R. Bras. Zootec., 51:e20210067, 2022
https://doi.org/10.37496/rbz5120210067

Biometeorology and animal welfare
Full-length research article

Revista Brasileira de Zootecnia

Brazilian Journal of Animal Science
e-ISSN 1806-9290
www.rbz.org.br

Interactive effects of digestible protein levels on thermal and physical stress responses in Nile tilapia

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ABSTRACT - The effects of dietary digestible protein (DP) levels (22, 26, 29, 32, and 34%) and different stressors (cold-induced stress, CIS; heat/dissolved oxygen-induced stress, HDOIS; transport-induced stress, TIS; and size-sorting-induced stress, SSIS) on hemato-biochemical parameters were evaluated. Four hundred and forty Nile tilapia fingerlings were distributed into 40-250 L aquaria and fed experimental diets for 110 days, and fed each of the five experimental diets, that were randomly distributed to eight replicates per treatment. Then, different groups of fish were subjected to one type of stress. Groups of 40 fish were used on CIS (17 °C), HDOIS (32 °C), and TIS (4 h), and a group of 140 fish on SSIS (15 min air exposure and 60 s handling). There was no effect on hemato-biochemical profile when DP levels were compared, neither before nor after stress; however, there was a significant stress effect. Digestible protein did not mitigate stress response under SSIS and CIS; lymphopenia and neutrophilia were the main cell-mediated immune response; dietary 22 and 26% DP impaired oxygenation on SSIS and TIS; fish under HDOIS and SSIS demanded more energy using triglycerides as an energy source; the diet formulated to contain 22% DP was not adequate to keep homeostasis under temperature stress. Cluster analysis showed that, for DP levels below the requirement for growth, SSIS and CIS were considered the most stressful conditions. At 34% DP level, HDOIS response was comparable to that of non-stressing conditions.

Keywords: Cluster analysis, fish health, *Oreochromis niloticus*, physiological response, stressors

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Received: April 12, 2021
Accepted: May 23, 2022

How to cite: Freitas, J. M. A.; Peres, H.; Carvalho, P. L. P. F.; Furuya, W. M.; Sartori, M. M. P.; Pezzato, L. E. and Barros, M. M. 2022. Interactive effects of digestible protein levels on thermal and physical stress responses in Nile tilapia. Revista Brasileira de Zootecnia 51:e20210067. https://doi.org/10.37496/rbz5120210067

1. Introduction

Intensive aquaculture production is accompanied by stressful situations, which may affect not only physiological responses and homeostasis, but also profitability, production, and sustainability of aquaculture industry worldwide (Hassan et al., 2021). Such stress response is characterized by hormonal, physiological, metabolic, molecular, and genetic changes, which provide additional energetic resources to face adverse situations (Barton and Iwama, 1991; Wendelaar Bonga, 1997). However, these adaptive mechanisms if not efficient, may lead to maladaptive responses such as low performance,

physiological imbalance, and eventually death (Tort, 2011). The direct cause-effect relationship between stress and fish adaptive responses requires investigation, mainly of sub-clinical aspects affecting growth performance, health, disease resistance, and consequently economic loss (Sung et al., 2011; Dawood et al., 2020; Xavier et al., 2020).

Protein, one of the elements of appropriate nutrition, plays an important role on growth, stress response, and health (Oliva-Teles, 2012; Peres et al., 2022). Indeed, it has been observed that protein and amino acids (AA) deficiency reduces plasma AA (Li et al., 2007; Aragão et al., 2008), thus impairing the synthesis of specific proteins with crucial roles on cell signaling, immune and antioxidant responses, cell adhesion, and cell turnover (Buxbaum, 2007). An adequate dietary protein level may reduce oxidative stress (Pérez-Jiménez et al., 2009; Castro et al., 2012) and improve stress resistance and immune response (Habte-Tsion et al., 2017; Zuo et al., 2017). Moreover, the adjustment of dietary protein and AA may provide precursors to sustain fish homeostasis and welfare (Costas et al., 2008; Wu, 2013). Besides, under adverse conditions, voluntary feed intake may be impaired, and a protein/amino acid-fortified diet may be required to avoid deficiency signs.

Indeed, studies have shown that fortification of the diets above the requirement level, with specific amino acids such as arginine, methionine, tryptophan, and taurine may increase stress and disease resistance (Costas et al., 2011; Bañuelos-Vargas et al., 2014; Machado et al., 2015; Huang et al., 2021). For Nile tilapia, dietary protein levels may modulate stress response induced by high stock density (Abdel-Tawwab, 2012; Hooley et al., 2014). Despite its importance, available data on the effects of protein and AA in health and stress resistance remain scarce. Therefore, the current study investigated the interactive effects of dietary digestible protein (DP) level, below and above Nile tilapia protein requirements, and common stressors in intensive aquaculture, such as thermal, transport, and size sorting, on hematological and biochemical response of Nile tilapia.

2. Material and Methods

This study was conducted in Botucatu, São Paulo State, Brazil (22°50'46.2" S and 48°25'51.94" W). Research on animals was conducted according to the institutional committee on animal use (protocol 163/2012-CEUA).

2.1. Experimental diets

A reference diet containing 29% DP was formulated to meet the protein requirement for growth of Nile tilapia according to Fernandes Junior et al. (2016) and NRC (2011). Then, four isoenergetic diets (16 MJ kg^{-1} digestible energy) were formulated to contain DP levels below (22 and 26% DP) and above (32 and 34% DP) the reference diet. The corresponding DP levels confirmed by *in vivo* digestibility assay (Peres et al., 2022) were 28.6% for the reference diet and 22.1, 25.6, 31.9, and 33.9% for the remaining diets, respectively (Table 1).

