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The effect of digestible protein to digestible energy ratio and choline supplementation on growth, hematological parameters, liver steatosis and size-sorting stress response in Nile tilapia under field condition

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ABSTRACT

This study evaluated growth performance, hematological parameters, histological liver analysis, and production costs of Nile tilapia fed increasing levels of digestible protein, digestible energy, and choline. Twelve thousand Nile tilapia (148 ± 6.7 g) were randomly distributed into 80 1 m^3 net cages, in a 5 × 2 × 2 factorial design with five digestible protein (DP) levels (24, 26, 28, 30, and 32% DP), two digestible energy (DE) levels (13.4 and 14.65 MJ DE kg^{-1} diet), and two choline levels (0.0 and 1000 mg kg^{-1} diet), with four replicates per treatment. Fish fed the higher energy level showed a sparing effect of protein; the higher protein level determined the highest fillet yield. Fish fed diets with 24% DP showed the highest liver lipid, and independently of treatment all analyzed fish showed hepatocyte degeneration. The best benefit cost ratio for whole fish production was achieved with 28% DP/13.4 MJ DE kg^{-1}, and for fillet production with 30% DP/13.4 MJ DE kg^{-1}. The results of the hematological assay showed alterations in red blood cells, mean corpuscular volume, albumin, Albumin:Globulin ratio, and glucose after size-sorting stress. Overall, these results indicate a lower resistance to stress, mainly for fish fed with no choline and oil supplementation. In this study we determined that the best performance was achieved with DP:DE ratios of 21.45 g MJ^{-1} (28.74% DP/13.4 MJ DE kg^{-1}) and 18.60 g MJ^{-1} (27.25% DP/14.65 MJ DE kg^{-1}). The highest fillet yield was obtained with 30% DP, regardless of the dietary energetic level. Sustained homeostasis was observed in this setting, and even though size-sorting stress altered some hematological parameters, they were still within the range recognized as healthy. Choline was not effective in protecting the liver against hepatic steatosis, but was able to buffer some of the negative effects of stress under these rearing conditions.

Statement of Relevance: This research has been approved by the Ethics Committee of our Institution. Our team has been working with nutrition and fish health since 2000. According to the NRC (2011), only a few studies have been conducted to estimate the dietary nutrient requirements of farmed fish under intensive culture conditions. Moreover, a number of factors may affect the dietary and nutritional requirements of fish differentially in the laboratory and under intensive culture. For example, climatic conditions may fluctuate widely in the field, directly affecting physiological responses, hence nutritional requirements. Similarly, fish densities are much higher under intensive farming. Establishing the appropriate nutritional requirements in these settings must also consider the trade-off between growth performance and production costs. Based on this, we investigated the effect of different levels of digestible protein and digestible energy on growth performance and hematological responses of the Nile tilapia in a commercial fish farm in Brazil, where fish farmers are known to use diets with excessive levels of crude protein throughout fish culture and hepatic steatosis is often reported. Therefore, we also investigated the potential hepatic protective effect of choline against such conditions. Finally, we analyzed the health status, as measured by hematological parameters, of fish subjected to handling-induced stress procedures that are usual on fish farms.

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1. Introduction

Dietary protein is one of the main factors that influence fish production and nitrogen waste into the water which, in excess, may impair fish

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growth and water quality under intensive fish farming (Mires et al., 1990, Tibbetts et al., 2000). Previous research conducted under laboratory conditions has estimated a dietary requirement of 25% (El-Sayed and Gaber, 2005) and 27.5% crude protein (Balarin and Haller, 1982) for tilapia. According to the NRC (2011), however, digestible protein to digestible energy ratio is a more rational way of expressing protein requirements. This is because dietary energy levels are also tightly linked to nutrient consumption. If energy is available in excess, it may impair nutrient consumption, whereas when in deficit protein can be catabolized as energy source (NRC, 2011). For the Nile tilapia, previous research conducted in the laboratory has determined optimal digestible protein:digestible energy ratios ranging from 21.4 to 32.7 g MJ^{-1} depending on fish size (Trung et al., 2011). However, the digestible protein:digestible energy ratio has not been evaluated under field and stress conditions.

According to the NRC (2011), only a few studies have been conducted to estimate the dietary nutrient requirements of farmed fish under intensive culture conditions. Studies developed in these conditions are needed especially considering the constant environmental stress that fish are subjected to in intensive commercial settings. Moreover, a number of factors may affect the dietary and nutritional requirements of fish differentially in the laboratory and under intensive culture. For example, climatic conditions may fluctuate widely in the field, directly affecting physiological responses, hence nutritional requirements. Similarly, fish density is much higher, and fish management, such as sorting, is also more severe under intensive farming. Therefore, the nutritional demands of these fish are likely much higher, and may impair performance and immune responses, and increase susceptibility to pathogens when not met. On establishing the appropriate nutritional requirements in these settings the trade-off between growth performance and production costs must also be considered.

Several metabolic syndromes, such as hepatic steatosis, have been associated with nutritional disorders. Since choline plays an important role on the maintenance of cell structure, lipid transportation and phosphatidilcholine precursor, which is responsible for hepatic steatosis prevention, its dietary inclusion is necessary since fish cannot synthesize this compound in adequate amount (Ketola, 1976; Halver, 2002; McDowell, 1989; NRC, 2011).

In this study we investigate the effect of different levels of digestible protein and digestible energy on growth performance and hematological responses of the Nile tilapia in a commercial fish farm in Brazil, where fish farmers are known to use diets with excessive levels of crude protein throughout fish culture and hepatic steatosis is often reported. Therefore, we also investigated the potentially protective effect of choline (Ogino et al., 1970; Osol et al., 1982; Wilson and Poe, 1988) against hepatic steatosis, a recurrent problem in fish raised under intensive conditions. Finally, we analyze the health status, as measured by hematological parameters, of fish subjected to size-sorting stress procedures that are typical of commercial practice.

2. Materials and methods

The study is made of two phases. In phase I, fish received diets with different levels of digestible protein, digestible energy and with or without supplemented choline. Growth performance, fillet yield, visceral fat, liver lipid concentration and economical analyses were evaluated. In phase II, we compared fish physiological condition considering their health status, before and after sorting-induced stress.

2.1. Study area (phases I and II)

This study was developed to evaluate the growth performance, physiological response, and production cost of Nile tilapia in net cages fed diets containing levels of digestible protein, digestible energy, and choline for 119 days in the autumn and winter (9 March to 5 July). The research was carried out in the Fernandes Fish Farm, Canoas II reservoir, São Paulo State, Brazil (22°56′41.47″ S and 50°10′39.06″ W). This reservoir is located between São Paulo and Parana State. The water average temperature is 23.3 ± 7 °C, with maximum and minimum temperatures of 30 and 16 °C, respectively.

2.2. Fish and experimental procedure (phase I)

Fish were stocked into ten 6 m^3 (2.0 × 2.0 × 1.5 m) net cages at a density of 2300 male Nile tilapia (Supreme Tilapia strain) per net cage during 25 days, in order to adapt to experimental conditions. Then, twelve thousand male Nile tilapia juveniles with an initial body weight of 148 ± 6.7 g (mean ± SD) were randomly stocked into 80 1 m^3 net cages (1.0 × 1.0 × 1.0 m) at a density of 150 fish per net cage (Ridha, 2006). The experimental design was a 5 × 2 × 2 factorial design with five digestible protein (DP) levels (24, 26, 28, 30, and 32% DP), two digestible energy levels (DE) (13.4 and 14.65 MJ DE kg^{-1} diet), and two choline levels (Cho) (0.0 and 1000 mg kg^{-1} diet) totalizing 20 treatments. All treatments were randomly distributed among net cages with four replicates per treatment. Fish were hand-fed two or three times daily, depending on the water temperature. Briefly, the amount of feed was previously weighed, and then distributed to each net cage using a boat. The feeding management took about 40 min, in which the mortality was recorded.

