S.A. of C.V., Guadalajara, Jalisco, México. [3]PROAQUA, S.A. de C.V. Mazatlán, Sinaloa, México. [4]Droguería Cosmopolita, S.A. de C.V. México, D.F., México. [5]Trouw Nutrition México S.A. de C.V. (by courtesy). [6]DSM Nutritional Products México S.A. de C.V., El Salto, Jalisco, México. *Vitamin premix composition: Vitamin A, 10 000 000 IU o mg g^{-1}; Vitamin D3, 2 000 000 IU; Vitamin E, 100 000 g; Vitamin K3, 4.00 g; Thiamine B1, 8.00 g; Riboflavin B2, 8.70 g; Pyridoxine B6, 7.30 g; Vitamin B12, 20.00 mg; Niacin, 50.00 g; Pantothenic acid, 22.20 g; Inositol, 153.80 g; Nicotinic Acid, 160.00 g; Folic acid, 4.00 g; 80 mg; Biotin, 500 mg; Vitamin C, 100.00 g; Choline 300.00 g, Excipient c.b.p. 2000.00 g. **Mineral premix composition: Manganese, 100 g; Magnesium, 45.00 g; Zinc, 160 g; Iron, 200 g; Copper, 20 g; Iodine, 5 g; Selenium, 400.00 mg; Cobalt 600.00 mg. Excipient c.b.p. 1500.00 g.

Ingredient	(% dry weight)
Fishmeal[1]	52.60
Squid meal[2]	6.00
krill meal[3]	7.59
fish oil[4]	8.78
Dextrine[4]	17.47
Wheat gluten[4]	2.00
Vitamin premix[5*]	0.60
Minerals premix[5**]	0.23
Carotenoids[6]	0.08
Antioxidant[6]	0.05
Soybean lecithin (70%)[6]	1.50
Vitamin C[6]	0.10
Alginate[4]	3.00
Proximate analyses	
Dry matter	92.91
Crude protein	43.06
Crude fat	13.86
Ash	14.01
Nitrogen free extract	15.27

dures were conducted at temperatures of 0-4°C. All segments were frozen individually at -64°C for 24 h and then lyophilized for four days and stored under dry conditions at 4°C until the assay was conducted. Prior to analysis, each lyophilized segment, diluted at a ratio of 1:10 (wet weight: volume) in a physiological saline solution (NaCl 9 g L^{-1}), was ice-cold-homogenized with an Ultra-Turrax homogenizer. All 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. The enzymatic reaction mixture consisted of 300 µL of substrate with 0.2 mol L^{-1} glycine-HCl buffer (pH 2) and 100 µL of enzymatic extract, incubated at 37°C and stopped by the addition of 600 µL of 5% (w/v) trichloroacetic acid (TCA). Alkaline protease activity was estimated by method of Walter (1984) using casein as substrate. The enzymatic reaction mixtures consisted of 250 µL of 0.1 mol L^{-1} Tris-HCl buffer, 0.01 M (pH 9) $CaCl_2$, 100 µL of enzymatic extract and 250 µL of 1% casein in Tris-HCl buffer, incubated at 37°C and stopped by adding 600 µL of 8% (w/v) TCA. The trypsin activity was determined by modified method of Erlanger *et al*. (1961). A Nα-benzoyl-L-arginine-4-p-nitroanilide hydrochloride (BAPNA 1 mmol L^{-1}) substrate was used. The enzymatic reaction mixtures consisted of 560 µL of substrate in 0.05 mol L^{-1} Tris-HCl, 0.01 mol L^{-1} (pH 8.2) $CaCl_2$ and 80 µL of enzymatic extract, incubated at 37°C and stopped by adding 160 µL of acetic acid at 30%. 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.

