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Coping with suboptimal water temperature: modifications in blood parameters, body composition, and postingestive-driven diet selection in Nile tilapia fed two vegetable oil blends

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ABSTRACT

The world tilapia production faces seasonal variations. However, very few nutritional studies have addressed suboptimal temperature. We evaluated the effect of two temperatures (20 or 30 °C) and two vegetable oil blends (one rich in corn oil (**COR**) and one rich linseed oil (**LIN**)) on tilapia growth, body composition, and blood parameters using a 2×2 factorial design with the following treatments: COR-20; LIN-20; COR-30; LIN-30 (**Trial 1**). In addition, we also evaluated the effect of postingestive signals of dietary oils when the organoleptic properties of diets were isolated (**Trial 2**). In the Trial 1, 256 fish (15.36 ± 0.14 g) were placed in 16 aquariums and submitted during 30 days to the 2×2 factorial designs: COR-20; LIN-20; COR-30; LIN-30. The temperatures were established in two independent water recirculation systems. In the Trial 2, 96 fish (34.02 ± 0.79 g) were placed in 12 aquariums and subjected to the same experimental design of Trial 1, but to evaluate fish feeding behavior. They were allowed to select the encapsulated diets provided in different feeding halls to evaluate if diet preferences are influenced by postingestive signals. As the Trial 1 results show, diets had no significant effects on growth, dietary protein use, and body centesimal composition, but 30 °C induced the best performance and protein deposition ($P < 0.05$). LIN-20 showed lower very-low-density lipoprotein and cortisol, but higher high-density lipoprotein (**HDL**), aspartate aminotransferase (**AST**), alanine aminotransferase (**ALT**), and triglycerides (**TG**) than COR-20 ($P < 0.05$). COR-30 presented higher HDL, AST, ALT, TG, and cortisol than LIN-30. The fish fed COR showed lower C20:5n-3 (**EPA**) and higher n-6 than fish fed LIN ($P < 0.05$). The fish fed LIN had high n-3 highly unsaturated fatty acid. $\sum$ polyunsaturated fatty acid was higher at 30 °C. Finally, the tilapia in Trial 2 showed clear diet intake regulation and preference for LIN ($P < 0.05$), regardless of temperature. In short, lipid sources had no influence on tilapia performance; however, temperature affects carcass lipid deposition as well as fatty acids profile. Notably, the preference for linseed oil can suggest nutritional metabolic issues, contributing to animal behavior knowledge.

Implications

In subtropical regions, the ability for tilapia to maintain homeostasis could be limited, due to suboptimal water temperature. Fatty acids are important modulators of biological membranes permeability and several physiological functions. We found a positive correlation between n6/n3 ratios and environmental temperature (suboptimal) on blood parameters and body composition. The n6/n3 ratios will not impact negatively on growth but in physiological adaptation to suboptimal temperature. The positive relationship between n6/n3 ratios intake target, and their link with physiological–behavioral responses (i.e., cortisol), could contribute to aquafeed use and animal health.

Introduction

Tilapia are widely cultured in the tropical and subtropical regions (El-Sayed, 1999; Corrêa et al., 2018). It is known that tilapia feeding and swimming decrease at 20 °C. Beyond the metabolic rate

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reduction effect, a stress response followed by changes in some biochemical and enzymatic blood parameters is usually observed (Dan and Little, 2000). Glucose, esterol, triglycerides, and transaminase enzyme activities were shown to alter in Nile tilapia facing cold stress (Shi et al., 2015). In addition, it is already known that plasma cortisol of Nile tilapia can be changed under low temperature condition (Barcellos et al., 1999).

Fatty acids are partly responsible for the cell membrane structure, fluidity, and functionality (Corrêa et al., 2017). Freshwater species can convert essential fatty acids, including linoleic acid (**LOA**, 18:2n-6) and alpha-linolenic acid (**α-LNA**, 18:3n-3), into their long-chain polyunsaturated fatty acid (**LC-PUFA**) homologs as arachidonic (**ARA**, 20:4n-6), eicosapentaenoic (**EPA**, 20:5n-3), and docosahexaenoic (**DHA**, 22:6n-3) (Tocher, 2010). Acute stress from low temperature exposure in tilapia favors the metabolism of saturated fatty acids for energy needs, but fish begin to metabolize LC-PUFAs during long stress periods. Polyunsaturated fatty acid retention, and its bioconversion into homologs of longer chain lengths, apparently indicates adaptations to lower temperature acclimation in Nile tilapia (Nobrega et al., 2017; Corrêa et al., 2018). Understanding the effects of diet ingredients on lipoprotein class distribution can ultimately prove a fundamental step to gain a better understanding of lipid metabolism (Ferreira et al., 2011). Few studies have considered to examine how temperature ranges affect tilapia nutrition according to farm conditions.

It is already known that the temperature affects the metabolism and feed intake which may affect the nutrients obtained by fish. In behavioral terms, fish can reveal nutritional needs by its ability to regulate specific nutrients intake (Raubenheimer et al., 2012). Rubio et al. (2003) have proposed innate behavioral processes to explain the existence of specific appetites for some macronutrients by sea bass (*Dicentrarchus labrax*) using encapsulation method. Subsequently, this method was valided in tropical species such as tilapia (*Oreochromis niloticus*) (Fortes-Silva et al., 2011) and tambaqui (*Colossoma macropomum*) (Pereira et al., 2018). The organoleptic properties are diminished enough in this method to prevent fish identify diet composition before ingestion. Recent studies have demonstrated the existence of taste receptors and signaling elements in the gastrointestinal tract of fish, suggesting that sensory properties of the diet might also have functional effects beyond oral taste sensations and palatability (Morais, 2016; Bertucci et al., 2019). These studies strongly suggest that diet preferences in these species are the direct result of postingestive influences of specific nutrients and fish nutritional needs.

Our objective was to investigate if changing diet lipid profile can affect how tilapia respond to suboptimal temperature (20 °C). We evaluated the effect of vegetable oil blends and suboptimal temperature on the growth, blood parameters, and fatty acid composition (Trial 1). The second purpose was to investigate if fish can use postingestive signals to show dietary preferences according with ambient temperature, using encapsulated food method (without organoleptic proprieties) (Trial 2).

Material and methods

Pre-experimental conditions

Sex-reversed males of tilapia (*O. niloticus*), obtained from a local hatchery, were maintained in two tanks (2000 l) in the Laboratory of Fish Nutrition and Feeding Behavior (AquaUFRB) and were fed at 3% of BW with a commercial diet (Pirá 36, Guabi, Brazil, 36% CP) for 2 weeks before the trials started. The tanks were equipped with a filter and aeration pump in a closed water recirculation system. The photoperiod was 12:12 h light/dark (**LD**) at 28 °C.

Table 1
Experimental tilapia diets formulation and composition.

