UNIVERSIDAD AUTÓNOMA DE BAJA CALIFORNIA

FACULTAD DE CIENCIAS MARINAS

Taurine requirement and its role in the physiology and metabolism of juvenile *Totoaba macdonaldi*

DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

BY

TONY BUDI SATRIYO

ENSENADA, BAJA CALIFORNIA, MEXICO. JUNE 2017

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DEDICATION

This dissertation is dedicated to my beloved family for their love, inspiration, encouragement and a never ending support. Thank you for showing me how important are efforts, perseverance and work ethics for an accomplishment.

DEDICATORIA

Esta tesis se dedica a mi amada familia por su amor, inspiración, ánimo y apoyo. Gracias por enseñarme la importancia de los esfuerzos, la perseverancia y la ética de trabajo para un logro.

ACKNOWLEDGEMENTS

It is my pleasure to acknowledge those who assist in the process of accomplishment of this dissertation.

Thanks to University of Baja California, Mexico for receiving me in the PhD program of Molecular Ecology and Biotechnology at the Faculty of Marine Science, as well as for granting me the UABC International Mobility fellowship 2016.

I am very grateful for the financial supports from Mexican Council for Science and Technology (CONACyT) to conduct the research, and also for PhD scholarship and Beca Mixta International Mobility fellowship 2016.

Many thanks to all the members of Fish Nutrition Lab FCM-UABC for all their friendship, help and assistance in various components of this work including lab analysis, feeding trial and sampling. Also, thanks to the members of Totoaba Hatchery FCM-UABC for their help and friendship.

I would like to express my sincere thanks to my advisors, Dr. Mario Alberto Galaviz Espinoza and Dr. Lus Mercedes López Acuña, for allowing me to join their research group, for their considerable guidance throughout the project, for letting me run with my ideas, for their understanding and helping me to finally reach the goal. Thank you also for giving me the opportunity to present my work in different international conferences and for encouraging me to do research stay abroad that have greatly enriched my scientific experience and strengthened this work.

I would like to thank Dr. Conal David True, Principal Investigator of endangered species *Totoaba macdonaldi* for his valuable contribution as a

committee member, for allowing me to conduct the experiment in Totoaba hatchery FCM-UABC, for his feedback regarding marine fish culture and support throughout this process. I am fortunate to work with this endangered marine species.

I would like to express my gratitude to Dr. Guillaume Salze for his kind collaboration and valuable contribution as a committee member, for his critical view and constructive suggestions which enabled me to develop an understanding of the subject. Thank you also for receiving me at Auburn University and providing me a great deal of knowledge about statistical analysis for nutrition studies.

I would like to extend my thanks to Dr. Sergio Rodríguez Morales for his valuable support as a committee member, for all the contributions of time, comments and scientific knowledge in the area of chemistry to develop this work.

AGRADECIMIENTOS

Es un placer para mí agradecer a quienes han ayudado en el proceso de cumplimiento de esta tesis doctoral.

Gracias a la Universidad Autónoma de Baja California, México por recibirme en el Doctorado de Ecología Molecular y Biotecnología de la Facultad de Ciencias Marinas, así como por otorgarme la beca de Movilidad Internacional 2016.

Estoy muy agradecido por los apoyos financieros del Consejo Nacional de Ciencia y Tecnología, CONACyT México para realizar mi investigación, así como por la beca de estudios de doctorado y Beca Mixta 2016.

Muchas gracias a todos los miembros del Laboratorio de Nutrición Acuícola de FCM-UABC por la amistad, ayuda y asistencia en varias partes de éste trabajo incluyendo análisis de laboratorio, bioensayo y muestreo. También, gracias a los miembros de la Unidad de Biotecnología en Piscicultura FCM-UABC por la ayuda y amistad.

Quisiera expresar mi sincero agradecimiento a mis asesores, el Dr. Mario Alberto Galaviz Espinoza y la Dra. Lus Mercedes López Acuña, por permitirme unirme a su grupo de investigación, por su orientación a lo largo del proyecto, por dejarme correr con mis ideas, por su comprensión y ayuda a lograr la meta. Gracias también por darme la oportunidad de presentar mi trabajo en conferencias internacionales y por apoyarme a hacer estancia en el extranjero que ha enriquecido mi experiencia científica y ha fortalecido mi formación profesional.

Quisiera agradecer al Dr. Conal David True, Investigador Principal de la especies en peligro de extinción *Totoaba macdonaldi*, por su valiosa contribución como miembro del comité, por permitirme llevar a cabo el experimento en la Unidad de Biotecnología en Piscicultura FCM-UABC, por sus comentarios sobre maricultura y el apoyo durante todo este proceso. Tengo la fortuna de haber trabajado con esta especie marina en peligro de extinción.

Quisiera expresar mi gratitud al Dr. Guillaume Salze por su colaboración y valiosa contribución como miembro del comité, por su visión crítica y sugerencias que me permitieron desarrollar la comprensión del tema. Gracias también por recibirme en la Universidad de Auburn, EUA y proporcionarme un gran conocimiento sobre el análisis estadístico para los estudios de nutrición.

Quisiera expresar mi agradecimiento al Dr. Sergio Rodríguez Morales por su valioso apoyo como miembro del comité, por todas las contribuciones de tiempo, comentarios y conocimientos científicos en el área de química para desarrollar el presente trabajo y mi conocimiento.

Taurine requirement and its role in the physiology and metabolism of juvenile *Totoaba macdonaldi*

ABSTRACT

Totoaba (*Totoaba macdonaldi*) is an endemic species inhabiting the Gulf of California. Because of overfishing its population declined. Currently, protocols for captive breeding, larval development and juvenile nutrition have been developed based on the dedicated research that pointed out the high potential of commercial totoaba aquaculture. The information regarding specific nutrients, such as taurine, is important in order to support a nutritionally balanced diet for marine species such as totoaba under culture conditions. Taurine (2-aminoethanesulfonic acid) is the end-product from the metabolism of sulfur-containing amino acid, which is involved in several physiological and metabolic processes. This study was conducted in order to evaluate the effect on growth performance, feed utilization, proximate composition of tissues, hematological parameters and *in vivo* digestibility of juvenile totoaba after supplementing taurine on the diet. Eight isoproteic (50%) and isolipidic (12%) experimental diets were formulated to contain increasing levels of taurine (0.23, 0.45, 0.91, 1.28, 1.76, 2.20, 2.72, 3.01% as-is) by using washed fishmeal (FM) as primary protein source. Ten weeks after initiating the feeding trial, the following observations were made in the basal diet (T-0.23): green liver occurance, low gallbladder-somatic index (GBSI), low apparent digestibility coefficient (ADC) of lipid, low erythrocyte turnover, low plasma cholesterol and triglycerides, as well as low visceral fat. The modelling of the thermal-unit growth

coefficient (TGC) best fitted a five-parameter saturation kinetic model (5-SKM, $P<0.001$) showing marginal differences ($R^2=0.13$) for juvenile totoaba (initial weight 10.0 ± 1.0 g). Low GBSI (0.095%) was concomitant with low ADC of lipid (78.9%) after a low-taurine diet (T-0.23) was provided. The modelling of GBSI and ADC of lipid best fitted a four-parameter SKM (4-SKM, $R^2=0.72$ and 0.87, respectively, $P<0.001$). Plasma cholesterol levels increased linearly with dietary taurine ($R^2=0.75$, $P<0.001$), whereas plasma triglycerides increased quadratically ($R^2=0.53$, $P<0.001$). This suggests modulations of lipid metabolism in totoaba. Plasma total bilirubin levels increased linearly and the lowest concentrations were observed when the basal diet (T-0.23) was consumed. However, hematocrit and hemoglobin levels were slightly different after comparing the treatments. This study demonstrates that supplementation with a low level of taurine (0.45% as-is) in a washed FM-based diet may prevent the occurrence of green liver in juvenile totoaba. Green liver, GBSI, ADC of lipid, plasma cholesterol and triglycerides may be informative diagnostic tools for taurine deficiency in totoaba.

Keywords: taurine, green liver, lipid digestibility, bile salts, bile pigments, saturation kinetic model

Requerimiento de taurina y su papel en la fisiología y metabolismo de juveniles de *Totoaba macdonaldi*

RESUMEN

Totoaba macdonaldi es una especie endémica del Golfo de California y la sobreexplotación pesquera de esta especie provocó un agotamiento de las reservas naturales. Hasta la fecha, se han desarrollado protocolos sobre reproducción en cautiverio, desarrollo de larvas y nutrición de juveniles, destacando el alto potencial para la acuicultura comercial de totoaba. La información sobre nutrientes específicos como la taurina es importante para apoyar una dieta formulada nutricionalmente balanceada para especies marinas como totoaba en condiciones de cultivo. Taurina (ácido 2-aminoetanosulfónico) es un producto final del metabolismo de aminoácidos azufrados que involucra varios procesos fisiológicos y metabólicos. Este estudio se realizó para evaluar el efecto de la suplementación de taurina en la dieta sobre el crecimiento, la utilización de alimento, la composición proximal de varios tejidos, los parámetros hematológicos y la digestibilidad *in vivo* de juveniles de totoaba. Se formularon 8 dietas experimentales isoproteicas (50%) e isolipídicas (12%) que contenían diferentes niveles de taurina (0.23, 0.45, 0.91, 1.28, 1.76, 2.20, 2.72, 3.01%) usando dieta a base de harina de pescado lavada como fuente principal de proteína. Después de 10 semanas de experimento, los organismos alimentados con la dieta basal (T-0.23) mostraron hígado verde, bajo nivel de índice bilisomático (GBSI), bajo digestibilidad aparente (ADC) de lípidos, bajo recambio de eritrocitos, bajo niveles

de colesterol y triglicéridos plasmáticos, así como bajo nivel de lípidos visceral. El coeficiente de crecimiento por unidad térmica (TGC) presentó el mejor ajuste por medio del modelo de cinética de saturación de cinco-parámetros (5-SKM, $P<0.001$) con diferencias marginales ($R^2=0.13$) entre los juveniles de totoaba (peso inicial 10.0 ± 1.0 g). Bajo nivel de GBSI (0.095%) fue acompañado con bajo nivel de ADC de lípidos (78.9%) en la dieta basal (T-0.23). GBSI y ADC de lípidos fueron mejor ajustado por el modelo de cuatro-parámetros SKM (4-SKM, $R^2=0.72$ y 0.87, respectivamente, $P<0.001$). Los valores de colesterol plasmático aumentaron linealmente con la suplementación de taurina en la dieta ($R^2=0.75$, $P<0.001$), mientras que los triglicéridos plasmáticos aumentaron cuadráticamente ($R^2=0.53$, $P<0.001$), lo que sugiere modulaciones del metabolismo lipídico en totoaba. Los niveles de bilirubina total plasmática aumentaron linealmente y la concentración más baja se observó en la dieta basal (T-0.23), sin embargo, los niveles de hemoglobina y hematocrito cambiaron ligeramente entre los tratamientos. Este estudio demuestra que la suplementación con un nivel bajo de taurina (0.45%) en una dieta a base de harina de pescado lavada puede prevenir el síndrome de hígado verde en juveniles de totoaba. El hígado verde, GBSI, ADC de lípidos, colesterol plasmático y triglicéridos podrían ser herramientas de diagnóstico de la deficiencia de taurina en totoaba.

Palabras clave: taurina, hígado verde, digestibilidad de lípidos, sales biliares, pigmentos biliares, modelo de cinética de saturación

Kebutuhan taurin dan fungsinya dalam fisiologi dan metabolisme juvenil *Totoaba macdonaldi*

RINGKASAN

Totoaba macdonaldi adalah spesies ikan laut endemik di perairan Teluk California di Meksiko. Penangkapan ikan secara berlebihan menyebabkan populasinya terancam punah. Sampai saat ini, berbagai macam penelitian meliputi teknologi pemijahan buatan, perkembangan fisiologi pencernaan larva dan pakan buatan bagi juvenil sedang dikembangkan sehubungan dengan prospek diversifikasi dan komersialisasi budidaya ikan totoaba. Informasi tentang nutrisi spesifik seperti taurin sangat penting dalam formulasi pakan dengan kandungan gizi seimbang untuk menunjang kegiatan budidaya ikan laut totoaba. Taurin (asam 2-aminoetanasulfonat) adalah produk akhir dari metabolisme asam amino yang mengandung sulfur dimana zat gizi ini terlibat dalam banyak proses fisiologis dan metabolisme dalam tubuh. Penelitian ini dilakukan untuk mengetahui pengaruh penambahan taurin pada pakan terhadap pertumbuhan, efisiensi pakan, kandungan proksimat pada berbagai organ, parameter hematologi dan daya cerna *in vivo* pada juvenil ikan totoaba. Perlakuan terdiri atas delapan pakan yang dibuat isoprotein (50%) dan isolipidik (12%) dengan dosis taurine (0.23, 0.45, 0.91, 1.28, 1.76, 2.20, 2.72, 3.01%). Pakan dibuat dengan menggunakan tepung ikan yang telah dicuci dengan etanol sebagai sumber protein utama. Setelah sepuluh minggu masa pemeliharaan, pakan dengan dosis taurin terendah (T-0.23) menunjukkan beberapa efek negatif yang meliputi sindrom hati hijau, indeks gallbladder-somatik

yang rendah, daya cerna lemak yang rendah, pergantian sel darah merah yang rendah, kadar kolesterol plasma dan trigliserida plasma yang rendah, serta kandungan lemak visceral yang rendah. Koefisien pertumbuhan unit termal (TGC) pada juvenil totoaba (berat awal 10.0 ± 1.0 g) paling sesuai dipresentasikan dengan model kinetik saturasi lima parameter (5-SKM, $P<0.001$) dengan perbedaan yang kecil ($R^2=0.13$). Indeks gallbladder-somatik yang rendah (0.095%) disertai dengan daya cerna lemak yang rendah (78.9%) pada perlakuan pakan dengan dosis taurin terendah (T-0.23). Kedua parameter ini paling sesuai dipresentasikan dengan model kinetik saturasi empat parameter (4-SKM, $R^2=0.72$ dan 0.87, berurutan, $P<0.001$). Kadar kolesterol plasma meningkat secara linear seiring dengan penambahan dosis taurin ($R^2=0.75$, $P<0.001$), sementara kadar trigliserida plasma meningkat secara kuadratik ($R^2=0.53$, $P<0.001$), hal ini menunjukkan efek modulasi metabolisme lemak pada ikan totoaba. Kadar bilirubin total plasma meningkat secara linear terhadap dosis taurin dan konsentrasi terendah ditunjukkan pada pakan kontrol (T-0.23), namun kadar hematokrit dan hemoglobin mengalami perubahan yang sangat sedikit antara perlakuan. Penelitian ini menunjukkan bahwa penambahan taurin dengan dosis (0.45%) pada pakan berbasis tepung ikan dapat mencegah terjadinya sindrom hati hijau pada juvenil totoaba. Sindrom hati hijau, indeks gallbladder-somatik, daya cerna lemak, kadar kolesterol plasma dan trigliserida plasma bisa digunakan sebagai parameter diagnosis kekurangan zat gizi taurin pada ikan totoaba.

Kata kunci: taurin, sindrom hati hijau, daya cerna lemak, garam empedu, pigmen empedu, model kinetik saturasi

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I. INTRODUCTION

1. *Totoaba macdonaldi*

Totoaba is an endemic species inhabiting the Gulf of California, Northwest of Mexico (Figure 1). Totoaba is a fish characterized by a long lifespan. This demersal species is the largest fish within the Scianidae family, having a maximum reported length of 2 m and up to 135 kg weight (Barrera-Guevara, 1990). Totoaba seasonally migrate from deep to shallow water. The temperature of surface water ranges between 23 and 29°C (True *et al.*, 1997). The distribution of totoaba comprises from the Colorado River mouth to: (a) the Fuerte River mouth along the continental coast of Mexico; and (b) Bahía Concepción along the Baja California Peninsula (Figure 2). Totoaba are iteroparous, which means the fish are capable of spawning more than once during their life. This fish spawn only one time each year and it is considered as an estuarine spawner. Adult totoaba spawn at the Colorado River estuary between late winter and early spring. Totoaba adults migrate towards the Colorado River delta for spawning whereas juveniles move towards the island regions (Tiburon and Angel de la Guarda). As they are large carnivorous fish, adult totoaba require large amounts of food, hence their migration may be linked to sardine availability on the Gulf (Cisneros-Mata *et al.*, 1995). Totoaba is a strict carnivorous species and it requires high protein and moderate lipid levels (López *et al.*, 2006). Wild juveniles eat small benthic organisms such as small crabs, fish, amphipods and shrimp, whereas wild adults eat pelagic fish, mainly sardines and adult crabs (Cisneros-Mata *et al.*, 1995).

Figure 1. Juvenile *Totoaba macdonaldi*.

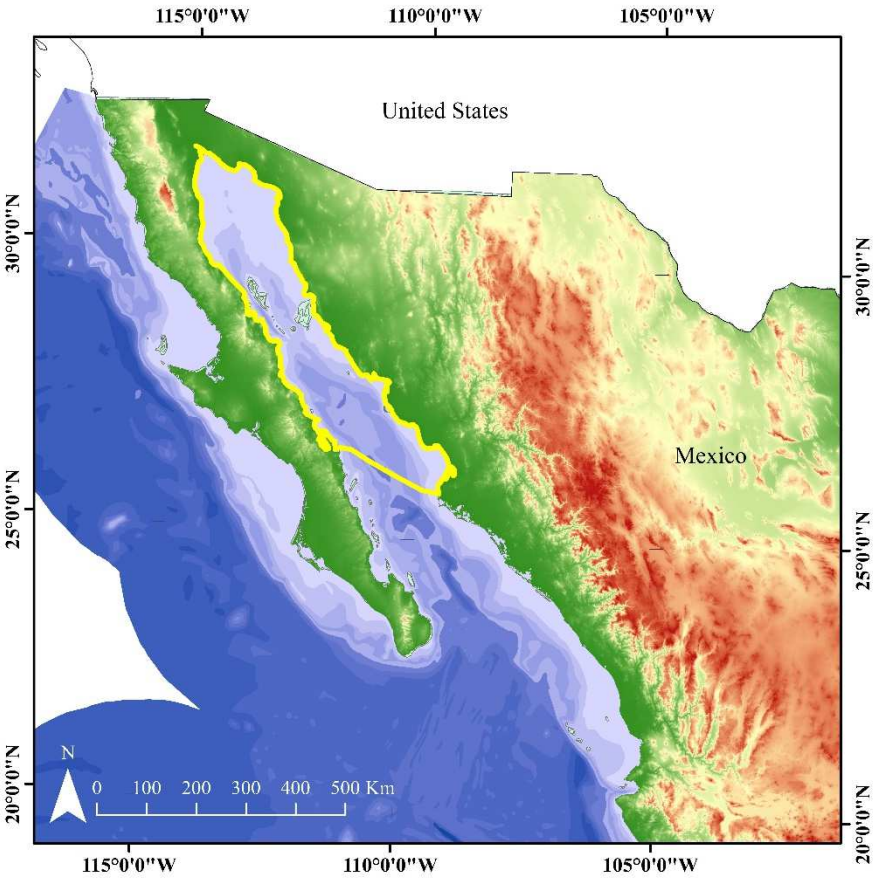

Figure 2. Map of totoaba's potential distribution (indicated in yellow line) on the Gulf of California, Mexico. (www.iucnredlist.org)

1.1 Endangered species

This species had been intensively overfished throughout its distribution range, thus leading to a depletion of its natural reserves. In 1942, totoaba catches

peaked at >2000 tons before declining to 52 tons in 1975 (Cisneros-Mata *et al.*, 1995). Totoaba fishing was banned in that year. In 1976 totoaba was placed on the endangered list issued by the Convention on International Trade in Endangered Species (CITES) and in 1979 it was included in the U.S. Endangered Species Act (ESA) (Barrera-Guevara, 1990). Totoaba are also listed as Critically Endangered by the International Union for Conservation of Nature (IUCN) (Findley, 2010). The underlying causes for the decline of totoaba population over the last 40 years may be: 1) the overfishing of adults by artisanal gillnet during their annual breeding migration to the Colorado delta; 2) the bycatch of juveniles in shrimp trawl nets on the northern Gulf, and 3) habitat loss caused by ecological alteration of its spawning and nursery grounds due to a decreased flow on the Colorado River (Barrera-Guevara, 1990; Cisneros-Mata *et al.*, 1995; Román Rodríguez and Gregory Hammann, 1997). The catching of this fish developed as a response to the demand for its swimbladder that has been exported to China and the United States, where a swimbladder soup is highly valued (Cisneros-Mata *et al.*, 1995).

