Soybean protein concentrate as a protein source for totoaba (*Totoaba macdonaldi*) juveniles: effect on intermediary metabolism and liver histological organization

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Abstract

This study aimed to investigate the effects of replacing fish meal (FM) with soybean protein concentrates (SPC) on the intermediary metabolism and health of *Totoaba macdonaldi* juveniles. Fish (initial weight 50 ± 1 g) were fed for 60 days with eight diets: a reference diet (RD) and seven experimental diets where FM was replaced gradually with 15 to 100% SPC (SPC15, SPC30, SPC45, SPC60, SPC75, SPC90, and SPC100, respectively). Alanine aminotransferase (ALT) activity significantly decreased ($P<0.05$) as SPC inclusion levels increased in diets. The aspartate aminotransferase AST/ALT ratio was significantly higher ($P<0.05$) in totoaba fed with the SPC100

diet. Activities of hexokinase (HK) and glucokinase (GK) significantly decreased ($P<0.05$) as the level of SPC inclusion increased in diets, and the lowest fructose 1-6 biphosphatase (FBPase) activities were found in the SPC100 group. The Pearson's correlation coefficient between the level of SPC inclusion in the diet showed a negative correlation to HK, GK and FBPase (r =-0.71, r =-0.84 and r =-0.73, respectively). The histological organization of the liver in totoaba juveniles fed RD, SPC15, SPC30 and SPC45 diets were similar. Totoaba fed with SPC90 and SPC100 showed histological alterations in hepatic and pancreatic parenchyma. Overall, according to the findings in this study, 45% of dietary FM could be replaced by SPC without causing adverse changes in metabolism, histological organization of liver, and health of juveniles of totoaba when cultured for 60 days. However, levels greater than 60% of SPC could compromise the health status of fish.

Keywords: soybean protein concentrate, intermediary metabolism, hepatocytes, health, fishmeal replacement.

1. Introduction

Plant-protein sources are generally considered the most viable potential alternative for fish meal (FM) replacement in aquafeeds due to their low-cost. Replacement has allowed the aquaculture industry to grow in a model less dependent on FM (Tacon et al., 2011; Anderson et al., 2016). Among different plant-protein sources, soybean protein concentrate (SPC) is one of the most commonly used in aquafeeds due to its high crude protein content (*ca.* 60-70%) (Lusas and Riaz, 1994) and an apparently adequate amino acid profile (Li et al., 2015). SPC is produced through aqueous ethanol or methanol extraction of defatted soybean flakes to decrease or remove anti-

nutritional factors (ANFs) (Hardy, 2010). Although this plant-protein ingredient has been successfully used to substitute dietary FM for several fish species (Deng et al., 2006; Ngandzali et al., 2011; Kalhoro et al., 2018; Tola et al., 2019; Liang et al., 2020; Arriaga-Hernández et al., 2021), there is still uncertainty about the effect of this ingredient included in the diet at high dietary levels on the metabolism and liver condition in marine carnivorous fish species (Kalhoro et al., 2018).

The liver is a metabolically active organ in fish and other organisms and plays a central role in metabolism and distribution of nutrients. It utilizes carbohydrates for the synthesis of cholesterol and stores glucose as glycogen (Klover and Mooney, 2004; Tacon et al., 2011). In particular, this organ is the center of intermediary metabolism, being involved in glucose uptake and release, ketone bodies and urea production, amino acid uptake and release, and lipid processing (Dabrowski and Guderley 2002; Potter, 2007). Therefore, it may be considered a good target tissue (Ardeshir et al., 2017) for intermediary metabolism enzyme activities, and the histomorphological organization of the liver accurately reflects any physiological disorder originating from a nutritionally unbalanced diet or fasting episode (Walton and Cowey, 1982; Fernández et al., 2007; Gisbert et al., 2008;). Several studies have addressed the response of key enzymes of the intermediate metabolism in several freshwater and marine species such as rainbow trout *Oncorhynchus mykiss* (Vilhelmsson et al., 2004), gilthead seabream *Sparus aurata* (Fernández et al., 2007), totoaba *T. macdonaldi* (Bañuelos-Vargas et al., 2014) and yellow perch *Perca flavescens* (Kumar et al., 2019) among others. In addition, other studies have evaluated the histological organization of the liver when fish were fed different diets, especially when FM was replaced by plant-protein sources (Caballero et al., 2004; López et al., 2015; Couto et al., 2016; Goda et al., 2019; Ogueji et al., 2020). By combining both approaches, researchers may provide a clear overview of the effects of the diet on fish at a hepatic level.

As a carnivorous fish species, *T. macdonaldi* has a high crude protein requirement (47-52%) (Bañuelos-Vargas et al., 2014; López et al., 2015; Trejo-Escamilla et al., 2017). Different nutritional studies have been conducted in this species to evaluate different protein sources as sustainable dietary strategies to replace FM, such as poultry by-product meal (Zapata et al., 2016), soybean meal (Fuentes-Quesada et al., 2018), and SPC (López et al., 2015; Trejo-Escamilla et al., 2017). Although the findings have shown that it is feasible to replace FM in compound diets for *T. macdonaldi* with different alternative protein sources, little information is available on how this species withstands these dietary strategies with regard to the hepatic condition and health. Therefore, this study aims to evaluate the effects of gradual substitution of FM by SPC on the condition of the liver utilizing different biomarkers from the intermediary metabolism.

2. Materials and methods

2.1 Diet formulation

A reference diet (RD) and seven isonitrogenous and isolipidic diets with increasing levels of SPC were formulated. The diets were designed to replace equivalent quantities of FM by SPC: 0, 15, 30, 45, 60, 75, 90, and 100% (RD, SPC15, SPC30, SPC45, SPC60, SPC75, SPC90, and SPC100, respectively). A fishmeal-based diet López et al. (2015) was used as a reference diet (RD). All dietary ingredients were thoroughly mixed in a food processor (Hobart, Troy, OH, USA), the wet mixture was pelleted through a commercial meat grinder and the pellets (3-5 mm) were dried in a convection oven overnight (65 ± 5°C) and stored at -20 °C until use. Proximate and amino acid compositions of diets are presented in Tables 1 and 2.

