UNIVERSIDAD AUTÓNOMA DE BAJA CALIFORNIA

FACULTAD DE CIENCIAS MARINAS

INSTITUTO DE INVESTIGACIONES OCEANOLÓGICAS

Absorción *in vitro* de aminoácidos en el intestino proximal de peces carnívoros de agua dulce: trucha arcoiris (*Oncorhynchus mykiss*) y agua salada: atún aleta azul (*Thunnus orientalis*) y totoaba (*Totoaba macdonaldy*)

T E S I S

QUE PARA CUBRIR PARCIALMENTE LOS REQUISITOS NECESARIOS PARA OBTENER EL GRADO DE

MAESTRO EN CIENCIAS EN OCEANOGRAFÍA COSTERA

PRESENTA

ANTONIO ROSAS SERVIN

ENSENADA, BAJA CALIFORNIA, MÉXICO. NOVIEMBRE 2006

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PREFACIO

El presente trabajo contribuye a la generación del conocimiento básico para el desarrollo de un sistema *in vitro* que sea capaz de monitorear la digestibilidad de peces. Es así que el trabajo está dividido en dos artículos: el primero que incluye el perfil y actividad de enzimas digestivas del atún aleta azul (*Thunnus orientalis*) y el segundo establece las tasas de absorción a absorción máxima obtenidas a través del desarrollo del modelo de "intestino invertido". En este último, se midieron a partir de un hidrolizado proteico de caseina la absorción de un pool de amino ácidos divididos entre los esenciales y no eseniales. Este trabajo se basó en la absorción de la porción proximal del intestino de peces carnívoros de agua dulce: Trucha arcoiris (*Oncorhynchus mykiss*) y marinos: Atún aleta azul (*Thunnus orinetalis*) y Totoaba (*Totoaba macdonaldy*).

RESUMEN

Se evaluó el potencial digestivo en el atún aleta azul (*Thunnus orientalis*) mediante la identificación y caracterización de las diversas enzimas digestivas, utilizando extractos crudos de estómago, masa cecal e intestinos proximal, medio y distal.

Se encontró un nivel relativamente mayor de actividad proteolítica en la masa cecal comparado con el intestino proximal. La actividad de la tripsina fue mayor en la masa cecal, mientras que la quimotripsina mostró una mayor actividad específica en el intestino proximal, la actividad que en general disminuyó a lo largo del intestino. Al estudiar la actividad a distintas temperaturas y pHs, se obtuvo que la actividad proteolítica (pepsina, proteasas alcalinas, tripsina, quimotripsina y leu-aminopeptidasa) así como de amilasa y lipasa tuvieron su máximo en el rango alcalino, con un pH máximo a pH 9.0 y a temperaturas entre 35-60° C, excepto para la pepsina, que mostró la actividad a la misma temperatura pero en el rango ácido de pH (3.0).

Complementariamente, se midió el potencial de absorción de aminoácidos mediante la técnica de intestino invertido (intestino proximal fresco) donde se obtuvieron valores de concentración máxima al minuto 20 y velocidad de absorción, se utilizaron tres especies de peces carnívoros, uno de agua dulce, trucha arcoiris (*Oncorhynchus mykiss*) y dos marinos, el atún aleta azul (*Thunnus thynnus*) y totoaba (*Totoaba macdonaldy*). Con el fin de estandarizar el homogenado de absorción se utilizó un digerido pancreático comercial de caseína (Bacto™ tryptone DIFCO). Los resultados mostraron que hay preferencias en el paso de aminoácidos Treonina y Serina a través del intestino, a pesar de su concentración en el medio de absorción en las tres

especies estudiadas. La trucha presentó mayor tasa de absorción por cm^2 para todos los aminoácidos en comparación a los peces marinos. Se observó que aún entre especies existe preferencia para el paso de algunos aminoácidos, lo que nos indica que cada especie presenta un patrón de absorción diferente para cada aminoácido.

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1. INTRODUCCIÓN

El proceso de domesticación de cualquier especie requiere del estudio de los requerimientos nutricionales que permita un óptimo crecimiento con una alta eficiencia nutricional, lo que deberá traducirse en una ganancia máxima de crecimiento con un costo mínimo. Este tipo de estudios es complejo y requiere del confinamiento de los organismos que permita el estudio *in vivo* de digestibilidad, absorción de nutrientes, el control de los nutrimentos ingeridos, así como su respuesta individual o en grupo. Dichos estudios resultan ser complejos en peces por la dificultad de la evaluación de los parámetros requeridos en un medio ambiente diferente (agua), más aun cuando el tamaño y comportamiento del organismo requiera de instalaciones costosas para mantenerlos bajo condiciones controladas.

El atún es una especie que hoy en día se explota comercialmente en engordas (ranchos atuneros) en el noroeste de México con resultados, poniendo a México como el segundo productor a nivel mundial y con grandes expectativas de crecimiento en menos de 10 años de su inicio. De tal manera que en el 2004 el atún aleta azul (*Thunnus orientalis*) fresco se convirtió en el segundo producto de exportación en el sector de alimentos y bebidas, donde el volumen de exportación tuvo un incremento del 103%, al registrar 3,849 toneladas métricas, frente a 1,896 del año 2003 (Foodex 2005). Hoy en día México cuenta con el rancho de atún aleta azul más grande del mundo.

El cultivo de atún se basa en la engorda de juveniles capturados del medio natural, los cuales son arrastrados desde los lugares de su captura hasta las zonas aledañas a la costa, lo que les permite cosecharlos durante varios meses al año y comercializar el producto en fresco transportado a cualquier

parte del mundo, repercutiendo favorablemente en su precio. Durante el proceso de engorda, de 4 a 5 meses, estos peces se alimentan de sardina fresca, cuya captura está regulada por cuotas, lo cual es una limitante para el crecimiento de esta industria. Por lo anterior, es necesario contar con un alimento seguro que no dependa de las fluctuaciones naturales de su pesquería para poder mantener un crecimiento sostenido en la producción del atún, objetivo importante para la domesticación de cualquier especie.

Para realizar estudios de nutrición de esta especie será importante, por lo expuesto anteriormente, a recurrir a modelos *in vitro* que simulen su digestibilidad desde el laboratorio. Estos métodos *in vitro*, tienen la finalidad de medir la digestibilidad sin la manipulación directa de los organismos. Los sistemas desarrollados se basan en incubar distintos alimentos en presencia de enzimas y así predecir el nivel de digestión. Dichos métodos varían en el tipo de enzimas utilizadas, muchas de ellas se agregan grupos de enzimas comerciales generalmente procedentes de bovinos y cerdos, o bien a través de homogenizados crudos pero procedentes de organismos silvestres sin contar con un historial alimenticio (Outzen et al., 1996, Kurokawa et al., 1998). El estudio de sistemas *in vitro* para especies domésticas terrestres a pesar de haber sido exhaustivos (Boisen y Eggum, 1991, Van Soest, 1994), por lo general solo consideran el potencial de hidrólisis de la mezcla enzimática sin resolver la absorción de nutrientes, donde generalmente la información es interpretada como la calidad proteica de un ingrediente para un organismo determinado (Tanaka, 1973, Ezquerra et al., 1997; Ezquerra et al., 1998). No obstante, se ha desarrollado toda una serie de metodologías que combinan desde la estandarización de la dieta para la obtención de los extractos

enzimáticos así como la definición exacta de pasos y tiempos para la simulación del tracto digestivo (Ezquerra et al., 1997).

Un método que ha sido ya utilizado es el de intestino invertido, donde aparte de evaluar el grado de hidrólisis del alimento, se mide la tasa de permeabilidad a través del intestino (Buddington et al., 1987).

El objetivo del presente trabajo fue generar el conocimiento básico de fisiología digestiva del atún aleta azul (*Thunnus orientalis*) bajo condiciones de engorda, para conocer el tipo y actividad de las enzimas presentes en las diversas regiones del aparato digestivo (Articulo 1), y cuantificar la dinámica de absorción de los diferentes aminoácidos en la porción proximal (inmediatamente después de los ciegos pilóricos) del intestino de dos peces carnívoros marinos y de uno de agua dulce (Articulo 2). Para los estudios de absorción se utilizó el modelo del intestino invertido (Karasov y Diamond 1983) que es una técnica que desde hace tiempo se a empleado para el estudio de absorción en monogástricos (Karasov et al, 1983) y aun más en el área de farmacología (Stelzner et al, 2001, Ingrosso et al, 2006, Sharma et al, 2005). No obstante también hay varios trabajos en peces (Buddington y Diamon 1986, Buddington et al, 1991) realizados con el objetivo de ver interacciones en la absorción de amino ácidos, dipéptidos (Nordrum et al, 2000), glucosa (Buddington et al, 1987), y diversos compuestos que pueden absorberse por el intestino, así como algunos tóxicos (Weber y Lanno 2001). Lo novedoso de este estudio será el utilizar esta técnica para medir la dinámica de absorción en una región del intestino a partir de un hidrolizado completo de proteína. De esta manera se podrá contribuir al desarrollo de alimentos balanceados altamente digestibles.

Se utilizó a la trucha arcoiris (*Oncorhynchus mykiss*) y totoaba (*Totoaba macdonaldy*) como modelos control para la estandarización de las técnicas y comparación de los procesos de absorción.

2. OBJETIVOS GENERALES

Conocer el potencial digestivo del atún aleta azul (*Thunnus thynnus*) para generar el conocimiento básico que sirva para desarrollar un modelo de digestibilidad dinámico *in vitro*.

2.1 OBJETIVOS PARTICULARES

a) Determinar la actividad específica de las enzimas presentes en las distintas regiones del intestino del atún aleta azul (pepsina, tripsina, quimotripsina, α-amilasa y lipasa).

b) Caracterizar la actividad de cada enzima a diferentes pHs y temperaturas.

c) Montar la técnica del intestino invertido utilizando el intestino proximal de la trucha arcoiris (*Oncorhynchus mykiss*) y totoaba (*Totoaba macdonaldy*).

d) Determinar la absorción específica y tasa de absorción de los aminoácidos esenciales y no esenciales: en la porción proximal (inmediatamente después de los ciegos pilóricos) del intestino de peces carnívoros de agua dulce: trucha arcoiris y marinos: atún aleta azul y totoaba.

3. DISCUSIÓN GENERAL

El patrón enzimático del atún aleta azul (*Thunnus orientalis*) al igual que la mayoría de los peces carnívoros tendió a presentar una mayor actividad enzimática para las enzimas pepsina, tripsina, quimiotripsina, lipasas y una actividad menor para la enzima degradadora de carbohidratos, la α-amilasa.

La distribución de la actividad de la tripsina fue mayor para la masa cecal, lo que es indicativo del sitio de producción de esta enzima. La quimotripsina presentó mayor actividad en el intestino proximal y dicha actividad fue en disminución a lo largo del intestino, ya que en esta región se necesita que el bolo alimenticio tenga más contacto con la enzima para ser hidrolizado.

Los análisis de temperatura y pH mostraron que las actividades de las proteasas alcalinas totales, tripsina, quimiotripsina y aminopeptidasa fueron mayores a un pH alcalino, así como la amilasa y lipasa obtuvieron su máximo de actividad en el rango alcalino pH 9 y temperaturas de 35-60°C, excepto para la pepsina, que mostró su máximo nivel de actividad a las mismas temperatura pero a un pH de 3.

Se consideró importante estudiar el ambiente de la hidrólisis de la proteína en los peces ya que al no controlar su temperatura corporal, estos estudios nos indican la flexibilidad de las enzimas para seguir degradando los alimentos balanceados.

En el presente trabajo se montó la técnica del intestino invertido para medir la absorción intestinal utilizando a la trucha arcoiris. Con el fin de validar el sistema, y establecer el rango de tiempo de vida media del intestino despues de extraído del pez grado de seguridad de utilizar los tejidos en estado fresco, se realizaron controles experimentales; uno bajo atmósfera de CO_2 en donde

se inhibe cualquier transporte activo y el otro con tejido 8 horas después del sacrificio (postmortem). En estos experimentos se observó, que el oxígeno es indispensable para que se lleve a cabo la absorción, y en segundo lugar que el grado de frescura afecta la tasa de absorción después de 8 horas. Diferencias que se observaron sólo en algunos amino ácidos, como el incrementando al doble en la absorción de Glu, así como el decremento (a la mitad) de la Arg, Thr y Tyr. Este margen de 8h nos permitirá establecer con cierta seguraridad la manipulación de los intestinos sin tener un efecto negativo en la absorción.

El transporte de los intestinos de atún desde los corrales hasta el laboratorio se logró en 2h, tiempo en el que podemos asegurar los tejidos permanecieron en estado fresco.

Este trabajo sólo consideró la absorción en la porción proximal del intestino, la más cercana al estómago y después de los ciegos pilóricos, donde la absorción no sólo dependerá de la capacidad intestinal, sino seguramente también, del grado de hidrólisis presente en el quimo.

Un aspecto importante a destacar es hecho de que se detectó una absorción específica y tasa de absorción del 90% más en el intestino de trucha que en totoaba y atún. La causa de esto puede explicarse con lo expuesto por Ferraris y Ahearn (1984) quienes establecen que debido a que las células que están expuestas a un medio ambiente marino presentan un mayor intercambio de moléculas orgánicas para osmorregular, los nutrientes siguen un patrón de un contraflujo que hace que el resultado sea un menor flujo de nutrientes absorbidos. Si bien esta explicación podría resultar del todo entendible, sería interesante estudiar cual será el mecanismo que presentan estos organismos marinos para compensar este "inconveniente". Buddington et al. (1987)

establecen que dichas adaptaciones evolutivas se basan en modificaciones genéticas que implican cambios en la anatomía (longitud y grosor del intestino, aparición de un mayor número de ciegos pilóricos,), tiempo de retención intestinal, etc.

Por otro lado, los resultados aquí presentados muestran que hay preferencia para el paso de algunos aminoácidos independientemente de su concentración en el medio de absorción, especie y ambiente (marino y de agua dulce) como pudo ser observado para la Serina y Treonina.

En un futuro, será interesante conocer el grado de influencia de otros factores que intervengan en la absorción como lo son la fuente de origen proteico (vegetal o animal), y la evaluación comparativa entre las distintas regiones del intestino. El potencial de aplicación de este modelo podría ser amplio al incluir estudios de absorción de ingredientes con potencial para incluirse en las dietas de los peces, así como aditivos alimenticios que en su conjunto se puedan formular dietas altamente digestibles y aprovechables que, aparte de incrementar la eficiencia alimenticia disminuyan la carga de materia orgánica en el medio ambiente marino donde se desarrollan estas engordas.

