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Effect of the dietary linoleic/α-linolenic ratio (n6/n3) on histopathological alterations caused by suboptimal temperature in tilapia (*Oreochromis niloticus*)

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ABSTRACT

Tilapias are produced worldwide, including subtropical areas. In turn, dietary fatty acids can enhance resistance under cold stress. The present study reports the nutritional effect on suboptimal temperature tolerance based on histopathological alterations in tilapia (*Oreochromis niloticus*). Fish (initial weight: 34.02 ± 0.79) were exposed to two different temperatures (20 °C and 30 °C) for 30 days. Under both conditions, fish were fed two different diets based on the linoleic/α-linolenic ratio (n6/n3 = 12.02 and n6/n3 = 3.85). The most important alterations in liver caused by temperature included high cellular hyperplasia in fish at 30 °C ($P < 0.05$). Suboptimal temperature also caused gills aneurysm, lamellar lifting and low hyperplasia ($P < 0.05$). Cytoplasmic vacuolization decreased and nuclear displacement increased in the fish fed n6/n3 = 3.85 ($P < 0.05$). Suboptimal temperature provided high gills aneurysm, epithelial lamellae lifting and low lamellar hyperplasia ($P < 0.05$). Conversely, the fatty acid content (n6/n3 = 3.85) in the diet provided low lamellar lifting and fusions ($P < 0.05$). Finally, tilapia showed an increased glycogen storage trend in gills and muscle at low temperature ($P < 0.05$). Our study suggests that when feeding tilapia a high dietary n3 content, this fish is able to respond to suboptimal temperature in subtropical aquaculture facilities.

1. Introduction

Adaptability and tolerance of tilapias to a wide range of environments and intensification of cultivation systems have resulted in the rapid expansion of tilapia farming and these fish being introduced into many subtropical and temperate regions worldwide (Santos et al., 2013). Although there are a considerable number of fish farms in lakes and small water bodies, the cage culture of tilapia (*Oreochromis niloticus*) in large reservoirs is an emergent aquaculture practice (David et al., 2015) and, in turn, temperature cannot be controlled under these conditions. Tilapia production in Brazil occurs almost nationwide (Brabo et al., 2016), and the annual increases in Brazilian fish production per area reveal the importance of considering temperature variation on fish farms. Tilapia farms in the Sao Paulo state, one in west Parana and one in south Minas Gerais, have reported water temperatures of 20–22 °C (Sebastião et al., 2017). In short, Nile tilapia has a high potential to be used as a model in thermal studies of histopathology alterations.

Although tilapia has been cultured or studied at a wide range of temperatures, it might be particularly sensitive to low temperature. Affected fish may appear to be in relatively good conditions, but could present typical macroscopic lesions. The main histopathological findings are also observed in the liver (Ibarz et al., 2010). The causes of histopathological changes can include more than one factor. Amino acid mobilization due to starvation during overwinters could lead to muscle changes in gilthead sea bream (*Sparus aurata*) (Gallardo et al.,

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2003). Sea bream *S. aurata* showed a severe fatty infiltration in hepatocytes, as has muscular fiber degeneration when cultivated for 4 months at 16–17 °C (Tort et al., 1998). A drop in temperature can alter the gill epithelium, which lowers the total number and apical membrane microvilli of chloride cells or can reduce gill, kidney and intestine Na^+/K^+-ATPase activity (Ibarz et al., 2010). Thus rapidly renovating fish epithelia surfaces, such as the gill epithelium, may be sensitive indicators of essential fatty acid deficiencies in fish (Bell et al., 1985). Therefore, few studies have focused to date on histopathological studies of fish overwinter, mainly in tilapia.

Water temperature also plays a particularly important role in influencing fatty acid metabolism in fish to help them adapt to environmental temperature variations (Ma et al., 2015). The fatty acid profile is important as it is responsible for cell membrane structure, fluidity and functionality (Corrêa et al., 2018). According to (NRC, 2011), tilapia has a PUFA requirement of n-6 and n-3 series. Freshwater fish generally convert linoleic acid (LOA, 18: 2n-6) and alpha-linolenic acid (α-LNA, 18: 3n-3) into arachidonic (ARA, 20: 4n-6), eicosapentaenoic (EPA, 20: 5n-3) and docosahexaenoic (DHA, 22: 6n-3) (Tocher, 2010). However, the n-3/n-6 ratios vary for body tilapia according to season, and are lower in autumn (0.5–0.6) and rise to 0.7–1.6 in winter (Rasoarahona et al., 2005). Acute low temperature-stressed tilapias are able to selectively metabolize saturated fatty acids for energy needs. However, as the duration of the stressor and loss of muscle fat increase, fish begin to metabolize long-chain polyunsaturated fatty acids (He et al., 2015). Tilapia under thermal stress influences may replace and convert body glycogen at the expense of fatty acid composition (He et al., 2015). The glycogen mobilization in cells has revealed recovering cell activity and has been related to histopathological changes (Rios et al., 2007). The effect of dietary lipid sources on tilapia resistance to cold stress is not well understood (Abdel-Ghany et al., 2019). Taken together, these observations suggest that fatty acids and their ratio play a significant role in the thermal adaptation of tilapia to environmental conditions. If this were applicable, the thermal-nutritional interaction could affect body tissues and, therefore, animal health.