	COR	LIN
Ingredients (g/kg)		
Soybean meal	370.0	370.0
Corn meal	222.0	222.0
Soy protein concentrate	150.0	150.0
Wheat flour	100.0	100.0
Linseed oil[1]	13.0	50.0
Corn oil[1]	56.0	14.0
Palm oil[1]	1.0	6.0
Yeast[2]	50.0	50.0
Dicalcium phosphate[3]	15.0	15.0
Vitamin and mineral premix[4]	10.0	10.0
L-Lysine HCl[3,5]	6.0	6.0
DL-Methionine[5]	4.0	4.0
L-Threonine[6]	2.0	2.0
Antioxidant (BHT)	1.0	1.0
Proximate composition (g/kg)		
DM	911.0	908.0
CP	354.0	360.0
Crude lipid	74.0	70.0
Ash	58.0	57.0
Energy[7] (MJ/kg)	19.9	21.0
Fatty acids composition (% of total FA)[8]		
C14:0	0.10	0.13
C16:0	12.61	12.51
C16:1	0.22	0.20
C17:0	0.11	0.10
C18:0	2.64	3.52
Σ SFA	16.38	17.13
C18:1n9	31.43	27.24
C20:1	0.21	0.22
C20:0	0.50	0.40
Σ MUFA	31.86	27.66
C18:2n6	46.75	43.10
C18:3n6	0.04	0.09
C18:3n3	3.90	11.20
C22:0	0.22	0.30
C24:0	0.20	0.17
Σ PUFA	50.70	54.40
Σ n6	46.80	43.20
Σ n3	3.90	11.20
n6/n3 ratio	12.02	3.86

COR is the diet elaborated with a vegetable oil blend, rich in corn oil, and LIN is the diet elaborated with a vegetable oil blend, rich in linseed oil.
[1] Refined oil Mundo dos Óleos, Cruzeiro, Brasília, DF, Brazil.
[2] From *Saccharomyces cerevisiae*, Grupo Ullmann, Belo Horizonte, MG, Brazil.
[3] Nutrimix, Campo Grande, MS, Brazil.
[4] Mix Vita/Min Omnivorous fish Cargill, Vila Cordeiro, São Paulo, SP, Brazil. Composition (mg/kg diet): iron sulfate, 196; copper sulfate, 28; zinc oxide, 280; manganese oxide, 52; sodium selenite, 1.2; cobalt sulfate, 0.4; potassium iodide, 1.2; vitamin A, 19 950 (UI/kg diet); vitamin D3, 7 980 (UI/kg diet); vitamin E, 199; vitamin K3, 10; vitamin C, 700; thiamin, 50; riboflavin, 50; pyridoxine, 50; cyanocobalamine, 0.1; niacin, 200; calcium pantothenate, 100; folic acid, 10; biotin, 1.6; inositol, 100; ethoxyquin, 247.
[5] Ajinomoto, Vila Mariana, São Paulo, SP, Brazil.
[6] Evonik, Santo Antônio, São Paulo, SP, Brazil.
[7] Gross energy: determined by direct combustion in an adiabatic bomb calorimeter.
[8] FA, fatty acids; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Experimental diets

Two diets were formulated with mixtures of vegetable oils and different proportions of linseed (**LIN**) and corn (**COR**) oils (Table 1) to provide different content of n-3 and n-6 fatty acids (Table 1). Experimental diets were formulated to have 36% of CP and 20 MJ/kg of energy, according with Almeida et al. (2019).

Diet ingredients were weighed and mixed with an automatic paddle mixer, followed by extrusion in a single-screw extruder (Inbramaq, Sao Paulo, Brazil) to produce 4-mm pellets. Pellets were dried at 65 °C in a convection drier for approximately 24 h until the moisture content was around 10% and then stored in a freezer (−20 °C) until use.

After fish adaptation in laboratory condition and diets preparation, two trials were designed to evaluate the relation of lipidic composition of diets and suboptimal temperature on growth and behavioral parameters as can be seen below.

Trial 1: effects dietary lipids and temperatures on growth, feed utilization, hematological parameters, whole-body composition, and fatty acids deposition

After adapting to laboratory conditions, 256 fish (average initial weight of 15.36 ± 0.14 g and 10.93 ± 0.12 cm of total length) were randomly placed in 16 aquariums (60 l each measuring 40 × 30 × 50 cm), which totaled 16 animals per aquarium in a 2 × 2 factorial arrangement (by comparing two diet effects at two temperatures). The aquariums were placed inside a insulate chamber designed for temperature and photoperiod control. The walls of chamber were made with thermal insulating material, and the inside light was controlled by a digital timer. At this time, two temperatures were set to predefined values in two independent systems each one with individual water recirculation and similar filter. Temperature system 1 (8 aquariums at 20 °C): suboptimal temperature was controlled indirectly by adjusting room temperature (Split 12 000 BTU/s Cold LG Smile TSNC122TNW5) and regulated to gradually decrease 2 degrees/day until it stabilized at 20 °C. After reaching equilibrium, the aquarium water temperature and ambient temperature were virtually the same (20 °C). This condition was maintained until the end of the experiment. Temperature system 2 (8 aquariums at 30 °C): to obtain a water temperature of 30 °C in the eight remaining aquariums of the same insulated thermal chamber, a digital temperature controller was fitted (Novus N480D, United States), coupled to two temperature sensors (PT 100). Temperature of 30 °C is inside the optimal range for tilapia growth (El-Sayed and Kawanna, 2008; Nivelle et al., 2019). This system heated water and allowed constant temperature control with aeration, mechanical and biological filters. To avoid temperatures fluctuations, maintenance renewal was done with water at the same temperature of the corresponding system.

In each temperature subsystems (20 and 30 °C), four aquariums per diet (LIN and COR) were used. Thus, the final experimental designs were devised as follows: COR-20; LIN-20; COR-30; and LIN-30. During the experiment, feed was provided three times a day (0800 h; 1200 h; 1700 h) to all the groups during the light phase. Animals were fed until apparent satiation, and uneaten food was collected (approximately 30 min after each meal), separated from feces, to be dried and weighed. Feed intakes were calculated as delivered food (dry weight) – uneaten food (dry weight). The water parameters for the system at 20 °C were daily measured with following results: temperature 20 ± 1 °C, dissolved oxygen (7.3 ± 0.40 mg/l), pH (6.6 ± 0.17), total ammonia (0.004 ± 0.002 mg/l), and nitrite (0.03 ± 0.08 mg/l). For the system at 30 °C, temperature was 30 ± 1 °C, dissolved oxygen 6.4 ± 0.19 mg/l, pH (6.8 ± 0.36), total ammonia (0.005 ± 0.001 mg/l and nitrite (0.06 ± 0.11 mg/l). Water temperature, dissolved oxygen, and pH were monitored with a multiparameter analyzer HANNA HI 9828 (Madrid, Spain). Ammonia and nitrite were monitored weekly with colorimetric test (ALFAKIT, Florianópolis, Brazil). All these parameters were adequate for the species according with Almeida et al. (2019).