2.2 Experimental conditions and fish sampling

Juveniles of totoaba were provided by the marine finfish hatchery of the Facultad de Ciencias Marinas, Universidad Autónoma de Baja California (México). Before the experiment, fish were acclimated to the experimental facility for two weeks. A total of 480 fish (initial body weight, BW = 50.0 ± 1.0 g) were randomly selected and stocked into 24 tanks of 100 L with a density of 20 fish per tank. Tanks were connected to a water recirculation system (flow rate = 1.6 L min^{-1}). During the experiment, physicochemical water parameters were monitored twice a day to maintain this species' recommended conditions. The temperature was kept at 23.0 ± 1.0 °C with thermo-controlled chillers. Salinity was measured with a refractometer and maintained at 35.0 ± 0.5‰. Photoperiod was kept at 12:12 light: dark. Oxygen concentration was higher than 6 mg L^{-1}. Total ammonia-nitrogen (NH_4^+-N) and total nitrite-nitrogen (NO_2^--N) were measured daily before the first meal of the day with colorimetric test kits (Aquarium Pharmaceutical, Mars, PA, USA) and their levels were maintained <0.2 and <0.1 mg L^{-1}, respectively. Triplicate groups of fish were fed with experimental diets to apparent satiation twice a day (08:00 and 18:00 h), seven days a week for 60 days. The uneaten feed was recovered from each tank, dried and their weight subtracted from the total feed administered for each tank after two hours of feeding to calculate feed intake. All experimental procedures related to fish husbandry were approved by the Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food (Mexican Official Standard NOM-062-ZOO-1999).

2.3 Growth performance

Fish were individually weighed to the nearest 0.1 g at the beginning, at the fourth week, and at the end of the experiment (24 h of fasting before the BW measurement). Fish were anesthetized with 0.3 mL of clove oil dissolved in 3 mL of 70 % ethanol per liter of filtered seawater. The following equations were used to evaluate fish growth performance and feed efficiency:

Daily growth index (DGI= [(final Body Weigth (BW_f)$^{1/3}$ – initial Body Weigth ($BW_i^{1/3}$)/time in days] x 100; Daily feed intake (DFI, g kg Average Body Weight (ABW^{-1}) day^{-1}) = [(daily food intake per fish per day x 1000) / (BW_f + BW_i) / 2]/days; Feed efficiency (FE) = wet weight gain/feed intake; protein efficiency ratio (PER) = wet weight gain/dry protein consumed.

2.4 Metabolic enzyme analyses

In order to evaluate the impact of diets with different levels of SPC, fish (n =2 per tank; 6 per dietary treatment) were sampled and sacrificed 4 h after the morning meal with an overdose of clove oil. Once the liver was excised, it was placed in dry ice and stored at -80 °C until the measurement of metabolic enzymes. Each liver was homogenized in 5 volumes of ice-cold 100mM-Tris–HCl buffer containing 0.1mM-EDTA and 0.1% (v/v) Triton X-100, pH 7.8 (0-4 ºC). Homogenates were centrifuged at 30,000 x *g* for 30 min at 4 °C and supernatants kept in aliquots and stored at -70 °C until their use (Pérez-Jiménez et al., 2012). All enzyme assays were carried out at 25 °C, and the changes in absorbance were monitored to determine the enzyme activity using a microplate reader (Thermo Scientific-Miltiskan Go, Finland). The optimal substrate and protein concentrations for the measurement of maximal activity for each enzyme were established by preliminary assays. The enzymatic reactions were initiated by the addition of the tissue extract. The molar extinction coefficients used for H_2O_2 and NADPH were 0.039 and 6.22 mM^{-1} cm^{-1}, respectively. The specific assay conditions for each enzyme were as follows: aspartate aminotransferase (AST; EC 2.6.1.1) and alanine aminotransferase (ALT; EC 2.6.1.2) activities were assayed with kits from Pointe Scientific, Inc. USA (ALT/GPT, ref. A7526; ASAT/GOT, ref. A7571) at λ = 340 nm. Glutamate dehydrogenase (GDH; EC 1.4.1.2) activity was performed using a reaction mixture containing 71.4 mM imidazole–HCl buffer (pH 7.4), 2.9 mM NADH, 14.3 mM ADP, 3.3 M ammonium acetate, 2 units LDH mL^{-1} (Morales et al., 1990). Hexokinase (HK; EC 2.7.1.1) and glucokinase (GK; EC 2.7.1.2) activities were determined according to Vijayan et al.

149 (1990). Reaction mixture contained 71.4 mM imidazole–HCl buffer (pH 7.4), 50 mM ATP, 100 mM
150 $MgCl_2$, 8 mM NADP, 2 units G6PDH mL^{-1} and 10 mM (HK) or 1M (GK) glucose. Fructose 1,6-
151 bisphosphatase (FBPase; EC 3.1.3.11) activity was determined according to Morales et al.
152 (1990). Reaction mixture consisting of 71.4mM imidazole–HCl buffer (pH 7.4), 100 mM $MgCl_2$,
153 240 mM 2-mercaptoethanol, 10 mM NADP, 2 units G6PDH mL^{-1}, 2 units PGI mL^{-1} and 0.5mM
154 fructose 1,6- bisphosphate. Glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity
155 was measured using a reaction mixture containing 71.4 mM imidazole–HCl buffer (pH 7.4), 100
156 mM $MgCl_2$, 20 mM NADP and 10 mM glucose-6-phosphate (Morales et al., 1990). Malate
157 deshydrogenase (EC 1.1.1.40) activity was performed using a reaction mixture containing 71.4
158 mM imidazole–HCl buffer (pH 7.4), 100 mM $MgCl_2$, 8 mM NADP, and 40 mM malate (Singer et
159 al., 1990)

160

161 *2.5 Liver histology*

162 At the end of the experiment, three fish from each tank (9 fish per experimental diet) were used
163 for histological purposes. Fish were sacrificed as previously indicated and placed on ice to remove
164 the liver, which was immediately fixed in 10% buffered Davidson (pH 7.2). Liver samples were
165 dehydrated in a graduated series of ethanol and embedded in paraffin (Tissue-tek II, mod. 4640B,
166 USA). Sections of 5 μm were cut (Leica RM2125 RTS) and stained with hematoxylin and eosin
167 (H&E). The histological sections were analyzed by Axioscop microscopy and photographs (600
168 dpi) were taken with a camera AxioVision 4.8.2 SP3 (08-2013).

169

170 *2.6 Statistical analysis*

171 Enzyme activity data among the different soybean levels were fitted to a 2nd non-linear quadratic
172 model ($Y = a + bX + cX^2$) using a Quasi-Newton iteration. In addition, treatment averages were

analyzed by one-way ANOVA and comparison between groups were compared by the orthogonal Bonferroni test. A significance value of 0.05 was used for all tests. All statistical analyses were performed with Prism V. 9.0 software. Additionally, a principal component analysis (CPA) was performed on the enzyme activity data concerning SPC inclusion.