4. CONCLUSIONES GENERALES

- El aparato digestivo del atún presenta enzimas catalíticas como proteasas (tripsina, quimiotripsina y aminopeptidadas) lipasas y α-amilasa con actividades específicas suficientes como para catalogar al atún con un potencial digestivo alto, además de poder modular la hidrólisis de materias primas i*n vitro* que permita determinar su digestibilidad.
- Se logró adaptar la técnica de intestino invertido para conocer el perfil y cinética de aminoácidos que se absorben a través del intestino de peces carnívoros a partir de un digerido pancreático de caseína (triptona), cuantificando su concentración por fluorescencia mediante HPLC.
- La trucha arcoiris y la totoaba (*Totoaba macdonaldi*) resultaron ser un buen modelo para el montaje de la técnica (por su manejo y disponibilidad) y posteriormente, mediante algunas adaptaciones necesarias para implementar la técnica de absorción *in vitro* en el Atún (*Thunnus orientalis*).
- Es indispensable el O_2 para el transporte de los aminoácidos al menos en esta porción del intestino estudiada, ya que al desplazar el oxígeno mediante el burbujeo con CO_2 la absorción disminuyó hasta un 90%
- Es importante contar con intestinos frescos ya que a las 8 horas postmortem hay una significativa alteración en el patrón de absorción.
- Los peces marinos presentaron una absorción específica por cm^2 equivalente al 10% de lo observado en la trucha arcoiris de agua dulce.

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6. ARTICULOS

6.1 ARTICULO 1

Partial characterization of the digestive enzymes of Pacific bluefin tuna *Thunnus orientalis* under culture conditions

A. Matus de la Parra · A. Rosas · J. P. Lazo and M. T. Viana

Sometido al Fish Biology 2006

6.2 ARTICULO 2

Comparative intestinal aminoacids absorption in Pacific bluefin tuna *Thunnus orientalis*, Rainbow trout (*Oncorhynchus mykiss*) and *Totoaba macdonaldi.*

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Partial characterization of the digestive enzymes of Pacific bluefin tuna *Thunnus orientalis* under culture conditions

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Abstract

Digestive enzyme activities of Pacific bluefin tuna *Thunnus orientalis* were evaluated and characterized in crude extracts of stomach, cecal mass, and proximal, middle and distal intestine. A relatively higher level of alkaline proteolytic activity was detected in the cecal mass compared to the proximal intestine. Trypsin like activity was higher in the cecal mass whereas Chymotrypsin showed higher specific activity in the proximal intestine, activity that in general decrease along the intestine. The temperature and pH analysis showed that proteolytic activity (pepsin, alkaline protease, trypsin, chymotrypsin and leu-aminopeptidase) as well as amylase and lipase were maximum in the alkaline range, with a maximum at pH 9.0 and temperatures between 35-60°C, except for the pepsin, that showed maximum activity at the same temperatures but in the acid range (pH 3.0). Lipase activity was evaluated in the presence or absence of various salts (NaCl, Na-taurocholate, Na-cholate and Na-deoxycholate) and a mixture of natural or commercial bile salts. Lipase activity was generally inhibited in the presence of bile salts and significantly increased in the presence of NaCl, suggesting that the principal tuna lipase is of the pancreatic type with no dependence on bile salts under our assay conditions. This knowledge will be of importance to design models to predict tuna fish digestibility under culture conditions.

Keywords Digestive enzymes · Pacific bluefin tuna · *Thunnus orientalis* · pancreatic lipase · bile-salts

Introduction

Pacific bluefin tuna *Thunnus orientalis* is one of the most important fishery resources in the north Pacific, where Japan is among the principal countries utilizing this resource. Within the fish market Pacific bluefin tuna can reach the highest market prices compared to other tuna fish (Tičina et al. 2004). Therefore, the culture of this valuable species in captivity has been one of special interest for marine fish farmers. Therefore, sea ranching has been developed, where fish are captured and transported alive to near-shore net-pens (50 m diameter) and fed fresh or frozen fish to recover by increasing the fat levels which results in high valuable fish for the international market.

The Pacific bluefin tuna is a carnivorous species with a well-defined stomach, a system of blind sacs called pyloric cecae and a very short intestine divided in proximal middle and distal.

The study of the fish digestive physiology will be used to develop novel diets for intensive farming of tuna species since a proper understanding of their digestive capacity to utilize different nutrients will be worth to design *in vitro* models to predict digestibility.

Several authors have performed research with several purposes comparing digestive enzymes activities in fish of different species and with different feeding habits (Simpson and Haard 1984; Martinez and Serra 1989; Chakrabarti et al. 1995; Hidalgo et al. 1999). Trypsin, chymotrypsin and pepsin are the most important digestive enzymes due to its high proteolytic activities; these enzymes have been characterized in several organs of different fish species (Gildberg and Raa 1983; Klomklao et al. 2004; Kishimura et al. 2005; Klomklao et al. 2006). Since the main purpose of tuna ranching is to increase

the lipid content of the muscle to a desirable level for the Japanese market, tuna are fed high lipid diets (fresh and frozen sardine and mackerel) often enriched with lipids and vitamins. Thus, the characterization of lipase activity would be of importance to improve lipid utilization.

To our knowledge little information is available with respect to the digestive physiology of the Pacific bluefin tuna under culture feeding, information that will be used to develop novel diets for intensive farming of tuna species through a proper understanding of their digestive capacity to utilize different nutrients and to design *in vitro* models to predict digestibility. Thus, the objective of this work is to characterize the digestive enzymes of the Pacific bluefin tuna under culture conditions as a first attempt to contribute to the developmental base of an *in vitro* digestibility.

Materials and Methods

Tuna Sampling

Pacific bluefin tuna *Thunnus orientalis* were obtained from a commercial farm "Maricultura del Norte, S.A. de C.V." in Ensenada, B.C., Mexico. Freshly dissected digestive tracts were collected from six Pacific bluefin tuna (24.15±7.58 Kg and 1.07±0.13 m) harvested from growout floating cages, immediately placed in iced-chilled containers and transported to the laboratory.

The digestive tract of each organism was individually dissected and divided into five segments: stomach, a well developed cecal mass (numerous pyloric cecae with an embedded pancreatic tissue, i.e., no discrete pancreatic tissue), proximal, middle and distal intestine, all procedures were conducted in the cold (0-4ºC). The stomach lumen and the three intestine segments were thoroughly scrapped, whereas the cecal mass was cut into small pieces. Each

section was individually ice-cold-homogenized with an ultra-Turrax homogenizer in physiological saline solution (NaCl 0.9%) and diluted in 1:3 (wet weight:volume) for stomach and cecal mass, 1:4 (w:v) for proximal and distal intestine and 1:7 (w:v) for middle intestine. All homogenates were centrifuged at 8,500 *g* for 15 min at 4°C. The supernatant (crude extracts) samples were frozen and stored at -79°C until enzyme assays.

Enzyme Activity Assays

Alkaline protease activity was determined according to a modified method of Sarath et al. (1989). Briefly, the incubation mixtures consisted of 100 µL 0.05 M Tris-HCl/10 m*M* $CaCl_2$ (pH 8.1) buffer, 100 µL crude extracts (cecal mass or intestine) and 150 µL 2% azocasein as substrate. The reaction was performed at 25°C for 1 h and terminated by adding 750 µL 10% TCA. The optical density of the supernatant was measured at 360 nm.

Trypsin activity was determined according to a modified method of Erlanger et al. (1961). BAPNA (Nα-benzoyl-L-arginine-4-*p*-nitroanilide hydrochloride) was used as substrate. The amount of *p*-nitroaniline liberated from BAPNA at pH 8.2 and 25°C was determined at 410 nm.

Chymotrypsin activity was assayed according to Hummel (1959) using BTEE (N-benzoyl-L-tyrosine ethyl ester) as substrate. The increase in absorbance at 256 nm resulting the hydrolysis BTEE at pH 8.1 at 25°C was recorded every 30s for 6 min.

Pepsin or acid protease activity was determined by the modified method of Sarath et al. (1989), with denatured haemoglobin (2% pH 2) as substrate. The reaction mixture consisted of 150 µL of substrate in 0.2 *M* HCl-KCl buffer at pH 2 and 20 µL crude extract. Optical density of the supernatant was

determined at 280 nm. Activity was calculated as tyrosine liberated by proteolysis into the supernatant after of 1 h incubation at 25°C and then TCA precipitation.

Leucine aminopeptidase (LAP) activity was determined according to Appel (1974), using 1.2 m*M* L-Leu-*p*-nitro-anilide as substrate. The activity was determined as the amount of *p*-nitroaniline formed at pH 8.0 and 25°C by measuring the increase in absorbance at 410 nm.

In order to characterize and establish optimum assay conditions for tuna lipase, cecal mass crude extracts were homogenized and assayed for lipase activity under various salt treatments. Lipase activity was evaluated in the presence or absence of salts; 1) a control with no salts added, 2) 6 m*M* Na-taurocholate (NaTC) + 100 m*M* NaCl, 3) 6 m*M* commercial bile salts (BS:Na-cholate and Na-deoxycholate, 1:1) + 100 m*M* NaCl, 4) 100 m*M* NaCl, 5) 6 m*M* NaTC without NaCl (Na-TC w/o NaCl), and 6) 6 m*M* BS without NaCl (BS w/o NaCl). In addition, the effect of natural bile (extracted from the gallbladder) at two concentrations (15 and 30 µL added to the reaction mixture) was evaluated. Activity was determined by the hydrolysis of 4-nitrophenyl-caproate (4-NPC) according to the modified method of Gjellesvik et al. (1992). Briefly, the reaction was initiated by adding the enzyme extract to 0.5 *M* Tris-HCl (pH 7.4) buffer, 4-NPC (100 m*M* in ethanol, with a final concentration 0.35 m*M* in assay mixture) and the particular salt evaluated. Temperature was maintained at 20°C and the increase in absorbance was recorded at 400 nm. The enzymatic activity was expressed as percent relative activity.

Amylase activity was determined by the method of Bernfeld (1955), with 1% starch as substrate. A solution of 3.5-dinitrosalicylic acid was used as the acid reagent to determine the amount of reducing sugar produce as maltose by measuring absorbance at 546 nm.

One unit (U) of activity was defined as the amount enzyme that liberated 1 µmol of product per min under the conditions described above for each enzymatic assay.

Protein Concentration

Protein concentration in all assays was determined according to Bradford (1976) using a bovine serum albumin (BSA) as standard and reported as mg protein equivalent to BSA.

All samples assays were done in 6 individual fish and reported as means with standard deviation.

Effect of Temperature and pH on Enzyme Activities

For theses assays crude extracts of each section (i.e., stomach, cecal mass and intestinal sections) were centrifuged at 8,500 *g* for 20 min at 4ºC and supernatants used in enzyme assays for pH and temperature optimum.

The effect of temperature on the digestive enzyme activities was evaluated by incubating the crude extracts at 4, 15, 25, 35, 45 and 60ºC under similar assay conditions as previously described. The effect of pH on the digestive enzyme activities was measured at 25ºC and the following buffer were used 0.2 *M* glycine-HCl, pH 3.0, 0.2 *M* citrate-phosphate pH 5.0 and 0.2 *M* Tris-HCl pH 7.0-9.0 without $CaCl_2$ for pepsin, lipase, α-amylase and leu-aminopeptidase activity, and with 15 m*M* $CaCl_2$ for alkaline protease, trypsin

and chymotrypsin activity. For pepsin activity, haemoglobin (pH 3.0) was used as substrate (Tanji et al. 1988).

The enzymatic activity for both assays (pH and temperature) was expressed as percent relative activity.

Statistical analyses

Since data from specific activity of enzymes from digestive tract lacked equal variances, non-parametric analyse were performed using a Kruskall-Wallis rank-mean test, followed by a Mann-Whitney test to determined significant differences between samples ($P< 0.05$) (Zar 1996). The data of relative activity were previously transformed by arcsine for analysis. SPPS version 12.0 was used for all statistical analyses.

Results and Discussion

Enzyme Activity Assays

The proteolytic activities of total alkaline and acid proteases, trypsin, chymotrypsin, and LAP found in the different sections of the digestive tract are given in Table 1. A high level of alkaline proteolytic activity was detected in the cecal mass, compared to the proximal intestine whereas chymotrypsin activity was higher in the proximal intestine compared to the cecal mass, at the contrary, several times higher trypsin activity was found in the cecal mass than those found in the different intestine sections ($P < 0.05$). This is expected since zymogens granules are stored in the cecal mass and released in the intestine until stimulated by the hormone cholecystokinin (CCK), which is in turn released into the blood stream upon ingestion of food (Liddle 1997). In previous studies with *T. thynnus*, trypsin and chymotrypsin activity were detected only in the pyloric cecae, whereas no activity was detected in intestine tissue (Stevens and

McLeese 1984), differences that could be due to different feeding regime. In the omnivorous fish *Clarias gariepinus*, chymotrypsin activity was relatively low in the pancreas, but relatively high in the different intestinal regions evaluated (Uys and Hecht 1987). The cecal mass, which included pancreatic tissue, and the intestinal extracts used in this study, contained a mixture of hormones, peptides and enzymes, which could rapidly activate zymogens during the dissection and homogenization process, activity that could be detected on any of the organs assayed (Hjelmeland and Raa 1982).

The stomach had a relative high pepsin-like specific activity (Table 1). Since digestive enzyme activities are known to be influenced by feeding activity, values obtained in the present study may reflect a different pattern than those wild tuna fish exposed to mix diets. Moreover, since different substrates to test one enzyme result in differences in Units reported it is difficult to compare results by absolute numbers. For example, BAPNA used in the present works usually gives lower Units than those obtained by TAME (Heu et al. 1995)

In the present work, leucine aminopeptidase activity was similar and low throughout the three intestine sections, which is in accordance to earlier results reported in the Atlantic salmon by Krogdahl and Bakke-McKellep (2005).

Lipase activity was significantly higher in the cecal mass compared with the intestinal sections ($P < 0.05$). In the intestinal region, the proximal region showed a higher level of activity decreasing towards the distal intestine. This is in agreement with several reports that have shown that lipid digestion and absorption in fish is performed in the proximal intestine (Borlongan 1990; see review by Rust 2002).