Thus the objective of this study was to evaluate the combined effect of two temperatures (suboptimal 20 °C and optimal 30 °C) and two fatty acids ratios [(linoleic acid, 18: 2n-6)/α-linolenic acid, 18: 3n-3)] on liver and gill histopathology alterations in Nile tilapia. The glycogen dynamics of the fish exposed to this experimental condition was also evaluated.

2. Material and methods

This research was conducted according to the principles of ethics in animal experimentation of the Ethics Commission on the Use of Animals (CEUA-UFRB: no. 23007.00014330/2018-97).

2.1. Animal housing

The sexually reverted male of tilapia obtained from the AguaVale (Bahia, Brazil) fish farm, were kept at the aquaculture experimental facility of the Laboratory of Fish Nutrition and Feeding Behaviour (AQUAUFRB) at the University of Bahia-UFRB, Brazil. They were acclimated for 10 days in two 5.0 m^3 indoor polyethylene tanks and fed a commercial diet (Pirá 36, Guabi, Brazil, 36% crude protein). After this period, 256 fish (mean weight ± SE: 34.02 ± 0.79 g and 13.62 ± 0.13 cm) were distributed in 16 aquariums of 60 L each (16 fish per aquarium). Aquariums were placed inside an insulated thermal chamber and a digital temperature controller was fitted (Novus N480D, USA), coupled to two temperature sensors (PT 100). The system consisted in recirculation water, supplied as a continuous flow to be aerated and filtered (mechanical filter and biofilters). The photoperiod was 12: 12 h light/dark (LD) at 28 °C.

Table 1
Composition, proximate analyses and fatty acids content of experimental diets for juvenile tilapia.

Ingredients (g/kg)	D1:n6/n3 = 12.02	D2:n6/n3 = 3.85
Soybean meal[a]	370.0	370.0
Corn meal[b]	222.0	222.0
Soy protein concentrate[c]	150.0	150.0
Wheat flour[d]	100.0	100.0
Linseed oil[e]	13.0	50.0
Corn oil[e]	56.0	14.0
Palm oil[e]	1.0	6.0
Yeast[f]	50.0	50.0
Dicalcium phosphate[g]	15.0	15.0
Vitamin and Mineral premix[h]	10.0	10.0
L-Lysine HCl[i]	6.0	6.0
DL-Methionine[j]	4.0	4.0
L-Threonine[k]	2.0	2.0
Antioxidant (BHT)	1.0	1.0
Proximate composition (g/kg)		
Dry matter	911.0	908.0
Crude Protein	354.0	360.0
Crude Lipid	74.0	70.0
Ash	58.0	57.0
Energy (MJ kg^{-1})[k]	19.9	21.0
Fatty acids composition (% of total FA)[l]		
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
LA/LNA (n6/n3) ratio	12.02	3.86

[a] Crude protein 46%, Crude lipids 3%, dry matter 12.5%, Cargill, SP, Brazil.
[b] Crude protein 7.9%, Crude lipids 3%, dry matter 13.9%, Bioquima, MG, Brazil.
[c] Crude protein 60%, Crude lipids 1.8%, dry matter 12.5%, Cargill, SP, Brazil.
[d] Crude protein 14%, crude fiber 11%, lipids 3%, dry matter 13.5%, Bioquima, MG, Brazil.
[e] Refined oil Mundo dos Óleos, DF, Brazil.
[f] From *Saccharomyces cerevisiae,* Grupo Ullmann, MG, Brazil.
[g] Nutrimix, MS, Brazil.
[h] Mix Vita/Min Omnivorous fish Cargill, SP, Brazil. Composition (mg kg-1 diet): iron sulphate, 196; copper sulphate, 28; zinc oxide, 280; manganese oxide, 52; sodium selenite, 1.2; cobalt sulphate, 0.4; potassium iodide, 1.2; vitamin A, 19 950 (UI kg-1 diet); vitamin D3, 7980 (UI kg^{-1} 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.
[i] Ajinomoto, SP, Brazil.
[j] Evonik, SP, Brazil.
[k] Gross energy: determined by direct combustion in an adiabatic bomb calorimeter.
[l] Myristic (C14:0). Palmitic (C16:0). Palmitoleic (C16:1). Margaric (C17:0). Stearic (C18:0). Oleic (C18:1n 9). Linoleic (C18:2n 6). Linolenic (C18:3n 3). Arachidic (C20:0). Gondoic (C20:1). Arachidonic (C20:4n 6) (AA). Eicosapentaenoic (C20:5n 3) (EPA). Behenic (C22:0). Docosahexaenoic (C22:6n 3) (DHA). Lignoceric (C24:0). Saturated fatty acid (SFA). Monounsaturated fatty acid (MUFA) and Polyunsaturated fatty acid (PUFA).