Pre-sampling procedures and performance parameters

At the end of the experiment, fish were submitted to a 24 h feed deprived period. Animals were anesthetized with eugenol solution (50 mg/l) (Simões et al., 2011). After deep anesthesia, were individually weighed (wet basis), measured, and blood sampled before being euthanized by medullary section to proceed with dissection. The whole-body samples for the centesimal composition and fatty acid profile were collected and stored at −80 °C until analyzed. Further details on sampling procedures are given in the subsequent sections.

The following growth parameters were evaluated: final length (cm); final BW (g); weight gain (**WG**, g); daily weight gain (**DWG**, g/day); specific growth rate (SGR, %); weight gain (WG, g) = final weight − initial weight; daily weight gain (DWG, g) = final weight − initial weight; specific growth ratio (**SGR**) = in final weight − in initial weight × 100/time (days).

The feed utilization indicators were also measured as follows:

- Feed intake (**FI** = daily feed intake (g)/of BW (kg))
- Feed efficiency ratio (**FER** = weight gain (g)/dry feed intake (g)).
- Protein efficiency ratio (**PER** = weight gain (g)/dry protein intake (g)).
- Apparent net protein utilization (**ANPU**, %) = 100 × (final-initial fish body protein/total protein intake

Survival (S) as a percentage was measured at the end of experiment (30 days) by counting individuals.

Hematological analysis

After the anesthetic, eight fish were randomly collected from each replicate to determine the hematological parameters. Blood was collected by caudal puncture and plasma separated and stored in a freezer (−20 °C). Briefly, a syringe (3 ml) was used with a 21G sterile hypodermic needle coated with anticoagulant ethylenediaminetetraacetic acid (50 IU). The tubes containing the blood samples were centrifuged for 5 min at 4000 rpm. Plasma was carefully pipetted out, aliquoted, and stored at −20 °C until analyzed. These samples were used to determine the biochemical parameters in blood plasma, i.e., glucose (**GLU**), cholesterol, serum aspartate aminotransferase (**AST**), alanine aminotransferase (**ALT**), total cholesterol, very-low-density lipoprotein (**VLDL**), high-density lipoprotein (**HDL**), LDL, AST, ALT, TG, cortisol, and total protein. Analyses of total protein, cholesterol, VLDL, HDL, LDL, and triglycerides were done using commercial quantitative assay kits from Doles Company (Goiânia, Brazil). Glucose and cholesterol were analyzed by the enzymatic method with an aliquot of 10 μl/sample. Readings were taken by spectrophotometer (BioPlus semi-automatic, 2000) with absorbance set at 510 nm. Transaminases (AST and ALT) were determined by the kinetic method using 100 μl/sample, with spectrophotometer readings taken at an absorbance of 340 nm and at 30 °C. The determination of plasma cortisol was obtained by a radioimmunoassay using a kit (Coat-A-Count cortisol, Diagnostic Products Corporation, Los Angeles, CA, USA).

Centesimal analysis

Four fish were randomly collected from each replicate to determine the whole-body composition. Diets were analyzed in triplicates. Diets and the whole-body composition, expressed as CP, total lipids and ash, were determined as described by the Association of Official Analytical Chemists (Association of Official Analytical Chemists (AOAC), 2005). Briefly, DM was determined by drying samples at 105 °C until constant weight; ash by incineration in a muffle furnace at 450 °C for 4 h; lipid by petroleum ether extraction in soxhlet System HT apparatus (after acid hydrolysis); CP by measuring nitrogen (N × 6.25) by the Kjeldahl method.

Fatty acid analysis

Eight fish were individually processed, and each sample was analyzed in duplicates. Samples were analyzed for total lipids and fatty acid profiles were determined. Moisture content was established by drying samples in an oven at 105 °C until constant weight. The total lipid content extraction was performed using chloroform:methanol (2:1) until gravimetric quantification. Nonadecanoic acid (19:0) was used as an internal standard. While performing the lipid and fatty acid analyses, samples were protected from oxidation by maintaining them in a nitrogen atmosphere and by using butylated hydroxyl toluene as antioxidant (100 mg/l of solvent). The fatty acid methyl esters were analyzed in a gas chromatograph (Hewlett Packard 5890; Hewlett-Packard Company, Wilmington, DE, USA) equipped with a flame

ionization detector and a Supelcowax fused "silica capillary column (30 m 9 0.32 mm ID, Supelco, Bellefonte, PA, USA)." Nitrogen was used as a carrier. The column temperature was maintained at 180 °C for 12 min to be thereafter increased to 212 °C at a rate of 2 °C/min and maintained at 212 °C for 13 min. Fatty acids were identified by comparing the retention times of the methyl ester standards by Supelco® 37 Component FAME mix (Sigma-Aldrich, USA) and by referring to a well-characterized fish oil.

Trial 2: behavioral approach of the postingestive signals to evaluate diet preferences and feed intake

Trial 2 was designed to evaluate fish diet preference when allowed to choose between LIN and COR experimental diets with no organoleptic proprieties effect for either the temperatures (20 or 30 °C) condition. For this purpose, the sensorial characteristics of diets were isolated by the encapsulation method (Rubio et al., 2003; Fortes-Silva et al., 2011). Uncolored capsules (size no. 4; Genix, Anápolis, Brazil) and a 0.2 ml volume were used in the encapsulation process of both diets. The diets were the same of the first trial. Forty-eight juveniles (34.02 ± 0.79 g and 13.62 ± 0.13 cm) were randomly distributed into six 60 l aquariums (eight fish per aquarium) under the 20 °C condition. The fish size was deliberately chosen so animals could ingest the capsules effortlessly. Forty-eight other fish were randomly distributed into six aquariums under the 30 °C condition. In each temperature group (20 or 30 °C), fish were allowed to select capsules containing experimental diets. This trial was carried out in the same thermal chamber and similar conditions of Trial 1. Capsules with experimental diets were supplied separately in each aquarium in equidistant feed hall (10 cm^2) made with PVC pipe (50 mm of diameter). These feeding halls with access from below were fixed in aquarium wall. Thus, feed hall did not allow the mixing of capsules. For consumption calculation, encapsulated diets were previously weighed (approximately 0.040 g each) and offered in excess (40 capsules of each diet) to allow feeding until satiation without restriction. After a 5-min feeding time, the capsules in each feed hall were counted. After 18 trial days, fish were fasted for 2 days and capsules were switched between feeding halls to isolate the local preference effect (Rubio et al., 2003). The trial was performed for 27 days, and the water quality parameters were similar to those in Trial 1. At the end of the experiment, feed intake was expressed as g100/g b.w. Diet preference or consumption of each diet was expressed by considering the total diet intake of COR and LIN diet as 100% of consumption.

