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Research Article

Effects of marine biofloc on the metabolism, hematology, and antioxidant capacity of juvenile red tilapia (*Oreochromis* sp.) fed commercial diets containing different protein levels

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ABSTRACT. This study evaluated the effects on the growth performance and physiological response (metabolism, hematology, and antioxidant capacity) of the juvenile hybrid red tilapia *Oreochromis* sp. (initial average weight of 2.82 ± 0.02 g) cultured in seawater with biofloc and fed commercial diets containing 25, 35 and 40% crude protein (P25, P35, and P40, respectively). After seven weeks of bioassay, fish fed P35 and P40 showed no significant differences in weight gain, specific growth rate, and condition factor. The P35 and P40 treatments showed the highest values of growth performance (SGR of 4.85 and 4.43% d^{-1}, respectively) compared to the P25 treatment (4.3% d^{-1}). Otherwise, fish from P35 showed lower activities for metabolic enzymes (ASAT, ALAT, GK, FBPase, G6PD, and FAS) than P40, which suggests that in P35, there was a better use of energy and protein content in the diets. On the other hand, P35 presented values similar to P40 regarding oxidative damage to lipids in the intestinal (5.62 and 5.77 nmol g^{-1}, respectively) and muscle (4.76 and 3.93 nmol g^{-1}, respectively) tissues, but higher than P25 in the liver (1.21 nmol g^{-1}). Finally, the P25 diet triggered a significant decrease in erythrocyte parameters, as well as total protein and albumin in the blood plasma of the fish. In conclusion, marine biofloc allows hybrid red tilapia juveniles to be fed commercial diets with 35% crude protein without compromising their productive performance, antioxidant system, and hematological parameters.

Keywords: *Oreochromis*; sea water; microbial protein; blood biochemistry; metabolic enzymes; antioxidant system

INTRODUCTION

Tilapia are among the most important groups of fish used in aquaculture worldwide due to their rapid growth, resistance to stress and disease, ease of reproduction, rapid production of fingerlings, and acceptance of formulated foods. Additionally, they are euryhaline organisms, allowing them to grow adequately in different saline conditions (El-Sayed 2020). These biological advantages are reflected in the rapid growth of related production activity and its economic value worldwide in recent years (FAO 2020). Among the different species and varieties of commonly cultivated tilapia, red hybrid tilapia (*Oreochromis* sp.)

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is of interest for aquaculture due to its morphological characteristics (shape and appearance), tolerance to saline conditions over a wide range of conditions (Barreto-Curiel et al. 2015, Putra et al. 2019), and marine systems with biofloc at high culture densities (Bañuelos-Vargas et al. 2021).

Biofloc technology (BFT) is a system with high bacterial productivity used in intensive and hyperintensive aquaculture in which the principle is to develop aggregates of microorganisms, mainly heterotrophic bacteria, through the manipulation of the carbon: nitrogen ratio (C:N ratio), which promotes the assimilation of inorganic nitrogenous compounds produced by organisms in culture into microbial proteins (Nisar et al. 2022). Thus, by using BFT, good quality culture water can be maintained (Khanjani et al. 2021) as it has been reported for tilapia culture (Avnimelech 1999, Crab et al. 2009, Ekasari et al. 2015, Putra et al. 2019, Khanjani & Sharifinia 2020). Moreover, microorganisms contained in biofloc are a potential source of nutrients (mainly protein, lipids, minerals, and vitamins) for animals in aquaculture production systems (Ballester et al. 2010, Sgnaulin et al. 2020, Khanjani et al. 2022). Considering this, BFT can contribute to the nutritional status of cultured species such as shrimp, carp, and tilapia, which results in a reduction in feed conversion rates (FCRs) due to a decrease in the consumption of formulated feed resulting from the ingestion of suspended particles in biofloc (Mansour & Esteban 2017, Green et al. 2019, Putra et al. 2019, Khanjani & Sharifinia 2020). For example, an improvement in growth performance immune and antioxidant status of Nile tilapia (*Oreochromis niloticus*) fingerlings cultured in BFT and fed 20% crude protein (CP) compared to those in clear water and fed 30% CP has been reported (Mansour & Esteban 2017). Also, using BFT allows the culture of Nile tilapia even at high densities, such as 350 fish m^{-3} (0.98 g initial weight), without compromising their growth and immune response (Haridas et al. 2017). For their part, Bañuelos-Vargas et al. (2021) reported that the combination of BFT plus the use of probiotics in the cultures improved the feed efficiency as well as antioxidant and immune responses of red tilapia reared at high-density in seawater (240 fish m^{-3}). In this study was also reported that hybrid red tilapia grown in seawater with biofloc at high densities exhibited low cell damage, as measured by lipoperoxidation by reactive oxygen species (ROSs), as well as globulin concentration (GB) values were lower (average of 1.26) in fish from BFT plus probiotics (at both densities), while the albumin: globulin ratio (AL:GB) values were highest (1.11-1.28) compared to the values (0.24-0.50 average) recorded in fish from control treatments (clear water). Authors suggested that fish grown in biofloc had better health status than those kept in seawater without biofloc (Bañuelos-Vargas et al. 2021). However, the study also showed that red tilapia grown in biofloc presented the highest viscerosomatic index (VSI) values and total lipid content in whole fish. Previous reports have indicated that an increase in available protein can promote its use as an energy source (NRC 2011), which, together with energy from lipids, could result in excess energy in organisms. High production costs in aquaculture are generated mainly by meeting food industry needs because the amount of protein in the diet still represents the most expensive and important nutrient for meeting growth needs as well as for maintaining fish health (NRC 2011, Montoya-Camacho et al. 2019, El-Sayed 2020). In freshwater-farmed Nile tilapia fingerlings and juveniles, the CP content in the diet was significantly reduced from a maximum of 36% to a minimum of 28 to 23% when the fish were kept in systems with biofloc (Hisano et al. 2020, Nguyen et al. 2021). Da Silva et al. (2018) reported that for tilapia juveniles weighing 10 to 60 g and 60 to 230 g in freshwater supplemented with biofloc, dietary protein could be reduced by up to 28% relative to CP or 22% relative to total CP, respectively; thus, this is a specific stage effect. In contrast to studies of tilapia kept in freshwater with biofloc, no reports evaluate the use of diets with different protein levels for juvenile hybrid red tilapia kept in seawater with biofloc. This study evaluated the growth performance and physiology (metabolism, hematology, and antioxidant capacity) response of juvenile red tilapia fed commercial diets containing different protein levels and maintained in seawater with biofloc.