All of the ingredients were finely ground, and then mechanically mixed with water in a multi-function mixer (Ação Científica®, Piracicaba, SP, Brazil). Extruded pellets were obtained through a single-screw extruder mill (Exteec®, Ribeirão Preto, SP, Brazil) and oven-dried overnight at 55 °C and stored at 4 °C until further use. Diet formulation and proximate analyses are presented in Table 1 and the amino acid composition in Table 2.

2.2. Fish and feeding

Nile tilapia juveniles obtained from a commercial fish farm (Piscicultura Fernandes, Palmital, SP, Brazil, 22°56'41.47" S and 50°10'39.06" W) were acclimatized to laboratory conditions for two weeks before starting the feeding trial and were fed the reference diet four times a day.

Then, groups of 11 uniform-sized Nile tilapia, with an initial body weight of 9.27±0.22 g, were randomly distributed into 40 250-L aquaria. Each of the five experimental diets was randomly distributed to

Table 1 - Ingredient composition and proximate analyses of the experimental diets

	Diet				
	22% DP	26% DP	29% DP	32% DP	34% DP
Ingredient[1] (g/kg dry weight)					
Soybean meal[2]	202.6	285.7	371.5	452.5	537.3
Wheat middling[3]	70	70	70	80	80
Meat and bone meal[4]	100	100	100	100	100
Poultry viscera meal[5]	50	50	50	50	50
Corn meal[6]	526.4	457.9	375	300.9	222.8
Soybean oil[7]	28	20	18	10	5
L-lysine	10	5.4	6.8	-	-
DL-methionine	3.8	3.2	2.5	1.8	1.3
L-threonine	5.7	4.6	3.5	2.4	1.4
L-tryptophan	1.3	0.9	0.5	0.1	-
BHT	0.2	0.2	0.2	0.2	0.2
Vitamin and mineral premix[8]	0.5	0.5	0.5	0.5	0.5
NaCl	1	1	1	1	1
Ascorbic acid[9]	0.5	0.5	0.5	0.5	0.5
Proximate analyses (g/kg dry matter)					
Dry matter	947.9	947.1	948.6	935.8	924.7
Digestible protein (DP)	221.1	256.4	285.9	319.2	339.8
Crude fat	100	90.3	85.9	89.4	71.5
Crude fiber	13.9	19.9	23.3	26.4	32.7
Ash	73.9	78.6	81.8	90.4	91.9
Digestible energy (DE; kJ/g)	17.1	16.9	16.1	16.1	15.0
DE:DP (kJ/g)	67.7	60.6	54.9	48.2	42.2

BHT - butylated hydroxytoluene; CE - crude energy; CL - crude lipid.
[1] All ingredients were obtained at FMVZ Animal Feed Processing Plant.
[2] 459.3 g kg^{-1} CP, 17.6 kJ g^{-1} CE, and 10.9 g kg^{-1} CL.
[3] 148 g kg^{-1} CP, 16.9 kJ g^{-1} CE, and 41.7 g kg^{-1} CL.
[4] 452.5 g kg^{-1} CP, 15 kJ g^{-1} CE, and 89 g kg^{-1} CL.
[5] 586.9 g kg^{-1} CP, 19.9 kJ g^{-1} CE, and 171.2 g kg^{-1} CL.
[6] 83.6 g kg^{-1} CP, 16 kJ g^{-1} CE, and 34 g kg^{-1} CL.
[7] 39.5 kJ g^{-1} CE.
[8] Vitamin and mineral supplement (levels per kg of product): vitamin A, 1,200,000 IU; vitamin D3, 200,000 IU; vitamin E, 12,000 mg; vitamin K3, 2,400 mg; vitamin B1, 4,800 mg; vitamin B2, 4,800 mg; vitamin B6, 4,000 mg; vitamin B12, 4,800 mg; folic acid, 1,200 mg; calcium pantothenate, 12,000 mg; vitamin C, 48,000 mg; biotin, 48 mg; choline, 65,000 mg; nicotinic acid, 24,000 mg; Mn, 4.000 mg; Zn, 6.000 mg; I, 20 mg; Co, 2 mg; Cu, 4 mg e Se, 20 mg.
[9] Vitamin C Rovimix® Stay-C 35®, DMS Nutritional Products, Switzerland.

eight replicates per treatment. Fish were hand-fed until apparent satiation four times a day, between 08:30 and 17:30 h, for 110 days. Due to this imposed feeding regime, at the end of 110-day feeding trial, the final body weight was: 190.1±32.2 (22% DP), 249.3±18.4 (26% DP), 272.6±25.8 (29% DP), 260.5±26.2 (32% DP), and 276.9±9.7 (34% DP) (Peres et al., 2022).

Each aquarium was supplied with 6 L min^{-1} dechlorinated tap water passed through a biological filter and a heater. The aquaria were connected in a closed, recirculating system. During the trial, the water temperature was kept at 26.1±1.1 °C and monitored twice a day; the pH (6.5±0.08), dissolved oxygen concentration (6.1±0.2 mg L^{-1}), and the ammonia level (0.002±0.0 mg L^{-1}) were monitored once a week with a YSI 556® multi-probe system (YSI Environmental, Yellow Spring, OH, USA) and a commercial test kit (Alcon®, Camboriú, SC, Brazil), respectively.