1.2 Stocking program

In 1994, the Faculty of Marine Science (FCM) of the University of Baja California (UABC) initiated a project intended to collect totoaba broodstock from the wild for captive breeding purposes and to assess the possible establishment of a fish stocking for this endangered species (True *et al.*, 1997). The successful breeding program implemented by FCM-UABC has elevated totoaba's potential for diversification and commercial aquaculture in Mexico. Interest in totoaba

mariculture comes at the time of growing awareness in Mexico to widen the range of marine species for sustainable farming.

1.3 Totoaba nutrition

Several studies focused on captive breeding, larval development and juvenile nutrition have been carried out as part of repopulation and commercialization efforts. In order to gain some basic knowledge on the nutritional requirements for this species, a proximate analysis and a fatty acid profile in muscle and viscera were evaluated on wild and cultured totoaba (López *et al.*, 2006). Based on the study describing the development of totoaba's digestive system, its stomach becomes functional within a period of 20 – 24 days post-hatch. This indicates that totoaba larvae may be able to digest inert food and to absorb nutrients (Galaviz *et al.*, 2015). Several studies have been conducted aiming to evaluate growth performance, proximate composition and survival rate of juvenile totoaba. An optimal growth was observed on fish fed with a diet containing 52% crude protein and 8% crude lipid that was characterized by a 25.7 mg protein/kJ energy (P/E) ratio (Rueda-López *et al.*, 2011). Other study reported that totoaba's diet may be formulated to contain 47% crude protein (Minjarez-Osorio *et al.*, 2012). Soy protein concentrate can replace fishmeal protein on the diet up to 34% without compromising totoaba's growth performance (Trejo-Escamilla *et al.*, 2016).

The use of plant protein in diets for carnivorous species such as totoaba presents a number of challenges associated with imbalances in essential nutrients, therefore studies to overcome the limiting factors of plant protein are required in

order to support the development of sustainable totoaba's diet. Some efforts have been undertaken in order to formulate a specific diet for totoaba. However, to this date, the available information on taurine supplementation favoring this species as well as the quality improvement of an alternative protein source (soy protein ceoncentrate) in combination with taurine is scarce (Bañuelos-Vargas *et al.*, 2014; López *et al.*, 2015).

2. Green liver syndrome in fish

When serious nutritional problems occur, they induce physiological abnormalities such as severe green liver, as previously observed on susceptible species fed with taurine-deficient diets that subsequently resulted in higher fish mortality rates (Takagi *et al.*, 2005; Takagi *et al.*, 2006a). The green pigment in liver originates from biliverdin, tetrapyrrole derived from the catabolism of the heme group. Taurine deficiency has been linked to green liver syndrome development in red sea bream *Pagrus major* and yellowtail *Seriola quinqueradiata*, (Takagi *et al.*, 2005; Takagi *et al.*, 2011). However, the underlying mechanism of green liver occurrence differs between these species considering the composition and concentration of bile pigments as well as hematological parameters.

The bile pigments synthesized by red sea bream *P. major* are biliverdin and taurobilirubin. These are excreted from hepatopancreas into the bile as biliverdin which has a high polarity, while the hepatopancreatic biliverdin that exceeds the excretion capacity is reduced to bilirubin that possess a low polarity (Sakai *et al.*, 1990). Subsequently, bilirubin is conjugated with taurine in order to increase its

polarity and it is excreted into the bile as taurobilirubin (Sakai *et al.*, 1987; Takagi *et al.*, 2006b). When taurine content in liver is low, its conjugation with bilirubin decreases and an excess of biliverdin production occurs. Consequently, a large amount of biliverdin is retained in the liver (Takagi *et al.*, 2006b). The occurrence of green liver in red sea bream *P. major* is the consequence of biliverdin overproduction and a gradual increase of hemolysis as erythrocytes become osmotically fragile (Takagi *et al.*, 2006b).

Regarding yellowtail *S. quinqueradiata*, bile pigments are converted from biliverdin to bilirubin. The latter is conjugated with taurine in order to increase its polarity before excretion. The bile pigments in yellowtail *S. quinqueradiata* are excreted from the liver into the bile mainly as taurobilirubin (Sakai *et al.*, 1987; Sakai *et al.*, 1990; Takagi *et al.*, 2005; Takagi *et al.*, 2008). Green liver appearance in this species is caused by a decreased excretion of bile pigments into the bile, as bilirubin is not conjugated with taurine, and biliverdin overproduction due to excessive hemolysis which is caused by both, low blood osmolality and a fragility of erythrocyte membrane (Takagi *et al.*, 2005; Takagi *et al.*, 2006a; Takagi *et al.*, 2008). Moreover, green liver symptom was concomitant with anemia in yellowtail *S. quinqueradiata* fed with a taurine-deficient diet (Takagi *et al.*, 2005; Takagi *et al.*, 2006a).

Green liver syndrome has been observed after totoaba were fed for more than 6 months with a commercial diet for trout (Figure 3).

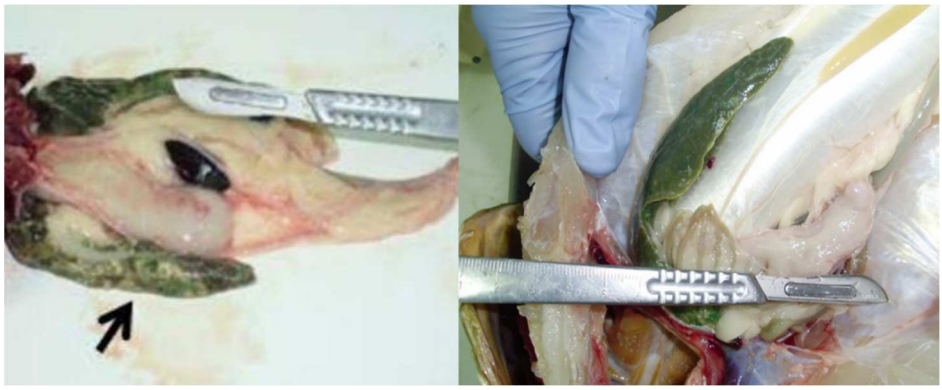

Figure 3. Acute green liver in totoaba fed with a commercial diet for trout.

3. Taurine

Taurine (2-aminoethanesulfonic acid) is the end-product from the metabolism of sulfur-containing amino acids. In mammalian tissue, taurine is synthesized from methionine to cysteine by a series of enzyme-catalyzed reactions (Kuzmina *et al.*, 2010). Having an amino group and acidic group, taurine is an amino acid which is specifically defined as a β-sulfonic acid. Taurine is a zwitterion at physiological pH.

Taurine is not incorporated into protein as well as oxidized as an energy substrate in animals. Instead, taurine is present as free form in most animal tissues and it is widely distributed in cytosol and plasma. This compound is highly polar and lipophobic, thus passive diffusion of taurine across the cell membrane is limited. The main absorption route into enterocytes occurs via taurine transporters (TauT). These cells contain specific Na^+/Cl^--dependent carriers to maintain a high intracellular to extracellular taurine ratio. TauT functions as an active transport system that moves taurine through a membrane against a concentration gradient (from low to high concentration region) and requires energy from the cell in the

form of ATP.

The studies focused on intestinal taurine transport in vertebrates described the high-affinity, low-capacity Na^+/Cl^--dependent taurine transporter (TauT) (O'Flaherty *et al.*, 1997). In fish, there are only a few studies focused on TauT characterization (Takeuchi *et al.*, 2000; Zarate and Bradley, 2007; Kozlowski *et al.*, 2008). Taurine absorption has been described in the digestive tract of Senegalese sole *Solea senegalensis* larvae, where TauT was highly expressed in the stomach and the hindgut, indicating that taurine is rapidly absorbed when digestion begins. It was also shown that taurine is endogenously used for bile acid conjugation and it may be recycled at the posterior end of the digestive tract. This suggests the existance of an enterohepatic recycling pathway intended to preserve steady levels of taurine in Senegalese sole *S. senegalensis* (Pinto *et al.*, 2012).

When taurine natural sources (e.g., fish meal) are inadequate, synthetic taurine may supplemented in order to meet the fish requirement in aquafeeds. Synthetic taurine is a white crystalline powder which is water soluble (10 g dissolves in 100 mL at 25°C) and the pH of a 5% aqueous solution is 4.1–5. It contains at least 98% taurine on a dry basis. There are several methods of manufacturing synthetic taurine, for example taurine is chemically produced from ethylene oxide and sodium bisulphite that are mixed by liquid ammonia and sulfuric acid, then the compound is purified, crystallized, dried and blend with the carrier to finally obtain the additive in its final form (Hayes and Sturman, 1981; EFSA, 2012).

3.1 Taurine function

Taurine participates in numerous physiological functions, e.g. membrane protection, anti-oxidation and detoxification in mammals, as well as osmoregulation in invertebrates and higher animals (Schaffer *et al.*, 2000), cholesterol degradation (Yokogoshi *et al.*, 1999), calcium level modulation, reproduction, cell membrane stabilization, cell volume regulation, anti-inflammatory agents, carrier of lipid-soluble vitamins in mammals, enhancing the production of bile salt in fish and bile acid conjugation (Huxtable, 1992).

Taurine is important for cholesterol metabolism as it stimulates (1) bile acid production (the degradation product derived from cholesterol metabolites) by stimulating the activity of cholesterol 7α-hydroxylase, the rate-limiting enzyme for bile acid synthesis, and (2) cholesterol synthesis by activating 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of cholesterol *de novo* synthesis pathway (Chen *et al.*, 2012). In lipid metabolism, the roles of taurine include their conjugation with bile acids in liver, thus increasing the use of bile acids and the micelle formation for fat absorption in the small intestine (Yokogoshi *et al.*, 1999).

3.2 Taurine biosynthesis

Taurine biosynthesis takes place mostly in liver. After that, it is absorbed by other tissues from plasma circulation in a process mediated by TauT. Moreover, taurine is synthesized in other tissues, such as brain (Huxtable, 1992) and pancreas, predominantly in α-islets (Bustamante *et al.*, 1998). Dietary taurine

deficiency is primarily identified in liver, as it is the main site where taurine synthesis takes place. Brain and muscles are metabolically active and produce high levels of free radicals, therefore these tissues are also important sites where taurine may exert an effect. If dietary taurine is insufficient, these tissues may synthesize it independently of liver or its absorption from the diet. This may result in an increased expression in these tissues. This was observed in mice, as non-hepatic tissues compensate the loss of hepatic taurine synthesis (Ueki *et al.*, 2012). These tissues may respond to the taurine levels present on the diet by up- or down- regulating gene expression in situations of low or high dietary taurine.

In mammals, taurine is synthesized from sulfur amino acids: methionine and cysteine. The latter is the key intermediate for the taurine biosynthesis pathway in mammals. Methionine is essential as it donates a sulfur atom to cysteine during the transsulfuration process, thus rendering cysteine a dispensable amino acid. The carbon skeleton of cysteine is provided by serine. Methionine is metabolized by three pathways: transmethylation, remethylation and transulfuration. Methionine is converted to cysteine by transmethylation and transsulfuration pathways (Courtney-Martin *et al.*, 2012).

Taurine is the end-product of methionine oxidation pathway. There are two possible taurine biosynthesis pathways that use methionine as a precursor (Figure 4): 1) the Cysteine sulfinic acid pathway and 2) the Cysteine acid pathway. In both pathways, the first step is an oxidation to produce cysteine and they diverge after this point. This first step is methionine transformation to cystathionine by cystathionine synthetase followed by the conversion of cystathionine to cysteine

by cystathionase (Griffith, 1987).

The sequence of reactions within the cysteine sulfinic acid route (pathway 1) are the following: oxidation of cysteine to cysteine sulfinate by cysteine dioxygenase (CDO, EC 1.13.11.20), decarboxylation of cysteine sulfinate to hypotaurine by cysteine sulfinate decarboxylase (CSD, EC 4.1.1.29) and further hypotaurine oxidation to taurine. In this pathway 1, cysteine dioxygenase (CDO) and cysteine sulfinate decarboxylase (CSD) are the key enzymes that confer the ability to biosynthesis taurine. CDO is the essential enzyme for cysteine degradation, whereas CSD is the rate-limiting enzyme for taurine biosynthesis (Griffith, 1987). In this process, pyridoxal-5′-phosphate (vitamin B6) is required as the coenzyme of CSD. This enzyme plays a crucial role in this pathway as it catalyzes the reaction that produces hypotaurine and certain species naturally lack thereof. Some observations suggest that CSD participates in the taurine synthesis pathway in teleost (Yokoyama *et al.*, 2001).

Another possible metabolic pathway is the cysteine acid route (pathway 2). Cysteine sulfinate is synthesized from cysteine and it is subsequently metabolized to cysteic acid. This compound is metabolized to produce taurine when hypotaurine is absent. In this pathway 2, cysteic acid is decarboxylated by cysteic acid decarboxylase (CAD) (Stipanuk, 1986). In carps, since it lacks CSD activity to produce hypotaurine (pathway 1), there is another possible route to produce taurine through cysteic acid (pathway 2).

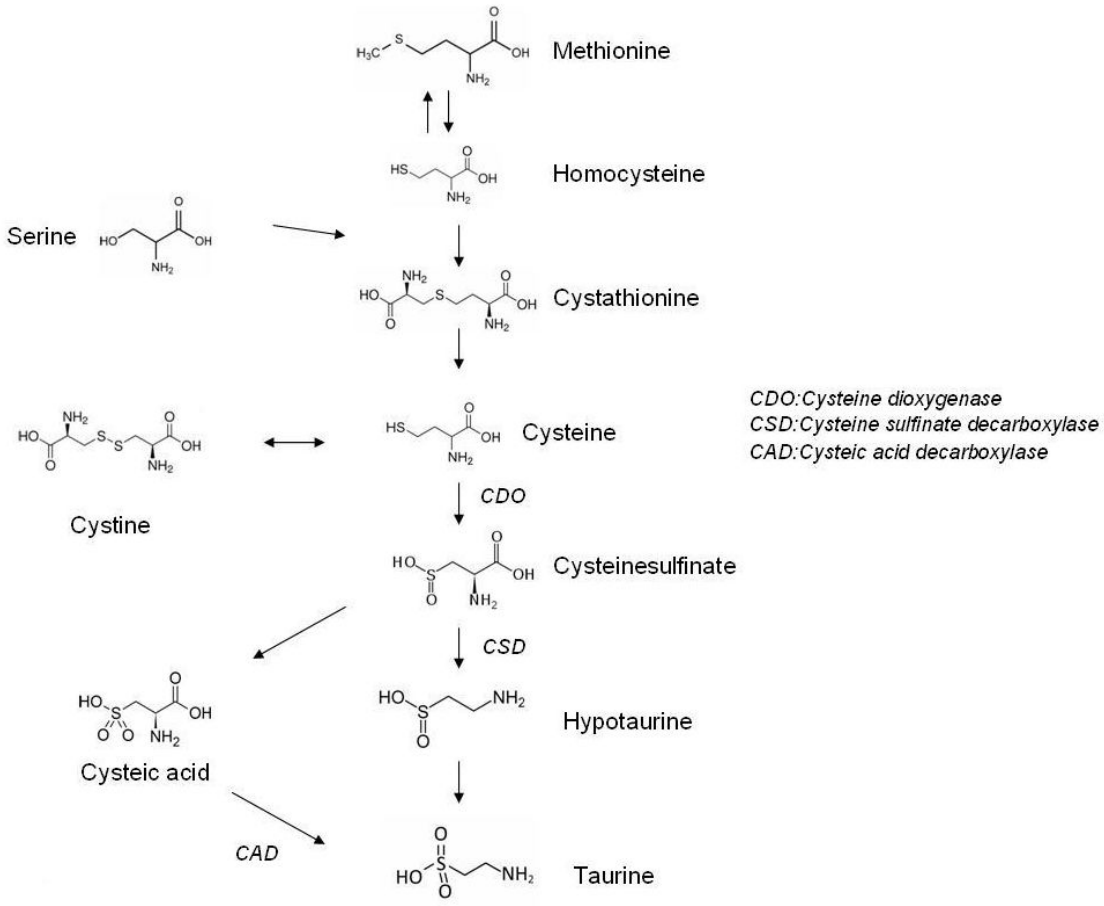

Figure 4. Metabolism of sulfur-containing amino acids and taurine biosynthesis pathways. Adapted from Stipanuk (1986).

The conversion efficiency of sulfur-containing amino acids to taurine has been assessed by estimating CSD and CDO activities in the livers of some marine fish (Goto *et al.*, 2001a; Yokoyama *et al.*, 2001; Takagi *et al.*, 2005). It has been reported that CSD activities were detected in rainbow trout *Oncorhynchus mykiss* and tilapia. The activity is less in Japanese flounder *Paralichthys olivaceus* and red sea bream *P. major*; whereas it is not detected in yellowtail *S. quinqueradiata*, bluefin tuna *Thunnus thynus*, skipjack tuna *Katsuwonus pelamis* and common carp (Yokoyama *et al.*, 2001). The CSD activity in red sea bream *P. major* and Japanese

flounder *P. olivaceus* is approximately 50% of that measured in rainbow trout *O. mykiss.* However, higher CSD activity in rainbow trout *O. mykiss* and tilapia are still inadequate to support maximum growth. The activity of CSD is variable among fish, depending on the species and their size. Furthermore, some observations suggest that an impaired synthesis may exist rather than a low substrate availability for taurine synthesis.

4. Taurine in ingredients

The difference regarding nutrient content between protein sources (i.e., plant vs fish meal) is an essential aspect to be taken into account to develop a sustainable aquafeed. Some nutrients absent in plant proteins may be found in fishmeal and, among these, taurine has received considerable attention. Taurine is found in animal tissue like fishmeal, but is scarce in plant protein sources such as soybean meal, wheat flour, soy protein concentrate and corn gluten (Spitze *et al.*, 2003). Consequently, when fishmeal is replaced by plant-derived ingredients taurine levels are low in the aquafeed. This situation, combined with the low methionine content of the alternative protein sources may restrict taurine function as nutrient in some species (Gatlin *et al.*, 2007), because some marine fish have a very limited ability to synthesize taurine (Yokoyama *et al.*, 2001). In this case, taurine could be described as an essential nutrient which the organism cannot synthesize it or not enough quantity to meet its needs.

The animal-derived ingredients contain appreciable amounts of taurine. However, the nutrient content may be different in fishmeal depending on the

species, the processing method and the fillet content before processing. White fishmeal has 0.34% taurine (Lim *et al.*, 2013). Brown fishmeal contain approximately 0.6% taurine (Kim *et al.*, 2008a). Menhaden fishmeal contains approximately 0.5% taurine and this level could be equivalent to 0.3% taurine in diet formulated to contain 40% crude protein (Gaylord *et al.*, 2006).

5. Taurine deficiency and its requirement in fish

Taurine deficiency occurs in fish species possessing a limited ability to synthesize it and also when natural taurine sources are excluded from the diet. Some studies have demonstrated that taurine deficiency may cause several deleterious effects such as: reduced growth performance (Takagi *et al.*, 2010; Jirsa *et al.*, 2014), increased mortality (Takagi *et al.*, 2006a), inferior feed performance (Kim *et al.*, 2005a). Some physiological abnormalities have beed detected, such as green liver syndrome in red sea bream *P. major* (Goto *et al.*, 2001b; Takagi *et al.*, 2006b; Takagi *et al.*, 2011), yellowtail *S. quinqueradiata* (Takagi *et al.*, 2005; Takagi *et al.*, 2006a; Takagi *et al.*, 2008) and totoaba *T. macdonaldi* (López *et al.*, 2015). Other effects include low plasma taurine levels, low osmotic tolerance on erythrocytes, hemolytic anemia as indicated by a low hematocrit value in yellowtail *S. quinqueradiata* (Takagi *et al.*, 2006a; Takagi *et al.*, 2008); low cholesterol level (hypocholesterolemia) in the latter species and red sea bream *P. major* (Goto *et al.*, 2001b; Maita *et al.*, 2006); decrease in hepatic mitochondrial protein content and cytochrome c oxidase (COX) activity (last enzyme of the electron transport chain in the mitochondria) in Florida pompano *Trachinotus carolinus* (Salze *et al.*,

2016); hepatocytes and adenocytes atrophy, including impaired zymogen granules in exocrine pancreas of red sea bream *P. major* (Amano *et al.*, 2012); poor spawning performance of a yellowtail *S. quinqueradiata* broodstock (Matsunari *et al.*, 2006). All these detrimental effects are counteracted by supplementing dietary taurine which suggests that it may be an essential nutrient for these fish species.

