UNIVERSIDAD JUÁREZ AUTÓNOMA DE TABASCO

División Académica de Ciencias Biológicas

CARACTERIZACIÓN PARCIAL DE ENZIMAS DIGESTIVAS EN LARVAS Y JUVENILES DE ROBALO BLANCO DEL PACIFICO *Centropomus viridis*

TESIS

Para obtener el título de:

Maestro en Ciencias Ambientales

PRESENTA:

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Villahermosa, Tabasco, México. Septiembre, 2020

Villahermosa, Tab., a 21 de Septiembre de 2020

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A mis padres

Agradecimientos

A Dios y a mi familia por apoyarme en todo para poder cumplir esta meta; a mis padres por esta gran herencia, a mis hermanas por creer en mí y apoyarme para continuar mi carrera.

A la Universidad Juárez Autónoma de Tabasco, División Académica de Ciencias Biológicas de la ciudad de Villahermosa, Tabasco México, la cual dio las facilidades otorgadas para la realización de la tesis.

Al Consejo Nacional de Ciencia y Tegnología por otorgarme la beca que facilitó mis estudios durante dos años.

Al Dr. Emyr Saúl Peña Marín, quien fue mi asesor en todo lo realizado, por darme la oportunidad de trabajar con él y recibir sus conocimientos para el desarrollo de la presente investigación, por su paciencia ,por compartirme su conocimiento, sus consejos y sus experiencias académicas

Al Dr. Carlos Alfonso Alvarez González, por su gran apoyo a cada paso de esta investigación, por darme acceso a las instalaciones a su cargo y facilitarme todo lo necesario para desarrollar el presente trabajo.

A mi comité tutorial y sinodales por sus valiosas observaciones, el Dr. Rafael Martínez García, a la Dra. Susana Camarillo Coop, y a la Dra. Rocío Guerrero Zarate

Al Centro de Investigaciones en Alimentación y Desarrollo, Unidad Mazatlan por recibirme, sobre todo al Dr. Leonardo Ibarra Castro y el Dr. Juan Manuel martínez Brown por apoyarme durante la estancia académica y facilitarme los recursos para la investigación.

Agradezco a los encargados de producción de peces en CIAD, Unidad Mazatlán en acuicultura y Manejo Ambiental, por facilitar los ejemplares utilizados para esta investigación; a mis compañeros y amigos que me apoyaron, por sus conocimientos y sugerencias en las diferentes actividades realizadas a lo largo de la investigación.

INDICE

INTRODUCCIÓN

La acuicultura es una importante actividad económica que provee medios efectivos para la producción intensiva de organismos acuáticos bajo condiciones controladas. Para que la acuicultura pueda desarrollarse de manera exitosa, es necesario hacer diferentes investigaciones con la finalidad de conocer las condiciones óptimas de las diferentes especies de interés (FAO, 2016). Para esto, es fundamental comprender la fisiología de las diferentes especies de peces, particularmente aquellas que presentan ontogenia indirecta, la cual se caracteriza por tener un sistema digestivo incompleto al momento de su eclosión, por lo que es una etapa crítica en su vida y que ocasiona cambios morfofisiológicos complejos que pueden afectar su transformación del periodo larval al de juvenil (Balon, 1990; Zambonino-Infante y Cahu, 2001).

Existen muchas especies emergentes de interés acuícola, dentro de las cuales, las de la familia Centropomidae, nombrados de manera común como robalos, poseen características que las posicionan como excelentes candidatos para su cultivo. Las diversas especies de Centropomidos habitan en aguas costeras someras, estuarios, ríos, lagunas salobres, y realizan migraciones hacia aguas dulces (FAO, 2016). Todas las especies de robalos son consideradas de alto valor económico, debido al sabor de su carne y al gran tamaño que alcanzan en la etapa adulta. La producción anual de robalos en México es una de las más importantes a nivel pesquero y acuícola y tiene una gran demanda entre los consumidores, por lo que se registró en 2017 un volumen de cosecha de 16,482 toneladas, con un valor de 755 millones de pesos (CONAPESCA, 2018).

El género *Centropomus* cuenta con 13 especies que componen la subfamilia Centropomidae (Carvalho-Filho *et al*., 2019), las cuales habitan el océano Atlántico occidental y el océano Pacífico oriental. En la región del océano Pacífico oriental, se registran seis especies de robalos (*C. armatus, C. medius, C. nigrescens, C. robalito, C. viridis y C. unionesis*), de los cuales *C. viridis* llega a medir hasta dos metros de longitud, aunque su talla comercial es de 60 cm (Tucker, 1987; Tringali *et al*., 1999; Perera-García *et al*., 2008).

Las estadísticas indican que la producción pesquera no satisface la demanda del mercado nacional y presenta una clara disminución a lo largo del tiempo, aunado a ser una especie de captura estacional (Anón, 1994). Debido a lo antes mencionado, es necesario el realizar estudios sobre la especie que permitan desarrollar la biotecnología de cultivo para la producción bajo condiciones controladas. En este aspecto, los estudios con base en la fisiología digestiva de las especies, permiten evaluar la capacidad enzimática digestiva durante diferentes etapas de vida, incluyendo la ontogenia temprana, por lo que esta información permite la generación de protocolos de alimentación, el desarrollo de dietas específicas, así como la determinación del protocolo para la deshabituación del consumo de presas vivas a alimentos balanceados y con ello, lograr una adecuada transformación de larva a juvenil (Moyano *et al*., 2005). En este sentido, existen técnicas bioquímicas que permiten determinar las capacidades enzimáticas digestivas de manera específica para cada etapa de vida y por ende, el potencial fisiológico digestivo, así como afinidad hacia ciertos insumos alimenticios (Zambonino-Infante *et al*., 2001).

De acuerdo a la Comisión de Enzimas (EC, Enzyme Commission) las enzimas se clasifican y se nombran según la clase de reacción que cataliza, a partir de lo cual se catalogan seis categorías principales de enzimas, las cuales son: 1) oxidorreductasas, 2) transferasas, 3) hidrolasas, 4) liasas 5) isomerasas, 6) ligasas (Álvarez-González *et al*., 2006). En este sentido, las enzimas digestivas son clasificadas dentro del grupo 3 de las hidrolasas, las cuales se encargan de romper macromoléculas (proteínas, lípidos y carbohidratos) en sus monómeros básicos (aminoácidos, ácidos grasos y monosacáridos), por lo que desde el punto de vista nutricional es proceso indispensable para la absorción de los nutrientes esenciales para que el organismo haga diversos procesos bioquímicos y metabólicos (Guillaume *et al*., 2004).

En la actualidad el INAPESCA está enfocando esfuerzos y recursos para detonar la maricultura en México, siendo las especies de la familia Centropomidae de gran interés dentro de este proceso. El primer cultivo a nivel piloto comercial se ha registrado para el robalo blanco (*C. viridis*) en al Pacífico mexicano, lo que engloba

la generación de zootecnia en reproducción, producción de juveniles y proceso de engorda, lo cual genera expectativas positivas para el cultivo de esta especie (Ibarra-Castro et al., 2017; INAPESCA, 2018). De esta forma, se requiere ampliar los conocimientos biológicos de las especies de esta familia, que ayuden a mejorar la zootecnia. En este aspecto, es fundamental el entender la capacidad digestiva, lo que incluye los mecanismos químicos del proceso digestivo en todas las etapas de desarrollo. Por lo tanto, conocer la funcionalidad de las enzimas digestivas en cada etapa de vida se convierte en una herramienta útil para mejorar la tecnología de cultivo (Álvarez-Lajonchére *et al*., 2002; Krogdahl *et al*., 2005).

ANTECEDENTES

La importancia del entendimiento de los cambios o variaciones de las enzimas digestivas durante la ontogenia temprana de peces, particularmente las de desarrollo indirecto, reside en que no poseen un tracto digestivo completo al momento de eclosionar, y por ende, la capacidad digestiva es limitada (Zambonino-Infante et al., 2001).