Table 2 - Amino acid composition (g/100 g dry matter) of experimental diets

	22% DP	26% DP	29% DP	32% DP	34% DP	Requirement[1]
Essential amino acids (EAA)						
Arginine	1.6	1.7	2.1	2.4	2.7	2.0
Histidine	0.6	0.7	0.8	0.9	1.0	0.5
Isoleucine	1.0	1.1	1.2	1.4	1.6	0.9
Leucine	2.2	2.4	2.6	2.9	3.1	1.5
Lysine	2.2	2.1	2.4	2.1	2.4	1.6
Methionine	0.9	0.9	0.8	0.8	0.8	
Met+Cys	1.2	1.3	1.2	1.2	1.3	1.0
Phenylalanine	1.3	1.4	1.6	1.8	2.0	
Phe+Tyr	2.2	1.9	2.7	3.0	3.4	1.6
Threonine	1.6	1.6	1.6	1.7	1.7	1.5
Tryptophan	0.4	0.4	0.4	0.4	0.4	0.4
Valine	1.2	1.3	1.4	1.6	1.8	1.2
Non-essential amino acids (NEAA)						
Alanine	1.5	1.7	1.8	1.9	2.1	
Aspartic acid	2.3	2.7	3.1	3.6	4.0	
Cystine	0.3	0.4	0.4	0.4	0.5	
Glutamic acid	4.4	5.0	5.6	6.4	7.0	
Glycine	1.9	2.1	2.2	2.5	2.7	
Serine	1.2	1.4	1.5	1.8	2.0	
Tyrosine	0.9	0.5	1.1	1.2	1.4	
∑ EAA	12.7	13.6	14.9	16.0	17.4	
∑ NEAA	12.5	13.8	15.7	17.8	19.6	
EAA/NEAA	1.0	1.0	0.9	0.9	0.9	

DP - digestible protein.
Proline was not determined.
[1] Values expressed according to Diógenes et al. (2016).

2.3. Stress challenge and analysis

After the 110-day feeding trial, fish were fasted for 12 h, and eight fish/treatment were bled for determination of hemato-biochemical profile. The data obtained at this point was labeled as non-stressed. To avoid the impact of handling the fish on their hematological responses, fish were weighed only after being bled. After one week, the remaining fish were anesthetized and randomly distributed into different groups, and each group was subjected to only one of the following stress challenges: cold-induced stress (CIS), heat/dissolved oxygen-induced stress (HDOIS), transport-induced stress (TIS), or size-sorting-induced stress (SSIS). The first group was subjected to CIS. After four days, the second group of fish was subjected to HDOIS. Both stresses took place in the same challenge room. On the very day fish were subjected to CIS, the third group was subjected to TIS. Then, in the following day, the fourth group was subjected to SSIS. After being subjected to the different stress conditions, fish were bled, and the same hemato-biochemical profile was determined. These data were labeled as stressed. Therefore, the potential effect of different DP levels on fish health was evaluated by comparing the effect of different DP levels before challenges (non-stressed), the effect of different DP levels after challenges (stressed), and by comparing their condition before and after stress considering the same DP level.

2.3.1. Cold-induced stress (CIS)

A group of 40 fish (204.53±54.39 g) was randomly assigned into 20 40-L aquaria (five treatments/four replicates) at a density of two fish per aquarium (eight/treatment) in an experimental challenge room with a cooling system that allowed a gradual decrease in the air temperature, thus reducing water temperature (1 °C h^{-1}) from 26 to 17 °C, as described by Guimarães et al. (2016). Fish were fed the corresponding diets used at the feeding trial. Water temperature (17.0±0.3 °C), pH (7.5±0.0), and dissolved oxygen concentration (7.9±0.3 mg L^{-1}), and saturation (82.2±1.9%) were monitored using a YSI 556® multi-probe system. After 30 h at 17 °C, the same hemato-biochemical parameters were evaluated, and the stressed profile was determined.

2.3.2. Heat/dissolved oxygen-induced stress (HDOIS)

Another group of 40 fish (207.92±56.23 g) was randomly assigned into 20 40-L aquaria (five treatments/four replicates) at a density of two fish per aquarium (eight/treatment) in a warm-water challenge system (Damasceno et al., 2016) that allowed a gradual increase in the water temperature (1 °C h^{-1}) from 26 to 32 °C. The heaters (80 watts) were connected to a temperature controller (Full Gauge Controls, Canoas, RS, Brazil). Fish were fed the same experimental diet as in feeding period. Water temperature (31.8±0.1 °C), pH (6.9±0.3) and dissolved oxygen concentration (2.4±0.2 mg L^{-1}) and saturation (83.2±2.2%) were monitored using a YSI 556® multi-probe system. After 30 h at 32 °C, the same hemato-biochemical parameters were evaluated, and the stressed profile was determined.

2.3.3. Transport-induced stress (TIS)

A third different group of 40 fish (258.47±71.59 g) was subjected to TIS as described by Barros et al. (2014). Briefly, eight fish/treatment were randomly assigned into five 11-L net cages distributed in a 600 L fish transport tank, and the transport was undertaken for four hours.

Before TIS started and for the following four hours, dissolved oxygen concentration and saturation, pH, electric conductivity, total dissolved solids, and temperature were monitored using a YSI 556® multi-probe system, and the un-ionized ammonia concentration was determined using a commercial test kit. The values were within the comfort range for the species (Boyd, 1990). Then, the same hemato-biochemical parameters were evaluated, and the stressed profile was determined.

2.3.4. Size-sorting-induced stress (SSIS)

A fourth group of 140 fish (250.09±71.59 g) was subjected to SSIS as described by Fernandes Junior et al. (2016). Briefly, groups of 28 fish/treatment were randomly collected and confined into an experimental net cage at a high stock density (180 kg m^{-3}) for 15 min, simulating the harvesting process. Each net cage (treatment) was kept inside an isolated 250-L tank. Then, fish were transferred to a fiberglass table and sorted into three sizes, i.e., small, medium, and large, as it is routinely carried out in a Nile tilapia fish farm. This practice lasted for 1 min per treatment, and then fish were immediately subjected to the sampling process. For such, eight fish/treatment were randomly sampled, and the same hemato-biochemical parameters were evaluated, and stressed profile determined.

2.4. Hematological and biochemical profile

At the end of the 110-day feeding trial and after fasting for 12 h, and at the end of each stress challenge, eight fish/treatment (one fish/tank) were randomly collected, anaesthetized with benzocaine solution (67 mg L^{-1}), and bled for evaluation of the hematological profile. Blood was collected from the caudal vein using a tuberculin syringe rinsed with anti-coagulant (3% EDTA, Vetec, Quimica Fina Ltda, Duque de Caxias, RJ, Brazil).