The requirement seems to be linked to the diverse physiological roles of specific nutrient in fish (Takagi *et al.*, 2006a). Nutrient requirements of fish have been assessed by quantifying the dose-response relationship (Shearer, 2000; Hernandez-Llamas, 2009; Jobling, 2015) where graded levels of nutrients are supplemented in diets and evaluated after providing daily similar feeding portions in the same period. Quantitative and qualitative responses are measured based on several parameters, such as growth, feed intake, survival, body composition, health indexes, enzymatic activities and morphological characteristics (Vedenov and Pesti, 2008).

Fish maintain their taurine levels through dietary intake and endogenous biosynthesis. The metabolic pathways for taurine synthesis and the ability to meet the metabolic requirements without dietary intake are species-dependent. The limited ability to synthesize taurine causes the requirement of this nutrient. Studies on the taurine requirement of marine fish such as red sea bream *P. major*, Japanese flounder *P. olivaceus* and yellowtail *S. quinqueradiata* have shown that taurine is an essential nutrient at several life-cycles stages of fish including larvae, juveniles and broodstock (Takeuchi *et al.*, 2001; Kim *et al.*, 2005b; Kim *et al.*, 2005a; Matsunari *et al.*, 2006; Takagi *et al.*, 2008). These studies suggest that

these species have a very low taurine biosynthesis capacity and essentially they require an external supply. Dietary taurine supplementation may be fundamental when taurine levels are below the minimum requirement. The dietary taurine requirements of some cultured fish have been estimated to be within the range 0.20% - 1.66% (Salze and Davis, 2015).

6. Bile acid and Bile salt synthesis

Lipids are considered important for fish to provide energy and structural components for growth and development. Taurine participates during lipid digestion as it is conjugated with bile acids in liver in order to form bile salts that subsequently are stored in the gallbladder and finally they are released into the intestine. In most teleost, conjugated bile acids are formed from taurocholic and taurochenodeoxycholic acids, that has been reported to occur in some marine fish such as Atlantic salmon *Salmo salar*, sole *Paraplagusia bilineata*, the verdeles *Aixos maru*, *Trachurus japonicus*, *Scomberomorus commerson* (Yeh and Hwang, 2001) and Japanese flounder *P. olivaceus* (Kim *et al.*, 2007). These taurine-conjugated bile acids increase concomitantly with dietary taurine levels, where taurocholic acid comprises over 95% of the overall conjugated bile acids (Kim *et al.*, 2007). This implies that taurine is the only compound bound to cholesterol derivatives forming taurocholic and taurochenodeoxycholic acids. In rainbow trout *O. mykiss*, plasma taurine concentration decreases a few hours after feeding them with taurine-supplemented diets. This may be caused by the conjugation of bile acids with taurine (Yamamoto *et al.*, 2005). It has been suggested that taurine may

interfere with fish lipid metabolism as it causes hypolipidemic effects (low lipid levels in blood) in other animals (Militante and Lombardini, 2004). These may be associated with an increased bile acid synthesis (Russell, 2003).

Bile acids are synthesized in hepatocytes (parenchyma liver cells) using cholesterol as precursor. Three terminal carbon atoms from cholesterol are removed to produce the 24-carbon-atoms bile acids (Figure 5). Bile acids have a steroid nucleus with the different molecular forms regarding the number and position of additional hydroxyl groups. Cholesterol-7-alpha-hydroxylase (CYP7A1) is the enzyme catalyzing cholesterol hydroxylation in position 7. This reaction is the initial and limiting step during bile acid synthesis. The enzyme introduces a hydroxyl group at position 7 on the cholesterol ring. Through two different routes, primary bile acids are produced: cholic acid and chenodeoxycholic acid. They differ by the absence of one hydroxyl group at position 12 on the chenodeoxycholic acid (Baynes and Dominiczak, 2009). In human, primary bile acids are conjugated with glycine or taurine by a peptide bond between the carboxyl group on the bile acid and the amino moiety of the corresponding amino acid (Figure 6). In some species of fish, bile acids are only conjugated with taurine by Bile acid-coenzyme A (CoA): amino acid N-acyltransferases in the fish liver (Vessey *et al.*, 1990). Taurine participates in bile salts production in liver in its conjugated form with bile acids (taurocholic and taurochenodeoxycholic acids, the major conjugated bile acids in most fish) before its secretion into the gallbladder bile (Yeh and Hwang, 2001; Kim *et al.*, 2007; Kim *et al.*, 2008b).

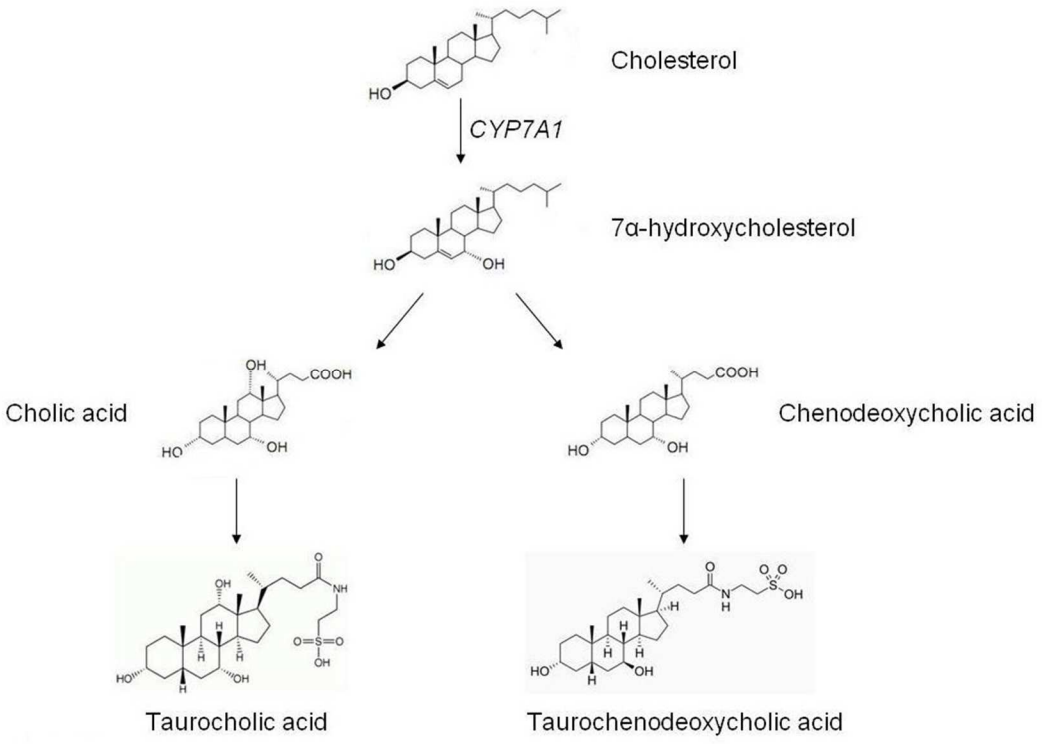

Figure 5. Synthesis of taurocholic acid and taurochenodeoxycholic acid. Adapted from Baynes and Dominiczak (2009)

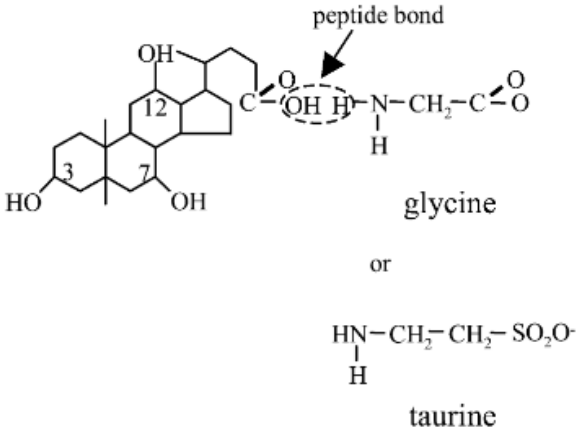

Figure 6. Peptide bond between the carboxyl group of bile acid and the amino moiety of amino acid. Adapted from Begley *et al.* (2005).

The role of bile salts is not restricted to the emulsification of ingested lipids, but they also increase the activity of certain pancreatic lipases. Bile salt-activated

lipase (BAL) is a multi-substrate digestive enzyme secreted by pancreas that hydrolyzes carboxyl ester bonds of acylglycerol, cholesterol esters, neutral lipid esters and esters of fat soluble vitamins (Murray *et al.*, 2003). The activity of BAL increases with the presence of bile salts. The relevance of this multi-substrate enzyme as a digestive lipase has been suggested for fish. BAL from teleost bears homology with mammalian BAL and it has been isolated from the pyloric caeca of Atlantic cod *Gadus morhua* (Gjellesvik *et al.*, 1989; Gjellesvik *et al.*, 1992) and from the hepatopancreas of red sea bream *P. major* (Iijima *et al.*, 1998).

Having carboxyl and hydroxyl groups, bile salts display amphipathic features. Their hydrophobic regions face towards the inner region of the micelle whereas their polar groups are directed towards its exterior, thus solubility is increased in aqueous environments (Figures 7 and 8). Emulsified lipids are susceptible to the hydrolytic activity of intestinal lipases and esterases (Brody, 1999).

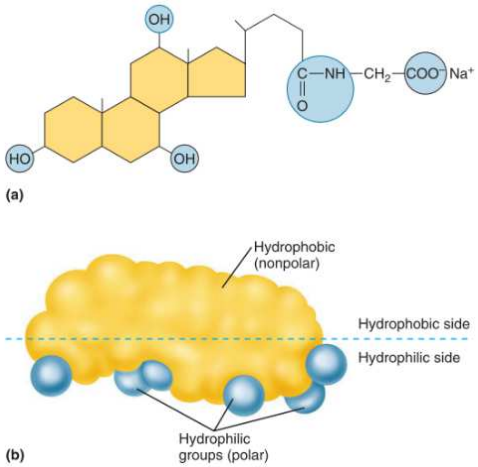

Figure 7. (a) Molecular structure of a bile salt; (b) Hydrophilic and hydrophobic regions. Adapted from Stanfield (2013).

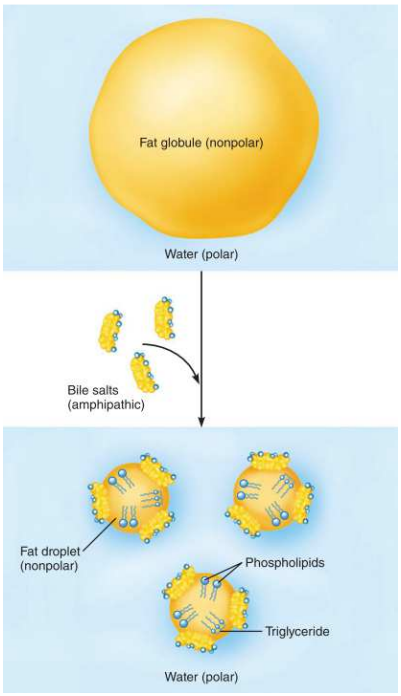

Figure 8. Bile salts emulsify fat droplets. Adapted from Stanfield (2013).

7. Red blood cell destruction and Bile pigment synthesis

A mature erythrocyte contains hemoglobin and its main function is to transport oxyhemoglobin to all tissues and to take up carbon dioxide from the tissue. Human erythrocytes have an average life span of 120 days. As they senesce the following processes occur: 1) the membrane becomes less flexible and its integrity is disrupted; 2) hemoglobin concentration increases; and 3) the activity of glycolytic enzymes decline. Furthermore, old erythrocytes are removed from the blood stream by phagocytic cells called macrophages. Spleen is site where phagocytosis of senescent cells occurs.

Red blood cell degradation is mainly extravascular (approximately 90%) and erythrocytes are submitted to phagocytosis and digestion by macrophages

located at reticuloendothelial system. Subsequently, hemoglobin molecules are cleaved in to two major components: heme and globin. The latter is catabolized in liver into its constituent amino acids that afterwards enter the circulating amino acid pool to finally be reutilized for *de novo* protein synthesis. Heme is degraded to iron, carbon monoxide and biliverdin (green pigment). In plasma, iron is transported by transferrin to be recycled as newly-synthesized hemoglobin on the red bone marrow. The alpha carbon is converted to carbon monoxide. The ring structure of the heme group is oxidatively cleaved by hemoxygenase to form a tetrapyrrole, biliverdin, a green pigment (Figure 9). Most vertebrates subsequently reduce biliverdin to the yellow-colored tetrapyrrole, bilirubin, because of the presence of the substrate-specific enzyme, biliverdin reductase (EC 1.3.1.24). The opening of the porphyrin ring generates tetrapyrrole, bilirubin, a yellow pigment. Unconjugated (pre-hepatic) and conjugated (post-hepatic) forms of bilirubin are present in plasma. After bilirubin enters plasma as its free non-conjugated form, it is bound to albumin and it is subsequently transported to liver where its linkage with glucuronic acid occurs. Finally, this conjugated bilirubin is excreted into the bile as a biliary pigment. Bilirubin is a lipophilic yellow pigment in its free non-conjugated form and it becomes hydrophilic in its conjugated form. Bilirubin is the product resulting from the catabolism of the heme group. When red blood cells degradation increases, unconjugated bilirubin levels exceed the liver capacity to perform the conjugation reaction. This condition results in high unconjugated bilirubin levels in blood plasma and eventually jaundice may occur (Turgeon, 2012).

Intravascular degradation is an alternative route for erythrocyte breakdown.

This process represents less than 10% of erythrocyte degradation by which hemoglobin is released directly into the bloodstream. Free hemoglobin binds immediately to haptoglobin to form a complex that prevents urinary excretion of plasma hemoglobin (Turgeon, 2012).

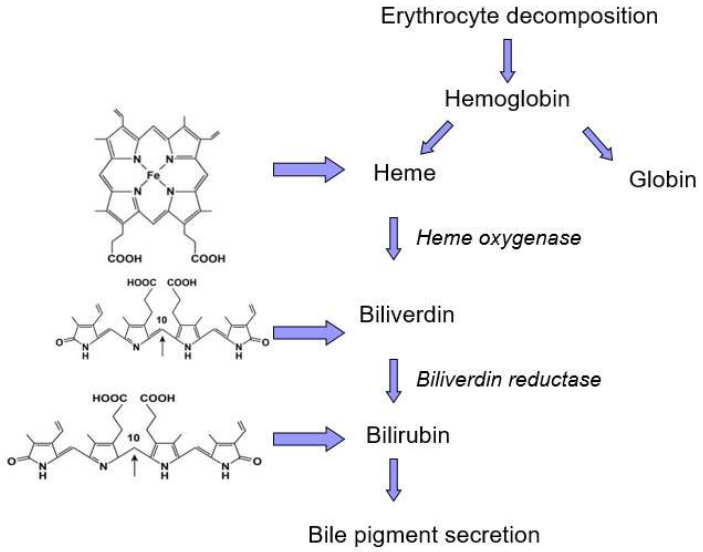

Figure 9. Heme catabolism and formation of bile pigments in human. Adapted from Dancygier (2009).

The erythrocyte life span in rainbow trout *O. mykiss* is approximately 500 days (Avery *et al.*, 1992). The light yellow coloration of serum in most fish is due to the presence of bilirubin (0.1-0.5 mg/dL) that is bound to albumin-like proteins. However, serum is blue-green in certain species because of the presence of biliverdin. Almost all fish excrete both biliverdin and bilirubin, while certain species excrete mainly either biliverdin or bilirubin (Cornelius, 1991). Fish characterized by blue-green plasma display different bile pigment pathways. Some of the biliverdin released from heme catabolism binds to specific serum proteins. Free biliverdin is excreted without modification and the protein-bound form is not metabolized to

bilirubin by biliverdin reductase. They are not removed from the blood stream by the liver or the kidney, but they remain, thus conferring a green coloration to plasma (Fang and Bada, 1988).

Based on the analysis performed on the bile of 39 fish species, bile pigment excretion is classified in two types (Figure 10). Type I fish excrete mainly bilirubin into the bile, whereast type II fish excrete most biliverdin with less bilirubin. Another relevant factor is that biliverdin reductase activity may control bile pigment composition. Additionally, there is a metabolic pathway shift between biliverdin and bilirubin excretion. Those that excrete mainly bilirubin may switch to biliverdin excretion during periods where energy deficit occurs. In contrast, those excreting mainly biliverdin lack the ability to shift to bilirubin excretion (Fang, 1987).

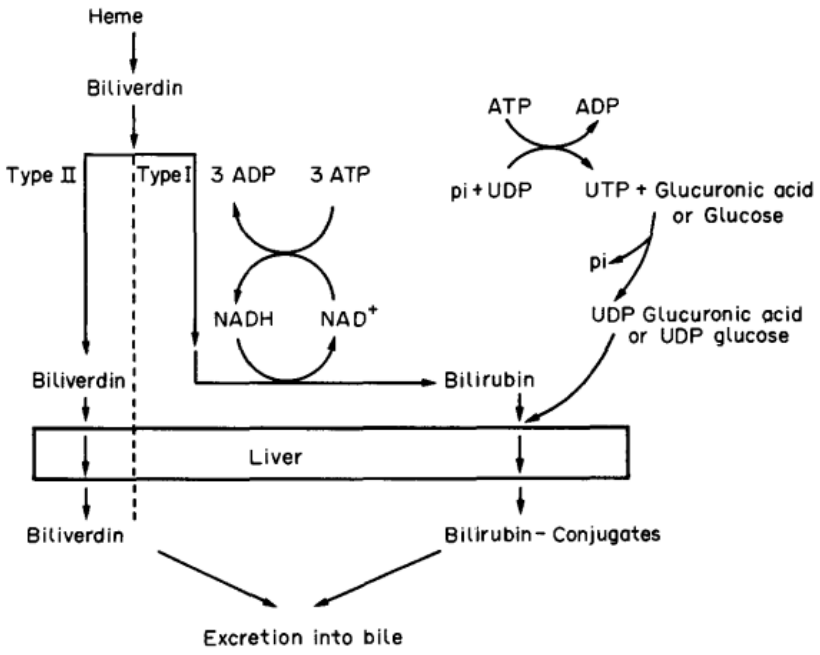

Figure 10. Pathways of heme catabolism and bile pigments production in fish. Adapted from (Fang, 1987).

In mammals, bile pigments are excreted from the liver into the bile mainly as a conjugate with glucuronic acid, but as a taurine-conjugate in fish (Sakai *et al.*, 1987; Sakai *et al.*, 1990). Ditaurobilirubin is the predominant bile pigment on the gallbladder bile of yellowtail *S. quinqueradiata* and pejerry fish. This conjugated bilirubin is also reported to occur in the gallbladder bile of red sea bream *P. major*, yellow fin pogy *Acanthopaqrus latus*, Japanese flounder *P. olivaceius* and tiger puffer *Takifugu rubripes*. The gallbladder bile of yellowtail *S. quinqueradiata* contains ditaurobilirubin, bilirubin diglucoronide, biliverdin and bilirubin monoglucuronide (Cornelius, 1991).

The gallbladder bile coloration changes gradually from yellow to dark green after a starvation period up to 8 days in eel *Anguilla japonica*, carp *Cyprinus carpio*, goldfish *Carassius carassius*, rainbow trout *O. mykiss*, Atlantic cod *G. morhua*, Atlantic salmon *S. salar*. Prolonged fasting of fish may possibly result in chemical alterations of bile pigments and changes in pigment composition if these are stored in the gallbladder for extended time periods (Fang, 1987; Cornelius, 1991; Avery *et al.*, 1992).

II. BACKGROUND

Several studies observed taurine accumulation in the whole body, liver and muscle of some fish species. A significant increase of taurine content in tissues was observed concomitantly with high taurine levels provided on the diet of juvenile Japanese flounder *P. olivaceus* (Park *et al.*, 2002; Kim *et al.*, 2003; Kim *et al.*, 2005a), European sea bass *Dicentrarchus labrax* (Brotons Martinez *et al.*, 2004), red sea bream *P. major* (Matsunari *et al.*, 2008b), rainbow trout *O. mykiss* (Yokoyama and Nakazoe, 1992) and yellowtail *S. quinqueradiata* (Matsunari *et al.*, 2005). This suggests that these species rely on dietary taurine in order to preserve their taurine body pool.