MATERIALS AND METHODS

Obtaining experimental organisms

Juveniles of hybrid red tilapia acclimated in seawater were obtained from a commercial farm (Productos Pesqueros de Topolobampo, Sinaloa, Mexico). The fish were transported to the Aquaculture Laboratory at the Faculty of Marine Sciences, Autonomous University of Sinaloa, Mexico, where the experiment was carried out. During conditioning and before the experiment, the fish were confined in a maternity tank with seawater from the Bay of Mazatlán, Sinaloa (35 g L^{-1} medium saline concentration) and biofloc, which was formed and then maintained, as described later. During this period, the fish were fed a commercial diet (Nutripec Purina™,

40% CP), considering a feeding rate of 8% of the biomass.

Experimental design and BFT system

The experiment consisted of a feeding bioassay with three commercial diets (Nutripec, Purina™) with different levels of CP: 25, 35, and 40% CP (P25, P35, and P40, respectively). The moisture, ash, and CP contents of the commercial diets were determined using AOAC standard methods (AOAC 2011), which consist following procedures: dry matter after drying at 105°C until constant weight; ash by incineration in a muffle furnace at 550°C for 24 h; protein content (N×6.25) by the Kjeldahl method after acid digestion using a Novatech™ digestion and distillation units. The total lipid was extracted by homogenizing the sample with 2:1 chloroform-methanol (v/v) and filtering the homogenate using a modified method of Folch et al. (1957). Nitrogen-free extract (NFE) was calculated as the difference between 100% (moisture + protein + lipids + ash) according to Hardy & Barrows (2002). The proximal composition of the diets is shown in Table 1.

Table 1. Proximal composition (%, dry mater) of commercial diets fed juvenile red tilapia hybrids farmed in seawater with biofloc. CP: crude protein; TL: total lipids; NFE: nitrogen-free extract: calculations based on the protein, lipid, ash, and moisture contents in feed. P25: 25% of CP, P35: 35% of CP, and P40: 40% of CP.

	P25	P35	P40
Dry matter	88	88	88
Moisture	12	12	12
CP	25	35	40
TL	4	7	9
Ash	15	13	12
NFE	44	33	27

One hundred and ten fish with an average individual body weight of 2.82 ± 0.02 g were confined to each experimental tank containing 1.5 m^3 of seawater (35 g L^{-1}) with biofloc. The experiment, a completely randomized design with three replicates per treatment, was carried out for seven weeks (August-September 2019) in nine independent tanks. The fish were fed three times a day (08:00, 13:00, and 17:00 h) using a daily feeding rate of 8% (based on the biomass in each experimental tank estimated weekly from a sample of 20 fish in each tank). Before the bioassay, biofloc formation was promoted according to the method of Pérez-Fuentes et al. (2016) modified by Bañuelos-Vargas et al. (2021); formation was achieved by maintaining a C:N ratio of 15:1 using a mixture of corn flour and molasses and adding commercial probiotics (Aquablend™ and Aquaprotec™) and prebiotics (Nutrimax™), according to the manufacturer's instructions.

The tanks were kept in a greenhouse under natural light and photoperiod. Aeration was continuous and provided by a 2.5 HP blower and an aerotube® hose installed at the bottom of each tank to keep solids in suspension. Once a week, the tanks were siphoned to remove excess solids; 5% of the water in each tank was replaced, and the water lost through evaporation was replaced with fresh water to maintain the salinity level at 35. Water quality parameters such as salinity, oxygen, and temperature were recorded daily using a multiparameter instrument (YSI PRO2030); alkalinity, pH, total ammonia, and nitrate content were measured weekly with a potentiometer and a digital multimeter (Hanna HI 83200). When the alkalinity decreased to less than 120 mg L^{-1}, calcium hydroxide ($Ca(OH)_2$) was added. The volume of the flocs was determined using Imhoff cones, as described by Avnimelech & Kochba (2009).

Biological parameters

At the end of the experiment, total body weight (± 0.01 g) and total length (± 0.1 cm) were obtained. Survival and biological indices were calculated using the following equations (Guillaume et al. 2004):

$$\text{Survival (\%)} = \frac{\text{final number of fish}}{\text{initial number of fish}} \times 100$$

$$\text{Specific growth rate (SGR; \% d-1)} = \frac{\text{Ln final weight} - \text{Ln initial weight}}{\text{number of days of bioassay}} \times 100$$

$$\text{Weight gain (WG; g)} = \text{final weight} - \text{initial weight}$$

$$\text{Feeding conversion rate (FCR)} = \frac{\text{food intake (g)}}{\text{WG}}$$

$$\text{Protein efficiency rate (PER)} = \frac{\text{ingested protein}}{\text{WG}}$$

The condition index was estimated by the Fulton condition factor (K), which was calculated as $K = (W \times L^{-3}) \times 100$, where W is the wet weight (g) and L is the standard length (cm).