En este aspecto, se han realizado diversos estudios relacionados con el desarrollo del tracto gastrointestinal y los cambios de las enzimas digestivas en larvas de peces durante la ontogenia temprana. Algunos de ellos han sido realizados en diferentes especies, teles como el pejelagarto (*Atractosteus tropicus*) (Frías Quintana *et al*. 2015), Lubina europea (*Dicentrarchus labrax*) y corvina roja (*Sciaenops ocellatus*) (Zambonino-Infante et al., 2001), lenguado de California (*Paralichthys californicus*) (Álvarez-González et al. 2006), atún rojo del Pacífico (*Thunnus orientalis*) (Murashita et al. 2014). Donde se estudió el tracto gastrointestinal de peces, describiendo el desarrollo del proceso gastrointestinal de larvas de peces marinos por medio de técnicas bioquímicas, histológicas y electroforéticas, con especial atención a especies de importancia comercial. Con este tipo de técnicas se ha logrado entender la capacidad enzimática digestiva, se demuestra la acción de enzimas que permiten al organismo obtener los nutrientes necesarios y lograr los cambios morfofisiológicos necesarios para su transformación

al periodo juvenil. Asimismo, este tipo de investigación permite comprender el momento más adecuados para realizar los cambios de alimentación. Por lo anterior, es imprescindible entender el papel que juegan las enzimas digestivas a lo largo de la ontogenia (Zambonino-Infante et al., 2001; Álvarez-González et al., 2006).

Existen diversos estudios enfocados en los cambios de las enzimas digestivas durante la ontogenia temprana de especies de peces de la familia Centropomidae como el de Teles *et al*. (2015), quienes reportan la ontogenia del tracto digestivo de larvas del chucumite (*Centropomus parallelus*), por lo que indican que las larvas pueden realizar el cambio de alimento vivo por alimento inerte a los 35 días después de la eclosión (DDE), lo que incrementa el éxito de su larvicultura. Por su parte, Yanes Roca *et al*. (2017), estudiaron las actividades enzimáticas digestivas durante el desarrollo larval de *Centropomus nigrescens*, por lo que se demostró la presencia de patrones generales en la actividad enzimática digestiva y se sugiere la posibilidad de realizar el cambio de alimento vivo por alimento inerte antes de los 32 DDE. El estudio de Jiménez Martínez *et al*. (2012) en las larvas del robalo blanco del Golfo de México (*Centropomus undecimalis*) mostró que se puede realizar la deshabituación a partir de los 34 DDE, edad en la que las larvas incrementan la actividad de las enzimas digestivas estomacales, por lo cual se considera el cambio de larva a juvenil.

Por otro lado, también se han realizado caracterizaciones de enzimas digestivas en etapa juvenil de especies de la familia Centropomidae. Concha Frías *et al*. (2016), realizaron la caracterización parcial de proteasas digestivas en *Centropomus undecimales*, por lo que concluyen que esta especie puede considerarse un pez marino con hábitos nutricionales carnívoros, mientras que Jesús Ramírez *et al*. (2017), realizaron la caracterización parcial de proteasas digestivas del chucumite (*Centropomus parallelus*), y consideran que esta especie tiene una alta capacidad digestiva similar a otros peces marinos carnívoros estrictos. Del mismo modo, se han realizado estudios de expresión de genes en la familia Centropomidae, como es el caso del estudio realizado por Asencio-Alcudia et al. (2018), quienes evaluaron la expresión génica de enzimas digestivas durante la ontogenia inicial del robalo

mexicano (*C. poeyi*), y reportan la sobreexpresión temprana de las enzimas digestivas, especialmente de tripsina, por lo que sugieren que el cambio de alimento vivo por inerte se puede realizar a partir de los 25 DDE y es más temprano en comparación con otras especies de Centropómidos.

JUSTIFICACION

Los peces de la familia Centropomidae poseen un gran valor económico y alimenticio ya que forman parte de las pesquerías artesanales de mayor importancia tanto en el Pacifico mexicano como en el Golfo de México. Los Centropómidos presentan características que los posicionan como especies marinas con alto potencial acuícola, lo que genera la necesidad de desarrollar estudios que impulsen su tecnología de cultivo. Dentro del ciclo de vida, el periodo larval es considerado el más crítico al ser organismos con una capacidad digestiva limitada, además que, desde el punto de vista del cultivo, se presentan altas mortalidades lo que es considerado un cuello de botella para potenciar la producción de juveniles. De esta forma, se requiere conocer el manejo de la especie a partir de su entendimiento fisiológico para el éxito de producción. Es así que el conocer la capacidad digestiva de la especie, ayudará a entender los mecanismos bioquímicos de la hidrólisis de las macromoléculas durante la ontogenia temprana de *C. viridis*.

OBJETIVOS

OBJETIVOS GENERALES

- Caracterizar parcialmente las enzimas digestivas durante la ontogenia temprana y etapa juveniles del robalo blanco (*Centropomus viridis*).

OBJETIVOS ESPECÍFICOS

- Determinar la actividad de enzimas digestivas (proteasas ácidas, proteasas alcalinas, tripsina, quimotripsina, leucina aminopeptidasa, lipasas y α-amilasa) durante la ontogenia temprana de *C. viridis* con el uso de técnicas bioquímicas.
- Determinar los óptimos de actividad de pH y temperatura de enzimas digestivas (proteasas, lipasas y carbohidrasas) en juveniles de *C. viridis* con el uso de técnicas bioquímicas.
- Determinar la cantidad, tamaños y tipos de enzimas digestivas (proteasas y lipasas) en juveniles de *C. viridis* con el uso de inhibidores específicos y técnicas electroforéticas.

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Characterization of digestive enzymes during early ontogeny of white snook (*Centropomus viridis*).

Running title: Digestive enzymes in the early stages of *C. viridis*

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ABSTRACT

The white snook (*Centropomus viridis*) is a species with a high economic value; therefore, aquaculture efforts include the biochemical characterization of its digestive enzymes in all life stages. In this work, digestive enzymes were characterized in larval and juvenile stages of *C. viridis*. At hatching, alkaline proteases, trypsin, chymotrypsin, LAP, and lipase activities were detected, and showed different activity patterns as the larvae developed. Pepsin activity increased on day 23 after hatching (DAH), showing the highest activity peak on juveniles. Maximum activity was detected at 40 °C. Meanwhile alkaline protease activity was high on juveniles with maximum activity at 60 °C and pH between 8 and 9; α-amylase and lipase activities had maximum activities at 50 °C and two maximum pH values at 7 and 9. Pepstatin A inhibited 96% of the total activity of acid proteases. Alkaline proteases showed high inhibitory effects with PHEN, TLCK, PMFS, and OVO (51.29, 30.16, 27.26, and 24.68%, respectively). Lipase-like activity was totally inhibited by Ebelactone B, and highly inhibited by Ebelactone A, Orlistat, 1% SDS, and PMFS (96.12, 77.16, 64.22, and 51.29%, respectively). Seven bands (102.6, 65.7, 58.9, 40.6, 27.4, 21.5 and 15.8 kDa) were detected with alkaline protease activity and were highly inhibited by OVO, PMFS, SBTI and EDTA, and three bands (57, 43.9 and 32.6 kDa) with lipase activity were highly inhibited by Ebelactone A, Ebelactone B, and PMFS. The results show that the ontogenetic development of *C. viridis* is of the indirect type, where the digestive system is fully developed on the 23rd DAH. Likewise, digestive enzymes' characterization in juveniles shows a pattern similar to other strictly carnivorous marine fish, the species shows a diversity of alkaline proteases that works in a wide range of pH and temperatures, joined to presence of digestive lipases-like enzymes and esterases as well as α-amylase isoforms, which can give advantages to the species in the use of alternative nutritional sources in aqua-feeds formulation.

Key words: Centropomidae; digestive enzymes; larval ontogeny; lipases; proteases; α-amylases.

1. Introduction

Fishes are the most diverse vertebrates with around 35,562 valid species (Fricke et al., 2020). This high species diversity also reflects a high diversity of ontogenetic development patterns with species-specific adaptations to embryonic and larval life, that are closely associated with the niche that each species occupies in different life periods (Kováč, 2002). In aquaculture applications, small pelagic larvae with long-lasting development represent one of the greatest challenges for establishing controlled seed production in many marine fish species (Hu et al., 2018). In this type of larvae, well-developed organ systems do not appear at the first-feeding stage, but organ systems develop through different morpho-physiological changes that culminate in metamorphosis from larva to juvenile (Balon, 1990). For example, in larvae at the first-feeding stage, the digestive system lacks gastric glands and other digestive functions (Yúfera and Darías, 2007). To achieve a successful production in species with long larval periods is fundamental to understand the critical and complex developmental processes that determine the success of the larviculture (Zambonino-Infante and Cahu, 2001).

In aquaculture fish species, understanding the enzymatic-digestive capacities throughout the development is essential to develop management protocols, select nutritional sources to plan specific diets, adjust feeding protocols, and determine the protocol for weaning larvae (Moyano et al., 2005).