Data normality to AST, ALT, AST/ALT ratio was checked with the Kolmogorov-Smirnov test and homoscedasticity with the Levene test. One-way analysis of variance (ANOVA) followed by multiple range Tukey test was used to compare experimental groups. Data expressed as percentages were arcsin-transformed. Pearson correlation test was used to evaluate the correlation between dietary levels of SPC with the activity of metabolic enzymes. A probability value of $P < 0.05$ was considered significant. These statistical analyses were carried out using the Sigma Stat program version 3.5 (Systat Sofware, San Jose, CA, USA).

3. Results

3.1 Growth performance

At the end of the experimental period, there were statistically significant differences in growth performance depending on the dietary SPC levels ($P < 0.001$). No significant differences were observed in weight gain (WG) and daily growth index (DGI) ($P>0.05$) in RD to SPC45 diets (17.5± 0.37 to 16.5± 0.35 g kg^{-1} ABW day^{-1} and 2.8± 0.03 to 2.6± 0.06%, respectively). These parameters statistically decreased even further in fish fed the highest levels of SPC (SPC90 and SPC100) as this group of fish showed the lowest WG and DGI values (11.9± 0.40 to 12.5 ± 0.23 g kg^{-1} ABW day^{-1} and 1.9 ± 0.09 to 1.8 ± 0.04%, respectively).

3.2 Intermediary metabolic enzymes

196 The non-linear fit model of the enzyme activities showed a highly significant ($P<0.01$)
197 inversely proportional fit with increasing soybean for GK and G6PDH enzymes ($R^2=0.70$),
198 followed by ALAT, FBPase and ASAT/ALAT ratio with inversely significant correlations ($P<0.05$).
199 Finally, malic, ASAT and GDH activities showed no significant fit for the quadratic model ($P>0.05$).
200 Comparison of the averages of HK, GK and ALAT enzyme activities showed highly significant
201 differences ($P<0.01$) between fish fed control treatment (0% soybean) compared to fish fed the
202 diets with 60, 75, 90 and 100% SPC, while comparison of enzyme activities of fish fed lower levels
203 of SPC (15, 30 and 45%) were similar to fish fed the control diet ($P>0.05$). ALAT enzyme shows
204 a highly significant ($P<0.01$) decrease in activity for fish fed 75, 90 and 100% SPC compared to
205 fish fed the control diet; likewise, the activity of fish fed the 30% SPC diet was statistically lower
206 compared to fish fed the control diet, and finally, fish fed 15 and 45% SPC were similar to fish fed
207 the control diet. Malic activity and the ASAT/ALAT ratio showed a significant increase in activities
208 for fish fed 100% SPC compared only with fish fed the control diet, but not with the rest of the
209 treatments. In the case of ASAT, G6DPH and GDH activities, no statistical differences were found
210 between dietary treatments ($P>0.05$) (Fig. 1).Results from the PCA showed a clear explanation
211 of the accumulated variance (61.92%) for the first two components estimated through the
212 eigenvalues (3.48 and 1.47). Thus, a high clustering is detected for the enzymes HK, GK, FBPase,
213 and ALAT towards the lower SPC values (0 and 15% SPC, purple dots), while the enzymes ASAT,
214 GDH, and G6PDH show a separation towards slightly higher inclusion (30 and 45% SPC, blue
215 dots). Finally, the enzyme malic activity separates, showing its maximum activity at the maximum
216 SPC dietary inclusion levels (90 and 100% SPC, green and yellow dots) (Fig. 2).

217

218 *3.3 Histological organization of the liver*

219 The main findings of dietary SPC's effect in the histological organization of the liver in totoaba
220 juveniles are summarized in Figures 3 and 4. Regarding the RD fed fish, the hepatic parenchyma

showed a typical organization that consisted of polyhedral hepatocytes with eosinophilic cytoplasm denoting glycogen and low accumulation of fat resulting in the central nuclei. Hepatocytes were arranged in tightly packed anastomosed plates around the veins (Fig. 3A), and the hepatic parenchyma was surrounded by a thin capsule of fibroconnective tissue. The histological organization of totoaba juveniles fed with different SPC levels was similar to that of the RD group, with differences only found in lipid deposition and glycogen accumulation (acidophilic cytoplasm) within hepatocytes.

In totoaba juveniles fed SPC15, SPC30, and SPC45 diets, the liver's histological organization was similar to the RD fed, except in the hepatocyte fat accumulation levels. In particular, hepatocytes from the groups mentioned above showed a major frequency of hepatocytes with their nuclei displaced to the cell periphery due to a large lipid vacuole occupying most cytoplasm area (Figs. 3B and 3C). The livers from fish fed SPC60 and SPC75 were similar to those fed the SPC15 diet, regardless of the reduced glycogen accumulation (decreased in hepatocytes' cytoplasm acidophily) (Fig. 4A). However, the livers from the SPC75 group showed a large infiltration of lymphocytes within the hepatic vascular system, along with the blood vessels that irrigated the disperse units of the exocrine pancreas (Fig. 4B). The livers of fish fed the SPC90 and SPC100 diets showed large variability depending on the area considered. Thus, some regions of the hepatic parenchyma were devoid of lipid vacuoles within hepatocytes. In contrast, the cells showed a homogeneous and slightly acidophil cytoplasm. Therefore, some other parts of the liver showed a large accumulation of lipids within hepatocytes with the nuclei displaced to the cell's periphery and lost their round shape. As consequence of the large accumulation of lipids within hepatocytes, they lost their typical shape and some pyknotic nuclei were also observed in some areas. Similar to the SPC75 group, infiltration of lymphocytes was a common characteristic in the vascular system irrigating the hepatic parenchyma and pancreatic acini (Figs. 4C and 4D).

The liver's overall histological organization in totoaba juveniles fed SPC90 and SPC100 diets indicated that these animals had some foci with severe hepatic steatosis.