At the end of the experiment, the fish were fasted for 24 h and then captured with a net. Blood samples were collected from eight randomly selected fish in each tank. Blood samples were immediately obtained by cardiac puncture using 1 mL syringes containing 0.16 mL of a 10% EDTA solution as an anticoagulant agent (Valenzuela et al. 2002). Finally, fish were randomly collected and euthanized following the recommendations of AVMA (2001) and AAZV (2006) with an overdose of 0.5 mg L^{-1} 2-phenoxyethanol

placed in an ice bath. Fish liver, muscle, and intestine samples were instantly frozen using liquid nitrogen and stored at -80°C for subsequent determination of the activity of metabolic and antioxidant enzymes. Also, the liver and digestive system were dissected and weighed to calculate the hepatosomatic (HSI) and viscerosomatic (VSI) indices:

$$\text{HSI (\%)} = \frac{\text{liver weight}}{\text{weight of whole fish}} \times 100$$

$$\text{VSI (\%)} = \frac{\text{viscera weight}}{\text{weight of whole fish}} \times 100$$

Enzyme activity and lipid peroxidation

The metabolic enzyme activity was determined in the liver samples, and the activity of antioxidant enzymes was measured in the liver, intestines, and muscles of the fish. All the samples were homogenized on ice in five volumes of cold 100 mM Tris-HCl buffer containing 0.1 mM EDTA and 0.1% (v/v) Triton X-100 (pH 7.8). The homogenates were centrifuged at 30,000 g for 30 min at 4°C, and the supernatants were immediately stored at -80°C until use (Pérez-Jiménez et al. 2012). All enzymatic assays were performed at 25°C and analyzed in duplicate in a 96-well microplate spectrophotometer (Multiskan GO, Thermo Scientific™). The soluble protein concentration was determined according to Bradford (1976) using a Bio-Rad protein assay kit with bovine serum albumin as the standard protein. The concentration of the substrate and the optimal protein for measuring the maximum activity of each enzyme was determined via preliminary tests. The activities of aspartate aminotransferase (ASAT/GOT; EC 2.6.1.1) and alanine aminotransferase (ALAT/GPT; EC 2.6.1.2) were analyzed with Pointe Scientific kits. Glutamate dehydrogenase activity (GDH; EC 1.4.1.2) was determined using a reaction mixture containing 71.4 mM imidazole-HCl (pH 7.4), 2.9 mM NADH, 14.3 mM ADP, 3.3 M ammonium acetate and 2 units mL^{-1} LDH (Morales et al. 1990). The activities of hexokinase (HK; EC 2.7.1.1) and glucokinase (HK-IV; EC 2.7.1.2) were determined according to methods described by Vijayan et al. (1990). The reaction mixture contained 71.4 mM imidazole-HCl (pH 7.4), 50 mM ATP, 100 mM $MgCl_2$, 8 mM NADP, 2 units mL^{-1} G6PD and 10 mM (HK) or 1 M (HK-IV) glucose. The activities of fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11) and glucose 6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) were determined according to methods described by Morales et al. (1990). The activity of the malic enzyme (ME; EC 1.1.1.40) was determined using a reaction mixture containing 71.4 mM imidazole-HCl buffer (pH 7.4), 100 mM $MgCl_2$, 8 mM NADP, and 40 mM L-malate (Singer et al. 1990). The activity of fatty acid synthase (FAS, EC 2.3.1.38.) was determined according to the methodology described by Chang et al. (1967) and modified by Chakrabarty & Leveille (1969).

The specific assay conditions for measuring the activity of the enzyme superoxide dismutase (SOD; EC 1.15.1.1) were determined following the methodology described by McCord & Fridovich (1969). Catalase activity (CAT; EC 1.11.1.6) was determined according to the method described by Aebi (1984). The activity of the enzyme glutathione peroxidase (GPX; EC 1.11.1.9) was measured following the methods described by Flohé & Günzler (1984); glutathione reductase activity (GR; EC 1.6.4.2) was determined as described by Morales et al. (2004). The levels of lipid peroxidation were determined based on the levels of malon-dialdehyde (MDA) according to the methodology described by Buege & Aust (1978) and modified by Pérez-Jiménez et al. (2009).

Hematological parameters

Blood samples were analyzed individually. The total erythrocyte count (EC, 10^6 cells μL^{-1}) was individually analyzed according to the modified method described by Blaxhall & Daisley (1973). The hematocrit (HT, %) was determined by microcapillary analysis with a glass tube and centrifugation (10,000 rpm for 5 min). Red cell indices, including the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), were calculated with EC, HT, and the concentration of hemoglobin (HB) (Torrens 2015). The remaining blood sample was centrifuged at 12,000 rpm for 5 min at 4°C to obtain the plasma (Atencio-García et al. 2007). The HB (g dL^{-1}), glucose (GLU, mg dL^{-1}), total protein (TP, g dL^{-1}), albumin (ALB, g dL^{-1}), total cholesterol (CHOL, mg dL^{-1}), and high-density lipoproteins (HDL, mg dL^{-1}) were measured by standardized procedures using Pointe Scientific kits. The globulin concentration (GB, g dL^{-1}) was calculated as the difference between the TP and serum ALB concentration.

Statistical analysis

The results are reported as the mean ± standard deviation. A completely randomized design was used in this study. The normality of distributions and homoscedasticity of variances were tested using the Kolmogorov-Smirnov and Levene tests, respectively. Data from growth performance, enzyme activities of liver metabolism and antioxidant systems, and hematology and biochemical of blood plasma were statistically analyzed by one-way analysis of variance

(ANOVA). The differences among the means were determined using Tukey's HSD test. Where the assumption of homoscedasticity was not satisfied even after data transformation (data from water quality parameters), a Kruskal-Wallis nonparametric test was performed. A significance level of $P < 0.05$ was used for all the statistical tests. The data were analyzed using the statistical program SigmaPlot™ 10.0.

RESULTS

In the present study, the total ammonia nitrogen content (TAN; Fig. 1a) was similar among the treatment groups during the first five weeks of cultivation; however, in the sixth week of feeding, the TAN content was significantly higher ($P < 0.05$) in the P40 (3.63 ± 0.82 mg L^{-1}) and P35 (3.40 ± 1.84 mg L^{-1}) treatment groups than in the P25 treatment group (2.26 ± 0.40 mg L^{-1}). The concentrations of nitrites (Fig. 1b) and nitrates (Fig. 1c) did not significantly differ among the treatments during the entire experimental period.