Many studies have identified different digestive enzymes (acid and alkaline proteases, trypsin, chymotrypsin, leucine aminopeptidase, carboxypeptidases, amylase, lipases, among others) during the ontogeny of marine and freshwater fishes like the red drum (*Sciaenops ocellatus*) (Lazo et al., 2000), California halibut (*Paralichthys californicus*) (Álvarez-González et al. 2006), Meagre (*Argyrosomus regius*) (Suzer et al. 2012), three spot cichlid (*Cichlasoma trimaculatum*) (Toledo-Solís et al. 2015) among others. These studies highlighted the importance to understand the digestive development during ontogeny, as a fundamental basis of its zootechnical management.

The diversity of emerging new species for commercial aquaculture is important and the Centropomidae family is included as a new option. This family has species inhabiting shallow coastal waters, estuaries, rivers, and brackish lagoons, and some of them migrate to freshwater. Some species from this family have a high economic value and it is related to their high meat quality and the size they can reach as adults (SAGARPA, 2010). Thirteen species of the *Centropomus* genus (Carvalho-Filho et al., 2019) inhabit the western Atlantic and eastern Pacific oceans, with six endemic species in eastern Pacific (*C*. *armatus*, *C*. *medius*, *C*. *nigrescens*, *C*. *robalito*, *C*. *viridis* and *C*. *unionesis*) (Tucker 1987; Tringali et al., 1999). Therefore, as a new species for commercial aquaculture, *C. viridis* basic knowledge is needed to understand the complex physical and biochemical changes in the gastrointestinal tract because biochemical adaptation could modulate the digestive tract enzyme systems. This knowledge is important to establish the larvae capacity to digest and absorb ingredients for different sources.

Previous studies on the *Centropomus* family species have characterized digestive enzymes during the larval development. For example, digestive enzyme activities during early ontogeny in the common snook (*C. undecimalis*) (Jiménez Martínez et al. 2012), the digestive tract and the development of accessory glands on fat snook (*C*. *parallelus*) larvae (Teles et al., 2015), the activity of digestive enzymes during larval development in black snook (*C*. *nigrescens*) (Yanes Roca et al. 2017), and gene expression of digestive enzymes during the ontogeny of the Mexican snook (*C*. *poeyi*) (Asencio-Alcudia et al., 2018). Other studies have characterized digestive proteases on *C. parallelus* (Jesús-Ramírez et al., 2017) and *C. undecimalis* juveniles (Concha-Frias et al., 2016).

Fishes digestive capacity can be improved if we understand the chemical mechanism by which food is digested from larvae to juveniles; thus, knowing the digestive enzymes at each stage becomes a helpful tool to know the animal's digestive ability. Consequently, this work aimed to characterize digestive enzymes during larval and juvenile stages of white snook (*Centropomus viridis*), as a new species for commercial aquaculture purposes.

2. Material and methods

2.1. Larval rearing and sample collection

Fertilized eggs of *C. viridis* were obtained from captivity brood stock (a female and three males) from the brood stock at the Food and Development Research Center (CIAD), Mazatlán unit. Oocyte maturation, ovulation, and spawn were induced using LHRHa in a cholesterol pellet (130 mg kg^{-1} dose), while, males were implanted with LHRHa in an EVAc Implant (635 ± 217 µg kg^{-1} dose) to produce eggs with 696 ± 3 µm diameters. Water quality parameters during egg incubation and larval rearing were temperature (26 ± 0.4 °C), salinity (35 g L^{-1}), dissolved oxygen (7.0 ± 0.6 mg L^{-1}; 105 ± 9 percent saturation), pH (7.9 ± 0.1) and total ammonia-nitrogen (≤ 0.5 mg L^{-1}). Larval rearing was handled in one 7-m^3 cylindrical fiberglass tank (black walls and a white bottom) with an initial stocking density of 82 eggs, based on the rearing protocols reported by Ibarra-Castro et al. (2017). Larvae total length (TL) measurements (±0.01 mm) were made with a binocular microscope.

During larviculture, *C. viridis* samples were taken at 0 (eggs), 1 (free embryo), 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 40, and 55 days after hatching (DAH). Samples were frozen and freeze-dried to preserve them until use. To characterize digestive enzymes during the early ontogeny of *C. viridis*, larvae samples from 3 to 40 DAH were dissected (cutting head and tail), while for eggs (0 DAH) and free embryo (1 DAH), whole body was used. The characterization of digestive enzymes in *C. viridis* juveniles was performed with fish samples of the 55 DAH, for which, the fish were dissected to extract the stomachs and intestines.

All samples previously described were homogenized with an ultrasonic processor 130 W (Daigger Scientific Inc., IL, USA), using a 1:10 dilution (tissue: saline solution) for all enzyme pools. The homogenates were centrifuged at 8,500 *g* for 15 min at 4 °C. The supernatant was recovered and distributed in aliquots and stored at -80 °C. Soluble protein concentration of each sample was determined by the Bradford technique (1976), using a standard solution of serum bovine albumin (2 mg ml^{-1}) for a calibration curve.

2.2. Activity quantification of digestive enzymes

Acid proteases activity was measured using the technique described by Anson (1938), using 1% of bovine hemoglobin as a substrate in 100 mM glycine-HCl, pH 2. Alkaline protease activity was determined using the technique described by Walter (1984), using 1% of casein as a substrate in 100 mM Tris-HCl, 10 mM $CaCl_2$, pH 9. Trypsin activity was quantified using the method described by Erlanger et al. (1961), with 1 mM Nα-Benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA) as a substrate in 100 mM Tris-HCl, 10 mM $CaCl_2$, at pH 8. Chymotrypsin activity was measured with the Del Mar et al. (1979) technique using 0.1 mM SAAPNA (N-succinyl-ala-ala-pro-phe p-nitroanilide) as a substrate in 100 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.8. The activity of leucine aminopeptidase (LAP) was measured with the technique described by Maroux et al. (1973), using 1 mM L-leucine *p*-nitroanilide as a substrate in 50 mM sodium phosphate, pH 7.2. The α-amylase activity was measured with the technique described by Robyt and Whelan (1968), using 2% potato starch as a substrate in 100 mM sodium dibasic phosphate and sodium citrate monohydrate buffer at pH 7.5. Lipase activity was measured with a modified technique described by Versaw et al. (1989), using 2-Naphthyl acetate (200 mM) as a substrate in 50 mM Tris-HCl, pH 7.2 and sodium taurocholate (100 mM). To reveal the enzymatic activity, a Fast-Blue B salt solution (100 mM) was added, and the mixture was clarified by adding ethanol: ethyl acetate solution (1:1 v/v).

One unit (U) of enzymatic activity was defined as the amount of enzyme that produced 1 µM of tyrosine using hemoglobin and casein as substrates, 1 µM of *p*-nitroanilide using BAPNA, SAAPNA and L-leucine-*p*-nitroanilide, 1 µM of p-naphthol using 2-Naphthyl acetate, and 1 µM of maltose using starch.

Total activity was calculated as Units ml^{-1} = [ΔAbs × final reaction volume (ml)]/[ε × time (min) × extract volume (ml)], and specific activity was calculated as Units mg $prot^{-1}$ = (Total activity)/(mg of soluble protein). ΔAbs represented the increase in absorbance and ε (molar extinction coefficient) for tyrosine, p-nitroanilide, p-naphthol and maltose was 0.005, 0.008, 0.02 and 0.0032 μM^{-1} cm^{-1}, respectively.

2.3. Effect of temperature and pH on enzymatic activity

Optimal temperature and pH of acid and alkaline proteases, lipases, and α-amylase were determined using an enzymatic pool extract from 10 juveniles (fish sampled on the 55 DAH). For optimal temperature, extracts were incubated in a temperature range between 10 to 70 °C with intervals of 10 °C, using the techniques described in the previous section for each enzyme type. The optimum pH of acid proteases was determined in the range of 1 to 5, while alkaline proteases, lipases and α-amylase in a pH range of 5 to 11 at 37 °C, with the following buffers previously reported by Matus de la Parra et al. (2007): glycine-HCl, pH of 1 to 3; acetate buffer, pH of 4 and 5; Tris-HCl, pH of 7 to 9; and glycine-NaOH, pH 10 and 11. The buffer pH was adjusted, but the compositions were the same as described in the section 2.2.