At the beginning of the experiment the water temperature was 24 °C (9 March); it gradually decreased to 21 °C (4 May), reached 17 °C on 4 June, and maintained until the end of the experiment (5 July, 2011). Therefore, the average water temperature was 20.5 °C, which is considered below ideal temperature for optimal growth and health (Lim and Webster, 2006). The amount of diet offered during the feeding trial considered the water temperature, as recommended for Nile tilapia (Lim and Webster, 2006). At above 21 °C fish were fed three times daily, and at lower temperatures twice daily. The average amount of feed fed to the fish was the same regardless of treatment (133.6 kg per net cage).

2.3. Water quality (phase I)

Water temperature, pH, dissolved oxygen (DO), dissolved oxygen saturation (DOS), total dissolved solids (TDS), and total ammonia were measured every morning through a multi probe system (YSI Environmental, Yellow Spring, OH, USA). The average water temperature was 20.5 °C ± 3.5 and other water quality parameters were: pH 7.25 ± 0.2; DO 7.23 ± 0.2 mg L^{-1}; DOS 76.0% ± 5.0; TDS 0.63 ± 0.6 mg L^{-1}; total ammonia 0.06 ± 0.01 mg L^{-1}; Secchi depth 3.67 ± 0.5 m; and cyanobacteria concentration <0.1 ppb. These values were within the comfort range for the species (Boyd, 1996).

2.4. Growth performance parameters (phase I)

At the end of the experimental period (119 days) final biomass (FB), specific growth rate (SGR), feed conversion ratio (FCR), visceral fat (VF), and fillet yield (FY) were determined.

Where:

FB (g) = weight of all fish in the net cage;

SGR $\left(\%day^{-1}\right)$ = (Ln final weight–Ln initial weight/total no.of the experimental days) ×100

FCR = dry feed intake (g)/wet weight gain (g);

VF (%) = [visceral fat weight (g) × 100]/fish weight (g); and

FY (%) = [weight of the fillet (g) × 100]/fish weight (g).

2.5. Diets

Two diets were formulated to contain 24 and 32% of DP with 13.4 and 14.65 MJ DE kg^{-1}. Others diets were obtained by the dilution

method. The nutritional requirements were based on Furuya (2010) and NRC (2011) (Table 1).

Composition of diets:

$$26\%DP/13.4\ MJ\ DE\ kg^{-1} = 75\%of\ 24\%DP/13.4 + 25\%of\ 32\%DP/13.4$$
$$26\%DP/14.65\ MJ\ DE\ kg^{-1} = 75\%of\ 24\%DP/14.65 + 25\%of\ 32\%DP/14.65$$
$$28\%DP/13.4\ MJ\ DE\ kg^{-1} = 50\%of\ 24\%DP/13.4 + 50\%of\ 32\%DP/13.4$$
$$28\%DP/14.65\ MJ\ DE\ kg^{-1} = 50\%of\ 24\%DP/14.65 + 50\%of\ 32\%DP/14.65$$
$$30\%DP/13.4\ MJ\ DE\ kg^{-1} = 25\%de\ 24\%DP/13.4 + 75\%of\ 32\%DP/13.4$$
$$30\%DP/14.65\ MJ\ DE\ kg^{-1} = 25\%de\ 24\%DP/14.65 + 75\%of\ 32\%DP/14.65.$$

Each diet comprised two treatments: without supplemented and supplemented with choline chloride, which was added at the expense of corn, totalizing 20 treatments. The diets were processed at Leben Foods® (Macatuba, São Paulo, Brazil). All ingredients were ground until sieved at a mesh diameter of 1000 μm and homogenized in a horizontal mixer and subsequently processed at 100–120 °C in a single screw industrial extruder (Tecnal®, Ribeirão Preto, São Paulo, Brazil) with capacity to process 1500 kg h^{-1} and exited in a 2 mm die to obtain a 5.0 mm pellet. The diets were dried in horizontal dryer (100–120 °C), cooled, and packaged in raffia bags.

2.6. Hematological assay (phases I and II)

At the end of the feeding period (119 days), and the period prior to size-sorting stress, seven fish per treatment were randomly collected and anesthetized with benzocaine (100 mg L^{-1}). Blood was collected from the caudal vein using a tuberculin syringe, rinsed with an anticoagulant (3% EDTA) (Barros et al., 2014).

Red blood cell (RBC) counts were determined by dilution and enumeration in a hemocytometer. Leukocyte differentiation was performed in blood extension stained with May–Grünwald–Giemsa–Wright according to Jain (1986). Differential counting was performed under a microscope at 100× in immersion oil. Two hundred cells were counted to establish percentages for each cellular component of interest.

Hemoglobin (Hb) was determined by the cyanometahemoglobin colorimetric method using a commercial kit (Analisa Diagnóstica, Belo Horizonte, MG, Brazil) according to Collier (1944). The hematocrit (Ht) percentage was determined using the microhematocrit method described by Goldenfard et al. (1971). The total plasma protein (TPP) was measured using a manual Goldberg refractometer by breaking the microhematocrit capillary just above the leukocyte layer after the hematocrit reading (Hrubec and Smith, 2010). The mean corpuscular volume [MCV = (Ht × 10) / RBC] and the mean corpuscular hemoglobin concentration [MCHC = (Hb × 100 / Ht] were calculated according to Wintrobe (1934).

The albumin concentration (ALB) was determined by the bromocresol method using the commercial kit Albumina Analisa Diagnóstica® for colorimetric determination. The albumin:globulin ratio (A/G) was determined using ALB and TPP values [Globulin = TPP − ALB; A/G = ALB/Globulin] (Jain, 1986). The glucose (Gluc) concentration was determined using the Analisa Diagnóstica® commercial kit.

Table 1
Ingredients and chemical composition of experimental diets (on dry matter basis)*.

Ingredients (g kg^{-1} dry diet)	24/13.4	24/14.65	26/13.4	26/14.65	28/13.4	28/14.65	30/13.4	30/14.65	32/13.4	32/14.65
Soybean meal	178	212	244	277	310	342	375	407	441	472
Wheat middlings	225	75	203	56	181	37	159	18	137	0
Poultry byproduct meal	200	200	200	200	200	200	200	200	200	200
Corn	368.9	440	326.5	395.6	284.3	350.9	242.9	306.5	200.6	261.1
L-Lysine	3.1	2.8	2.3	2.1	1.6	1.4	0.8	0.7	0	0
DL-Methionine	1.5	1.7	1.2	1.3	0.9	1	0.5	0.6	0.2	0.2
Tryptophan	0.2	0.3	0.2	0.2	0.1	0.2	0.1	0.1	0	0
Threonine	2.1	2	1.6	1.5	1.1	1	0.5	0.5	0	0
Poultry viscera oil	0	45	0	45.1	0	45.3	0	45.4	0	45.5
NaCl	5	5	5	5	5	5	5	5	5	5
Premix vit/min[a]	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3	13.3
Banox[b]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Moldzap[c]	1	1	1	1	1	1	1	1	1	1
Choline chloride	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7
Chemical composition (g kg^{-1} dry diet)										
Digestible energy (MJ kg^{-1})	13.4	14.7	13.4	14.7	13.4	14.7	13.4	14.7	13.4	14.7
Digestible protein	240	240	260	260	280	280	300	300	320	320
Crude protein[d]	291	292.9	314.8	306.5	340.8	336.2	355.2	345.1	373.3	373.2
Crude fiber[d]	44.8	40.1	50.8	43.5	49.2	44.2	42.8	44.4	52.3	48.1
Ether extract[d]	22.5	90	20	82.1	21.9	81	25.3	81.8	29.3	81
Total calcium	8.9	8.7	9	8.9	9.1	9	9.2	9.1	9.3	9.2
Available phosphorus	6.4	6.2	6.4	6.3	6.5	6.3	6.5	6.4	6.6	6.4
Methionine	5.4	5.5	5.4	5.5	5.4	5.5	5.4	5.4	5.4	5.4
Sulfur amino acids	9.1	9.1	9.4	9.3	9.7	9.6	10	9.9	10.3	10.1
Lysine	15.2	15.2	16.1	16.2	17.1	17.2	18.1	18.2	19	19.2
Tryptophan	2.8	2.8	3.1	3.1	3.4	3.4	3.7	3.6	3.9	3.9
Threonine	10.5	10.5	10.9	10.9	11.3	11.3	11.6	11.7	12	12.1
Choline (mg kg^{-1})	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
DP:DE[e]	17.9	17.57	19.4	17.74	20.89	19.11	22.37	20.46	23.86	21.83