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

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

Tissue activity (Units wet $tissue^{-1}$) = Total activity *total tissue (g)

where Δabs represent 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 following buffers were used:

Table 2. Biometric parameters for three juvenile stages of spotted rose snapper *Lutjanus guttatus*. DSI: (Digestive tract weight (g)/fish weight (g))*100. Digestive tract represents the sum of stomach, pyloric caeca and intestine weight. EJ, MJ and LJ represent early, middle and late stage juveniles, respectively. Different superscript within columns indicate significant differences ($P < 0.05$).

Stage	Fish weight (g)	Digestive tract weight (g)	DSI
EJ	21.3 ± 2.6	0.25 ± 0.03	1.17 ± 0.12^{b}
MJ	190.0 ± 4.4	2.5 ± 0.21	1.32 ± 0.11^{b}
LJ	400.0 ± 11.5	7.6 ± 0.42	1.91 ± 0.09^{a}

glycine-HCl at a pH of 1 to 3; acetate buffer at a pH of 4 and 5; Tris-HCl at a pH of 7 to 9; and glycine-NaOH at a pH of 10. The buffers molarities were 0.2 mol L^{-1} for acid proteases, 0.1 mol L^{-1} for alkaline proteases and 0.05 mol L^{-1} for trypsin activity, with $CaCl_2$ (0.01 mol L^{-1}) for alkaline protease and trypsin activities (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 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).

Statistical analysis

Eight juveniles of each stage of spotted rose snapper were handled individually to maintain eight replicates per analysis. For comparison, the percent inhibition and percent relative activity in enzyme characterization 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 ($P \leq 0.05$). 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 the three juvenile stages are presented in Table 3. The stomach acid proteolytic activity showed significantly higher specific and tissue 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 ($P \leq 0.001$), however, tissue activity showed higher values in PC than other intestine sections for all juvenile stages. Meanwhile, significantly higher specific and tissue 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 4), nevertheless, tissue activity values increase with increasing life stage ($P \leq 0.001$).

Temperature effect on acid and alkaline protease activity

The three juvenile stages presented optimum temperature of acid proteases at 45°C (Fig. 1a) ($P \leq 0.001$). Acid proteases relative activity at 30°C showed differences between EJ, MJ (70%) and LJ (40%) ($P \leq 0.001$), while relative activity at 50°C showed differences between EJ (80%) and MJ and LJ (60%) ($P \leq 0.001$). The optimum temperature of total alkaline proteases was 55°C for EJ, 50°C for MJ and LJ (Fig. 1b) ($P \leq 0.001$). Differences were found in the relative activity percent at 20, 30, 40 and 60°C between EJ and the other stages ($P \leq 0.001$). In general terms, LJ showed higher relative activities (%) than EJ and MJ in total alkaline protease activity, when individual sections were compared.

Effect of pH on acid and alkaline protease activity

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. 2a) ($P \leq 0.001$). Significant differences in relative activity at pH 4 were found between EJ, LJ (30%) and LJ (50%) ($P \leq 0.001$). Alkaline protease activity showed high relative activity (%) over a wide pH range

Table 3. Protease activity in the stomach (ST), pyloric caeca (PC), proximal (PI), middle (MI) and distal intestine (DI) in three juvenile stages of spotted rose snapper *Lutjanus guttatus*. EJ, MJ and LJ represent early, middle and late stage juveniles, respectively. Lower-case show differences in columns, upper-case show differences in rows.

Stage	*Specific activity (U mg protein^{-1}) of crude extract **Tissue activity (U wet tissue)				
	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}
**	269.2 ± 28.7 ^{c}	2.15 ± 0.6 ^{A,c}	0.46 ± 0.1 ^{B}	0.46 ± 0.9 ^{B}	0.67 ±0.2 ^{B}
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}
**	1002.3 ± 161.4^{b}	5.24 ± 0.6 ^{A,b}	2.61 ± 0.7 ^{B}	2.05 ± 0.5 ^{B}	2.52 ± 0.6 ^{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}
**	1746.6 ± 203.6^{a}	13.95 ± 1.6 ^{A,a}	4.33 ± 0.5 ^{BC}	3.12 ± 0.5 ^{C}	4.59 ± 0.8 ^{B}

Table 4. Trypsin-like activity in the pyloric caeca in three juvenile stages of spotted rose snapper *Lutjanus guttatus*. EJ, MJ and LJ represent early, middle and late stage juveniles, respectively. Different superscript within rows indicate significant differences ($P < 0.05$).