Statistical analysis

All the statistical analyses were performed using version 9.0 of the Statistical Analysis Software (SAS), with a significance threshold (P) of 0.05. The data subjected to the statistical tests were first checked for normality (Cramér-von Mises test) and homoscedasticity (Levene test). All the data were analyzed by a two-way ANOVA, followed by Tukey's post hoc test. The diet selection data were first converted into arcsine (√) to achieve the homogeneity of variance before being subjected to the *t*-test.

Results

The output of statistical analysis for Tables 2–5 as well mathematical equations and codes of statistical models are showed in Supplementary Materials, Tables S1–S4, and Supplementary Material S1, respectively.

Trial 1: effects dietary lipids and temperatures on growth, feed utilization, hematological parameters, whole-body composition, and fatty acids deposition

No mortality was observed during the trial. As expected at the suboptimal temperature (20 °C), fish displayed depressed performance, and the growth parameters were higher in the fish cultivated at 30 °C ($P < 0.05$) (Table 2). The final length, final weight, WG, DWG, and SGR values were higher in the fish fed at 30 °C ($P < 0.05$). Similarly, the best values of FI, FER, PER, and ANPU were obtained in the fish cultivated at 30 °C ($P < 0.05$). In addition, the centesimal analysis revealed higher lipids in the whole body ($P < 0.05$) in the fish cultivated at 20 °C (Table 3). Conversely, CP was higher in the fish cultivated at 30 °C. Ash and DM showed no differences ($P > 0.05$), and no effects of diets were found for the centesimal body composition ($P > 0.05$). When considering the effect of diets, no differences were found for any of the growth and feed utilization parameters ($P > 0.05$).

Hematological parameters

When considering the serum biochemical variables and temperature effect, significant differences were found for total cholesterol with higher values at 20 °C ($P < 0.05$) (Table 4). Similarly, VLDL, AST, TG, and glucose were all higher in the fish maintained at 20 °C ($P < 0.05$), while HDL was higher in the fish maintained at 30 °C ($P < 0.05$).

When examining the diet effect, VLDL was higher, but only in the fish fed COR diet at 20 °C ($P < 0.05$). HDL had no differences between temperatures or diets ($P < 0.05$). Low-density lipoprotein was not

Table 2
Growth and feed utilization of juvenile Nile tilapia (*O. niloticus*) fed two different oil blends (COR or LIN) reared under two different temperatures (20 or 30 °C).

Parameters	Diets					*P*-value		
	COR-20[1]	LIN-20[2]	COR-30[3]	LIN-30[4]	RMSE	Diet	Temp.	Int.
Final length (cm)	11.99^{aB}	11.97^{aB}	14.84^{aA}	14.92^{aA}	0.168	NS	0.00	NS
Final body weight (g)	26.92^{aB}	26.82^{aB}	51.51^{aA}	53.94^{aA}	1.412	NS	0.00	NS
Weight gain (WG, g)	11.56^{aB}	11.27^{aB}	36.30^{aA}	38.64^{aA}	1.421	NS	0.00	NS
Daily weight gain (DWG, g/d)	0.39^{aB}	0.38^{aB}	1.21^{aA}	1.29^{aA}	0.047	NS	0.00	NS
Specific growth ratio (SGR, %)	1.87^{aB}	1.82^{aB}	4.07^{aA}	4.20^{aA}	0.118	NS	0.00	NS
Feed intake (FI, g/kg)	9.02^{aB}	9.15^{aB}	21.22^{aA}	21.26^{aA}	0.112	NS	0.00	NS
Feed efficiency ratio (FER, g)	1.47^{aB}	1.53^{aB}	1.09^{aA}	1.03^{aA}	0.010	NS	0.00	NS
Protein efficiency ratio (PER, %)	1.90^{aB}	1.83^{aB}	2.54^{aA}	2.70^{aA}	0.129	NS	0.00	NS
Apparent net protein utilization (ANPU, %)	21.97^{aB}	22.35^{aB}	36.5^{aA}	35.41^{aA}	1.103	NS	0.00	NS
Survival (%)	100	100	100	100	–	–	–	–

Data are presented as mean ($n = 4$). Equal lowercase letters (a–b) in the same line represent absence of statistical difference between diets and different capital letters (A–B) in the same line represent significant differences between optimal and suboptimal temperatures (20 and 30 °C) by two-way ANOVA followed by the Tukey test ($P < 0.05$). NS = not significant, Temp. = temperature, Int. = interaction.

[1] COR-20 = fish subjected to temperature of 20 °C condition and fed with diet elaborated with a vegetable oil blend, rich in corn oil.
[2] LIN-20 = fish subjected to temperature of 20 °C condition and fed with diet elaborated with a vegetable oil blend, rich in linseed oil.
[3] COR-30 = fish subjected to temperature of 30 °C condition and fed with diet elaborated with a vegetable oil blend, rich in corn oil.
[4] LIN-30 = fish subjected to temperature of 30 °C condition and fed with diet elaborated with a vegetable oil blend, rich in linseed oil.

Table 3
Whole-body composition of juvenile Nile tilapia (*O. niloticus*) fed two different oil blends (COR or LIN) reared under two different temperatures (20 or 30 °C).

Variables (g/kg of wet weight)	Diets						*P*-value		
	Initial	COR-20[1]	LIN-20[2]	COR-30[3]	LIN-30[4]	RMSE	Diet	Temp.	Int
Protein	162.0	183.7^{aB}	182.3^{aB}	203.0^{aA}	205.9^{aA}	1.561	NS	0.00	NS
Lipid	58.4	88.0^{aA}	84.7^{aA}	76.3^{aB}	73.2^{aB}	2.085	NS	0.02	NS
Ashes	39.9	53.7	82.8	60.0	68.3	2.034	NS	NS	NS
Moisture	90.1	89.6	87.6	91.1	89.0	0.296	NS	NS	NS

Data are presented as mean ($n = 4$). Equal lowercase letters in the same line represent absence of statistical difference between diets and different capital letters in the same line represent significant differences between optimal and suboptimal temperatures (20 and 30 °C) by two-way ANOVA followed by the Tukey test ($P < 0.05$). NS = not significant, Temp. = temperature, Int. = interaction.
[1] COR-20 = fish subjected to temperature of 20 °C condition and fed with diet elaborated with a vegetable oil blend, rich in corn oil.
[2] LIN-20 = fish subjected to temperature of 20 °C condition and fed with diet elaborated with a vegetable oil blend, rich in linseed oil.
[3] COR-30 = fish subjected to temperature of 30 °C condition and fed with diet elaborated with a vegetable oil blend, rich in corn oil.
[4] LIN-30 = fish subjected to temperature of 30 °C condition and fed with diet elaborated with a vegetable oil blend, rich in linseed oil.

affected by of temperature or diets ($P > 0.05$). Higher AST activity was observed in the fish fed LIN diet at 20 °C (LIN-20), in contrary fish revealed higher AST when fed COR diet at 30 °C condition ($P < 0.05$). The fish fed LIN at 30 °C condition had lower alanine aminotransferase (**ALT**) values than all other treatments ($P < 0.05$). Moreover, glucose was higher in the fish at 20 °C ($P < 0.05$), regardless of diet. Similarly, TG and cortisol were higher in the fish at 20 °C ($P < 0.05$). Fish fed COR diet had higher cortisol in both temperatures ($P < 0.05$). Finally, diets had no influence on total protein, regardless of temperature ($P > 0.05$).