One of the most interesting results obtained in this study was the activity of lipase against the substrate used (4-nitrophenyl-caproate). The lipase activity increased significantly in the presence of 100 m*M* NaCl without any bile salts (Fig. 1) and also activity was increased, but to a lesser extent in the presence of NaTC without NaCl. Increasing concentrations of natural bile salts from tuna bile resulted in a significant decrease in activity. Sodium chloride concentration has been found to be important for the activity of pancreatic lipase in rats (Borgstrom and Erlanson 1973). Maximal activity of rat lipase was obtained in a rather narrow concentration ranging between 100 and 150 m*M* NaCl, similar to the concentration used in the present study. To date, most of the studies suggest that fish lipase in teleosts is a non-specific and bile salt dependent lipase, which has activity only in the presence of bile salts whereas the activity of 1,3 specific pancreatic lipase is low or non-existent (Patton et al. 1977; Lie and Lambertsen 1985; Gjellesvik et al. 1992). In addition, Gjellesvik et al. (1992) was able to demonstrate that bile salt-dependent lipase was the only pancreatic enzyme involved in the lipid digestion in cod. Results from the present study suggest that bile salt-dependent lipase in tuna fish is not the main enzyme involved in the lipid digestion since a 60% reduction in enzyme activity was observed in the presence of bile salts. It is likely that several other types of lipases like activities could be involved in the digestion of lipids in tuna. Furthermore, the effect of the putative co-lipase factor in lipase activity needs to be addressed to clarify the interaction among lipase, co-lipase, bile salts and substrate in Pacific bluefin tuna.

Natural bile obtained from the gallbladder inhibits the lipase activity tested here. The only explanation could be that at both bile salts concentrations

used here were higher than the desire, but still strange that this could occur. Several studies have reported an inhibitory effect of bile salts on rat lipase activity (Borgstrom and Erlanson 1973; Morgan and Hoffman 1971). The authors have attributed this effect to the build up of a detergent monolayer above a critical micellar concentration (up to 4 m*M* depending on the bile salt) that prevents lipase from reaching its substrate.

Significantly higher levels of α-Amylase activity were found in the cecal mass and proximal intestine compared to the middle and distal intestine (Table 1). Similar to the other enzymes studied, amylase activity progressively decreases along the intestine, in which starch digestion and glucose absorption mainly occur in the anterior part, with decreasing amylase activity from anterior towards the posterior end (Kuz'mina 1985; Uys and Hecht 1987; Lundstedt et al. 2004). However, several studies shown higher carbohydrases activities in different intestine sections than those found in the proximal intestine (Chakrabarti et al. 1995; Uys and Hecht 1987; Hidalgo et al. 1999). It is important to mention that most of the carnivorous fish exhibit a low or moderate amylase activity in the intestine and pancreas (Munilla-Morán and Saborido-Rey, 1996b; Kuz'mina et al. 2003), which is in agreement with a natural diet, low in carbohydrates (Harpaz and Uni 1999). However, a higher amylase activity in tuna fish under culture will be positive in order to introduce balanced diets with a certain amount of carbohydrates.

The Effect of Temperature

The temperature dependence profile of the stomach showed that pepsin activity reaches up to 70% of the maximum activity at 25ºC, and a maximum at 45ºC (Fig. 2a). The effect of temperature on enzyme activity was similar to that

reported by several authors in other fish species, such as *Mallotus villosus* and *Gadus morhua*, with highest pepsin I activity found at 38ºC and 43ºC in pepsin II (Gildberg and Raa 1983) and *Scophthalus maximus* with optimum temperature range 40-50ºC for stomach protease (Wang et al. 2006). Higher values of relative activity were found at higher temperatures for trypsin and total alkaline proteases (Fig. 2b). Similar results were found for trypsin specific activity in pyloric cecae of the *T. thynnus*; this enzyme that increase in activity at 65ºC (Stevens and McLeese 1984) and trypsin A and B from yellowfin tuna spleen (Klomklao et al. 2006). The optimum temperature found for trypsin in *T. orientalis* was higher than the optimum temperature observed in other fish species (40 to 50ºC) such as *Gadus ogac* (Simpson and Haard 1984), *C. carpio* (Cao et al. 2000) and *Sardinops sagax caerulea* (Castillo-Yañez et al. 2005). In the present study, the maximum chymotrypsin activity was observed at 35ºC, whereas at 60ºC a 13% decrease from its maximum was reported. However, the total alkaline protease activity shows a similar trend to that in trypsin, which probably means that spite of the difference in optimum temperature between trypsin and chymotrypsin the highest activity of these enzymes in *T. orientalis* is higher at higher temperature; therefore the *in vitro* analysis could be held at higher temperatures for hydrolysis without affecting the overall results.

Amylase activity between 4 and 25ºC was approximately 60% of the maximum activity found; while an increase of 10ºC (25 to 35ºC) enhanced the activity to 90% of the maximum. This is in agreement with other studies characterizing amylase activity in other fish species, where highest amylase activity was found in temperatures between 25 to 55ºC (Uys and Hecht 1987; Munilla-Moran and Saborido-Rey 1996b, Hidalgo et al. 1999; Kuz'mina et al.

2003). Maximum activity for cecal mass lipase was found at 45ºC, similar to that reported for *Chanos chanos* (Borlongan 1990). LAP highest activity was observed at 45ºC for the proximal intestine while the middle and distal intestines showed a maximum activity at 60ºC (Fig. 3). These results were similar to that obtained in intestinal homogenates of *Solea solea*, in which highest activity was found at 50ºC (Clark et al. 1987). Recent tagging studies assessing thermal adaptation of Atlantic and Pacific bluefin tuna have shown that, in both the peritoneal cavity and the muscles can achieve and conserve a higher temperature than the surrounding water temperatures (Stevens et al. 2000; Kitagawa et al. 2006). The temperature profile of digestive enzymes activity suggests the great efficiency of enzymes to maintain high levels of activity in a broad temperature range, with a putative beneficial effect on the overall food digestion.

The Effect of pH

Pepsin showed hydrolytic activity of 2% acidified haemoglobin at pH 3.0 with no activity detected at pH 5.0 or higher (Fig. 4a). Highest acid protease activity was observed in the stomach region and highest alkaline activity in the cecal mass and intestine (Gildberg and Raa 1983; Uys and Hecht 1987; Martinez and Serra 1989; Chong et al. 2002; Lundstedt et al. 2004; Wang et al. 2006). Pepsin has been identified as the major gastric protease in fish stomach breaking down large peptide chains after HCl hydrolysis (Gildberg 1988; Chong et al. 2002; Wang et al. 2006). It is important to stress that only fish species with a well-defined and functional stomach (i.e., fully functional oxytocynic cells) posses pepsin activity (Jónás et al. 1983; Uys and Hecht 1987; Gildberg 1988; Lundstedt et al. 2004).

The activity of α-amylase assayed with 1% starch at pH ranging from 3.0 to 9.0 showed high activity at pH 3.0 (close to 60% of maximum), increasing slowly thereafter to a maximum at pH 8.0 and decreasing at pH 9 (Fig. 4b). Optimum pH for fish amylases seems to be species dependent, with reported optimum activity in the range of 4.5-8.0; for example, highest activity of intestinal and pyloric cecae amylase of *S. aurata* and *Scophthalmus maximus* has been found in the neutral pH region (7.0-7.5), while for *Sebastes mentella* highest activity was observed in a more acidic environment (pH of about 4.5-5.0) (Munilla-Moran and Saborido-Rey 1996b; Fernandez et al. 2001). Rather interesting, tuna bluefin cecal mass amylase still posses more than 60% of maximal activity at quite acid pH (pH 3), suggesting a rather broad pH stability and activity for this type of enzyme. Most fish amylases have a maximum activity in the alkaline region and almost no activity has been reported below pH 4 (Munilla-Moran and Saborido-Rey 1996b; Fernandez et al. 2001). It is possible that more than one isoenzyme is involved in the amylase digestion in bluefin tuna, and therefore this activity will be of importance for the development of novel balanced diets.

Cecal mass lipase, trypsin and chymotrypsin were more sensitive to pH changes than amylase, with a null activity (lipase and trypsin) or 20% residual activity at pH 3.0-5.0 (Fig. 4b). Similar results have been observed in other fish species, where no activity was found for trypsin and chymotrypsin in an acidic environment and low temperatures (Simpson and Haard, 1984). In this study, highest trypsin and chymotrypsin activity was found at pH 8.0. In contrast, in the Atlantic bluefin tuna highest activity for trypsin was obtained at pH higher than 9.0 (Stevens and McLeese 1984). Total alkaline proteases showed maximum

activity at pH 9.0, slightly higher than that obtained in trypsin and chymotrypsin, suggesting the presence of other proteases like carboxypeptidases, elastases and collagenases, which showed the maximum activity in the alkaline pH range (9.0 to 10.0) (Hidalgo et al. 1999). However, since optimal pH varies depending on substrate specificity (Klomklao et al. 2006) implications should be taken with caution. The effect of pH on proteases (trypsin and chymotrypsin) from intestinal extract of several fishes has been reported to be optimum in the neutral alkaline range (Munilla-Moran and Saborido-Rey 1996a; Chong et al. 2002; Kishimura et al. 2005).

Among lipases, Borlongan (1990) detected activities peaks at pH 6.4-6.8 and 8.0-8.6 in the anterior intestine and in pancreas of milkfish, *C. chanos*, suggesting the presence of two lipases. In the present work the highest activity for cecal mass lipase was observed at pH 9.0, decreasing at pH 8 and 7, but no picks could be detected to suggest the presence of two principal lipases, however it could be possible the presence of more lipases in lower amount with different specificities as mentioned before.

Highest activity of LAP was found in the middle and distal intestine at pH 8.0 and in the proximal intestine at pH 9.0. This is in agreement with other LAP from intestinal homogenates of *S. solea*, showing optimum activity at pH 8.3 using leucinamide as substrate (Clark et al. 1987). The presence of different peaks of maximal activity in the intestinal section evaluated suggests the possible presence of isoenzymes in the crude extract. For example, LAP from proximal intestine with maximum activity at 45ºC and pH 9.0 could be different from that in the middle and distal intestine (maximum activity at 60ºC and pH 8.0).

Based on the information obtained so far, it can be conclude that the digestive system of bluefin tuna is highly efficient in the utilization of different nutrients from a wide range of food. Bluefin tuna seem to be capable of digesting high protein and high lipid diets; and a carbohydrate inclusion seems feasible. The pepsin activity found indicates a good potential for the digestion of hard digestion connective tissues (after HCl hydrolysis) present in the actual feed (fresh and frozen fish) utilize by most tuna farmers.

The most interesting result reported here was on the lipase activity which was inhibited in the presence of bile salts and significantly increased in the presence of NaCl. Based on these preliminary results it is suggest that the principal tuna lipase is of the pancreatic type with no dependence on bile salts under our assay conditions. Further studies are needed in order to fully understand the role of bile salt in bluefin tuna to optimize the use of lipids in diet formulation, in particular since high lipid diets are needed in the tuna farming industry and new lipid sources should be supplied to make more efficient low cost diet formulation in the future. In this way, the present research will be helpful to continue the studies to develop an *in vitro* system to evaluate the apparent digestibility under culture systems to search novel diets to be used for bluefin tuna farmers.

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Table 1 Specific activity from the various enzyme presents in the digestive tract of *Thunnus orientalis*

Enzymes	pH	Specific Activity (U/mg protein) of crude extracts				
		Stomach	Cecal Mass	Proximal Intestine	Middle Intestine	Distal Intestine
Trypsin	8.2	-	13.95±8.0^{a}	0.26±0.1^{b}	0.25±0.1^{b}	0.12±0.0^{b}
Chymotrypsin	8.1	-	265.7±42.3^{a}	317.1±84.2^{a}	204.6±60.4^{ab}	114.2±29.1^{b}
Alkaline proteases	8.1	-	16.9±5.8^{a}	9.6±2.8^{a}	4.4±2.0^{a}	0.7±0.3^{b}
Pepsin	2.0	41.3±15.3	-	-	-	-
α-Amylase	6.9	-	37.6±5.9^{a}	23.8±6.8^{ab}	16.1±3.8^{bc}	9.7±2.9^{c}
Lipase	7.4	-	27.5±4.6^{a}	3.9±1.1^{b}	2.1±0.2^{c}	1.5±0.5^{c}
Aminopeptidase	8.0	-	-	0.01±0.0	0.01±0.0	0.01±0.0

values are given as the average and standard errors of six individuals. Different superscript indicates significant differences ($P < 0.05$)

Figures Legends

Fig. 1 NaCl effect on the lipase activity of cecal mass crude extracts from the Pacific bluefin tuna.

(1) control (without nacl and bile salts), (2)NaTC (Na-taurocholate + NaCl), (3)BS (mixed of Na-cholate and Na-deoxycholate + NaCl), (4) 35 µLof natural bile, (5)15 µL of natural bile, (6)NaCl (without bile salts), (7)NaTc w/o NaCl (na-taurocholate without bile salts) and (8)BS w/o NaCl (bile salts without NaCl) were tested for activity. Bars indicate mean ± s.d (n=3). Lower case indicate statistical differences ($P < 0.05$).

Fig. 2 Effect of temperature (**a**): on pepsin activity of extract from bluefin tuna stomach and (**b**): on trypsin, chymotrypsin, total alkaline proteases, lipase and α-amylase activities of extract from bluefin tuna cecal mass.

Fig. 3 Effect of temperature on leucine-aminopeptidase activity from proximal, middle and distal intestine of Pacific bluefin tuna.

Fig. 4 Effect of pH (**a**): on pepsin activity of extract from bluefin tuna stomach and (**b**): on trypsin, chymotrypsin, total alkaline proteases, lipase and α-amylase activities of extract from bluefin tuna cecal mass.

Fig. 5 Effect of pH on leucine-aminopeptidase activity from proximal, middle and distal intestine of Pacific bluefin tuna.