The pH values varied significantly among the treatment groups throughout the experiment, with a decreasing trend in the P40 group during weeks 2 (7.6 ± 0.3) and 5 (7.3 ± 0.1) for the P25 group (8.3 ± 0.1 and 7.6 ± 0.1, respectively) (Fig. 2a). The total alkalinity concentration (Fig. 2b) did not significantly differ among the treatment groups throughout the experiment. The floc volume showed a trend toward a significant increase ($P < 0.001$) for cultivation time in all the treatment groups (Fig. 2c).

Regarding the productive parameters (Table 2), the red tilapia juveniles in the P35 and P40 treatment groups presented significantly equal final weights, weights gained, SGR, HSI, and K indices, which were also significantly greater ($P < 0.05$) than those in the P25 treatment group. In contrast, the FCR was higher in the P25 group (1.37 ± 0.02) than in the P35 and P40 groups (0.90 ± 0.06 and 1.00 ± 0.02, respectively). The PER values were significantly greater in the P35 group (3.20 ± 0.21) than in the P40 group (2.49 ± 0.07). Similarly, the VSI values were significantly higher in the P35 group than in the P25 and P40 groups, but there were no significant differences in HSI among the treatment groups.

The activities of key enzymes involved in hepatic intermediate metabolism are shown in Table 3. The activities of the enzymes ASAT, ALAT, FBPase, and FAS in the P40 treatment group were significantly greater than those in the P35 treatment group. The activities of the enzymes GDH, HK, and ME did not significantly differ among the treatment groups. However, the P25 treatment group had the highest GK enzyme activity among the treatment groups. Similarly, G6PD activity was higher in the P25 treatment than in the P35 treatment, but its values were similar to the P40 treatment.

Regarding the response of the fish in the P35 treatment group to the antioxidant defense system, the activities of SOD, CAT, GPX, and GR in the liver were significantly lower than those in the fish in the P25 treatment group (Table 4). In contrast, the level of lipoperoxidation (as measured by the MDA content) in this organ significantly increased with increasing protein content in the diet. Concerning the intestine, a trend toward increased CAT activity was observed with increasing protein intake. Still, there were no differences in the activities of SOD, GPX, GR, or MDA among the treatment groups. In muscle, there were no significant differences in the activity of the different antioxidant enzymes or the MDA levels among the treatment groups.

Table 5 shows the juvenile red tilapia's hematological and blood biochemical data. A significant tendency toward an increase in the EC and HT values was observed with decreasing CP content in the diets; in contrast, the MCH and MCHC parameters showed a significant decreasing trend in their values in the fish-fed diets with lower protein content. No significant differences regarding plasma biochemistry were observed in GLU concentration among the treatment groups. However, the TP, ALB concentrations, and the ALB:GB ratio decreased in fish fed a diet with a lower protein content, in contrast to the concentration of GB, for which the P25 treatment group presented significantly greater values of this parameter. The concentrations of CHOL and HDL did not significantly differ among the treatment groups.

DISCUSSION

Previous studies carried out with Nile tilapia and red tilapia grown in biofloc in freshwater have indicated that variation in the levels of CP in their diets does not influence water quality parameters such as pH or the concentrations of TAN, nitrites, and nitrates during the first weeks of cultivation (Green et al. 2019, Hisano et al. 2020). Similar results were observed in the present study, which worked in seawater with biofloc. Also, the pH and floc concentration were kept within the recommended ranges for tilapia cultivation (Emerenciano et al. 2017). Otherwise, to maintain alkalinity above 100 mg L^{-1}, it was necessary to add calcium hydroxide

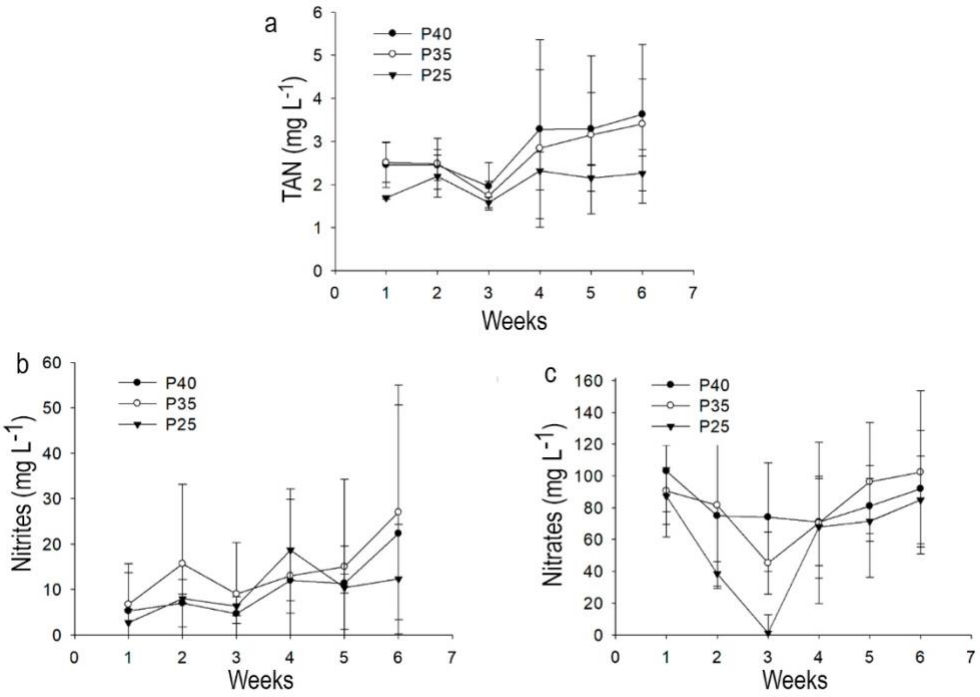

Figure 1. a) Total ammonium nitrogen (TAN), b) nitrites, and c) nitrates in the seawater with biofloc of juvenile red tilapia hybrids fed with different levels of crude protein (CP) in commercial diets where P25: 25% of CP, P35: 35% of CP, and P40: 40% of CP. Different lowercase superscripts indicate significant differences between protein levels when ($P < 0.05$).