Fatty acids

Regarding tilapias whole body fatty acids profile, no differences were observed for Σ SFA (saturated fatty acids) in fish under both temperatures or fed with experimental diets ($P > 0.05$) (Table 5). Moreover for MUFA (monounsaturated fatty acids), no differences were noted ($P > 0.05$). Compared to fish fed LIN, the fish fed the COR diet showed a smaller C20:5n-3 (EPA) proportion ($P < 0.05$) at both temperatures. A larger C22:6n-3 (DHA) proportion was observed in the fish at 20 °C ($P < 0.05$). The fish cultivated at 30 °C had a larger ∑ PUFA proportion than those at 20 °C ($P < 0.05$), while the opposite was observed for DHA/EPA ratio ($P < 0.05$). Fish fed COR diet showed higher body proportion of n-6, mainly under 30 °C condition ($P < 0.05$). The fish allowed to feed LIN diet presented larger n-3 and n-3 highly unsaturated fatty acid proportions, but temperature did not seem to affect these fatty acid proportions ($P > 0.05$).

Trial 2: behavioral approach of the postingestive signals to evaluate diet preferences and feed intake

No mortality was observed during the trial. The final weight was 65.83 ± 3.27 g for the fish fed at T30°C and 61.68 ± 2.31 g for the T20°C condition. Initially, the fish under the T30°C condition showed a clear preference for encapsulated COR diet (Fig. 1A). After 4 days, a clear selection pattern began to be defined. From day 15, a significant diet preference for LIN was observed for both temperature conditions ($P < 0.05$). This pattern was generally maintained after switching capsules between feeding halls. This diet selection pattern was similar when fish were reared at T20°C (Fig. 1B). When considering total feed intake, the fish reared at 30 °C showed higher consumption ($P < 0.05$) (Fig. 1C). The average intake for each diet at the end of the experimental period revealed a higher intake for the LIN diet at both temperatures ($P < 0.05$).

Discussion

Growth rates were significantly higher at 30 °C than at 20 °C. Moreover, the diets with LIN or COR did not affect growth and feed use in both temperatures. In other study, the performance was the same regardless of oil source used (linseed or soy) when under optimal temperature (28.8 °C) (Ng et al., 2013). It has been known that n-6 is required at low amount by tilapia, around 0.5% of dry diet (Takeuchi et al., 1983, National Research Council (NRC), 1993), on the other hand, n-3

Table 4
Serum biochemical parameters of juvenile Nile tilapia (*O. niloticus*) fed two different oil blends (COR or LIN) reared under two different temperatures (20 or 30 °C).

Parameters	Diets					*P*-value		
	COR-20[1]	LIN-20[2]	COR-30[3]	LIN-30[4]	RMSE	Diet	Temp.	Int.
Total cholesterol (mg/dl)	152.27^{aA}	157.75^{aA}	96.75^{aB}	100.00^{aB}	23.068	NS	0.00	NS
HDL (mg/dl)	11.00^{aA}	16.5^{bA}	20.00^{aB}	12.00^{bA}	2.877	0.02	0.09	NS
LDL (mg/dl)	79.00^{aA}	102.5^{aA}	64.75^{aA}	78.25^{aA}	26.049	NS	NS	NS
VLDL (mg/dl)	62.25^{aA}	38.75^{bA}	12.00^{aB}	11.25^{aB}	6.303	0.00	0.00	NS
Triglycerides (mg/dl)	193.13^{aA}	301.75^{bA}	61.00^{aB}	51.97^{bB}	30.975	0.00	0.00	NS
AST (U/L)	56.25^{aA}	102.75^{bA}	51.75^{aB}	34.25^{bB}	22.970	0.00	0.00	NS
ALT (U/L)	8.50^{aA}	8.87^{aA}	11.09^{aA}	4.00^{bB}	4.812	0.05	0.08	NS
Total protein (mg/dl)	4.95^{aA}	4.49^{aA}	4.68^{aA}	4.73^{aA}	2.698	NS	NS	NS
Cortisol (mg/dl)	8.2^{aA}	3.65^{bA}	3.25^{aB}	1.3^{bB}	0.795	0.00	0.00	NS
Glucose (mg/dl)	56.40^{aA}	60.40^{aA}	36.90^{aB}	42.90^{aB}	7.733	NS	0.04	NS

Data are presented as mean ($n = 4$). Equal lowercase letters in the same line represent absence of statistical difference between diets and different capital letters in the same line represent significant differences between optimal and suboptimal temperatures (20 and 30 °C) by two-way ANOVA followed by the Tukey test ($P < 0.05$). NS = not significant, Temp. = temperature, Int. = interaction.
High-density lipoprotein (HDL), low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), aspartate aminotransferase (AST), alanine aminotransferase (ALT).
[1] COR-20 = fish subjected to temperature of 20 °C condition and fed with diet elaborated with a vegetable oil blend, rich in corn oil.
[2] LIN-20 = fish subjected to temperature of 20 °C condition and fed with diet elaborated with a vegetable oil blend, rich in linseed oil.
[3] COR-30 = fish subjected to temperature of 30 °C condition and fed with diet elaborated with a vegetable oil blend, rich in corn oil.
[4] LIN-30 = fish subjected to temperature of 30 °C condition and fed with diet elaborated with a vegetable oil blend, rich in linseed oil.

Table 5
Whole-body fatty acid composition (% of total FA) of juvenile Nile tilapia (*O. niloticus*) fed two different oil blends (COR or LIN) reared under two different temperatures (20 or 30 °C).