*Specific activity (U mg protein^{-1}) **Tissue activity (U wet tissue)		
EJ	MJ	LJ
*82.50 ± 2.24^{a}	*23.18 ± 2.47^{b}	*22.77 ± 9.66^{b}
**0.35 ± 0.01^{b}	**3.68 ± 0.47^{b}	**13.11 ± 5.63^{a}

(5-10) and an optimum at pH 9 in the three juvenile stages (Fig. 2b) ($P \leq 0.001$) Differences were found in relative activity percent at pH 5 between LJ (80%) and EJ, MJ (50%) ($P \leq 0.001$).

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 ($P \leq 0.001$). In general, EJ presented higher relative activities (%) than MJ and LJ (Fig. 3a). Trypsin activity showed optimum activity at pH 9 for all juvenile stages. Remnant activity showed significant differences ($P < 0.001$) at pH 10 (between 80 and 90%) versus pH 8 (between 40 and 60%) (Fig. 3b).

Specific inhibitors effects

Pepstatin A inhibited the total activities in stomach extracts in all juvenile stages (Fig. 4). The percent of alkaline protease inhibition are summarized in Table 5. 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 ($P = 0.240$).

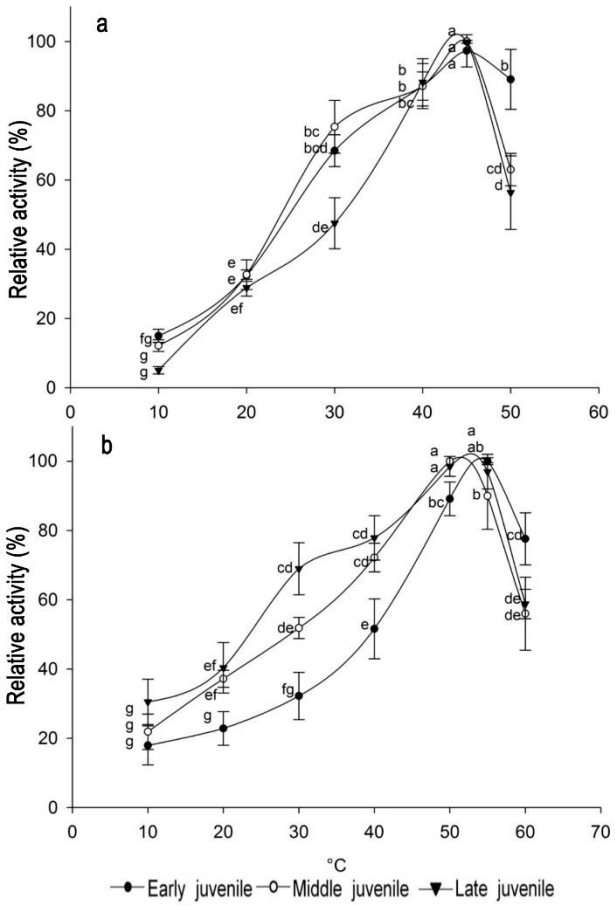

Figure 1. Temperature effects (°C) on the relative activity of a) acid, and b) alkaline proteases in three juvenile stages of *Lutjanus guttatus*.