to the water for all the treatment groups and, consequently, to maintain the buffering capacity of the seawater and pH values greater than 7 during the experiment, as recommended by Furtado et al. (2011) and Miranda-Baeza et al. (2017). The maximum TAN levels recorded (2.2-3.6 mg mL^{-1}) in the last week of the present study were similar to those reported for BFT systems with early juvenile red tilapia by Miranda-Baeza et al. (2017), who reported average values of 3 to 5 mg L^{-1}. Notably, the levels of ammonium, nitrites, and nitrates recorded in this study were above the recommended (ideal) levels for the cultivation of juvenile tilapia (<1, <1, and 0.5-20 mg L^{-1}, respectively) (Emerenciano et al. 2017). However, it has been reported that the tolerance of tilapia to different levels of nitrogenous residual compounds is related to different factors, but mainly to the water's pH and the organism's size. Thus, Timmons et al. (2002) indicated that a maximum TAN concentration of 3 mg L^{-1} is safe for temperate water fish if the pH is between 6.5 and 8.5, conditions under which ammonia is ionized (NH_4^+), the least toxic form for fish (López-Elías et al. 2015). Interestingly, at week 6 of the bioassay in the present study, the P25 and P40 treatment groups had significantly different TAN concentrations. These results could be related to the high catabolic activity of the amino acids in the P40 treatment group, which will be discussed later.

In euryhaline fish such as tilapia, osmoregulation may affect the efficiency and feed conversion rate (Rubio et al. 2005, Ridha 2015). Previous studies such as Barreto-Curiel et al. (2015) and Bañuelos-Vargas et al. (2021) reported they used a commercial diet with high protein content (40% CP) to feed early (3-42 g) juveniles of hybrid red tilapia when culture in marine systems, even with biofloc. Of course, the amount of protein required also depends on the quality (e.g. amino acid profile) and bioavailability of the nutrients. Moreover, it is known that an excessive dietary protein level is not economically feasible for fish culture because the ingredients that provide this nutrient are responsible for a large part of the feed cost. Since BFT can contribute to the nutritional status of tilapia as an additional protein source to that provided by commercial food for tilapia grown in freshwater (Da Silva et al. 2018, Hisano et al. 2020, Zablon et al. 2022) and brackish water (Durigon et al. 2020), therefore, dietary protein levels can be reduced without compromising fish growth, resulting in a significant reduction in production costs (Ekasari et al. 2023). Under this context, the present research focused on evaluating the adjustments of red tilapia hybrids fed

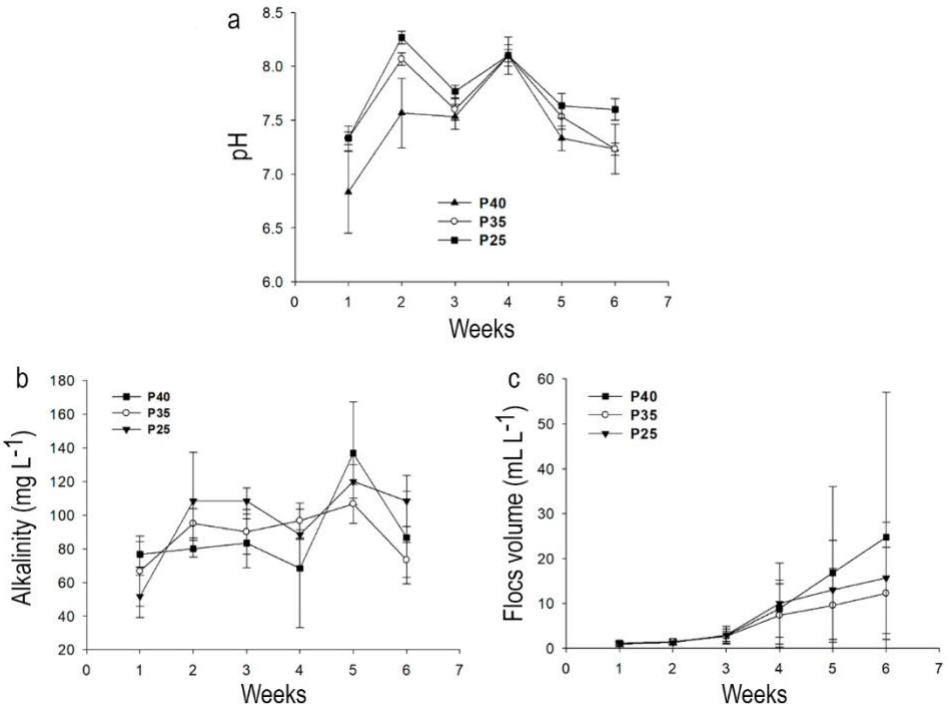

Figure 2. a) pH, b) total alkalinity, and c) flocs volume in the seawater with biofloc of juvenile red tilapia hybrids fed with different levels of crude protein (CP) in commercial diets where P25: 25% of CP, P35: 35% of CP, and P40: 40% of CP. Different lowercase superscripts indicate significant differences between protein levels when ($P < 0.05$).

different CP levels from commercial diets cultured in seawater with biofloc.

Our first results showed no significant differences in the survival of red tilapia among the treatments with different protein levels in the diet (25 to 40%). However, the organisms' weight increase was mainly influenced by the lower dietary protein, where fish fed a diet with 25% CP exhibited the least weight gain. In comparison, fish fed a diet containing 35 and 40% CP presented similar weight gain. Hisano et al. (2020) reported that the growth performance of early juveniles (6-30 g) of Nile tilapia grown in freshwater with biofloc was similar when diets with CP levels of 28 to 36% were used, thus allowing an 8% reduction in dietary protein. However, in the present study, the fish fed only 25% CP presented lower productive performance and feed efficiency than the tilapia fed 35% CP. Therefore, it is possible that the protein requirement of early juveniles of tilapia maintained in seawater (salinity of 35) is greater than that of Nile tilapia cultivated in freshwater due to increased metabolic energy costs. These processes involve the osmoregulation of tilapia grown in saline water (Nassar et al. 2021). Such energy costs could be partly covered by the use of microbial proteins provided by biofloc, which has been reported for Nile tilapia grown in brackish water with biofloc (Durigon et al. 2020) and for red tilapia in seawater (Bañuelos-Vargas et al. 2021).

