Centro de Investigación en Alimentación y Desarrollo, A.C

Estudio de la capacidad digestiva proteica en la ontogenia juvenil del pargo flamenco *Lutjanus guttatus*

POR

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Resumen

La acuicultura es un sector con una alta velocidad de crecimiento, donde nuevas especies como el pargo flamenco, *Lutjanus guttatus* se encuentran en proceso de desarrollo e implementación de tecnologías de cultivo. El pargo flamenco es una especie con aceptación y valor comercial altos en Latinoamérica. Además, el ciclo reproductivo de dicha especie ya es conocido. Si bien el ciclo de vida ya se completó, el entendimiento profundo de los procesos fisiológicos digestivos durante su etapa de engorda, es importante con la visión de generar un rendimiento mayor en la industria. En este contexto, el objetivo principal de este trabajo fue el generar conocimiento sobre la fisiología digestiva en juveniles de *L. guttatus*, con enfoque en la eficiencia proteica de diferentes tallas juveniles, haciendo uso de técnicas *in vitro* (enzimáticas, pH Stat y electroforéticas). Los resultados indican una tendencia positiva en el aumento de actividad de proteasas ácidas y alcalinas totales con relación a la edad. En el estómago de *L. guttatus* se presentan dos isoformas de pepsina durante toda su etapa juvenil, mientras que en ciegos pilóricos e intestino, una diversificación del número y tipo de enzimas existe con el aumento de edad. Los juveniles tempranos presentan cinco bandas con actividad proteolítica alcalina, mientras en etapas juveniles tardías se presentan cuatro bandas adicionales con capacidad proteica. Ensayos realizados de digestibilidad *in vitro*, muestran una capacidad digestiva estomacal comparable durante toda la etapa juvenil, mientras que la actividad digestiva en ciegos pilóricos e intestino cambia de acuerdo a la talla juvenil y la fuente proteica sometida. Esto también se refleja en la cantidad total de amino ácidos liberados. Dado los resultados anteriores, se realizó una validación

de los resultados *in vitro* por medio de un ensayo dosis respuesta, en donde se formularon dos dietas experimentales sustituyendo el 100% de harina de pescado por mezclas de harinas vegetales o mezclas de subproductos de animal terrestre. Juveniles tempranos y tardíos fueron alimentados con las mismas dietas experimentales, donde se midió el crecimiento, rendimiento del alimento, índices corporales y parámetros sanguíneos. Los resultados obtenidos, validan los cambios y diferencias (de utilización de distintas fuentes proteicas) de la capacidad de digestión proteica encontradas en ensayos *in vitro*, donde juveniles tempranos mostraron un crecimiento diferencial entre las dietas experimentales, mientras juveniles tardíos no mostraron diferencias en crecimiento entre tratamientos. Es así que el pargo flamenco presenta cambios en su capacidad de digestión proteica relacionada al desarrollo de su ontogenia juvenil.

Palabra clave: *Lutjanus guttatus*, Enzimas, pH Stat, Electroforesis, Aminoácidos libres, Crecimiento, Parámetros sanguíneos

Abstract

The aquaculture sector shows a high increase in production, where new species such as spotted rose snapper, *Lutjanus guttatus*, a species that has been in focus to constant development. Spotted rose snapper presents a high acceptability value in Latin-America, besides that its reproductive cycle is well known. Even though a deep understanding on the digestive physiology processes to produce commercial size organisms is still lacking. Thus, the aim of the present work was the knowledge generation about the digestive physiology of juveniles *L. guttatus*. Within this context the protein digestibility was comparatively studied among different size organisms (20 to 450 g average weight), using *in vitro* techniques (enzymatic, pH Stat, electrophoretic). Results obtained shows a significant positive tendency to increase the acid and alkaline protease specific activity with age. Spotted rose snapper stomach presents two pepsin isoforms along the juvenile stage, whereas in the pyloric caeca and intestine a diversification on type and number of digestive enzymes increases. In early juveniles five alkaline are presents bands, while late juveniles present four additional bands with alkaline proteolytic activity. In vitro digestibility assays shows equivalent gastric digestive activity among all juvenile stages, while the digestive activity in the pyloric caeca and intestine changes accordingly their age and protein source exposed, which simultaneously, is reflected with the total free amino acids produced. Given the above results, a validation with an in vitro technique was performed through a dose response trial. Then, two experimental diets were formulated substituting 100% of fishmeal by vegetable and renders mixtures to feed comparatively early and late juveniles. The response

parameters like growth, somatic index and blood chemistry were evaluated. Results validate what previously was obtained where changes and differences in digestive activity where observed among the juvenile stages. Early juveniles resulted in a differential growth between both experimental diets, whereas the late stages were unable to show differences in growth. Therefore it can be concluded that rose spotted snapper presents changes in protein digestive capacity related to ontogeny juvenile development.

Key words: *Lutjanus guttatus*, Enzymes, pH Stat, Electrophoresis, Total free amino acids, Growth, Blood chemistry

CAPÍTULO 1

Introducción general

Los peces son clasificados como detritívoros, herbívoros, omnívoros o carnívoros, ya que presentan adaptaciones morfológicas digestivas de acuerdo a sus hábitos alimenticios. Independiente de los hábitos alimenticios de la especie, los peces pueden modificar su comportamiento y metabolismo digestivo en respuesta a la disponibilidad de alimento (Rust, 2002; Pérez-Jiménez *et al*., 2009). Es así que el crecimiento y la eficiencia provocada por la ingesta de un alimento en peces, dependerá de su capacidad fisiológica y bioquímica para digerir y transformar nutrientes, sin embargo muchos factores bióticos y abióticos pueden influenciar el estado fisiológico del animal y los procesos relacionados con la digestión, absorción y transformación de dichos nutrientes (Furnè *et al*., 2008).

En la actualidad existe una investigación amplia para el desarrollo de dietas formuladas, así como la búsqueda de nuevas fuentes de ingredientes de bajo costo con digestibilidad alta. Conocimiento que será importante para la formulación y manufactura de dietas, por lo que el entender la capacidad digestiva de las especies será importante para el diseño de nuevas formulaciones. Los peces presentan un paquete enzimático complejo que incluye gran variedad de proteasas, lipasas y carbohidrasas que les permite llevar a cabo la digestión de diferentes nutrientes.

Dentro de las enzimas digestivas, las proteasas juegan un papel fundamental en la digestión para que se traduzca en un crecimiento y supervivencia altos. Las proteasas encontradas dentro de los órganos digestivos de peces, son las responsables de catalizar la hidrolizan de los enlaces peptídicos (Klomklao, 2008), donde se incluye pepsina, gastricinas, tripsinas, quimotripsinas, colagenasas, elastasas, carboxipeptidasas y carboxilesterasas (Haard, 1994; Simpson, 2000) de

las cuales tripsina, quimotripsina y pepsina son las enzimas digestivas más importantes debido a su abundancia y alta actividad proteolítica de acuerdo a estudios de caracterización de enzimas digestivas reportados en diferentes peces (Castillo-Yáñez et al., 2004, 2005, 2006; Klomklao et al., 2004, 2007).

Dentro de las enzimas digestivas en peces carnívoros, las lipasas constituyen un grupo de enzimas definido como carboxilesterasas, encargadas de catalizar la hidrólisis de acilgliceroles de cadena larga (Smichi et al., 2013), donde se incluye la fosfolipasa A2, lipasa pancreática y lipasas dependientes de sales biliares (Izquierdo et al., 2000). Aunado, la digestión de carbohidratos en peces se lleva a cabo por enzimas endógenas denominadas α-amilasa y disacaridasas, de origen pancreático e intestinal, las cuales presenta una baja o moderada actividad en peces carnívoros.

Los procesos enzimáticos digestivos suceden de forma secuencial y/o en conjunto, donde dichos procesos se encuentran mediados por detección luminal de las células epiteliales tanto por señales paracrinas / autocrinas como señales endócrinas por medio del sistema vascular sanguíneo. Los compuestos de señalización son producidos por células del sistema gastroenteropancreatico. Los procesos regulados por hormonas que forman parte del proceso digestivo incluyen, la estimulación de la ingesta de alimento, estimación de secreciones gástricas, movilidad gástrica, estimulación de secreción de enzimas pancreáticas, contracciones de la vesícula biliar, alteración de la movilidad intestinal de vesícula biliar, así como el control de la inhibición de la ingesta de alimento. Dichos procesos son regulados principalmente por miembros de familias de hormonas

gastroenteropancreaticas, donde sus representantes más estudiados y conocidos son gastrina, colecistoquinina (CCK), insulina, secretina, glucagón y somatostatina. (Buddington y Krogdahl 2004; Takei y Loretz 2011).

Como parte de un esquema descriptivo del proceso digestivo de nutrientes, la digestión en un pez juvenil carnívoro con todos sus órganos desarrollados comienza en el estómago. Las células G secretan gastrina, lo que estimula la secreción combinada de ácido clorhídrico y la principal enzima proteolítica gástrica (pepsina) a partir de su forma inactiva, llamada zimógeno o pro-enzima (pepsinógeno), secretados a partir de las glándulas oxínticas gástricas, incrementando a su vez la movilidad estomacal. Ya una vez que el alimento está en el estómago, la porción solubilizada (quimo) pasará a través del píloro hacia los ciegos pilóricos e intestino (Rust, 2002; Buddington y Krogdahl, 2004; Bakke et al., 2011). El píloro es el músculo que controla el tiempo de salida de los jugos gástricos hacia ciegos pilóricos e intestinos y por ende el responsable de retener el alimento en estómago. Una vez que el quimo pasa a los ciegos pilóricos e intestino, la vesícula y el páncreas secretan sales biliares y jugos pancreáticos respectivamente, inducido por la hormona CCK, encargada de mediar esta ruta metabólica. Las enzimas proteolíticas y co-lipasas son secretadas como zimógenos, las cuales son activadas en el lumen intestinal, mientras que lipasas y amilasas son liberadas en su forma activa. La cascada de eventos que genera la activación de los zimógenos es iniciada por la enteroquinasa, secretada por células intestinales. Las enteroquinasas activan a la tripsina a partir de su zimógeno (tripsinógeno) y a su vez la tripsina posee la importante función de activarse a ella misma así como a otras enzimas digestivas

(elastasas, carboxipeptidasas A y B, quimotripsina y co-lipasa en algunos casos) (Cao et al., 2000; Bakke et al., 2011). Aunado al proceso antes descrito y para contrarrestar el pH ácido que es vertido a ciegos pilóricos e intestino por el paso del quimo, el páncreas se encarga de secretar jugos gástricos cargados de bicarbonatos, lo que genera un cambio en el pH a alcalino lo que inactiva a la pepsina y deja las condiciones idóneas para el funcionamiento de otras enzimas (proteasas, lipasas y carbohidrasas) (Rust, 2002; Bakke et al., 2011).

En peces, se ha llevado a cabo un gran esfuerzo en el entendimiento de las adaptaciones y cambios fisiológicos digestivos durante la ontogenia temprana de diferentes especies, la cual ha mejorado a través de numerosas investigaciones en diferentes especies de peces marinos (Kolkovski, 2001; Zambonino-Infante y Cahu, 2001; Lazo et al., 2007; Rønnestad et al., 2007; Álvarez-González et al., 2008; Galaviz et al., 2012; Salze et al., 2012; Moguel-Hernández et al., 2013). Por otro lado, otras investigaciones enfocan sus esfuerzos en estudios de digestibilidad de diferentes nutrientes proteicos, utilizando paquetes enzimáticos o enzimas purificadas propias de los peces, con la finalidad de conocer su afinidad a diferentes fuentes nutricionales. Estas investigaciones se basan en el uso de la técnica pH Stat, tanto en peces como crustáceos (Dimes y Haard, 1994; Dimes et al., 1994; El-Mowafi et al., 2000; Alarcón et al., 2002; Lemos et al., 2009; Martínez-Montaño et al., 2010, 2011; Tibbetts et al., 2011a, 2011b).

Es así que la comprensión de la capacidad de digestión en especies de interés es de gran utilidad para la formulación de alimento específicos, los cuales son esenciales para el desarrollo sostenible de la acuicultura. Es por ello que la mejora

de los alimentos acuícolas, tanto en la combinación de diferentes fuentes, así como la búsqueda de nuevas fuentes de ingredientes es necesaria para incrementar la eficiencia de producción comercial de diversas especies de peces. Lo anterior, se debe principalmente a que la oferta mundial de harina de pescado y aceite de pescado es finita y plenamente utilizada como principales fuentes nutricionales para la elaboración de dietas para engorda animal (Tacon y Metian, 2008). Las fuentes alimenticias alternativas, pueden contener perfiles inadecuados de amino ácidos, así como compuestos y factores anti nutricionales que afecten el proceso digestivo. Por ende, es necesario entender los procesos y la regulación de las funciones fisiológicas en peces de cultivo (Rust, 2002).

La investigación a nivel mundial sobre la utilización de diferentes ingredientes, ya sean productos o subproductos de peces, animales y plantas ha sido constante. Fuentes que actualmente se encuentran disponibles en el mercado para la formulación de alimentos para peces (Hardy, 2010; Hardy y Barrows, 2002). Ingredientes que varían considerablemente en su calidad proteica y perfil de nutrientes de acuerdo a su frescura, origen, especie, temporada de cosecha y otros factores (Lemos y Tacon, 2011) que dan lugar a resultados variables en digestibilidad (Klomklao, 2008). El entender los procesos digestivos en peces contribuye a la optimización y retención de la proteína dietaría, lo que se traduce en crecimiento y al mismo tiempo en la reducción de los desechos nitrogenados (Ahmed y Khan, 2004; Crab *et al*., 2007).

La especie en estudio es el pargo flamenco (*L. guttatus*), el cual forma parte de la familia Lutjanidae, constituida por peces depredadores con hábitos de alimentación

variable, donde todos son carnívoros, alimentándose principalmente de peces y crustáceos bentónicos (Allen 1987; Vázquez *et al*., 2008). En la especie, ya se han realizado grandes esfuerzos en la búsqueda de fuentes proteicas alternativas para la manufactura de alimentos formulados en el pargo flamenco, con la finalidad de disminuir la dependencia hacia la harina de pescado. En la Tabla 1 se muestra un resumen de las fuentes alternativas utilizadas previamente en dietas experimentales y su porcentaje de sustitución sin mostrar efectos negativos en crecimiento.

Tabla 1. Fuentes proteicas alternativas utilizadas para la formulación de dietas para el pargo flamenco *L. guttatus*.

Experimento	Fuente	Peso (g)	% sustitución
García-Ortega, 2010	Concentrado de soya	16.7	50
Silva-Carrillo *et al.,* 2012	Pasta de soya	17.7	20
Hernández *et al*., 2014	Subproductos harina de ave- pet grade	11.0	90
Hernández *et al*., 2014	Subproductos harina de ave- food grade	11.0	50
Hernández *et al*., 2014	Subproductos de atún	5.4	25-30

Si bien, los esfuerzos generados en la especie han arrojado información valiosa que ha optimizado su cultivo y engorda, es necesario dilucidar los procesos fisiológicos digestivos que generan los resultados obtenidos y si éstos varían de acuerdo a su etapa de vida juvenil, que representa la etapa de engorda del pargo flamenco.