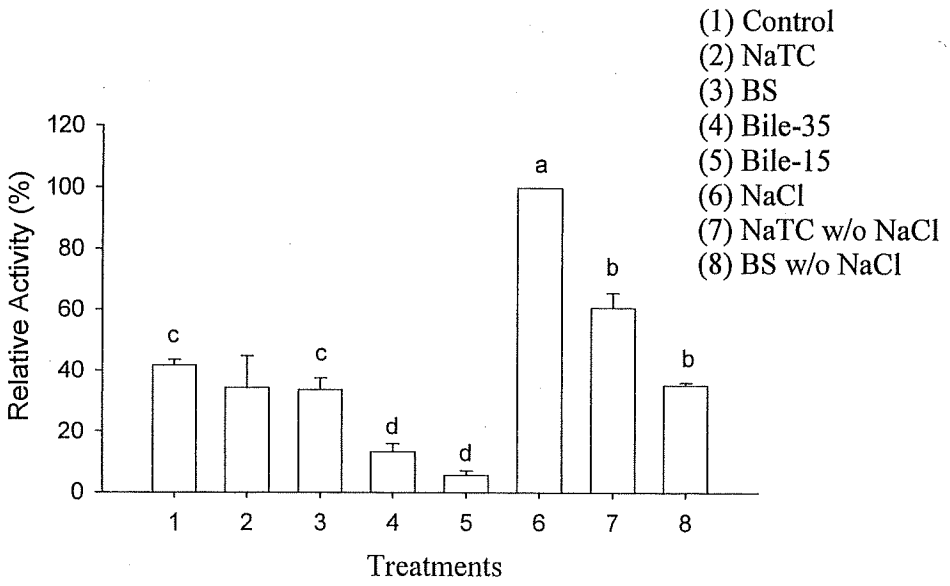
(1) Control
(2) NaTC
(3) BS
(4) Bile-35
(5) Bile-15
(6) NaCl
(7) NaTC w/o NaCl
(8) BS w/o NaCl
Relative Activity (%)
120
100
80
60
40
20
0
a
b
c
c
b
d
d
1
2
3
4
5
6
7
8
Treatments

Fig. 1

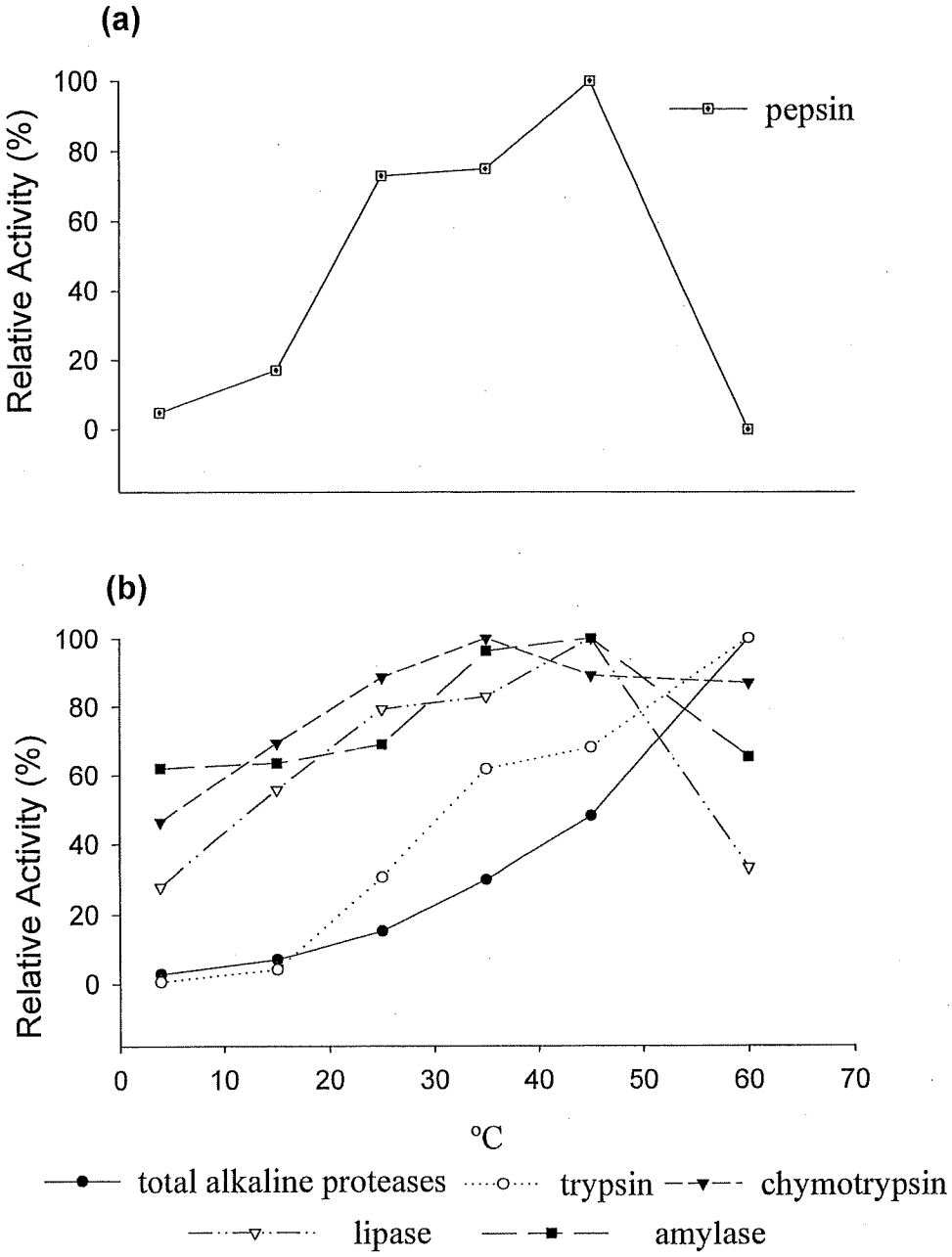
(a)
Relative Activity (%)
100
80
60
40
20
0
pepsin
(b)
Relative Activity (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
°C
total alkaline proteases
trypsin
chymotrypsin
lipase
amylase

Fig 2

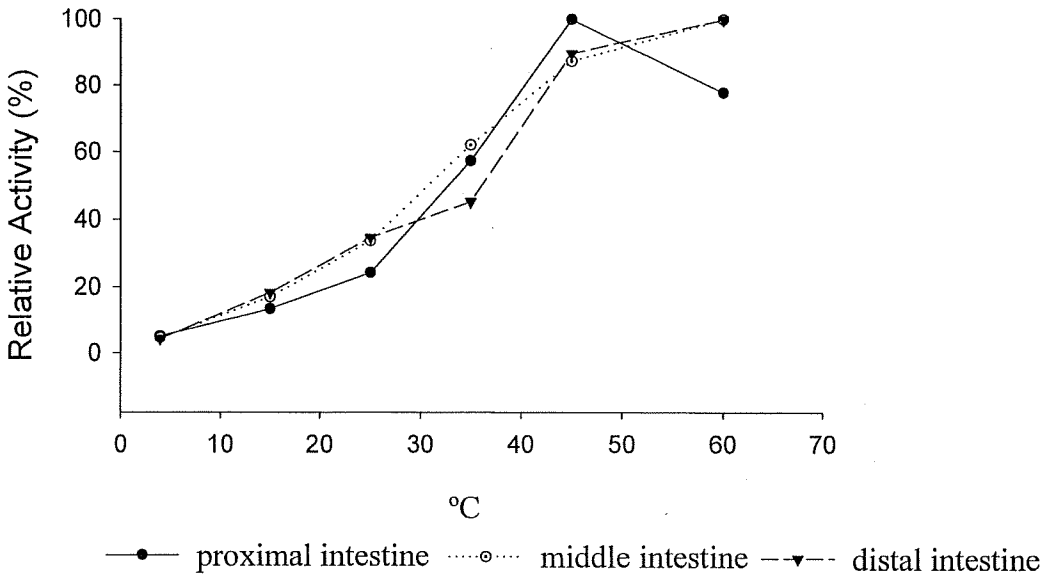
Relative Activity (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
°C
proximal intestine
middle intestine
distal intestine

Fig 3

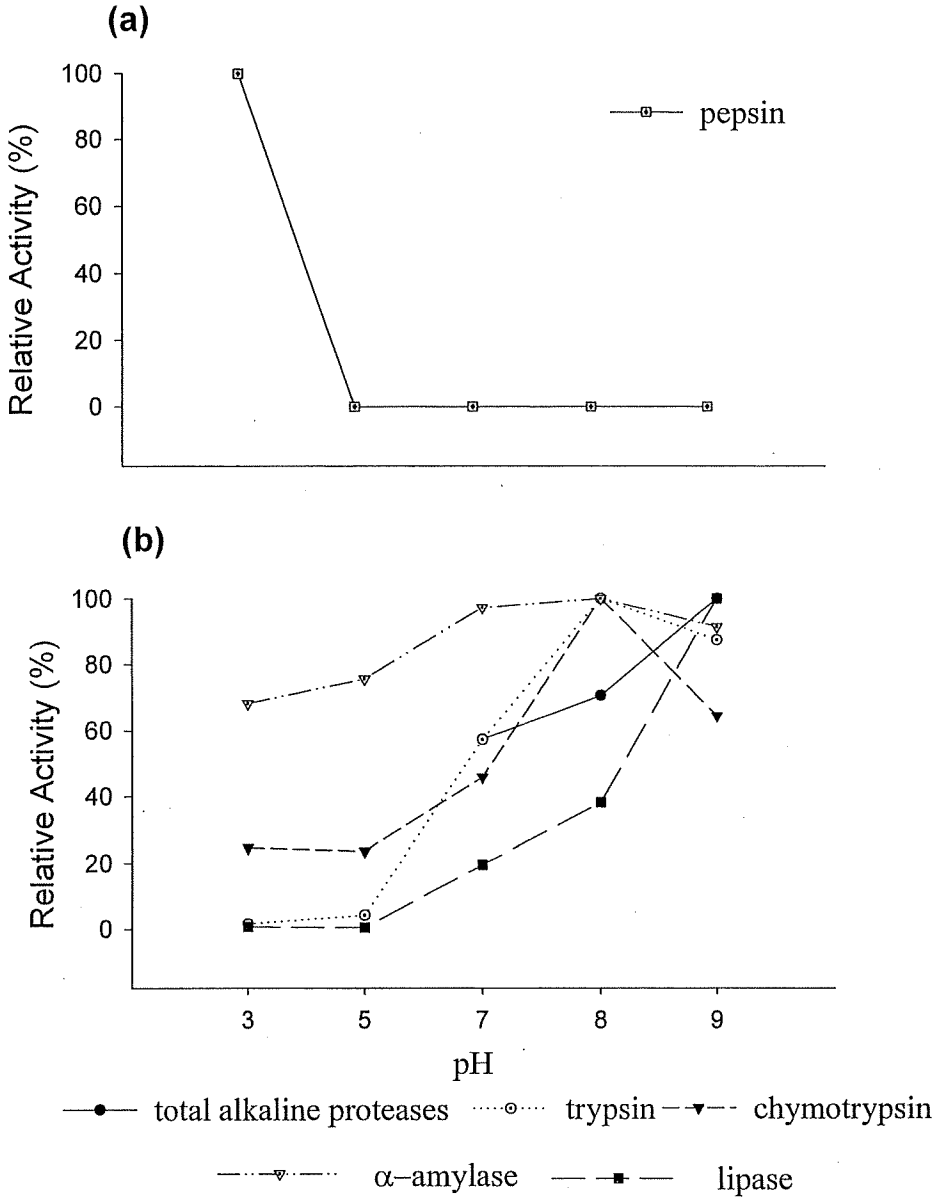
(a)
Relative Activity (%)
100
80
60
40
20
0
pepsin
(b)
Relative Activity (%)
100
80
60
40
20
0
3
5
7
8
9
pH
total alkaline proteases
trypsin
chymotrypsin
α–amylase
lipase

Fig 4

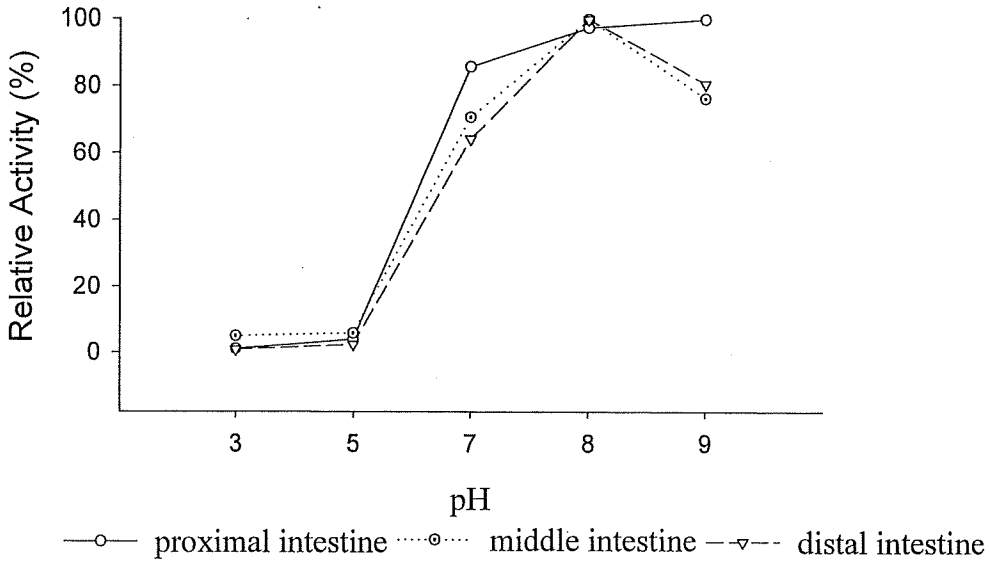
Relative Activity (%)
100
80
60
40
20
0
3
5
7
8
9
pH
proximal intestine
middle intestine
distal intestine

Fig 5

Comparative intestinal amino acid absorptions in Pacific bluefin tuna *Thunnus orientalis*, Rainbow trout (*Oncorhynchus mykiss*) and *Totoaba macdonaldi*.

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Keywords: everted intestine, amino acid absorptions, digestibility

Short running title: Comparative intestinal amino acids absorption

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ABSTRACT

The everted intestine technique was used to estimate amino acid absorption in the proximal section of three fish species. A pancreatic digest of casein (Tryptone) ultra-filtered to 10,000 Da was the standard amino acid mixture. Rainbow trout (*Oncorhynchus mykiss*) intestine was used to set up the experimental system, and the results were compared with those obtained with intestines from *Totoaba macdonaldi* and bluefin tuna fish. Amino acid absorption kinetics by the everted intestine showed a sigmoid shape for all amino acids and fish tested. Initial experiments showed that the intestinal tissue should be fresh and healthy to perform the absorption measurements. In general, specific absorption of essential amino acids was higher than those of non-essential ones. No correlation between amino acid initial concentration in Tryptone solution and the specific absorption was found. The maximum specific absorption of trout was 10-times higher than those observed in marine fish. The relative amount of the different amino acids absorbed in all three species showed somehow different preferential orders. The absorption rates for all species were also estimated. Absorption rates in trout for all amino acids were one order of magnitude higher than those observed in totoaba and bluefin tuna. Leu was absorbed at 208.1 pmol cm^{-2} min^{-1} in trout, whereas totoaba and bluefin tuna showed rates of 17.4 and 12.4 pmol cm^{-2} min^{-1}, respectively. Moreover, some differences in the absorption rate sequence between species were observed, where for trout the speed sequence was Leu >Lys > Ser, in totoaba was Ala > Lys > Thr, and in bluefin tuna was Glu > Ala > Lys. The possibility of using the everted technique to evaluate and then design artificial diets for large fish species with commercial value is discussed.