2.4. Effect of inhibitors on enzyme activity

Samples of the stomach and intestine were incubated at 37 °C for 1 h with the following inhibitors: 1 mM of Pepstatin A for acid proteases (only stomach samples), and 10 mM tosyl-phenylanyl-chloromethyl ketone (TPCK), 10 mM 1,10-phenanthroline (PHEN), 10 mM ethyl-diamine tetra-acetic acid (EDTA), 10 mM tosyl-lysyl-chloromethyl ketone (TLCK), 250 mM ovalbumin (OVO), 250 mM soybean trypsin inhibitor (SBT1), and 100 mM phenyl methyl sulphonyl fluoride (PMSF) for alkaline proteases (only intestinal samples), and residual activity was quantified with the previously described techniques (Anson, 1938; Walter, 1984) in the section 2.2. The inhibition percentage of lipase was measured by incubating 55 DAH samples with 1 mM Ebelactone A (Ebe A), 1 mM Ebelactone B (Ebe B), 1 mM Orlistat, 100 mM PMSF at 37 °C for 1 h, and 2-Naphthyl acetate as substrate as previously described by Versaw et al. (1989) in the section 2.2.

2.5. Zymograms

Activity bands of alkaline proteases and lipases were revealed by PAGE electrophoresis 10% using samples of intestinal extracts of juveniles sampled on the

55 DAH. For alkaline proteases, samples were run in 4% stacking and 10% resolving gels, and 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3 buffer for 120 min and 100 volts (Laemmli 1970; García-Carreño et al., 1993). At the end of the electrophoresis, the gels were washed and incubated for 30 min at 5 °C in a 2% Hammerstein casein solution with 100 mM Tris-HCl, 10 mM $CaCl_2$, pH 9. Then the gels were washed and incubated in the same solution at 37 °C for 90 min. The gels were washed with distilled water and fixed in trichloroacetic acid (12%) solution for 15 min.

For lipase zymograms, two discontinuous gels (4% stacking and 10% resolving gel) were used. The electrophoresis was run in a 5 mM Tris-HCl, 0.62 M Glycine, pH 7.0 buffer with 100 volts for 120 min. At the end of the electrophoresis, the gels were washed and incubated in a 50 mM Tris solution at pH 7.5, with 25 mM of substrates (2-Naphthyl acetate or 4-Nitrophenyl myristate) and 50 mM sodium taurocholate for 90 min. Next, the gels were transferred to a 50 mM Tris solution with 100 mM Fast Blue. Lipase activity was identified as bands with a strong color. SDS-PAGE markers of low range (Cat# 161-0304, BIO-RAD, Hercules, CA) were used to calculate molecular weights of the bands with proteolytic and lipase activity using the software Quality One V 4.6.5 program (Bio-Rad, Hercules, CA).

2.6. Statistical analysis

Data on enzymatic activities during early ontogeny and effect of temperature, pH and inhibitors type were submitted to normality (Kolmogorov-Smirnov) and homoscedasticity (Levene test). When the assumptions complied, a one-way analysis of variance (ANOVA) was applied. To test the differences between treatments, a posteriori Tukey test was performed. All tests were carried out using a level of significance of 95% using Sigma Plot (Systat Software, San Jose, CA).

3. Results

3.1. Digestive Enzymes during the larval development

Mean total length of *C. viridis* larvae at first feeding step was 2.75 ± 0.09 mm and grew to 24.35 ± 4.35 mm on the 42 DAH, where standard length growth was adjusted to an exponential model (R^2 = 0.993) (Fig. 1).

The specific activity of acid proteases was detected from the first days at very low levels, increasing on the 11 DAH and decreasing on the 13th DAH, then showed a tendency to increase from the 21st DAH reaching maximum activity on the 35th DAH ($p \leq 0.05$). High digestive enzymatic activity of alkaline proteases was observed from the time of hatching with activity peaks on the 3rd, 7th, 13th and 40th DAH ($p \leq 0.05$) (Fig. 2A).

Trypsin activity was detected from the 1st DAH and showed a maximum activity on the 11th, 27th and 35th DAH. Chymotrypsin was detected on the 1st DAH with activity peaks on the 3rd, 13th and 27th DAH maintaining its activity until the end of the sampling period ($p \leq 0.05$). Leucine aminopeptidase activity was detected from the 1st DAH, increasing on the 7th and 23rd DAH, with a maximum activity between the 35th and 40th DAH ($p \leq 0.05$) (Fig. 2B).

The activity of α-amylase was detected from the 3rd DAH, showing a maximum activity on the 9th, 23rd and 35th DAH, without changes until the 40th DAH ($p \leq 0.05$). Lipase activity was detected from the 1st DAH, showing activity peaks on the 5th, 19th and 27th DAH with the highest activity on the 35th DAH ($p \leq 0.05$) (Fig. 2C).

3.2. Effect of temperature and pH on juvenile's digestive enzymatic activity

The maximum temperature of acid proteases was found at 40 °C, showing the lowest values at 10 °C (40%) and 70 °C (50%) ($p \leq 0.05$) (Fig. 3A). The maximum acid protease activity was found at pH 2.0, showing 84.71% of relative activity at pH 3, decreasing to 17.34% at pH 4, however showed an increase in activity at pH 5 (68.17%) ($p \leq 0.05$) (Fig. 3B).

Digestive alkaline proteases showed maximum activity at 60 °C, while α-amylases and lipases showed the same maximum activities at 50 °C ($p \leq 0.05$) (Fig. 3C). The maximum alkaline protease activity was found between pH 8 and 9, while lipase and

α-amylase activities showed two maximum peaks of activities at pH 7 and 9 ($p \leq 0.05$) (Fig. 3D).

3.3. Effect of inhibitors on juvenile's enzymatic activity

Digestive alkaline proteases were not inhibited with TPCK (90.97% of residual activity) and EDTA (99.11% of residual activity), while PHEN, TLCK, PMFS, and OVO showed a reduction of 51.29, 30.16, 27.26, and 24.68% of the activity ($p \leq 0.05$), respectively (Fig. 4A). Digestive acid protease activity showed that pepstatin A inhibited 96% the total activity (Fig. 4B).

Lipase results showed that Ebelactone B eliminated the activity (100% inhibition), while Ebelactone A (96.12%), Orlistat (77.16%), 1% SDS (64.22) and PMFS (51.29) decreased the activity (Fig. 4C), all this inhibitors showed significant differences ($p \leq 0.05$) compared with the control sample (without inhibitors).

3.4. Zymograms of juvenile's digestive enzymes

The zymogram of alkaline digestive proteases from intestine samples of juveniles (55 DAH) showed seven bands with digestive protease activity (102.6, 65.7, 58.9, 40.6, 27.4, 21.5 and 15.8 kDa) in the control sample (without inhibitors) (Fig. 5). On the other hand, OVO and PMFS inhibited six activity bands (65.7, 58.9, 40.6, 27.4, 21.5, and 15.6 kDa), SBTI inhibited four bands (58.9, 40.6, 27.4, and 21.5 kDa), TLCK inhibited four bands (65.7, 27.4, 21.5 and 15.8 kDa), EDTA inhibited four bands (102.6, 65.7, 58.9, 40.6 kDa), TPCK inhibited two bands (65.7 and 27.4 kDa) and PHE inhibited one band (102.6 kDa).

The zymogram of lipase, using 2-Naphthyl acetate as substrate, showed three bands with lipase-like activity (57, 43.9 and 32.6 KDa) in the control (Fig. 6A). Ebelactone A inhibited the 43.9 kDa band, Ebelactone B inhibited the 57 kDa band, while PMFS inhibited the 32.6 kDa band. Orlistat did not inhibit any band. Likewise, the zymogram of lipase using myristate as a substrate showed the same three bands with lipolytic activity (57, 43.9 and 32.6 kDa) in the control (Fig. 6B). The addition of

Ebelactone A and PMFS inhibited the same 43.9 kDa band, while Ebelactone B and Orlistat did not inhibit any band.

4. Discussion

4.1. Characterization of Digestive Enzymes During Early Ontogeny|

The detection of alkaline enzyme activity from the first days of hatching in *C. viridis* agrees with other reports of active alkaline digestive enzymes before the digestive tract is complete (Moyano et al., 1996), where an increase in activity may be regulated by transcriptional factors (Yúfera and Darias 2007; Zambonino-Infante and Cahu, 1994). In this sense, previous studies with fasted and daily fed larvae of *C. nigrescens* confirmed the regulation by transcriptional factors (Yanes Roca et al., 2017), which lead to the increase in activity associated with high growth rates, fast anatomical, and physiological changes, and the stomach development (Zambonino-Infante and Cahu 2001; Zambonino-Infante et al., 2008).