Increasing evidence indicates that taurine may be essential in fish starting from the early stages of their life cycle (larvae stage). Several studies describe the positive effect on larvae and juveniles' growth and survival when fish are fed with a taurine-supplemented diet. Accordingly, the physiological requirement for taurine may be high during these stages. The rotifers provided to fish larvae during the intensive production of marine fish contain lower taurine levels when compared to their natural prey, e.g. copepods (Yamamoto *et al.*, 2008). Some strategies have been suggested such as rotifers enriched with taurine along with its supplementation through a microdiet during the larval stage. This technological development greatly increased taurine content in rotifers and subsequently improved fish larvae growth, survival and morphological development in some species: cobia *Rachycentron canadum* (Salze *et al.*, 2011; Salze *et al.*, 2012), northern rock sole *Lepidopsetta polyxystra* (Hawkyard *et al.*, 2014) and

Senegalese sole *S. senegalensis* (Pinto *et al.*, 2010).

Although the physiological functions and processes in which taurine participate are not fully understood, increasing number of studies have shown that taurine supplementation on the diet improves growth performance, feed efficiency, feed intake of plant-based diets of several highly valued aquaculture species (Table 1).

Table 1. Taurine studies of some aquaculture species

Species	References
Japanese flounder *P. olivaceus*	Park *et al.* (2002); Kim *et al.* (2003); Kim *et al.* (2005b); Kim *et al.* (2005a); Kim *et al.* (2007); Kim *et al.* (2008b)
Summer flounder *Paralichthys dentatus*	Enterria *et al.* (2011)
Red sea bream *P. major*	Takagi *et al.* (2006b); Matsunari *et al.* (2008b); Matsunari *et al.* (2008a); Takagi *et al.* (2010; 2011)
Yellowtail *S. quinqueradiata*	Matsunari *et al.* (2005); Takagi *et al.* (2005); Takagi *et al.* (2006b); Takagi *et al.* (2008); Khaoian *et al.* (2014)
European sea bass *D. labrax*	Brotons Martinez *et al.* (2004)
Cobia *R. canadum*	Lunger *et al.* (2007); Salze *et al.* (2010); Watson *et al.* (2013); Watson *et al.* (2014)
Common dentex *Dentex dentex*	Chatzifotis *et al.* (2008)
Florida pompano *T. carolinus*	Rossi and Davis (2012)
Turbot *Scophthalmus maximus*	Qi *et al.* (2012)
Rainbow trout *O. mykiss*	Gaylord *et al.* (2006); Gaylord *et al.* (2007)
Parrot fish *Oplegnathus fasciatus*	Lim *et al.* (2013)
White seabass *Atractoscion nobilis*	Jirsa *et al.* (2014)
Sablefish *Anoplopoma fimbria*	Johnson *et al.* (2015)

Taurine supplementation intended to improve growth and feed performance

has become the most popular research topic regarding taurine in fish nutrition. Taurine has been correlated with the stimulation of the olfactory system in fish as its amino acid structure possesses the main features of a feeding stimulant (i.e. low molecular weight, the presence of nitrogen, water solubility, acid and base properties) (Carr *et al.*, 1996). Several physiological studies reported that dietary taurine supplementation impacts on the composition of conjugated-bile acids in Japanese flounder *P. olivaceus* (Kim *et al.*, 2007; Kim *et al.*, 2008b) and in Korean rockfish *Sebastes schlegeli* (Kim *et al.*, 2015). Moreover, it affects sulfur amino acids metabolism (Park *et al.*, 2002), it enhances the activity of digestive enzymes in cobia *R. canadum* larvae (Salze *et al.*, 2012), it preserves a normal morphology development of larvae (Salze *et al.*, 2011), it alters lipid metabolism and storage in Atlantic salmon *S. salar* (Espe *et al.*, 2012) and it increases skin thickness whereas scale loss is restricted in red sea bream *P. major* (Kato *et al.*, 2013). Furthermore, dietary taurine prevents erythrocyte osmotic fragility in yellowtail *S. quinqueradiata*. This indicates that its role for hemolytic suppression occurs through osmoregulation and biomembrane stabilization (Takagi *et al.*, 2006a). It has been shown that taurine supplementation induces a metabolic modulation in totoaba *T. macdonaldi* by stimulating intermediary metabolism and by reducing oxidative damage to the liver (Bañuelos-Vargas *et al.*, 2014). Taurine plays a role to alleviate green liver incidence in yellowtail *S. quinqueradiata* (Takagi *et al.*, 2008) and red sea bream *P. major* (Takagi *et al.*, 2010).

III. JUSTIFICATION

The ability to synthesize taurine *in vivo* is species-specific, therefore taurine may be classified as essential nutrient, if the body cannot make it in sufficient quantity to meet its needs, and non-essential nutrient. Fish may require taurine from the diet in the absence of endogenous production or when it is not sufficient to meet physiological needs and to achieve specific goals such as maximum growth and/or normal health. The benefits from taurine supplementation on diets based on both fish meal and plant protein, has been assessed in a variety of teleost fish. All of these studies concluded that increased taurine levels result in improved growth, survival, palatability, feed conversion and normal physiological condition when compared to the control diet that contains very low taurine levels. This suggests that taurine may be an essential nutrient.

Although many aspects regarding the role of taurine on physiology and metabolism remain to be elucidated in fish species, it is considered a limiting nutrient when replacing fishmeal. The impact of taurine deficiency on the physiological condition is different among fish species. Green liver syndrome was observed in certain marine species fed with taurine-deficient diets based on plant protein. Considering that taurine biosynthesis is variable among fish species and that most of the marine species require dietary taurine and in order to identify those physiological processes limited or blocked when taurine is lacking, it is necessary to identify the essentiality of taurine for specific species of marine fish. Expansion of totoaba aquaculture is still limited by the incomplete knowledge on totoaba nutritional requirements. It is necessary to examine if taurine deficiency is the

underlying cause of physiological abnormalities and poor growth performance in totoaba in order to develop a grow-out diet able to sustain the high growth rate of this candidate species for diversification and commercial mariculture purposes.

IV. HYPOTHESIS

1). *Totoaba macdonaldi* require taurine on its diet as this nutrient is involved on the production of bile salts that improve lipid digestion and metabolism.

2). Green liver is an indicator of taurine deficiency in *Totoaba macdonaldi* and this abnormal physiological condition results from impairments of bile pigment metabolism caused by taurine deficiency.

V. OBJECTIVE

The main objective of this study was to evaluate dietary taurine essentiality in juvenile *Totoaba macdonaldi* in order to improve the current knowledge of this nutrient emphasizing its role on fish physiology and metabolism. To achieve this goal, the following specific objectives were established:

1. To define taurine requirements for juvenile *Totoaba macdonaldi* in a washed fishmeal-based diet.
2. To assess physiological changes occuring in juvenile *Totoaba macdonaldi* derived from taurine deficiency.

VI. MATERIALS AND METHODS

1. Experimental diet

Eight experimental diets were formulated to be isoproteic (50%), isolipidic (12%) and isoenergetic (20 kJ g^{-1}) and to contain increasing taurine levels by using washed fishmeal as the primary protein source (Table 2). Taurine was removed from fishmeal by washing it with 70% ethanol before its use for diet formulation. A basal diet was used as a control (unsupplemented taurine) and another 7 diets were supplemented with increasing taurine levels at the expense of cellulose. Taurine concentrations on the eight experimental diets were 0.23 (basal diet), 0.45, 0.91, 1.28, 1.76, 2.20, 2.72 and 3.01 (% as-is). As the fishmeal washing treatment may deplete other soluble molecules, attractants (2%) were added to the diets. Experimental diets were prepared at the Fish Nutrition Laboratory of FCM at the UABC. All dry ingredients including taurine were thoroughly mixed in a food mixer (Kitchenaid) for 10 min. Then, fish oil was slowly added to the dry ingredients and mixed for another 5 min. Boiling water was added in order to obtain the dough used for pelleting. The latter process was carried out by passing the dough through a 3 mm die in a meat grinder (Kitchenaid). Pellets were dried in an oven at 65°C for 24 h. Dry diets (Figure 11) were packed and stored at –20°C until further use.

Table 2. Formulation and chemical composition of experimental diets with different levels of taurine

Ingredients (% as-is)	**T-0.23**	**T-0.45**	**T-0.91**	**T-1.28**	**T-1.76**	**T-2.20**	**T-2.72**	**T-3.01**
Fishmeal [a]	65.6	65.6	65.6	65.6	65.6	65.6	65.6	65.6
Gelatin	4.0	3.7	3.4	3.1	2.8	2.4	2.1	1.8
Starch	10.5	10.5	10.5	10.5	10.5	10.5	10.5	10.5
Fish hydrolysate [b]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Fish oil	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Mineral mix [c]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Vitamin mix [d]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Binder [e]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Choline chloride	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Vitamin C	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Vitamin E	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Cellulose	5.0	4.9	4.8	4.6	4.5	4.4	4.3	4.1
Taurine	0.0	0.22	0.68	1.05	1.53	1.97	2.49	2.78
Marker (silica)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Chemical analysis (%)								
Protein	51.2	50.0	50.1	50.4	50.6	49.9	50.6	50.9
Lipid	12.4	11.5	11.7	11.6	11.4	11.8	11.8	11.3
Ash	11.4	13.5	14.2	14.0	14.5	13.7	13.3	13.5
Moisture	3.3	2.4	2.1	1.4	2.0	2.3	2.2	1.5
Taurine [f]	**0.23**	**0.45**	**0.91**	**1.28**	**1.76**	**2.20**	**2.72**	**3.01**
Gross Energy (kJ g^{-1}) [g]	20.7	20.2	20.2	20.4	20.1	20.3	20.4	20.4
P/E (mg kJ^{-1})	24.7	24.7	24.8	24.7	25.1	24.6	24.8	25.0

[a] Washed fishmeal Rangen (protein: 68%, lipid: 11%, ash: 14%); [b] Soluble fish protein concentrate CPSP (protein: 70%, lipid: 22%, ash: 6%, taurine: 0.33%); [c] Mineral mix (g kg^{-1}): KH_2PO_4, 320; NaH_2PO_4, 250; $Ca(H_2PO_4)_2$, 200; $MgSO_4.7H_2O$, 150; $C_6H_{10}CaO_6$, 35; $C_6H_5FeO_7$, 25; NaCl, 10; $ZnSO_4.7H_2O$, 3.53; $MnSO_4.H_2O$, 1.62; $CuSO_4.5H_2O$, 0.31; $CoCl_2.6H_2O$, 0.01; [d] Vitamin mix (mg kg^{-1}): inositol, 256.39; niacin, 51.28; riboflavin; ρ-aminobenzoic acid, 25.53; pantothenic acid, 17.92; β-carotene, 9.39; menadione, 6.11; thiamine, 3.85; pyridoxine, 3.06; folic acid, 0.96; biotin, 0.39; cholecalciferol, 25793 IU; cyanocobalamin, 5.59; choline chloride, 20; [e] Nutrikelp Albiomar (protein: 2%, lipid: 0.1%, fiber: 1%, ash: 4%); [b,f] Analyzed at University of Missouri, Agricultural Experiment Station Chemical Laboratories; [g] Gross energy (kJ g^{-1}) calculated based on protein, lipid and carbohydrate at 23.6, 39.5, 17.2 kJ g^{-1}, respectively.

Figure 11. Sample of experimental diet

2. Feeding trial

The experiment was carried out in the Marine Fish Hatchery of FCM at UABC (Figure 12). The experimental units consisted of 24 rectangular fiberglass tanks of 100 L capacity supported by continuous recirculated seawater, bead biofilter, UV disinfection system and a 3000 L reservoir tank. Temperature was controlled with a chiller and aeration was supplied to each tank with an airstone connected to a regenerative blower. The photoperiod of 12 h light: 12 h dark was maintained. Twenty juvenile totoaba (10.0 ± 1.0 g) were randomly distributed in each experimental tank (8 treatments, n=3). Totoaba were acclimatized for two weeks in the experimental units and were fed a commercial diet during this period. The experiment was performed in triplicate and Diet 1 (T-0.23) was used as basal diet. Fish were fed with the experimental diets to visual satiety twice a day at 08:00 and 16:00 h for ten weeks. Temperature and dissolved oxygen were monitored daily. Water quality parameters such as total ammonia nitrogen, nitrite and nitrate were measured three times a week for 10 weeks. During the feeding period, water

temperature was 23 ± 1°C, dissolved oxygen was 6.0 ± 1.0 mg/L, salinity was 35 ± 0.5 g/L, pH was 7.6 ± 0.1, total ammonia nitrogen was <0.25 mg/L, nitrite was <0.5 mg/L and nitrate was <40 mg/L.

Figure 12. Rearing facility and experimental unit

3. Sampling

At the beginning and at the termination of the experiment, fish were measured (total length) and weighed (g) using an Ictiometer and electronic balance (Ohaus PAJ4102). At the end of the trial, blood samples were collected from 8 fish from each tank previously anesthetized with a solution of clove oil (10%) diluted with 70% ethanol. Blood was collected via heart with 1 ml syringes pre-filled with 0.1 mL EDTA as anticoagulant. Hematocrit and hemoglobin were measured immediately after sample collection. Plasma was obtained after centrifugation at 12,000 rpm for 5 min at 4°C (Eppendorf 5415R) for analysis of total protein, albumin and glucose. The remaining plasma was stored at -80°C for subsequent analysis of total cholesterol, triglycerides and total bilirubin. Those fish were euthanized and then dissected to obtain liver, gallbladder and viscera (stomach, pyloric caeca and intestine). For glycogen analysis, different fish were fed 6 h

before they were sacrificed with a quick blow to the head. The liver was frozen on dry ice immediately and stored at -80°C.

4. Growth evaluation

Metrics of growth and tissue replacement were calculated according to the following equations:

$$WG = FBW - IBW$$

$$PWG = \frac{WG}{IBW} \times 100$$

$$CF = \frac{100 \times FW}{FL^3}$$

$$TGC = \frac{\left(FBW^{1/3} - IBW^{1/3}\right)}{\sum_d T} \times 100$$

where: WG: weight gain (g); FBW: final body weight (g); IBW: initial body weight (g); PWG: percent weight gain (%); CF: condition factor; FW: fish weight (g); FL: fish total length (cm); TGC: thermal-unit growth coefficient; T: temperature (°C); d: experiment duration (days).

5. Analysis of green liver syndrome

Occurrence of green liver in fish liver was evaluated with respect to different levels of dietary taurine by direct observation of freshly dissected liver in fish from each dietary treatment.

6. Biological parameters

Somatic indices were obtained by calculating hepatosomatic index, viscerosomatic index, and gallbladdersomatic index.

$$HSI = \frac{LW}{FW} \text{ x } 100$$

$$VSI = \frac{VW}{FW} \text{ x } 100$$

$$GBSI = \frac{GBW}{FW} \text{ x } 100$$

where: HSI: hepatosomatic index (%); LW: liver weight (g); FW: fish weight (g); VSI: viscerosomatic index (%); VW: viscera weight (g); GBSI: gallbladdersomatic index (%); GBW: gallbladder weight (g).

7. Feed evaluation

Feed efficiency and nutrient utilization efficiency were calculated according to the following equations:

$$FE = \frac{WG}{FI}$$

$$PER = \frac{WG}{PI}$$

$$LER = \frac{WG}{LI}$$

$$PR = (FBW \text{ x } FBP) - (IBW \text{ x } IBP)$$

$$LR = (FBW \text{ x } FBL) - (IBW \text{ x } IBL)$$

$$PRE = \frac{PR}{PI} \text{ x } 100$$

$$LRE = \frac{LR}{LI} \text{ x } 100$$

where: FE: feed efficiency (%); FI: feed intake (g); PER: protein efficiency ratio; PI: protein intake (g); LER: lipid efficiency ratio; LI: lipid intake (g); PR: protein retention (g); FBP: final body protein (%); IBP: initial body protein (%); LR: lipid retention (g); FBL: final body lipid (%); IBL: initial body lipid (%); PRE: protein retention efficiency (%); LRE: lipid retention efficiency (%).

8. Chemical analysis

Proximate analyses of the experimental diets, muscle, whole fish, viscera and liver were performed according to AOAC (2000). Micro-kjeldahl digestion (Labconco) using copper catalyst for crude protein analysis was performed according to AOAC method 984.13. Lipid analysis was performed according to a modified Folch method using dichloromethane-methanol (2:1) (Cequier-Sánchez *et al*., 2008). Moisture was determined by drying at 105°C for 15 h. Ash was obtained from incineration samples in a muffle (Barnstad Thermolyne) at 550°C for 6 h. Taurine content of diets was analyzed using cation-exchange chromatography (cIEC-HPLC) coupled with post-column ninhydrin derivatization and quantitation by Agricultural Experiment Station Chemical Laboratories (ESCL), University of Missouri, USA.

9. Hematological and blood biochemistry

Hematological analyses were conducted to determine levels of hematocrit, hemoglobin, total protein, glucose, albumin, total cholesterol, triglyceride and total bilirubin. For hematocrit analysis (%) a microhematocrit method was used by centrifuging capillary at 10,000 rpm for 10 min (Clinical VWR 200). Subsequently, the capillary was placed in a hematocrit reader for determining packed cell volume percentage. Hemoglobin concentration was proportional to the color intensity of cyanmethemoglobin formation due to hemoglobin oxidation by potassium ferricyanide, which was measured at 540 nm (Pointe Scientific H7504). Total protein concentration was proportional to the color reaction of protein molecules with cupric ions which is known as Biuret reaction and the absorbance was read at 540 nm (Pointe Scientific T7528). Glucose concentration was proportional to the color intensity of a quinoneimine dye formed by series of reactions catalyzed by glucose oxidase and peroxidase, which was read at 500 nm (Pointe Scientific G7521). Albumin concentration was measured by a bromocresol green (BCG) dye-binding procedure which was read at 630 nm (Pointe Scientific A7502). The absorbance of hemoglobin, total protein, glucose and albumin was read in a spectrophotometer (HACH DR5000). Total cholesterol concentration was proportional to the color intensity which was read at 500 nm after series of reactions catalyzed by cholesterol esterase, cholesterol oxidase and peroxidase (Pointe Scientific C7510). Triglycerides concentration was proportional to the color intensity of a quinonimine dye by series of reactions catalyzed by lipase, glycerol kinase, glycerol phosphate oxidase and peroxidase, which absorbs at 500 nm

(Pointe Scientific T7532). Total bilirubin concentration was proportional to the color intensity of azobilirubin formed after bilirubin couples with diazotized sulfanilic acid (diazo), which was measured at 555 nm (Pointe Scientific B7576). The absorbance of total cholesterol, triglycerides and total bilirubin was read in a microplate reader (Thermoscientific MultiskanGO).

10. Glycogen determination in liver

Liver glycogen content was determined following the method reported by Plummer (1987). Frozen liver samples were weighed and homogenized with 2 mL potassium hydroxide 30%. The samples were then placed in a 100°C water bath for 20 min, stirring every 5 min. They were then placed in an ice water bath and 0.2 mL saturated sodium sulfate and 5 mL ethanol 95% were added. After vortexing the samples, they were let stand for 5 min. The homogenates were then centrifuged at 2,000 g for 10 min to obtain a pellet, which was resuspended in 5 mL distilled water. At room temperature, samples were brought up to 10 mL, and 1 mL aliquot were transferred and mixed with 1 mL of 1.2 M HCl. Samples were placed in a water bath at 100°C for 2 h again and were brought to pH 7 using 0.5 M NaOH and phenol red as an indicator. Finally, the released glucose content from glycogen was quantified according to the color intensity of a quinoneimine dye formed by series of reactions catalyzed by glucose oxidase and peroxidase, which was read at 500 nm (Pointe Scientific G7521) using microplate reader (Thermoscientific MultiskanGO).

11. Fecal collection and apparent digestibility coefficients

To minimize nutrient leaching in our study, feces were removed by siphoning after feeding. Feces were collected by an appropriate mesh screen and pooled by tank. Fecal samples were rinsed with distilled water to remove salt and stored at –20°C for subsequent analysis. Feces collections were terminated after 3 weeks, when approximately 5 g of dry weight feces were obtained from each tank.

The indirect method was used to calculate the apparent digestibility coefficients (ADC) of dry matter and nutrient, with acid-insoluble ash (AIA) as the internal marker (Goddard and McLean, 2001). AIA content of all experimental diets and fecal samples was analyzed to determine apparent digestibility coefficients of dry matter and nutrient using the following equations:

$$ADC_{DM} = 100 - \left(\frac{AIA_d}{AIA_f} x100 \right)$$

$$ADC_N = 100 - \left(\frac{N_f}{N_d} x \frac{AIA_d}{AIA_f} x100 \right)$$

where: ADC_{DM}: apparent digestibility coefficients of dry matter (%); AIA_d: acid-insoluble ash in diet; AIA_f: acid-insoluble ash in feces; ADC_N: apparent digestibility coefficients of nutrient (%); N_f: nutrient (protein or lipid) in feces (%); N_d: nutrient in diet (%).