INTRODUCTION

Fish supplies from conventional fisheries are fast approaching a practical limit (FAO, 2004), therefore improvements in production will have to come throughout aquaculture, activity that has had the higher increase in production rate (10% yearly) among food-producing animals (FAO, 2004). However, aquaculture is highly dependent on the availability of fish meal and oil for aqua-feeds. Thus, research is needed to assure a more efficient use of alternative ingredients.

Feed ingredients used in aquaculture can be evaluated through a variety of chemical and biological tests. Doubtless, the best method is to feed them with the experimental diet and compare the results with those obtained from a control group fed with a diet of which nutritional value is known. Moreover, with fecal collection, digestibility measurements could be performed to estimate how utilizable the feed components are.

The diversity of cultured species and requirements to meet different sizes and conditions makes, so far, impossible to perform biological evaluations for many of the species with economical value. *In vitro* studies are an alternative for feed performance evaluation, which implicate the measurement of feed products or each ingredient alone, after an enzymatic degradation under the expected conditions for a particular organism. These *in vitro* techniques do not consider protein absorption, and therefore the methodology is mainly used to evaluate protein quality or eventually, the potential feed degradation by the fish in study, but not feed digestibility *per se*.

Nutrient absorption occurs in the intestine mainly by the enterocyte cells throughout more or less specific transporters, either by carrier-independent diffusion, facilitated transport or active transport (Mailliard *et al.*, 1995). Different types of transporters have been suggested for neutral, basic, acidic, and imino amino acids, and it is generally accepted that active amino acid transport in the small intestine, involves an initial Na-dependent association or binding to a carrier (Nassar, 1989). Nevertheless, among fish, it has been reported that amino acid absorption differs between fresh and marine fish (Ferraris and Ahearn, 1984), with changes in the transporters, possibly due to ultrastructural and electrophysiological changes in euryhaline fish intestines (Oide and Utida 1967).

Several *in vitro* and *in vivo* methods have been reported to measure amino acid absorption, including the incubation of gut samples, everted intestines, or plasma amino acid studies in a radio-labeled medium (Ferraris and Ahearn, 1984; Buddington *et al.*, 1984; Berge *et al.*, 2004), where amino acids have been studied individually or in groups.

The everted intestine system has been used for some time as a model to study nutrient absorption (Bamford *et al.*, 1972) and mainly in pharmacology to modulate drug uptake for the development of new drugs. The system has been rather useful and consists in using segments from different parts of the intestine, to measure the molecules as they move across the barrier under different conditions. However, no studies have been reported on the absorption of all amino acids from a given protein source.

Tuna fish aquaculture has become popular in many countries, especially Australia and Mexico, where several grow-out concessions have proliferated;

however the fish's size and pelagic behavior makes rather difficult to perform *in vivo* studies. *Totoaba macdonaldi*, a large endemic fish specie from the Sea of Cortes, although not a pelagic one, still need a large space in the lab to perform nutrition research. However, rainbow trout (*Oncorhynchus mykiss*), a fresh water specie, because of its size and availability, might be used as a research model for the development of alternate techniques to study fish nutrition.

They all are carnivores and poikilothermic; have quite similar digestive systems, but differ in the development of their pyloric cecae as absorptive organs, the type of pancreatic tissue (from diffuse, to organized), the length of the digestive tract (from short, to long). Tuna fish are able to increase and maintain higher body temperatures than those of the environment. Totoaba has a broad spectrum of salinity and temperature tolerance.

Thus, the aim of the present work was to compare the absorption rates (both the extent of the transport and their kinetics), from different carnivore, fresh water and marine fishes, to develop a dynamic gastro-intestinal model for amino acid digestibility studies.

MATERIALS AND METHODS

Fish samples

Viscera from bluefin tuna fish (28 to 32 kg weight) were obtained from a commercial sea farm (Operadora Pesquera de Oriente SA de CV, Ensenada BC, Mexico); the organs were dissected, placed in ice and transported about 15 km for further processing. Live rainbow trout (450-600 g body weight) from a nearby commercial farm and Totoaba (400g weight) (Facultad de Ciencias Marinas, UABC) were transported to the respective laboratories, where they

were immediately killed and the intestines dissected less than an hour afterwards.

The intestinal section just after the pyloric cecae (referred as the proximal intestine) was sliced and handled in iced solution. For trout and totoaba, 5 cm long pieces were used, whereas for tuna fish the length of intestine segments was 10 cm. A segment from an individual fish was considered as a replicate. All experiments were done in triplicate including the control group.

Experimental procedure

The technique used for everted intestine, adapted from other species, was as reported by Budington *et al.* (1987), and further modified here. In summary, the fresh sleeves were closed at one end with surgical string, and from that end, they were everted. The open part of each sleeve was tied to a glass tube coming from the lock of a flask designed for this purpose (Fig. 1), so that samples could be taken over time. Oxygen was bubbled throughout a thin glass tube located at one side (Fig. 1).

All sleeves were pre-incubated for 5 min at 18°C in a Ringer solution (117 mM NaCl, 25 mM $NaHCO_3$, 5.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, and 2.5 mM $CaCl_2$, pH 7.4) for fresh or marine organisms (Budington *et al.,* 1987 and Berge *et al.,*2004). To compensate the ion strength differences in marine organisms, 150 mM NaCl were added to the Ringer solution. The solutions were constantly gassed with 95:5 (%) O_2-CO_2 mixture. After being bubbled, the Ringer solution was changed to the filtered Tryptone-hydrolysed solution described below, whereas the control group consisted in the

Ringer solution only. To standardize the protein-hydrolysate solution, Tryptone (Bacto™ tryptone DIFCO cat. number 211705), a pancreatic digest of casein filtered with an Amicon ultrafiltration cell (10,000 Daltons cut-off), was chosen. After performing several tests with different solution concentrations (results not shown), a concentration of 50 mg mL^{-1} was chosen for all experiments.

The general experimental procedure was as follows: intestinal sleeves were dry- blotted, measured in length, and weighed before mounting them in the glass tube as explained before (Figure 1). The Ringer solution was added inside the intestine (1 mL for rainbow trout and totoaba, and 2 mL for bluefin tuna), then the everted intestines were immediately submerged in the flask containing the Ringer solution, and O_2 was gassed throughout a needle to maintain the solution saturated. Both Ringer solutions, inside the intestine and outside with the Tryptone solution, were isosmotically balanced by adding NaCl. Single 60 µL samples were taken from each experimental unit every 5, 10, 15, 20, 25, 30, and 60 min, individually collected, and frozen at -80°C until analyzed. Controls with only Ringer solution and no amino acid solution, were used to correct for the initial amino acid concentration. After incubation the intestine sleeves were dry-blotted and weighed to measure the final volume to adjust for sample concentration.

To evaluate the effect of intestinal tissue viability on the amino acid transport, trout everted intestine sleeves in triplicate groups were assayed either with optimum conditions and bubbling O_2 (control), bubbling CO_2 instead, and tissues after 8 hours *post-mortem* in optimal conditions (O_2). All intestines were tested simultaneously under a similar Tryptone solution (50mM) at 18°C.

Amino acid concentrations were reported as nmol mL^{-1}, whereas amino acid specific absorptions were reported as pmol cm^{-2}. Moreover, absorption rates were calculated as pmol cm^{-2} min^{-1}. Amino acid percentages were given either, as the percentage of total absorbed amino acids ( ), or as the percentage of absorbed amino acid in relation to the initial amino acid concentration in the Tryptone solution [ ].

To calculate the absorption rates from the 60 min sampling time, the values obtained from the log phase were considered up to their maximal concentration, being in all cases at min 20, time with best linear fitting (R^2 > 0.80).

Amino acid analysis

Individual samples were evaluated for amino acid content using an Agilent HPLC equipped with a florescence detector (Agilent, 1100 series). Samples were chromatographied through a reverse phase column (3.9 x 150 mm) 4 µm Nova Pak ™ C_{-18}, using the water-acetonitrile gradient recommended by Waters AccQ•Tag™ system. The fluorescence detector was setup for an excitation wavelength of 250 nm and emission wavelength of 395 nm. Analyses were run at constant temperature of 40°C. HPLC signal calibration and standard curves were obtained by using a standard amino acid solution containing from 12.5 to 75 pmol of each amino acid.

Statistical Analysis

All values obtained from the experiments, both, as maximum absorption rates or specific absorptions for each amino acid, were analyzed by one-way analysis of

variance, and multiple comparisons of means were performed using the Tukey test. Differences were reported as statistically significant with a $P<0.05$. All statistical analyses were performed using the statistical program SIGMA STAT (3.1).

RESULTS

The amino acid absorption kinetics obtained from the everted intestine samples showed a sigmoid shape for all amino acids and fish tested. Amino acid absorption rates were estimated fitting data from log phase (up to 20 min.) to a linear model, which showed high R^2 values. Saturation was attained after 20 min, thus, maximum absorption was estimated, for all cases, as the absorption reached after 20 min, nevertheless experiments were followed up to 60 min.

In general, the specific absorptions were higher for the essential amino acids than for the non-essential ones (Table 1). For trout under optimum conditions, 69.2% of the absorbed amino acids were essential, compared to 30.8% for the non-essential. Leu and Lys accounted for the highest proportion in the final absorbed amino acid mixture (17.0 and 10.1%, respectively) followed by Ala, Ser and Glu (7.0, 6.3 and 6.3%, respectively), even though their initial concentration in the Tryptone solution was low for Ala and Ser (Table 1). From table 1 it appears that the amino acids, both essential and non essentials, were not absorbed in proportion to their initial concentration in the Tryptone solution; in other words, there was no correlation between the initial amino acid concentrations in the solution and their specific absorption. Two extreme cases

could exemplify this: Asp, having a high concentration in the initial solution, showed low specific absorption, whereas Ser being one of the lowest initial concentrations, showed high specific absorption. It is important to point out that Cys could not be detected as an absorbed amino acid.

In terms of the proportion of each amino acid that is absorbed, in general the lower the initial concentration, the higher the absorbed proportion: *e.g.* the two amino acids with the lowest initial concentrations, Thr and Ser (3.3 and 6.2 nmol mL^{-1}, respectively), showed the highest absorption proportions (89.1 and 49.8 % respectively).

In order to prove that amino acid transport is mainly metabolism-mediated, two control experiments were carried out: one bubbling CO_2 which provoke tissue death by inducing anoxia (Kraus et a., 2005), and another using tissue after 8 hrs *postmortem*. In both cases the amino acid transport was significantly lower, especially in the experiment bubbling CO_2, in which only one tenth of the transport in optimal conditions could be found (Table 1). At 8 hours *postmortem*, compared to fresh intestine with O_2, the maximum absorption was also lowered, but was still higher than those found when CO_2 was bubbled. These results may suggest that the intestinal tissue used for this type of experiments, should be freshly harvested.

The specific amino acid absorptions and the final amino acid profiles were determined, in addition of trout, in intestines form Totoaba and bluefin tuna (Table 2). Comparing the maximum specific amino acid absorptions between the different species, both marine fish resulted in a 90% lower rates of that observed in trout. The relative amount of the amino acids absorbed in all three species showed somehow different preferential orders; whereas in trout the

absorption sequence was Leu > Lys > Thr, in totoaba they were Leu > Lys > Val, and in tuna fish Lys >Leu > Thr. In the case of non-essential amino acids the absorption sequences were Ala >Glu > Ser for trout; Ala > Ser > Gly > Glu for totoaba, and Ala > Ser > Gly for bluefin tuna fish. The relative proportion of each absorbed amino acid compared to their initial concentration, followed the same trend.

The absorption rates for all three species were estimated (Table 3). It was observed that in trout all amino acids were absorbed at significant higher rates (one order of magnitude higher) than those observed in totoaba and bluefin tuna. Leu was absorbed at 208.1 pmol cm^{-2} min^{-1} in trout, whereas totoaba and bluefin tuna showed rates of 17.4 and 12.4 pmol cm^{-2} min^{-1}, respectively. Moreover, some differences in the absorption rate sequences between species were observed, where for trout the sequence order was Leu >Lys > Ser (208.1, 194.1 and 149.1 pmol cm^{-2} min^{-1}, respectively), in totoaba it was Ala > Lys > Thr (27.3, 21.3 and 19.8 pmol cm^{-2} min^{-1}, respectively), and in bluefin tuna it was Glu > Ala > Lys (30.9, 23.2 and 15.7 pmol cm^{-2} min^{-1}, respectively).

DISCUSSION

To our knowledge, this seems to be the first work reporting the specific absorption and absorption rates of amino acids from a solution containing a pool of essential and non-essential amino acids, in fish gut. With the aim of standardizing a system for large fish (such as tuna fish), that are difficult to be raised in laboratory conditions, rainbow trout and juvenile totoaba were used.

Intestines from rainbow trout were used to determine how the freshness of the tissue affect amino acid transport. The CO_2 results indicate that fresh tissues are necessary to measure amino acid transport. Boge et al. (1979) observed that transport of Gly was effectively inhibited under anoxia in rainbow trout. However, the 8-hours postmortem experiments showed that tuna tissues, that were transported to the laboratory facilities in a three hours trip, were fresh enough to perform the amino acid transport experiments.

In the 8-postmortem treatment, differences in sensitivity of the different amino acid transporters were found. Although most of the amino acids were not affected, His, Arg and Thr were reduced in half. On the opposite, Glu showed a double increase in the amount transported. Glutamine is an important fuel for the intestinal mucosa. van der Hulst et al. (1997) found in intestine rats that glutamine is quantitatively more important substrate for the proximal intestine than for the distal gut, being important when the supply of essential amino acids are diminished.

When all three species were compared, a significant higher absorption rate was observed in rainbow trout, compared with totoaba and bluefin tuna. In all cases the absorption rates followed a quadratic trend, where saturation was reached at min 20, independently to the maximum concentration. Bluefin tuna

and totoaba showed one order of magnitude less transport rates than those obtained from trout. According to Ferraris and Aheran (1984) fresh water fish are more efficient for absorbing amino acids since they have a relatively lower apical membrane permeability than found in marine teleosts. Marine organisms have a large diffusion process for organic solutes in the brush border membrane, resulting in a relatively low transepithelial nutrient transfer as a result of significant backflux of organic solutes to the gut lumen from the epithelial cells. This apparent less efficiency in amino acid specific absorptions in marine fish compared to fresh water could be compensated by adaptations fixed genetically, like larger pyloric (Buddington et al.,1987), gut thickness or slower transit times.