Reports in *P. californicus* showed that trypsin and other alkaline proteases secretion is similar during larval ontogeny, indicating that a large part of the proteases present was trypsin-like (Álvarez-González et al., 2006), which agrees with this study, where trypsin showed similar activity peaks compared to the total activity of alkaline proteases. In *C. undecimalis*, trypsin activity peaks were detected on the 1st and 7th DAH and were reduced on the 34th DAH, while chymotrypsin reached its peak on the 25th DAH and then decreased (Jiménez Martinez et al., 2012). In our results, the increase in trypsin activity agrees with the opening of the mouth and the appearance of the first granules of secreted trypsin zymogens that has been observed in other marine fish larvae (Teles et al., 2015).

The relationship between trypsin and chymotrypsin is used as a nutritional index. In normal conditions, trypsin increases due to the demand for protein hydrolysis, but under inadequate feeding or food restriction, trypsin decreases while chymotrypsin secretion increases (Moyano et al., 1996; Cara et al., 2003). The secretion of pancreatic enzymes (α-amylase, trypsin, chymotrypsin, and lipase) evidenced by the enzyme activity has been used as a maturation indicator of the digestive system and

support glands of the digestive system during larval development, where the secretion of these enzymes is higher in larvae with good growth and healthy development, indicating an excellent nutritional status (Zambonino-Infante and Cahu, 1994, 2001).

In this work, leucine aminopeptidase (LAP) activity was detected from the 1st DAH, increasing on the 7th and 23rd DAH, with a maximum activity peak on the 35th DAH, remaining until the 40th DAH. However, in *C*. *undecimalis*, LAP activity peaks by 2 and 12 DAH, decreased until the end of the fish development (Jiménez Martinez et al., 2012). In the black snook (*Centropomus nigrensces*), the highest peak of activity was detected on the 5th DAH and then decreased (Yanes Roca et al., 2017). A high LAP activity is related to the stomach's absence in the first development stages, where protein micropinocytosis and its intracellular digestion is active. Nevertheless, in *C*. *viridis* the activity was maintained until the 40th DAH, suggesting a high digestive performance by the species (Zambonino-Infante and Cahu, 2001, Zambonino-Infante et al., 2008).

The activity of α-amylase was detected from 3rd DAH, showing different activity peaks. In the case of *C*. *undecimalis*, amylase activity was detected from hatching, showing peaks of maximum activity on the 7th DAH, decreasing until the end of the fish development (Jiménez Martinez et al., 2012). Studies in the California halibut, α-amylase activity is detected from the embryonic stage, being maintained until the 30th DAH (Álvarez-González et al., 2006). These differences could mean that α-amylase at the beginning could be genetically encoded and its expression depends on whether it is required and the food composition (Álvarez-González et al., 2008).

The α-amylase activity during larval development suggests that the metamorphosis process has not ended, while decreasing activity, even before fed offering, it is a reflex of the stomach maturation (Zambonino-Infante and Cahu, 2001; Mata-Sotres et al., 2016). However, high levels of α-amylase activity could serve as a strategy to use carbohydrates from feeds in a carnivore fish (Álvarez-González et al., 2008; Jiménez-Martinez et al., 2012).

In this study, lipase activity was detected from the first days of development, showing three activity peaks on the 5th, 19th and 27th DAH, reaching the highest peak on the 35th DAH. These results agreed with reports in *C*. *undecimalis* (Jiménez-Martinez et al., 2012). The presence of lipases in the first days of hatching is related with the lipid absorption from the yolk sac, and in the following days to changes in feeding and enrichment of the food with lipid supplies (Green and McCormick 2001).

During the development of the digestive system in gastric fish, protein digestion is modified by acidification of the medium by secreting HCl from the gastric glands in the stomach and the production of pepsin-like enzymes (Kvåle et al., 2007). In this study, pepsin activity showed an increase on the 11th and 21st DAH until reaching a maximum activity on the 35th DAH, suggesting the development of a functional stomach, corroborated with a decreased of alkaline proteases activity on the 23rd DAH. Therefore, the appearance of gastric cells marks the beginning of the juvenile stage as well to the weaning process in larvae management (Salze et al., 2012). Something remarkable that we found in *C. viridis* is the increase of acid protease activity on the 23rd DAH, meanwhile in *C*. *poeyi*, *C*. *parallelus*, *C*. *nigrensces*, and *C undecimalis*, acid protease activity increased until the 25th, 30th, 32nd, and 34th DAH (Jiménez-Martinez et al., 2012; Teles et al., 2015; Asencio-Alcudia et al., 2018; Yanes Roca et al., 2017), being *C*. *viridis* the species that shows the shortest larval period comparing to other *Centropomidae* species.

4.2. Digestive Enzymes Characterization

In this study, the maximum pH value for acid proteases was 2, coinciding with reports for most carnivorous species, like *C*. *parallelus* (Jesús-Ramírez et al. 2017), *C*. *undecimalis* (Concha-Frías et al., 2016), and the spotted sand bass *(Paralabrax maculatofasciatus*) (Álvarez-González et al., 2008), where pepsin-like enzymes have an optimum pH between 2 and 4, among several fish carnivorous fishes (Klomklao et al., 2008). The stomach is the most important organ for protein digestion in carnivorous fish species. This organ needs low pH values to generate protein denaturalization because of the secreted HCl, where pepsin cleaves peptide

bonds formed by aromatic amino acids (phenylalanine and tyrosine) from the proteins N–terminus to release peptides (Zhao et al., 2011), to subsequently be exposed in the pyloric caeca and/or intestine to a wide variety of enzymes from the pancreatic and luminal origin (endo- and exo-peptidases). This process complements protein hydrolysis to release small peptides (di- and tri-peptides) and free amino acids, which are absorbed by the enterocytes (Yúfera and Darias 2007).

Acid protease activity was completely inhibited by pepstatin A, suggesting that pepsin A is the main digestive protease in the acid phase of *C*. *viridis*, just like in *C*. *parallelus* (Jesús-Ramírez et al., 2017) and *C*. *undecimalis* (Concha-Frías et al., 2016), species in which, only one activity band was detected with Rf of 0.35 and 0.45 respectively.

The maximum pH for alkaline proteases found for *C*. *viridis* was between pH 7 and 9, which could be an effect of the mixture of several isoforms of serine proteases (trypsin-like and chymotrypsin-like) (Hidalgo et al., 1999; Álvarez González et al., 2006). In agreement, *C*. *parallelus* shows two peaks at pH 8 and 10 (Jesús-Ramírez et al., 2017) and *C*. *undecimalis* has two peaks, at pH 7 and 11 (Concha-Frías et al., 2016).

In some cases, alkaline proteases showed activity at pH 5 and 6 (70 to 80% of relative activity), suggesting the presence of pancreatic and/or intestinal proteases that work better in acid conditions (Moyano et al., 1996). Thiol proteases (mainly cathepsins) from pancreatic or intestinal origin display their maximum activity over a broad pH range (3.5 to 8.0) (Zeef and Dennison, 1988). The presence of such digestive enzymes possesses physiological importance because this species can hydrolyze proteins during the switch of acid to alkaline pH from the stomach to the intestine, resisting these conditions until it changes to a more alkaline pH (7 to 9) in the intestine where other alkaline enzymes are capable of acting (Álvarez-González et al., 2008).

Some inhibitors such as SBTI, TLCK, OVO, and PMFS reduced 27.98, 30.16, 24.68 and 27.26% of the residual activity of *C*. *viridis* juvenile's alkaline proteases respectively, these results agreed with reports in the fat snook (*C*. *paralellus*), where

SBTI, TLCK, OVO, and PMFS inhibited 25, 15, 10 and 40%, respectively (Jesús-Ramírez et al., 2017). In *C*. *viridis*, alkaline proteases showed some resistance to the inhibitors, since the highest percentage of inhibition was with PHEN, reducing 50% of the relative activity that could be related to the presence of metalloproteases (exopeptidases) such as aminopeptidase and carboxypeptidase (Moyano et al., 1996).

The zymogram showed that PMFS and OVO inhibited six of seven bands; however, these inhibitors do not discriminate between trypsin, chymotrypsin, and elastases. For example, *C. parallelus* shows five alkaline digestive activity bands where PMFS and OVO inhibited three of them (Jesus-Ramirez et al., 2017). The addition of TLCK inhibited the four bands with lower molecular weight meanwhile SBTI inhibited the five bands with lower molecular weight in *C. viridis*, these inhibitors have been used for trypsin-like identification (Klomklao et al., 2006) that are specific digestive enzymes with a molecular mass between 22 to 30 kDa (Bougatef, 2013). Previous studies in *C. parallelus* demonstrated the importance of trypsin for digestion to highlight the species' carnivorous nature (Jesús-Ramirez et al., 2017). The TPCK inhibitor only inhibited two bands (27.4 and 65.7 kDa) and is used to identify chymotrypsin-like enzymes. In the sailfin catfish (*Pterygoplichthys disjunctivus*), a chymotrypsin-like enzyme was purified from the viscera that showed a molecular weight similar to *C. viridis* chymotrypsin-like enzymes (29 kDa), which was also inhibited by TPCK (Villalba-Villalba et al., 2013). Even though TPCK inhibited the activity band of 65.7 kDa, we do not have enough information to classify it as a chymotrypsin-like enzyme, only as an alkaline protease, since there are no reports that identified chymotrypsins with a similar molecular weight.