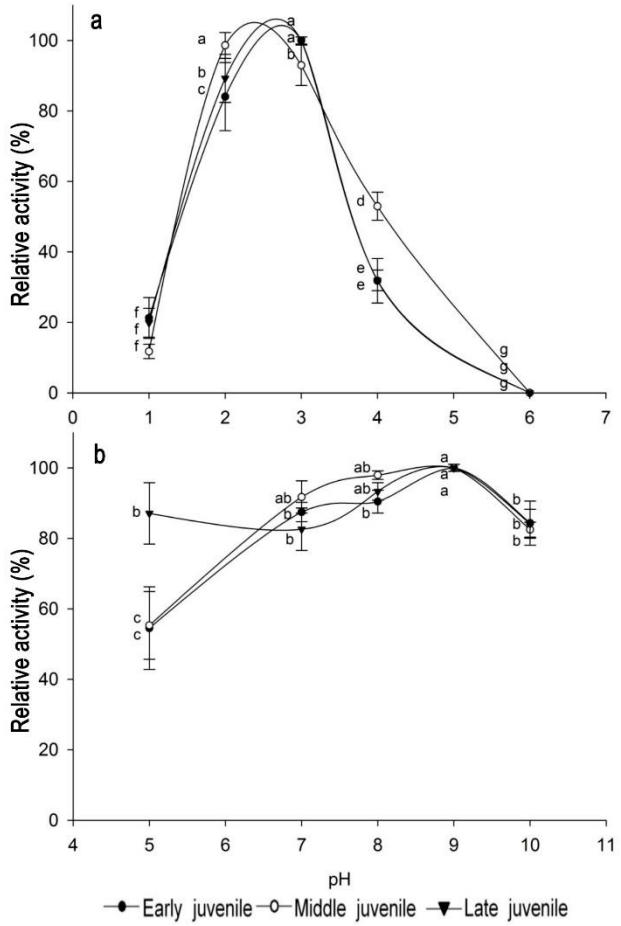

Figure 2. pH effects on the relative activity of a) acid and b) alkaline proteases in three juvenile stages of *Lutjanus guttatus*.

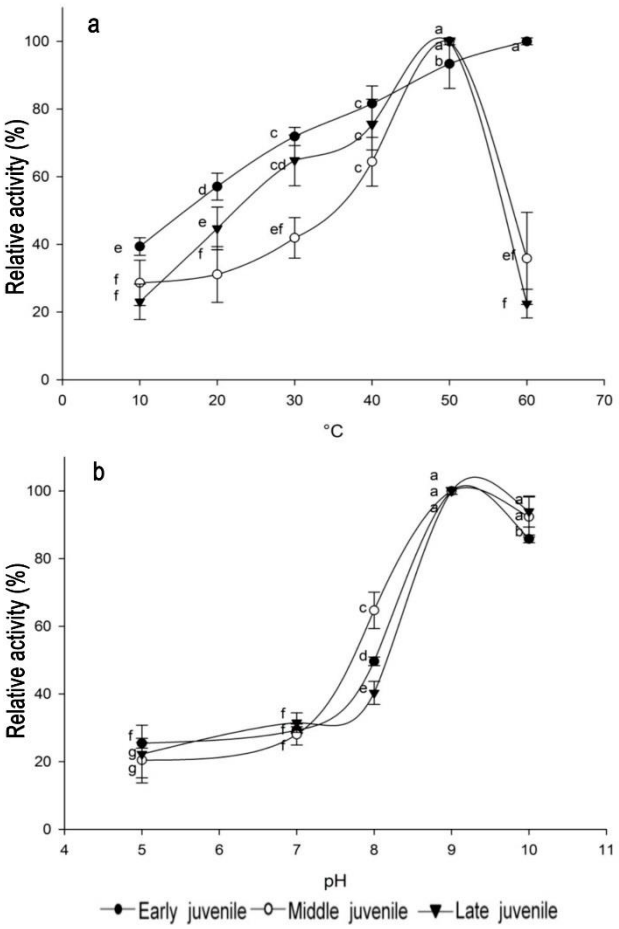

Figure 3. a) Temperature and b) pH effects on the relative trypsin-like activities in three juvenile stages of *Lutjanus guttatus*.