2.2. Experimental design

The experimental design was set up in a 2 × 2 factorial arrangement (by comparing two diet effects at two temperatures). Temperatures were re-established in two independent systems. Thus the thermal condition was adjusted to a suboptimal temperature of 20 °C (common in subtropical zones) and one of 30 °C (common in tropical zones). Under both conditions, fish were fed two different diets based on the linoleic/α-linolenic ratio (n3/n4) (Table 1), with four combinations: C1 – diet at the n-6/n-3 ratio (12.02)/20 °C; C2 − diet at the n-6/n-3 ratio (3.85)/20 °C; C3 – diet at the n-6/n-3 ratio (12.02)/30 °C; C4 – diet at the n-6/n-3 ratio (3.85)/30 °C. Fish were fed until apparent satiation (08: 00 h; 12:00 h; 17: 00 h) for 30 days.

Water renewal was 10% per day and new water temperatures were corrected before exchanges. During the experimental period, the physico-chemical variables of the water used for the system at 20 °C were: dissolved oxygen 7.3 ± 0.40 mg L^{-1}, pH 6.6 ± 0.17, ammonia 0.004 ± 0.002 mg L^{-1} and nitrite 0.03 ± 0.08 mg L^{-1}. The system at 30 °C comprised dissolved oxygen 6.4 ± 0.19 mg L^{-1}, pH 6.8 ± 0.36, ammonia 0.005 ± 0.001 mg L^{-1} and nitrite 0.06 ± 0.11 mg L^{-1}. Water temperature, dissolved oxygen and pH were monitored daily with a multiparameter HANNA HI 9828 (Madrid, Spain). Ammonia and nitrite were monitored weekly with an ALFAKIT meter.

2.3. Sample procedure

At the end of experiment (30 days), survival was measured by counting individuals. Animals were anesthetized with eugenol solution (50 mg L^{-1}). After deep anesthesia, fish were euthanized by medullary section. Fish were sampled from all the groups to determine the histopathology alterations of liver and gill. Liver and gill tissues (3.0 ± 0.2 mg each) were removed and immediately stored in 10% formaldehyde (fixed) for 24 h.

2.4. Histopathological analysis of liver and gill tissue

Liver and gill samples (second gill arch) were chemically fixed in 10% formaldehyde for 24 h. Tissues were processed in an automatic Leica TP 1020 tissue processor. Samples were dehydrated using ethanol solutions at increasing degrees (70%, 80%, 90% and 100%). This was followed by the diafanization of samples with a xylene solution for 2 h. Finally, samples were embedded in paraffin wax (56–58 °C). After paraffin embedding, molds were sectioned (4 μm) on the parasagittal plane using a manual microtome (Hyrax M40 −Zeiss) before being subsequently stained with hematoxylin-eosin and Periodic Acid-Schiff-PAS- (analysis glycogen) mounted in DPX. Preparations were analyzed under a compound light microscope (Infinita – Global Optics NO 229T) coupled to a USB digital camera (Infinita Global Optics). The photomicrographs of gills and livers were taken at the magnifications of 100–400 × and 400–1000 × .

The type and extent of histological alterations were described by the method developed by Bernet et al. (1999). Histopathological alterations were classified into three reaction patterns (rp): circulatory, regressive and progressive.

Histological circulatory alterations are result from a pathological condition of blood and tissue fluid flow. Regressive alterations are defined as processes that lead to reductions in organ function or loss, while progressive alterations are defined as increased activity of cells or tissues.