Otherwise, EDTA inhibited the four bands with higher molecular weight; meanwhile, PHE inhibited only the band with the highest molecular weight in *C. viridis*. In particular, metalloproteases include amino and carboxypeptidases that are intestinal brush-border membrane proteases. Both types of digestive enzymes display an essential role in the final protein digestion, liberating amino acids that are absorbed by enterocytes (Chong et al., 2002). These exopeptidases have been

detected in some marine (Jesús-Ramírez et al., 2017, Peña et al., 2017) and freshwater fishes (Chong et al., 2002; Guerrero-Zárate et al., 2014). As an example, the Atlantic cod (*Gadus morhua*) has a collagenolytic serine protease that belongs to Zn^{2+} metalloproteinase family with the possibility to display trypsin-like and chymotrypsin-like activities (Kristjánsson et al., 1995). The inhibition (inactivation) results with chelates suggest that metalloproteases in *C*. *viridis* could have a role as digestive enzymes; however, more studies are needed to elucidate the dependence of a divalent metal cofactor (Mn^{2+}, Co^{2+}, instead of Zn^{2+}) (Kim and Lipscomb, 1993) and if those metal ions need to be supplemented in the diet.

The optimal temperature of acid proteases in *C. viridis* juveniles (55 DAH) was at 40 °C, which is consistent with reports in *C*. *parallellus*, showing its optimum at 45 °C (Jesús-Ramirez et al., 2017), nevertheless, *C*. *undecimallis* reports an optimum temperature of 30 °C of acid proteases (Concha-Frías et al., 2016). Low temperatures decrease the activity of many physiological processes. Therefore, some fish species compensate the low activity values by increasing the stomach's food retention, which in conjunction with peristaltic movements and digestive substances, enhances protein digestibility (Álvarez-González et al., 2006).

The α-amylase activity is related to carbohydrate digestion, and it develops in the intestine by pancreatic secretions under a pH close to the pH of the organs (Hidalgo et al., 1999; Natalia et al., 2004). In our work, two optimal activities were found at pH 7 and 9, which is consistent with reports in Common pandora (*Pagellus erythrinus*) (pH 7 and 9), Annular seabream (*Diplodus annularis*) (pH 6 and 9), blackspot seabream (*Pagellus bogaraveo*) (pH 4, 6, and 8) (Fernandez et al., 2001), in predatory and non-predatory fishes of Lake Chany in West Siberia (pH 7 and 9) (Solovyev et al., 2015). Herbivores and omnivores fishes have a higher α-amylase activity than carnivores, showing the importance of digestible carbohydrates in the nutrition of herbivores and omnivores (German et al., 2016). Furthermore, the presence of several amylase isoforms in the digestive system is typical of many species including carnivores (Champasri and Champasri, 2017), where the existence of these enzymes also means that fish can digest a broader range of

vegetable ingredients, helping to develop cost-effective formulated diets for farming (Natalia et al., 2004). However, the number of amylase isoforms does not precisely correlates to higher enzyme activity, while other factors could regulate such processes (German et al., 2016).

On other order of ideas, lipase activity showed two pH optimums (7 and 9) in *C. viridis* juveniles, while lipase zymograms showed three bands with a lipolytic activity using two substrates (acetate and myristate). Results showed the presence of lipase-like (dependent bile salts lipases), and esterase-like enzymes in the juvenile stage in our species, similar to *C. undecimalis* (Jiménez-Martínez et al., 2012), where both are capable of hydrolyzing the ester bonds of the carboxylic acids and release the fatty acids (Nazemroaya et al., 2015).

The main function of dependent bile salts lipases in mammals is the hydrolysis of cholesterol and retinyl fatty acid esters (Rudd and Brockman, 1985), and by possessing a wide range of substrate specificity, those lipases have a high affinity for di- and triglycerides with long-chain polyunsaturated fatty acids (Bornscheuer, 2002; Gjellesvik et al., 1992), while many esterases easily hydrolyze short-chain fatty acids (C_2 and C_4) without the presence bile salts (Solovyev et al., 2015), and could act in non-lipidic substrates (Bornscheuer, 2002). In this sense, *C. viridis* has an advantage on its lipid digestive capacity, where lipases act on fatty acids with ≥ 10 carbon atoms, while esterases acts among fatty acids with ≤ 10 carbon atoms (Gilham and Lehner, 2005), generating a wide range of digestible lipid substrates for the species. Considering the above-mentioned, the importance of lipase activity to provide high quality fatty acids is essential because lipids are an important part of the energy intake of marine carnivorous fishes (Watanabe, 1982) since the availability to use carbohydrates is low compared to the terrestrial species (Gjellesvik et al., 1992; Solovyev et al., 2015).

Some animals respond to internal and external environmental conditions with phenotypic plasticity, which means that they can modulate their biochemical, morphological, and physiological performance, even their behavior, as an adaptive response by phenotypic adjustments. In this sense, the digestive tract is the interface

of energy supply from the environment, where the digestive plasticity is related to energetic demands (Zaldúa and Naya, 2014; Ruthsatz et al., 2019). In fishes, the activity of pancreatic and intestinal digestive enzymes can show flexibility as a digestive plasticity by experiencing a temporal change in food composition (German and Horn, 2004) and/or feeding schedule (Lazado et al., 2017). Therefore, the digestive enzyme set, including proteases, lipases, and amylases on *C. viridis* shows digestive functionality in a wide range of conditions, and highlights the digestive plasticity potential of the species.

5. Conclusion

C. viridis presents high activity of alkaline proteases and lipases since hatching. In contrast, acid protease activity reaches the maximum value on the 23rd DAH, marking the beginning of the juvenile stage. The juvenile stage showed characteristics of a carnivore species with higher acid protease activity, presenting a wide diversity of serine and metallo-proteases on the alkaline digestive phase (intestine). The digestive repertory of the species shows the presence of lipases and esterases enzymes, and some α-amylases isoforms, digestive characteristics that can give the species a digestive advantage on the aqua-feeds formulation. All this new information can be used as a tool for design specific diets in this new species for aquaculture.

ACKNOWLEDGMENTS

Authors thank Consejo Nacional de Ciencia y Tecnología (CONACyT) for the fellowship grants. The authors are thankful to the staff of the CIAD-Mazatlán Fish Plant for their essential assistance.

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Table 1. Feeding schedule and food types during white snook (*Centorpomus viridis*) larval rearing.

Food item	Type	Amount	Period (DAH)
Microalgae	*Nannochloropsis oculata*	0.5–1.0 × 106 cells ml^{-1}	0–12
*Enriched rotifer	*Brachionus sp*	15 rotifer ml^{-1}	2–24
Artemia nauplii	*Artemia sp*.	0.2–2 nauplii ml^{-1}	13–22
*Enriched *Artemia* metanauplii	*Artemia sp.*	2–5 metanauplii ml^{-1}	20–35
Compound dry diet	OTOHIME™/SKRETTING	Ad libitum	19–55

*Rotifer and *Artemia* enriched with DHA-Protein Selco and S. Presso respectively (INVE Aquaculture, Belgium) applied according to the manufacturer.

Table 2. Digestive enzyme activity (U ml^{-1} and U mg $protein^{-1}$; mean ± SD, n = 9) in enzymatic extracts of white snook (*Centropomus viridis*) juveniles.