DISCUSSION

Previous studies in early ontogeny of the present species report the presence of wide battery of digestive enzymes, such as pancreatic (*i.e.*, trypsin, chymotrypsin, amylase, and lipase) and intestinal (*i.e.*, acid and alkaline phosphatases and leucine aminopeptidase) present from hatching, joined to appearance of pepsin activity between 20-25 days after hatching, considered as onset of juvenile period (Galaviz *et al.*, 2012; Moguel-Hernández *et al.*, 2013). However, this is the first work focused in changes over digestive proteases during ontogeny of juvenile stages, where differences in some parameters suggest the presence of other proteases type in larger juvenile stages and at same time, the use of a variety of specific inhibitors confirm the presence of wide range of proteases in the species.

As a rule, total digestive activity increases with fish age due to the increase of digestive tract size and mucosa weight (activity * total intestinal mucosa weight)

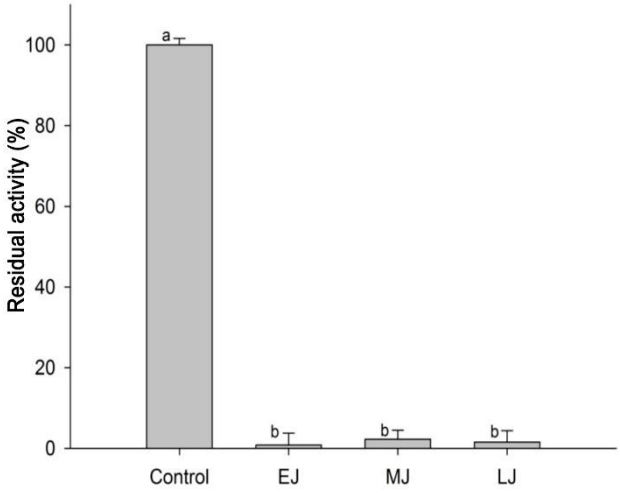

Figure 4. Percent of residual activity in stomach extract after incubation with pepstatin A in three juvenile stages of *Lutjanus guttatus*. EJ, MJ and LJ represent early, middle and late stage juveniles, respectively.

Table 5. The percent of activity inhibition in pyloric caeca after incubation with enzyme specific inhibitors in three juvenile stages of spotted rose snapper *Lutjanus guttatus.* EJ, MJ and LJ represent early, middle and late stage juveniles, respectively. *SBTI (soybean trypsin inhibitor), TPCK (N-tosyl-L-phenyl-chloromethyl ketone), PMSF (phenylmethylsulfonyl fluoride), TLCK (N_{α}-tosyl-L-lysine chloromethyl ketone hydrochloride), Phen (1.10-Phenanthroline), Ovo (Type II-T: Turkey egg ovomucoid). Different superscript within columns indicate significant differences ($P < 0.05$).

Inhibitor concentration ($mmol^{-1}$)	Percentage of activity inhibition					
	10	10	100	250	10	250
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}

(Kuzʾmina, 1996). In the present work a higher relation between digestive tract weight and total fish weight was found when increasing fish age as we detected in (Table 2), represented by higher DSI values. Therefore, increasing activities in all digestive sections related with increasing juvenile stage is in accordance with above mentioned. Some reports in certain fish species indicate that changes in specific enzyme activity (U mg $prot^{-1}$) vary at different ages (Chiu & Pan, 2002; Falcon-Hidalgo *et al*., 2011), which was found in this study and represents a higher capacity for protein breakdown. Some authors have reported comparative activities between fish stages, but the results are attributed to adaptations in feeding habitats (Kuzʾmina, 1996; Falcon-Hidalgo *et al.*, 2011). In this work, changes in specific activity (U mg $prot^{-1}$) and tissue activity (U wet tissue) for proteases at different juvenile stages were present, even though the three juvenile stages are from same batch culture and were conditioned for 20 days with the same diet and feeding frequency. Furthermore, differences at varying temperatures and pH were attributed to ontogenetic digestive changes and adaptations. *L. guttatus* shows adequate adaptive changes in enzyme activities that correspond to other carnivorous species, with proteolytic activity increasing with growth (Kuzʾmina, 1996; Falcon-Hidalgo *et al*., 2011).