Therefore, research on adjustments or changes in nutrient metabolism under different cultures or feeding conditions is important for obtaining a better understanding of the growth responses of fish. Thus, to our knowledge, this study is the first to analyze the activity of metabolic and antioxidant enzymes in red tilapia hybrids grown in seawater supplemented with biofloc and fed different levels of proteins. The activity of the hepatic enzymes ASAT and ALAT (responsible for amino acid catabolism) increased in P40-fed fish compared to P25-fed fish. Similar results were reported by Gaye-Siessegger et al. (2006) and Figueiredo-Silva et al. (2013) in juvenile Nile tilapia fed increasing levels of dietary protein. Therefore, the results suggest that the juveniles of hybrid red tilapia fed 40% CP receive excess dietary protein, which is not deposited as tissue protein but is directed toward amino acid catabolism.

On the other hand, the activity of GK, a key enzyme in glycolysis, increased significantly in the 25% CP treatment group (and with a greater content of carbohydrates in the diet). Several studies have shown that the partial replacement of proteins by carbohydrates can

Table 2. Growth performance of juvenile red tilapia hybrids fed with different levels of crude protein (CP) in commercial diets and farmed in seawater with biofloc. The values are mean ± standard deviation. Diet P25: 25% of CP, P35: 35% of CP, and P40: 40% of CP. WG: weight gain; SGR: specific growth rate; FCR: food conversion rate; PER: protein efficiency ratio; VSI: viscerosomatic index; HSI: hepatosomatic index; K: Fulton's condition factor. Values in the same row with different superscripts mean significant difference ($P < 0.05$).

	P25	P35	P40
Initial weight (g)	3.18 ± 0.63	3.19 ± 0.57	3.05 ± 0.53
Final weight (g)	33 ± 0.4^{b}	41 ± 2.3^{a}	42 ± 2.0^{a}
WG (g)	30 ± 0.4^{b}	38 ± 2.3^{a}	40 ± 2.0^{a}
SGR (% d^{-1})	4.30 ± 0.03^{b}	4.85 ± 0.15^{a}	4.43 ± 0.13^{a}
FCR	1.37 ± 0.02^{a}	0.90 ± 0.06^{b}	1.00 ± 0.02^{b}
PER	2.92 ± 0.04^{ab}	3.20 ± 0.21^{a}	2.49 ± 0.07^{b}
VSI	16.8 ± 1.28^{b}	19.6 ± 2.03^{a}	16.1 ± 1.38^{b}
HSI	2.63 ± 0.88^{b}	3.19 ± 0.78^{ab}	4.17 ± 0.38^{a}
K	2.20 ± 0.20^{b}	2.34 ± 0.22^{a}	2.36 ± 0.20^{a}

Table 3. Enzyme activity (mU mg protein^{-1}) of liver metabolism from juvenile red tilapia hybrids fed with different levels of crude protein (CP) in commercial diets and farmed in seawater with biofloc. The values are mean ± standard deviation. Diet P25: 25% of CP, P35: 35% of CP, and P40: 40% of CP. ASAT: aspartate aminotransferase. ALAT: alanine aminotransferase. GDH: glutamate dehydrogenase. GK: glucokinase. HK: hexokinase. G6PD: glucose-6-phosphate dehydrogenase. FBPASE: fructose-1,6-bisphosphatase. FAS: fatty acid synthase. ME: malic enzymes. Values in the same row with different superscripts mean significant difference ($P < 0.05$).

	P25	P35	P40
ASAT	297 ± 26^{ab}	265 ± 65^{b}	417 ± 31^{a}
ALAT	185 ± 31^{ab}	144 ± 55^{b}	254 ± 39^{a}
GDH	18.1 ± 1.5	17.3 ± 4.7	16.4 ± 9.3
GK	10.3 ± 1.25$^{\mathbf{a}}$	3.48 ± 1.64$^{\mathbf{b}}$	5.09 ± 2.96$^{\mathbf{b}}$
HK	3.59 ± 2.77	1.37 ± 0.75	2.86 ± 2.53
FBPase	3.75 ± 0.54^{ab}	2.81 ± 0.70^{b}	3.87 ± 0.49^{a}
G6PD	437 ± 42^{a}	303 ± 58^{b}	332 ± 64^{ab}
FAS	3.73 ± 0.86$^{\mathbf{ab}}$	1.63 ± 0.80$^{\mathbf{b}}$	5.64 ± 1.27$^{\mathbf{a}}$
ME	6.5 ± 4.3	15.7 ± 2.89	11.7 ± 4.68

Table 4. Antioxidant enzyme activity and lipoperoxidation (like MDA) of tissues from juveniles of red tilapia fed with different levels of crude protein (CP) in commercial diets and farmed in sea water with biofloc. The values are mean ± standard deviation. Diet P25: 25% of CP, P35: 35% of CP, and P40: 40% of CP. SOD (U mg protein^{-1}): superoxide dismutase; CAT (U mg protein^{-1}): catalase; GPX (mU mg protein^{-1}): glutathione peroxidase; GR (mU mg protein^{-1}): glutathione reductase; MDA (nmol g liver^{-1}): malondialdehyde. Values in the same row with different superscripts mean significant difference ($P < 0.05$).