The pathological importance of the observed alteration (alt) was defined as an “importance factor” (w), classified as 1, 2 or 3, corresponding to minimal (reversible pathological lesions), moderate (lesions that mostly revert after stressor agent neutralization) and severe (often irreversible lesions that cause the partial or total loss of the affected organ's function) pathological importance, respectively. Each alteration was also assessed using a “score value” (a) that ranged from 0 (absence of alteration) to 6 (mild to severe occurrence), depending on the degree and extent of the alteration (percentage of areas, in gills or liver, this specific alteration is exhibited). To obtain the organ index value, the importance factor and score value were multiplied according to the following formulae (Bernet et al., 1999):

The reaction index of an organ (I org cat) and the organ index (I org) were calculated following the equations:

$$\text{I orgcat} = \sum \text{alt}\,(a_{orgrpal} \times w_{orgrpalt}) \text{ and I org} = \sum \text{rp} \sum \text{alt}\,(a_{orgrpal} \times w_{orgrpalt}).$$

where: org = organ (constant: IL-liver; IG-gills); rp = reaction pattern; alt = alteration; a = score value; w = importance factor.

Frequency of lesions (FQ) was expressed by dividing the number of fish in which the change was found by the number of analyzed fish:

$$FQ\ \%= (N_{lesion} \times N_{total}{}^{-1}) \times 100$$

2.5. Microscopy analysis of glycogen

Samples from the gills, muscle and liver were fixed with 10% formaldehyde to be processed for historesin and colored by PAS. To quantify glycogen, five distant regions from large vessels were chosen to analyze as many hepatocytes as possible. A histopathological analysis was conducted with a Infinita – Global Optics NO 229T) microscope coupled to a USB digital camera (Infinita Global Optics). Images were captured by the software ToupView 3.7 and analyzed with the software ImageJ 1.44e. The averages obtained from the 4-μm sections were calculated for each individual.

2.6. Statistical analysis

Statistical analyses of the results were performed by using the version 9.0 of the Statistical Analysis Software (SAS), with a significance threshold (P) of 0.05. Data distribution normality was tested by the Kolmogorov-Smirnov test. The log transformation was used to transform skewed data or data in percentage to conform to normality. In those cases in which data followed normal distribution, homogeneity of variance was tested by Levene's test, and significant differences were tested using a two-way ANOVA followed by the Tukey test ($P < 0.05$).

3. Results

No mortality was recorded during the experiments. The fish livers exhibited a sinusoidal space (reversible pathological lesions) and nuclear displacement (revert after stressor agent neutralization). However, no differences were observed for thermal or dietary effluences (Table 2). The livers from the fish fed n6/n3 = 3.85 had low cytoplasmic vacuolization for both temperatures ($P = 0.002$) (Fig. 1 E, F, G and H). Even though some alterations were observed for nuclear degeneration and necrosis, no nuclear hypertrophy of hepatocyte or statistical differences were found. In addition, high cellular hyperplasia was observed in fish at 30 °C ($P = 0.002$).

By considering FQ, the fish livers presented a minor increase of the sinusoidal space at 20 °C ($P = 0.004$) (Table 2 and Fig. 1 A, B, C and D). This pattern was not observed for cytoplasmic vacuolization when considering the temperature effect, but diet n6/n3 = 3.85 induced a low FQ ($P = 0.004$). Nuclear degeneration (moderate lesion) and necrosis (severe lesion) were found for some groups, but the FQ for these parameters gave no differences. Similar to that previously observed, no FQ was found for nuclear hypertrophy of hepatocyte. Finally, the 30 °C temperature led to a high FQ for cellular hyperplasia (Table 2 and Fig. 1 I, J, K, L), with no effect for dietary fatty acid.

No effect of the fatty acids ratio or temperature on vasodilation and lamellar hypertrophy of gills was found (Table 3). However, gills aneurism was evident in fish at the lower temperature of 20 °C

Table 2
Liver alterations assigned to each category in juvenile tilapias reared at suboptimal and optimal temperatures (20 °C/30 °C) and fed the n6/n3 ratio (12.02/3.85), respectively. Alterations were calculated as histopathological indices (HP) and Frequency of lesions (FQ).