Red blood cell (RBC) and leukocyte counts were determined by dilution and enumeration using a hemocytometer. Leukocyte differentiation was performed using blood extension stained with May-Grunwald-Giemsa stain according to Jain (1986). Differential counting was performed under a microscope at 100X in immersion oil. To establish the percentage of each cellular component, 200 cells were counted on each extension. Hemoglobin (Hb) was determined by the cyanomethemoglobin colorimetric method using a commercial kit (Gold Analisa Diagnostica, Belo Horizonte, MG, Brazil) according to Collier (1944). Hematocrit (Htc) percentage was determined using the microhematocrit method described by Goldenfarb et al. (1971). Mean corpuscular volume [MCV = (Htc × 10)/RBC] and mean corpuscular hemoglobin concentration [MCHC = (Hb × 100)/Htc] were calculated according to Wintrobe (1934). Total plasma protein (TPP) level was measured using a manual Goldberg refractometer (Model SPR – N, Atago CO LTD, Japan) after the blood was centrifuged at 5000 rpm for 15 min.

For the biochemical analyses, blood was collected from the caudal vein, as aforementioned, using a tuberculin syringe with no anticoagulant. After that, blood samples were centrifuged at 5000 rpm for 20 min, and supernatant serum was collected. Albumin concentration (ALB) was determined by the bromocresol method using the commercial kit Albumina Analisa Diagnostica® for colorimetric determination. Albumin:globulin ratio (A:G) was determined using the ALB and TPP values [Globulin = TPP – ALB; A:G = ALB:Globulin]. Glucose (GLU), triacylglycerol (TGL), and cholesterol (CHOL) concentration were determined by enzymatic method using commercial kits (Labtest Diagnóstica, Lagoa Santa, MG, Brazil).

2.5. Statistical analysis

Hematological and biochemical parameters were tested using one-way ANOVA and Tukey test for comparison of effects of different DP levels before and after challenges. To compare the different groups (non-stressed and stressed) data were subjected to a Kruskal-Wallis non-parametric test and complemented with a Mann-Whitney multiple comparisons test. Differences were considered to be significant at the 0.05 probability level. All analyses were performed after checking the normality of data. A cluster analysis was carried out on hemato-biochemical data of all samples including non-stressed and stressed fish to discriminate the different types of stress by proportion of similarity. The analyses were conducted using the software Minitab® 16.1.1.0 (Minitab Inc., State College, Pennsylvania, USA).

3. Results

3.1. Digestible protein levels

The comparison among the different digestible protein levels did not show any effect on hemato-biochemical profile, before or after stress. On the other hand, there were effects when comparing values between non-stressed and stressed fish in each digestible protein level.

3.2. Stress

As for the different induced stress, results are shown as comparison between non-stressed and stressed fish within each DP level.

3.2.1. Cold-induced stress (CIS)

Fish fed 22% DP showed a significant decrease in A:G and an increase in GLU concentration (Table 3 and Figure 2A, respectively). Fish fed 26% DP showed an increase in Hb and MCHC (Table 3). Fish fed 32% DP showed an increase in MCHC and a decrease in TGL (Table 3 and Figure 2A, respectively). Fish fed 34% DP showed an increase in GLU (Figure 2A). There was a decrease

in lymphocytes (LYMP) and an increase on neutrophil (NEUT) for fish fed all experimental diets (Figure 1A).

3.2.2. Heat/dissolved oxygen-induced stress (HDOIS)

As for the erythrogram, fish fed 22% DP showed an increase in Htc, Hb, and MCHC (Table 3). In the leukogram, there was a decrease in LYMP and an increase in NEUT, and the biochemical results showed an increase in GLU (Figures 1B and 2B, respectively). Fish fed 26% DP showed an increase in Hb and MCHC (Table 3), a decrease in LYMP, and an increase in NEUT, and also an increase in GLU and

Table 3 - Hematological parameters of Nile tilapia fed different dietary digestible protein (DP) levels for 110 days and subjected to different acute stress[1]

	Stress	22% DP	26% DP	29% DP	32% DP	34% DP
RBC (10^6 μL^{-1})	Non-stressed	2.00	1.97	1.91	1.87	2.09
	CIS	2.00	2.10	2.01	1.91	2.01
	HDOIS	2.08	1.97	2.00	2.00	2.06
	TIS	1.94	2.06	2.08	2.11*	1.95
	SSIS	1.97	1.85	1.89	2.03*	1.73*
Htc (%)	Non-stressed	28.75	28.88	28.50	27.88	30.63
	CIS	29.13	30.63	27.88	26.63	28.25
	HDOIS	34.13*	30.50	32.38	30.88	29.50
	TIS	28.50	30.50	32.25	30.38	29.75
	SSIS	29.00	30.38	28.25	29.50	25.88*
Hb (g dL^{-1})	Non-stressed	6.31	5.76	6.77	5.93	6.53
	CIS	7.16	7.59*	7.33	7.02	6.89
	HDOIS	8.83*	8.63*	8.46*	8.54*	8.17
	TIS	7.57*	8.07*	8.03	8.21*	8.27
	SSIS	7.43	7.80*	7.39	8.23*	6.93
MCV (fL)	Non-stressed	143.24	143.40	156.40	145.37	153.24
	CIS	144.70	141.00	140.10	147.00	149.20
	HDOIS	165.79	161.00	156.78	159.34	148.72
	TIS	143.62	144.36	143.03	145.94	139.40
	SSIS	150.40	156.78	156.85	149.75	146.41
MCHC (%)	Non-stressed	21.66	19.76	23.75	20.43	23.92
	CIS	24.63	25.21*	24.87	26.13*	24.49
	HDOIS	25.89*	29.29*	27.24*	28.22*	28.18*
	TIS	25.73*	27.25*	26.05*	25.58*	26.29*
	SSIS	26.06*	26.30*	25.24	26.8*	26.64*
A:G (mg dL^{-1})	Non-stressed	0.47	0.52	0.61	0.66	0.47
	CIS	0.30*	0.38	0.38	0.51	0.48
	HDOIS	0.54	0.48	0.46	0.45	0.72
	TIS	0.38	0.42	0.43	0.52	0.50
	SSIS	0.33*	0.36	0.40	0.40*	0.52

RBC - red blood cell; Htc - hematocrit; Hb - hemoglobin; MCV - mean corpuscular volume; MCHC - mean corpuscular hemoglobin concentration; A:G - albumin:globulin ratio.
[1] Values are medians of eight replicates. For each dietary protein level, * denotes statistically differences between non-stressing and stressing conditions (Kruskal-Wallis test by ranks; $P<0.05$).