Enzyme Type	U ml^{-1}	U mg protein $^{-1}$
Acid proteases	18333.5±151.5	89.34±0.738
Alkaline proteases	3713.7±103.5	18.10±0.504
Lipase	2347.9±123	11.44±0.599
α-amylase	6608.7±140.5	32.21±0.684

Figure legends

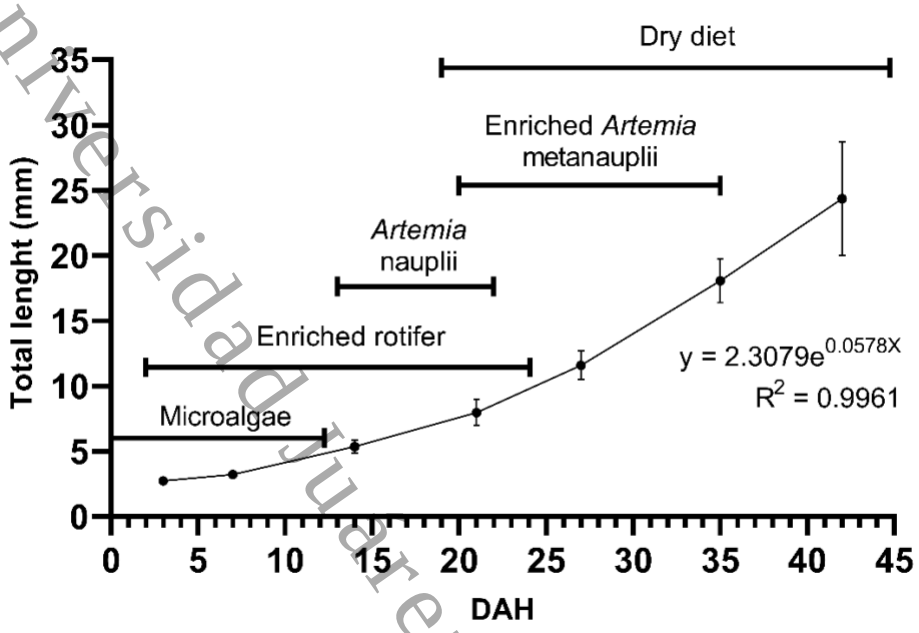

Figure 1. Larval growth in total length (mean ± SD) of white snook (*Centropomus viridis*) from hatching to 40 DAH.

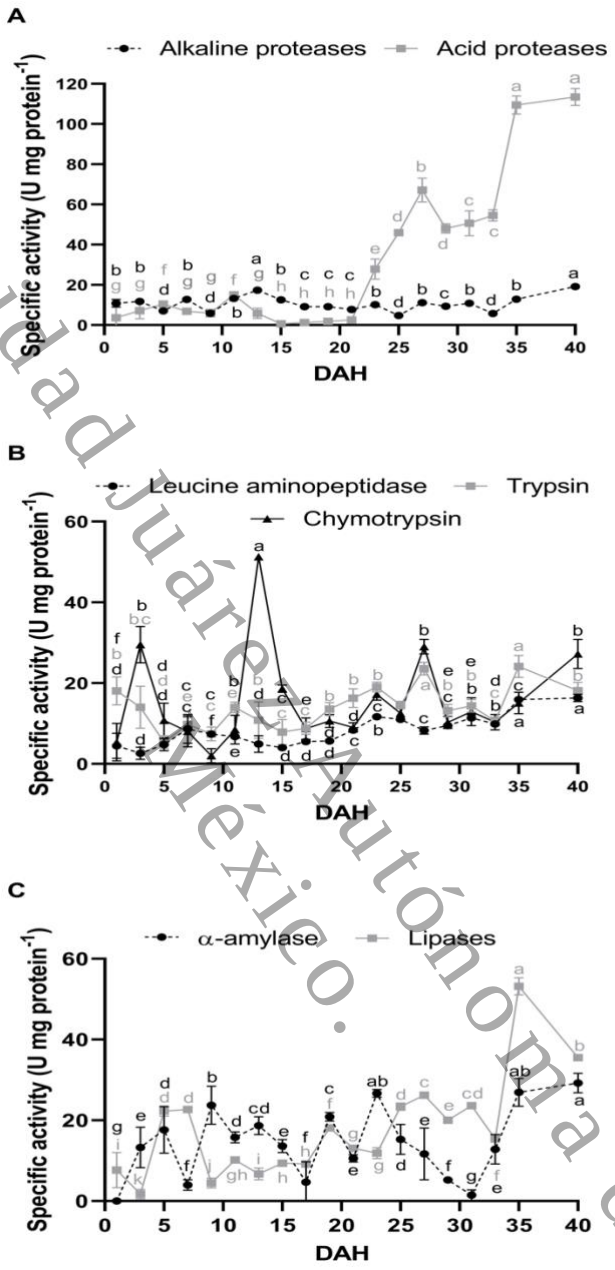

Figure 2. Specific digestive enzyme activity (U mg protein^{-1}) during larval development of white snook (*Centropomus viridis*) (mean ± SD), superscript letters indicate statistical differences among ages ($p < 0.05$) DAH. A) Acid and alkaline proteases; B) Trypsin, chymotrypsin, and leucine-aminopeptidase; C) Lipase and α-amylase.

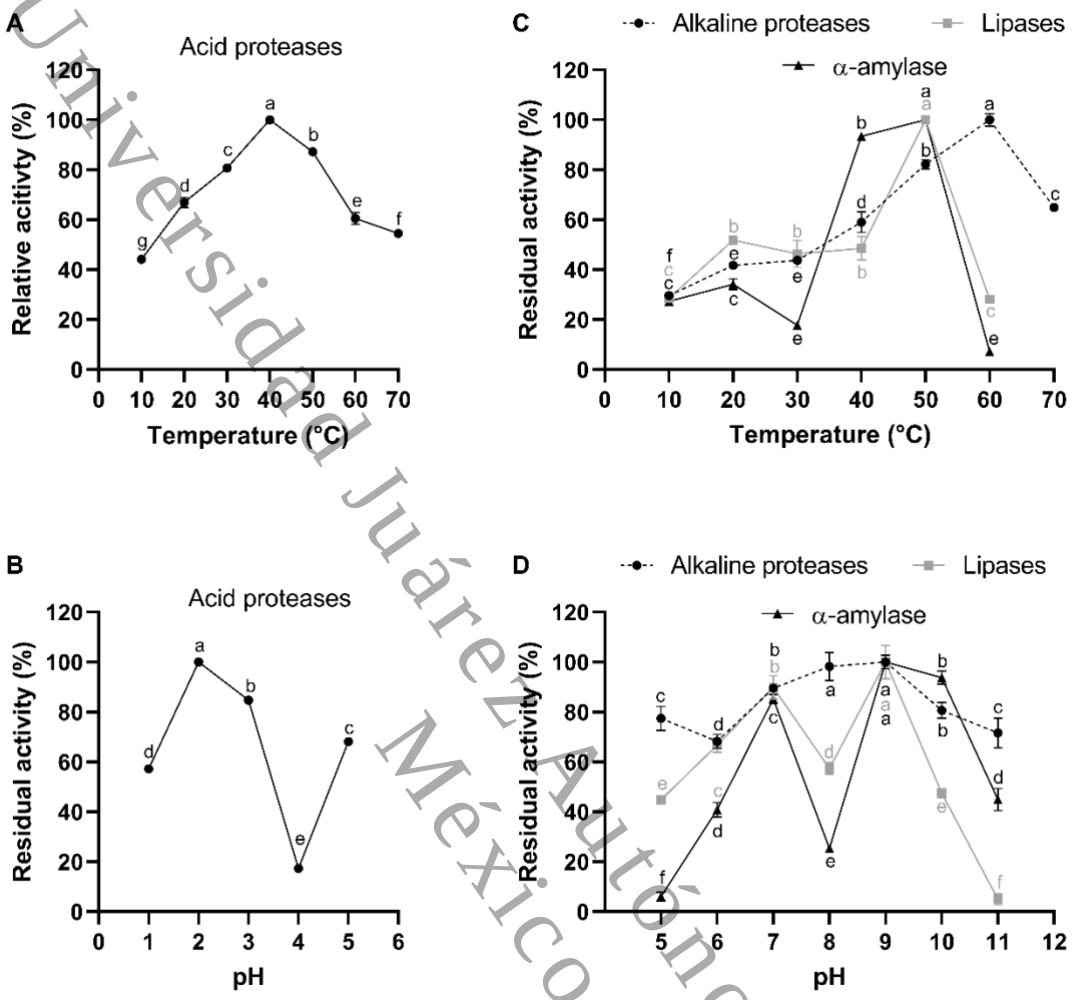

Figure 3. Effect of temperature (°C) and pH on white snook (*Centropomus viridis*) juvenile digestive enzymes. A) Temperature's effect on acid proteases; B) pH effect on acid proteases; C) Temperature's effect on total alkaline proteases, lipase, and α-amylase activities; D) pH effect on total alkaline proteases, lipase, and α-amylase activities.