VII. Statistical Comparison of Nutritional Response Models

All experimental diets were assigned by a completely randomized design.

The relationship between response parameters and dietary taurine levels as the independent variable were examined by regression analysis. Data were evaluated using five regression models, including three linear models (linear, logarithmic and quadratic) and two nonlinear models (4- and 5-parameter saturation kinetic models).

1. Linear models

The commonly used models for dietary evaluation are the linear model or straight-line equation, the logarithmic equation and the quadratic equation (Belal, 2005).

1.1 Straight-line or linear

The straight-line model is valuable in describing a linear range of responses to nutritional intake. The linear model equation is:

$$r = \beta_0 + \beta_1 X$$

where r: response; β_0: intercept (the response at zero intake level); β_1: slope of line; X: dietary taurine level.

1.2 Logarithmic

Over a wide range of nutrient intake levels, the growth response is not linear. Therefore, the logarithmic form of the linear equation is used to describe the curvilinear relationship over a wide range of nutrient intake levels. The equation of the logarithmic model is:

$$r = \beta_0 + \beta_1 \log X$$

where r: response; β_0: intercept; β_1: slope of line; log X: logarithm value of dietary taurine level.

1.3 Quadratic

A second-order polynomial or quadratic can fit well the increase and decrease in performance, but not the plateau (Pesti *et al.*, 2009). The quadratic model equation is:

$$r = \beta_0 + \beta_1 X + \beta_2 X^2$$

where r: response; β_0: intercept; β_1: regression coefficient for linear term; β_2: regression coefficient for quadratic term; X: dietary taurine level.

2. Nonlinear models

Nonlinear regression is useful when response parameters are not linear or cannot be linearized by transformation. Besides, nonlinear models are more meaningful than polynomial models. Biological systems rarely fit linear patterns, therefore nonlinear models are applied to describe more accurately biological responses than other models that force responses to match linear functions (Pesti *et al.*, 2009). Physiological responses to nutrients are graded and can be measured on a continuous scale. The main characteristic of nutrient-response curves is that they increase to a point and then reach a plateau, so a model which considers these observations is required to describe physiological response to graded dietary nutrient. The derivation of suitable model allows fish nutritionists to characterize

nutrients-responses relationship with numerical measures.

Saturation kinetic model (SKM) has been proposed for the characterization of nutrient-response relationships as an improvement and to overcome some limitations of linear model. SKM is developed based on enzyme kinetics concept including rate limiting and saturation steps (Mercer *et al*., 1989). The model is descriptive of a wide range of physiological responses considering various sections of SKM curve as follows (Figure 13):

a) *Threshold*: the lower range of nutrient level that produces zero or negligible response.
b) *Deficient*: the range where the slope of response increases. Maximum slope and efficiency occur in this portion.
c) *Adequate*: the section in which the slope goes from positive toward zero (a plateau has zero slope).
d) *Optimal*: the range of maximal response as indicated by plateau section.
e) *Inhibited (toxic)*: the range where response is diminished.

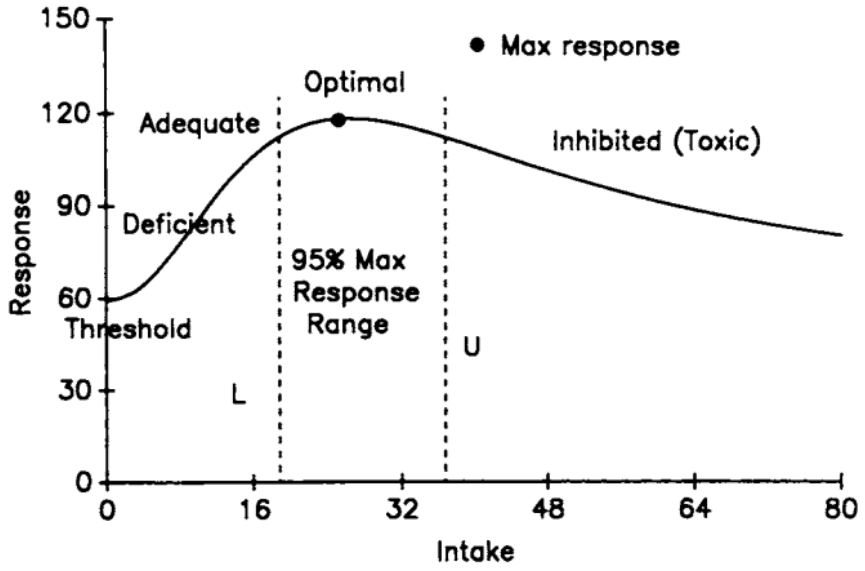

Figure 13. Nutrient-response curve following Saturation Kinetics Model (SKM). Adapted from Mercer *et al.* (1989).

4- and 5- SKM give a theoretical, mathematical and biological basis of nutrient-response relationships (Figure 14).

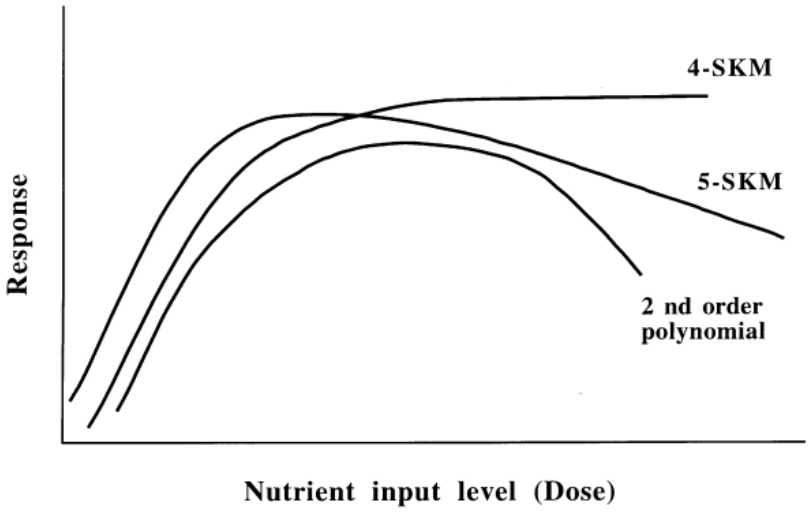

Figure 14. Nutrient-response curve of 4-SKM, 5-SKM and second order polynomial. Adapted from Shearer (2000).

2.1 4-SKM

4-SKM is parsimonious model which defines simple model with a minimum predictor variables but accomplishes great explanatory predictive power. This model follows sigmoid-shaped curve which reaches a plateau. Four derived parameters (b, n, $K_{0.5}$, R_{max}) are calculated to generate a theoretical response curve (Mercer *et al.*, 1989). The equation of this model is:

$$r = \frac{b(K_{0.5})^n + R_{max} I^n}{(K_{0.5})^n + I^n}$$

where r: response; b: intercept on the response axis; $K_{0.5}$: taurine level for half-maximum response; n: kinetic order; R_{max}: maximum response; I: dietary taurine level.

2.2 5-SKM

At higher nutrient levels, responses may become diminished from plateau phase. The inhibited section of the nutrient-response curve can be predicted by integrating an inhibition constant (Ks) to the SKM equation. 5-SKM produces an asymmetric curve which describes all regions of the nutrient-response curves including threshold, increase, plateau and decline response areas. Five derived parameters (b, n, $K_{0.5}$, R_{max}, Ks) are calculated to generate a theoretical response curve (Mercer, 1992). The equation used for this model is:

$$r = \frac{b(K_{0.5})^n + R_{max} I^n + bI^{2n}/(Ks)^n}{(K_{0.5})^n + I^n + I^{2n}/(Ks)^n}$$

where r: response; b: intercept on the response axis; $K_{0.5}$: taurine level for half

maximum response; n: kinetic order; R_{max}: maximum response; I: dietary taurine level; Ks: inhibition constant.

Subsequently, accuracy of fitting nonlinear models are applied and the curves were fitted using NLIN Procedure of SAS. Computational test for convergence with iterative method for nonlinear model is applied and good initial parameter estimates are required to obtain final model parameters for each measured response.

3. Model selection

The choice of a statistical model is critical for interpreting the relationship between nutrient intake and nutrient response. The selected model should have two principle characteristics: statistical significance and biological significance. Mathematical significance explains response variations as a function over wide ranges of nutrient intake, while biological significance has a theoretical basis in biological structure and function (Mercer, 1992). In this study, model selection was performed as described in (Salze *et al.*, 2017a) using the Akaike's Information Criterion (AIC) (Anderson *et al.*, 2000; Salze *et al.*, 2017b). All regression models were compared by considering the coefficient of determination statistics (R^2), AIC and sum of square error (SSE). The model with minimum SSE and AIC is the best overall fit to the data. A significance level of $P<0.05$ was used. Quadratic regression was chosen over a linear model when the P-value of the quadratic term was significant. All statistical analyses were performed using SAS University Edition (SAS Institute Inc., Cary, NC, USA).

VIII. RESULTS

1. Growth, morphological index and green liver occurrence

Growth performance was found to be affected by taurine inclusion levels as demonstrated by WG and TGC ($P<0.001$, Table 3). WG was higher (82.1 g) in fish fed dietary taurine at 0.45% (T-0.45) than those fed with other experimental diets, and the lowest growth (74.8 g) was observed in fish fed the basal diet (T-0.23). PWG ranged from 714-787% with corresponding TGC of 0.146-0.155. Figure 15 shows the relationship between final weight vs dietary taurine and TGC vs dietary taurine. TGC was best modeled by 5-SKM with marginal differences as indicated by low R^2 (0.13).

HSI and CF were not affected by varying taurine levels in totoaba ($P>0.05$). The experimental diets significantly affected other somatic parameters such as VSI and GBSI of totoaba ($P<0.05$, Table 3). VSI decreased quadratically ($R^2=0.31$, $P=0.019$). GBSI was best modeled by 4-SKM ($R^2=0.72$, $P<0.001$). The lowest GBSI value (0.095%) was observed for the group fed the basal diet (T-0.23) when compared to groups fed taurine supplemented diets, then plateaued at the taurine levels above 0.45% (T-0.45). GBSI attained the highest value (0.155%) for the group fed dietary taurine at 2.20% (T-2.20). Some physiological abnormalities have been found in this study. The appearance of green liver (Figure 16) was observed in fish fed the basal diet (T-0.23) after 10 weeks of feeding trial. Furthermore, darkening green bile also occurred in totoaba with green liver (Figure 17).

Table 3. Growth performance, somatic parameters and green liver occurrence of *Totoaba macdonaldi* fed diets containing different levels of taurine

Taurine (%)	T-0.23	T-0.45	T-0.91	T-1.28	T-1.76	T-2.20	T-2.72	T-3.01	PSE	Model	R^2	P
WG (g)	74.8	82.1	78.4	77.1	77.0	77.2	75.9	76.9	0.633	5-SKM	0.15	<0.001
PWG (%)	714	787	745	733	739	734	724	733	5.996	5-SKM	0.15	<0.001
TGC	0.146	0.155	0.150	0.149	0.148	0.149	0.148	0.148	0.001	5-SKM	0.13	<0.001
CF	1.06	1.11	1.05	1.06	1.08	1.08	1.10	1.10	0.009	Linear	0.05	NS
HSI (%)	1.75	1.61	1.58	1.48	1.42	2.00	1.61	1.86	0.040	Linear	0.06	NS
VSI (%)	2.35	2.07	2.18	2.07	2.17	2.15	2.14	2.43	0.025	Quadratic	0.31	0.019
GBSI (%)	0.095	0.144	0.147	0.135	0.139	0.155	0.144	0.145	0.002	4-SKM	0.72	<0.001
Green liver	+	–	–	–	–	–	–	–				

Linear, logarithmic, quadratic, 4-SKM and 5-SKM regression analyses were used for each parameter, then the best model regression was chosen according to methods described in the text. Statistically significant ($P<0.05$).

WG: weight gain, PWG: percent weight gain, TGC: thermal-unit growth coefficient, CF: condition factor, HSI: hepatosomatic index, VSI: viscerosomatic index, GBSI: gallbladdersomatic index; NS: not significant at the $\alpha=0.05$ level.

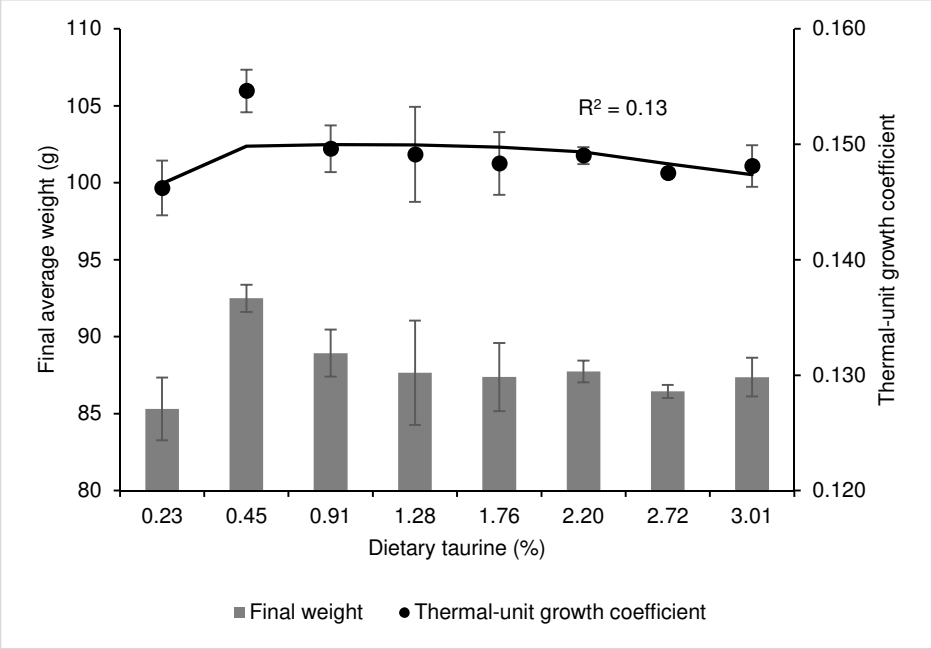

Figure 15. Final weight and TGC of *Totoaba macdonaldi* fed different taurine levels. TGC was best modeled by 5-parameter saturation kinetic model (5-SKM) (R^2=0.13, P<0.001). Model parameter estimates of TGC (I=0, $K_{0.5}$=0.10, R_{max}=0.15, n=4.44, K_s=7.44).

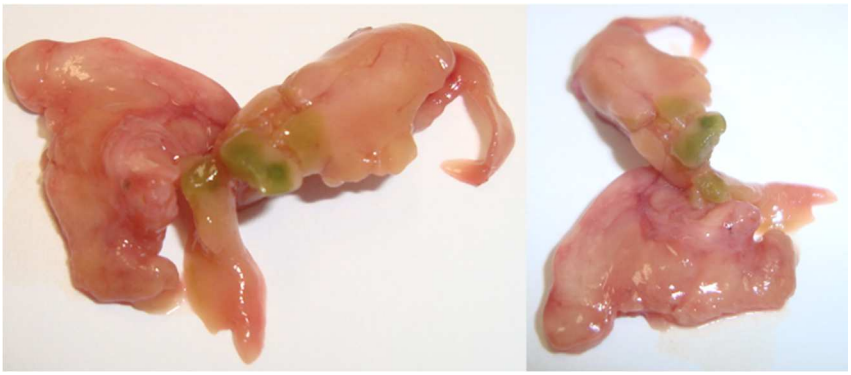

Figure 16. Occurrence of green liver in *Totoaba macdonaldi* fed taurine unsupplemented diet (T-0.23) after 10 weeks.

Figure 17. Dark green bile of *Totoaba macdonaldi* fed basal diet (T-0.23) (left side) and normal light yellow bile of *Totoaba macdonaldi* fed taurine supplemented diet (right side).

2. Nutrient utilization

Graded levels of dietary taurine were linearly correlated with FE (R^2=0.26; P=0.011) and PER (R^2=0.26, P=0.012, Table 4). Fish fed dietary taurine (T-0.23, T-0.45, T-0.91) had the highest FE (1.17). The highest PER (2.34) was recorded for the groups fed dietary taurine (T-0.45, T-0.91). PRE was fitted by 5-SKM (R^2=0.45, P=0.019). Increased PRE was noted between the basal diet (T-0.23) and diet 2 (T-0.45), and highest PRE (40.6%) was recorded on fish fed dietary taurine at 0.45% (T-0.45). Increasing levels of taurine did not influence FI, LER, PR, LR, LRE (P>0.05).

Table 4. Feed utilization of *Totoaba macdonaldi* fed diets containing different levels of taurine

Taurine (%)	T-0.23	T-0.45	T-0.91	T-1.28	T-1.76	T-2.20	T-2.72	T-3.01	PSE	Model	R^2	P
FI (g/fish)	64.1	70.1	67.0	66.9	66.9	68.3	67.1	67.6	0.416	Linear	0.02	NS
FE	1.17	1.17	1.17	1.15	1.15	1.13	1.13	1.14	0.006	Linear	0.26	0.011
PER (%)	2.28	2.34	2.34	2.29	2.27	2.27	2.24	2.23	0.012	Linear	0.26	0.012
LER (%)	9.40	10.22	10.02	9.93	10.06	9.62	9.62	10.07	0.051	Linear	<0.01	NS
PR (g)	10.4	14.3	12.2	12.0	10.0	10.2	10.7	10.2	0.365	Linear	0.15	NS
LR (g)	2.8	3.9	3.2	3.5	2.5	2.8	3.0	2.9	0.112	Linear	0.08	NS
PRE (%)	31.6	40.6	36.5	35.6	29.4	29.8	31.7	29.4	0.950	5-SKM	0.45	0.019
LRE (%)	34.9	48.0	41.3	44.4	32.5	34.6	38.5	37.2	1.320	Linear	0.07	NS

Linear, logarithmic, quadratic, 4-SKM and 5-SKM regression analyses were used for each parameter, then the best model regression was chosen according to methods described in the text. Statistically significant ($P<0.05$).

FI: feed intake, FE: feed efficiency, PER: protein efficiency ratio, LER: lipid efficiency ratio, PR: protein retention, LR: lipid retention, PRE: protein retention efficiency, LRE: lipid retention efficiency; NS: not significant at the $\alpha=0.05$ level.

3. Tissue proximate composition

Whole body, muscle, viscera and liver compositions of fish fed varying levels of dietary taurine are presented in Table 5. An absence of significant differences with the addition of dietary taurine was observed in whole body protein, lipid, ash and moisture; muscle protein, lipid, ash and moisture; liver protein, lipid, ash, moisture and liver glycogen; as well as viscera protein ($P>0.05$). However, significant differences among dietary treatments were recorded in viscera ash and lipid. Viscera ash decreased quadratically ($R^2=0.36$, $P=0.009$) and viscera lipid was best fitted by 4-SKM ($R^2=0.37$, $P=0.008$) in response to dietary taurine concentrations. The lowest viscera lipid content (3.7%) was found in fish fed the basal diet (T-0.23). Plateau response of viscera lipid was observed at the dietary taurine levels of more than 0.45% (T-0.45).

Table 5. Tissue proximate composition (wet weight) and liver glycogen content of *Totoaba macdonaldi* fed graded taurine levels

Taurine (%)	T-0.23	T-0.45	T-0.91	T-1.28	T-1.76	T-2.20	T-2.72	T-3.01	PSE	Model	R^2	P
Wh. body %												
WB protein	16.8	17.2	17.5	17.2	17.1	17.2	16.9	16.8	0.108	Linear	0.02	NS
WB lipid	4.3	4.5	4.4	4.8	4.1	4.4	4.6	4.5	0.076	Linear	0.00	NS
WB ash	3.7	3.6	3.7	3.5	3.7	3.7	3.7	3.6	0.028	Linear	<0.01	NS
WB moisture	73.6	72.9	73.1	72.8	73.7	73.1	72.8	72.7	0.120	Linear	0.07	NS
Muscle %												
M protein	18.5	19.4	19.2	18.9	18.9	19.3	19.6	18.8	0.107	Linear	0.03	NS
M lipid	1.6	1.9	1.9	2.1	1.7	2.3	1.9	1.9	0.061	Linear	0.04	NS
M ash	1.3	1.3	1.3	1.2	1.3	1.3	1.4	1.3	0.010	Linear	0.07	NS
M moisture	77.2	76.2	76.2	76.4	76.7	76.0	76.4	76.8	0.114	Linear	0.01	NS
Liver %												
L protein	10.2	10.4	11.6	11.6	11.1	11.8	11.4	10.4	0.385	Linear	0.01	NS
L lipid	27.9	36.6	31.4	30.0	36.1	29.6	32.6	31.3	0.551	Linear	<0.01	NS
L ash	1.1	1.1	1.2	1.1	1.1	1.2	1.2	1.1	0.041	Linear	0.01	NS
L moisture	55.0	48.7	52.8	53.3	48.6	55.1	52.6	53.3	0.617	Linear	0.01	NS
GLG	4.69	5.30	5.21	4.97	4.86	5.12	4.93	4.65	0.125	Linear	0.02	NS
Viscera %												
V protein	13.3	14.1	13.9	14.5	14.0	14.0	14.0	14.6	0.150	Linear	0.09	NS
V lipid	3.7	6.4	5.2	5.4	6.3	6.1	4.9	5.7	0.143	4-SKM	0.37	0.008
V ash	1.2	1.1	1.2	1.2	1.1	1.2	1.2	1.3	0.015	Quadratic	0.36	0.009
V moisture	79.3	76.2	77.6	76.7	76.2	75.9	77.3	76.5	0.240	Logarithmic	0.20	0.029

Linear, logarithmic, quadratic, 4-SKM and 5-SKM regression analyses were used for each parameter, then the best model regression was chosen according to methods described in the text. Statistically significant ($P<0.05$). NS: not significant at the $\alpha=0.05$ level. GLG: liver glycogen (g/100 g tissue).