Objetivos

Objetivo general

Generar conocimiento sobre la fisiología digestiva en juveniles de pargo flamenco (*Lutjanus guttatus*) mediante el estudio de las principales proteasas digestivas y aplicar dicho conocimiento para el desarrollo de técnicas de digestión *in vitro* que permitan valorar y caracterizar diferentes fuentes proteicas (animales y vegetales) con el fin de generar dietas altamente específicas para la especie.

Objetivos particulares

1) Determinar la actividad y el efecto del pH, temperatura e inhibidores en las principales proteasas digestivas (pepsina, proteasas alcalinas totales y tripsina) del pargo flamenco en 5 segmentos digestivos (estómago, ciegos pilóricos, intestino distal, intestino medio e intestino proximal), comparativo en tres tallas (20, 200 y 400 g de peso).

2) Determinar mediante electroforesis el número/tipo de enzimas digestivas en dos tallas del pargo flamenco (20 y 400 g) y su efecto en el grado de hidrólisis y cinética de liberación de AA en ensayos *in vitro* utilizando diversas fuentes proteicas (animales y vegetales) con las enzimas propias del organismo.

3) Realizar un ensayo dosis-respuesta para la validación de resultados obtenidos en ensayos previos, utilizando organismos de 60 y 450 gramos con 3 dietas experimentales con una sustitución del 100% del total de la proteína de harina de

pescado por una combinación de harinas vegetales y/o combinación de harinas de subproductos animales.

Esquema general

La presente tesis se integra en seis capítulos. La mayoría conformados por documentos preparados para ser publicados en revistas indizadas, que a su vez cubren los objetivos particulares de la presente investigación. Dentro del esquema, se incluyó una introducción general (Capitulo 1; presente capitulo), seguida por tres documentos en formato de publicación (Capitulo 2, 3 y 4), seguida por la discusión general y las conclusiones (Capitulo 5 y 6). Los documentos se anexan en el formato e idioma en el cual fueron aceptados, con estatus de sometidos o por someter. Una descripción general de los documentos en formato publicación que integran esta tesis es la siguiente:

Capítulo 2: Se realizó el estudio del efecto del pH y temperatura sobre las principales proteasas digestivas de manera comparativa entre tres tallas de juveniles de la especie en estudio, así como la identificación del tipo de proteasas presentes con el uso de inhibidores específicos.

Capítulo 3: Se realizó la continuación de estudio previo a partir de las diferencias encontradas en la primera investigación entre dos tallas de juveniles, donde se caracterizó el número y tipo de bandas enzimáticas durante el proceso digestivo ácido y alcalino. Además de comprobar que dichas diferencias poseen efecto directo sobre el grado de digestibilidad *in vitro* de diferentes fuentes proteicas, lo que también generó un efecto sobre la liberación total de aminoácidos.

Capítulo 4: Se realizó un ensayo dosis-respuesta de manera comparativa entre dos tallas de juveniles, donde se elaboraron dos dietas experimentales con la sustitución

total de harina de pescado por mezclas de subproductos animales o vegetales, con la finalidad de probar el efecto de los cambios enzimáticos entre tallas, sobre parámetros de crecimiento, índices somáticos y química sanguínea.

CAPÍTULO 2

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

Capitulo publicado en:

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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.

Key words: digestive enzymes, pepsin, trypsin, protease inhibitors

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 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 estadios 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 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 la capacidad digestiva de Lutjanidos.

Palabras clave: enzimas digestivas, pepsina, tripsina, inhibidores de proteasas

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 size for the market (450 g) (Herrera-Ulloa 2010). Reproduction techniques for juvenile mass production in hatcheries have been developed on a pilot scale for this species in Mexico (Ibarra-Castro and 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 (*Lutjanus argentiventris*) and Pacific dog snapper (*Lutjanus novemfasciatus*). Additionally, Khantaphant and Benjakul (2008;

2010) reported the skin gelatin hydrolyzation capability in brown stripe red snapper (*Lutjanus 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 (Perez-Jimenez *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 et al., 1987), and these variations are related and could affect growth rate and/or feed conversion efficiency (Torrissen and 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 2).

Table 2. Composition and proximate analyses of diet for spotted rose snapper *L. guttatus*.

Tabla 2. Composición y análisis proximales de dieta para pargo flamenco *L. guttatus.*

Ingredients (% 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 lecitine (70%)[6]	1.50
Vitamin C[6]	0.10
Alginate[4]	3.00
Proximate analyses (% dry weight)	
Dry matter	7.09
Crude protein	43.06
Crude fat	13.86
Ash	14.01
Nitrogen free extract	15.27

[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 Drogueria Cosmopolita, S.A. de C.V. México, D.F., México.
5 Trouw Nutrition México S.A. de C.V. (by courtesy). *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; 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.
6 DSM Nutritional Products México S.A. de C.V., El Salto, Jalisco, México.

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. The parameters and biometric indices of fish used in the assays are summarized in Table 3.

Table 3. Biometric parameters for three juvenile stages of spotted rose snapper *Lutjanus guttatus*.

Tabla 3. Parámetros biométricos para tres estadios juveniles de pargo flamenco *Lutjanus guttatus*.

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]

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.

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 for 24 hours 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 9g 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)

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 following buffers were used: 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 4.

Table 4. 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*.

Tabla 4. Actividad de proteasas en estómago (ST), ciegos pilóricos (PC), intestino proximal (PI), medio (MI) y distal (DI) en tres estadios juveniles de pargo flamenco *Lutjanus guttatus*.

	*Specific Activity (U mg protein^{-1}) of crude extract **Tissue Activity (U wet tissue)				
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}
**	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}

EJ, MJ and LJ represent early, middle and late stage juveniles, respectively.
Lower-case show differences in columns, upper-case show differences in rows.

The stomach acid proteolytic activity showed significantly higher specific and tissue activities (P≤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≤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≤0.001) were found

between stages when individual sections were compared. The trypsin-like specific activity showed a significantly higher (P≤0.001) value in the EJ stage than MJ and LJ stages (Table 5), nevertheless, tissue activity values increase with increasing life stage (P≤0.001).

Table 5. 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).

Tabla 5. Actividad tipo tripsina de ciegos pilóricos en tres estadios juveniles de pargo flamenco *Lutjanus guttatus*. Diferentes superíndices en la misma fila, indican diferencias significativas (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}

EJ, MJ and LJ represent early, middle and late stage juveniles, respectively.

Temperature effect on acid and alkaline protease activity

The three juvenile stages presented optimum temperature of acid proteases at 45°C (Fig. 1a) (P≤0.001). Acid proteases relative activity at 30°C showed differences between EJ, MJ (70%) and LJ (40%) (P≤0.001), while relative activity at 50°C showed differences between EJ (80%) and MJ and LJ (60%) (P≤0.001).

The optimum temperature of total alkaline proteases was 55°C for EJ, 50°C for MJ and LJ (Fig. 1b) (P≤0.001). Differences were found in the relative activity percent at 20, 30, 40 and 60 °C between EJ and the other stages (P≤0.001). In general terms, LJ showed higher relative activities (%) than EJ and MJ in total alkaline protease activity, when individual sections were compared.

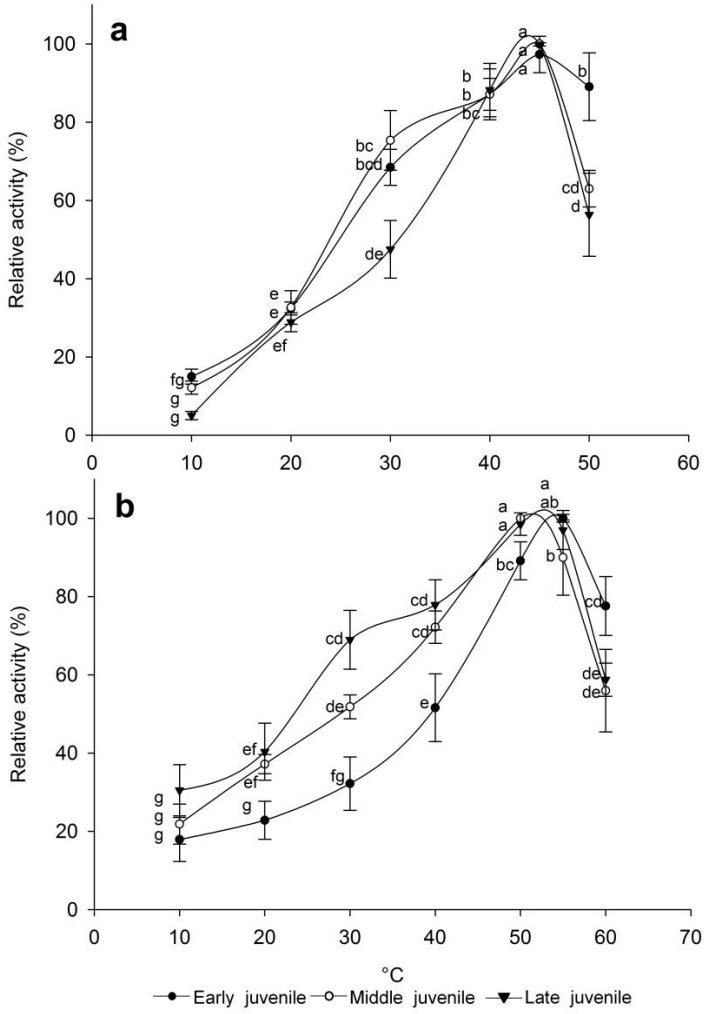

Figure 1. Temperature effects (°C) on the relative activity of acid (a) and alkaline proteases (b) in three juvenile stages of *Lutjanus guttatus*.

Figura 1. Efecto de la temperatura (°C) sobre la actividad relativa de proteasas ácidas (a) y alcalinas (b) en tres estadios juveniles de pargo flamenco *Lutjanus guttatus*.

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 (5-10) and an optimum at pH 9.0 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%) ($P \leq 0.001$).

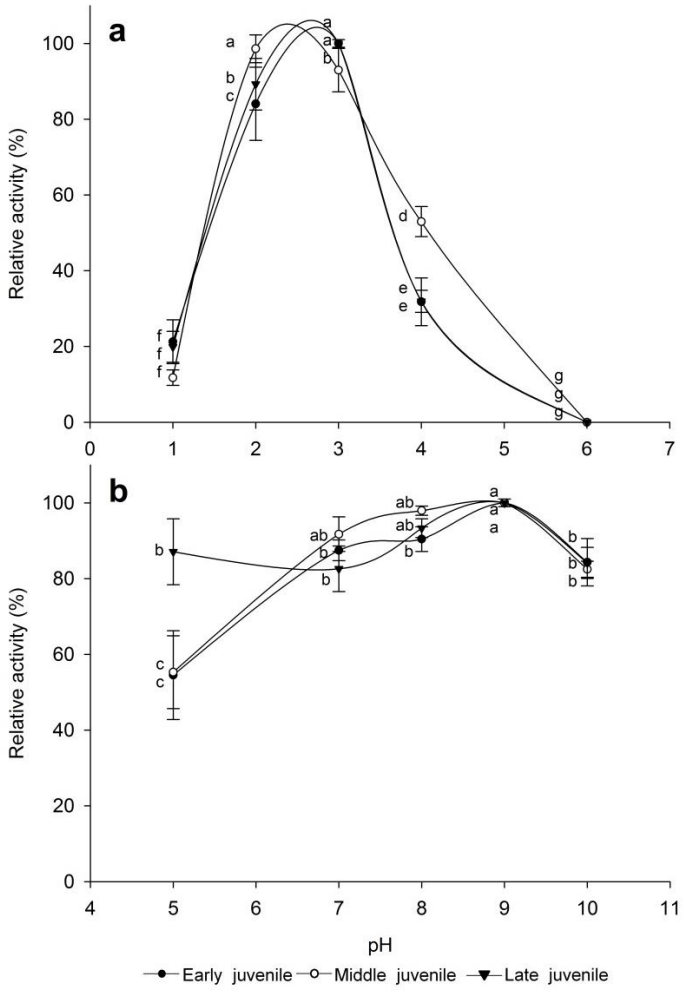

Figure 2. pH effects on the relative activity of acid (a) and alkaline proteases (b) in three juvenile stages of *Lutjanus guttatus*.

Figura 2. Efecto del pH sobre la actividad relativa de proteasas ácidas (a) y alcalinas (b) en tres estadios juveniles de pargo flamenco *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≤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).

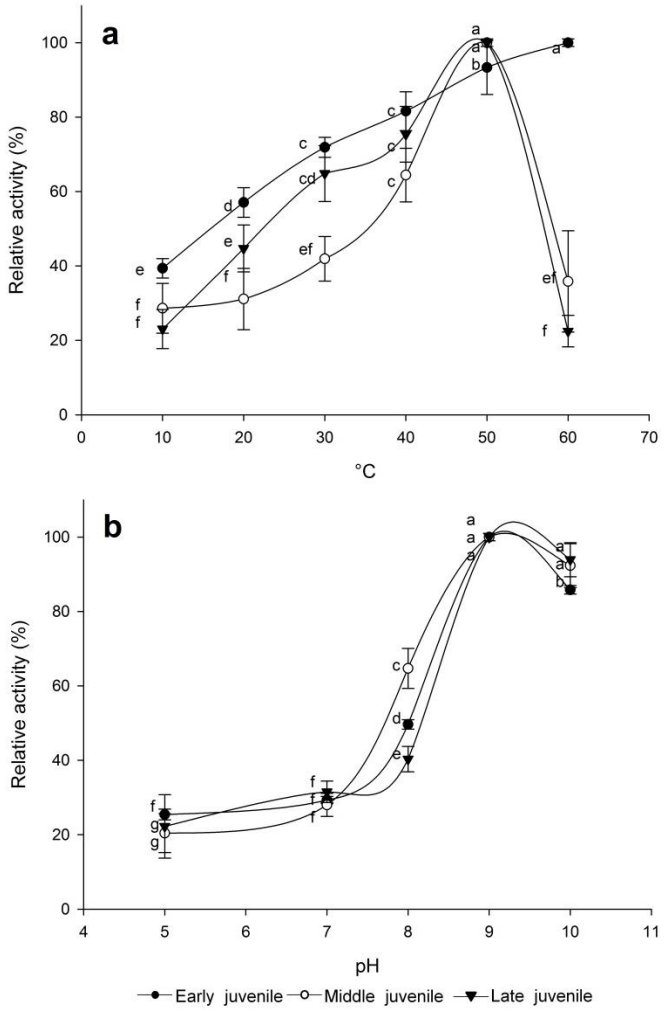

Figure 3. Temperature and pH effects on the relative trypsin-like activities in three juvenile stages of *Lutjanus guttatus*.