[a] Premix vitamins and minerals (Mogiana Alimentos) – Guaranteed minimum levels per kilogram of diet: Folic Acid: 10 mg; Copper: 20 mg; Vit. B1: 32 mg; Vit. E: 250 UI; Vit. K3: 30 mg; Niacin: 170 mg; Manganese: 50 mg; Selenium: 0.70 mg; Vit. A: 16,000 UI; Vit. B2: 32 mg; Vit. C: 325 mg; Vit. D3: 4500 UI; Vit. B6: 32 mg; Iron: 150 mg; Iodine: 1 mg; Cobalt: 0.50 mg; Vit. B12: 32 mcg; Calcium Pantothenate: 80 mg; Zinc: 150 mg; Biotin: 10 mg.
[b] Antioxidant.
[c] Antifungal.
[d] Analyzed values.
[e] Digestible protein and digestible energy ratio (g MJ^{-1}).
* Ingredients and chemical composition of the diets supplemented with choline.

2.7. Size-sorting stress (phase II)

After hematological profile and growth evaluation, fish were submitted to size-sorting stress. All fish were put on a fiberglass table where they were classified into three sizes. This procedure is routinely carried out in commercial fish farm in an attempt to restrict size ranges within rearing groups and thereby simplify feeding operations (Jobling and Reinsnes, 1987). This practice lasts for two days. Afterwards, blood samples were collected from seven anesthetized fish (benzocaine 100 mg L^{-1}) per treatment from different net-cages to determine the same hematological parameters previously described.

2.8. Lipid concentration and liver histological analysis (phase I)

The livers from five anesthetized fish (benzocaine 100 mg L^{-1}) per treatment were collected for lipid analysis. The livers were lyophilized and lipid concentration was determined according to AOAC methods (AOAC, 2000).

For histological analysis, fish were killed by a lethal dose of benzocaine solution (300 mg L^{-1}). A postmortem internal examination of the liver was performed and liver samples from three fish per treatment were collected for histological examination, fixed in buffered formaldehyde (10%). The samples were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin (HE).

Descriptive analysis was performed and the degenerative change was classified according to morphology based on Roberts and Rodger (1988). Scores in cellular degeneration were attributed as follows: 1 = 0 to 25% of degenerate hepatocytes; 2 = 26 to 50% of degenerate hepatocytes, 3 = 51 to 75% of degenerate hepatocytes, and 4 = more than 76% of degenerate hepatocytes. Histological changes were evaluated by a pathologist in a blind manner.

2.9. Economic analysis (phase I)

Prices for ingredients used in calculating the cost of diets were provided by Leben Food® (Macatuba, São Paulo, Brazil) based on the purchase price of the product by the company. The following values were used to calculate the cost of the experimental diets ($ kg^{-1} diet, quoted in March 2012; $1.00 = R$ 1.73): soybean meal: $0.43 kg^{-1}; wheat middlings: $0.25 kg^{-1}; Poultry-byproduct meal: $0.58 kg^{-1}; Corn: $0.29 kg^{-1}; Poultry viscera oil: $1.22 kg^{-1}; common salt: $0.22 kg^{-1}; Premix vitamin/mineral: $2.89 kg^{-1}; Moldzap: $2.72 kg^{-1}; Banox: $4.83 kg^{-1}, L-Lysine: $3.24 kg^{-1}, DL-Methionine: $6.36 kg^{-1}, L-Tryptophan: $28.73 kg^{-1}, and L-Threonine: $4.05 kg^{-1}. The cost of processing and bagging was included in the price of feed, with values of $0.14 kg^{-1} diet and $0.14 bag^{-1}, respectively. The value of transportation was added to the final product using $28.90 per ton diet.

The following variables were used to calculate the economic analysis:

1. Feed intake: total feed supplied;
2. Final biomass (kg): weight of all the fish in the net cage at the end of the experiment;
3. Fillet yield (%): [weight of the filet (g) × 100] / fish weight (g); and
4. Weight of fillet (kg): Yield fillet × final biomass.

Other information was also used, such as: cost of juveniles, feed prices, cost of filleting, the selling price of whole fish, and selling price of the fillet. From this data set were estimated economic indicators for the marketing of whole fish and fillets, presented in $/net cage (NC):

Gross revenue whole fish (GRwf) = selling price of the fish biomass ($ kg^{-1}) × final biomass (kg);

Gross revenue fillet (GRf) = selling price × quantity of the fillet (kg);

Production cost of whole fish (PCwf): (juvenile cost) + (feed intake × feed prices); and

Production cost of fillet (PCf) = (juvenile cost) + (feed intake × feed prices) + (quantity of fillet × cost of filleting).

Other calculations included:

Benefit/Cost Ratio of whole fish (BCRwf) = GRwf / PCwf; and

Benefit/Cost Ratio of fillet (BCRf) = GRf / PCf.

The purchase price of 148 g juveniles ($404.62 per thousand), sale of whole fish ($2.08 kg^{-1}) and fillet ($9.25 kg^{-1}), as well as the cost of processing the fillets including labor, ice, and packaging ($1.16 kg^{-1} of fillet) were indicated by the processing industry, based on values prevailing in March 2012.

2.10. Statistical analysis

Percentage data were arcsine transformed before statistical analysis. The variables were submitted to analysis of variance (ANOVA) ($\alpha < 0.05$); when significant differences were observed, a Tukey's test was applied ($P < 0.05$) for comparison of means. When possible, regression analysis was performed using the SAS software (Statistical Analysis Systems Institute, USA) (Zar, 1999). The paired t-test was used for analyzing differences before and after size-sorting stress.

2.11. Ethics statement

All experimental procedures were approved by the Animal Ethics Committee of the Veterinary and Animal Science College, São Paulo State University (protocol 44/2010 — CEUA).

3. Results

3.1. Growth performance and feed utilization

Survival rate (97.3 ± 1.4%) was not affected by digestible protein, digestible energy, and choline. Our results showed main effects of both DP and DE, with a significant interaction between DP and DE for FB, FCR and FY ($P < 0.05$) (Table 2). Overall, the highest FB, and the best FCR values were achieved with lower protein content only when fish were fed higher energy diets. Generally, the higher the dietary protein content, the higher the FY for both energy levels (Fig. 1). Protein effects were linear for FY and quadratic for FB, and FCR (Fig. 2).

Specifically, fish fed 24% DP with 14.65 MJ kg^{-1} showed better FCR than fish fed 13.4 MJ DE kg^{-1}. Moreover, fish fed 24 and 26% DP with 14.65 MJ kg^{-1} showed higher FB than fish fed 13.4 MJ DE kg^{-1} ($P < 0.05$). Within those fish fed lower energy diets (13.4 MJ DE kg^{-1}),

Table 2
Mean values of initial biomass (IB), final biomass (FB), specific growth rate (SGR), feed conversion ratio (FCR), fillet yield (FY), visceral fat (VF), and liver lipid (LL) of Nile tilapia fed digestible protein (DP), digestible energy (DE), and choline (Cho) levels for 119 days.