4. Discussion

Soybean protein concentrate is one of the most attractive alternative protein sources in aquafeed formulation due to its high protein content, amino acid profile, low levels of non-digestible carbohydrates, antinutritional factors (ANFs) and market availability (Brown et al. 2008). However, different studies have evidenced that its successful use in aquafeeds majorly depends on the fish species considered, and especially on the species' physiology, such as in Atlantic halibut *Hippoglossus hippoglossus* (Berge et al., 1999); rainbow trout *Oncorhynchus mykiss* (Kaushik et al., 1995); cod *Gadus morhua* (Colburn et al., 2012); black sea bream *Acanthopagrus schlegelii* (Ngandzali et al., 2011); starry flounder *Platichthys stellatus* (Li et al., 2015); *T. macdonaldi* (Bañuelos-Vargas et al., 2014); and longfin yellowtail *Seriola rivoliana* (Kissinger et al., 2016). In the current study, we evaluated the effect of different dietary levels of SPC on the hepatic health condition in *T. macdonaldi* by analysis of selected intermediary metabolic enzyme activities and the assessment of the histological condition of this organ, whereas the results of the tested diets on growth performance and feed efficiency parameters were discussed elsewhere (XXXX).

The liver is the key organ of the body, which controls many biological functions and plays a prominent role in critical physiological processes (*i.e.,* immunity, digestion, vitellogenesis, among others), as well as in anabolism (proteins, lipids and carbohydrates), catabolism (nitrogen, glycogenesis and detoxication (Bruslé and Gonzàlez I Anadon, 1996). Due to its important physiological role, different hepatic enzyme activities have been regularly used as liver condition and host health biomarkers (Wang et al., 2017). The liver, especially the hepatic parenchyma, is the main place for amino acid (AA) transamination in fish. The transaminases AST and ALT activities are responsible for transamination (Enes et al., 2006; Kumar et al., 2019). In particular,

ALT plays an essential role in AA metabolism and gluconeogenesis. ALT and AST catalyze the reductive transfer of an amino group from alanine or aspartate, respectively, to α-ketoglutarate to yield glutamate and pyruvate or oxaloacetate (Ozer et al., 2008). These two metabolic enzymes, particularly their ratio (AST/ALT), are reported as good hepatic damage biomarkers (Wang et al., 2014; Kalhoro et al., 2018). Under present experimental conditions, ALT levels decreased in totoaba juveniles fed diets with SPC levels higher than 45%. These results may be attributed to different factors: I) a reduction of diet digestibility due to the high content of SPC; II) a change in diet palatability and a reduction feed intake values; and III) a low tolerance to ANFs present in SPC. These hypotheses correlated to the lower growth performance of fish fed diets containing >45% SPC (Trejo-Escamilla et al., 2017). These diets may result in a reduction of AA availability for transamination (Lin and Luo, 2011), which may explain such low hepatic ALT levels. In contrast, ALT activity increased with the inclusion of SPC for *P. stellatus* and *A. schlegelii* fed diets containing >45% of SPC (Kalhoro et al., 2018; P. Li et al., 2015). These authors suggested a dysfunction or damage in the liver. In silvery-black porgy (*Sparidentex hasta*) juveniles, the effects of protein-free and essential amino acid-deficient (EAAD) diets were evaluated. The authors found that while plasma and liver ALT, AST, lactate dehydrogenase, and alkaline phosphatase were significantly increased, whereas superoxide dismutase decreased (Mozanzadeh et al., 2018). Additionally, in comparison to the RD, fish fed with SPC60 up to SPC100 diets presented lower red blood cell counts, hemoglobin and hematocrit levels, which indicate possible non-regenerative anemia in totoaba (Trejo-Escamilla et al., 2017).

In totoaba juveniles, the AST/ALT ratio showed ranging from 2.4 to 4.0 (RD and SPC100, respectively) increased due to high SPC inclusion. Fish that were fed diets containing the highest levels of SPC (75% and 100% soybean protein) showed accumulation of microvacuoles indicating lipids deposits. In addition, at a histological level, the livers of fish fed with SPC90 and 100 diets showed some regions of the hepatic parenchyma containing hepatocytes with large lipid, their

nuclei displaced to the cell's periphery, loss of its typical round shape, and in some cases, pyknotic nuclei were also observed. These results may be correlated to changes in the values of the AST/ALT ratio. For example, when membrane integrity is damaged or cell necrosis occurs in hepatocytes, the AST/ALT ratio increases in plasma (Adams, 2005; Fournier et al., 2004; Oost et al., 2003; Sandnes et al., 1988; Trenzado et al., 2006). These damages may be provoked by pathological episodes such as steatosis, toxicological damage, infectious diseases, and leakage of the cytosolic enzymes into the circulatory system.

The AST/ALT ratio can be used as an indicator of liver damage, and as such it is widely used in nutritional studies in livestock and toxicological studies in fish. However, there is partial information on its use for nutritional studies in warm water farmed fish (Sandnes et al., 1988; Attalla and Mikhail, 2008; Mozanzadeh et al., 2018;). The main reason for such limited use of the AST/ALT ratio as a health indicator in aquaculture is the absence of reference values under different farming scenarios and unsuitable nutritional conditions. Regardless of the lack of the reference values in totoaba, we found an increase of values in the AST/ALT ratio with increased SPC levels coupled with the detection of a large accumulation of fat in the hepatic parenchyma of fish. This fat accumulation indicates that the inclusion of high SPC levels in compound diets for juvenile totoaba resulted in liver damage. In a previousstudy, totoaba juvenile fed up to 60% of SPC without taurine inclusion showed changes in hepatocytes such cytoplasmatic vacuolation and displacement of nuclei toward periphery and tissue vascular congestion (López et al. 2015). This same study indicated alterations in serologic parameters such as albumin and globulins by a high SPC level. Bañuelos-Vargas et al. (2014) attributed the changes in AST and ALT to the higher inclusion of SPC 60% without taurine. In contrast, Attalla and Mikhail (2008) did not find relevant changes in the AST/ALT ratio when evaluating the effect of different soybean/algae protein percentages in tilapia diets. However, differences between current data and the study

mentioned above may be related to different diet formulation, ingredient properties, fish feeding habits and physiological capacity for both species regarding dietary protein alternative sources.

Dietary carbohydrates may have a crucial indirect influence on the intermediary metabolism of fishes (Hemre et al., 2002). The carbohydrate inclusion level and fish physiological capacity influence glucose metabolism in fish. In particular, glucokinase is an enzyme involved in synthesizing glycogen from glucose in the liver (Véron et al., 2016). Meanwhile, hexokinase is the first enzyme in the glycolysis pathway, where phosphorylation of glucose by ATP to glucose-6-phosphate occurs, and this molecule can be used in other metabolic pathways, such as pentose synthesis and gluconeogenesis (Enes et al., 2009). Under present experimental conditions, both enzymes decreased in parallel to the increase of SPC inclusion in diets, which may be attributed to low availability of digestible dietary carbohydrates due to the presence of ANFs in SPC (XXX). This hypothesis is supported by data from another study with the same species in which the digestibility of the diet was assessed by *in vitro* methods (Trejo-Escamilla et al., 2017). That study reported that diet digestibility decreased as SPC increased in compound diets. Similar results have been reported by Bañuelos-Vargas et al. (2014) in totoaba juveniles fed diets containing 60% SPC without taurine supplementation, where HK and GK were lower in comparison to other dietary groups. These results were attributed to low insulin stimulation and glucose transport into cells.