Figura 3. Efecto de la temperatura y pH sobre la actividad relativa de tripsinas en tres estadios juveniles de pargo *flamenco Lutjanus guttatus*.

Specific inhibitors effects

Pepstatin A inhibited the total activities in stomach extracts in all juvenile stages (Fig. 4).

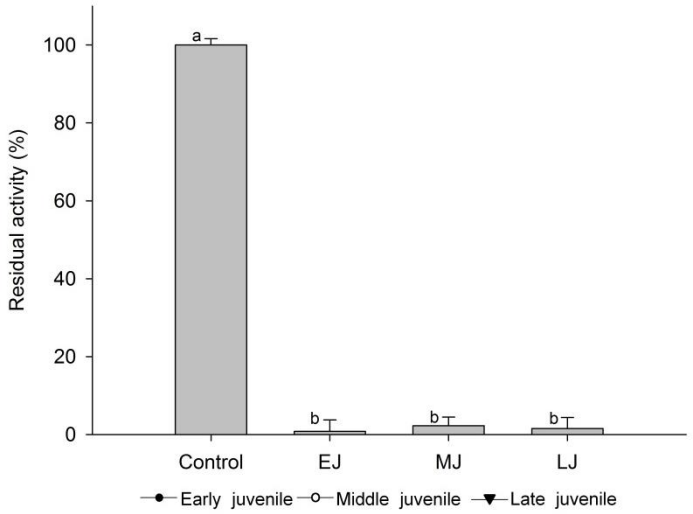

Figure 4. Percent of residual activity in stomach extract after incubation with pepstatin A in three juvenile stages of *Lutjanus guttatus*. EJ, MJ and LJ represent early, middle and late stage juveniles, respectively.

Figura 4. Porcentaje de actividad residual en extracto estomacal después de incubación con pepstatin A en tres estadios juveniles de pargo flamenco *Lutjanus guttatus*. EJ, MJ y LJ representan juveniles tempranos, medios y tardíos, respectivamente

The percent of alkaline protease inhibition are summarized in Table 6. 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 6. The percent of activity inhibition in pyloric caeca after incubation with enzyme specific inhibitors in three juvenile stages of spotted rose snapper *Lutjanus guttatus.* Different superscript within columns indicate significant differences (P<0.05).

Tabla 6. Porcentaje de inhibición de actividad en ciegos pilóricos después de incubación con inhibidores específicos en tres estadios juveniles de pargo flamenco *Lutjanus guttatus*. Diferentes superíndices en la misma columna, representan diferencias significativas (P<0.05).

	Percentage of activity inhibition					
*mmol^{-1}	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}

EJ, MJ and LJ represent early, middle and late stage juveniles, respectively.
*Inhibitor concentration
** TPCK (N-tosyl-L-phenyl-chloromethyl ketone), TLCK (N_{α}-tosyl-L-lysine chloromethyl ketone hydrochloride), PMSF (phenylmethylsulfonyl fluoride), SBTI (soybean trypsin inhibitor), Phen (1,10-Phenanthroline), Ovo (Type II-T: Turkey egg Ovomucoid)

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) (Kuz'mina, 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 3), 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 and 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 (Kuz'mina, 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 (Kuz̀mina, 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 and 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 didn't 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 and 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 *et al*. 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 pHs 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 and 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 and 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 and 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 and 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 *et al*. 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 5), where the specific activity reported for MJ and LJ are in accordance with the trypsin-like activity reported for *L. vitta* (Khantaphant and Benjakul 2010), with 21.9 Units 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; Kuzmina, 1996; Bassompierre *et al.* 1998; Chiu and 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.

ACKNOWLEDGMENTS

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CAPITULO 3

Comparative in vitro protein digestibility between juvenile stages of spotted rose snapper (*Lutjanus guttatus*)

Capitulo por someter:

Peña E, Hernández C, Ibarra-Castro L, Álvarez-González CA. 2015. Comparative in vitro protein digestibility between juvenile stages of spotted rose snapper (*Lutjanus guttatus*). Sometido a Aquaculture Nutrition.

Abstract

This study aimed to develop in vitro comparison of protein digestibility between two juvenile stages of *L. guttatus*, using crude extracts from stomach, pyloric caeca and intestine. The in vitro degree of protein hydrolysis of 13 feed ingredients including marine, animal and vegetables were measured by a pH Stat system. Total amino acid release and electrophoresis where used as complementary techniques. Degree of hydrolysis showed differences in sources between juvenile stages, mainly in alkaline hydrolysis phase. Best results in degree of hydrolysis and total amino acid release were present in fishmeal and squid meal as marine products, meat porcine meal and poultry by products meal as renders and soybean meal and canola meal as vegetable products that represents better protein sources to be use in practical diets as base of dietary protein

Stomach zymogram showed two pepsin isoforms in both juvenile stages. Pyloric caeca and intestine zymograms showed five bands with proteolytic activity in early juvenile, meanwhile four additional bands were found in late juvenile. Alkaline proteases were identified as serine and metalloproteases. Thus, *L. guttatus* modify alkaline enzyme pattern with juvenile development that modify hydrolysis degree and amino acid release of different protein sources.

Keywords: Proteases, digestibility, pH-Stat, amino acids, electrophoresis, snapper

INTRODUCTION

The spotted rose snapper (SRS) as all Lutjanidae family are carnivores marine fish species distributed in tropical zones that primarily feeds on demersal organisms, such as crustaceans and fish (Allen 1995). SRS presents a high potential for intensive culture in Latin America countries (Davis *et al*. 2000), where reproductions techniques to juvenile mass-produce are well developed at pilot level in Mexico (Ibarra-Castro & Alvarez-Lajonchere 2011; Alvarez-Lajonchere *et al*. 2012). For a sustainable aquaculture of species, constant juvenile mass produce is required in fish species industry, the next approach is to obtain sufficient information in digestive physiology and nutritional capacities with the view to manufacture specific diet. In that sense, aquaculture research is facing a challenge to find suitable and economically feasible protein sources to compound diets that substitute fish meal as the main protein source. In SRS, great efforts have been made in the search of alternative protein sources that replace fishmeal, including soybean meal, canola meal, fed grade and pet grade poultry by products and tuna by products meal (Silva-Carrillo *et al*. 2012; Hernández *et al*., 2014a, b, c; Hernandez *et al*. 2015). Digestibility of nutrient or diet depends on its chemical composition, type of ingredients (mainly protein and lipids) 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 is obtained 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, exist great interest and efforts 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 *et al*. 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/modification/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), nevertheless reported changes are attributed to several factors, including age, feeding habit of fishes, diet composition changes and sexual maturity. By the other side, some consistent examples about advantages related to the presence of some digestive isoenzymes in aquatic organisms exist. 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). 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. Previous studies in SRS, address an initial protease comparative characterization between juvenile stages of SRS (20, 190 and 400 g). Stomach extract characterization showed to optimum pH peaks, suggesting the presence of at least two pepsin isoforms, and pepsin activity in all juvenile stages increase with growth. Joined, final pyloric caeca and intestinal alkaline digestion presents a wide range of alkaline proteases, where a substitution or diversifications in the type of alkaline enzymes is proposed, based on previous enzymatic inhibition assays results (Peña *et al*. 2015). Therefore the aim of the presents study was to evaluate differences in acid (stomach extract) and alkaline (pyloric caeca and intestine extract) digestibility, over different marine-based, animal and plant proteins in comparative manner between two juvenile stages of spotted rose snapper.

MATERIALS AND METHODS

Experimental Ingredients

Casein substrate was obtained by Hammarsten quality casein, Research Organics (# Catalog 1082C). Bovine erythrocytes US Biological (# Catalog H1850) was used as hemoglobin substrate. Premium grade fishmeal was obtained from Selecta de Guaymas, S.A. de C.V., Guaymas, Sonora, México. Tuna by -products was obtained by Maz Industrial, S.A de C.V., Mazatlán, Sinaloa, México. Krill meal and squid meal were obtained in PROAQUA, S.A. de C.V., Mazatlán, Sinaloa, México. Meat porcine meal, meat bovine meal and poultry by products meal were obtained by Proteínas Marinas y agropecuarias S.A. de C.V., Guadalajara, Jalisco, México. Wheat gluten meal, corn gluten meal and canola meal were obtained in Droguería Cosmopolita, S.A. de C.V., México, D.F., México. Control diet was manufactured in CIAD for snapper feeding as previously reported (Peña *et al*., 2015).

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.

Experimental Animals

Fish were obtained from the Laboratory of Reproduction and Marine Finfish Hatchery of the Centro de Investigación en Alimentación y Desarrollo (CIAD), Sinaloa, Mexico, from a single spawning of our broodstock. Larviculture, was conducted as previously described by Alvarez- Lajonchère *et al*. (2012). At the end of larviculture management, juveniles where place in nursery step until they reach 20 g average weight (age of 120 days post-hatching). After nursery step, fish were weaning in commercial culture sea cages and where collected when was required to be place in tanks for diet adaptation by 20 days. According to their wet weight, fish where classified in two groups (considered in the juvenile stage): early juvenile (EJ; 21.3±2.6 g; 3 month after hatchery, MAH) and late juvenile (LJ; 400±11.5 g; 12 MAH), as previously reported by Peña *et al*. (2015). Diet adaptation was performed in fiberglass tanks (1500 L) with a constant water flow and fed twice at day (9:00 and 16:00 h) to apparent satiation with a control diet containing fish meal as a main protein source, as previously reported in number of references with *L. guttatus* juveniles (Silva-Carrillo *et al*. 2012; Hernández *et al*. 2014 a, b, c; Peña *et al*. 2015). To obtain viscera for in vitro assays, twenty eight fish per juvenile size were killed and immediately dissected to extract the digestive tract; they were starved for 24 hours to ensure the emptiness of the gut.

Dissection and extract preparation

Digestive tract of each fish was divided in two sections: stomach (St), corresponding to acid digestion phase and pyloric caeca and intestine (PC-I), corresponding to alkaline digestion phase. All procedures were conducted under ice at cold

temperatures (0-4 °C). Dissected segments were frozen at -64 °C for 24 hours and then lyophilized, with the objective to simplify storage and management of organs, where enzyme activity is not affected. Three pools of eight individual St or PC-I were used by both juvenile stages. Lyophilized segment were ice-cold-homogenized with an Ultra-Turrax homogenizer in distilled water, diluted in a ratio of 1:10 (wet weight: volume) for all segments. All homogenates were centrifuged at 16000 g for 15 min at 4 °C, and supernatant were pH adjusted to pH 2.9 to St extracts and pH 8.1 to PC-I extracts and then frozen for future assays.

Enzyme activity determination

Enzymatic activity of acid and alkaline digestive proteases were measured as previously described by Peña *et al*. (2015). Briefly, pepsin-like activity was measured by a modified method of Sarath *et al*. (1989), with denatured hemoglobin (2 % pH 2) as substrate, while total alkaline protease activity was estimated using casein-hydrolysis method by Walter (1984). In both enzymatic reactions, the amount of tyrosine liberated from hemoglobin or casein was determined at 280 nm. One unit (U) of enzymatic activity was defined as the amount of enzyme that produced 1 µg of product released per minute under the conditions described above for each assay. The principal objective to measure acid and alkaline enzyme activity was to adjust activity (Units ml-1) between extracts of juvenile stages to perform digestibility assays and electrophoretic techniques under same enzymatic Units. Total activity (Units ml^{-1}) = (Δabs*reaction final volume (ml))/(MEC*time (min)*extract volume (ml)). Where Δabs represent the increase in absorbance and MEC represents the molar extinction coefficient of tyrosine (0.005 mL/µg/cm).

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 7. *In vitro* hydrolysis assays where performed with crude extracts from St or PC-I pools. To determine protein degree of hydrolysis (DH), every single protein source was incorporated in 5 ml of distilled water 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, every protein source was prepared in substrate solution, nevertheless 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. DH was calculated using volume of HCl needed to maintain acid hydrolysis and alkaline hydrolysis in constant pH values. To calculate possible titrant spent by buffer capacity of the different ingredients, hydrolysis reaction was performed by every ingredient without enzyme extract addition, with the objective to adjust data. All assays were performed by triplicated and procedure was performed for both juvenile stages under same parameters. The DH was calculated using the following algorithm according to Adler-Nissen (1986); $DH\ (\%) = B \times NB \times 1/\alpha \times 1/MP \times 1/h_{tot} \times 100\%$.

Where B (mL) is the volume of NaOH or HCl needed to maintain constant pH of the reactions, NB is the normality of the titrants (0.1 N), 1/α is the average degree of dissociation of the α-amino groups related with the pK of the amino groups at pH and temperature of the reaction, MP is the mass (g) of protein in the reaction and h_{tot} is the total number of peptide bonds for a specific protein source. 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. Samples were frozen to -20 °C until total amino acid quantification technique.

Table 7. 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). This technique is based on conjunction of amino terminal group of AA with *o*-phtaldialdehyde (OPA). 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; solutions were incubated 5 min at room temperature and absorbance were read at 340 nm. TAAR was calculated using standard curve made with decrees L-leucine concentrations. All assays were performed by triplicated.

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). For the analysis of total alkaline proteases, pyloric caeca and intestine where separated and individually carried out in electrophoresis

technique. 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 gels were removed from the cell and soaked in 0.1 mol l^{-1} HCl to lower the pH to 2.0 with the objective to become enzyme active. After 15 min, 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 distilled water washed and 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, while destaining was carried out in a solution of methanol– acetic acid–water (35:10:55). Clear zones revealed the activity of proteases within a few minutes, although well-defined zones were obtained only after 2–4 h of staining.

Electrophoretic techniques were complemented with the use of specific inhibitors, by the method described by Dunn (1989). The CP-I extracts were 1 hour pre-incubated with following inhibitors (1:1 v/v): soybean trypsin inhibitor 250 mmol

l^{-1} (SBT1), N-tosyl-L-phenyl-chloromethyl ketone 10 mmol l^{-1} (TPCK), phenylmethylsulfonyl fluoride 100 mmol l^{-1} (PMSF), *N*α-Tosyl-L-lysine chloromethyl ketone hydrochloride 10 mmol l^{-1} (TLCK), 1,10-Phenanthroline 10 mmol l^{-1} (Phen), Type II-T: Turkey egg Ovomucoid 250 mmol l^{-1} (Ovo) and ethylenediaminetetraacetic acid 10 mmol l^{-1} (EDTA). Stomach extract were 1 hour pre-incubated with pepstatin A 1 mmol l^{-1}.

Molecular weight marker was applied to each SDS-PAGE (five µl per well); (kDa): Fosforilasa b (97.4 kDa), albumin (66.2 kDa), Ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin soybean inhibitor (21.5 kDa), a-Lactoalbúmin (14.4 kDa).The relative electromobility (Rf) was calculated for all zymograms (Igbokwe & Downe 1978). 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, using Quality One version 4.6.5 (Hercules, CA) software program.