Group: Diet		Temperature				*P-value*		
		20 °C		30 °C				
		n6/n3 = 12.02	n6/n3 = 3.85	n6/n3 = 12.02	n6/n3 = 3.85			
Histopathological Changes		*HP indices*	*HP indices*	*HP indices*	*HP indices*	*Diet*	*Temp.*	*Int.*
Circulatory alterations[a]	Sinusoidal Space (*)	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	NS	NS	NS
Regressive alterations[b]	Nuclear Displacement (*)	2.88 ± 0.35	3.00 ± 0.53	2.88 ± 0.35	2.88 ± 0.83	NS	NS	NS
	Cytoplasmic Vacuolization (*)	5.25 ± 0.46 aA	5.00 ± 0.76bA	5.50 ± 0.53 aA	4.13 ± 1.13bA	0.002	NS	NS
	Nuclear Degeneration (**)	0.50 ± 1.41	–	–	2.50 ± 2.07	–	–	–
	Necrosis (**)	0.50 ± 1.41	–	–	–	–	–	–
Progressive alterations[c]	Nuclear Hypertrophy of Hepatocyte (*)	–	–	–	–	–	–	–
	Cellular Hyperplasia (**)	4.25 ± 0.71 aB	4.25 ± 0.71 aB	5.75 ± 0.71 aA	4.50 ± 0.93 aA	NS	0.002	NS
	Histopathological Changes	*FQ (%)*	*FQ (%)*	*FQ (%)*	*FQ (%)*	*Diet*	*Temp.*	*Int.*
Circulatory alterations[a]	Sinusoidal Space (*)	2,98 ± 0,80 aB	3,64 ± 0,86 aB	3,84 ± 0,48 aA	5,14 ± 2,86 aA	NS	0.004	NS
Regressive alterations[b]	Nuclear Displacement (*)	16,62 ± 3,15bA	22,33 ± 5,19 aA	16,13 ± 1,53bA	23,50 ± 9,72 aA	0.003	NS	NS
	Cytoplasmic Vacuolization (*)	68,34 ± 9,34 aA	61,11 ± 4,43bA	63,09 ± 3,04 aA	54,76 ± 9,28bA	0.004	NS	NS
	Nuclear Degeneration (**)	–	–	–	3,87 ± 3,38	–	–	–
	Necrosis (**)	0,51 ± 1,44	–	–	–	–	–	–
	Cellular Hyperplasia (**)	10,09 ± 5,17 aB	12,00 ± 2,27 aB	16,95 ± 2,92 aA	12,93 ± 2,34 aA	NS	0.003	NS

The type and the extent of histological alterations were described by using a method developed by Bernet et al. (1999).
The pathological importance of the observed alteration was defined as an "importance factor", classified as (*) or (**) corresponding to minimal (reversible pathological lesions), moderate (lesions that in most cases revert after neutralization of the stressor agent) and severe (often irreversible lesions that cause partial or total loss of function of the affected organ). A score value ranging from 0 (absence of alteration) to 6 (severe occurrence of alteration) is specified in accordance with the degree and extent of a specific alteration. To obtain value of the organ index (HP indice) to "fator de importância" e valor do escore foram multiplicados, de acordo com as seguintes fórmulas (Bernet et al., 1999): I orgcat = Σalt ($a_{orgrpal} \times w_{orgrpalt}$) and I org = Σrp Σalt ($a_{orgrpal} \times w_{orgrpalt}$). Frequency of lesions (FQ) was expressed by dividing the number of fish on which the change was found with the number of fish analyzed:
Frequency of lesions (FQ %) = (N lesion x N total lesion $^{-1}$) ×100
Data are presented as mean and standard error (SE) (n = 8). Different lowercase letters in the same line represent differences between diets (n6/n3 ratio) and different capital letters in the same line represent differences between optimal and suboptimal temperatures (20 °C and 30 °C), two-way ANOVA followed by the Tukey test ($P < 0.05$), NS = not significant.
[a] Disturbances result from a pathological condition of blood and tissue fluid flow.
[b] Breakdown of tissue and/or cells which terminate in a functional reduction or loss of an organ. Changes in tissue architecture.
[c] Increase in the number of specific cell types or structures.

($P = 0.001$) (Table 4 and Fig. 2A, B, C, D). The fish fed n6/n3 = 3.85 showed low epithelial lifting of lamellae ($P = 0.001$) (Table 3 and Figure E, F, G, H). Similarly, diet had a clear effect on lamellar fusions with low values in the fish fed n6/n3 = 3.85 ($P = 0.000$). Hemorrhage, edema, desquamation and necrosis were not found in any group.

For the histopathological condition of gills, no significant differences in FQ were observed for vasodilation and lamellar hypertrophy, both of which were classified as reversible pathological lesions (Table 3). Suboptimal temperature induced FQ for aneurism (Table 3 and Fig. 2A, B, C, D) and epithelial lifting of lamellae (Table 3 and Fig. 2 E, F, G, H) ($P = 0.005$), both considered reversible pathological lesions. The fish fed n6/n3 = 12.02 had a high FQ for lamellar fusions ($P = 0.001$) (Table 3). High hyperplasia was observed in fish at 30 °C (Table 3 and Fig. 2 I, J, K, L). Reversible pathological lesions, such as hemorrhage, edema and lamellar epithelial desquamation and necrosis (severe lesion), were not found when considering FQ. A brief summary of the significant results of temperature and diets are shown in Figs. 3 and 4, respectively.