	IB (kg)	FB (kg)	SGR	FCR	FY (%)	VF (%)	LL (%)
DP (%)							
24	22	98.8	1.29	1.57	34.7	5.3	44.2 [b]
26	22.7	101.5	1.28	1.51	35.4	4.7	27.7 [a]
28	22.2	104.9	1.31	1.43	35.2	4.3	24.3 [a]
30	22	103.3	1.32	1.45	36.6	4.8	26.3 [a]
32	22	98.4	1.30	1.55	36.5	4.3	26.3 [a]
DE (MJ DE kg^{-1})							
13.40	22.2	100.4	1.29	1.52	35.7	4.3 [b]	30.1
14.65	22.2	102.3	1.31	1.48	35.7	5 [a]	29.4
Choline (mg kg^{-1})							
0	22.3	101.5	1.30	1.5	35.9	4.6	28.6
1000	22.1	101.2	1.30	1.5	35.5	4.8	30.9
CV (%)	5	3.31	3.64	3.87	2.48	25	24.4
Probability							
DP	ns	<0.0001	ns	<0.0001	<0.0001	ns	<0.0001
DE	ns	0.0149	ns	<0.0052	ns	0.0045	ns
DP*DE	ns	0.0095	ns	0.0022	0.0469	ns	ns

Mean values of four replicates, except liver lipid which is five replicates. The same superscript is not significantly different (Tukey $P > 0.05$). CV = coefficient of variation; Cho = ns; DP*Cho = ns; DE*Cho = ns; DP*DE*Cho = ns; ns = not significant.

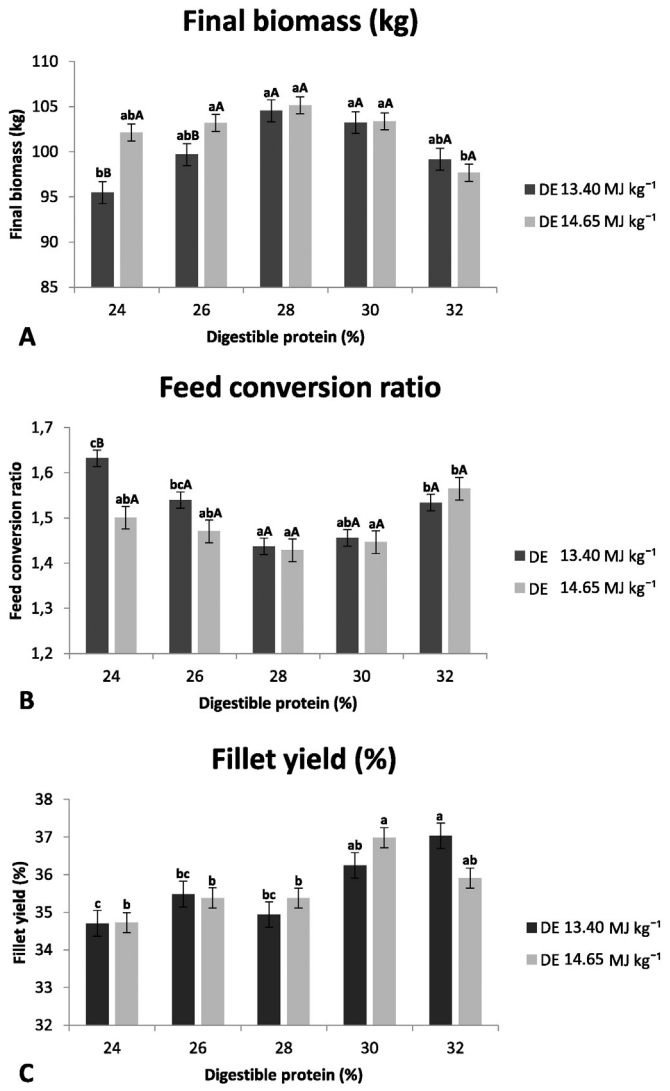

Fig. 1. A, B & C. Final biomass (A), feed conversion ratio (B) and fillet yield (C). Mean values of eight replicates ± standard deviation sharing the same lowercase superscript are not significantly different (Tukey $P < 0.05$) for digestible protein levels; mean values of eight replicates ± standard deviation sharing the same uppercase superscript are not significantly different (Tukey $P < 0.05$) for digestible energy levels.

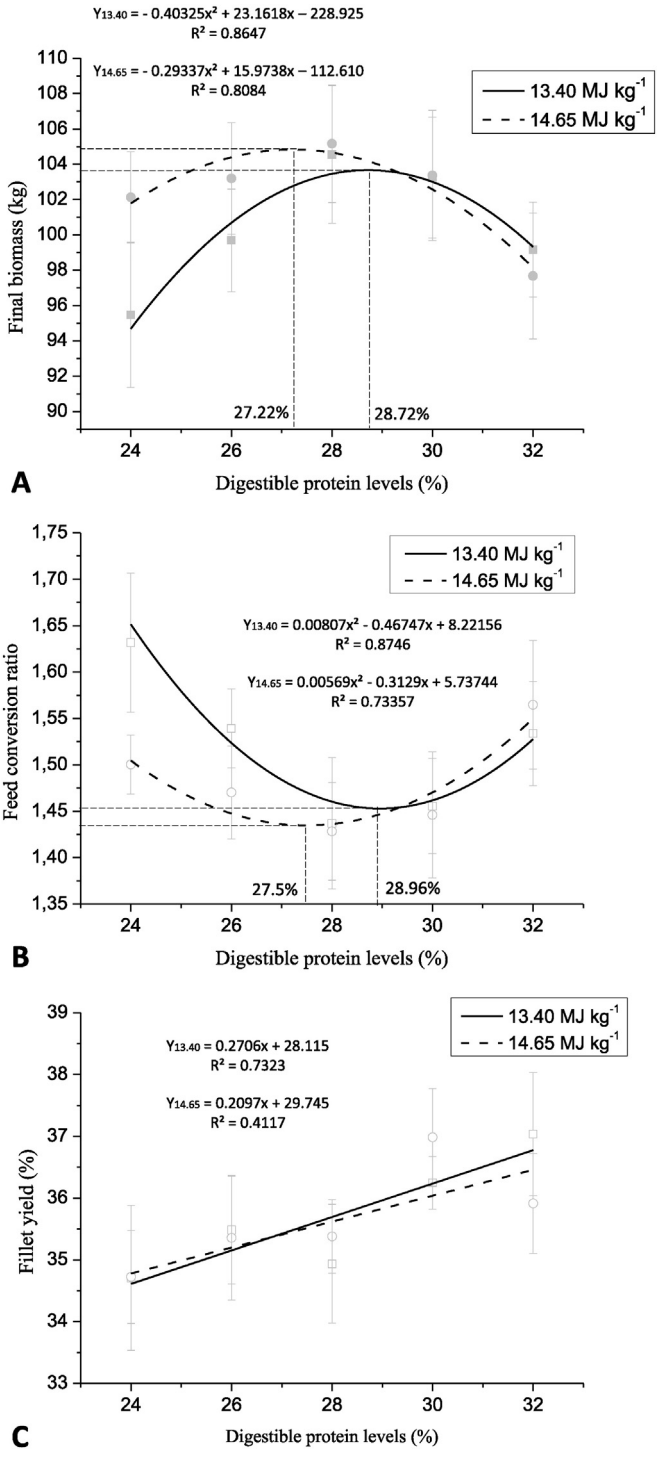

Fig. 2. Digestible protein requirement of Nile tilapia by quadratic relationship of digestible protein and digestible energy as a function of final biomass (A) and feed conversion ratio (B), and linear relationship as a function of fillet yield (C). Each point is the mean of eight replicates.

higher FB was achieved with 28 and 30% DP than with 24% DP ($P < 0.05$). For the high energy diets (14.65 MJ DE kg^{-1}) FB was higher for fish fed 26, 28, and 30% DP than for those fed 32% DP ($P < 0.05$) (Fig. 1). The best FCR values were observed for fish fed 28% DP/13.4 MJ kg^{-1}, whereas for 14.65 MJ kg^{-1}, the best ratio was achieved with 24% DP ($P < 0.05$). The analysis also showed that FY did not differ between dietary energy levels when protein content was fixed, but in lower energy fish fed 32% DP showed higher FY than those fed 24, 26, and 28%, although it did not differ from 30% DP. However, for fish fed 14.65 MJ kg^{-1}, 30% DP diets showed higher FY than 24, 26, and 28%, while it did not differ from 32% DP (Fig. 1).