It is known that amino acid transport systems in fish can be generally comparable to those of mammals, with small differences that could be regarded as distinct metabolic characteristics in the 8-postmortem treatment (Stortelli *et al.*, 1989). For example, it has been reported that absorption is affected by the relative concentration of specific amino acids in the intestinal tissue (Fearn and Hirst, 2006), besides the enzymes involved in the active transport, previous to the amino acid coupling to the cell membranes (Nassar, 1989), resulting in metabolic differences inside the epithelial cells (Wu, 1998). In humans and pigs it has also been demonstrated that certain gluconeogenic amino acids are more important sources of energy in the intestinal epithelium than glucose, and therefore the differences could be also attributed to differences in metabolic needs inside the tissue (Battezzati et al, 1995). The total amount of amino acids able to cross the intestine represent the available amino acids for the whole fish metabolism. A direct amino acid uptake by the intestine tissue could

be expected. However, the amount needed for intestinal metabolism was not measured.

The use of fresh intestine sleeves might be a useful tool to measure individual fish since the system showed a high degree of repeatability. The aim of this experimental setup was to simulate the gastrointestinal tract under laboratory conditions.

In the present work casein hydrolyzed (Tryptone) solution was used in order to standardize the experimental conditions. Nevertheless, hydrolyzates from artificial diets by gastric enzymes from a given fish could be used. Interference on growth and performance due to amino acid imbalance may occur at the absorption level, the metabolic level, or both (Berge *et al.*, 1999). High concentrations of one amino acid may affect the rate of absorption of another specific amino acid (Shimada, 2003).

The amino acid profile of vegetable proteins used in fish feed differs significantly from marine proteins such as fish meal; the most prominent differences relate to their content of Arg, Lys, Try and Met. A better understanding of amino acid interaction and intestinal adaptations concerning their absorption is of major importance, and therefore more studies are recommended using different protein sources and concentrations in order to have a better information for the fish farming industry, regarding feed design and optimization, particularly when it comes to replacement of dietary protein of marine origin with alternative sources, but also when feeds are supplemented with specific crystalline amino acids to optimize their amino acid profile. Finally, it has also been documented that sugars can interfere with the amino acid absorption (Smith, 1969) and therefore the importance to test whole feeds.

In the present work it was demonstrated the capability of using everted intestine system shown here, to simulate the amino acid absorptions by using their own fresh intestine segments. Moreover, these results should encourage the creation of mathematical models to predict the absorption capacity, by using different protein sources or even whole hydrolyzed feeds, to evaluate together possible interactions or synergies between different amino acids and their concentrations in a real pool.

1 ACKNOWLEDGEMENTS

2

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8

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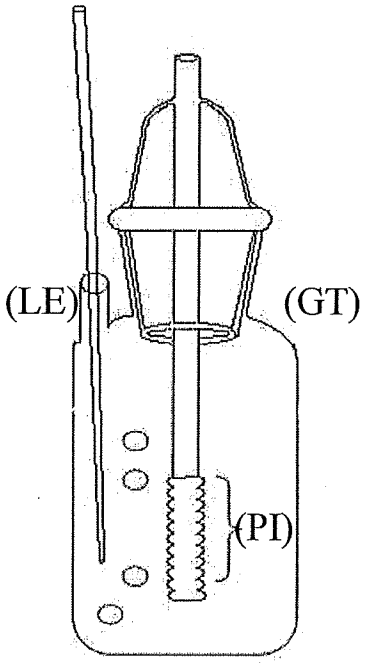
1 FIGURE TITLE

2 Figure 1. Flask used to measure the everted intestine where the proximate
3 intestine (PI) was adjusted to a glass tube (GT) from the lock. Oxygen was
4 bubbled throughout the lateral entrance (LE).


(LE)
(GT)
(PI)

using the proximal everted intestine from the rainbow trout (*Oncorhynchus mikiss*) at optimal conditions (O_2 and fresh intestine), and under CO_2 or O_2 but after 8 hours postmortem.

Amino acid	Initial aa concentration (tryptone)[1]	O_2			CO_2			8 hr postmortem		
		Specific average absorption[2]	Amino acid final profile[3]	Percentage of single adsorbed aa[4]	Specific average absorption[2]	Aa final profile[3]	Percentage of single adsorbed aa[4]	Specific average absorption[2]	Aa final profile[3]	Percentage of single adsorbed aa[4]
	nmol/mL	pmol cm^{-2}	(%)	[%]	pmol cm^{-2}	(%)	[%]	pmol cm^{-2}	(%)	[%]
Essential										
His	7.8	1714.3 ± 32 [a]	(3.5)	22.0	126.8 ± 19.7 [c]	(2.2)	1.6	762.2 ± 52 [b]	(1.8)	9.8
Arg	21.5	3416.5 ± 242 [a]	(7.0)	15.9	156.2 ± 7.9 [b]	(2.7)	0.7	626 ± 107.1 [b]	(1.4)	2.9
Thr	3.3	2939.9 ± 319.4 [a]	(6.0)	89.1	201.6 ± 35 [b]	(3.5)	6.1	642.8 ± 46.6 [b]	(1.5)	19.5
Val	26.1	4465.2 ± 104.5 [a]	(9.2)	17.1	694. 8 ± 32.6 [b]	(12.2)	2.7	3922.7 ± 373.5 [a]	(9.1)	15.0
Met	16.3	1651.5 ± 66.2 [a]	(3.4)	10.1	172.7 ± 35 [c]	(3.0)	1.1	1030.7 ± 149.6 [b]	(2.4)	6.3
Lys	62	4930 ± 255.6 [b]	(10.1)	8.0	491.6 ± 159 [c]	(8.6)	0.8	7818.2 ± 477.7 [a]	(18.0)	12.6
Ile	24.6	2900.6 ± 72.1 [a]	(6.0)	11.8	384.3 ± 40.8 [b]	(6.7)	1.6	2368.6 ± 396.4 [a]	(5.5)	9.6
Leu	110.7	8262.2 ± 653.8 [a]	(17.0)	7.5	946.5 ± 140.5 [b]	(16.6)	0.9	7227.8 ± 728. 5 [a]	(16.7)	6.5
Phe	73.7	3421.2 ± 292.6 [a]	(7.0)	4.6	359.5 ± 18.3 [b]	(6.3)	0.5	3516.1 ± 376.4 [a]	(8.1)	4.8
Non-essential										
Asp	32.8	1082 ± 88.4 [a]	(2.2)	3.3	145.4 ± 8.2 [b]	(2.6)	0.4	899.2 ± 41.2 [a]	(2.1)	2.7
Ser	6.2	3085.1 ± 334 [a]	(6.3)	49.8	357.4 ± 36 [b]	(6.3)	5.8	2150.6 ± 194.1 [a]	(5.0)	34.7
Glu	55.3	3062.5 ± 208 [b]	(6.3)	5.5	787.2 ± 181.3 [c]	(13.8)	1.4	7031.3 ± 183.9 [a]	(16.2)	12.7
Gly	9.7	1665.5 ± 86.5 [a]	(3.4)	17.2	214.6 ± 24.6 [c]	(3.8)	2.2	1128.1 ± 45.4 [b]	(2.6)	11.6
Ala	17.3	3384.6 ± 282.8 [a]	(7.0)	19.6	445.7 ± 70.1 [b]	(7.8)	2.6	2164.4 ± 499.6 [b]	(5.0)	12.5
Pro	8.5	1662.2 ± 159.6 [a]	(3.4)	19.6	136.2 ± 1.7 [b]	(2.4)	1.6	1491.1 ± 224 [a]	(3.4)	17.5
Tyr	7.8	1023.5 ± 34.3 [a]	(2.1)	13.1	76.8 ± 11.8 [c]	(1.3)	1.0	544.8 ± 23.1 [b]	(1.3)	7.0
Cys	6.1	nd	(0.0)	0.0	nd	(0.0)	0.0	nd	(0.0)	0.0

[1] Amino acids concentration of the initial Tryptone solution. .

Table 2. Maximum specific absorption (20 min) and standard error from the amino acids solution (50 mg mL^{-1} Bacto™ Tryptone) using the proximal everted intestine from the rainbow trout (*Oncorhynchus mikiss*), totoaba(*Totoaba macdonaldi*) and bluefin tuna (*Thunnus thynnus*).

		Trout			Totoaba			Bluefin tuna		
Amino acid	Initial aa concentration (tryptone)	Maximum average absorption[a]	Aa final profile[b]	percentage of single aa adsorbed[c]	Maximum average absorption[a]	Aa final profile[b]	percentage of single aa adsorbed[c]	Maximum average absorption[a]	Aa final profile[b]	percentage of single aa adsorbed[c]
	nmol/mL	pmol cm^{-2}	(%)	[%]	pmol cm^{-2}	(%)	[%]	pmol cm^{-2}	(%)	[%]
Essential										
His	7.8	769.8 ± 6.4[a]	(2.4)	9.9	216.8 ± 59.5[b]	(6.1)	2.78	220.1 ± 13.8[b]	(6.8)	2.82
Arg	21.5	1761.5 ± 90.6[a]	(5.5)	8.2	nd	(0.0)	0.00	144.2 ± 0.4[b]	(4.5)	0.67
Thr	3.3	2986.8 ± 355.2[a]	(9.3)	90.5	288.0 ± 14.4[b]	(8.1)	8.73	186.5 ± 13.3[b]	(5.8)	5.65
Val	26.1	656.9 ± 20.9[a]	(2.0)	2.5	289.8 ± 29.9[b]	(8.2)	1.11	157.1 ± 17.1[b]	(4.9)	0.60
Met	16.3	1215.3 ± 2.1[a]	(3.8)	7.5	86.6 ± 4.3[b]	(2.4)	0.53	90.5 ± 5.8[b]	(2.8)	0.56
Lys	62.0	3046.2 ± 750.6[a]	(9.5)	4.9	320.9 ± 47[b]	(9.0)	0.52	368.0 ± 65[b]	(11.4)	0.59
Ile	24.6	1970.1 ± 358.6[a]	(6.1)	8.0	143.0 ± 5.6[b]	(4.0)	0.58	153.4 ± 41[b]	(4.7)	0.62
Leu	110.7	4420.2 ± 348.7[a]	(13.7)	4.0	348.7 ± 14.6[b]	(9.8)	0.31	191.4 ± 2.5[b]	(5.9)	0.17
Phe	73.7	2033.0 ± 77.2[a]	(6.3)	2.8	111.2 ± 1.8[b]	(3.1)	0.15	137.9 ± 33.7[b]	(4.3)	0.19
No essential										
Asp	32.8	826.1 ± 72.9[a]	(2.6)	2.5	203.1 ± 31.1[b]	(5.7)	0.62	232.5 ± 42.9[b]	(7.2)	0.71
Ser	6.2	2872.6 ± 400[a]	(8.9)	46.3	349.2 ± 33.4[b]	(9.8)	5.63	315.5 ± 42.2[b]	(9.7)	5.09
Glu	55.3	3018.0 ± 87[a]	(9.4)	5.5	278.8 ± 49.3[b]	(7.8)	0.50	80.2 ± 46.3[c]	(2.5)	0.15
Gly	9.7	1101.9 ± 330[a]	(3.4)	11.4	282.5 ± 57.6[b]	(7.9)	2.91	287.5 ± 57.6[b]	(8.9)	2.96
Ala	17.3	3017.9 ± 109.8[a]	(9.4)	17.4	388.7 ± 23. 4[b]	(10.9)	2.25	412.3 ± 60.6[b]	(12.7)	2.38
Pro	8.5	1410.4 ± 80.2[a]	(4.4)	16.6	159.3 ± 20.7[b]	(4.5)	1.87	260.5 ± 20.3[b]	(8.0)	3.06
Tyr	7.8	1078.0 ± 94.5[a]	(3.3)	13.8	67.5 ± 11.1[b]	(1.9)	0.87	1.1 ± 0.2[b]	(0.03)	0.01
Cys	6.1	Nd	(0.0)	0.0	20.2 ± 6.9	(0.6)	0.33	nd	(0.0)	0.00

[1] Amino acids concentration of the initial Tryptone solution.
[2] Maximum specific amino acid absorbed concentration inside the everted intestine after 20 minutes. Values shown are means ± standard error.
[3] Amino acid percentage from the total absorbed amino acids in 20 min.
[4] Percentage for each amino acid in relation to their initial concentration in the tryptone solution
[a,b, c] For specific average absorption values, different superscripts indicate significant differences between treatment ($P<0.05$).
nd: no detected

Table 3. Average absorption rate (20 min) and error standard from the amino acid solution from a pancreatic digest casein (Bacto™ Tryptone; 50 mg ml^{-1}), using the proximal everted intestine from the rainbow trout (*Oncorhynchus mikiss*) totoaba (*Totoaba macdonaldi*) and bluefin tuna (*Thunnus thynnus*).

Amino acid	Trout pmol cm^{-2} min^{-1}	Totoaba pmol cm^{-2} min^{-1}	Bluefin tuna pmol cm^{-2} min^{-1}
Essential			
His	45.9 ± 7.5 [a]	10.9 ± 1.2 [b]	12.0 ± 1.0 [b]
Arg	64.7 ± 14.6 [a]	0.6 ± 0.2 [b]	9.1 ± 2.4 [b]
Thr	123.2 ± 24.7 [a]	19.8 ± 3.3 [b]	10.9 ± 1.5 [b]
Val	135.3 ± 14.8 [a]	13.5 ± 0.8 [b]	9.7 ± 1.8 [b]
Met	56.7 ± 9.3 [a]	4.3 ± 0.1 [b]	3.5 ± 0.5 [b]
Lys	194.1 ± 54.6 [a]	21.2 ± 4.6 [b]	15.7 ± 2.4 [b]
Ile	111.3 ± 19.8 [a]	7.9 ± 0.2 [b]	6.3 ± 1.5 [b]
Leu	208.1 ± 15.9 [a]	17.4 ± 0.4 [b]	12.4 ± 2.8 [b]
Phe	98.1 ± 2.8 [a]	5.7 ± 0.5 [b]	5.2 ± 1.2 [b]
Non-essential			
Asp	34.3 ± 7.1 [a]	10.3 ± 1.2 [b]	12.2 ± 2 [b]
Ser	149.1 ± 27 [a]	16.9 ± 0.9 [b]	12.5 ± 2.5 [b]
Glu	120.5 ± 18.1 [a]	19.4 ± 4.8 [b]	30.9 ± 2.8 [b]
Gly	72.9 ± 21.6 [a]	14.3 ± 1.7 [b]	13.7 ± 2.3 [b]
Ala	120.8 ± 18.8 [a]	27.3 ± 4.4 [b]	23.2 ± 2.5 [b]
Pro	55.3 ± 14.7 [a]	12.8 ± 0.2 [b]	8.7 ± 1 [b]
Tyr	22.2 ± 0.72 [a]	3.4 ± 0.2 [b]	4.4 ± 0.3 [b]
Cys	nd	1.1 ± 0.1	nd

A,b For any given amino acid, different supercripts indicate statistical differences between fish species ($P<0.05$). Values shown are means ± standard errors.
nd: no detected

7. ANEXOS

7.1 Cromatogramas

7.1.1 Perfil de aminoácidos en la triptona

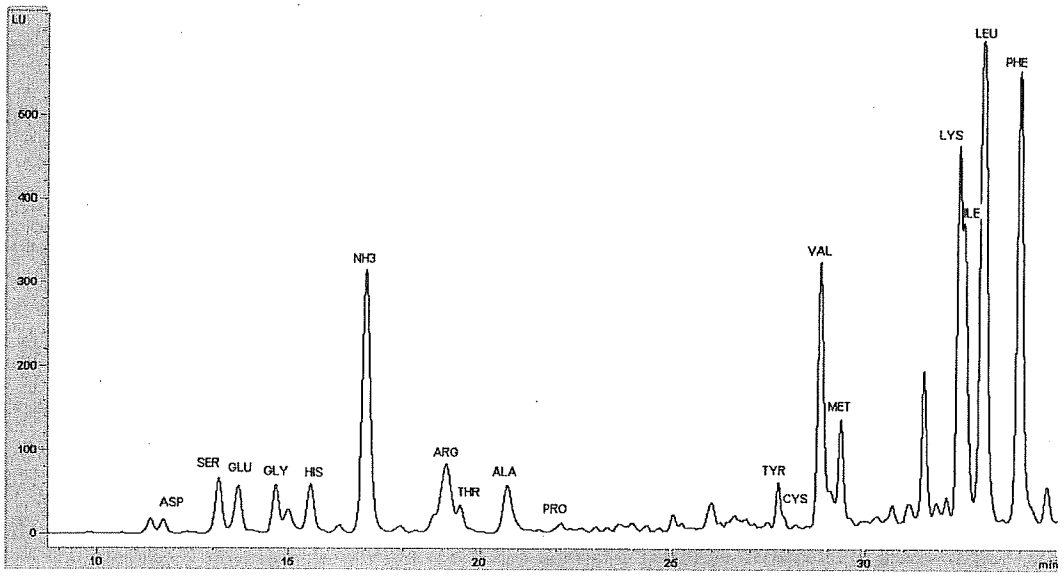


Fig 1. Cromatograma de la triptona.