A factor important that involved in gluconeogenic enzyme regulation in fish may be dietary protein (Enes et al., 2009). There is a positive effect in the FBPase activity with a good balance of protein for the fish (Enes et al., 2006; Kirchner et al., 2005). In this study, the activity of FBPase decreased in the liver of juveniles fed diets with SPC inclusion higher than 45%. FBPase is a key enzyme in gluconeogenesis that catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate, a precursor to glucose 6-phosphate. Therefore, this enzyme is a regulator of glucose synthesis from non-carbohydrate sources. Thus, the high dietary inclusion of SPC

(>45%) may negatively affect gluconeogenesis due to the low availability of AA present in soybean protein. In *S. hasta*, liver malfunctioning was provoked by a protein deficiency and low AA availability. Additionally, a disturbance in hepatocytes' integrity and protein synthesis was detected (Mozanzadeh et al., 2018). This disturbance could affect the metabolism of fish liver. In our study, the diets contained similar levels of essential and some non-essential AA. However, alanine decreased with respect to levels >45% of substitution, which affects the FBPase activity of totoaba in SPC60 to SPC100 experimental groups, indicating a disturbance in the metabolism and integrity of hepatocytes. Similar results were reported in *O. mykiss,* where lower FBPase activity showed a relation with low alanine in the diet (Kirchner et al., 2003). Therefore, we suggest that the activity of FBPase was limited for gluconeogenesis purposes, and this can also be attributed to the lower digestibility of proteins (Trejo-Escamilla et al., 2017), which might affect the availability of alanine (Salway 2013).

5. Conclusions

In summary, based on the present study results, totoaba juveniles fed with up to 45% of SPC-based diets during 60 days showed no effect on the activity of enzymes involved in intermediary metabolism. In activity, the catabolism levels of the key AA enzyme as ALT decreased in totoaba juveniles fed diets with SPC greater than 45% due to the reduction in AA availability induced by low availability for ALT transamination. The AST/ALT ratio increased with the high inclusion of SPC in juveniles fed with SPC100, indicating a possibility of liver damage such as the lipid accumulation in livers of fish fed with 100% of SPC. The histological organization of livers in totoaba juveniles fed RD to SPC45 diets was similar, showing fat accumulation within hepatocytes as a normal characteristic of nutrient availability. The liver of *T. macdonaldi* juveniles fed SPC75 to SPC100 showed infiltration of lymphocytes irrigating the hepatic parenchyma and pancreatic acini. Further, fish fed SPC90 and SPC100 diets showed severe hepatic steatosis and

abnormalities related to the presence of ANFs in SPC. In this study we concluded that totoaba juveniles could be fed with <45% of SPC in the diet without affecting the metabolism or health of the fish, indicating the possibility of formulating a sustainable diet for aquaculture of this species. The information obtained from the analyses in this study may be used to better understand the impact of nutritional protein imbalances in fish, as well as to provide reference values for a large series of physiological parameters under inadequate nutritional conditions in totoaba.

Acknowledgements

The research reported here was supported by the Universidad Autónoma de Baja California (UABC), México; the Consejo Nacional de Ciencia y Tecnología (CONACyT) (SADER-CONACYT No. 247698); and scholarship no. 206339 (Idaly Trejo Escamilla). The authors are grateful to M.Sc. Olivia Tapia for the assistance in the histological analysis. Special appreciation is given to Dr. Conal True from UABC for the supply of totoaba juveniles. Collaboration between Ibero-American researchers has been done under the framework of the network LARVAplus “Strategies for the development and improvement of fish larvae production in Ibero-America” (117RT0521) funded by the Ibero-American Program of Science and Technology for Development (CYTED, Spain).

Conflict of interest

The authors declare that there is no conflict of interests regarding the information reported in the publication of this paper.

Ethics statement

Fish were handled and treated following the technical specifications for the production, care and use of laboratory animals issued in the Official Mexican Standards (NOM-062-ZOO-1999) and according to the Research Ethics Committee (CEI-UABC-GU2010) of the Autonomous University of Baja California, Mexico based on international guidelines. In addition, all procedures and experimentation conducted with organisms produced at the marine finfish hatchery (Registration Number: DGVS-CR-IN-1084-B.C./09) are reported and evaluated by the DGVS Dirección General de Vida Silvestre, in Spanish (General Management for Wildlife)] in an annual basis.

Credit authorship contribution statement

Idaly Trejo-Escamilla: conceptualization, methodology, formal analysis, visualization, writing the original draft. Lus M López: conceptualization, supervision, formal analysis, review & editing, funding acquisition. Enric Gisbert: formal analysis, review & editing. Samuel Sanchez: review & editing. Deyanira Rodarte-Venegas: methodology, review & editing. Leticia Olivera-Castillo: methodology. Carlos A Álvarez: review & editing. Mario A Galaviz: conceptualization, formal analysis, review & editing the manuscript, project administration. All authors have read and approved the final manuscript.

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608 Table 1. Ingredients (g 100^{-1}, dry weight basis) and chemical composition of experimental diets
609 with different levels of soybean protein concentrate (SPC) for juvenile *Totoaba macdonaldi.*