Statistical analysis

For assays, three pools of eight fishes per size were used. Data of pH-Stat degree of hydrolysis (%) was arcsin ($x^{1/2}$) transformed, checked for normality and homogeneity, and submitted two-way ANOVA analyses between juvenile stages and the protein ingredient sources ($P \leq 0.05$). 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≤0.05) (Zar 1984).

RESULTS

In vitro degree of hydrolysis (DH)

Hemoglobin presented the highest DH among all ingredients in acid digestion for both juvenile stages (P≤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≤0.05) (Fig. 5A).

Alkaline hydrolysis showed that FM (fish meal) 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≤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≤0.05) (Fig. 5B).

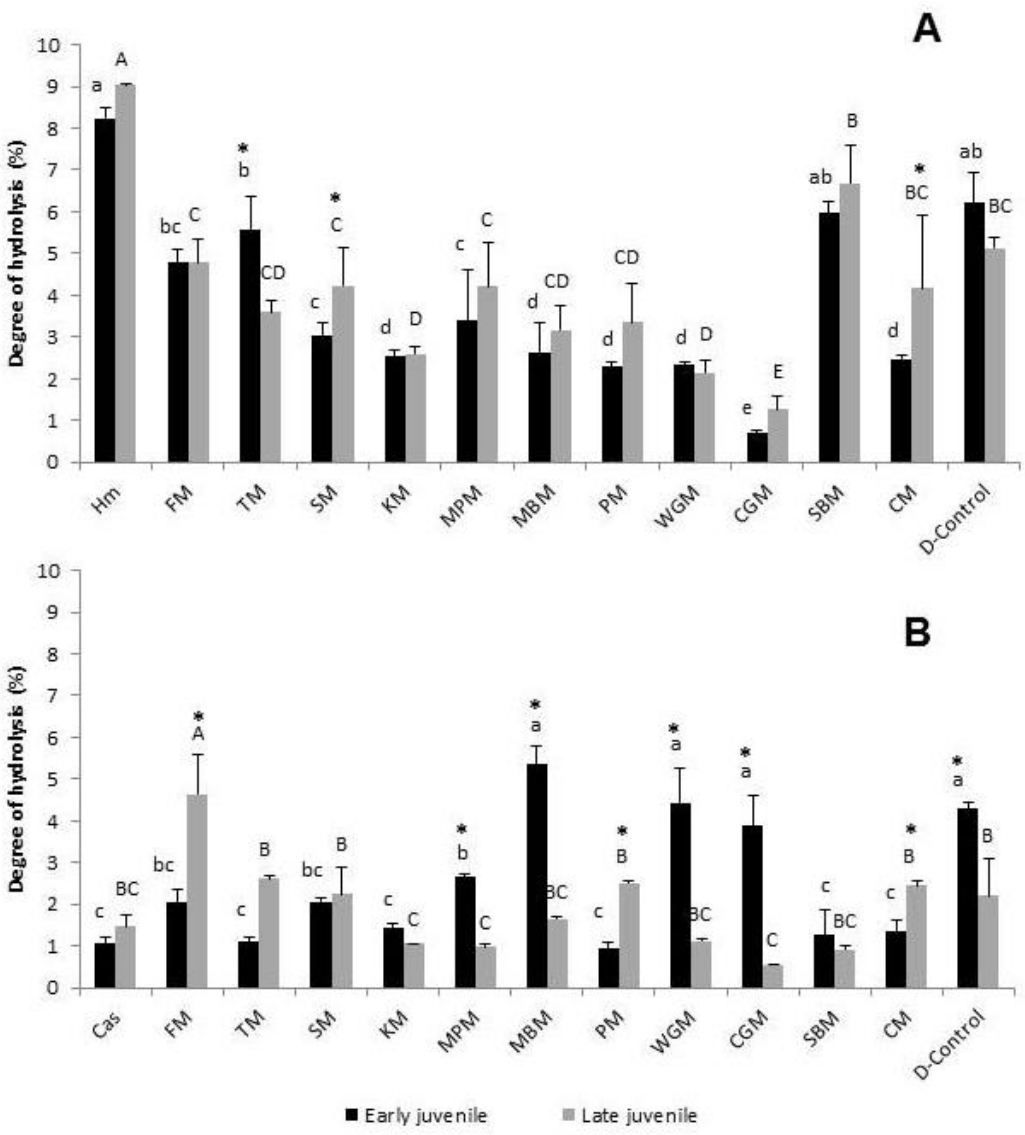

Figure 5. In vitro pH-stat degree of protein hydrolysis (DH) of feed ingredients using digestive enzyme extracts from *L. guttatus* early (20 g) and late juveniles (400g). Ingredients (8 mg ml^{-1} crude protein) were hydrolyzed for 15 min with (A) stomach, or 45 min with (B) pyloric caeca. Hm: hemoglobin; Cas: casein; FM: fish meal; TM: tuna by product meal; SM: squid meal; KM: krill meal; MPM: meat porcine meal; MBM: meat and bovine meal; PM: poultry by product meal; WGM: wheat gluten meal; CGM: corn gluten meal; SBM: soybean meal; CM: canola meal; D-control:

control diet used for *L. guttatus*. 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. 6A). 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 ($P\leq0.05$) (Fig. 6). 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 ($P\leq0.05$) (Fig. 7A). Alkaline hydrolysis in LJ stage showed higher TAAR in MPM, followed by FM, while Cas and SBM showed the lowest TAAR values ($P\leq0.05$) (Fig. 7B).

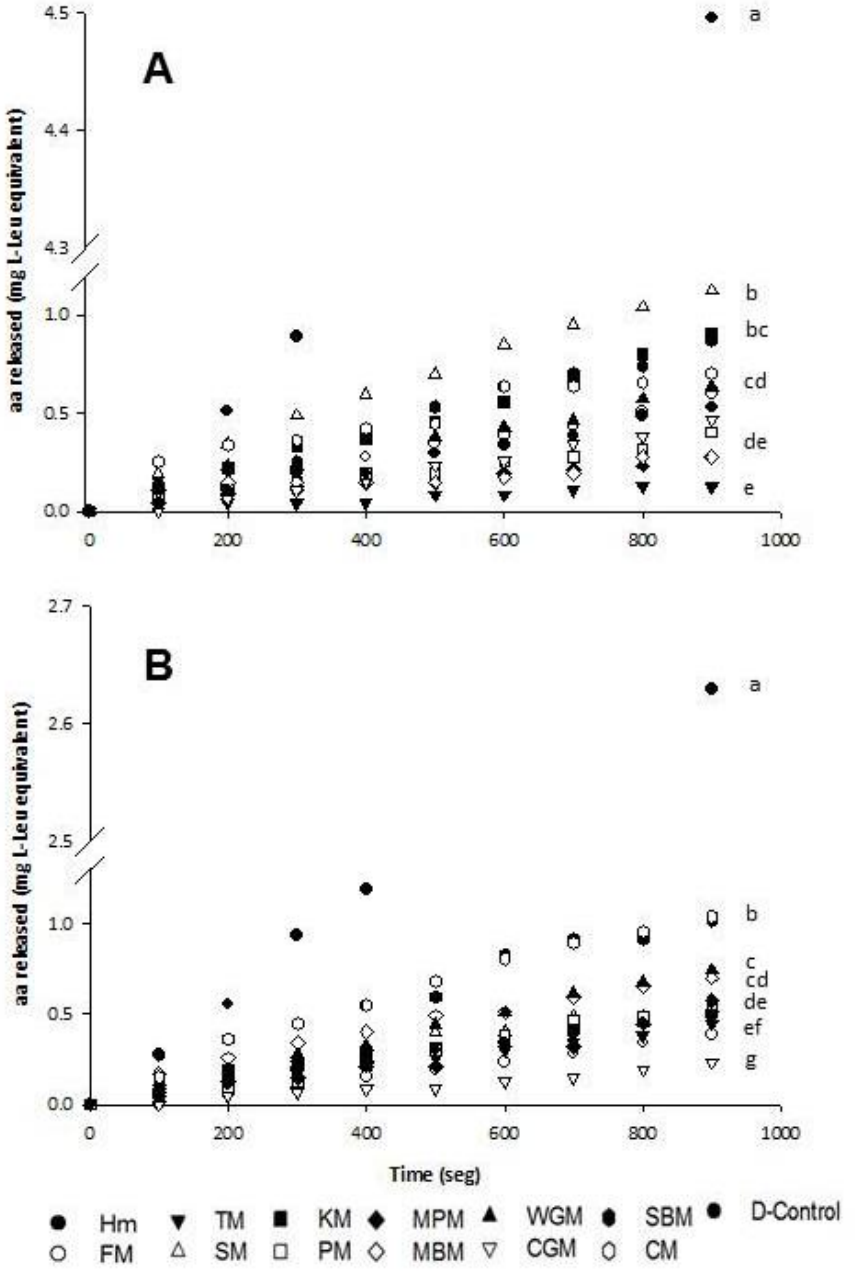

Figure 6. Kinetic of free amino acid released from ingredients using stomach enzyme extracts from *L. guttatus* a) early (20 g) and b) late juveniles (400g). Data points and regression lines of cumulative values against time for each meal are represented with the same symbol. Letters to the right of regression lines indicate differences (P≤0.05) among slopes. FM: fish meal; TM: tuna by product meal; SM: squid meal;

KM: krill meal; MPM: meat porcine meal; MBM: meat and bovine meal; PM: poultry by product meal; WGM: wheat gluten meal; CGM: corn gluten meal; SBM: soybean meal; CM: canola meal; D-control: control diet used for *L. guttatus.*

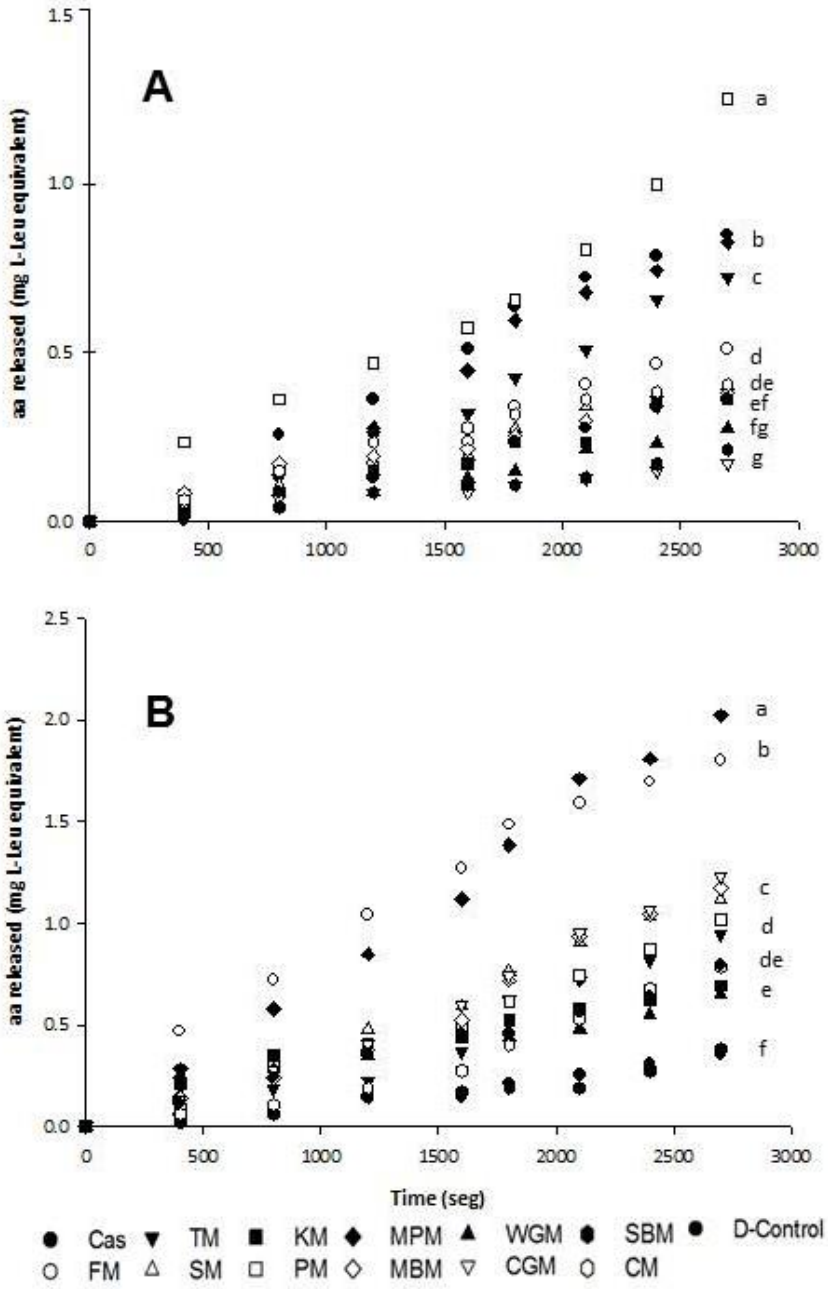

Figure 7. Kinetic of free amino acid released from ingredients using pyloric caeca-intestine enzyme extracts from *L. guttatus* a) early (20 g) and b) late juveniles (400g). Data points and regression lines of cumulative values against time for each meal are represented with the same symbol. Letters to the right of regression lines indicate differences (P≤0.05) among slopes. Cas: casein; FM: fish meal; TM: tuna by product meal; SM: squid meal; KM: krill meal; MPM: meat porcine meal; MBM: meat and bovine meal; PM: poultry by product meal; WGM: wheat gluten meal; CGM: corn gluten meal; SBM: soybean meal; CM: canola meal; D-control: control diet used for *L. guttatus*

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. 8).

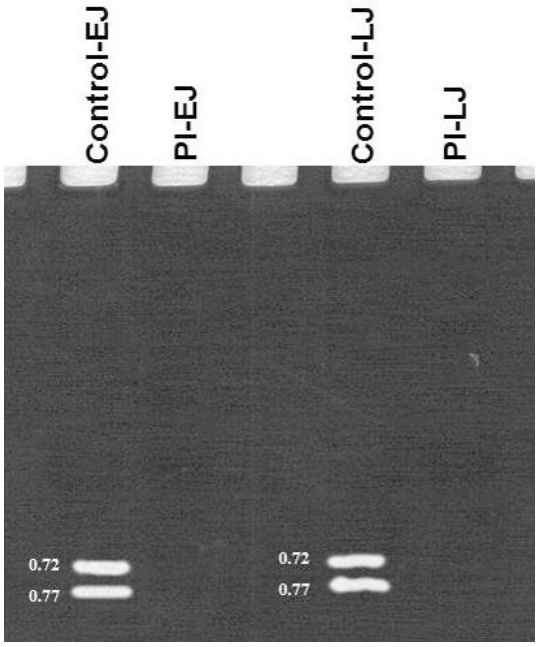

Figure 8. Zymogram of acid proteases from the multienzymatic stomach extracts of early juvenile (EJ; 20g) and late juvenile (LJ; 400g) stages of *L guttatus*, with the action of pepstatin A inhibitor (PI) on the isoforms.