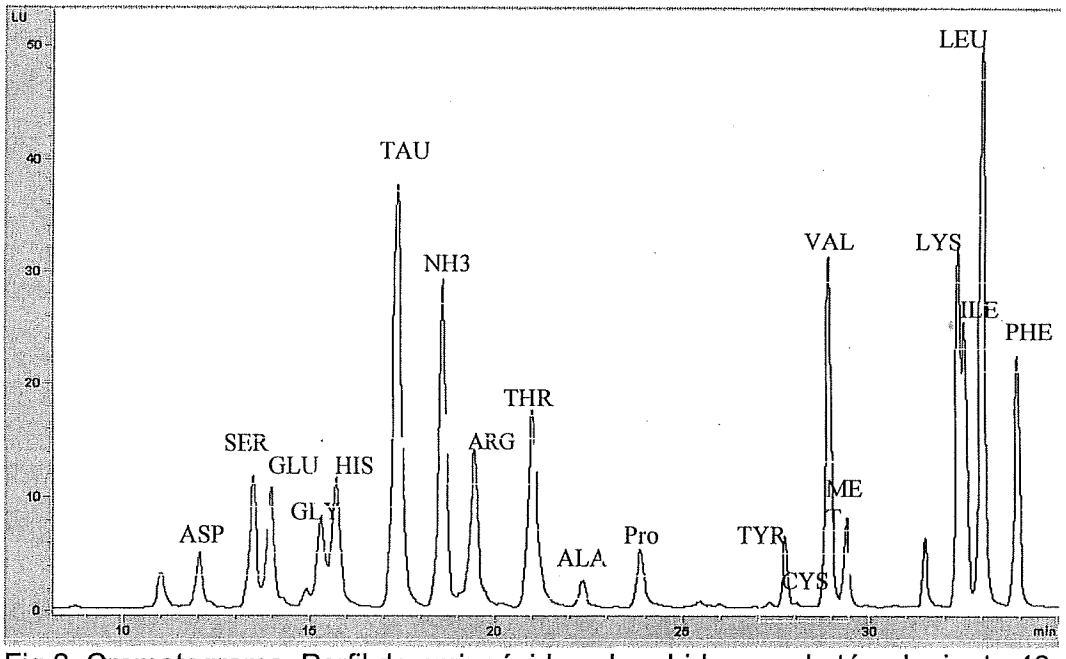


Fig 2. Cromatograma. Perfil de aminoácidos absorbidos en el atún al minuto 40

7.2 Absorción de aminoácidos en el tiempo Trucha
7.2.1 Aminoácidos esenciales

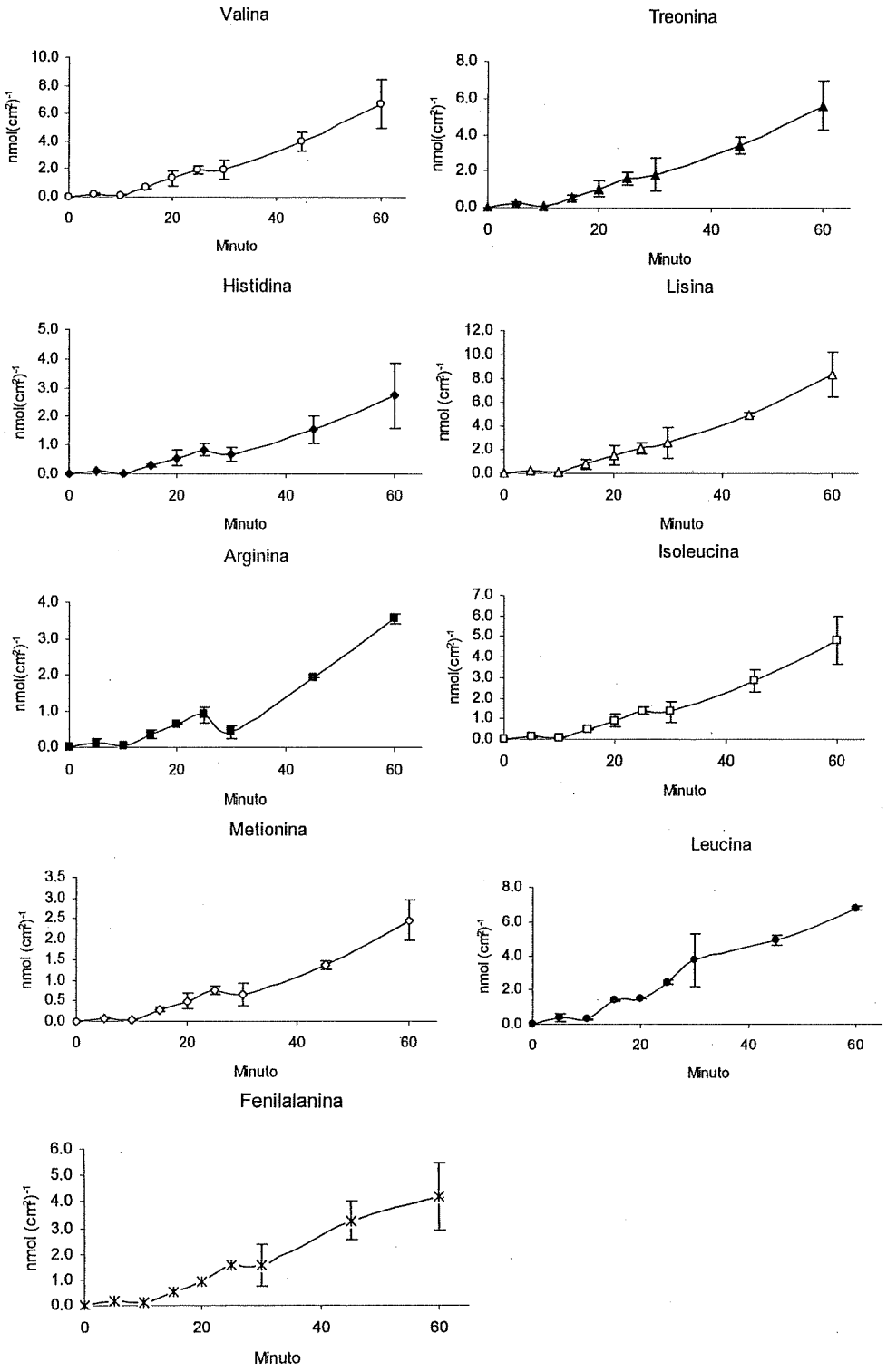


Fig.3. Cinética de absorción de aminoácidos esenciales en el tiempo para trucha arcoiris (*Oncorhynchus mykiss*). His ♦, Arg ■, Thr ▲, Val +, Met ◊, Lys Δ, Iso □, Leu x, Phe ж.

7.2.2 Aminoácidos no esenciales

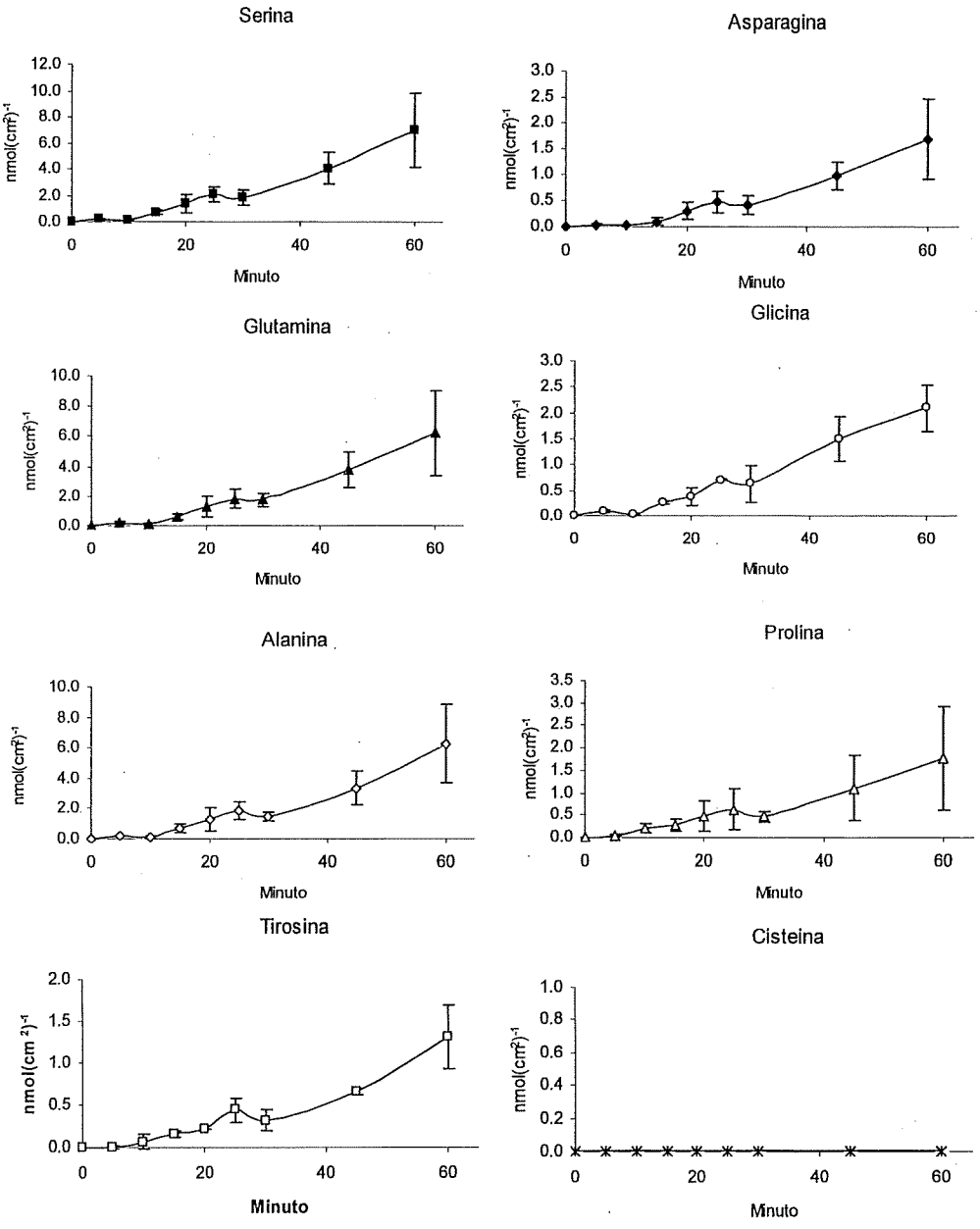


Fig 4. Cinética de absorción de aminoácidos no esenciales en el tiempo para trucha arcoiris (*Oncorhynchus mykiss*) Asp ♦, Ser ■, Glu ▲, Gly +, Ala ◊, Pro Δ, Tyr □, Cys ж.