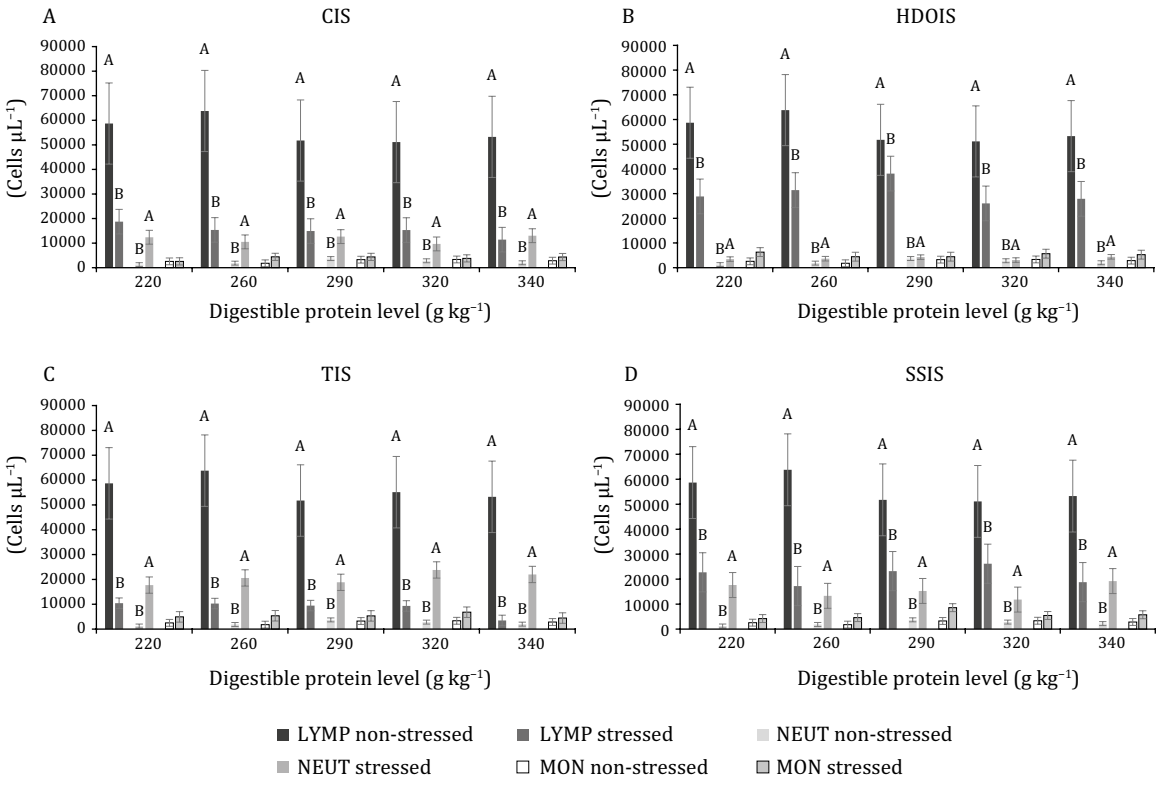
CHOL (Figures 1B and 2B, respectively). Fish fed 29 and 32% DP showed an increase in Hb and MCHC (Table 3), and fish fed 32% DP showed a decrease in LYMP (Figure 1B). Fish fed 34% DP showed an increase in MCHC (Table 3), NEUT, and monocyte (MON) (Figure 1B). Heat/dissolved oxygen-induced stress determined a decrease in TGL for fish fed all experimental diets (Figure 2B).

3.2.3. Transport-induced stress (TIS)

Red blood cell count increased in fish fed 32% DP, and Hb increased for fish fed 22, 26, and 32% DP (Table 3). Transport-induced stress determined a higher MCHC and GLU for fish fed all experimental diets (Table 3 and Figure 2C, respectively). Fish fed 34% DP showed a decrease in TGL (Figure 2C). As for white blood cell, TIS determined a decrease in LYMP and an increase in NEUT for fish fed all experimental diets and an increase in MON for fish fed 22, 26, and 32% DP (Figure 1C).