	P25	P35	P40
Liver			
SOD	237 ± 17^{a}	178 ± 38^{b}	231 ± 16^{a}
CAT	89.4 ± 34.7^{a}	34.6 ± 4.80^{b}	38.9 ± 3.31^{b}
GPX	4.54 ± 1.18^{a}	2.45 ± 0.47^{b}	3.22 ± 1.04^{ab}
GR	2.08 ± 0.67^{a}	0.82 ± 0.17^{b}	2.11 ± 0.45^{a}
MDA	1.21 ± 0.42^{b}	3.13 ± 1.26^{a}	4.33 ± 0.71^{a}
Intestine			
SOD	145 ± 48	141 ± 77	167 ± 52
CAT	19.6 ± 4.89^{b}	25.7 ± 7.88^{ab}	38.8 ± 7.66^{a}
GPX	3.53 ± 0.55	4.71 ± 0.65	4.32 ± 1.06
GR	1.74 ± 0.41	2.28 ± 1.23	3.03 ± 0.67
MDA	5.89 ± 1.42	5.62 ± 1.15	5.77 ± 1.40
Muscle			
SOD	84.1 ± 32	103 ± 17	104 ± 34
CAT	1.31 ± 0.55	1.32 ± 0.33	1.80 ± 0.46
GPX	2.64 ± 0.40	2.18 ± 0.34	2.19 ± 0.47
GR	0.81 ± 0.12	0.79 ± 0.14	0.86 ± 0.31
MDA	3.36 ± 1.08	4.76 ± 1.43	3.93 ± 0.98

increase the metabolic activity of liver glycolysis (Figueiredo-Silva et al. 2013, Xiong et al. 2014, Boonanuntanasarn et al. 2018), which indicates that tilapia adapt and regulate their metabolism to increase the levels of digestible carbohydrates (e.g. starch) intheir diets (Azaza et al. 2015, Boonanuntanasarn et al. 2018), the latter of which are adaptations of herbivorous fishes and omnivores. However, the decreased growth and protein efficiency observed in the P25 treatment group also indicated that the increase in glycolysis was insufficient to meet the metabolic requirements of these fish. Notably, the GK activity was similar between the P35 and P40 treatments; these treatments also resulted in similar weight gain values but presented differences in the PER, which was greater in the P35 group than in the P40 group. In addition, compared with those in the P40 and P25 treat-

Table 5. Hematology and biochemical of blood plasma from juvenile red tilapia hybrids fed with different levels of crude protein (CP) in commercial diets and farmed in seawater with biofloc. The values are mean ± standard deviation. Diet P25: 25% of CP, P35: 35% of CP, and P40: 40% of CP. EC: total erythrocyte count, HT: hematocrit, HB: hemoglobin, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, and MCHC: mean corpuscular hemoglobin concentration. GLU: glucose; TP: total protein; ALB: albumin; GB: globulins; AL:GB, albumin:globulins ratio; CHOL: cholesterol; HDL: high-density lipoproteins. Values in the same row with different superscripts mean significant difference ($P < 0.05$).

	P25	P35	P40
EC (10^6 cells μL^{-1})	2.28 ± 0.3^a	2.00 ± 0.42 ^{b}	1.9 ± 0.5^b
HB (g dL^{-1})	12.6 ± 1.49	13.0 ± 1.34	13.0 ± 1.3
HT (%)	45.0 ± 10.5	43.8 ± 6.4	39.3 ± 6.1
MCV (fL)	197 ± 35.2	239 ± 108	235 ± 128
MCH (pg)	55.6 ± 4.7	70.4 ± 28.2	77.5 ± 40.6
MCHC (%)	28.7 ± 3.6^b	30.4 ± 3.2^b	33.4 ± 2.9^a
GLU (mg dL^{-1})	44 ± 14^b	60 ± 19^a	51 ± 15^{ab}
TP (g dL^{-1})	3.65 ± 0.7 7^a	2.49 ± 0.45^b	2.50 ± 0.28^b
ALB (g dL^{-1})	0.93 ± 0.33^b	1.81 ± 0.86^a	1.63 ± 0.24^a
GB (g dL^{-1})	2.60 ± 0.53^a	0.68 ± 0.59^b	0.86 ± 0.34^b
ALB:GB	0.35 ± 0.09^b	2.36 ± 2.08^a	2.71 ± 2.97^a
CHOL (mg dL^{-1})	171 ± 84	187 ± 82	156 ± 89
HDL (mg dL^{-1})	76.1 ± 20	91.6 ± 30	82.9 ± 25

ment groups, the gluconeogenesis (FBPase) and lipogenesis (FAS and G6PD) values were lower in the P35 treatment group. This finding suggested, on the one hand, that the sum of the nutrients in the P40 diet plus those provided by the biofloc induced greater catalytic activity toward amino acids, an effect that was previously reported in tilapia juveniles kept in biofloc in brackish water (Durigon et al. 2020), but also generated a surplus of energy that was directed toward the production of lipids (increased lipogenesis was observed through increased FAS activity). The increased activity of ALAT and ASAT serves as a stress indicator in tilapia (Haridas et al. 2017).

Interestingly, the ASAT and ALAT levels in liver tissue were lower in the fish fed the P35 diet than in the fish fed the other diets, an effect related to lower stress levels in these organisms. However, for the P25 treatment group, even when the available protein levels were insufficient to maintain the maximum protein efficiency, the lipogenesis activity in these fish seemed to have been stimulated by excess carbohydrates in the diet. These results are consistent with previous findings of research carried out with juvenile Nile tilapia (Azaza et al. 2015, Boonanuntanasarn et al. 2018) and red hybrid tilapia (Green et al. 2019, Bañuelos-Vargas et al. 2021). Thus, in the present study, the analysis of the key enzymes involved in hepatic intermediate metabolism showed that the best balance of nutrients was observed in the P35 treatment group.