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. 9 and Fig .10, 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.

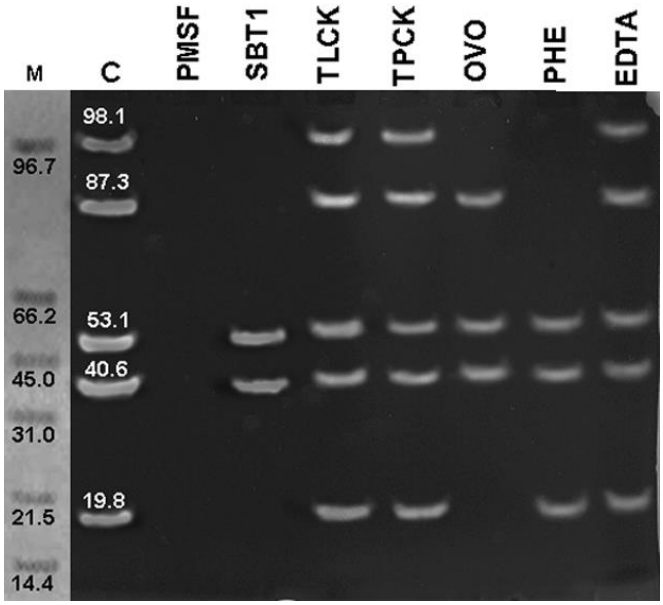

Figure 9. Zymogram of alkaline proteases from the multienzymatic pyloric caeca and intestine extracts of Early juvenile of *L guttatus* (20 g), with the action of the respective inhibitors on the isoforms. PMSF: phenylmethylsulfonyl fluoride, SBT1: trypsin soybean inhibitor TPCK: Tosylphenylalanine- methyl ketone, TLCK: Tosyl-lysine-methyl ketone, Ovo: Ovalbumin, Phen: Phenanthroline, EDTA: ethylenediaminetetraacetic acid, M: molecular weight marker (kDa): Fosforilasa b (97.4 kDa), albumin (66.2 kDa), Ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin soybean inhibitor (21.5 kDa), a-Lactoalbúmin (14.4 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. 9). Inhibition zymogram showed that PMSF, SBTI, turkey and PHE made first (98.1 kDa) and third band (87.3 kDa) disappear. The use of PMSF made the fifth (53.1 kDa) and sixth band (40.6 kDa) disappears. The octave band (19.8 kDa) only was inhibited by PHE. The five bands (98.1, 87.3, 53.1, 40.6 and

19.8 kDa) were inhibited when PMFS was used, while no inhibition was observed with TLCK and TPCK (Fig. 9).

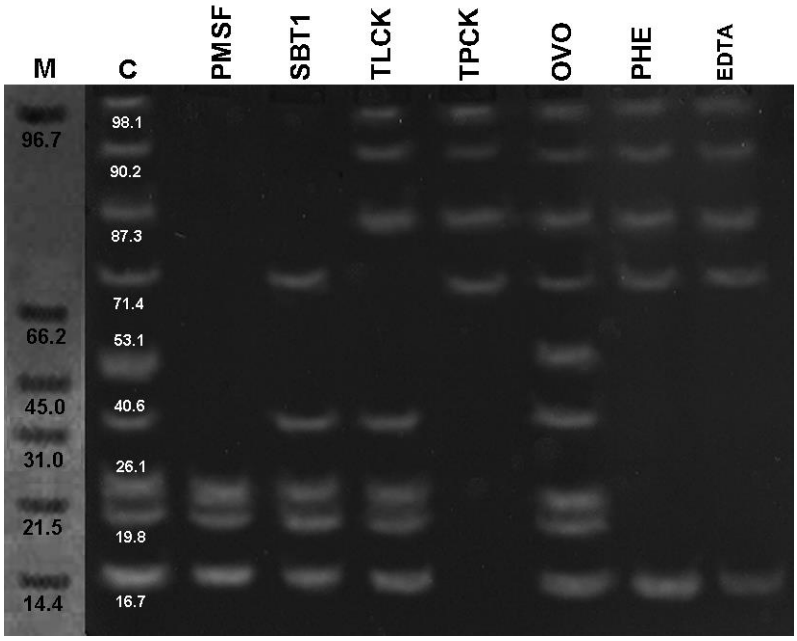

Figure 10. Zymogram of alkaline proteases from the multienzymatic pyloric caeca and intestine extracts of Late juvenile of *L guttatus* (400 g), with the action of the respective inhibitors on the isoforms. PMSF: phenylmethylsulfonyl fluoride, SBT1: trypsin soybean inhibitor TPCK: Tosylphenylalanine- methyl ketone, TLCK: Tosyl-lysine-methyl ketone, Ovo: Ovalbumin, Phen: Phenanthroline, EDTA: ethylenediaminetetraacetic acid, M: molecular weight marker (kDa): Fosforilasa b (97.4 kDa), albumin (66.2 kDa), Ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin soybean inhibitor (21.5 kDa), a-Lactoalbúmin (14.4 kDa).

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. 10). Inhibition zymogram of alkaline proteases in LJ stage showed that PMFS and SBTI made first, second and third bands (98.1, 90.2 and 87.3 kDa) disappeared. The use of PMFS and TLCK made the fourth band disappeared (71.4 kDa). The fifth band (53.1 kDa) was inhibited by PMFS, SBT1, TLCK, TPCK, PHE and EDTA. The use of PMFS, TPCK, PHE and EDTA made the sixth band (40.6 kDa) disappeared. The use of TPCK, PHE and EDTA made the seventh and eighth bands (26.1and 19.8 kDa, respectively) disappeared. The ninth band (16.7 kDa) only disappeared with the use of TPCK (Fig. 10).

DISCUSSION

In vitro degree hydrolysis

Lutjanidae fishes are carnivorous species, where the stomach represents the first step in protein digestion, partially hydrolyzing proteins in to oligo-peptides, that contribute to elevate pyloric caeca and intestine DH (Yasumaru & Lemos 2014) and at same time increase protein solubility by inactivation of protease inhibitors by pepsin activity and low pH as previously reported for other snapper species (Alarcón *et al*. 2001). Previous reports in SRS juveniles shows high pepsin specific activity (U mg protein^{-1}) in the specie, where that specific activity increase with fish growing during juvenile development, representing an indispensable digestion step for *L. guttatus* (Peña *et al*. 2015). In the present study, an acid pre-hydrolysis previous to perform alkaline digestion was not implemented, nevertheless is well characterized that acid *in vitro* pre digestion enhance DH in alkaline digestion for some ingredients, including fish meal, soybean meal, meat and bovine meal, poultry by products, wheat gluten and corn gluten (Alarcón *et al*. 2002; Yasumaru & Lemos 2014).

The highest DH in acid digestion was obtained by hemoglobin in both juvenile stages. Hemo-derivates (hemoglobin and blood meals) has shown high DH and ADC in marine and fresh water carnivore fish species (Bureau *et al*. 1999; Yasumaru & Lemos 2014). Nevertheless, high haemo-derivates inclusion in fish diets is reported to be low (5- 10%), because of low growth attributed to amino acid imbalance (Martínez-Llorens *et al*. 2008; Nogueira *et al*. 2012).

By the other side, ADC values of five protein sources (FM, TBM, PM, SBM and CM) have been reported for *L. guttatus* juveniles (average 90 g), concluding that animal and fish based products present better ADC values than vegetables sources, however, vegetal sources are well digested by the species (Hernández *et al*. 2015). Stomach DH between EJ and LJ stages, presented differences just between three protein sources (TM, SM and CM (Fig. 5A). Peña *et al*. (2015) demonstrate the presence of two pH optimums in pepsin-like enzyme activity (pH 2 and 3) in SRS juveniles that represents two pepsin isoforms. Therefore, DH differences between protein sources cold be attributed by quantitatively changes of the concentration of the different isoforms of the two pepsin isoforms, as all *in vitro* assays were random under same conditions and standardized in enzyme activity Units.

Biology of SRS and other Lutjanidae family members are a carnivorous fish that feeds primarily on fish and benthic crustaceans (Allen 1995; Thomson *et al*. 2000). Between all tested protein sources, KM presents high chitin content that could mimetize natural feed of snappers. Results don't show differences between DH of both juvenile stages, and at same time presented well DH values in acid and alkaline hydrolysis. Therefore, constant DH values in KM could be related to chitinase activity, which as a hydrolytic enzyme contributes to titrant spent in pH Stat assays.

Reports in Atlantic cod (*Gadus morhua*) presents higher chitinase activity in stomach than pyloric caeca (Danulat & Kausch 1984),while cobia (*Rachycentron canadum*) only shows chitinase activity in stomach (Fines &Holt 2010),where in both species a substantial endogenous production is suggested, and bacteria chitinolytic activity is not significant.

On the other hand, alkaline hydrolysis with PC-I extracts showed high variation in DH between juvenile stages in eight of thirteen protein sources probed, related to differences in enzyme pattern between stages. Degree of hydrolysis quantifies the number of peptide bonds hydrolyzed in a given protein, where relative hydrolysis contribution by endo or exo-proteases is not differentiated by pH Stat method (Alarcón *et al.* 2002). In contrast with these finds, Yasumaru and Lemos (2014) did not report differences in DH values between different sizes of same species, such as rainbow trout (*Oncorhynchus mykiss*), cobia (*Rachycentron canadum*), and Nile tilapia (*Oreochromis niloticus*).

Total amino acid release (TAAR)

As previous mentioned, first step in protein digestion is develop by stomach, partially hydrolyzing proteins, to generate numbers of oligo-peptides in reaction mixture, that provides a suitable substrate for pancreatic and/or intestinal exo-proteases (amino- and/or carboy-peptidases), where enzymatic actions are independent of specific amino acids within peptides (Alarcón *et al*. 2002), optimizing total amino acid release. After enzymatic protein digestion, free amino acids are the main way to be absorbed by specific enterocyte transporters from brush border membrane (Bakke

et al. 2011). Therefore quantification of TAAR as a complementary technique tool is important for selection of best ingredients for this specie.

Amino acid released by stomach extract hydrolysis (Fig. 6A and 6B), from hemoglobin was higher than all ingredients, results that are in agreement with higher values obtained in hemoglobin degree of hydrolysis. Hemoglobin has been the most frequent substrate for pepsin activity assays determination, due to high affinity to this protein (Klomklao *et al*. 2004). Vegetal protein sources SBM and CM and animal protein sources SM and KM presented high TAAR with stomach extract hydrolysis in both SRS stages. Soybean and canola presents high anti-nutrient factors content that affects nutritional quality of plant proteins by action of proteinase inhibitors (Ebrahimi-Mahmoudabad & Taghinejad-Roudbaneh 2011; Moyano-López *et al*. 1999), nevertheless, Alarcón *et al*. (2001), reported in yellow snapper (*Lutjanus argentiventris*) and Pacific dog snapper (*Lutjanus novemfasciatus*), that anti-nutrients don't affect acid stomach enzyme activity.

Alkaline protease hydrolysis with PC-I extract, show differences in TAAR between protein sources. Best TAAR with EJ extract was obtained with PM, Cas, MPM and TM, while LJ extracts generate higher TAAR in MPM and FM. Contrast with results in stomach extracts, SBM hydrolysis showed the lower TAAR values, indicating that SBM is not an adequate substrate alkaline phase digestion of SRS juveniles, supporting previous results in other snapper, where alkaline proteases are high sensitive to anti-nutrient factors of soybean meal extract that inhibits more than 50% activity in two snappers. (Alarcón *et al*. 2001). Previous studies in SRS juveniles demonstrate low tolerance (up to 20% material inclusion) for the use of SBM in dose response experiment (Silva-Carrillo *et al*. 2012).

Zymogram analyses

In the present study, a variety of general and specific inhibitors were used to determine the type of proteases from stomach, pyloric caeca and intestine present in two different juvenile stages of *L. guttatus* juveniles. Pepstatin A was used for the aspartic proteases (pepsin). Inhibitors such as PMSF, SBTI and turkey ovomucoid were used for alkaline serine proteases. EDTA and PHE were used as chelating deactivators to detect metalloproteases. For trypsin and chymotrypsin detection, specific inhibitors were used; TLCK and TPCK as respectively inhibitors.

Most fish species have two or three major pepsins, as aspartic protease in fish stomach is the first proteolytic enzyme in charge of the hydrolysis of long chain polypeptides (Guerrero-Zárate *et al*. 2014; Klomklao 2008). In this study, Native-PAGE electrophoresis showed that both bands were presents in both juvenile stages and where completely inhibited by pepstatin A presence. Therefore, the two bands correspond to pepsin isoenzymes as reported for other species such as giant grenadier (*Coryphaenoides pectoralis*) (Klomklao *et al*. 2007), Japanese eel (*Anguilla japonica*) (Chiu &Pan 2002), and tropical gar (*Atractosteus tropicus*) (Guerrero-Zárate *et al*. 2014). The Rf of the two pepsin isoenzymes reported in the present study were 0.71 and 0.77. Reports in tropical gar show the presents two pepsin isoforms with Rf of 0.35 and 0.71 (Guerrero-Zárate *et al*. 2014), gilthead seabream (*Sparus aurata*) and common dentex (*Dentex dentex*) present two mayor pepsin isoenzymes with Rf of 0.811 and 0.66 (Alarcón *et al*. 1998), while spotted sand bass (*Paralabrax maculatofasciatus*) presents only one acid protease with an Rf of 0.75, closer to porcine Rf (0.72) (Álvarez-González *et al*., 2010).

The use of casein as non-specific substrate during gel incubation in SDS-PAGE electrophoresis, enables the view of a wide battery different proteases, including trypsin, chymotrypsin, carboxypeptidases, aminopeptidases, elastases and collagenases as the main pancreatic and/or intestinal proteases, as reported in several fish species (Torrissen *et al*. 1987; Chong *et al*. 2002; Klomklao 2008; Unajak *et al*. 2012). The enzymatic inhibition assays on alkaline proteases, showed that the common five bands present in EJ and LJ (98.1, 87.3, 53.1, 40.6 and 19.8 kDa), were inhibited totally by PMFS, and partially by SBTI, TLCK and OVO, showing a prevalence of serine proteases type enzymes. Major serine proteases are represented by trypsin and chymotrypsin, where ranges of molecular masses are reported between 18 and 90 KDa for different fish species (Dimes *et al*. 1994; Alarcón *et al*. 1998; Chong *et al*. 2002; Klomklao 2008).