	Diets						*P*-value		
Fatty acids (FA)	Initial	COR-20[1]	LIN-20[2]	COR-30[3]	LIN-30[4]	RMSE	Diet	Temp.	Int.
C16:0	20.0	17.23	17.11	17.29	17.94	0.393	NS	NS	NS
C18:0	8.0	7.74	7.91	7.24	6.96	0.534	NS	NS	NS
∑ SFA	31.0	27.46	27.46	26.66	27.29	0.981	NS	NS	NS
C16:1n-7	1.1	2.12^{aA}	1.82^{aA}	1.61^{aB}	1.48^{aB}	0.243	NS	0.003	NS
C16:1n-9	0.5	0.23^{aA}	0.60^{bA}	0.17^{aA}	0.57^{bA}	0.087	0.001	NS	NS
C18:1n-9	20.1	23.03	21.63	22.75	22.08	1.100	NS	NS	NS
C18:1n-7	0.5	2.84	2.87	2.26	2.42	0.229	NS	NS	NS
∑ MUFA	26.0	30.85	29.71	28.64	28.51	1.329	NS	NS	NS
C18:2n-6	14	19.30^{aA}	17.64^{aA}	24.08^{aB}	21.05^{aB}	1.418	NS	0.002	NS
C18:3n-3	0.4	2.01^{aA}	4.11^{bA}	2.82^{aA}	5.98^{bA}	0.787	0.001	NS	NS
C20:4n-6 (AA)	6.3	4.22	3.67	3.57	3.39	0.695	NS	NS	NS
C20:5n-3 (EPA)	1.1	0.40^{aA}	0.45^{bA}	0.39^{aA}	0.48^{bA}	0.045	0.001	NS	NS
C22:5n-3	1.2	1.08	1.21	0.85	1.12	0.104	NS	NS	NS
C22:5n-6	5.2	4.74	3.98	2.90	2.58	0.629	NS	NS	NS
C22:6n-3 (DHA)	3.2	3.42^{aA}	3.53^{aA}	2.52^{aB}	3.24^{aB}	0.280	NS	0.001	NS
∑ PUFA	40.0	41.69^{aA}	42.83^{aA}	44.20^{aB}	44.70^{aB}	1.434	NS	0.006	NS
n-9	22.1	24.20	24.20	24.16	23.96	1.096	NS	NS	NS
n-6	20.0	29.24^{aA}	26.08^{bA}	31.61^{aB}	27.76^{bB}	2.055	0.006	0.001	NS
n-3	10.2	7.55^{aA}	10.33^{bA}	7.45^{aA}	12.39^{bA}	2.066	0.007	NS	NS
n-3 HUFA	7.2	5.39^{aA}	6.02^{bA}	4.50^{aA}	6.15^{bA}	0.724	0.007	NS	NS
n-6/n-3	1.7	3.88^{aA}	2.53^{bA}	4.25^{aA}	2.28^{bA}	0.824	0.000	NS	NS
DHA/EPA	6.1	8.66^{aA}	7.94^{aA}	6.49^{aB}	6.80^{aB}	0.785	NS	0.002	NS

Data are presented as mean ($n = 4$). Equal lowercase letters (a–b) in the same line represent absence of statistical difference between diets and different capital letters (A–B) in the same line represent significant differences between optimal and suboptimal temperatures (20 and 30 °C) by two-way ANOVA followed by the Tukey test ($P < 0.05$). NS = not significant, Temp. = temperature, Int. = interaction.
Palmitic (C16:0), stearic (C18:0), palmitolic (C16:1n-7), cis-7 hexadecenoic (C16:1n-9), oleic (C18:1n-9), vaccenic (C18:1n-7), linoleic (C18:2n-6), linolenic (C18:3n-3), arachidonic (C20:4n-6) (AA), eicosapentaenoic (C20:5n-3) (EPA), docosapentaenoic (C22:5n-3), docosapentaenoic (22:5n-6), docosahexaenoic (C22:6n-3) (DHA).
Fatty acids (FA), saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA), highly unsaturated fatty acid (HUFA).
[1] COR-20 = fish subjected to temperature of 20 °C condition and fed with diet elaborated with a vegetable oil blend, rich in corn oil.
[2] LIN-20 = fish subjected to temperature of 20 °C condition and fed with diet elaborated with a vegetable oil blend, rich in linseed oil.
[3] COR-30 = fish subjected to temperature of 30 °C condition and fed with diet elaborated with a vegetable oil blend, rich in corn oil.
[4] LIN-30 = fish subjected to temperature of 30 °C condition and fed with diet elaborated with a vegetable oil blend, rich in linseed oil.