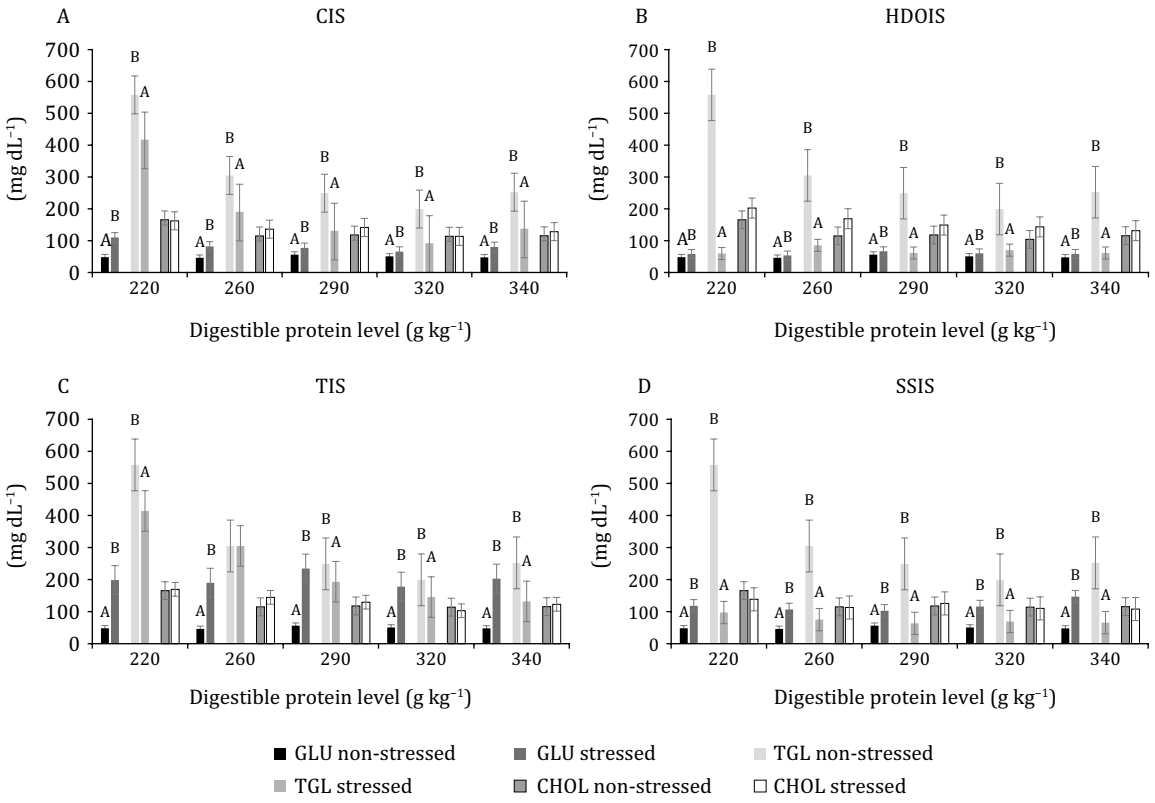
3.2.4. Size sorting induced stress (SSIS)

Size-sorting-induced stress determined an increase in RBC and a decrease in A:G in fish fed 32% DP, an increase in Hb of fish fed 26 and 32% DP, and an increase in MCHC of fish fed 22, 26, 32, and 34% DP (Table 3). Fish fed 22% DP showed a decrease in A:G (Table 3). A reduction in RBC and Htc was observed in fish fed 34% DP (Table 3). As for WBC, there was a decrease in LYMP and an increase in NEUT in fish fed all experimental diets (Figure 1D). A higher GLU and lower TGL were observed in fish fed all experimental diets under SSIS (Figure 2D).



LYMP - lymphocyte; NEUT - neutrophil; MON- monocyte.
Bars represent medians and minimum – maximum of eight replicates.
Different uppercase letters indicate significant difference between non-stressed and stressed groups by Mann-Whitney test (P<0.05).

Figure 1 - Effect of cold-induced stress, CIS (A), heat/dissolved oxygen induced stress, HDOIS (B), transport-induced stress, TIS (C), and size-sorting induced stress, SSIS (D) on white blood cell count of Nile tilapia juveniles fed different digestible protein levels.



GLU - glucose; TGL - triglycerides; CHOL - cholesterol.
Bars represent medians of eight replicates.
Different uppercase letters indicate significant difference between non-stressed and stressed groups by Mann-Whitney test (P<0.05).

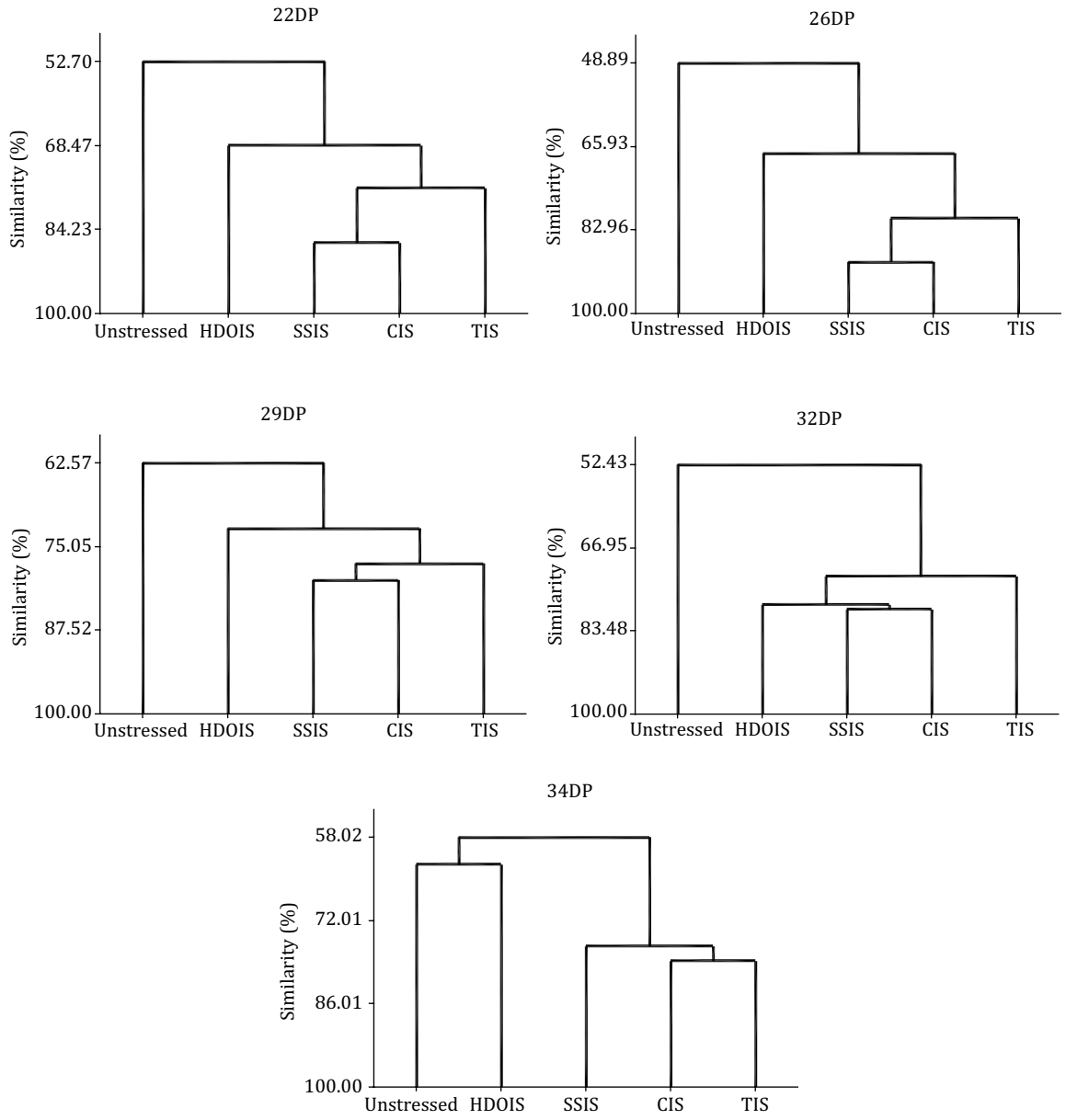
Figure 2 - Effect of cold-induced stress, CIS (A), heat/dissolved oxygen induced stress, HDOIS (B), transport-induced stress, TIS (C), and size-sorting induced stress, SSIS (D) on serum biochemical parameters of Nile tilapia juveniles fed different digestible protein levels.