Some authors report enzyme digestive changes during the ontogenesis of fish, suggesting that specific types of protease could be expressed at specific fish age by means of fish ontogenesis (Chiu & Pan 2002; Kuz'mina 1996; Unajak *et al*. 2012). The preset study shows a clear difference in number of bands between EJ and LJ stages, where four additional bands appear in LJ stage. Inhibitors TPCK, PHE and EDTA made the seventh and eighth band (26.1 kDa and 19.8 kDa) disappeared, and ninth band (16.7 kDa) only disappeared with the use of TPCK (chymotrypsin-type inhibitor). Previous reports in Cuban Gambusia show the appearance of chymotrypsin isozyme in adult stage that is not present in juvenile stage (Falcón-Hidalgo *et al*. 2011).

Inhibition by PHE and EDTA in fifth, sixth, seventh and eighth bands in both juvenile stages, it is explain by presence of metalloproteases, that are important digestive

enzymes (aminopeptidases and carboxypeptidades) from intestinal lumen, that plays an important role in nutrient digestion, previously reported for other marine carnivorous species (Alarcón *et al*. 1998) and carnivorous and herbivorous freshwater fishes (Dimes *et al*. 1994; Chong *et al*. 2002; Guerrero-Zárate *et al*. 2014). Collagenolytic serine proteases type, belong to zinc metalloproteinase and physiological function in several organisms is attributed to their digestive power, displays trypsin-like and chymotrypsin-like activities (Haard 1994), with molecular masses ranging from 35 to 40 kD (Chong *et al*. 2002; Falcón-Hidalgo *et al*. 2011), previously characterized in Atlantic cod (*Gadus morhua*) (Kristjansson *et al*. 1995). In species with different phylogenetic lines such as Atlantic salmon (*Salmo salar L*.) and spiny lobster (*Panulirus argus*), isoenzyme pattern is not affected by developmental stage but genetically isozymes pattern variation is present, where this variations are correlated with digestion capacity, growth rate and/or feed conversion efficiency (Torrissen *et al*. 1987; Bassompierre *et al*. 1998; Perera *et al*. 2010). The fact that *L. guttatus* presents a diversification in enzyme pattern between EJ and LJ stages related to juvenile ontogeny makes more complex the understanding of digestive adaptations in the specie, where possible genetically isoenzyme pattern variations could exist, and patters of different phenotypes needs to be clarified. High number of alkaline proteases could be present in fish by evolutionary gene duplication, followed by sequence divergence, that provide a mechanism for the evolution of isoenzymes and eventually new functional properties (Taylor and Raes 2004; Perera *et al*. 2015)

In conclusion, the spotted rose snapper presents two pepsin isoforms during juvenile ontogeny that confer them similar acid digestion capacity, while alkaline proteases pattern is modify with juvenile development, that affects degree of hydrolysis of different protein sources and total free amino acids kinetics. Better results in DH and TAAR are present in fishmeal and squid meal as marine products, meat porcine meal and poultry by products meal as renders and soybean meal and canola meal as vegetable products, which represents better protein sources to be use in practical diets as base of dietary protein. Future studies needs efforts in validate *in vitro* finds by *in vivo* trial that let us probe performance and at same time clarified possible existence of genetically isoenzyme pattern variations in the species.

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CAPITULO 4

Effects of fishmeal replacement by renders or vegetable sources on growth parameters, somatic index and blood chemistry in two juvenile stages of spotted rose snapper *Lutjanus guttatus*

Abstract. Effect of total fish meal replacing with vegetable protein source mix (soybean meal concentrate and corn gluten meal) or renders meal mix (poultry by products meal and meat porcine meal) in two juvenile stages of spotted rose snapper on growth performance, somatic index, blood chemistry and proteases activity. According to their wet weight, fish where classified in two groups (considered in the juvenile stage): early juvenile (EJ; 61.2±8.8 g) and late juvenile (LJ; 467.8±38.8 g; 12). A control diet and two experimental diets were formulated to be nearly isonitrogenous (45% crude protein) and isolipidic (10%). Control diet (DC) was based on fishmeal (FM) protein. One experimental diet name as vegetable diets (DV) with total substitution of FM protein by vegetable protein mix (soybean meal concentrate and corn gluten meal; relation 1:1) and second experimental diet name as render diet (DR) with total substitution of FM by renders meal mix (poultry by products meal and meat porcine meal; relation 1:1). Trial was performed during 60 days, where fish where feed three times a day to apparent satiety. Results show a differential growth in EJ stage, where best growth parameters was obtained by fish feed DR. By the other side, LJ stage did not show effects in growth parameters by protein source change in diet. Fish in EJ stage, showed changes in FCR, PER and CF in fish feed DV, and at same time, DV diet generates differences in HIS, VSI and RIL. Nevertheless, fish in LJ stage only showed effect by DV in CF, HSI and MSI. Therefore, juveniles of different stages present differences in diet utilization that could be attributed to digestive process changes as well as metabolic adaptations.

Keywords: Fishmeal replacement, Growth, vegetable protein, Renders, Blood chemistry, *Lutjanus guttatus*,

INTRODUCTION

Fish meal (FM) is the major protein source for most carnivorous fish species, nevertheless, use of fish meal and fish oil (derived from wild capture fisheries) by the aquaculture sector will decrease in the long term (Tacon and Metian 2008; Kumaraguru-Vasagam *et al*. 2015).

FM presents balanced amino acids profile, high digestibility and palatability, presence of potential growth factors, and it is highly improbable that complete replacement will be possible with a single alternative protein source (Zhou et al., 2011). Therefore, aquaculture production will require increasing use of alternative feed ingredients such as vegetable sources and by-products from terrestrial animal processing industries (Hardy, 2010).

Soy protein concentrate (SPC) is well utilized by fish species, and most carnivorous fish can grow well with SPC as a main or only source of protein with well supplemented diets with limiting amino acids (Storebakken et al. 2000). Previous reports showed that 50% of FM substitution by SPC in diets for *L. guttattus* juveniles does not affect growth (Garcia-Ortega et al., 2010). Corn gluten meal (CGM) is a high protein ingredient (60% – 70% dry matter) produced as a byproduct during the corn starch processing. CGM is lack of anti-nutritional factors, low in fiber and, except for lysine and arginine and to a lesser extent methionine, has an adequate indispensable amino acid profile (Pereira and Oliva-Teles, 2003; Luo et al., 2013; Men et al., 2014).

By the other side, rendered animal protein ingredients are good sources of amino acids, with high protein content, total digestible dry matter (DM) and digestible protein and energy similar to fish meal (Bureau et al., 1999; Zhou et al., 2011). Poultry

by-product meal (PBM) is one potential rendered animal protein which has been tested in diets for some fish species (Hernández et al., 2010; El-Sayed, 1994; Kureshy et al., 2000; Turker et al., 2005; Wang et al., 2006; Zhou et al 2011). PBM is mostly used in pet foods by its palatability, protein quality and essential amino acids, essential fatty acids, vitamin and mineral contents (Hernandez et al., 2010). Two previous studies in *L guttatus* juveniles showed that feed grade PBM can be replaced up to 50% by fishmeal, while pet grade PBM can be replaced up to 90% by fishmeal in balance diets (Hernández et al., 2014a, b). Otherwise, porcine meat meal PMM has been not widely tested, where studies in fingerling Nile tilapia (*Oreochromis niloticus*) showed equal growth results as commercial reference diets, showing a promising meal (Hernández et al., 2010) Nevertheless, it is difficult that a sole protein source could replace total fish meal in diets for *L. guttatus* juveniles. Therefore, the use of two or three protein sources in fish feed formulation can reduce the effects of nutrient imbalance, excessive levels of anti-nutritional factors or lower palatability (Bureau et al., 2000).

By the other side, previous studies has shown differences between specific activity and alkaline enzymatic band pattern between early juvenile and late juvenile stage *of L guttatus*, where these differences are directly related to *in vitro* protein digestibility and total amino acids liberation between different vegetables and animal protein sources (Capítulo 2 y 3), therefore, validation of in vitro previous finds by *in vivo* trial that let us probe performance is required.

Consequently, the objective of this research was to compare the effects of total fish meal replacing with vegetable protein source mix (soybean meal concentrate and corn gluten meal) or renders meal mix (poultry by products meal and meat porcine

meal) between two juvenile stages of spotted rose snapper on growth performance, somatic index and blood chemistry.

MATERIALS AND METHODS

Experimental diets

Two experimental diets and one control diets were formulated to be isonitrogenous (45% crude protein) and isolipidic (10%) (Table 8). Control diet (DC) was based on fishmeal (FM) protein. One experimental diet name as vegetable diets (DV) with total substitution of FM protein by vegetable protein mix (soybean meal concentrate and corn gluten meal; relation 1:1) or other experimental diet name as render diet (DR) with total substitution of FM by renders meal mix (poultry by products meal and meat porcine meal; relation 1:1). Diets were balanced for methionine content using *L. guttatus* carcass amino acid profile adjusted to 45%, as a reference. Control diets were manufactured as described by Silva-Carrillo et al. (2012). Briefly, dry ingredients were ground in a hammer mill to a particle size of 250 µm. The macro ingredients were mixed in a Hobart mixer (model A-200 Troy, OH, USA) followed by micro ingredients mix and fish oil until a homogeneous mixture was obtained, and diet pellet were manufactured in Brabender extruder with Twin Screw, model TSE 20/40. The pellets were stored in labeled, sealed containers and were held at -20 °C until analysis of the proximate and amino acid compositions.

Table 8. Ingredients and proximate composition of experimental diets for *Lutjanus guttatus.* (DC: Control diet; DR: Render diet; DV: vegetable diets)

Ingredients*(g Kg^{-1} wet wt.)	DC	DR	DV
Fish meal	480.3	0.0	0.0
Poultry by products meal	0.0	266.7	0.0
Meat porcine meal	0.0	287.8	0.0
Soybean meal concentrate	0.0	0.0	268.7
Corn gluten meal	0.0	0.0	236.5
Krill meal	75.9	75.9	75.9
Squid meal	60.0	60.0	60.0
Wheat gluten meal	20.0	20.0	20.0
Fish oil	50.6	17.9	70.8
Corn starch	254.9	213.4	207.8
Alginate	30.0	30.0	30.0
Mineral premix	2.3	2.3	2.3
Vitamin premix	6.0	6.0	6.0
Carotenoids	1.0	1.0	1.0
Soy Lecithin	15.0	15.0	15.0
Vitamin C	1.0	1.0	1.0
Choline chloride	2.0	2.0	2.0
Antioxidants	1.0	1.0	1.0
CaP dibasic[d]	0.0	0.0	2.0
Methionine[d]	0.0	5.15	4.6
Composition (% DM)[g]			
Crude protein	46.25±0.88	45.21±0.33	46.87±0.59
Crude lipids	10.25±0.15	10.81±0.27	10.86±0.11
Ash	11.43±0.06	12.25±0.24	5.11±0.09
NFE	32.06±0.84	31.73±0.35	37.15±.0.49
Gross energy(kJ g^{-1})	19.86±0.0	19.53±0.36	21.39±0.13

[a] "Premium" grade fish meal was obtained from Selecta de Guaymas, S.A. de C.V. Guaymas, Sonora, México.
[b] Proteínas marinas y Agropecuarias, S.A. of C.V., Guadalajara, Jalisco, México.
[c] Droguería Cosmopolita, S.A. de C.V. México, D.F., México.
[d] Sigma-Aldrich Chemical, S.A. de C.V. Toluca, México State, México.
[e] Trouw Nutrition México S.A. de C.V. (by cortesy). *Vitamin premix composition: Vitamin A, 10,000,000 IU o mg/g; 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; 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.
[f] DSM Nutritional Products Mexico S.A. de C.V., El Salto, Jalisco, Mexico.
[g] mean±SD, number of determinations=3.
[h] Nitrogen-free extract (including fiber)=100−(% protein+% lipid+% ash).

Fish rearing and feeding

Spotted rose snapper were produced in a pilot pilot-scale hatchery at Food Research and Development Center A.C. (CIAD) Mazatlán, Mexico, following established protocols for spawning and larval rearing conducted as described by Alvarez-Lajonchère *et al*. (2012). Fish were weaning in commercial culture sea cages, and where collected as required. According to their wet weight, fish where classified in two groups (considered in the juvenile stage): early juvenile (EJ; 61.2±8.8 g) and late juvenile (LJ; 467.8±38.8 g). Fish in EJ stage were randomly distributed at a stocking density of 12 fish in 300 L fiber glass tanks, while fish in LJ stage were distributed in stocking density of 7 fish in 1500 L fiber glass tanks, all experimental treatment by triplicate replicates. Fish were feed three times a day (9:00, 13:00 and 18:00 h) to apparent satiation with corresponding experimental diet. Both systems presented constant aeration and water flow (8 and 16 L min^{-1}, EJ and LJ systems respectively), where sea water was pumped from the seashore, passed through two parallel sand filters and delivered to four 25-m^3 high-density polyethylene (HDPE) holding cisterns (4 m x 15 m). Over the duration of the study, water quality parameters average (±SD): temperature, 29 ± 1.5 °C; dissolved oxygen, 6.4±0.5 mg L^{-1}.

The fish were caught with scoop nets and anesthetized with 2-phenoxyethanol (Sigma®, St. Louis, MO, USA) at a concentration of 0.3 ml L^{-1}, to be weighed every 15 days to calculate mean body weight and the biomass in each tank. Growth and

feed efficiency of were assessed by calculating the weight gain (WG), feed intake (FI), feed conversion ratio (FCR), specific growth rate (SGR), survival (SUR), protein efficiency ratio (PER) and condition factor (CF). At the end of the experiment a sample of 9 fish in EJ stage and 7 fish in LJ stage per treatment were sacrificed. Each fish was dissected to obtain viscera, liver, intestine and visceral fat weight to determine viscerosomatic index (VSI), hepatosomatic index (HSI), mesenteric fat index (MSI) and relative intestine length (RIL) .The formulas used are shown below:

WG = final mean weight (g) - initial weight (g)

FI = $\sum$ [(total feed consumption (g)) / (number of fish)] / number of days

FCR = feed intake (g) / weight gain (g)

SGR = [(Ln final weight- Ln initial weight) / number of days] x 100

Survival = (Final number / initial number) x 100

PER = weight gain / protein intake

CF= 100 x [BW (g)/BL^3 (cm)]

HSI= Liver weight (g) x 100/BW (g)

VSI= [viscera weight (g)/ BW (g)] x 100

MSI= Mesenteric fat weight (g) x 100/BW (g)

RIL= [Intestine length (cm)/ BL (cm)] x 100

Where, BW indicates the total body weight (g) and BL the total body length (cm) of the fish.