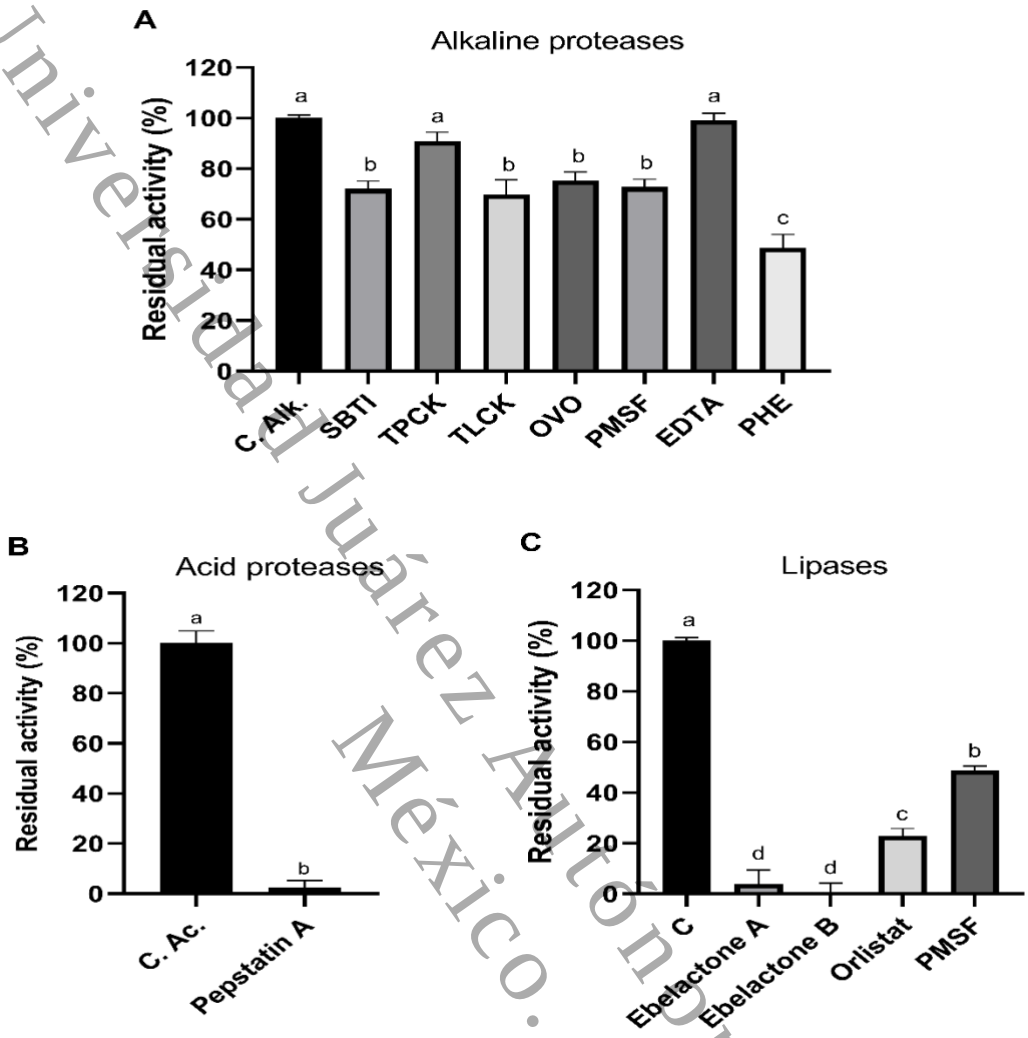

Figure 4. Effect of inhibitors on relative activity (%) of white snook (*Centropomus viridis*) juvenile A) Alkaline proteases with no inhibitor (C. Alk.), tosyl-phenylanyl-chloromethyl ketone (TPCK), 1, 10 phenanthroline (PHEN), ethyl-diamine tetra-acetic acid (EDTA), tosyl-lysyl-chloromethyl ketone (TLCK), ovalbumin (OVO), soybean trypsin inhibitor (SBTI), phenyl methyl sulphonyl fluoride (PMSF). B) Acid proteases with no inhibitor (C. Ac.), and acid proteases incubated with Pepstatin A. C) Digestive lipases: Lipase without inhibitor (Control); inhibitors: Ebelactone A, Ebelactona B, Orlistat and Phenyl methyl sulphonyl fluoride (PMFS).

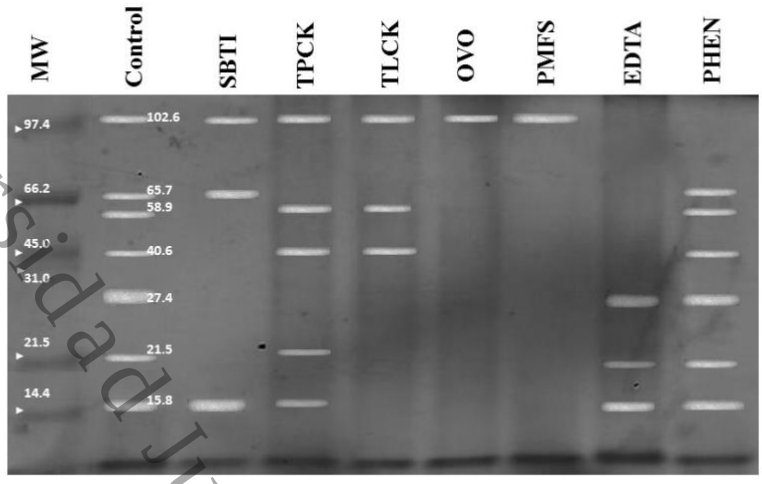

Figure 5. SDS-PAGE electrophoresis analysis of alkaline digestive proteases of white snook (*Centropomus viridis*) juveniles with different inhibitors. SDS-PAGE Markers: Phosphorylase B (97.4 kDa), Serum albumin (66.2 kDa), Ovalbumin (45.0 kDa), Carbonic anhydrase (31.0 kDa), Trypsin inhibitor (21.5 kDa), Lysozyme (14.4 kDa) (Cat# 161-0304). Alkaline proteases with no inhibitor (Alk control), tosyl-phenylanyl-chloromethyl ketone (TPCK), phenanthroline (PHEN), ethyl-diamine tetra-acetic acid (EDTA), tosyl-lysyl-chloromethyl ketone (TLCK), ovalbumin (OVO), soybean trypsin inhibitor (SBT1), phenyl methyl sulphonyl fluoride (PMSF).

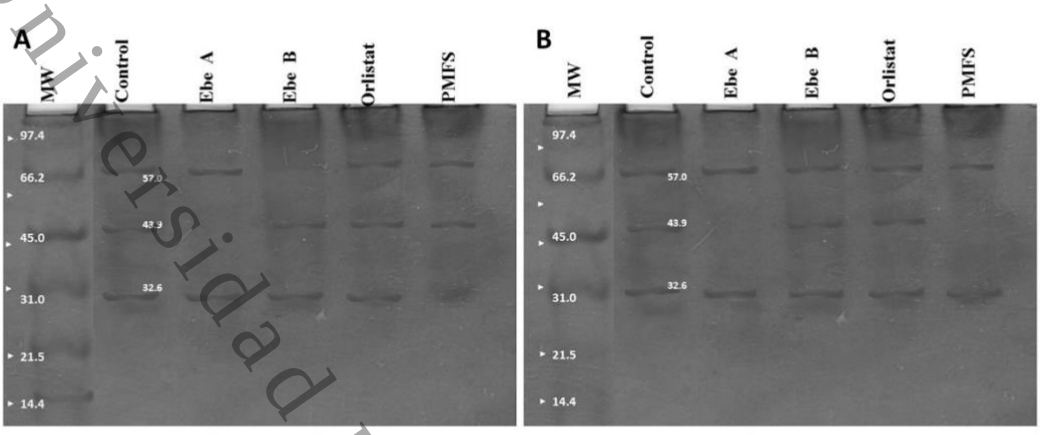

Figure 6. Native-Page electrophoresis analysis of lipases of white snook (*Centropomus viridis*) juvenile using 2-Naphthyl acetate and 4-Nitrophenyl myristate as substrate and exposed to different inhibitors. SDS-PAGE Markers: Phosphorylase B (97.4 kDa), Serum albumin (66.2 kDa), Ovalbumin (45.0 kDa), Carbonic anhydrase (31.0 kDa), Trypsin inhibitor (21.5 kDa), Lysozyme (14.4 kDa) (Cat# 161-0304). Control (without inhibitors), Ebelactone A (Ebe A), Ebelactone B (Ebe B), Orlistat, and Phenyl methyl sulphonyl fluoride (PMFS).