Finally, the amount of glycogen tended to decrease in the hepatocytes of tilapia at 30 °C compared to 20 °C ($P = 0.001$) (Table 4). Similar patterns were found for white muscle and gills ($P = 0.001$). A statistical difference appeared between the fish fed the fatty acids ratio. Those fed n6/n3 = 12.02 displayed decreased muscle glycogen ($P = 0.002$) and gills glycogen ($P = 0.001$), regardless of the thermal condition.

4. Discussion

No interaction effect between dietary fatty acids ratio and water temperatures were detected by interaction analysis for histological changes in liver and gills of tilapia. According to Atwood et al. (2003), low temperature or dietary fatty acid had no interaction to many physiological characteristics of stress. Some studies had also no interaction effect (temperature *vs*. fatty acids), suggesting that different factors can affect its interaction as specified metabolic pathways of different tissue as liver, whole body, muscle, gills and brain in sea bass (*Dicentrarchus labrax*) (Skalli et al., 2006) or similar digestibility of diets in different temperatures for Atlantic salmon (*Salmo salar*). These factors may mask the interaction effect analysis; however the specific effects of the temperature as well as the diets were clear.

Similar to that observed in the present study, neither suboptimal 22 °C or lipid sources had any effect on tilapia survival (Corrêa et al., 2017), which suggests adaptation mechanisms to thermal challenge. Regarding the liver structure, circulatory alterations were found in the fish subjected to either 20 °C or 30 °C, but statistical differences was found so when considering the FQ of histopathological change indices (HP). The fish at 30 °C obtained higher sinusoidal space values, which has a maximal importance factor. By considering the inflammation processes hypothesis, the fish cells at 20 °C could swell, which would reduce sinusoidal spaces (Hines and Spira, 1974). Changes in sinusoidal spaces may affect the movement of nutrients, chemical signals or other physiological components in the livers of *Labeo rohita* (Marigoudar et al., 2013). Although tilapia is a tropical fish, liver circulatory adaptations could occur at the cellular level. Rainbow trout (*Salmo gairdneri*) acclimated to 5 °C have shown an enlarged and more developed hepatocyte Golgi apparatus than other fish at 18 °C, which suggests an adaptive secretory function (Berlin and Dean, 1967).

The most prevalent liver alterations were regressive and progressive changes. Regressive alterations are defined as processes that may reduce organ function or loss, while progressive alterations are defined as