3.2. Visceral fat deposition, lipid concentration, and histological analyses in the liver

VF was positively associated with 14.65 MJ DE kg^{-1} diet. Independently of DE, the highest levels of liver lipid accumulation were observed with 24% DP diets (Table 2). Moreover, macroscopic examination of the liver showed that alterations in color and consistency were observed only for fish fed this protein level. However, histological alterations were observed in the liver for all diets, showing hydropic and fatty degeneration (Fig. 3).

3.3. Hematological assay — size-sorting stress

Generally, the results of the hematological assay showed alterations in RBC, MCV, Alb, A:G, and glucose, after stress (Tables 3 and 4). Fish fed diets with no choline and oil at 24% and 32% DP showed a decrease in RBC and an increase in MCV ($P < 0.05$), and fish fed 26% DP showed a

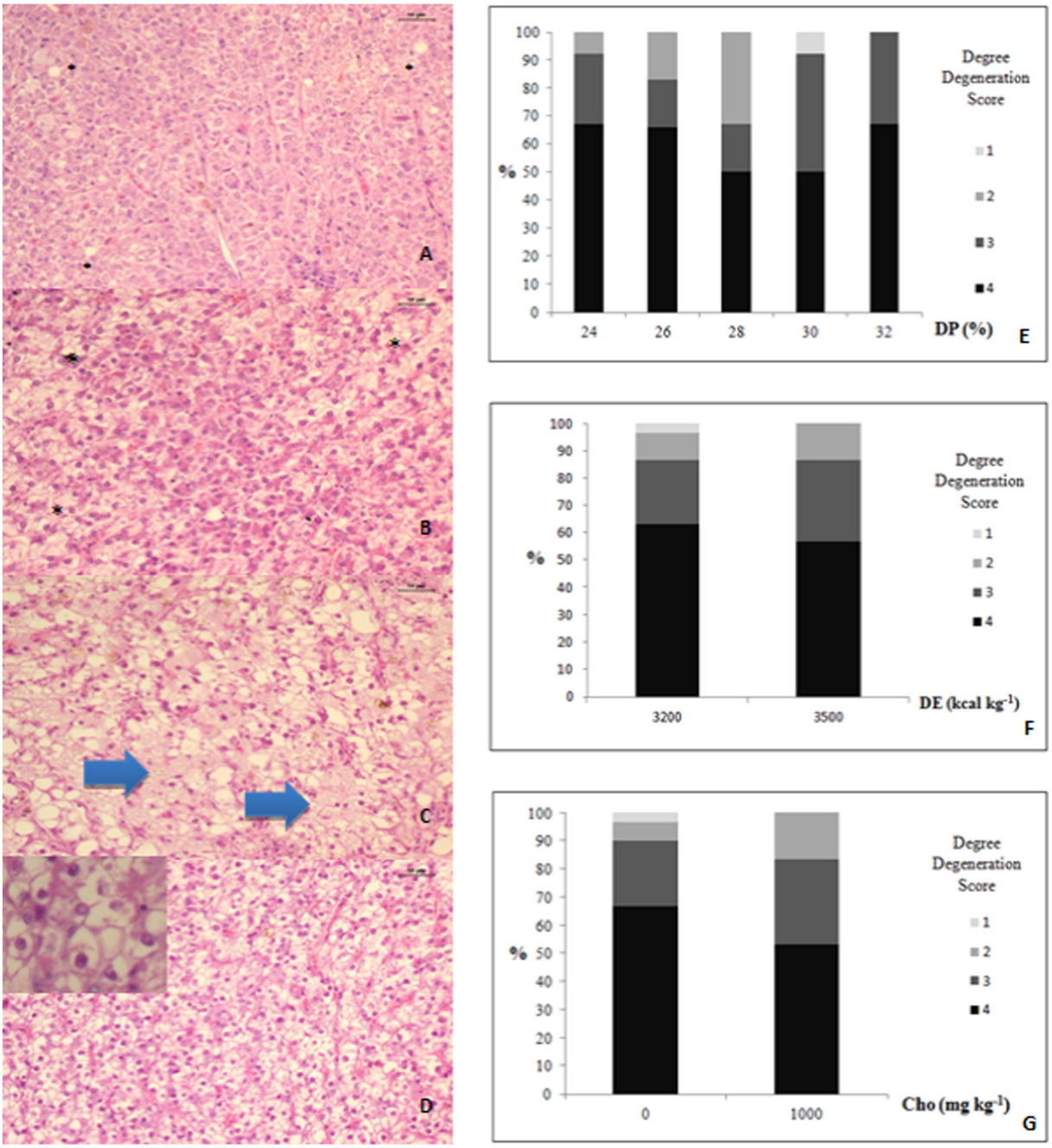

Fig. 3. Fish Liver. (A) Cell degeneration (*), score 1 (1 to 25% of hepatocytes with cellular degeneration). (B) Areas with cell degeneration (*), score 2 (26 to 50% of hepatocytes with cellular degeneration). (C) Cell degeneration, score 3 (51 to 75% of hepatocytes with cellular degeneration). Arrow shows normal hepatocytes. (D) Cell degeneration, score 4 (more than 75% of hepatocytes with cellular degeneration). There is no normal liver tissue. Inset: close aspect of the hepatocytes with cytoplasmic vacuoles. HE, Bar = 50 μm. (E) Percentage of livers with scores 1, 2, 3 and 4 considering digestible protein (DP) levels; (F) digestible energy (DE) levels and (G) choline (Cho) levels.

decrease in RBC. The only exception was an increase in MCV values with choline supplementation ($P < 0.05$) (Table 5) for fish fed 26% DP/13.4 MJ kg^{-1}. Similar effects were observed for albumin and A:G ratio, which were also affected ($P < 0.05$) by size-sorting stress regardless of DP levels and choline supplementation. Glucose concentration was also affected, showing higher values ($P < 0.05$) after stress, except for fish fed 24% DP diets. However, the comparison of values before and after size-sorting stress showed a DP effect ($P < 0.05$) on Alb and A:G ratio for fish fed 26 and 28% DP (Table 5). Size-sorting stress also determined a significant effect ($P < 0.05$) on lymphocyte, neutrophil, and monocyte counts. Lymphopenia, neutrophilia, and an increase in monocyte count were observed for all treatments. However, leukocyte differentiation was not affected ($P > 0.05$) by protein and energy levels, or by choline supplementation The mean values for lymphocytes, neutrophils, and monocytes, before and after size-sorting stress, were 97.01, 1.48, 1.51% and 76.33, 20.05, 3.49%, respectively (Table 6).

3.4. Economic analysis

Economic analysis showed the highest profitability for whole fish production using 28% DP/13.4 MJ kg^{-1}, with BCRwf of 1.52. However, for fillet production, the highest BCRf (1.85) was obtained using 30% DP/13.4 MJ (Table 7).

Table 3
Hematological parameters of Nile tilapia fed digestible protein, digestible energy, and choline levels for 119 days and subjected to size-sorting stress.