	Diets							
	RD	SPC15	SPC30	SPC45	SPC60	SPC75	SPC90	SPC100
Ingredients (% dry weight)								
Fish meal[1]	57.7	49.0	39.6	31.3	23.3	17.2	6.6	0.0
Soybean protein concentrate[1]	0.0	8.6	16.9	25.4	33.6	40.0	51.0	58.0
Krill meal[2]	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Gelatin	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Corn Starch	14.0	14.0	13.5	13.0	12.5	12.2	12.0	9.0
Cellulose	4.1	3.7	2.7	2.6	2.6	2.4	1.6	1.6
Fish oil[1]	5.0	5.5	8.0	8.3	8.5	8.6	9.0	9.5
Mineral mix[3]	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Vitamins mix[4]	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Choline chloride	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Lysine[5]	1.0	1.0	1.0	1.1	1.1	1.2	1.3	1.3
Methionine[5]	0.5	0.5	0.6	0.6	0.7	0.7	0.8	0.8
Taurine[5]	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Proximate analyses (% dry weight)								
Crude protein	51.4	51.4	50.3	50.8	51.5	51.5	50.9	50.5
Crude lipid	12.8	11.9	12.3	12.4	12.0	11.9	11.8	11.8
Ash %	14.7	14.1	12.9	12.9	12.3	11.1	10.2	9.2
Starch %	13.8	13.6	13.1	12.8	12.2	11.8	11.4	8.6
NFE%[6]	7.3	8.4	10.0	11.2	11.5	13.5	15.5	19.9
Gross energy (kJ/g^{-1})	19.6	19.3	19.2	19.0	19.1	19.0	18.8	18.2
P/E ratio (g kJ^{-1})	24.6	24.9	24.2	24.2	24.5	24.3	23.9	23.6

610 [1]Rangen, commercial HighPro fish meal, U.S.A. (protein: 72%; lipids: 12%; Ash: 16%) and
611 Soybean Protein Concentrate (SPC, protein: 71%, lipids: 5%, ash:5%), [2]Krill meal from
612 Skretting, Vancouver, British Columbia, Canada; CP: 60.0%; CL: 20%). [3] ASA Premix (g kg^{-1}):
613 thiamin HCl, 0.5; riboflavin, 8.0; pyridoxine HCl, 5.0; Capantothenate, 20.0; niacin, 40.0; biotin,
614 0.040; folic acid, 1.80; cyanocobalamin, 0.002; vitamin A acetate (500,000 IU g^{-1}) 2.40; vitamin
615 D3 (400,000 IU g^{-1}), 0.50; DL-α-tocopheryl acetate, 80.0; α cellulose 834.26. [4] ASA Premix (g
616 100 g^{-1}): cobalt chloride, 0.004; cupric sulfate pentahydrate, 0.250, ferrous sulfate heptahydrate,
617 4.0, manganous sulfate anhydrous, 0.65; potassium iodide, 0.067; sodium selenite, 0.010; zinc

618 sulfate heptahydrate 13.19, α-cellulose 81.83. [5]Amino acids from Pharmaceutical Co., Ltd.,
619 U.S.A, [6]%Nitrogen Free Extract:100-(Protein+Lipid+Ash+Starch).

Table 2. Amino acid (AA) composition (g 100 g diet^{-1}, on a dry weight basis) with different levels of soybean protein concentrate for juvenile *Totoaba macdonaldi*

	RD	SPC15	SPC30	SPC45	SPC60	SPC75	SPC90	SPC100
Essential AA								
His	3.94	4.06	4.11	3.87	3.72	3.48	3.61	3.83
Thr	4.13	3.99	4.09	3.74	3.57	3.24	3.36	3.36
Arg	6.54	6.37	7.52	6.96	6.53	5.95	6.67	6.78
Val	4.46	4.35	4.42	4.05	3.95	3.56	3.72	3.70
Met	1.88	1.72	2.01	1.80	1.64	1.13	1.41	1.20
Ile	4.00	3.68	3.49	3.78	3.60	3.45	3.51	3.74
Leucine	7.00	6.96	6.38	6.54	6.48	6.12	6.31	6.52
Phe	3.95	4.10	4.18	4.04	4.19	4.10	4.33	4.71
Lys	8.63	8.05	7.74	7.80	6.73	6.08	6.25	6.08
Trp	0.11	0.10	0.12	0.09	0.10	0.11	0.10	0.09
Tau	1.32	1.30	1.27	1.18	1.10	1.21	1.16	1.01
Non- essential AA								
Asp	8.44	9.00	10.65	8.85	8.86	11.03	8.98	15.52
Ser	4.04	4.12	4.55	4.42	4.20	4.21	4.34	4.27
Glu	15.63	16.17	17.72	17.67	17.07	16.92	17.95	19.31
Gly	5.24	5.02	5.59	5.35	4.69	4.66	4.53	4.70
Ala	5.93	5.33	5.79	5.21	4.50	3.17	4.09	3.35
Pro	4.17	4.41	4.68	4.67	4.67	4.74	4.96	5.21
Tyr	1.51	1.46	2.16	1.85	1.90	1.23	1.90	1.70

Table 3. Specific activity (mU mg protein^{-1}) of amino acid catabolic in the liver (ALT, AST, AST/ALT RATIO) of *Totoaba macdonaldi* fed diets containing different levels of soybean protein concentrate (SPC).

Diets	RD	SPC15	SPC30	SPC45	SPC60	SPC75	SPC90	SPC100	PC
Amino acids catabolism									
Aspartate aminotransferase (ALT)	399.8±16.4^{a}	354.7±11.3^{b}	332.2±10.9^{b}	356.7±39.7^{b}	365.1±10.2^{b}	273.3±22.0^{c}	266.8±9.8^{c}	247.2±10.9^{c}	-0.73
Alanine aminotransferase (AST)	972.6±18.0	828.6±35.7	894.4±52.1	828.9±60.8	963.4±78.6	798.9±49.6	799.7±35.5	923.9±57.0	ns
AST/ALT	2.4±0.1^{a}	2.3±0.1^{a}	2.7±0.1^{a}	2.4±0.1^{a}	2.6±0.2^{a}	3.0±0.2^{a}	3.0±0.2^{a}	4.0±0.2^{b}	-0.71

Values are mean ±SEM (n=6). Significant differences within diets are indicated by different letters (P<0.05). Abbreviation: n.s. not significant (P > 0.05). PC (Pearson Correlation).