7.3 Absorción de aminoácidos en el tiempo Totoaba (*Totoaba macdonaldy*)

7.3.1 Aminoácidos esenciales

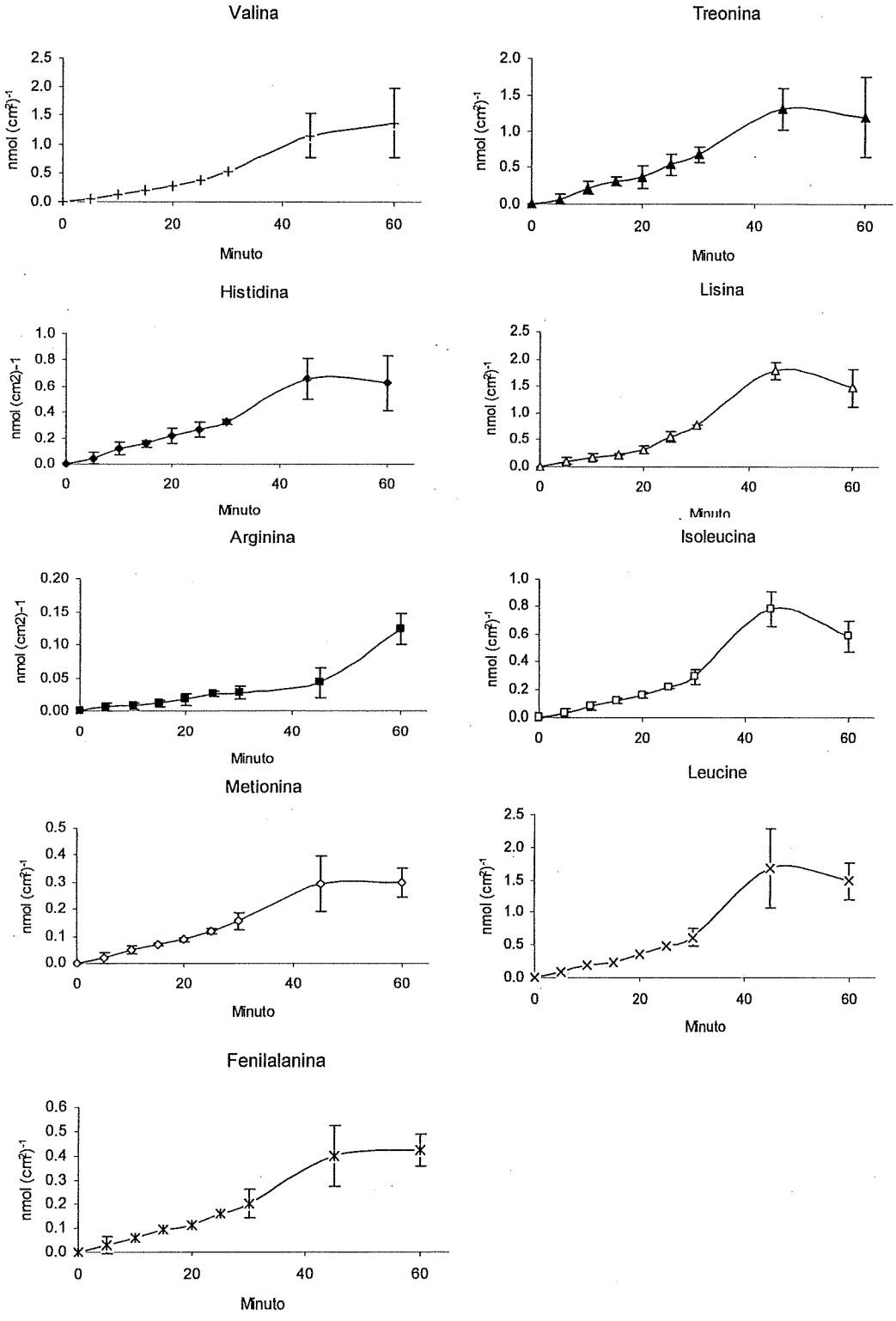


Fig.5. Cinética de absorción de aminoácidos esenciales en el tiempo para la totoaba (*Totoaba macdonaldy*). His ♦, Arg ■, Thr ▲, Val +, Met ◊, Lys Δ, Ile □, Leu x, Phe ж.

7.3.2 Aminoácidos no esenciales

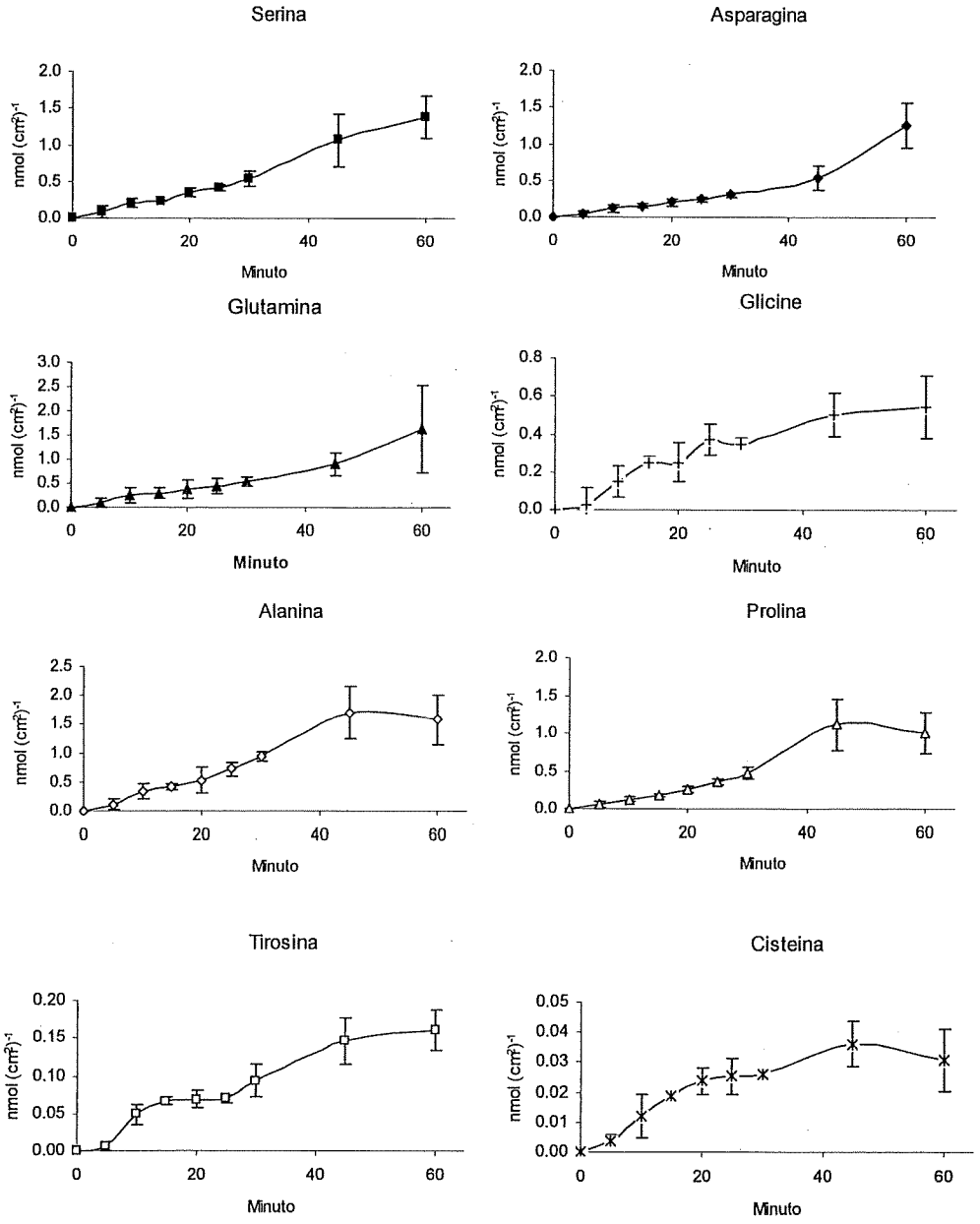


Fig.6. Cinética de absorción de aminoácidos no esenciales en el tiempo para la totoaba (*Totoaba macdonaldy*). Asp ♦, Ser ■, Glu ▲, Gly +, Ala ◊, Pro Δ, Tyr □, Cys ж.

7.4 Absorción de aminoácidos en el tiempo Atún (*Thunnus orientalis*)

7.4.1 Aminoácidos esenciales

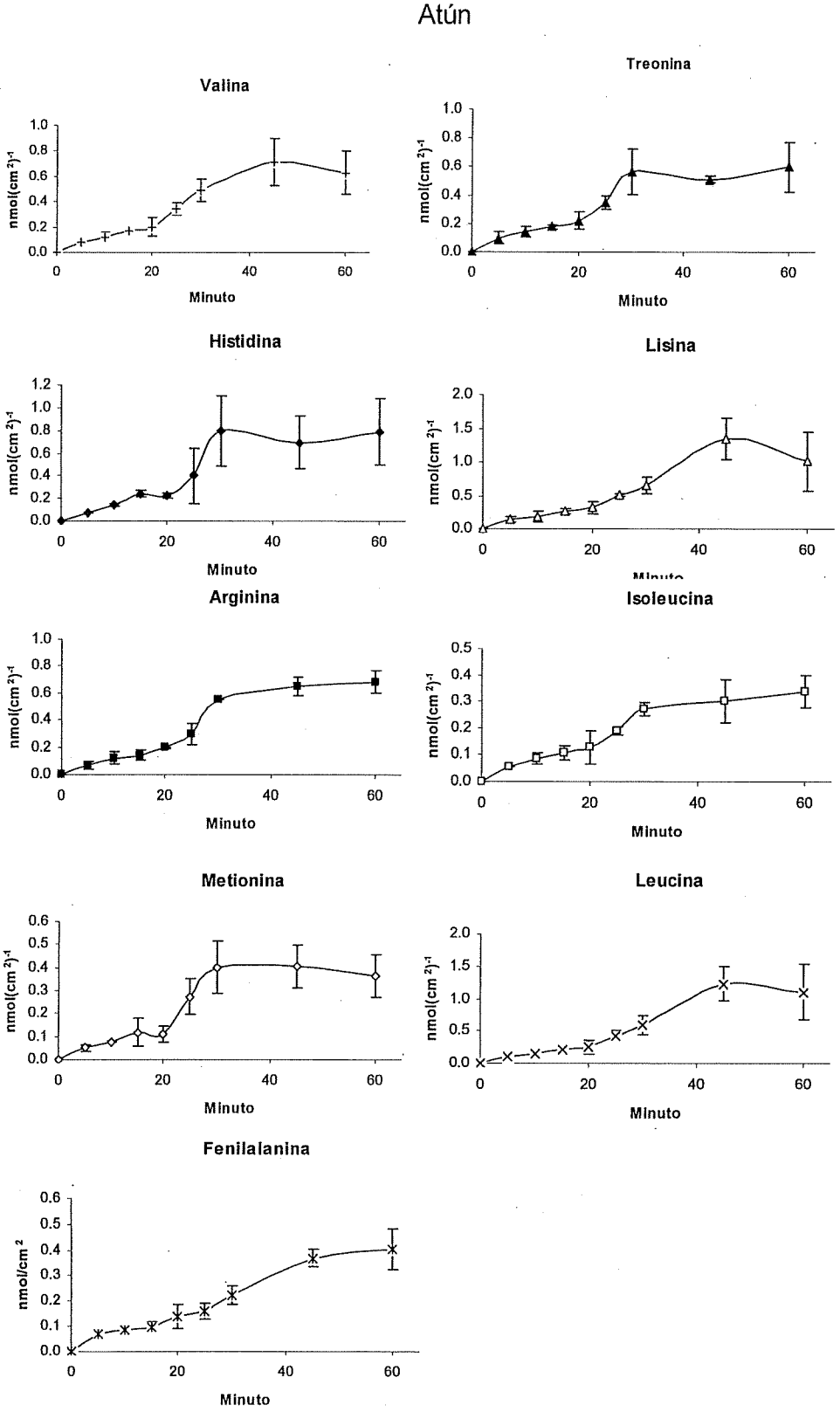


Fig.7. Cinética de absorción de aminoácidos esenciales en el tiempo para el atún (*Thunnus thynnus*). His ♦, Arg ■, Thr ▲, Val +, Met ◊, Lys Δ, Ile □, Leu x, Phe ж.

7.4.2 Aminoácidos no esenciales

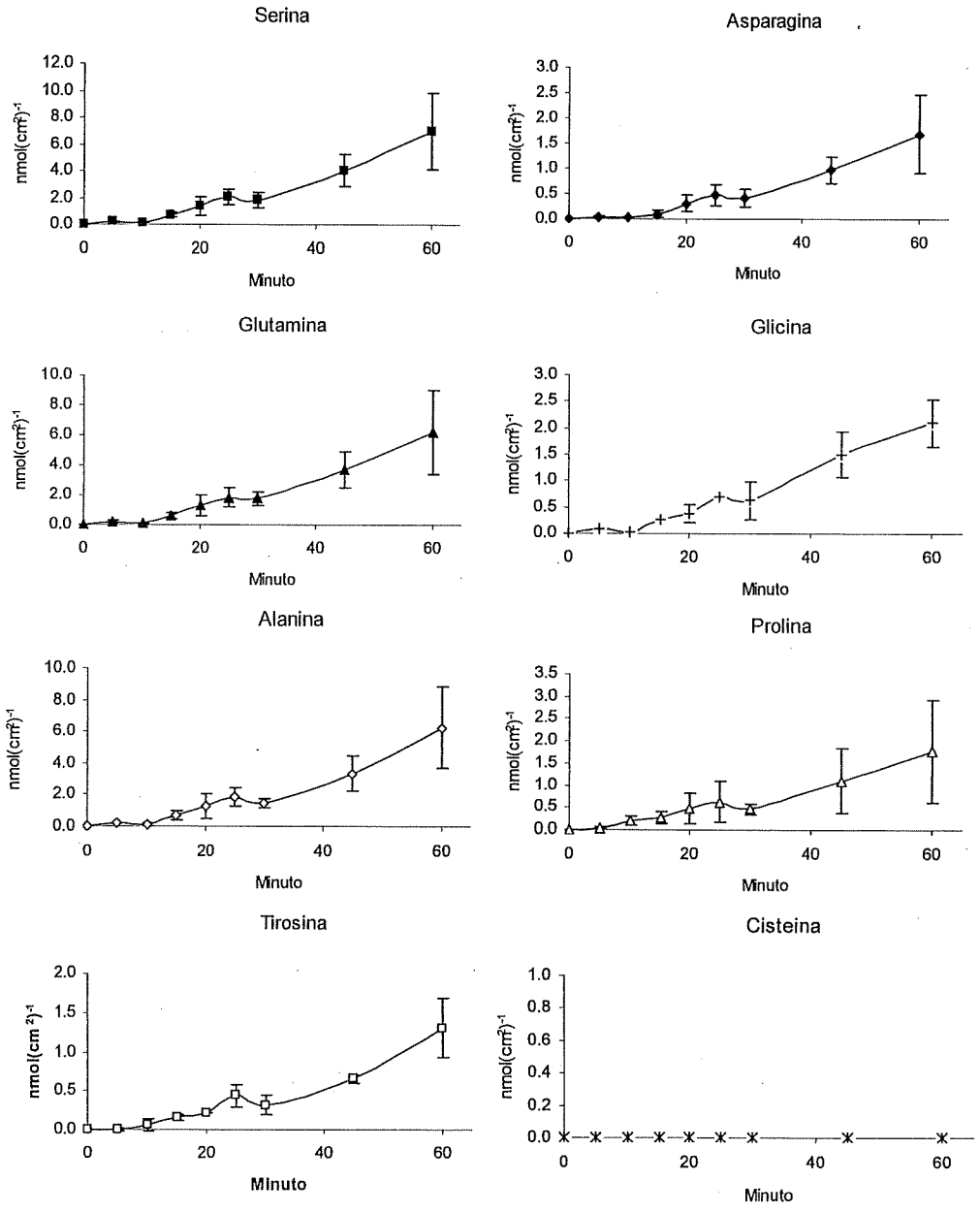


Fig.8. Cinética de absorción de aminoácidos no esenciales en el tiempo para el atún (*Thunnus thynnus*). Asp ♦, Ser ■, Glu ▲, Gly +, Ala ◊, Pro Δ, Tyr □, Cys ж.