		RBC (10^6 μL^{-1})		Ht (%)		Hb (g dL^{-1})		MCV (fL)		MCHC (%)	
		Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
DP (%)	24	2.16	1.93	25.96	26.30	6.88	7.45	122.44	138.39	26.49	28.24
	26	2.11	1.95	25.73	26.19	6.86	6.93	123.82	134.00	26.73	26.63
	28	2.05	1.91	24.93	24.80	6.56	7.02	124.16	131.65	26.33	28.24
	30	2.09	1.79	24.15	23.98	6.44	6.73	119.65	136.14	26.37	28.24
	32	1.96	1.82	23.73	24.54	6.28	6.72	124.14	136.11	26.54	27.40
DE (MJ kg^{-1})	13.40	2.10	1.85	25.15	24.92	6.71	6.92	121.64	135.39	26.69	27.85
	14.65	2.05	1.91	24.67	25.36	6.50	7.02	124.13	135.11	26.30	27.67
Choline (mg kg^{-1})	0	2.10	1.87	25.24	25.00	6.64	6.97	122.69	135.02	26.27	27.96
	1000	2.05	1.89	24.60	25.28	6.57	6.97	123.08	135.49	26.71	27.56
	CV (%)	19.12	13.76	13.87	11.93	15.99	13.77	16.64	13.23	9.65	9.44
Probability											
Initial × final		0.0001		ns		ns		<0.0001		ns	

Mean values of seven replicates. The absence of letters indicates no significant difference (Tukey $P > 0.05$). RBC, red blood cells; Ht, hematocrit; Hb, hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; DP, digestible protein; DE, digestible energy; Cho, choline rate; initial = values obtained before handling-induced stress; final = values obtained after handling-induced stress; CV = coefficient of variation; DP = ns; DE = ns; Cho = ns; DP*DE = ns; DP*Cho = ns; DE*Cho = ns; DP*DE*Cho = ns; ns = not significant.

4. Discussion

We sought to investigate the nutritional requirements of the Nile tilapia under intensive rearing conditions representative of those employed in commercial fish farms, as well as the putative protective effect of supplementation with choline on hepatic damage. To this end, we tested 20 diets including five levels of DP, two levels of DE, supplemented or not with choline and subjected fish to size-sorting stress. Differences in DP:DE ratio significantly affected growth performance. Moreover, although choline did not protect the liver against lipid accumulation, it was able to buffer some of the negative effects of stress.

The higher growth observed in diets containing low protein (24 and 26% DP) but high energy can be attributed to a protein sparing effect, as previously observed (De-Silva et al., 1992; Meyer and Fracalossi, 2004; NRC, 2011). To ensure the efficiency of low protein diets, the amount of energy supplied must be sufficient to meet energetic requirements, allowing the use of protein sources to sustain adequate growth. Accordingly, fish fed low energy diets used the protein fraction as energy source, as evidenced by the lower growth performance.

A poor growth performance was also achieved when the highest protein content was added to the diet, regardless of the energy level. Since, protein and free amino acid excess can be catabolized and excreted determining an unnecessary metabolic cost, we could infer that this nitrogen excretion may be associated with lower growth performance. Here, the best performance was achieved for the DP:DE ratios 21.45 g MJ^{-1} (28.74% DP/13.4 MJ DE kg^{-1}) and 18.60 g MJ^{-1} (27.25% DP/14.65 MJ DE kg^{-1}) at the low and high energy levels, respectively.

Consistent with the results of growth performance, our economic analysis showed that the best BCRwf is obtained with 28.0% DP/13.40 MJ. In general, diets with 14.65 MJ DE were less profitable because of the high cost of oil, although they were efficient in terms of growth performance. Still, the use of oils as energy sources might be a cost-effective alternative depending on the market value of protein sources.

FY was higher at the highest DP contents (30 and 32%), regardless of the energy level supplied. In our study the growth of muscle tissue was directly affected by the dietary protein, as previously reported by Li et al. (2000). Likewise, this result is consistent with previous research showing a positive association between fillet yield and amino acid content (Furuya et al., 2004). This response might be related to the high levels of lysine and threonine in diets with higher protein concentration, and may also explain the higher BCRf for the 30.0% DP/13.40 MJ diet. The cost–benefit analyses indicated that the optimal dietary protein level relies on the final product desired. Regarding whole fish production,

Table 4
Plasmatic parameters of Nile tilapia fed digestible protein, digestible energy and choline levels for 119 days and subjected to size-sorting stress.

		TPP (mg dL^{-1})		Alb (mg dL^{-1})		Glob (mg dL^{-1})		A:G		Gluc (mg dL^{-1})	
		Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
DP (%)	24	3.67	3.83	0.73^{a}	1.05	2.90	2.77	0.26^{ab}	0.40	37.70	63.89
	26	3.77	3.98	0.93^{b}	1.12	2.85	2.86	0.34^{b}	0.38	36.90	69.68
	28	3.73	3.77	0.92^{b}	1.33	2.81	2.51	0.34^{b}	0.55	32.35	89.24
	30	3.42	3.85	0.82^{ab}	1.26	2.65	2.60	0.32^{ab}	0.50	28.33	78.24
	32	3.61	3.68	0.69^{a}	1.11	2.96	2.61	0.24^{a}	0.43	31.69	91.82
DE (MJ kg^{-1})	13.40	3.66	3.76	0.84	1.12	2.89	2.64	0.30	0.44	32.81	80.49
	14.65	3.62	3.88	0.81	1.24	2.78	2.72	0.30	0.47	34.14	74.47
Choline (mg kg^{-1})	0	3.67	3.81	0.84	1.21	2.84	2.71	0.31	0.45	32.72	71.82
	1000	3.62	3.83	0.80	1.14	2.83	2.65	0.29	0.45	34.28	82.40
	CV (%)	13.14	13.16	25.75	24.97	15.56	16.57	33.67	32.20	41.64	58.37
Probability											
Initial × final		ns		<0.0001		ns		<0.0001		<0.0001	
DP		ns	ns	0.0001	ns	ns	ns	0.0001	ns	ns	ns

Mean values of seven replicates. Mean values sharing the same superscript are not significantly different (Tukey $P > 0.05$). TPP, total plasma protein; Alb, albumin; Glob, globulin; A:G, albumin:globulin ratio; Gluc, glucose; DP, digestible protein; DE, digestible energy; Cho, choline rate; initial = values obtained before handling-induced stress; final = values obtained after handling-induced stress; CV = coefficient of variation; DE = ns; Cho = ns; DP*DE = ns; DP*Cho = ns; DE*Cho = ns; DP*DE*Cho = ns; ns = not significant.

Table 5
Hematological parameters of Nile tilapia fed digestible protein, digestible energy and choline levels for 119 days and subjected to size-sorting stress.