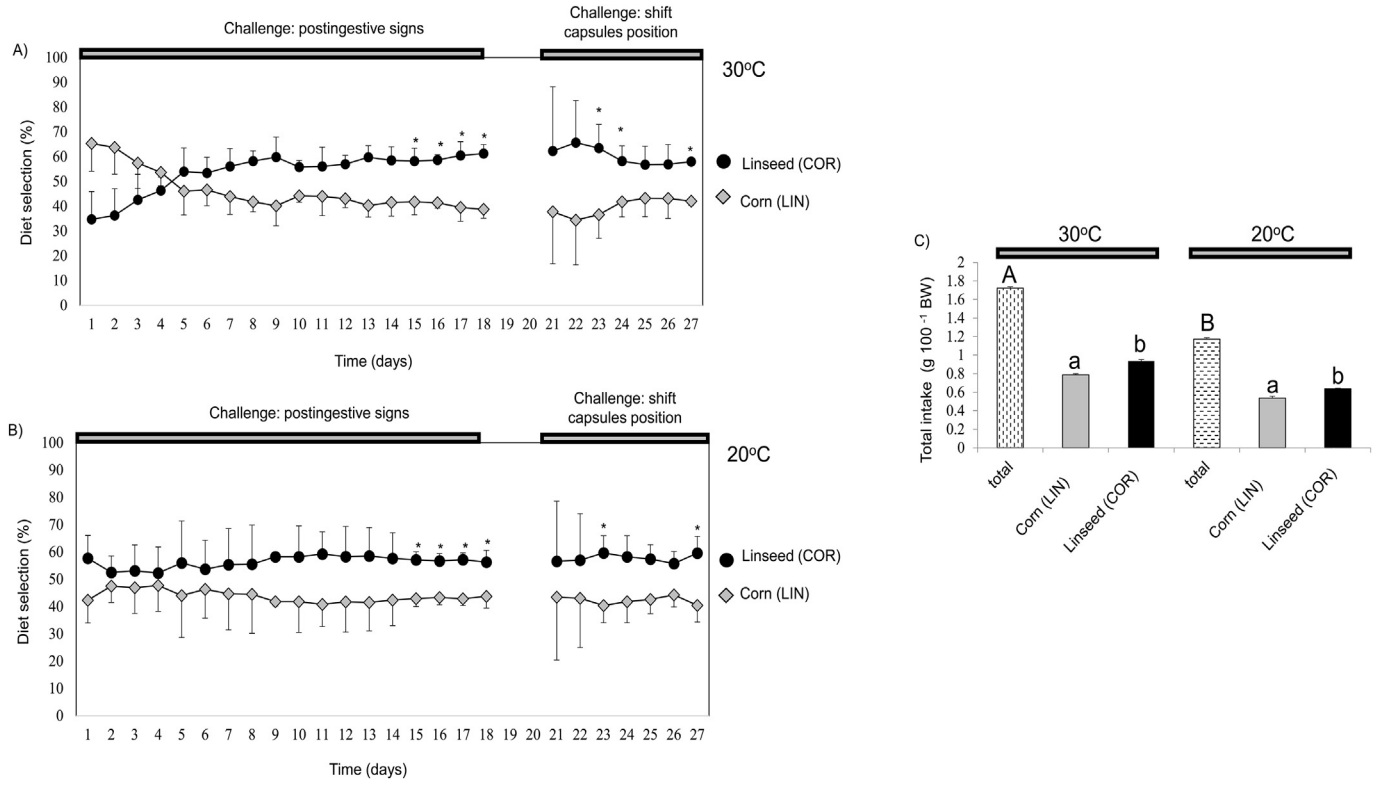

Fig. 1. Daily evolution of diet selection in the absence of organoleptic properties (encapsulated diets) of juvenile Nile tilapia (*O. niloticus*) fed two different oil blends (COR or LIN) reared under two different temperatures: (A) 30 °C and (B) 20 °C. COR is the diet elaborated with a vegetable oil blend, rich in corn oil and LIN is the diet elaborated with a vegetable oil blend, rich in linseed oil. Values (mean ± SD) represent the percentage of total diets selected as 100%. The daily averages with an asterisk differ significantly after the arcsine transformation of data, *t*-test, $n = 3$ ($P < 0.05$). (C) Total feed intake. The means followed by different capital letters indicate significant differences between diet intake, *t*-test, $n = 3$ ($P < 0.05$).

requirement is not clear (Ng, 2001). In absolute terms, LOA had similar amount in both experimental diets of the present study. Linoleic is the principal essential fatty acid for tilapia; therefore, lack of difference in growth was expected. According to El-Husseiny et al. (2010), poor growth only was observed for fry tilapia fed diets containing high LA/LNA ratios (above 13).

A lack of significant effect for temperature and dietary lipid profile on growth or feed efficiency was also observed by Corrêa et al. (2018) in tilapia at 22 or 28 °C. However, Nobrega et al. (2017) reported a different outcome with worsened performance in tilapia at 20 °C when the dietary α-LNA was increased up to 0.99% (n-6/n-3 = 0.0.55) compared to 0.03% (n-6/n-3 = 21,6); 0.21% (n-6/n-3 = 2,37); 0.37% (n-6/n-3 = 1,43); and 0.67% (n-6/n-3 = 0.83). Despite of these diverged studies, there is currently a paucity of information concerning the lipid requirements at different temperatures. Thus, we can hypothesize that absence of effects of the oil blends on the performance at low temperature may also have occurred because all fish's metabolism is depressed by lower temperature.

When examining body composition, the tilapia fed at 20 °C obtained lower protein and higher lipid contents. Obviously, fish size affects body composition, but our aim was to evaluate the effect of diet within each temperature during the same cultivation period. Fish fed experimental diets had no significant effects on body composition. Similarly, Ng et al. (2013) were able to demonstrate that the dietary lipid source (linseed, soy, and fish oil) had no significant effect on moisture, CP, crude lipid, or ash in tilapia (*Oreochromis* sp.) at 28 °C. According to Ng et al. (2001), the whole-body moisture, protein, and ash of tilapia are not significantly affected by the dietary lipid source. However, Nobrega et al. (2017) reported that an increase in dietary α-LNA levels leads to heavier body lipid contents in tilapia, without effect on protein or ash when juvenile are raised at cold suboptimal temperatures (Nobrega et al., 2017).

Regarding the temperature effect on body composition, tilapia's metabolic rate may be responsible for the observed effects. Normally, body crude lipid content rises when increased body weight is observed in fish at optimal temperature (El-Sayed et al., 1996). We assume that at 20 °C all metabolism should be running at a slower rate and maybe what is happening is that the energy expenditures were below dietary intake favoring lipid deposition. Similarly, Cichlid (*Symphysodon aequifasciatus*) exposed to low temperatures presents increased muscle lipids over time (Wen et al., 2018). Desaulniers et al. (1996) suggested the increase in muscle lipid contents partially offsets the impact of cold on the intracellular diffusion of oxygen in striped bass (*Morone saxatilis*). It has been proposed that in low temperature, the capacities of lipid substrate oxidation would not be maximized, perhaps to avoid excessive proton leak or damage to the polyunsaturated mitochondrial membranes (Guderley, 2004).

In our study, a clear effect of temperature on circulating cholesterol was found. The fish at low temperature showed higher total cholesterol. Cholesterol is a major constituent of the plasma membrane and has pronounced effects on the physical properties of membranes to minimize effects of temperature on membrane structure and function (Crockett, 1998). In addition, LIN diet provided higher HDL at suboptimal temperature, but the opposite was observed in fish at 30 °C condition. HDL plays a central role in transferring cholesterol from the extrahepatic tissues to the liver for metabolism (Wang et al., 2016). Under cold stress condition, HDL can transport the cholesterol from peripheral cells to the liver to reduce the lipid peroxidation damage in tilapia (Shi et al., 2015). However, this response mechanism remains to be elucidated (Ferreira et al., 2011). In our study, higher values of TG were observed in the fish fed LIN diet. According to Ferreira et al. (2011), the TG level was higher in the tilapia fed linseed oil rather than olive or fish oil. Moreover, our results revealed that VLDL was higher at 20 °C when compared to fish kept at 30 °C, but lowered when fish were fed LIN instead of COR diet. Higher VLDL is usually related to heightened lipogenesis since it is the main endogenous lipid transporter. However, it is suggested that some n-3 lipids can diminish lipogenesis via nuclear proliferator-activated receptors (**PPARs**) in the rainbow trout (Coccia et al., 2014). Diets prepared with higher proportions of linseed oil led to lower VLDL concentrations and less fatty acid removal to the blood stream in freshwater fish (*Tor tambroides*) (Ramezani-Fard et al., 2014).