Previous investigations carried out in fish revealed that the intake of diets with high protein or lipid contents can induce a greater generation of free radicals and, as a consequence, increase the activity of the enzymes of the antioxidant defense system (Azaza et al. 2020, Ebrahimi et al. 2020). Therefore, an increase in the tissue concentration of MDA, a product of oxidative damage caused by lipids (lipoperoxidation), is an important indicator of an increase in cellular damage due to oxidative stress (Flores-Méndez et al. 2022). In the present study, the range of MDA values (1.21-4.23 nmol g $liver^{-1}$) was lower than that previously reported in our study with hybrid tilapia grown in marine biofloc at different densities (Bañuelos-Vargas et al. 2021). These findings suggest that the cultivation conditions and variety of tilapia cultivated influence the MDA levels of the fish. The low levels of MDA observed in the P25 treatment group could be related to the significant increase in the activity of the antioxidant enzymes (CAT and SOD) as previously has been reported for hepatic tissues of tilapia fish (Flores-Méndez et al. 2022).

Similarly, the decrease in MDA in the P25 treatment group could be the result of an additive effect derived from the high G6PD activity because feeding organisms high levels of carbohydrates induces an increase in the activity of this enzyme (Hemre et al. 2002), which plays an important role in the ability of GR enzymes to regenerate the reduced glutathione that is

used by GPX, preventing damage caused by free radicals (Hoseinifar et al. 2020). In this sense, the increase in the activity of the G6PD enzyme, as observed in this study, could be integral to the antioxidant defense system in hybrid red tilapia. However, despite the significantly lower CAT activity in the intestinal tissues of the fish fed the P25 diet than in those fed the other diets, there were no significant differences in the MDA levels among the treatment groups. Regarding the fillet (or muscle), there were no significant differences in the response of the antioxidant system or MDA levels of the fish-fed diets with different protein levels. Similarly, Battisti et al. (2020) mentioned that cultivating *Rhamdia quelen* catfish in biofloc favors decreasing MDA levels in muscle tissue and gills, even when maintained at high densities. Likewise, the antioxidant activity and MDA results for the intestinal and muscular tissue of the red hybrid tilapia juveniles in this study may have been affected by the biofloc in which they were cultivated, which may have prevented cellular damage caused by a reduction in the protein content of the diet. However, because these are the first results of the evaluation of the antioxidant response in the tissues of juvenile red tilapia hybrids under marine biofloc conditions and different nutritional conditions, additional in-depth studies are needed to gain a better understanding of the effects of the variation in nutrients in the diet when different ontogenetic stages of hybrid red tilapia are cultivated in marine conditions with biofloc.

Hematological and blood biochemical parameters are recognized as important indicators of the health and well-being of fish under different environmental, physiological, and nutritional conditions (Hrubec & Smith 2010, Lopez et al. 2015, Román-Reyes et al. 2020, Bañuelos-Vargas et al. 2021). In the present study, the EC, HB, HT, MCV, MCH, and MCHC values were similar between the fish-fed diets containing 35 and 40% CP; however, the fish fed the P25 diet had increased EC and HT values, but the MCH and MCHC values decreased. Erythrocytes are known to decrease in volume as they age (lower MCV values), with immature erythrocytes exhibiting greater volume (Hrubec & Smith 2010); therefore, the results of the present investigation suggest that the MCH and MCHC parameters indicate a deficit of dietary protein in hybrid tilapia fed only 25% CP, even with the benefits of marine biofloc. These conclusions are further reinforced by the PT and ALB concentrations and the ALB:GB ratio, which decreased in the fish-fed diets with a lower protein content. ALB concentration is an important indicator of the physiological condition of farmed fish (Román-Reyes et al. 2020, Bañuelos-Vargas et al. 2021) because one of the main functions of albumin is to store amino acids; therefore, a decrease in ALB concentration is usually related to a protein deficit in the diet (Chernyavskikh et al. 2019). Also, Javed & Usmani (2015) reported that ALB:GB values less than 0.8 are indicators of metabolic adjustments caused by different stressors. In our study, the concentration of GB in the P35 and P40 treatment groups was significantly higher compared with those in the P25 treatment groups. These results were reflected by the significant decrease in the ALB:GB ratio in the P25 treatment group. In a previous study, we found similar results in hybrid tilapia subjected to high cultivation densities in marine systems without biofloc, which were related to the high-stress conditions at a very high culture density (Bañuelos-Vargas et al. 2021). However, because in the present research, the main factor was the reduction in the CP in the diet, our results seem to indicate that early juveniles of red tilapia hybrids may have different essential nutrient requirements, so further studies are necessary for a better understanding of the effects of the nutritional contributions of marine biofloc in the feeding of hybrid tilapia in different ontogenic stages.

CONCLUSIONS

Cultured early juveniles of red tilapia in seawater with BFT improve their protein efficiency by reducing the requirement of CP concentration in the diet by more than 10%, allowing the replacement of 40% CP commercial diets with those diets containing only 35% CP without altering growth, the antioxidant defense capacity of the fish or their health conditions. The above reduction seems related to a more efficient metabolic adjustment of nutrients. Nevertheless, biofloc did not compensate for further reducing diet to 25% CP, which negatively affects the growth performance of tilapia. Further studies are necessary to identify a minimum protein concentration contained in diets without compromising the growth and health of red tilapia in different ontogenic stages cultured in BFT and to determine if other non-protein compounds provided by the biofloc are limiting nutrients. Further research is needed to understand the role of the antioxidant system in the gut and muscle of these fish, as well as the effects of marine biofloc on digestive capacity and fillet quality.

Credit author contribution

E. Martínez-Montaño: methodology, data curation, formal analysis, writing-original draft, review, and editing; G.A. Rodríguez-Montes de Oca, J.C. Román-

Reyes, R. Gómez-Ávila & D. Castañeda-Farias: methodology, validation, supervision, review, and editing; J.A. Salazar-Leyva, C. Hernández, & D.J. López-Peraza: formal analysis, review and editing; I. Bañuelos-Vargas: conceptualization, validation, funding acquisition, project administration, supervision, formal analysis, methodology, data curation, original draft, review and editing. All authors have read and accepted the published version of the manuscript.

Conflict of interest

The authors declare no potential conflict of interest in this manuscript.

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