Biochemical analysis

To analyze final proximal composition, two fish were selected at random from each replicate tank, for a total sample size of six fish per treatment group. Moisture,

protein, lipid and ash levels in the diets were determined using standard methods according to AOAC (2000). The samples were homogenized and dried at 105 °C for 24 hours prior to chemical analyses. The level of crude protein was analyzed using micro-Kjeldahl method by Labcocnco System (Labconco, Kansas City, MO). The lipid content was obtained after extraction with petroleum ether, using a micro Foss Soxtec Avanti 2050 Automatic System (Foss Soxtec, Hoganäs, Sweden). The ash content was determined by calcination of the samples in a muffle furnace at 550 °C (Fisher Scientific International, Inc. Pittsburgh, PA, USA). The gross energy content in diets was measured by combustion in a Parr bomb semimicro-calorimeter 6725 (Parr, Instrument Company, Moline, IL, USA) using benzoic acid as the standard. Amino acids profile in the experimental diets was quantified following (Vázquez-Ortiz *et al*., 1995) with high performance liquid chromatography (HPLC, Varian 9012, Walnut Creek, CA, USA).

Blood chemistry parameters

At the end of the experiment, fish were carefully handled to minimize stress. The fish were anesthetized and in less than 3 minutes, blood samples were collected from the caudal vein using 1-mL non-anticoagulant insulin syringes 21 G x 32 mm (Terumo Mexico, DF, Mexico). Ten fish were selected randomly from each experimental diet. A volume of 400 µL of blood was extracted. The blood sample was divided into 200-µL samples and placed into 2 Eppendorf tubes. The first tube, with no anticoagulant, was immediately centrifuged for 10 min at 7000 rpm in a Clay-Adams micro centrifuge, and the serum was stored in a -20 °C freezer for further analysis of the total protein concentration, glucose, triglycerides and cholesterol

levels. The second tube included K_2 EDTA (BD Microtainer, Franklin Lakes, NJ, USA) to prevent coagulation. This tube was used to determine the hematocrit and hemoglobin concentrations. To calculate the hematocrit levels, tubes were placed for 10 min in a microhematocrit centrifuge (SOL-BAT P600, Mexico, DF, México). The packed cells were measured using a hematocrit reader and reported as a percentage (Del Rio-Zaragoza et al., 2008). The hemoglobin concentration in erythrocytes was determined using the cyanmethemoglobin method (hemogloWiener reactive, Wiener Lab., Riobamba, Rosario, Argentina) following the manufacturer's instructions. The total protein concentration, triglyceride and cholesterol levels were determined using commercial kits supplied by Randox and Biosystem Laboratories LTD (Admore, Diamond Road, Crumlin, Co. Antrim, United Kingdom).

Statistical analysis

The data for each parameter were tested for normality and homoscedasticity. The data was separately analyzed by life stage; using one-way analysis of variance (ANOVA) with diet as the independent variable. Tukey's HSD test was used for post-hoc identification of significant differences among the dietary treatment groups at a significance level of 5% (Zar, 1984). All of the statistical procedures were performed using SigmaPlot 12.0 software.

RESULTS

Proximate and amino acid composition of diets

All of the diets were isonitrogenous and isolipidic and nearly isoenergetics (19.53-21.39 kJ g1). The ash content was lower in DV than DR and DC, while DV presented higher NFE content (Table 8).

Amino acid profile of experimental diets and body composition of *L guttatus* juveniles is presented in table 9. Diets where only supplemented with methionine to adjust deficiency. Therefore, compared with amino acid profile of *L. guttatus* body composition adjusted to 45% protein (as percent of protein in diets), DC showed lower values in Arg and Thr, DR showed lower values in Iso, Leu, Phe and Val, while DV showed lower values in Arg and Val.

Table 9. Total amino acid composition of experimental diets (g AA/ 100 g of diet DW) for *Lutjanus guttatus* juveniles. (DC: Control diet; DR: Render diet; DV: vegetable diets).

Amino acids	Body composition	DC	DR	DV
Essential				
Arg	4.39	2.63	11.72	3.90
His	0.83	1.28	2.54	2.43
Iso	1.93	1.80	0.71	1.16
Leu	3.15	4.26	2.34	4.82
Lys	2.06	4.32	1.96	2.00
Met	1.11	1.38	1.11	1.11
Phe	1.96	2.25	1.30	2.23
Thr	2.63	1.37	4.89	2.42

Val	2.20	2.14	0.97	1.40
Non-essential				
Ala	0.68	3.58	2.00	2.74
Ásp	4.18	4.62	2.31	3.25
Glu	5.91	8.14	4.99	8.70
Gly	1.02	2.36	5.31	2.33
Ser	0.65	2.60	1.74	2.41
Tyr	3.21	1.59	0.84	1.53

Values are means±standard deviation (n=3). Different superscript letters within rows indicate significant (P<0.05). DW—dry weight; Arg—arginine; His—histidine; Ile—isoleucine; Leu—leucine; Lys—lysine; Met—methionine; Phe— phenylalanine; Thr—threonine; Val—valine; Ala—alanine; Asp—aspartate Glu—glutamate; Gly—glycine; Ser—serine; Tyr—tyrosine; Tau—taurine. Amino acid in body composition is adjusted to 45% of protein.

Growth parameters and somatic index

Growth parameters of EJ and LJ stages are summarized in Table 10. In EJ stage higher WG and SGR was obtained by fish fed DR, followed by DC and DV (P≤0.05), nevertheless in LJ stage fish didn't show differences in WG and SGR between treatments (P≥0.05). FCR in EJ stage showed higher values in DV, while PER and FI showed higher values in DR and DC diets (P≤0.05). Fish in LJ stage didn't show differences in FCE, PER and FI between treatments (P≥0.05). Fish in EJ stage showed differences in CF between all treatments, with higher values in DC, followed by DR and DV, while, fish in LJ stage showed a decrease in fish CF in treatment DV (P≤0.05).

Somatic index of EJ and LJ stages are summarized in Table 10. In both juvenile stages, HSI showed a decrease only in fish feed DV (P≤0.05). In EJ stage, higher values in VSI were obtained by DR treatment (P≤0.05), while in LJ state fish didn't show differences in VSI between treatments (P≥0.05).Survival didn't show

differences between treatments in both juvenile stages (P≥0.05). MSI in EJ stage didn't show differences (P≥0.05), meanwhile, in LJ stage, fish fed DV showed lower values (P≤0.05). RIL in EJ stage showed lower values in fish fed DV (P≤0.05), without effects in LJ stage in RIL (P≥0.05).

Table 10. Growth parameters and somatic index of *Lutjanus guttatus juveniles* fed the experimental diets for 60 days. (DC: Control diet; DR: Render diet; DV: vegetable diets).

	EJ Stage			LJ Stage		
	DC	DR	DV	DC	DR	DV
Initial weight (g)	61.32±0.24	61.21±0.24	61.14±0.04	468.4±3.20	467.3±0.90	467.8±2.10
Final weight (g)	120.71±3.09[b]	132.6±3.79[a]	98.5±2.18[c]	520.2±2.30	518.1±9.0	510.1±8.6
WG (g)[1]	59.39±3.33[b]	71.46±4.0[a]	37.36±2.14[c]	51.83±1.36	50.76±8.1	42.27±7.45
SGR (% d^{-1})[2]	1.13±0.05[b]	1.29±0.05[a]	0.79±0.04[c]	0.17±0.01	0.17±0.03	0.14±0.02
FCR[3]	1.46±0.16[b]	1.35±0.10[b]	1.89±0.06[a]	2.98±0.24	3.46±0.30	3.67±0.48
PER[4]	1.54±0.17[a]	1.65±0.12[a]	1.18±0.03[b]	0.75±0.06	0.65±0.06	0.61±0.08
FI(g)[5]	86.29±4.49[a]	96.31±4.11[a]	70.4±3.51[b]	154.05±8.59	173.87±11.61	152.64±7.05
CF[6]	1.89±0.09[a]	1.78±0.08[b]	1.63±0.08[c]	1.70±0.14[a]	1.67±0.09[a]	1.47±0.16[b]
Survival (%)	87.27±6.30	97.22±4.81	97.22±4.81	100.0±0.0	100.0±0.0	100.0±0.0
HSI[7]	2.67±0.30[a]	2.25±0.41[a]	1.37±0.20[b]	1.57±0.19[a]	1.71±0.29[a]	1.10±0.15[b]
VSI[8]	3.73±0.50[b]	4.45±0.39[a]	3.21±0.36[b]	2.79±0.16	3.11±0.40	2.63±0.31
MSI[9]	6.38±1.40	5.94±1.15	6.95±1.42	12.04±1.57[a]	13.20±1.53[a]	7.57±2.50[b]
RIL[10]	71.62±4.18[a]	63.26±9.81[a]	52.41±4.5[b]	74.0±9.88	66.26±6.13	79.26±9.0

Within a line value with same superscripts do not differ significantly (P<0.05)
[1] WG = final mean weight (g) - initial weight (g)
[2] Specific growth rate= [In final BW (g) - In initial BW (g)] /time days) x 100
[3] Feed conversion ratio= Dry feed consumed (g)/ wet BW gain (g)
[4] Protein efficiency ratio= [final BW (g) - initial BW (g)]/weight of protein consumed (g)
[5]Total individual feed intake
[6] Condition factor= 100 x [BW (g)/BL3 (cm)]
% S= [Final organisms number/ initial organisms number] x 100
[7] Hepatosomatic index= Liver weight (g) x 100/BW (g)
[8] Viscerosomatic index= [viscera weight (g)/ BW (g)] x 100

[9] Mesenteric fat index= Mesenteric fat weight (g) x 100/BW (g)
[10] RIL= [Intestine length (cm)/ BL (cm)] x 100

Whole-body composition

Final body composition of EJ and LJ stages are presented in Table 11. Fish in EJ stage did not show differences in protein, lipid, ash and NFE between treatments (P≥0.05). However, LJ fish showed differences in protein between DR and DV, and lipid content showed differences between all treatments; with higher values in fish feed DR diet (P≤0.05).

Table 11. Whole-body composition of *Lutjanus guttatus juveniles* fed experimental diets for 60 days

	EJ Stage			LJ Stage		
	DC	DR	DV	DC	DR	DV
Moisture	34.13±1.03	35.98±1.49	34.59±1.84	36.72±1.95	37.04±3.78	35.27±1.94
Lipids	21.02±0.05	21.58±0.01	22.21±0.29	24.06±0.12[b]	24.73±0.46[a]	23.63±0.22[c]
Ash	8.95±0.10	8.40±0.05	8.11±0.09	7.93±0.03	7.65±0.07	8.63±0.39
Protein	30.54±0.43	30.12±0.40	31.62±0.73	29.65±0.61	28.42±0.70	31.18±0.41

Proximal results are in wet basis.
Mean± SD, number of determinations = 3.
Nitrogen-free extract (including fiber) = 100 - (% protein + % lipid + % ash).

Blood chemistry

Blood chemistry parameters of EJ and LJ stages are summarized in Table 12. Total protein and glucose do not show differences between treatments in both juvenile stages (P≥0.05). Hematocrit in EJ stage showed differences between treatments, with higher values in fish fed DV, followed by DR and DC (P≤0.05), while fish in LJ stage didn't show differences in hematocrit between treatments (P≥0.05). Same

pattern was obtained in hemoglobin values in both juvenile stages, showing differences between treatments, where higher values were obtained in fish fed DR, followed by DV and DC (P≤0.05). Cholesterol in EJ stage showed lower value in fish fed DR (P≤0.05), by the other side, cholesterol in LJ stage showed higher value in fish fed DR (P≤0.05). Triglycerides in EJ stage showed higher values in fish fed diet DR (P≤0.05), while do not differences were observed in LJ stage (P≥0.05).

Table 12. Blood Chemistry of *Lutjanus guttatus* juveniles fed experimental diets

Juvenile stage	EJ Stage			LJ Stage		
Parameters	DC	DR	DV	DC	DR	DV
Total protein ($g \cdot L^{-1}$)	67.1±9.2	63.0±3.8	69.3±13.3	51.9±8.0	60.2±7.5	60.9±9.1
Glucose ($g \cdot L^{-1}$)	103.8±15.13	93.47±9.38	102.56±27.32	121.8±17.9	103.7±16.3	113.4±29.5
Hematocrit (%)	53.11±7.15 ^{b}	57.75±4.2 ^{ab}	63.71±7.18 ^{a}	53.20±9.25	60.00±3.65	56.00±7.55
Hemoglobin ($g \cdot dL^{-1}$)	6.37±1.75 ^{b}	8.63±0.95 ^{a}	6.96±0.92 ^{ab}	7.02±1.73 ^{b}	9.56±1.82 ^{a}	7.19±1.79 ^{ab}
Cholesterol ($mg \cdot dL^{-1}$)	400.1±53.8 ^{a}	327.8±25.4 ^{b}	445.1±57.8 ^{a}	311.1±30.42 ^{c}	444.4±52.34 ^{a}	368.7±30.71 ^{b}
Triglycerides ($mg \cdot dL^{-1}$)	232.8±8.15 ^{b}	295.9±14.9 ^{a}	229.4±11.7 ^{b}	472.76±53.21	500.09±40.33	471.79±49.19

Values are means ± standard deviation (n=10).
Treatments in the same row with different superscripts differ significantly ($P < 0.05$)

DISCUSSION

The present study makes a challenge in two juvenile stages of *L. guttatus*, through contrasting dietary protein sources, with the view to better understand differences in the use of different protein sources based diets, taking as principal parameter fish growth. Results showed that change in protein source affected growth in EJ stage, but not in LJ stage, representing differences of assimilation potential and nutrient metabolization, confirming by *in vivo* trial that differences exist in digestive capacity between *L. guttatus* juveniles in different stages, as previously reported by *in vitro* assays (Chapter 2 and 3).

Previous study in *L guttatus* juveniles, showed that FM can be replace by tuna by products meal (TBM) up to 30% (Hernández et al. 2014a), feed grade PBM can be replaced up to 50% by FM, while pet grade PBM that presents higher nutritional value can be replaced up to 90% by FM in *L. guttatus* diets (Hernández 2014b, 2014c). By the other side, soy bean meal (SBM) and soy protein concentrate (SPC) have been prove for *L. guttatus* juveniles (Silva-Carrillo et al., 2012; García-Ortega, 2010), where acceptance of SPC and SBM are up to 50% and 20% respectively. Therefore, results obtained in EJ stage with 100% FM replacement by render mix (DR) showed better growth performance than control diet, representing a goal of the present study. However, LJ stage didn't show effect in growth performance by protein source change in diet, representing same growth performance by LJ fish fed alternative protein sources, which means good result, due too experimental diets show same performance than fish feed control diets. Despite, in the present study 100% of FM was substituted, representing 35% of dietary protein content, all diets presented a high quality constant protein basis (Krill meal, squid meal and wheat gluten meal), representing 10 % of dietary protein content that could balance deficiencies in some nutrients in all diets.