It has been demonstrated that plasma glucose increased when tilapias were exposed to cold stress (11 12 °C) for 60 min, but no effect was found in the fish acclimated to 22 and 35 °C (Kindle and Whitmore, 1986). Although the total plasma protein did not differ between groups, the TG and glucose values were significantly higher in the fish maintained at the suboptimal temperature, which could also suggest a thermal stress some degree Regarding to diet effect, our results showed that linseed rich diet lead to higher TG at 20 °C, but lower at 30°C. An recent study, revealed that increased amount of dietary linseed oil provided higher content of serum triglyceride and improvement oxidative status in cold-water salmonid (*Brachymystax lenok*) (Yu et al., 2019). Our findings revealed that cortisol also increase in tilapia under cold-water temperature but decreased when fish fed LIN. Similarly, stressed sea bream (*Sparus aurata*) fed linseed oil recovered basal cortisol levels after 1 week compared to fish oil or soybean oil (Ganga et al., 2011). Eicosapentaenoic acids promoted cortisol production in sea bream interrenal cells in *S. aurata*, though the physiological mechanisms by which these HUFA regulate the hormone-induced plasma cortisol levels are not clear (Ganga, 2006). it has been suggested that eicosanoids could be a potential modulators of the hypothalamus-pituitary-inter-renal axis in fish, which is directly reflected in stress parameter responses (Ganga et al., 2010). In addition, fatty acids and their derivatives have been suggested to affect stress-coping ability by signal nuclear receptors and transcription factors (such as PPAR) by interaction with steroidogenic acute regulatory protein (**STAR**) and glucocorticoid receptors GR via energetic metabolism in teleost fish (Martins et al., 2012).

In general, it was observed in the present study that fish at 30 °C showed lower plasmatic ALT and AST activity, mainly in fish fed LIN diet. Alanine aminotransferase and AST may be released into the bloodstream following hepatic cells damage in fish. The increased levels of AST and ALT activities in plasma of pufferfish (*Takifugu obscurus*) under cold stress when temperature decreased to 13 °C indicated that organ dysfunction occurred (Cheng et al., 2017). According to Wen et al. (2018), the activities of ALT and AST were generally increased with decreased temperatures in discus fish (*S. aequifasciatus*),

Regarding the diet effect, Babalola et al. (2008) associated the increased activity of serum ALT and AST with the release of transaminase from the cytoplasm due to hepatic cellular damage in *Heterobranchus longifilis* fed alternatives oils (sheabutter oil, palmkernel oil, sunflower oil, pork lard, and poultry fat). Kenari et al. (2010) observed a reduction in ALT activity in caspian brown trout (*Salmo trutta caspius*) fed with dietary blend of canola and soybean. In general way, due to various metabolic responses between species, it is difficult to conclude a generalized metabolic pattern of organ dysfunction in response to cold acclimation (Wen et al., 2018). In short, our findings indicate that environmental stressors can lead to an increase in the plasma ALT and AST activities; however, it is not possible to infer a disease process.

In our study, $\sum$ PUFA increased in tilapia at 30 °C. It is interesting to note that colder temperatures favored 18:2 n-6 incorporation in body lipids, whereas 18:3n-3 deposition was not affected. Thus, 18:2n-6 was mainly responsible for higher PUFA at higher temperatures. This could suggest a direct effect of temperature on the substrate preference. The selective retention of specific fatty acids as 18-carbon PUFA may be a strategy for tilapia homeostasis (Corrêa et al., 2018). However, more future studies are needed. Our results also revealed that tilapia under both temperature conditions presented increased total n-3 PUFA and less n-6 PUFA when fed LIN diet compared to those fed COR. Similarly,

the n-3 PUFA proportion was higher in the tilapia fed higher dietary α-LNA levels, but no differences were found for n-6 PUFA (Nobrega et al., 2017). Our results agree with the study by Tocher et al. (2002), who reported that Nile tilapia was able to perform desaturation and the elongation of n3 and n6 PUFA. While n-3 HUFA increased in the whole body of the tilapia fed COR compared to LIN diet COR had more n-3 HUFA than LIN. In addition, diets had no effect on DHA/EPA content; however, we observed a higher DHA/EPA ratio in the tilapia at 20 °C than at 30 °C. This result also corroborated with what Nobrega et al. (2017) reported as these authors found that a suboptimal temperature also provided the preferential accumulation of DHA than EPA in tilapia. It has been demonstrated that elongase and desaturases transcription can be heightened at lower temperatures (Ren et al., 2013). Also, EPA usually responds more intensely than DHA to dietary 18:3n-3 because it is synthetized earlier in the elongation and desaturation pathway and its formation involves less enzymes and intermediates.

Our findings reveal that tilapia prefer LIN diet compared to COR diet, regardless of temperature. As previously discussed, LIN clearly favored the reduction of cortisol and increased EPA in fish, regardless of temperature. This result could be related to food preference, suggesting welfare status. According to Fortes-Silva et al. (2010), the average intake and preference of linseed diet were higher than for the soy or fish oil diets in Nile tilapia. This behavior seems to be also associated with each species' feeding habit. Preferences for sunflower and linseed oils were lower than for fish oil in carnivorous sea bass (*D. labrax*) (Luz et al., 2017). However, in all these cases, fish had been in contact with the taste and smell of diets. The complete exclusion of diet-related organoleptic properties in our study and the physical shift of capsules to confuse fish support the hypothesis of a nutritional endogenous effect on tilapia choice. Using the capsules method of organoleptic properties isolation, tambaqui (*C. macropomum*) show a nutritional target to regulate their protein and energy (Filho et al., 2018). Similarly, after a 10-day nutritional feed deprivation challenges, tambaqui have the ability to select fish oil rather than linseed or corn oil without orosensorial properties, probably because it is a fast energy source (Pereira et al., 2018). In short, our findings could support the theory of feed intake regulation related with physiological and nutritional needs in fish exposed to low temperature stress.

Conclusion

Both lipid profiles tested were equally able to promote normal growth without compromising performance confirming that essential fatty acids requirements are probably easily achieved under practical formulations. The lower temperature did not trigger a significant elevation of PUFAs on tilapia carcass as an mechanism to keep membrane fluidity, although cholesterol may have an important role in coping with suboptimal temperatures, since a clear elevation was observed on the animals kept under 20 °C. At which level and what mechanisms are involved are subjects to further investigations, but it is a relevant finding to better understand how lipid metabolism of tilapia reacts under cold stress situations. Temperature had a significant effect on carcass lipid profile, but as previously suggested, changes were not directed toward membrane fluidity maintenance since unsaturation index was heightened in warmer environment. A clear preference toward 18:2n-6 deposition was evidenced at 30 °C although the biological significance of this remains to be elucidated. Finally, tilapia prefers diet with linseed than corn diet, regardless of its sensory properties, which suggests an endogenous effect in its choice. N-3 rich oils such as linseed oil may have a strategical use in tilapia industry to reduce the impact of stress caused by suboptimal temperatures during the winter improving well-being and health during cold exposure.

Supplementary materials

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2020.100092.

Ethics approval

The fish husbandry and experimental protocols conducted in this study were approved by the Ethics Commission on Animal Use of the Federal University of Bahia and were in accordance with Protocol number 23007.00014330/2018–97 (CEUA/UFRB).

Data and model availability statement

None of the data were deposited in an official repository.

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Declaration of interest

Authors and co-authors of this study declare that they have not any conflicts of interest in the publication of this study.

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