4. Hematological and blood biochemical parameters

Hematocrit and hemoglobin concentrations were best modeled using 5-SKM ($P<0.001$) with a slight difference indicated by low R^2 for hematocrit ($R^2=0.05$) and hemoglobin ($R^2=0.18$) (Table 6). Bilirubin increased linearly ($R^2=0.58$, $P<0.001$) with the lowest value (0.108 mg/dL) found in fish fed the basal diet (T-0.23). The relationship between hematocrit, hemoglobin and plasma bilirubin as a part of bile pigment metabolism with increased taurine levels is shown in Figure 18. Plasma total cholesterol level in totoaba increased linearly ($R^2=0.75$, $P<0.001$) as dietary taurine increased with the lowest level (82.3 mg/dL) in the basal diet (T-0.23). Plasma triglyceride of totoaba increased significantly with dietary taurine and followed a quadratic pattern ($R^2=0.53$, $P<0.001$) with the lowest concentration (50.7 mg/dL) in the basal diet (T-0.23) and highest value when fish were fed dietary taurine 2.20% (T-2.20). Using 5-SKM, significant differences with low R^2 were observed for plasma glucose ($R^2=0.07$, $P<0.001$). Plasma total protein and albumin were not significantly different between the dietary treatments ($P>0.05$). The correlation between dietary taurine levels and plasma lipid metabolism as well as visceral fat is shown in Figure 19.

Table 6. Hematological parameters of *Totoaba macdonaldi* fed graded taurine levels.

Taurine (%)	T-0.23	T-0.45	T-0.91	T-1.28	T-1.76	T-2.20	T-2.72	T-3.01	PSE	Model	R^2	P
HT (%)	19.00	19.95	18.85	19.29	19.96	19.09	19.31	18.99	0.182	5-SKM	0.05	<0.001
HB (g/dL)	4.78	5.08	5.37	5.22	5.48	4.96	5.16	5.25	0.080	5-SKM	0.18	<0.001
BR (mg/dL)	0.108	0.189	0.195	0.125	0.193	0.245	0.243	0.242	0.003	Linear	0.58	<0.001
GLU (mg/dL)	47.75	59.85	42.09	57.37	52.26	46.21	55.21	43.72	1.695	5-SKM	0.07	<0.001
TP (g/dL)	2.54	2.61	2.62	2.57	2.65	2.68	2.59	2.69	0.027	Linear	0.07	NS
ALB (g/dL)	1.33	0.91	1.14	1.20	0.98	1.25	1.30	0.97	0.036	Linear	<0.01	NS
CHO (mg/dL)	82.3	84.9	93.4	100.3	98.0	106.3	107.5	111.3	1.221	Linear	0.75	<0.001
TG (mg/dL)	50.7	67.9	73.8	72.3	84.2	101.4	79.4	75.0	2.170	Quadratic	0.53	<0.001

Linear, logarithmic, quadratic, 4-SKM and 5-SKM regression analyses were used for each parameter, then the best model regression was chosen according to methods described in the text. Statistically significant ($P<0.05$). NS: not significant at the $\alpha=0.05$ level.
HT: hematocrit, HB: hemoglobin, BR: total bilirubin, GLU: glucose, TP: total protein, ALB: albumin, CHO: total cholesterol, TG: triglycerides.

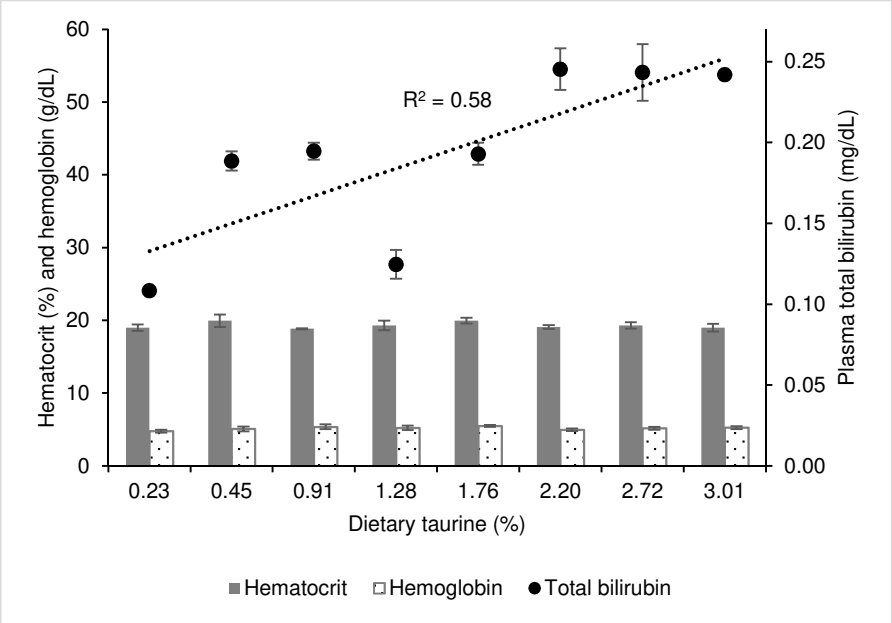

Figure 18. Plasma total bilirubin (R^2=0.58, P<0.001), hematocrit and hemoglobin of *Totoaba macdonaldi* fed graded levels of taurine.

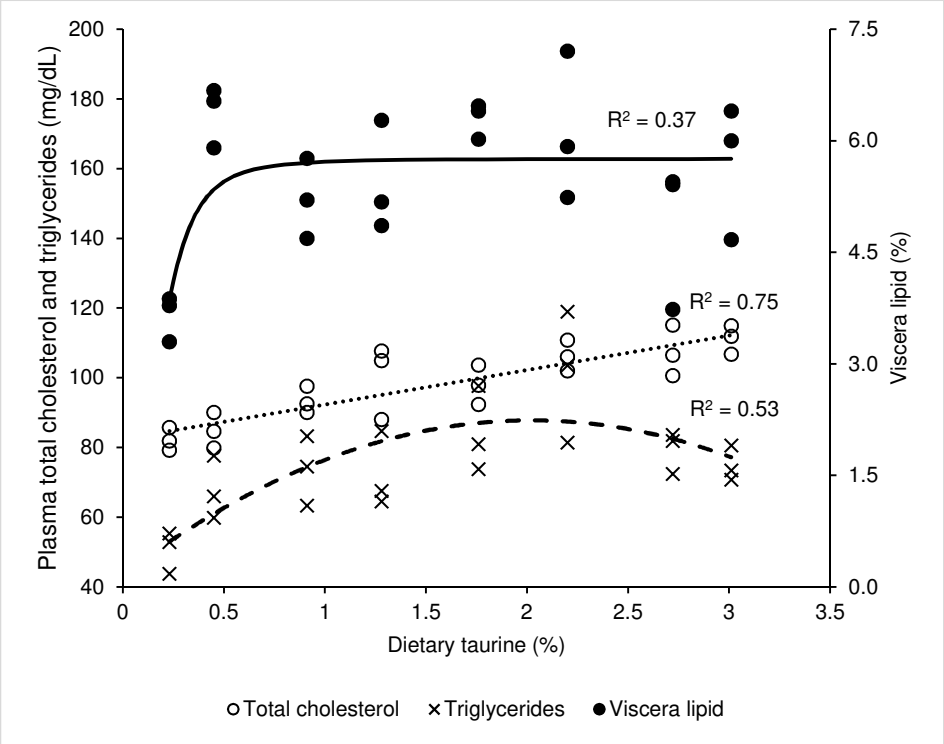

Figure 19. Relationship between dietary taurine vs plasma total cholesterol (R^2=0.75, P<0.001), dietary taurine vs plasma triglycerides (R^2=0.53, P<0.001) and dietary taurine vs viscera lipid of *Totoaba macdonaldi*. Viscera lipid was best fitted by 4-parameter saturation kinetic model (4-SKM) (R^2=0.37, P=0.008). Model

parameter estimates of viscera lipid (I=1.85, $K_{0.5}$=0.01, R_{max}=5.76, n=3.09).

5. Apparent dry matter, protein and lipid digestibility

The results of ADC of dry matter, protein and lipid were all affected by experimental diets (Table 7). ADC of protein was best fitted with 5-SKM (R^2=0.63, P<0.001), with the value between 89–92%. ADC of dry matter decreased quadratically (R^2=0.50), with the highest level (65.1%) noted in the basal diet (T-0.23). ADC of lipid was best modeled by 4-SKM (R^2=0.87, P<0.001). The basal diet (T-0.23) was found to have the lowest ADC of lipid (78.9%) and the highest (92.6%) was noted when fish fed taurine 2.2% (T-2.2). Plateau response of ADC of lipid was observed at the dietary taurine levels of more than 0.45% (T-0.45). There is the same pattern between ADC of lipid and GBSI with increased taurine levels. Both response parameters were best modeled by 4-SKM and this correlation is shown in Figure 20.

Table 7. Apparent digestibility coefficient (ADC) of *Totoaba macdonaldi* fed diets containing different levels of taurine

Taurine (%)	T-0.23	T-0.45	T-0.91	T-1.28	T-1.76	T-2.20	T-2.72	T-3.01	PSE	Model	R^2	P
ADC (%)												
Protein	89.5	92.0	92.1	92.1	90.7	91.6	91.7	90.0	0.122	5-SKM	0.63	<0.001
Lipid	78.9	88.5	88.9	88.2	90.4	92.6	92.0	90.8	0.198	4-SKM	0.87	<0.001
Dry matter	65.1	59.6	60.0	57.7	56.7	59.7	57.8	58.6	0.342	Quadratic	0.50	<0.001

Linear, logarithmic, quadratic, 4-SKM and 5-SKM regression analyses were used for each parameter, then the best model regression was chosen according to methods described in the text. Statistically significant ($P<0.05$). NS: not significant at the $\alpha=0.05$ level.

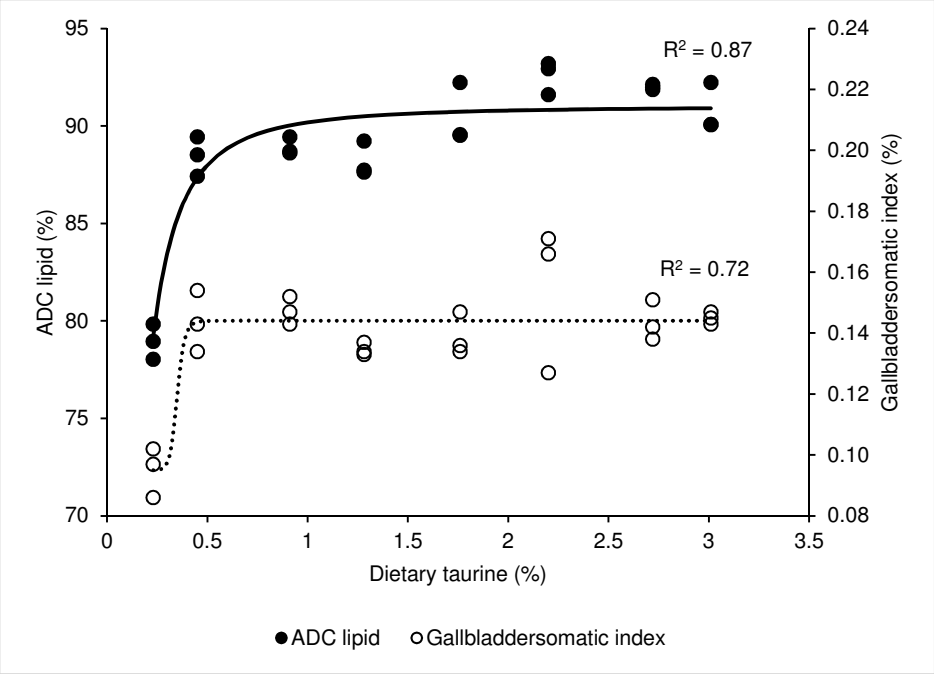

Figure 20. Relationship between ADC of lipid vs dietary taurine and GBSI vs dietary taurine in *Totoaba macdonaldi*. 4-parameter saturation kinetic model (4-SKM) of ADC of lipid (R^2=0.87, P<0.001) and GBSI (R^2=0.72, P<0.001) of *Totoaba macdonaldi* fed different levels of taurine. Model parameter estimates of ADC lipid (I=0, $K_{0.5}$=0.009, R_{max}=91, n=1.89) and GBSI (I=0.095, $K_{0.5}$=2E-09, R_{max}=0.14, n=18.9).

IX. DISCUSSION

1. Growth

This experiment was designed to determine any biological relevance of taurine in juvenile totoaba. It is necessary to elucidate the physiological roles and the dietary taurine requirement in order to develop a sustainable diet for this candidate species for diversification and commercial aquaculture purposes. The ability to synthesize taurine is species-specific and it is scarce in strictly carnivorous species such as bluefin tuna *T. thynus*, yellowtail *S. quinqueradiata*, Japanese flounder *P. olivaceus* and red sea bream *P. major* (Goto *et al.*, 2001b; Yokoyama *et al.*, 2001). Therefore, taurine supplementation is mandatory in diets formulated for these carnivorous fish species. A positive effect on growth has been reported for some marine and freshwater fish species when they were fed with a taurine-supplemented diet (Salze and Davis, 2015). A previous study on totoaba fed with two levels of SPC-based diets supplemented with taurine showed similar growth performance in comparison to fish fed with a fishmeal-based diet used as reference (López *et al.*, 2015). Studies conducted on Japanese flounder *P. olivaceus* (Park *et al.*, 2002) and yellowtail *S. quinqueradiata* (Matsunari *et al.*, 2005; Khaoian *et al.*, 2014) reported that taurine concentration in a fishmeal-based diet is inadequate for these fish species. This suggested that different fish species have a different taurine requirement level.

Different types of fishmeal such as jack mackerel meal, white fishmeal, menhaden fishmeal and brown fishmeal contain different amounts of taurine, 0.23, 0.34, 0.5, 0.6%, respectively (Kim *et al.*, 2005a; Gaylord *et al.*, 2006; Kim *et al.*,

2008a; Lim *et al.*, 2013). These quantities may be reduced by a half in a formulated diet containing 50% crude protein. In this study, fishmeal was used as protein source in order to reduce the palatability problem posed by the use of plant feedstuff for totoaba. Fishmeal was washed with ethanol to remove taurine and it was assumed that the basal diet contained very low level of this nutrient.

Based on the TGC, a plateau response was observed on diets 2 (T-0.45) through 8 (T-3.01). This suggests that the taurine requirement was met in these treatment groups. However, the growth of fish fed with the basal diet (T-0.23) was significantly lower when compared to the other groups. This points out an increased response regarding the basal diet (T-0.23) and the diet 2 (T-0.45). Additionally, green liver was detected as physiological abnormality in fish fed only with the basal diet (T-0.23). This demonstrates that such diet was taurine-deficient. However, a quantitative requirement cannot be estimated from this data because there is no responsive phase before reaching the plateau in the dose-response experiment. Consequently, totoaba's taurine requirement may be estimated to be within the 0.23 - 0.45% range.

2. Green liver and bile pigments excretion in *Totoaba macdonaldi*

Green liver has been observed in susceptible species fed with taurine-deficient diets, typically where taurine-rich ingredients, such as fishmeal, were replaced by ingredients lacking thereof, e.g. SBM (Takagi *et al.*, 2008; Takagi *et al.*, 2010) or SPC (López *et al.*, 2015). Green liver is the consequence of taurine deficiency in some species such as Japanese yellowtail *S. quinqueradiata* and red

sea bream *P. major* (Goto *et al.*, 2001b; Takagi *et al.*, 2005; Takagi *et al.*, 2006b; Sarker *et al.*, 2012). A previous study performed on totoaba showed the occurrence of green liver when fish were fed with SPC with no taurine supplementation (López *et al.*, 2015). In this study, green liver was observed as physiological abnormality after totoaba was fed with a diet lacking taurine (T-0.23). The low taurine level contained in the control diet (T-0.23) was possibly the triggering factor for green liver in totoaba. This indicates that the taurine level contained in the basal diet (T-0.23) was insufficient to preserve the physiological condition of juvenile totoaba. Therefore, green liver may be recognized as indicator of taurine deficiency in totoaba.

In red sea bream *P. major* and yellowtail *S. quinqueradiata,* the underlying mechanism for green liver occurrence was reported based on hematological parameters and also by considering bile pigments composition and concentration (e.g. biliverdin and bilirubin). In these two species, green liver was induced as the consequence of hepatic bile pigment accumulation derived from increased hemolysis (Takagi *et al.*, 2005; Takagi *et al.*, 2006a; Takagi *et al.*, 2006b; Takagi *et al.*, 2008). Green liver syndrome appears to be caused by an increase in hepatopancreatic biliverdin content, a green-colored bile pigment. As observed in yellowtail *S. quinqueradiata* and red sea bream *P. major*, hepatic bile pigment accumulation is a factor possibly contributing to green liver incidence in totoaba as a consequence of dietary taurine deficiency, since bile pigment needs to be conjugated with taurine in order to increase its polarity before its excretion into the bile occurs.

In this study a low incidence of green liver was observed in totoaba after a 10-week period providing the basal diet (T-0.23). Green liver occurrence is highly probable when a taurine-deficient diet is provided for a long time period because of the hepatic bile pigment accumulation that is not excreted into the bile, as reported for red sea bream *P. major* and yellowtail *S. quinqueradiata*. A low green liver incidence (20%) was observed on red sea bream *P. major* after 29 weeks on a deficient diet. This value increased after 34 weeks (45%) (Takagi *et al*., 2006b). Regarding yellowtail *S. quinqueradiata*, green liver was observed after 21 weeks (Takagi *et al*., 2006a). In this study, it was not observed when fish were fed with the diet 2 (T-0.45) through diet 8 (T-3.01). This indicates that taurine was sufficient in these diets. Thus, approximately 0.45% of dietary taurine may counteract this physiological abnormality in totoaba.

Some studies conducted on Atlantic salmon *S. salar*, cod *G. morhua*, eel *A. japonica*, carp *C. carpio*, goldfish *C. carassius* and rainbow trout *O. mykiss* reported that bile color gradually changes from light yellow to dark green after 2-8 days' starvation (Fang, 1987; Cornelius, 1991; Avery, 1992). Fang (1987) classified fish bile pigment excretion into the bile as two types. Type I: fish mainly excrete bilirubin, and type II: fish mainly excrete biliverdin and also bilirubin in a lesser extent. In this study, a dark green bile was linked to taurine levels but not to starvation. This physiological change only occurred when fish were fed with the basal diet (T-0.23). The normal light yellow coloration of bile was observed in the other treatments. In totoaba, taurine deficiency may induce alterations of the metabolic pathways occurring between biliverdin and bilirubin excretion. As bile

color changed from yellow to dark green, this may indicate that bilirubin excretion switched to biliverdin. The different activity of biliverdin reductase might be another factor that may impact on bilirubin levels in totoaba. Regarding bile pigment metabolism, in totoaba there are two possible explanations considering taurine levels: (1) fish fed with the basal diet (T-0.23) may be classified as type II (they excrete mainly biliverdin), as it was observed that bile shifted from yellow to dark green color concomitantly with the lowest plasma bilirubin level; (2) those fish fed with taurine-supplemented diets may be classified as type I (fish excrete most bilirubin), as a light yellow bile was observed along with high plasma bilirubin levels.