Total replacement of fish meal for carnivorous species by a sole protein source is low probable, thus combination of various animals or plant ingredients have demonstrated good nutritional value (Bureau et al., 2000; Guo et al., 2007). Studies in mangrove red snapper juveniles (*L. argentimaculatus*) with average initial body weight of 30g, showed low tolerance for substitution of FM by animal by-product mixture (25% cow liver meal, 20% leather meal, 20% meat and bovine meal (MBM), 15% blood meal (BM), 10% poultry feather meal (PFM) and 8% poultry manure

dried), with optimal substitution of 23% of protein (Jamil et al., 2007). Nevertheless, species such as cunate drum (*Nibea miichthioides*) showed acceptance up to 80% of FM replacement with blend of PBM, MBM, feather meal (FEM) and BM or blend of PBM, MBM and BM (Guo et al., 2007), while Lu et al., (2015), reported blend of PBM, FEM and BM that can substitute 100% of FM with comparable growth performance to rainbow trout (*Oncorhynchus mykiss*) feed the control diet.

Substitution of vegetable mix in the presents study results in lower WG, SGR, PER and FI while FCR showed higher values in EJ fish; nevertheless, results in LJ fishes didn't show effects in all parameters after mentioned. Condition factor in both juvenile stages showed lower values in fish fed DV. Studies in Atlantic cod (*Gadus morhua*), with partial and total substitution of FM protein (25, 50, 75 and 100%) by a plant protein mixture (50% wheat gluten, 36% soy protein concentrate and 14% bioprocessed soybean meal), showed that substitution up to 75% produce only marginal effect on fish growth (Olsen et al., 2007). A mixture of blend of soybean meal, peas, corn gluten and wheat was able to subtituute75% of FM in diets for Solea senegalensis (*Senegalese sole*) without impairing feed intake, growth performance and protein utilization (Cabral et al., 2013). Diets with replacement of FM by mixture of proteins (soy, peas, corn and wheat) for gilthead seabream (*Sparus aurata*) showed acceptance up to 60% without affecting weight gain, however PER presented lower values (Dias et al., 2009). Sitjà-Bobadilla et al., (2005) report partial and total substitution of FM protein (50, 75 and 100%) by a plant protein mixture (corn gluten, wheat gluten, extruded peas, rapeseed meal and sweet white lupin), in diets for gilthead seabream, where all inclusion showed differences in WG, FI, SGR, and feed efficiency up to 50% . Defatted soybean meal (DSM) and corn gluten meal

(CGM) where use to replace 75% fish meal in diets for rainbow trout, without differences with FM diet in WG and SGR and showing higher values of FCR and PER (Lu et al., 2015)

Hepatosomatic index showed lower values in fish fed DV in both stages juvenile stages compared to other treatments. Contrasting with presents results, where increasing HSI in other fish species fed with partial or total plant protein sources are reported, such as gilthead seabream (De Francesco et al., 2007), rainbow trout (Lu et al., 2015) and Solea senegalensis (Cabral et al., 2013). Liver weight depends on energetic reserves of glucose in form of glycogen and fat as lipid reserves, therefore fish feed DV in both stages presented low energetic reserves in liver, induced by switch in metabolism of nutrients by change of principal protein source in diet. Even both stages showed lower HSI, growth where deprived in EJ stage, while LJ stage showed same growth as other diets.

Differences in MSI where found in LJ stage, with lower values in fish fed DV, as a reflex that fish feed diet DV, made use of mesenteric fat as an energetic source. Nevertheless, fish in EJ stage didn't show effects in MSI. By the other side RIL showed a decrease by fish fed DV in EJ stage, while LJ stage didn't show effects in intestine longitude, where reduction in total intestine length could be due by presence of ant nutritional factors, presented in soy meal concentrate, where EJ could be more sensible than LJ state to presence of those factors.

Total replacement of protein source in experimental diets, did not affect carcass content in EJ stage, however LJ stage showed lower protein content in fish feed DR, while fish feed DV showed lower lipid values Previous reports in other fish species show decreasing lipid carcass values when are feed with partial or total replacement

with vegetable protein sources (Cabral et al., 2013; Luo et al., 2013; Yan et al., 2014).

In the present study, blood serum protein and glucose levels do not show effects by change of protein source in diets in both juvenile stages. Previous reports in the *L. guttatus*, show a constant level in plasma protein content (between 55 g L^{-1} to 46 g L^{-1}), without variation between treatments feeding poultry by-products base diets, while glucose serum is reported to low levels in *L. guttatus* feed diets with 75% of FM substitution by feed grade PBM (Hernández et al. 2014b, 2014c). Contrasting, gilthead seabream show protein serum content changes when are feed with high levels (75% to 100%) of plant protein blend in mixture, while glucose content didn't showed effect (Sitjá-Bobadilla et al., 2005). Although glucose levels were constant in the present study, HSI of fish feed DV in both stages where lower than other treatments. As previous mentioned, glycogen is the liver form of glucose reserve, therefore, fish feed DV uses all digestible carbohydrate as a source of energy as a physiological strategy in energetic resources distribution, caused by protein source change.

Hemoglobin and hematocrit between other blood parameters are related to health and immune response (Zhou et al., 2005). Hematocrit in EJ stage showed higher values in fish feed DV than DC, however, LJ stage did not showed effect in hematocrit content. Fish feed DA in EJ stage showed higher values in hemoglobin than fish feed DC, while LJ stage didn't show effects. Reports in other species such as Atlantic cod, rainbow trout and meagre (*Argyrosomus regius*) did not show effects in hematocrit and hemoglobin serum content in fish feed diets with partial or total FM substitution by plant protein blends or animal by-products (Lu et al., 2015; Olsen et

al., 2007; Ribeiro et al., 2015). However, hematological parameters of fish feed with experimental diets indicate that health status were comparable to those reported for clinically healthy snappers of the same species (Del Rio-Zaragoza et al. 2011), and at same time are in the range of previous reports in the specie feed with render based diets (Hernandez et al., 2014b; 2014c).

Cholesterol in EJ stage showed higher values in fish feed DC and DV, nevertheless, contrasting results in LJ stage where obtained, with higher values of cholesterol in fish feed DA. Dietary protein source has been shown to affect blood cholesterol levels in species such as *gilthead seabream, Oncorhynchus mykiss Japanese flounder (Paralichthys olivaceus)* and European seabass (*Dicentrarchus labrax*) feed with diets based on plant protein showed hypocholesterolemia effect (Deng et al., 2010; Kaushik et al., 1995, 2004; Sitka-Bobadilla et al., 2005). However, in the present study, fish feed DV didn't show a hypocholesterolemia effect compared with other treatments.

Triglycerides are the main reserve lipids and easily transported from blood to various organs and tissues (Peres et al. 2014).Triglycerides in EJ stage showed higher values in fish feed DA, while LJ stage didn't show effects in triglyceride. By the other side, triglycerides levels in LJ are in the range of 471- 500 $mg \cdot dL^{-1}$, while EJ triglyceride range between 229- 295 $mg \cdot dL^{-1}$, that represents higher dependence of lipid as energy source by fish in LJ stage in all treatments compared with EJ fish.

In conclusion, diets with total substitution of FM by vegetal or render mix proteins, influence differences in growth parameters, somatic index and blood chemistry mainly in EJ stage than LJ stage of *Lutjanus guttatus*, representing a higher influence in digestive and metabolic processes. A mixer of poultry by products meal and

porcine meat meal was able to substitute total FM, with better growth results in EJ stage. However, future studies in enzyme activity and SDS-Page electrophoretic techniques will provide more information about digestive changes and adaptations as a response or FM substitution by vegetable and renders mix proteins.

CAPITULO 5

Discusión general

Si bien, existen muchas investigaciones en caracterizaciones de enzimas digestivas en diferentes especies, gran parte de esas investigaciones se han enfocado en la ontogenia temprana y/o la caracterización en una talla juvenil de dicha especie. Durante la ontogenia temprana de muchas especies de peces, existen cambios sucesivos en la actividad y/o expresión de diferentes enzimas en conjunto con un rápido desarrollo del sistema digestivo y órganos auxiliares (Zambonino-Infante y Cahu, 2001). Lo anterior para generar entendimiento en esta etapa, y así cerrar ciclos de cultivo de especies con potencial acuícola.

Por otra parte, la caracterización de enzimas digestivas que se han llevado a cabo durante etapas juveniles, dan por hecho que dicho número, tipo y/o actividades de enzimas digestivas no cambian durante toda la etapa juvenil y el conocimiento que existe se ha llevado a cabo de forma comparada durante la ontogenia juvenil de diferentes especies (Unajak et al., 2012; Yasumaru y Lemos, 2014), resultando limitado para otras especies. Si bien, el entendimiento de los procesos fisiológicos digestivos durante la ontogenia temprana de las especies con interés de cultivo es de suma importancia, el conocer los cambios digestivos con detalle durante etapas juveniles necesita ser atendido. Esto porque la etapa juvenil corresponde en la mayoría de las especies a la mayor fase de cultivo antes de cosecha. Por ende, el entender los cambios y/o adaptaciones digestivas servirá como base para la formulación de dietas específicas para etapas juveniles en engorda. Además de poder adecuar las fórmulas al tipo de manejo y estado fisiológico (época del año, entre otros), con la finalidad de aumentar el rendimiento productivo de la especie.

Por otro lado, las enzimas de origen marino presentan diferencias en la estabilidad y cinética comparadas con enzimas equivalentes provenientes de animales superiores terrestres (Klomklao, 2008; Rustad et al., 2011). Las vísceras son una rica fuente de enzimas hidrolíticas, especialmente proteasas, con una alta actividad en un amplio rango de actividad a diferentes pHs y temperaturas (Gildberg, 1992; Castillo-Yañez et al., 2006). Debido a sus diferentes funciones, las enzimas de origen marino son cada vez más usadas en industrias con enfoque biotecnológico y bioquímico (Khantaphant y Benjakul et al., 2008, 2010). Es así que los resultados presentados en el capítulo 2 y parte del capítulo 3 sirven como base para futuras investigaciones que busquen la aplicación de los desperdicios (vísceras) de *L. guttatus* provenientes de cultivo. Esto si llegara a expandirse el cultivo de esta especie. Sin embargo el enfoque que se buscó generar en dicho capítulo, fue realizar una caracterización de manera comparada entre edades de la misma especie, con la finalidad de resaltar las posibles diferencias fisiológicas tales como actividad de proteasas, óptimos en pH y temperatura. Así como caracterizar el tipo de enzimas encontradas dentro del grupo de proteasas alcalinas mediante el uso de inhibidores específicos. Resultados que dieron origen a la realización de la segunda investigación descrita en el capítulo 3.

Los resultados obtenidos en el capítulo 3, complementan en gran parte la caracterización de proteasas realizada con anterioridad, donde el supuesto de una diversificación de las enzimas digestivas en fase alcalina existía, lo cual se corroboró a través del uso de técnicas electroforéticas. Ahí se observó la aparición

de cuatro bandas adicionales en juveniles tardíos, además de demostrar la presencia de dos isoformas de pepsina en el estómago durante la etapa juvenil.

Los resultados obtenidos en los capítulo 2 y 3, muestran una alta actividad enzimática tipo pepsina durante toda la etapa juvenil de *L. guttatus*, aunado a la obtención de un grado de hidrólisis (DH) alto en la mayoría de las fuentes proteicas utilizadas, lo que corrobora la importancia del estómago en la especie, resaltando la naturaleza carnívora del pargo flamenco.

El pargo flamenco presenta un estómago bien definido con un píloro estrecho, lo que sugiere que el estómago retiene los alimentos para sólo dejar pasar la parte solubilizada a la fase digestiva alcalina en ciegos pilóricos e intestino. Es así que la combinación de la alta actividad de la pepsina presente en *L guttatus*, aunado al tiempo de retención del alimento, genera una pre hidrolisis suficiente para generar un buen aprovechamiento de los nutrientes. Ya que el tamaño del intestino de esta especie es corto, al representar entre el 52 y el 79 % de la longitud total del pez, supone un tiempo de retención corto, en donde los ciegos pilóricos también juegan un papel importante en la fase digestiva alcalina de esta especie, tal como se demostró en el capítulo 2.

En conjunto los resultados obtenidos del capítulo 2 y 3 generan pautas de investigación, así como la necesidad de validar de manera *in vivo* las diferencias en especificidad hacia diferentes fuentes proteicas de acuerdo a la talla juvenil. Por lo que con la finalidad de generar un reto fisiológico que pudiera reflejar un crecimiento diferencial, se decidió sustituir el 100% de la harina de pescado (equivalente al 75%

de la proteína) por otras fuentes. Es así que se formularon tres dietas; una con harina de pescado, otra con la mezcla de dos harinas con subproductos animales y la tercera con una mezcla de proteínas vegetales. Esto con la finalidad de observar un posible aumento o detrimento de su digestibilidad como ha sido reportado en diferentes especies de peces (Bureau et al., 2000; Guo et al., 2007).

A partir de este experimento (capítulo 4), se observó un efecto diferencial en el crecimiento de la talla juvenil temprana, como resultado del cambio de la fuente proteica, sin embargo y de manera contrastante, en la talla juvenil tardía no se muestra diferencia en crecimiento entre los diferentes tratamientos. Estos resultados de alguna manera corroboran la teoría de poseer una capacidad digestiva alta (capitulo 3) en las tallas juveniles tardías. Sin embargo es necesario cuestionar el aumento en tasa de conversión alimenticia (FCR) y la disminución de la tasa específica de crecimiento (SGR) entre las tallas juvenil temprana y tardía, donde se muestra el requerimiento del doble de alimento para generar un kilogramo de biomasa cuando el organismo se acerca a la talla adulta. Dichos resultados generan otras preguntas de investigación sobre los requerimientos energéticos y metabólicos entre las diferentes tallas.

CAPITULO 6

Conclusiones

- La actividad específica de proteasas digestivas ácidas y alcalinas incrementa con el crecimiento de los juveniles, aumentando y diversificando las enzimas en la fase alcalina, con menor número de enzimas en juveniles tempranos que juveniles tardíos.
- Las diferencias en número y tipo de enzimas digestivas en la fase alcalina entre juveniles de 20 y 400 gramos, modifican el grado de hidrólisis de diferentes fuentes proteicas y la cinética de liberación de amino ácidos de dichas fuentes.
- Los mejores resultados en grado de hidrólisis y liberación total de aminoácidos se presentaron en harina de pescado y calamar para las fuentes marinas; harina de carne de cerdo y harina de subproductos de ave para fuentes de subproducto animal; harina de pasta de soya y harina de canola para las fuentes vegetales.
- El efecto de la sustitución total de la harina de pescado (representando el 75% de la proteína dietaría) por fuentes vegetales y subproductos de origen animal, generan diferencias en parámetros de crecimiento, índices

corporales e índices sanguíneos entre juveniles tempranos (60 g) y juveniles tardíos (450 g).

- La mezcla de subproductos de ave y harina de carne de cerdo en relación 1:1 proteína, pueden sustituir el total de harina de pescado (representando el 75% de la proteína dietaría) en juveniles tempranos y tardíos (60 y 450 g, respectivamente)
- La sustitución total de harina de pescado (representando el 75% de la proteína dietaría) por mezclas de harinas vegetales o de subproductos de animales terrestres, puede ser utilizada en dietas para juveniles tardíos, mayores a 450 gramos.

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