3.2.5. Cluster analysis

The cluster analysis (Figure 3) demonstrated the degree of similarity between stressed and non-stressed fish according to each dietary DP level. Fish fed 22, 26, and 29% DP showed the highest similarity between SSIS and CIS challenges (86.75, 89.50, and 80.09%, respectively), which showed the highest difference from non-stressed fish. The following cluster comprised TIS (76.45, 80.60, and 77.57%, respectively) and then HDOIS (68.42, 67.36, and 72.32%, respectively). For fish fed 32% DP, the cluster with the highest similarity was SSIS and CIS (79.20%), which presented the highest difference from non-stressed fish. The following cluster comprised HDOIS (78.21%) and TIS (72.58%). On the other hand, fish fed 34% DP showed CIS and TIS as the cluster with the highest similarity (78.77%) presenting the highest difference from non-stressed fish. The following clusters were SSIS (76.34%) and HDOIS (58.01%).

4. Discussion

Stress responses vary among fish species, leading to a wide range of physiological and biochemical mechanisms to overcome the situation or compensate the imbalance (Tort, 2011). Stress responses affect overall protein and energy metabolism, inducing mobilization of body energy reserves (Diógenes et al., 2019). A nutritionally complete diet can help fish to cope with stress, providing the nutrients and energy to support the stress response. Thus, fish nutritional history may have a



DP - digestible protein.

Figure 3 - Proportion of similarity between non-stressed and fish under different types of stress by cluster analysis of hemato-biochemical data.

protective effect counteracting some of the consequences of the stress response (Tort et al., 2004; Conceição et al., 2012).

In our study, we evaluated graded dietary DP levels under different types of stress and the capacity to maintain health standards. The stressors were chosen based on common aquaculture practices aiming to simulate both physical and environmental-related types of stress. As for environmental stressors, temperature-induced stress determined different hematological and metabolic responses. The main effect of CIS was on the leukogram and the main effect of HDOIS was on energy source and oxygenation.

Lymphopenia and neutrophilia were determined at CIS in all fish sampled, regardless the dietary DP levels. These results have been consistently observed in Nile tilapia, subjected to different stressors, irrespectively of the immunonutrition (Bly and Clem, 1992; Falcon et al., 2007; Barros et al., 2009;

Fernandes Junior et al., 2010; Barros et al., 2014; Araújo et al., 2017). Although Nile tilapia has been described as a fish species that stands broad variation of temperature, its thermal comfort ranges from 27 to 30 °C (Boyd, 1990; El-Sayed, 2006). In the present study, a short period (30 h) under low temperature (17 °C) was enough to trigger the cell-mediated immune response. The neutrophilia observed was probably necessary to keep the fish healthy, since neutrophils are a critical component of the host defense system, forming the first line of cellular defense against invading organisms (Smith, 2000). Since MON count did not increase and no signs of bacteria were observed, we assumed that neutrophils were able to maintain health. Similar results were described by Bly and Clem (1992), Barros et al. (2009), and Araújo et al. (2017). Studies have shown that leukocyte numbers can vary according to intensity and time of stress. According to Tort (2011), acute stress determines a significant reduction in lymphocytes and monocytes and an increase in neutrophils, whilst a chronic stress determine an initial increase, followed by a general decrease in blood leukocyte numbers.

Conversely, under HDOIS, an increase in metabolism with higher demand for energy and oxygen were expected. Regardless of dietary DP levels, fish showed higher Hb values, although their values were within the normal range for healthy Nile tilapia (Hrubec and Smith, 2010; Damasceno et al., 2016; Xavier et al., 2020). Consequently, MCHC was generally higher, thus demonstrating higher hemoglobin concentration in the erythrocytes. Alterations in Hb and MCHC were expected because of the lower oxygen level under high water temperature. The absence of significance in RBC and Htc could be attributed to the length of the induced-stress period. The increase in Htc and MCV values under HDOIS, although not significantly different, can be considered beyond the normal range (Hrubec and Smith, 2010; Teixeira et al., 2012). Together, these results may suggest that immature RBC could have been released to counteract hypoxia induced by this stressor, maintaining tissue oxygenation (Fernandez and Grindem, 2000).