Variables			RBC (10^6 µL^{-1})		MCV (fL)		Alb (mg dL^{-1})		A:G		Gluc (mg dL^{-1})	
Protein	Energy	Choline	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
24	13.40	0	2.25 ± 0.36^{a}	1.76 ± 0.22^{b}	119.16 ± 22.71^{b}	146.61 ± 13.39^{a}	0.75	1.06	0.07	0.41	33.08	32.87
24	13.40	1000	2.10	1.96	124.24	133.59	0.88	0.78	0.16	0.38	42.36	67.34
24	14.65	0	2.05	1.90	127.57	140.89	0.68 ± 0.19^{b}	1.34 ± 0.39^{a}	0.24 ± 0.10^{b}	0.53 ± 0.14^{a}	35.98	64.60
24	14.65	1000	2.24	2.10	118.78	131.49	0.64 ± 0.33^{b}	1.10 ± 0.26^{a}	0.21 ± 0.08^{b}	0.36 ± 0.07^{a}	39.70	73.32
26	13.40	0	2.22 ± 0.37^{a}	1.83 ± 0.20^{b}	124.47	125.61	0.92	1.12	0.36	0.37	23.37 ± 8.20^{b}	66.81 ± 19.41^{a}
26	13.40	1000	2.07	1.96	116.47 ± 18.19^{b}	138.81 ± 17.43^{a}	1.02	0.94	0.38	0.31	40.11 ± 13.79^{b}	67.91 ± 13.97^{a}
26	14.65	0	2.08	1.98	130.48	139.62	0.95	1.17	0.32	0.41	48.41	66.70
26	14.65	1000	2.07	2.02	123.86	130.2	0.85 ± 0.22^{b}	1.26 ± 0.34^{a}	0.29 ± 0.05^{b}	0.47 ± 0.12^{a}	35.90 ± 17.26^{b}	77.87 ± 35.88^{a}
28	13.40	0	2.02	1.99	127.50	134.84	0.96 ± 0.15^{b}	1.32 ± 0.27^{a}	0.35 ± 0.13^{b}	0.51 ± 0.17^{a}	35.12	68.87
28	13.40	1000	2.09	1.91	126.40	126.95	0.96 ± 0.25^{b}	1.44 ± 0.21^{a}	0.31 ± 0.12^{b}	0.63 ± 0.21^{a}	29.73 ± 6.26^{b}	95.96 ± 61.46^{a}
28	14.65	0	2.11	1.86	120.23	137.07	1.00	1.14	0.42	0.41	32.90	86.65
28	14.65	1000	1.97	1.88	122.51	127.73	0.80 ± 0.15^{b}	1.27 ± 0.36^{a}	0.29 ± 0.11^{b}	0.54 ± 0.19^{a}	31.38 ± 7.91^{b}	102.57 ± 66.13^{a}
30	13.40	0	2.07	1.74	118.12	128.92	0.81 ± 0.18^{b}	1.10 ± 0.04^{a}	0.29 ± 0.09^{b}	0.48 ± 0.15^{a}	38.25 ± 12.11^{b}	76.12 ± 30.48^{a}
30	13.40	1000	2.10	1.85	122.77	135.14	0.83	1.19	0.29	0.43	29.78 ± 8.59^{b}	82.82 ± 40.96^{a}
30	14.65	0	2.21	1.85	110.95	130.38	0.87 ± 0.20^{b}	1.40 ± 0.40^{a}	0.34	0.47	27.90 ± 8.69^{b}	66.38 ± 32.05^{a}
30	14.65	1000	1.94	1.71	128.93	150.12	0.80 ± 0.26^{b}	1.46 ± 0.35^{a}	0.33 ± 0.13^{b}	0.61 ± 0.18^{a}	24.46 ± 6.76^{b}	82.87 ± 26.79^{a}
32	13.40	0	2.13 ± 0.17^{a}	1.78 ± 0.25^{b}	117.48 ± 18.87^{b}	139.86 ± 15.94^{a}	0.75	1.21	0.25 ± 0.09^{b}	0.48 ± 0.13^{a}	25.01 ± 5.48^{b}	144.04 ± 64.28^{a}
32	13.40	1000	1.96	1.71	119.34	142.21	0.48 ± 0.09^{a}	1.06 ± 0.22^{b}	0.16 ± 0.08^{b}	0.43 ± 0.12^{a}	31.74 ± 7.64^{b}	100.91 ± 41.80^{a}
32	14.65	0	1.84	2.01	132.28	125.07	0.71 ± 0.09^{a}	1.18 ± 0.45^{b}	0.24	0.42	33.76	37.66 ± 4.51
32	14.65	1000	1.92	1.78	127.47	137.30	0.78	1.07	0.28	0.40	41.24	68.38 ± 21.56

Mean values of seven replicates ± standard deviation. Mean values sharing the same superscript are not significantly different (t-test $P > 0.05$). RBC, red blood cells; MCV, mean corpuscular volume; Alb, albumin; A:G, albumin:globulin ratio, Glu, glucose.

the highest profitability was reached with 28% DP/13.40 MJ kg^{-1}. However aiming fillet production, the best result was achieved with 30% DP/13.40 MJ kg^{-1} emphasizing the importance of protein content for muscle synthesis, since protein deposition appears to be the main determinant of live weight gain in fish (Dumas et al. 2007). Our results also showed that significantly lower growth performance of fish fed 24 and 26% DP diets offsets the lower feed costs and reduced profitability. Despite the fact that protein is the most costly macronutrient in aquaculture diets, in this study, the higher inclusion of crystalline amino acids on lower protein diets does not impact as much as oil inclusion, which clearly represents the main source of feed cost fluctuation, as described above.

The lowest protein level, at which concentration of carbohydrates in the diet was highest, induced lipid accumulation in the liver (44.16%), as observed macroscopically and histologically. Overall, the percentage of lipids in the liver ranged from 24.32 to 44.16%, a percentage higher than previously observed by some authors (3.9 to 16.0%; Shiau and Lo, 2000; Peres et al., 2004; Shiau and Su, 2005), yet within the range (38.2 to 49.8%) observed by Kasper et al. (2002). The high level of carbohydrate as an energy source might have led to fat deposition by lipogenesis once energy requirements were met, as previously reported (Helland and Grisdale-Helland, 1998; Gaye-Siessegger et al., 2006; Young et al., 2006; Leaver et al., 2008).

In contrast to previous findings (Ogino et al., 1970; Osol et al., 1982; Wilson and Poe, 1988), choline did not protect fish from fat accumulation in the liver. In fact, there was a high degree of degeneration in fish livers regardless of the diet, possibly due to the higher intake of food intake in commercial farming conditions such as those used in this study. In these settings, higher amounts of choline might be needed for a protective effect to be detected. Likewise, the high VF observed in fish fed high energy diets likely resulted from excessive energy not used for growth (Jauncey and Ross, 1982; Brown et al., 1992; Mohanta et al., 2009).

Fish dynamic equilibrium is constantly threatened by fluctuations in intrinsic and extrinsic factors, which may determine deleterious effects on growth, reproduction, and disease resistance. Although temperatures below the comfort zone along with high stock density may cause physiological stress, in this study these sources of stress, inherent to intensive culture system, did not affect fish hematological profile at the initial phase, as presented in Table 3, probably due to the gradual decrease in water temperature. Accordingly, hematological parameters obtained before the size-sorting stress procedure were close to those determined in the laboratory for this species (Hrubec and Smith, 2010; Barros et al., 2014), and under culture condition (Hrubec et al., 2000).

Hematopoietic tissue needs high and constant protein supply, so qualitative and quantitative cellular alterations have been described under inappropriate protein nutrition (Hrubec and Smith, 2010). It has also been showed that erythropoietin, the major physiological regulator of erythropoiesis, is closely related to protein level, mainly due to erythrocytes precursor synthesis. Protein dietary levels maintained erythropoiesis, even after size-sorting stress. However, in those diets without oil and choline, erythropoiesis was impaired, resulting in release of immature cells, following the stress challenge. This response is consistent with the observation that choline is a component of phospholipids in the fragile cellular membranes of tissue (Halver, 2002). Therefore, in our study, the size-sorting stress could have increased the demand for tissue oxygenation, resulting in the observed alterations in blood parameters. Indeed, anemia caused by choline deficiency was determined in rainbow trout (Kitamura et al., 1967). To our knowledge, however, few studies have analyzed the relationship between choline and erythropoiesis.

The increase of TPP and decrease of globulin levels found in our study reinforce the hypothesis that, regardless of the experimental diet, the acute size-sorting stress did not allow fish to reach homeostasis. Stress can also be evaluated by changes in albumin and glucose

Table 6
Mean values of the percentage of lymphocytes, neutrophils and monocytes from the Nile tilapia fed digestible protein (%), digestible energy (MJ kg^{-1}) and choline (mg kg^{-1}) levels for 119 days and subjected to size-sorting stress.