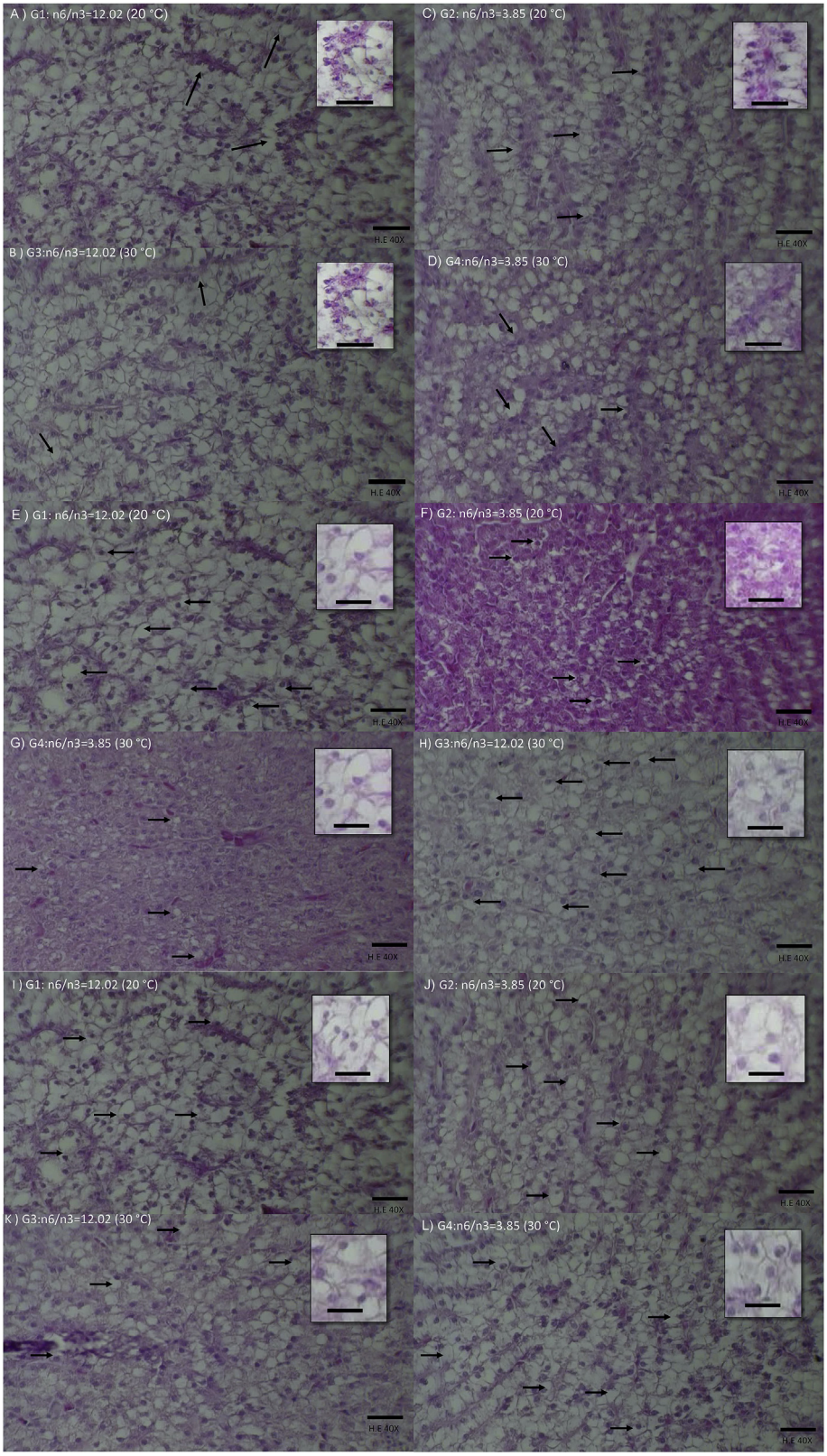

Fig. 1. Liver alterations assigned to each category in juvenile tilapias. Nuclear displacement: A "G1: n6/n3 = 12.02 (20 °C)", B "G3: n6/n3 = 12.02 (30 °C)", C: "G2 n6/n3 = 3.85 (20 °C)", D "G4: n6/n3 = 3.85 (30 °C)"; Cytoplasmic vacuolization: E "G1: n6/n3 = 12.02 (20 °C)", F "G3: n6/n3 = 12.02 (30 °C)", G "G2: n6/n3 = 3.85 (20 °C)", H "G4: n6/n3 = 3.85 (30 °C)"; Cellular hyperplasia: I "G1: n6/n3 = 12.02 (20 °C)", J "G3: n6/n3 = 12.02 (30 °C)", K "G2: n6/n3 = 3.85 (20 °C)", L "G4: n6/n3 = 3.85 (30 °C)". Black arrows represent alterations.

Table 3
The gill alterations assigned to each category in juvenile tilapias reared at suboptimal and optimal temperatures (20 °C/30 °C) and fed the n6/n3 ratio (12.02/3.85), respectively. Alterations were calculated as histopathological indices (HP) and Frequency of lesions (FQ).

Group: Diet		Temperature				*P-value*		
		20 °C		30 °C				
		n6/n3 = 12.02	n6/n3 = 3.85	n6/n3 = 12.02	n6/n3 = 3.85			
Histopathological Changes		*HP indices*	*HP indices*	*HP indices*	*HP indices*	*Diet*	*Temp.*	*Int.*
Circulatory alterations[a]	Vasodilation (*)	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	NS	NS	NS
	Hemorrhage (*)	–	–	–	–	–	–	–
	Aneurysm (*)	2.00 ± 0.00 aA	2.00 ± 0.00 aA	0.75 ± 1.04 aB	1.50 ± 0.93 aB	NS	0.001	NS
	Oedema (*)	–	–	–	–	–	–	–
Regressive alterations[b]	Epithelial Lifting of Lamellae (*)	2.25 ± 0.46 aA	1.50 ± 0.93bA	2.13 ± 0.35 aA	1.00 ± 1.07bA	0.001	NS	NS
	Lamellar Epithelial Desquamation (*)	–	–	–	–	–	–	–
	Lamellar Fusions (*)	2.00 ± 0.00 aA	1.00 ± 1.07bA	1.50 ± 0.93 aA	0.25 ± 0.71bA	0.000	NS	NS
	Necrosis (***)	–	–	–	–	–	–	–
Progressive alterations[c]	Lamellar Hypertrophy (*)	3.13 ± 0.83	3.13 ± 0.83	3.50 ± 1.07	2.75 ± 0.46	NS	NS	NS
	Lamellar Hyperplasia (**)	4.75 ± 1.04 aB	4.75 ± 1.04 aB	8.75 ± 1.49 aA	7.25 ± 1.83 aA	NS	0.000	NS
Histopathological Changes		*FQ (%)*	*FQ (%)*	*FQ (%)*	*FQ (%)*	*Diet*	*Temp.*	*Int.*
Circulatory[a]	Vasodilation (*)	7.38 ± 4.20	6.56 ± 3.44	3.99 ± 1.97	6.03 ± 7.04	NS	NS	NS
Alterations	Aneurism (*)	3.47 ± 1.91 aA	6.98 ± 4.39 aA	1.28 ± 2.10 aB	1.55 ± 1.21 aB	NS	0.005	NS
Regressive[b]	Epithelial Lifting of Lamellae (*)	14.57 ± 9.78 aA	11.26 ± 8.17 aA	8.72 ± 5.07 aB	7.40 ± 9.14 aB	0.001	NS	NS
Alterations	Lamellar Fusions (*)	6.69 ± 2.01 aA	3.10 ± 4.42bA	4.27 ± 3.14 aA	0.25 ± 0.71bA	0.0001	NS	NS
Progressive alterations[c]	Lamellar Hypertrophy (*)	41.00 ± 14.27	42.08 ± 18.38	35.51 ± 14.48	33.95 ± 7.51	NS	NS	NS
	Lamellar Hyperplasia (**)	26.89 ± 7.96 aB	30.02 ± 9.78 aB	45.74 ± 14.06 aA	50.70 ± 15.92 aA	NS	0.0001	NS