The high activities found in acid proteases of all juvenile stages of *L. guttatus* is an important characteristic leading to a more efficient breakdown and utilization of feed protein. The acid protease activities reported for EJ are comparable with those reported in gilthead seabream (*Sparus aurata*), common dentex (*Dentex dentex*) (Alarcón *et al*., 1998) and *L. novemfasciatus* and *L. argentiventris* (Alarcón *et al.*, 2001) (sampled fish weighed between 25 and 50 g). Gastric digestion increase intestinal hydrolysis, leading to a significant shift in soluble polypeptides to oligo- and dipeptides (Yasumaru & Lemos, 2014). Therefore, because acid protease activities are higher with growth in *L. guttatus*, fishmeal could be reduced in the balanced diets of larger fish, and a higher amount of plant or animal by-products as protein sources in feeds could be used.

Specific activity between PC and the three intestine sections did not show variation, however, tissue activity showed higher activity in PC that other intestine section, related to tissue size. Pyloric caeca in fish is an organ with principal function of increase surface area and hence the nutrient uptake (digestion and absorption) capacity of fish, where PC is reported as the major site of uptake, even than the entire remaining alimentary tract (Buddington & Diamond, 1986), as reported in the present study.

The total alkaline protease activity at 37°C for *L. guttatus* in the three juvenile stages are comparable to those reported for *L. argentiventris* (52.3 ± 3.9 U mg $prot^{-1}$) and *L. novemfasciatus* (17.2 ± 1.1 U mg $prot^{-1}$) (Alarcón *et al.*, 2001). The use of a non-specific technique (Walter, 1984) at a neutral and basic pH enables the quantification of activities of different proteases, such as trypsin, chymotrypsin, carboxypeptidases, aminopeptidases, elastases and collagenases as the main proteases that acts together as reported in several fish species (Torrissen,1987; Klomklao, 2008; Unajak *et al*., 2012). This demonstrates the real digestion capacity of the species over a wide range of parameters. In this sense, the extracts use from the digestive system of the species of interest is more suitable, because a complex battery of digestive enzymes catalyses digestion (Alarcón *et al*., 2002). On the other hand, temperatures and pH used in the assays are only operational parameters used to understand changes in enzymatic activities among juvenile *L. guttatus* stages and are not exactly the same as natural conditions. Moreover, similar to other poikilothermic fish species, *L. guttatus* possess a maximum and minimum tolerance for some parameters.

Most fish species have two or three major pepsins with an optimum haemoglobin digestion at a pH between 2 and 4 (Gildberg & Raa, 1983; Klomklao, 2008). In this study, the optimum pepsin-like enzyme activity occurred at pH 2 for MJ and at pH 3 for EJ and LJ, coupled to total inhibition of pepsin with pepstatin A in the three juvenile stages and changes in relative activity (%) at different temperatures and pH indicates the existence of at least two pepsin isoforms. Klomklao *et al.* (2007) reported pepsin A and pepsin B characterization from giant grenadier (*Coryphaenoides pectoralis*) with different optimum pH (3.0 and 3.5, respectively) and an optimum temperature of 45°C. Chiu & Pan (2002) report that two pepsins, designated PI and PII, isolated from stomach of juvenile and adult of Japanese eel (*Anguilla japonica*) and differences in optimum pH and total activity between isoforms were found.