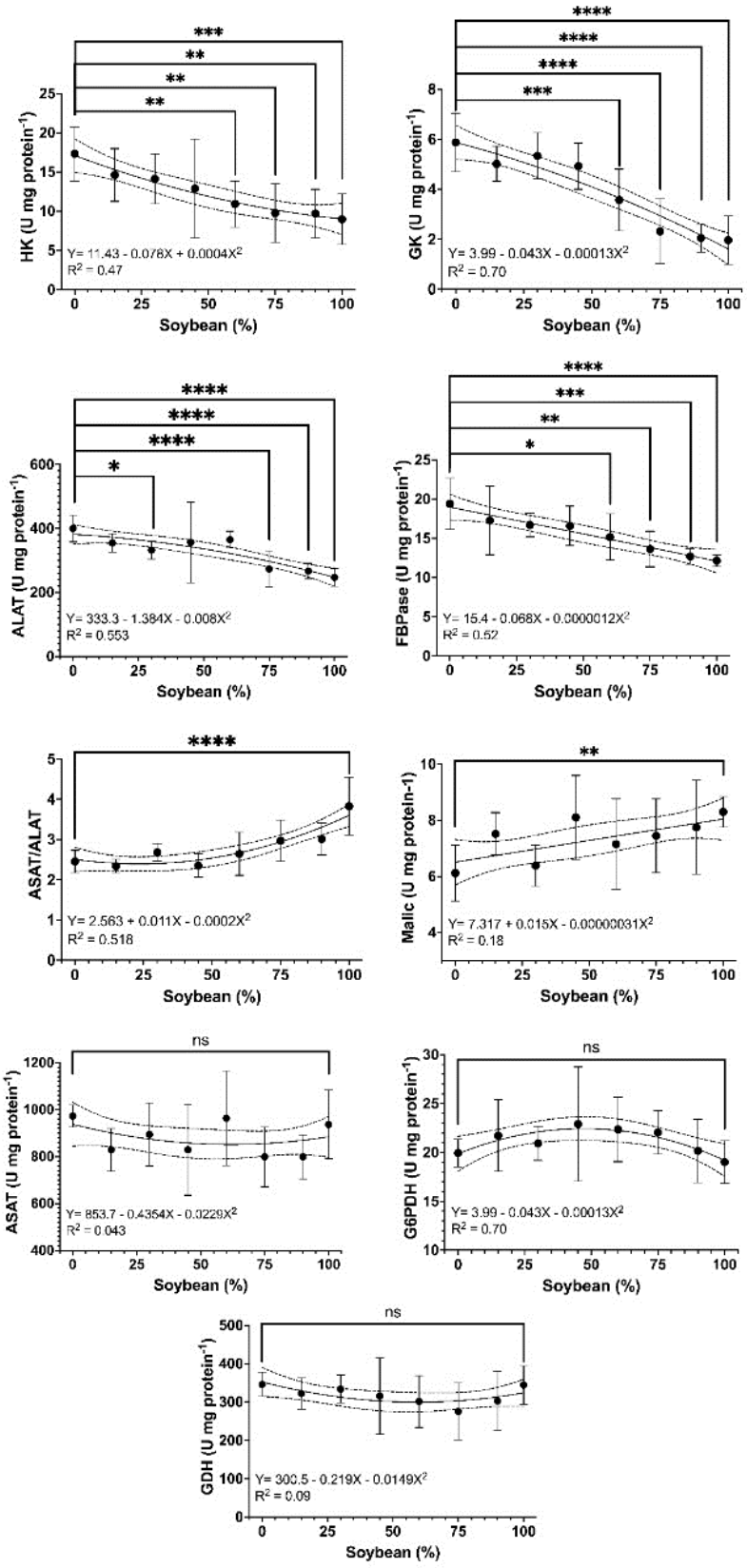

Figure 1. Non-linear fit models for the enzymatic activities according to the inclusion of soybean meal.

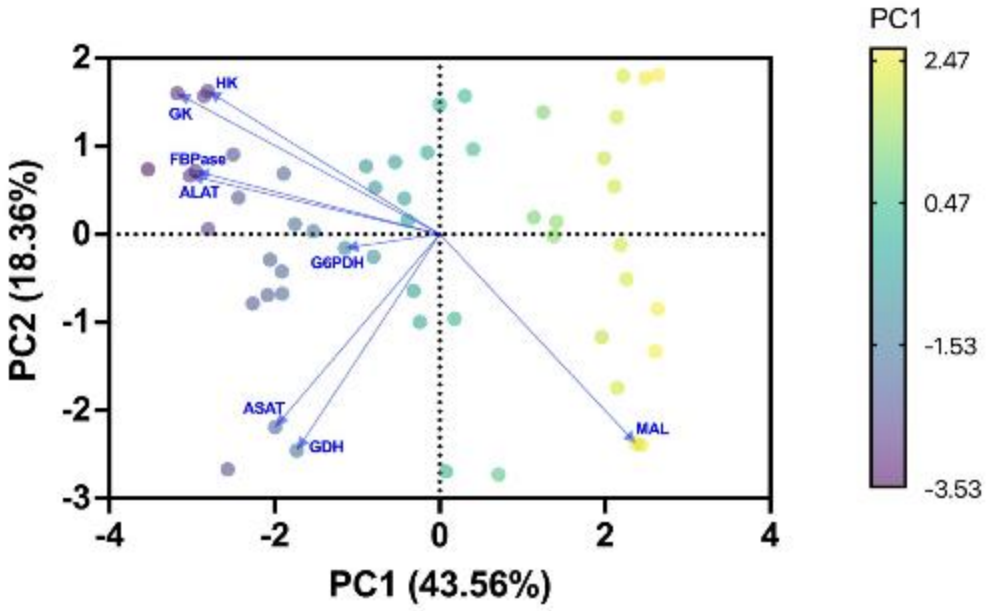

Figure 2. Principal Component Analysis (PCA) of enzymatic activities according to the soybean meal inclusion. Each dot represents the average of six biological replicate analyses of samples in the plot. Factors F1 and F2 used in this plot explain 69.89% of the total variance, which allows confident interpretation of the variation.