3. Somatic parameters

Morphological parameters are often used to assess fish nutritional status and they may provide an indication of their physiological condition. In this study, alterations of the viscerosomatic index (VSI) were detected on juvenile totoaba after feeding them with the experimental diets. Significant VSI differences suggest that dietary taurine affects the weight of digestive organs. VSI correlates with weight and lipid content in viscera. Fat accumulation was probably caused by an energy excess that is subsequently converted to visceral fat. In fish, several organs store lipids, including the mesenteric membranes, the liver and muscles. A substantial amount of dietary lipid is incorporated as fat that is mainly accumulated in visceral tissue when compared to the rest of the body (Sheridan, 1994). Therefore, taurine may participate in lipid metabolism and storage in

juvenile Atlantic salmon *S. salar* when taurine availability was low in diets containing high levels of plant protein (Espe *et al.*, 2012). However, a clear relationship between taurine levels and HSI could not be observed in this experiment. Other studies reported that HSI was not affected by increasing taurine levels in turbot *S. maximus* (Qi *et al.*, 2012), rainbow trout *O. mykiss* (Gaylord *et al.*, 2006; Gaylord *et al.*, 2007) and cobia *R. canadum* (Watson *et al.*, 2014). HSI value is species-specific and provides an indication of the energy status reserve in the form of glycogen and lipid storage. A high HSI value indicates that fish receive enough energy from the diets and they store the energy and lipid surplus in the liver.

Bile acids are synthesized from cholesterol in the liver and they are conjugated with taurine as a component of bile that is stored in the gallbladder (Goto *et al.*, 1996; Kim *et al.*, 2007; Kim *et al.*, 2008b). C_{24} bile acids are the most common in many teleost, i.e.: cholic acid and chenodeoxycholic acid (Hofmann *et al.*, 2010). These are exclusively conjugated with taurine forming the bile salts, taurocholic and taurochenodeoxycholic acids (Goto *et al.*, 1996; Yeh and Hwang, 2001; Kim *et al.*, 2007; Kim *et al.*, 2008b; Kim *et al.*, 2015). On a study conducted with radiolabeled taurine on Senegalese sole *Solea senegalensis* fed with plant-based diets supplemented with taurine, it was pointed out that the latter was accumulated in the liver and the gallbladder, thus suggesting the importance of taurine for bile salts synthesis (Richard *et al.*, 2017). In this study, juvenile totoaba GBSI was modified by dietary taurine levels. The lowest GBSI value observed after providing the basal diet (T-0.23) may indicate nutritional status problems and

physiological condition deficiencies in juvenile totoaba. This low GBSI level was possibly caused by the decreased production of bile salts stored in the gallbladder caused by dietary taurine deficiency.

4. Nutrient utilization

In this study, no significant difference in FI was found during the feeding trial. This is in agreement with the experiment conducted on red sea bream *P. major* fed with fishmeal-based diets supplemented with taurine (Kato *et al.*, 2013). Possibly, a factor that affected feed intake is palatability. Additionally, fish may adapt their feed intake in order to meet the protein or amino acids requirements. An excessive taurine supplementation on the diets did not exert inhibitory effects on feed intake. This does not agree with other study conducted on turbot *S. maximus* fed with dietary taurine supplementation (Qi *et al.*, 2012). A decreased FE was observed in totoaba when taurine levels were above 1.28% (T-1.28). An increased FE was reported on juvenile turbot *S. maximus* fed with diets containing up to 1% taurine (Qi *et al.*, 2012). Studies on juvenile cobia *R. canadum* (Watson *et al.*, 2014) and turbot *S. maximus* (Qi *et al.*, 2012) showed that dietary taurine supplementation impacts on PER. In this trial, increased in PER was noted when basal diet (T-0.23) and diet 2 (T-0.45) were compared. This demonstrates that dietary taurine has an effect on protein utilization in totoaba.

5. Tissue composition

Fish carcass composition is often used as an indicator of its quality. Several

factors, including diet, are known to affect fish carcass composition. Data regarding fish body composition allows to assess the efficiency of nutrient transfer from feed to fish tissues and it is also useful to predict the overall nutritional status. Previous studies on totoaba reported that dietary composition do not affect carcass composition after the fish were fed with SPC supplemented with taurine (López *et al.*, 2015). Conversely, it was reported that dietary taurine modifies crude protein on the whole body and it promotes growth on juvenile turbot *S. maximus* (Qi *et al.*, 2012), probably by increasing protein synthesis and protein productive value. In this trial, protein composition in totoaba's whole body and muscle was not modified and it was not affected by externally added taurine levels. Seemingly, fish regulate their feed consumption in order to achieve an optimal body protein deposition rate and the P/E ratio (24.8) of the diets was adequate. The lack of significant differences regarding lipid content in totoaba liver when dietary taurine increased is opposite to the finding observed on red sea bream *P. major*, in which significant differences regarding hepatic lipids were observed when fish were fed with dietary taurine supplementation in casein-based diets (Matsunari *et al.*, 2008b). A statistical difference was observed regarding ash content in totoaba's viscera. The higher value of viscera ash was probably due to elevated proportions of mineral content. The lowest value for viscera lipid composition observed after providing a basal diet (T-0.23) could be caused by an impairment of lipid digestion and absorption.

6. Hematological parameters and erythrocyte turnover

The analysis of blood parameters serves as indicators to evaluate the status of aquatic animals in response to stress conditions, different pollutant types, nutrition, as well as ecological and physiological changes. Hematological parameters are responsive to dietary modifications, thus they are considered important tools to assess fish health status (Buentello *et al.*, 2007) and physiological conditions (Kader *et al.*, 2010). Hematocrit and hemoglobin values are indicators of general health in fish and they may change in response to deficiencies of essential nutrients (Buentello *et al.*, 2007; López *et al.*, 2015). Generally, a low hematocrit value indicates anemia. A low hematocrit level has been reported as symptom of taurine deficiency in yellowtail *S. quinqueradiata* (Takagi *et al.*, 2006a; Takagi *et al.*, 2008; Khaoian *et al.*, 2014) and parrot fish *O. fasciatus* (Lim *et al.*, 2013). Dietary taurine modifies the hematocrit value as it can prevent hemolysis in yellowtail *S. quinqueradiata*. This suggests that taurine plays a role in hemolytic suppression through osmoregulation as it increases blood osmolality and it stabilizes the membrane in order to decrease erythrocytes fragility (Takagi *et al.*, 2006a). However, this effect may be specific to some species, as a taurine deficient diet did not modify hematocrit values on red sea bream *P. major* (Takagi *et al.*, 2006b) and Florida pompano *T. carolinus* (Salze *et al.*, 2016). Other study reported that red sea bream *P. major* fed with low-fishmeal diet without taurine supplementation was not affected by anemia, based on the red blood cell count, as well as the hemoglobin and hematocrit values (Takagi *et al.*, 2006b). A previous study conducted on juvenile totoaba showed that taurine

supplementation did not change hematocrit and hemoglobin levels when they were fed with SPC-based diets supplemented with taurine (López *et al*., 2015). In this study, totoaba fed with low taurine levels did not show low hematocrit and hemoglobin levels. It is not possible to determine if either taurine deficiency simply does not affect hematocrit, as occurred on Florida pompano *T. carolinus* and red sea bream *P. major*, or if such effect was not observed because taurine levels on the basal diet were only marginally deficient, thus allowing fish to preserve erythrocytes homeostasis.

Bile pigments such as bilirubin and biliverdin are the end-product of hemoglobin metabolism. In specific cases, bile pigments are conjugated with taurine as a bile component and they are subsequently stored in the gallbladder. Feeding low taurine diets to red sea bream *P. major* and yellowtail *S. quinqueradiata* results in decreased hepatic taurine content and in excessive bile pigment accumulation in this organ (Takagi *et al*., 2005; Takagi *et al*., 2006b). This study showed that by modifying taurine levels, plasma total bilirubin increased linearly in totoaba and the lowest concentration was observed in the basal diet (T-0.23). However, hematocrit and hemoglobin levels were maintained in all treatments. Erythrocyte turnover indicates the rate of red blood cells removal from circulation whereas those recently produce enter. Considering the increased bilirubin level when the liver breaks down hemoglobin as part of the red blood cells renewal process, these results suggest that totoaba may adjust erythrocyte turnover in response to taurine. Thus, erythrocyte life span is extended in taurine-deficient fish in order to overcome the impaired capacity to produce new cells,

leading to a reduced bilirubin production while maintaining hematocrit. Conversely, the capacity to produce new erythrocytes is enhanced in taurine-replete fish, enabling the organism to increase turnover as suggested by the increased bilirubin production. Moreover, although a slight change was observed regarding hematocrit and hemoglobin values, the lowest plasma total bilirubin level and the low incidence of green liver were observed on fish fed with a control diet (T-0.23).

Plasma triacylglycerol and total cholesterol were significantly lower on tiger puffer *T. rubripes* fed with a diet containing 0.29% taurine in comparison to those measured on fish fed with 1.19% taurine (Lim *et al.*, 2011). Increased plasma cholesterol levels caused by high levels of dietary taurine were reported by Watson *et al.* (2014) in cobia *R. canadum* fed with a fishmeal-based diet supplemented with taurine. A low level of plasma total cholesterol was the underlying cause of low lipid digestibility in Atlantic salmon *S. salar* (Torstensen *et al.*, 2000). The low plasma lipid values, including total cholesterol, triglycerides and the low value of visceral fat that were observed in our basal diet group could be attributed to an impaired lipid digestion and the scarce absorption of dietary lipids in the intestine caused by dietary taurine deficiency.

Plasma analysis including total protein, albumin and glucose were not significantly altered by the dietary treatments. This is in agreement with previous studies conducted on totoaba fed with SPC-based diets supplemented with taurine (López *et al.*, 2015). In contrast, other results showed that cobia *R. canadum* fed with fishmeal-based diets supplemented with taurine displayed a significant difference regarding plasma glucose (Watson *et al.*, 2014). However, the same

authors reported no significant change of plasma albumin.

7. *In vivo* digestibility

Feed digestibility is considered an important factor to assess feed utilization, as it represents the amount of nutrients absorbed on the animal's intestinal tract. Digestibility is considered as the sum for each individual ingredient on the diet. Thus, ADC of a specific nutrient included on a diet should be calculated by summing the proportional ADCs for that nutrient in each one of the individual ingredients composing the diet (Glencross *et al.*, 2007). It is critical to know nutrient digestibility of all feed ingredients in order to effectively evaluate the overall nutritive value of ingredients intended to be used on specific species. In our study, feces were removed by siphoning after the feeding in order to minimize the impact of environmental factors. Therefore, leaching originated from feces should not interfere with the accuracy of our digestibility determinations. An elevated ADC of dry matter was observed on the control group (T-0.23). It has been suggested that ADC of dry matter provides an accurate estimation of the total amount of indigestible material in feedstuffs (Brunson *et al.*, 1997). In this study, ADC of protein best fitted a 5-SKM with good levels after comparing all treatments (90-92%).

Fishmeal-based diets supplemented with methionine and cysteine increased taurine plasma levels and improved lipid digestibility in Atlantic salmon *S. salar* (Nordrum *et al.*, 2000). Another study conducted on this species reported that the low ADC of lipid may also be attributed to the low levels of sulfur-containing

amino acids such as methionine, cysteine and taurine contained on a SBM-based diet (Krogdahl *et al.*, 2003). Bile salts play an important role for lipid digestion and absorption as they are required for lipid emulsification, micelle formation and activation of certain lipases (Gjellesvik *et al.*, 1989; Romarheim *et al.*, 2006). Fish diets supplemented with bile salts have been suggested to improve lipid digestion (Romarheim *et al.*, 2006; Yamamoto *et al.*, 2007; Iwashita *et al.*, 2008; Nguyen *et al.*, 2011). The necessity for a low FM-based diet (FM 35) supplemented with taurine has been reported for yellowtail *S. quinqueradiata* in order to preserve normal lipid digestion and absorption (Khaoian *et al.*, 2014). Poor lipid digestion and absorption were observed on juvenile Senegalese sole *S. senegalensis* when they were fed with a plant-based diet containing low taurine levels (Richard *et al.*, 2017). In this study, the functionality of the bile process from the liver to the intestine may have been disrupted in fish fed with the basal diet (T-0.23). Interesting results were observed regarding ADC of lipid and GBSI as they showed the same trend. Low GBSI was concomitant with low lipid digestibility after providing a basal diet (T-0.23). Taurine may promote lipid utilization in totoaba through the bile acid pathway in order to produce bile salts. The decrease in ADC of lipid observed on fish fed with the basal diet (T-0.23) might be explained by a low bile salts synthesis and the consequent limited availability of bile salts in the intestinal chyme that promote lipid emulsification, digestion and absorption to ultimately increase lipid utilization. Taurine supplementation on the diet probably assisted lipid digestion and absorption in this species. The low lipid digestibility observed after providing the basal diet (T-0.23) suggests a limited bile salts

availability and/or low lipase activity. As totoaba fed with the basal diet (T-0.23) exhibited physiological changes, based on ADC of lipid and GBSI, dietary taurine should be formulated at 0.45% or higher in order to improve physiological condition when they are fed with washed FM.

Furthermore, the incidence of green liver was observed after providing the control diet (T-0.23) that was concomitant with low lipid digestibility. This is in agreement with another experiment conducted on red sea bream *P. major* fed with non-fishmeal diets that revealed green liver syndrome and impaired lipid digestion and absorption (Goto *et al.*, 2001b).

X. CONCLUSION

Totoaba is a fish with moderate energy requirements and, as lipid levels were similar in all diets and they met this requirement, the increased lipid digestibility, GBSI, viscera lipids, as well as plasma cholesterol and triglycerides observed on the taurine-supplemented groups demonstrate that taurine modulates lipid digestion, absorption and metabolism in totoaba.

Taurine deficiency possibly caused bile pigment was not transported into the bile in the gallbladder, thus remaining sequestered in the liver of fish fed with the basal diet (T-0.23). This hepatic bile pigment accumulation possibly contributed to onset of green liver in totoaba. The incidence of green liver and the low bilirubin concentration observed after providing the basal diet (T-0.23) possibly resulted in low bile production as indicated by a low GBSI, because one of the bile components stored in the gallbladder is bile pigment.

It has been hypothesized that taurine might be an integral part of bile pigment and bile acid metabolism in totoaba. Accordingly, taurine seems to be shared for conjugation purposes with bile pigments and bile acids. Several factors may impact on the low bile production in totoaba fed with a taurine deficient diet (T-0.23), considering the bile pigment status: 1) Insufficient conjugation of bile pigment (bilirubin) with taurine; and 2) Low erythrocyte turnover resulting in low bilirubin levels. Concerning the bile acid status, it may be caused by insufficient conjugation of bile acids with taurine for bile salt synthesis. Consequently, bile production was decreased as both bile pigments and bile salts as bile components were not transported to the gallbladder.

Taurine level is an important consideration to develop totoaba's diet. The results obtained in this study demonstrate that by supplementing a washed FM-based diet with low taurine levels (0.45%) may improve physiological changes in juvenile totoaba, as evidenced by the absence of green liver, low GBSI, low lipid digestibility, low erythrocyte turnover, low plasma cholesterol and triglycerides, as well as low visceral fat. Green liver, GBSI, ADC of lipid, plasma cholesterol and triglycerides may be promising diagnostic tools for taurine deficiency in totoaba. Moreover, dietary taurine may enhance digestive enzyme activities in this species that deserves further research.

XI. RECOMMENDATION

Taurine is considered as an essential nutrient for totoaba therefore the supplementation of this nutrient is important in practical diet of totoaba under culture condition. It could be suggested that taurine requirement of totoaba is between 0.23-0.45%. In addition, it might be useful to investigate the possibility to determine quantitative requirement of this nutrient by replacing with plant protein sources to obtain a diet with very deficient taurine level in order to obtain preceding phase before plateau section following SKM curve.

The supplementation of taurine 0.45% may prevent green liver appearance in totoaba. However, the information about green liver in totoaba is limited and needs to be investigated. Histological study of green liver can help to understand better any changes in cellular level of this physiological abnormality.

Investigating taurine biosynthetic genes expression: CDO, CSD as a common pathway in teleost or CAD as an alternative pathway, as well as their enzymatic activities in liver as main synthetic organ, at graded taurine levels would be an interesting topic to know their expressions in response to dietary taurine supplementation, in order to contribute on taurine knowledge in totoaba.

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XIII. APPENDICES

A. Statistical analysis and model comparison of response parameters using five regression models (linear, logarithmic, quadratic, 4-SKM and 5-SKM)

Table 8. Statistical analysis and model comparison of growth and somatic parameters

Response	Linear			Logarithmic			Quadratic			4-SKM			5-SKM		
	R^2	P	SSE	R^2	P	SSE	R^2	P (quad)	SSE	R^2	P	SSE	R^2	P	SSE
WG (g)	0.04	NS	239	0.01	NS	246	0.04	NS	238	0.09	NS	227	**0.15**	**<0.001**	**212**
PWG (%)	0.05	NS	22515	0.02	NS	23163	0.05	NS	22456	0.09	NS	21564	**0.15**	**<0.001**	**20151**
TGC	0.05	NS	0.00034	0.02	NS	0.00035	0.05	NS	0.00034	0.07	NS	0.00035	**0.13**	**<0.001**	**0.00033**
CF	**0.05**	**NS**	**0.039**	0.02	NS	0.040	0.08	NS	0.037	0.01	NS	0.040	0.03	NS	0.039
HSI (%)	**0.06**	**NS**	**1.33**	0.01	NS	1.40	0.16	NS	1.19	0.02	NS	1.39	0.34	NS	0.93
VSI (%)	0.04	NS	0.57	0.00	NS	0.59	**0.31**	**0.008**	**0.40**	0.14	NS	0.51	0.52	0.005	0.28
GBSI (%)	0.25	0.013	0.007	0.42	<0.001	0.005	0.40	0.034	0.005	**0.72**	**<0.001**	**0.002**	0.72	<0.001	0.002

Statistically significant (P<0.05), NS: not significant at the α=0.05 level, P (quad): P-value of the quadratic term, SSE: sum of square error.
WG: weight gain, PWG: percent weight gain, TGC: thermal-unit growth coefficient, CF: condition factor, HSI: hepatosomatic index, VSI: viscerosomatic index, GBSI: gallbladdersomatic index.

Table 9. Statistical analysis and model comparison of feed utilization

Response	Linear			Logarithmic			Quadratic			4-SKM			5-SKM		
	R^2	P	SSE	R^2	P	SSE	R^2	P (quad)	SSE	R^2	P	SSE	R^2	P	SSE
FI (g/fish)	**0.02**	**NS**	**124**	0.04	NS	121	0.03	NS	122	0.00	NS	125	0.00	NS	125
FE	**0.26**	**0.011**	**0.015**	0.23	0.017	0.016	0.27	NS	0.015	0.28	NS	0.014	0.28	NS	0.014
PER (%)	**0.26**	**0.012**	**0.066**	0.16	NS	0.074	0.27	NS	0.064	0.23	NS	0.067	0.37	0.025	0.055
LER (%)	**<0.01**	**NS**	**2.70**	0.02	NS	2.66	0.03	NS	2.63	0.00	NS	2.70	0.27	NS	1.97
PR (g)	**0.15**	**NS**	**83**	0.10	NS	89	0.15	NS	83	0.22	NS	77	0.31	<0.001	67
LR (g)	**0.08**	**NS**	**8.1**	0.05	NS	8.3	0.09	NS	8.1	0.14	NS	7.6	0.20	NS	7.0
PRE (%)	0.21	0.023	547	0.14	NS	599	0.21	NS	547	0.21	NS	552	**0.45**	**0.019**	**386**
LRE (%)	**0.07**	**NS**	**1170**	0.04	NS	1212	0.07	NS	1170	0.07	NS	1171	0.20	NS	1009

Statistically significant (P<0.05), NS: not significant at the α=0.05 level, P (quad): P-value of the quadratic term, SSE: sum of square error.
FI: feed intake, FE: feed efficiency, PER: protein efficiency ratio, LER: lipid efficiency ratio, PR: protein retention, LR: lipid retention, PRE: protein retention efficiency, LRE: lipid retention efficiency.