Alkaline proteases present a wide range of activity; over 80% of the relative activity occurred in the pH range of 7 to 10 for the three juvenile stages. EJ and MJ present a relative activity that fell to 50% at a pH of 5, while LJ conserve relative activity (80%). The presence of other alkaline protease type such as thiol protease-type called cathepsin, which appears to be pancreatic or intestinal in origin (Kirschke & Barret, 1987) could explain these results. Cathepsins from different species display maximum activity over a broad pH range from 3.5 to 8.0 (Zeef & Dennison, 1988).

Four serine-protease inhibitor types were used (TLCK, PMFS, SBTI and Ovo), where TLCK and SBTI showed a more trypsin-like affinity for enzyme inhibitors. Strong relative inhibition of SBT1 was found in EJ (54.9 ± 6.6%), while other inhibitors showed a lower relative contribution (14.2 ± 1.3%, 15.7 ± 2.5% and 18.5 ± 1.2% for TLCK, PMFS and Ovo, respectively). For all of the serine protease inhibitors, a decreased tendency was found with growth. Serine-proteases are found in different isoforms in the pyloric caeca and intestine in some fishes (Falcon-Hidalgo *et al.*, 2011; Unajak *et al.*, 2012); therefore, differences in affinity with inhibitor type could exist, which could explain variations in the relative contributions of enzyme type over different juvenile stages in *L. guttatus*.

A metalloproteinase type inhibitor (Phen) showed a tendency to decrease with age, and fluctuated between 32.7 ± 2.0 and 23.3 ± 1.1 for EJ and LJ, respectively. Collagenolytic serine proteases differ from muscle collagenases, which belong to zinc metalloproteinase, and physiological function in several organisms is attributed to their digestive power (Kristjansson *et al*., 1995), and they display both trypsin-like and chymotrypsin-like activities (Haard, 1994) and have been previously characterized in Atlantic cod (*Gadus morhua*) (Kristjansson *et al*., 1995). Other authors report in mammalian and fish pancreases existence of two zinc carboxypeptidases, previous reported in marine organisms (Hajjou *et al.*, 1995; Kishimura *et al*., 2006).

Total alkaline and trypsin-like optimum activities at different temperatures show differences between the EJ stage and other juvenile life stages. A different type of enzyme or isozyme can be expressed in the EJ stage and not in other juvenile stages, results that are in accordance with other reports (Torrerissen, 1987; Unajak *et al*., 2012). In conjunction with the above-mentioned, total specific trypsin-like activity was four times higher in EJ than other juvenile stages (Table 4), where the specific activity reported for MJ and LJ are in accordance with the trypsin-like activity reported for *L. vitta* (Khantaphant & Benjakul, 2010), with 21.9 U mg protein^{-1} from pyloric caeca extract. Nevertheless, the optimum trypsin activity differs from the total alkaline protease optimum in the EJ stage, and combined with the decrease of inhibition average by serine-inhibitors with fish growth, indicate that trypsin-like enzymes are not the main digestive alkaline enzymes and other types of enzymes are present in this species.

In this study, no gonad was found in 400 g fish represented by the LJ stage, therefore, changes found in protease activity under different conditions could not be attributed to the onset of sexual maturity. In addition, some authors report enzyme changes during the ontogenesis of fish, suggesting that specific types of protease could be produced at a specific fish age by means of fish ontogenesis (Torrissen, 1987; Kuz'mina, 1996; Bassompierre *et al.*, 1998; Chiu & Pan, 2002; Rathore *et al*., 2005; Chakrabarti *et al*., 2006; Unajak *et al*., 2012).

In conclusion, the digestive system of spotted rose snapper is highly efficient in the breakdown of protein. The high pepsin activities 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 substitution or diversification in the type of alkaline enzymes exists. The present study represents the first research conducted on digestive proteases activities with comparative objective in snapper juveniles and that will serve as a basis for future studies in SDS-Page electrophoresis and *in vitro* digestibility assays with different protein sources, that will provide more information about the digestive physiology of *L. guttatus* at different juvenile stages, which will be useful to develop efficient diets to optimize growth under cultural conditions.

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