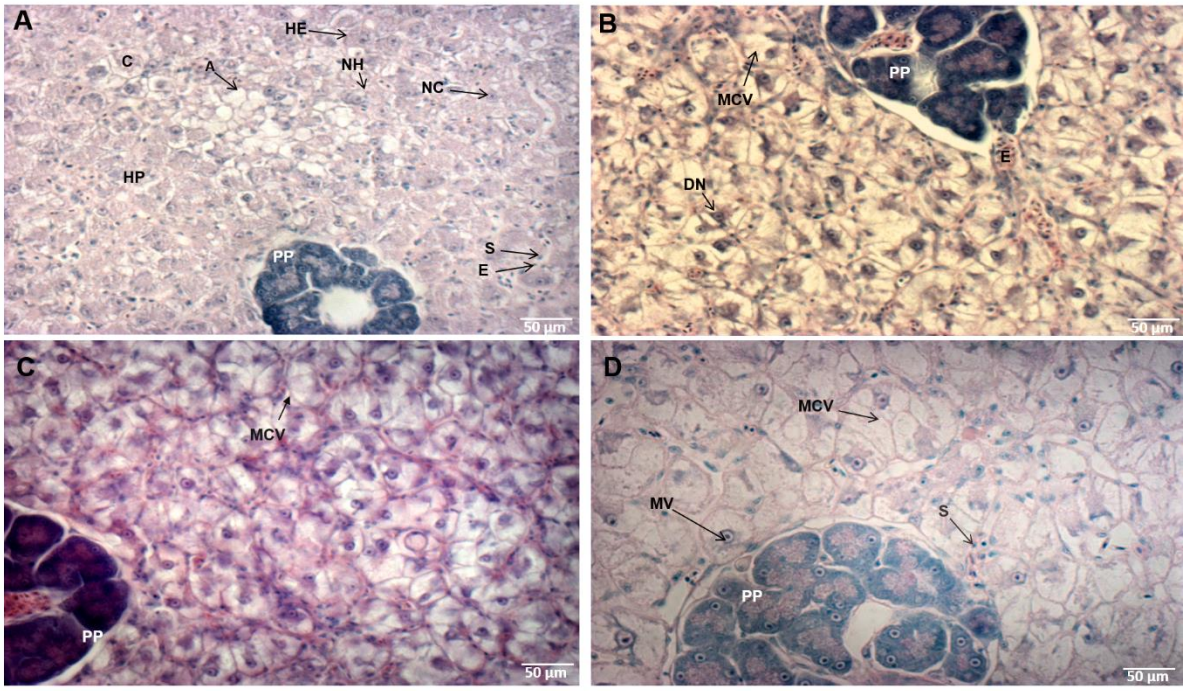

Fig. 3. (A) Sagittal section of liver of juvenile *Totoaba macdonaldi* fed reference diet (RD), showing the nucleus with normal appearance. (B) SPC 15. Hepatic parenchyma (HP) denotes normal appearance. Appearance of a fatty liver (non-pathogenic). (C). SPC30. Accumulation of lipid deposits, similar to the SPC15 diet. Most hepatocytes show contraction of the cytoplasm, denoting a fatty liver (non-pathogenic). (D). SPC45. Accumulation of glycogen and lipids within hepatocytes, which induces a fatty liver. In the pancreatic parenchyma, no damage or pyknotic nuclei are detected. Abbreviations: Hepatic parenchyma (HP); Parenchyma pancreatic (PP); Adipocytes (A); Hepatocytes (HE); Cytoplasm (C); Hepatocyte nucleus (HN); Nucleolus (NC); sinusoids (S); Erythrocytes (E); lipid inclusions (IL); Nucleus towards the periphery (DN). Hematoxylin & Eosine staining

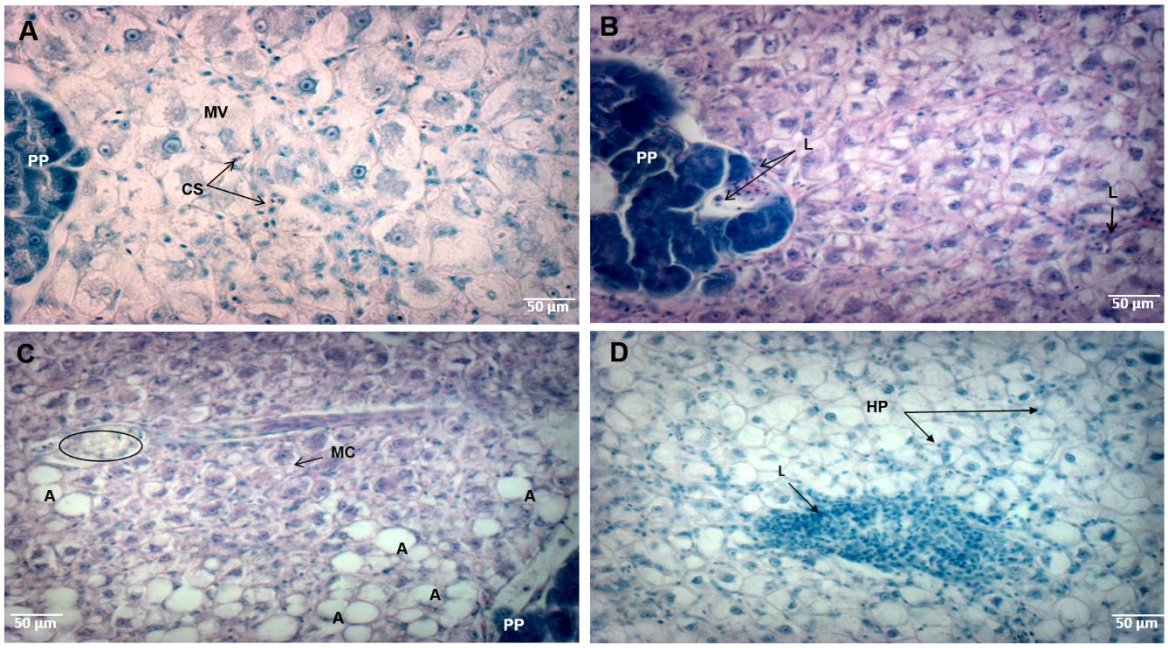

Fig. 4. (A) Sagittal section of liver of juvenile *Totoaba macdonaldi* fed with SPC60. (B) SPC75. In the hepatic parenchyma, the accumulation of reserves (glycogen and lipids) prevails compared to the control diet (RD), lymphocyte infiltrations are detected in both the liver and pancreatic parenchyma in response to some abnormal organ damage. (C). SPC90. Accumulation of lipid deposits, some areas with a large accumulation of fat, large vacuoles that involve the movement of nuclei of hepatocytes to the periphery, eventually having an atrophy in the same cell. Meanwhile, in other areas there is no deposition of energy reserves. The size of the hepatocytes in this treatment is smaller compared to the control diet (RD). The liver is not healthy, there is a greater infiltration of lymphocytes. (D). SPC100. There is a large accumulation of fat in hepatocytes, displacing the nucleus towards the periphery, denoting the presence of pyknotic nuclei. A large infiltration of lymphocytes is observed in the liver parenchyma, as well as in the pancreatic parenchyma, with greater infiltration in the central vein and periphery of the pancreatic acinus. The liver of fish fed this treatment does not appear healthy compared to the control diet (RD). Abbreviations: Hepatic parenchyma (HP); Parenchyma pancreatic (PP); Adipocytes (A);

Hepatocytes (HE); Cytoplasm (C); Hepatocyte nucleus (HN); Nucleolus (NC); sinusoids (S); Erythrocytes (E); lipid inclusions (IL); Lymphocytes (L). Hematoxylin & Eosine staining.