Table 10. Statistical analysis and model comparison of tissue proximate composition

Response	Linear			Logarithmic			Quadratic			4-SKM			5-SKM		
	R^2	P	SSE	R^2	P	SSE	R^2	P (quad)	SSE	R^2	P	SSE	R^2	P	SSE
Whole body (%)															
WB protein	**0.02**	**NS**	**5.4**	<0.001	NS	5.5	0.10	NS	4.9	0.05	NS	5.2	0.07	NS	5.1
WB lipid	**0.00**	**NS**	**3.05**	0.01	NS	3.04	0.00	NS	3.05	0.02	NS	2.97	0.01	NS	3.01
WB ash	**<0.01**	**NS**	**0.436**	<0.001	NS	0.436	0.00	NS	0.436	0.01	NS	0.433	0.02	NS	0.429
WB moisture	**0.07**	**NS**	**7.71**	0.07	NS	7.71	0.08	NS	7.62	0.03	NS	8.01	0.11	NS	7.36
Muscle (%)															
M protein	**0.03**	**NS**	**6.8**	0.06	NS	6.6	0.05	NS	6.7	0.09	NS	6.4	0.05	NS	6.7
M lipid	**0.04**	**NS**	**2.3**	0.09	NS	2.2	0.11	NS	2.1	0.13	NS	2.0	0.14	NS	2.0
Muscle ash	**0.07**	**NS**	**0.08**	0.02	NS	0.09	0.23	NS	0.07	0.00	NS	0.08	0.32	NS	0.06
M moisture	**0.01**	**NS**	**8.4**	0.05	NS	8.1	0.14	NS	7.3	0.07	NS	7.9	0.32	NS	5.8
Liver (%)															
L protein	**0.01**	**NS**	**65**	0.04	NS	63	0.10	NS	59	0.08	NS	61	0.12	NS	58
L lipid	**<0.01**	**NS**	**311**	0.01	NS	309	0.02	NS	305	0.00	NS	311	0.19	NS	252
L ash	**0.01**	**NS**	**0.702**	0.01	NS	0.703	0.01	NS	0.702	0.01	NS	0.698	0.04	NS	0.682
L moisture	**0.01**	**NS**	**274**	<0.001	NS	277	0.03	NS	268	0.01	NS	275	0.08	NS	254
GLG	**0.02**	**NS**	**7.0**	0.00	NS	7.1	0.05	NS	6.8	0.04	NS	6.9	0.05	NS	6.8
Viscera (%)															
V protein	**0.09**	**NS**	**11.0**	0.13	NS	10.5	0.11	NS	10.8	0.12	NS	10.7	0.18	NS	10.0
V lipid	0.05	NS	24	0.14	NS	21	0.21	NS	20	**0.37**	**0.008**	**16**	0.46	0.014	13
V ash	0.11	NS	0.15	0.03	NS	0.17	**0.36**	**0.009**	**0.11**	0.01	NS	0.17	0.31	NS	0.12
V moisture	0.11	NS	42	**0.20**	**0.029**	**38**	0.23	NS	36	0.16	NS	40	0.39	0.044	29

Statistically significant (P<0.05), NS: not significant at the α=0.05 level, P (quad): P-value of the quadratic term, SSE: sum of square error.
GLG: glycogen (g/100 g tissue)

Table 11. Statistical analysis and model comparison of hematological parameters

Response	Linear			Logarithmic			Quadratic			4-SKM			5-SKM		
	R^2	P	SSE	R^2	P	SSE	R^2	P (quad)	SSE	R^2	P	SSE	R^2	P	SSE
HT (%)	0.01	NS	16.5	<0.001	NS	16.6	0.02	NS	16.2	0.01	NS	16.4	**0.05**	**<0.001**	**15.7**
HB (g/dL)	0.03	NS	3.40	0.08	NS	3.22	0.11	NS	3.12	0.16	NS	2.93	**0.18**	**<0.001**	**2.88**
BR (mg/dL)	**0.58**	**<0.001**	**0.026**	0.55	<0.001	0.028	0.58	NS	0.026	0.25	NS	0.047	0.40	0.036	0.037
GLU (mg/dL)	0.02	NS	1971	0.01	NS	1992	0.03	NS	1951	0.01	NS	1984	**0.07**	**<0.001**	**1867**
TP (g/dL)	**0.07**	**NS**	**0.318**	0.08	NS	0.315	0.07	NS	0.316	0.08	<0.001	0.315	0.08	NS	0.315
ALB (g/dL)	**<0.01**	**NS**	**1.039**	0.01	NS	1.034	<0.001	NS	1.039	0.00	NS	1.037	0.14	NS	0.895
CHO (mg/dL)	**0.75**	**<0.001**	**731**	0.76	<0.001	712	0.77	NS	670	0.77	<0.001	659	0.77	<0.001	659
TG (mg/dL)	0.28	0.008	4407	0.42	<0.001	3580	**0.53**	**0.003**	**2888**	0.46	0.006	3346	0.55	<0.001	2791

Statistically significant (P<0.05), NS: not significant at the α=0.05 level, P (quad): P-value of the quadratic term, SSE: sum of square error.
HT: hematocrit, HB: hemoglobin, BR: total bilirubin, GLU: glucose, TP: total protein, ALB: albumin, CHO: total cholesterol, TG: triglycerides.

Table 12. Statistical analysis and model comparison of Apparent Digestibility Coefficient (ADC)

Response	Linear			Logarithmic			Quadratic			4-SKM			5-SKM		
	R^2	P	SSE	R^2	P	SSE	R^2	P (quad)	SSE	R^2	P	SSE	R^2	P	SSE
ADC prot (%)	0.00	NS	28	0.03	NS	27	0.27	0.012	20	0.38	0.020	17	**0.63**	**<0.001**	**10**
ADC lip (%)	0.53	<0.001	188	0.74	<0.001	106	0.71	0.002	117	**0.87**	**<0.001**	**51**	0.87	<0.001	51
ADC dm(%)	0.27	0.009	134	0.46	<0.001	99	**0.50**	**0.005**	**91**	0.64	<0.001	66	0.63	<0.001	68

Statistically significant (P<0.05), NS: not significant at the α=0.05 level, P (quad): P-value of the quadratic term, SSE: sum of square error.

B. Presentation and publication

Some studies which are reported in this thesis, have been presented in the following communications:

Poster presentation:

Satriyo T.B., López L.M., Galaviz M.A., Salze G. (2016) Effects of different taurine levels on growth performance and feed utilization of juvenile *Totoaba macdonaldi***.** Aquaculture 2016. World Aquaculture Society. February 22-26, 2016. Las Vegas, Nevada, USA. Poster presentation.

Satriyo T.B., Galaviz M.A., López L.M., Salze G, Rodriguez S. (2015) Growth performance and biological parameters of *Totoaba macdonaldi* fed with diets containing different level of taurine. XII International Symposium on Aquatic Nutrition. November 11-13, 2015. Hermosillo, Sonora, Mexico. Poster presentation.

Oral presentation:

Satriyo T.B., López L.M., Galaviz M.A., Salze G. (2016) Evaluation of dietary taurine on blood parameters, somatic indices and liver status of *Totoaba macdonaldi*. Aquaculture 2016. World Aquaculture Society. February 22-26, 2016. Las Vegas, Nevada, USA. Oral presentation.

Journal publication:

Satriyo T.B., Galaviz M.A., Salze G., López L.M. (2017) Assessment of dietary taurine essentiality on the physiological state of juvenile *Totoaba macdonaldi*. Aquaculture Research. https://doi.org/10.1111/are.13391

Lipid digestion and metabolism

Fish bile is released from the gallbladder only when food reaches the hind gut. After a 24-hour fasting period, the gallbladder expands up to 20% of the liver weight and 6 hours after refeeding it returns to 23% of liver weight (Cornelius, 1991). The chyme (partially digested acidified food) produced by the stomach is emulsified by bile salts in the duodenum and it forms 1 µm-diameter droplets, thus increasing the available surface for lipase activity. Gallbladder bile enables lipid digestion by providing bile salts that act as surfactants capable to emulsify them. Another function of bile is to regulate intestinal pH. In the foregut neutrality prevails, whereas the hindgut is alkaline. Neutrality may change in the foregut when it is reached by the acid digesta, thus it is necessary to provide the alkaline conditions (pH 7 to 10) required for the optimal function of digestive enzymes, especially for proteases. These alkaline conditions are attained because of bicarbonate and bile secretion from pancreas and gallbladder, respectively (Sanz, 2009).

Lipase is a glycerol ester hydrolase and it is the only enzyme secreted by pancreas in its active form, as it requires the presence of colipase in order to fully activate its hydrolytic activity. At physiological levels, bile salts inhibit lipase and thus they prevent its binding to the lipid-water interface of droplets. In order to overcome this inhibition, colipase binds to lipase and it replace bile salts at the interface enabling triglyceride hydrolysis. Colipase is a small protein secreted by the pancreas that binds to the triglyceride ester bond region and subsequently to lipase through electrostatic interactions. It is a cofactor that activates lipase. It is secreted as procolipase that is subsequently activated by trypsin. Its function is to

prevent the inhibitory effects of bile salts on lipase activity. In the intestine, bile salts emulsify lipids and afterwards the pancreatic lipase breaks down the triglyceride ester bonds 1 and 3 thus releasing free fatty acids and 2-monoglycerides (Figure 21).

Esterase (non-specific lipase or bile salt-activated lipase) is secreted by the pancreas and it is activated by bile salt (its optimum pH 7.7-8.3). This enzyme acts in the intestine and it hydrolyzes ester bonds from different lipids (cholesterol esters, fat soluble vitamins) and all three bonds in triglycerides (thus releasing glycerol and fatty acids). The non-specific lipase combines with pancreatic lipase to achieve complete lipid digestion thus releasing unseparated fatty acids (C2 of triglyceride) by pancreatic lipase and hydrolyzing waxex more efficiently (Figure 21).

Phospholipase A2 is secreted by intestinal cells as a proenzyme (zymogen) that is further activated by trypsin in the presence of bile salts. This enzyme catalyzes the hydrolysis of the ester linkages of fatty acids at the position 2 in phosphoacylglycerols to release free fatty acids and lysophospholipids (Figure 21).

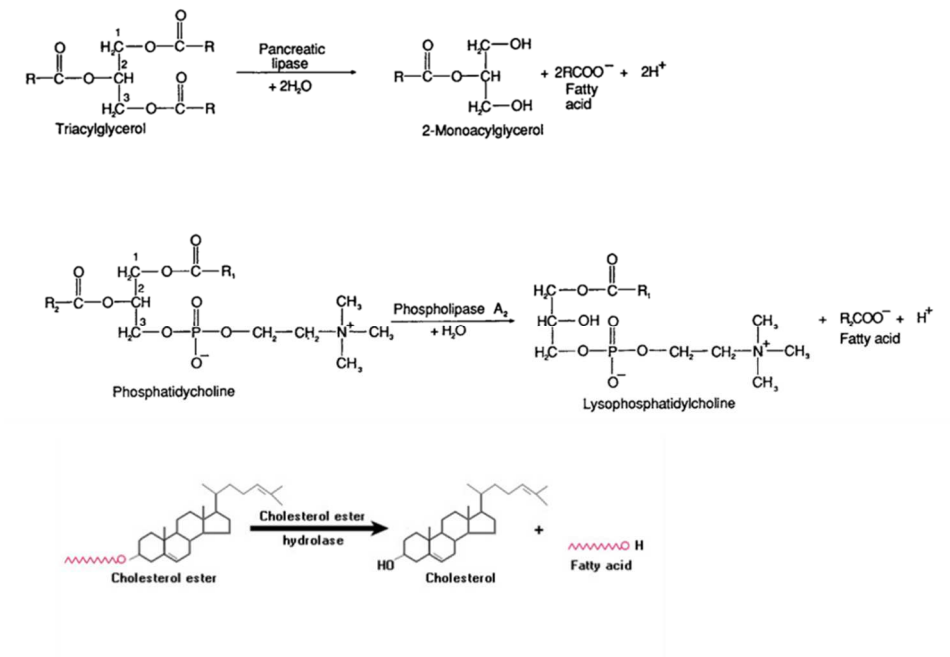

Figure 21. Specific lipolytic enzymes involved in lipid digestion. Adapted from Bhagavan (2002); Harvey *et al.* (2011).

Because of their hydrophobic character, the products from dietary lipid digestion such as fatty acids, 2-monoglyceride, lysophospholipids, cholesterol and fat-soluble vitamins, must be solubilized in the intestinal lumen so they are transported to enterocytes, their absorption site. Bile salts play a crucial role during luminal transport and absorption of lipid hydrolysates by forming micelles that cross the enterocyte's unstirred water layer and the brush border (apical membrane) (Figure 22). In the absence of bile salts, fatty acids will not be efficiently transported into the intestinal cell. As the high levels of hydrolysis products are fat soluble, they diffuse across the enterocyte's membrane. Short chain fatty acids (2-10 carbons) do not need to be solubilized by micelles and they cross the enterocyte's membrane, to be subsequently released directly into the blood. Whereas absorbed monoglycerides, mostly re-esterified as triglycerides, are

assembled into chylomicrons and they are absorbed by enterocytes and afterwards transported through the lymphatic system to the blood.

Other absorption mechanisms have been reported for the products from lipid digestion where transporters possessing several degrees of specificity are involved. These transporters belong to the family of fatty acid binding proteins (FABP) and their translocation mechanism is Na^+-dependent. Cholesterol diffuses into the enterocyte and once inside all absorbed lipids bind to specific binding proteins: the Fatty Acids Binding Protein (FABP) and the Sterol Carrier Protein (SCP). This prevents their accumulation in cytoplasm because of their hydrophobic character and they are transported into the smooth endoplasmic reticulum where re-esterification occurs in order to synthesize again triacylglycerols, phospholipids and cholesterol esters. All these lipids bind to apoproteins (apo B, C and A) and they are modified in the Golgi complex thus forming chylomicrons that are excreted from the enterocytes by exocytosis to subsequently enter the lymphatic capillaries (Garrido Pertierra, 2006).

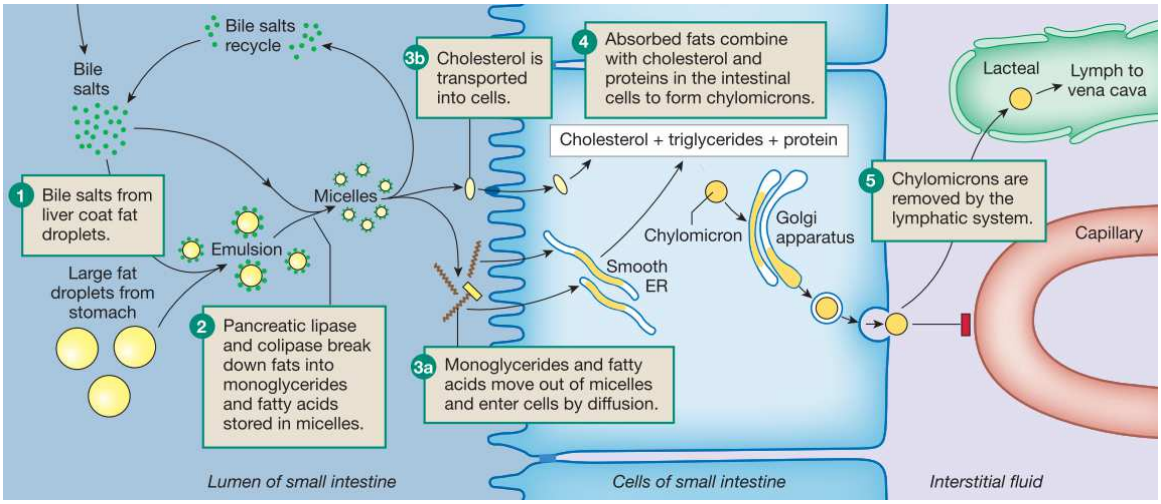

Figure 22. Absorption of ingested lipid, triglycerides synthesis (re-esterification), chylomicron formation and exocytosis. Adapted from Silverthorn *et al.* (2013).

Lipoproteins constitute the lipid transport system through the blood stream and they are formed by proteins (apolipoproteins), phospholipids, triglycerides and cholesterol. Lipoproteins are particles that contain lipid droplets surrounded by a single phospholipid layer. They are amphipatic molecules in which the polar ends of phospholipid face outwards whereas nonpolar regions face inside in the inner region of the membrane at the lipoprotein center. As lipids are less dense than water, a lipoprotein's density decreases as the lipid:protein ratio is higher (Table 13). Lipoproteins transport lipids from intestine in the form of chylomicrons and as Very Low Density Lipoprotein (VLDL) from liver to various tissues for oxidation and to adipose tissue for storage purposes. Chylomicrons and VLDL are triglyceride enriched molecules, whereas Low Density Lipoprotein (LDL) and Intermediate Density Lipoproteins (IDL) contain high cholesterol levels (Brody, 1999). The exogenous pathway defines the transport of ingested lipid from the intestine towards other tissues. The endogenous pathway outlines the transport of lipids produced inside the body among its tissues and it includes VLDL, IDL, LDL and HDL (Figure 23). The five major lipoprotein groups are the following: (Dancygier, 2009).

1) Chylomicron: the largest and least dense of all lipoproteins. They are derived from the intestine and they display the highest triglyceride content. They are formed after the digestion and absorption of ingested lipids. These lipoproteins transport exogenous triglycerides from the intestine towards liver, muscle and

adipose tissue.

2) Very low density lipoproteins (VLDL): they are originated in the liver and they transport endogenous triglycerides. IDL are formed from VLDL whereas chylomicrons are transformed into chylomicron remnants. Both of them are taken up by the liver through their interaction with specific receptors and they are subsequently degraded in liver lysosomes. VLDL is converted to IDL and LDL by removing apoproteins.

3) Intermediate density lipoproteins (IDL): they are produced after VLDL metabolism after most triglycerides have been removed, thus cholesterol and phospholipid concentrations are increased.

4) Low density lipoproteins (LDL): all triglycerides are removed and a high concentration of cholesterol and phospholipids remain. They represent the final stage of VLDL catabolism and they contain mainly cholesterol. Free cholesterol and cholesteryl esters are transported by LDL.

5) High density lipoproteins (HDL): they contain a high proportion of proteins and a lower concentration of cholesterol and phospholipids. Its function is to remove the cholesterol excess from the tissues in order to convert it to esters and to transport it back to the liver to carry out its metabolism or degradation. In consequence, blood cholesterol levels decrease.

Table 13. Properties of the main types of human plasma lipoproteins

Class	Density (g/mL)	Protein (%)	Phospholipid (%)	Esterified Cholesterol (%)	Unesterified Cholesterol (%)	Triglycerides (%)
Chylomicrons	<0.95	1.5 – 2.5	7 – 9	3 – 5	1 – 3	84 – 89
VLDL	0.95 – 1.006	5 – 10	15 – 20	10 – 15	5 – 10	50 – 65
IDL	1.006 – 1.019	15 – 20	22	22	8	30
LDL	1.019 – 1.063	20 – 25	15 – 20	35 – 40	7 – 10	7 – 10
HDL	1.063 – 1.210	50 - 55	20 – 25	12	3 – 4	3

Source: Devlin (1997)

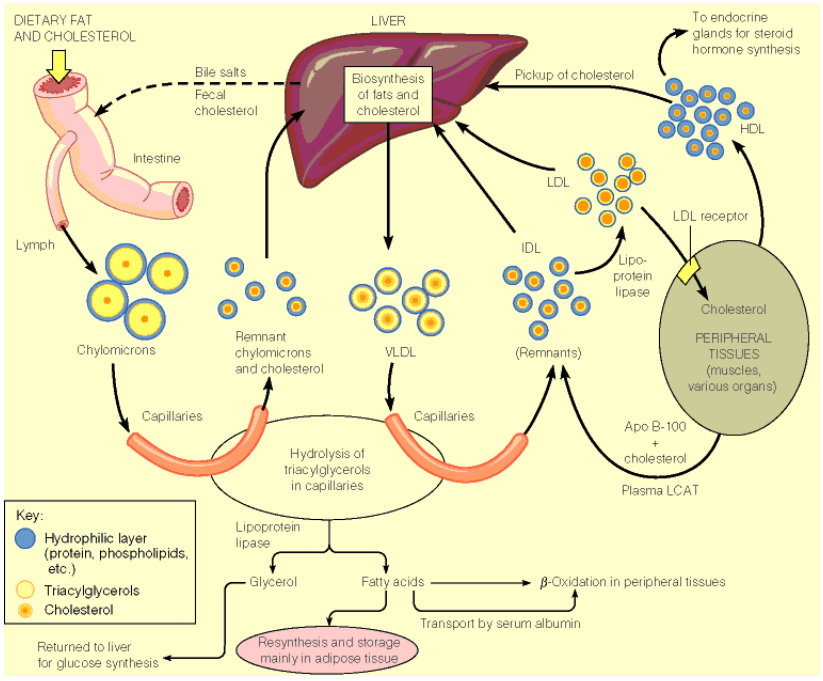

Figure 23. Lipoprotein transport pathways. Adapted from Mathews *et al.* (2000).