Even though no signs of bacteria were observed in Nile tilapia under HDOIS, fish fed 34% DP showed significant neutrophilia and monocytosis, which could be a result of the enhanced non-specific immune function. Arginine and phenylalanine levels above the dietary requirement (Diógenes et al., 2016) could have helped nitric oxide (NO) production, since arginine is the precursor for the biosynthesis of NO and phenylalanine can regulate NO production, being essential as a cofactor for NO synthase (Wu, 2013). Therefore, further studies assessing the innate immune response such as leukocyte activity and the production of oxygen and nitrogen reactive species could provide a better understanding on how the different stressors could affect fish physiology under challenging situations.

These results highlighted the importance of evaluating the thermal stress effect, even at higher water temperatures and longer periods, as it is commonly observed in tropical countries. Although no signs of bacteriosis were observed in the present study, the relationship between changes in water temperature and the incidence of bacteriosis has been previously reported (Kersters et al., 1996; Rodkhum et al., 2011).

Stress responses are energy-draining processes that uses triglycerides as the main energy source (Benton et al., 1994). Triglycerides are broken down into glycerol and fatty acids by lipase, and stress hormones contribute for lipolysis (Voet et al., 2002). Accordingly, the significant reduction observed in TGL under HDOIS (decrease in average 4.8-fold) supports the idea that Nile tilapia under high temperature requires extra energy to restore homeostasis. Under CIS, a significant decrease in TGL was also observed, but only in fish fed 32% DP (declining 2.17-fold). It is important to emphasize that, because Nile tilapia is a tropical fish species well-adapted to warm temperature (Zerai et al., 2010), under low temperature the metabolic rate decreases, and all physiological responses can be compromised. In CIS, fish fed the lowest and the highest DP levels showed significantly higher GLU, indicating stress, and the same occurred for fish fed the lowest DP level under HDOIS.

In summary, digestible protein ranging from 26 to 32%, at low temperature, and 26 to 34% at high temperature, seems to be able to keep fish homeostatic for a short term, probably as an energy-

yielding nutrient. Indeed, under HDOIS, fish mobilize more TGL as energy source than in CIS. The diet formulated to contain 22% DP was not adequate to keep homeostasis, either at low or high temperature. Additionally, this diet did not meet arginine requirement, an important functional amino acid to synthesize nitric oxide, as aforementioned. According to Iwama et al. (2011), an imbalance in dietary protein concentration could impair the immune response and increase their susceptibility to diseases. Therefore, we believe that this level of DP was inadequate for meeting all physiological demands.

Transport and size-sorting are considered physical stressors that involves capturing, loading, transporting or size-sorting, unloading, and stocking. Fish subjected to these multiple-phase operations generally take a long time to recover and may suffer severe physiological consequences (Barton, 2002; Conte, 2004; Sutthi and Doan, 2020; Obirikorang et al., 2020). The production of glucose with stress is a source of energy for animals and a tool to help them cope with the increased energy demand (Balasch and Tort, 2019; Rudneva, 2013). Such condition, as highlighted in our study, can be confirmed by the increase in GLU and decrease in TGL after both physical stressors. Similar results were obtained for Nile tilapia under commercial condition after fish being subjected to size-sorting stress (Fernandes Junior et al., 2016).

As for oxygen demand, both stressors required high oxygenation, as evidenced by the overall increased MCHC and Hb values. Fish fed diets that meet their DP requirement for growth were able to maintain the reference values for healthy Nile tilapia erythrogram. In addition, alterations occurred in non-specific immune response leading to an increase in cell-mediated defense, mostly NEUT under TIS and SISS, and MON under SISS, mainly for DP levels below the nutritional growth requirement. The importance of protein on immune response has been previously described, since cytokines, immunoglobulins, and other components of the immune system are proteinaceous (Gatlin, 2002). Although the erythropoiesis was maintained under both SSIS and TIS, the oxygenation demand increased especially under deficient DP levels and TIS. This relationship between dietary DP levels and both red blood cells and hemoglobin syntheses suggest that fish increased Hb concentration rather than red blood cells number, since both are protein compounds and the dietary protein concentration was not even appropriate for growth. On the other hand, the white blood cells response was directly correlated with stress, independently of the dietary DP levels.

Cluster analysis was applied to each DP level to identify the induced-stress responses comparable to the non-stressing condition, considering all analyzed parameters. Among the stress conditions studied, SSIS and CIS were the most stressful for fish fed a dietary DP lower or equal to 32% DP. At 34% DP, CIS and TIS were the most stressful. On the other hand, HDOIS is clustered with non-stressing conditions. As it was previously mentioned, TIS and SSIS are considered multiple-phase-operations stress, associated with long time recovering and severe physiological consequences (Barton, 2002; Conte, 2004). Interestingly, HDOIS can be considered less stressful than CIS, probably because Nile tilapia is a tropical fish species well-adapted to warm temperature, as aforementioned. Due to the severity of SSIS and CIS, producers should avoid handling fish during low-temperature periods to reduce deleterious effects. Based on that, producers should consider meeting Nile tilapia protein requirement, especially the digestible concentration, aiming not just the growth performance, but also fish health, higher energy reserve, and better immune response.

5. Conclusions

Dietary protein does not mitigate stress response under SSIS and CIS; lymphopenia and neutrophilia are the main cell-mediated immune responses; dietary digestible protein below the nutritional requirement for Nile tilapia growth under SSIS and TIS impairs oxygenation; fish under HDOIS and SSIS demand more energy using triglycerides as an energy source; the diet formulated to contain 22% digestible protein is not adequate to keep homeostasis under temperature stress. Cluster analysis showed that, for digestible protein levels below the requirement for growth, SSIS and CIS are considered

the most stressful conditions. At 34% digestible protein level, HDOIS response is comparable to that of non-stressing conditions.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

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Acknowledgments

The authors are grateful to the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; 2012/15849-9) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; 141761/2012-1) for the financial support and scholarship granted.

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