Variables			Lymphocytes (%)		Neutrophils (%)		Monocytes (%)	
Protein	Energy	Choline	Initial	Final	Initial	Final	Initial	Final
24	13.40	0	95.13[a]	81.88[b]	2.75[a]	15.5[b]	2.13[a]	2.63[a]
24	13.40	1000	95.75[a]	84[b]	2.5[a]	12.88[b]	1.75[a]	3.13[a]
24	14.65	0	97.88[a]	75.13[b]	1[a]	22.13[b]	1.13[a]	2.75[a]
24	14.65	1000	97.25[a]	71.75[b]	1.38[a]	23.25[b]	1.38[a]	5[b]
26	13.40	0	97.88[a]	76.88[b]	0.63[a]	17.13[b]	1.5[a]	3.5[a]
26	13.40	1000	95.63[a]	73.5[b]	2.13[a]	21.88[b]	2.25[a]	4.63[a]
26	14.65	0	97.25[a]	75.88[b]	1.38[a]	21.38[b]	1.38[a]	2.75[a]
26	14.65	1000	96.75[a]	71[b]	1.88[a]	25.88[b]	1.38[a]	3.13[a]
28	13.40	0	97[a]	78.88[b]	1.5[a]	18[b]	1.5[a]	3.13[b]
28	13.40	1000	96.38[a]	73.13[b]	1.63[a]	23.13[b]	2[a]	3.75[b]
28	14.65	0	96.38[a]	77.75[b]	1.38[a]	18.63[b]	2.25[a]	3.63[b]
28	14.65	1000	98.88[a]	73.13[b]	0.63[a]	25[b]	0.5[a]	1.88[b]
30	13.40	0	96.75[a]	76.75[b]	1.25[a]	18[b]	2[a]	5.25[b]
30	13.40	1000	98[a]	81.38[b]	0.88[a]	13.75[b]	1.13[a]	4.63[b]
30	14.65	0	96.75[a]	75.38[b]	2[a]	20.75[b]	1.25[a]	3.88[b]
30	14.65	1000	96.25[a]	74.5[b]	1.88[a]	22.25[b]	1.88[a]	3.25[a]
32	13.40	0	96.38[a]	71.38[b]	2.13[a]	25.86[b]	1.5[a]	2.75[a]
32	13.40	1000	98.13[a]	73.88[b]	0.88[a]	22[b]	1[a]	4.13[b]
32	14.65	0	97.88[a]	76.5[b]	1[a]	20.38[b]	1.13[a]	3.13[b]
32	14.65	1000	98[a]	83.88[b]	0.88[a]	13.25[b]	1.13[a]	2.88[a]

Values are means of seven replicates. Means followed by the same letters within a row, no significant difference (t-test $P < 0.05$).

concentration (Nardocci et al., 2014). Increase of albumin levels may be related to the transport of fatty acids required as an energy source in stressful situations, especially in the liver and muscle (Kaneko et al., 1997). Our observation of higher glucose concentrations after stress further confirmed this effect, in line with the expectation that energy can be obtained by glycogenolysis for a short period (Sumpter, 1997), as previously observed (Carneiro and Urbinati, 2002; Evans et al., 2006; Falcon et al., 2007; Barros et al., 2014).

Higher susceptibility to disease is one of the outcomes of immunosuppression caused by stress. Handling, especially during winter time, is considered one of the major stressors for fish. As a consequence, increased fish susceptibility to opportunistic pathogens has been reported in winter by fish farmers. The changes in lymphocytes, neutrophils, and monocytes observed in this study after the size-sorting stress confirm this effect.

Lymphopenia after stress has been previously reported in fish by Barton and Iwama (1991). Here, lymphopenia was also observed, together with neutrophilia and increase in monocytes following handling. Similar results were observed by Falcon et al. (2007); Barros et al. (2009), Under stress, the release of catecholamine and cortisol can cause constriction of the spleen, increasing blood flow and triggering the migration of leukocytes. The total number of white cells and lymphocytes may also decrease, together with phagocytic impairment of cellular functions (Barton and Iwama, 1991; McDonald and Milligan, 1992). Such a decrease in circulating lymphocytes might underlie the association between stress responses and disease outbreak due to the increased levels of plasma cortisol (Pickering and Pottinger, 1985). Likewise, Pickering (1986) reported that the decrease in lymphocytes may be related to fish resistance to pathogens. Here, the evaluation of leukocyte differentiation showed that size-sorting stress determined an increase of phagocytic cell production possibly aimed at the elimination of invading microorganisms, independently of the diet.

The cost–benefit analyses indicated that the optimal dietary protein level relies on the final product desired. Regarding whole fish production, the highest profitability was reached with 28% DP/13.40 MJ kg^{-1}. However aiming the fillet production, the best result was achieved

Table 7
Economic indicators of fillet production and production of the Nile tilapia fed digestible protein (%), digestible energy (MJ kg^{-1}), and choline (mg kg^{-1}) levels produced in net cages (NC) for 119 days.

Diets	Diet cost kg^{-1} ($)	Biomass NC^{-1} (kg NC^{-1})[a]	CPwf ($ NC^{-1})[b]	GRwf ($ NC^{-1})[c]	BCRwf[d]	Qt fillet NC^{-1} (kg NC^{-1})[e]	CPf ($ NC^{-1})[f]	GRf ($ NC^{-1})[g]	BCfR[h]
24/13.40	0.61	95.47	142.65	198.66	1.39	33.14	180.96	306.46	1.69
24/14.65	0.67	102.13	149.93	212.53	1.42	35.46	190.93	327.98	1.72
26/13.40	0.62	99.69	143.00	207.46	1.45	35.37	183.89	327.14	1.78
26/14.65	0.67	103.20	150.17	214.75	1.43	36.51	192.38	337.62	1.75
28/13.40	0.62	104.55	143.34	217.56	1.52	36.52	185.57	337.78	1.82
28/14.65	0.67	105.17	150.42	218.84	1.45	37.21	193.43	344.14	1.78
30/13.40	0.62	103.24	143.69	214.84	1.50	37.42	186.95	346.09	1.85
30/14.65	0.67	103.37	150.66	215.10	1.43	38.24	194.86	353.63	1.81
32/13.40	0.62	99.16	144.03	206.35	1.43	36.72	186.49	339.65	1.82
32/14.65	0.67	97.67	150.90	203.25	1.35	35.08	191.46	324.43	1.69

[a] Biomass (kg of fish per net $cage^{-1}$).
[b] Cost of whole fish production ($ net $cages^{-1}$).
[c] Gross revenue of whole fish ($ $cages^{-1}$).
[d] Benefit/cost ratio of whole fish.
[e] Fish fillet per net cage (kg net $cage^{-1}$).
[f] Cost of fillet production ($ net $cage^{-1}$).
[g] Gross revenue fillet ($ $cages^{-1}$).
[h] Benefit/cost ratio of fillet.

with 30% DP/13.40 MJ kg^{-1} emphasizing the importance of protein content for muscle synthesis, since protein deposition appears to be the main determinant of live weight gain in fish (Dumas et al. 2007). Our results also showed that significantly lower growth performance of fish fed 24 and 26% DP diets offsets the lower feed costs and reduced profitability. Despite the fact that protein is the most costly macronutrient in aquaculture diets, in this study, the higher inclusion of crystalline amino acids on lower protein diets does not reflect as much as oil inclusion, which clearly represents the main source of feed cost fluctuation.

In summary, this study showed that the best performance of Nile tilapia reared under intensive commercial farming conditions was achieved with DP:DE ratios of 21.45 g MJ^{-1} (28.9% DP/13.4 MJ DE kg^{-1}) and 18.60 g MJ^{-1} (27.5% DP/14.65 MJ DE kg^{-1}). The highest fillet yield was obtained with 30% DP, regardless of the dietary energetic level. Sustained homeostasis was observed in this setting, and even though size-sorting stress altered some hematological parameters, they were still within the range recognized as healthy. Choline was not effective in protecting the liver against hepatic steatosis, but was able to buffer some of the negative effects of stress under these rearing conditions. Overall, our study provides the best DP:DE ratio for Nile tilapia under intensive farming condition, which is essential to the fish feed industry, as well as to scientific community, especially considering health status and production costs. Based on unclear results for choline, higher levels should be tested, aiming at the appropriate dietary supplementation to avoid hepatic steatosis.

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