The type and the extent of histological alterations were described by using a method developed by Bernet et al. (1999).
The pathological importance of the observed alteration was defined as an "importance factor", classified as (*), (**) or (***) corresponding to minimal (reversible pathological lesions), moderate (lesions that in most cases revert after neutralization of the stressor agent) and severe (often irreversible lesions that cause partial or total loss of function of the affected organ) respectively. A score value ranging from 0 (absence of alteration) to 6 (severe occurrence of alteration) is specified in accordance with the degree and extent of a specific alteration. To obtain value of the organ index (HP indice) to "fator de importância" e valor do escore foram multiplicados, de acordo com as seguintes fórmulas (Bernet et al., 1999): I orgcat = Σalt ($a_{orgrpal} \times w_{orgrpalt}$) and I org = Σrp Σalt ($a_{orgrpal} \times w_{orgrpalt}$). Frequency of lesions (FQ %) = (N lesion x N total lesion $^{-1}$) ×100
Data are presented as mean and standard error (SE) (n = 8). Different lowercase letters in the same line represent differences between diets (n6/n3 ratio) and different capital letters in the same line represent differences between optimal and suboptimal temperatures (20 °C and 30 °C), two-way ANOVA followed by the Tukey test ($P < 0.05$), NS = not significant.
[a] Disturbances result from a pathological condition of blood and tissue fluid flow.
[b] Breakdown of tissue and/or cells which terminate in a functional reduction or loss of an organ. Changes in tissue architecture.
[c] Increase in the number of specific cell types or structures.

Table 4
Average glycogen (μm) in liver, white muscle and gills of juvenile tilapias reared at suboptimal and optimal temperatures (20 °C/30 °C), and fed two n6/n3 ratio (12.02/3.85), respectively.

Group: Diet	Temperature				*P-value*		
	20 °C		30 °C				
	n6/n3 = 12.02	n6/n3 = 3.85	n6/n3 = 12.02	n6/n3 = 3.85	Diet	Temp.	Int.
Liver	6499.12 ± 598.05 aA	7237.84 ± 690.95 aA	4748.47 ± 948.18 aB	3796.83 ± 444.87 aB	NS	< .0001	0.001
White muscle	3159.33 ± 158.90bA	3450.29 ± 236.90 aA	2574.18 ± 196.88bB	2822.09 ± 309.31 aB	0.002	< .0001	NS
Gills	1678.53 ± 137.41bA	2497.69 ± 416.19 aA	587.17 ± 166.24bB	945.17 ± 64.06 aB	< .0001	< .0001	0.001

Data are presented as mean calculated under magnification of 400× and standard error (SE) (n = 8). Different lowercase letters in the same line represent differences between diets (n6/n3 ratio) and different capital letters in the same line represent differences between optimal and suboptimal temperatures (20 °C and 30 °C), two-way ANOVA followed by the Tukey test ($P < 0.05$), NS = not significant.

increased cell or tissue activity (Kostić et al., 2017). Regarding regressive alterations, the nuclear displacement value was similar between temperatures. Nuclear displacement is a physiological response to the liver's energy storage (Rios et al., 2007). The tilapia fed a different plant oil or fish oil at an optimal temperature displayed similar hepatosomatic indices (Peng et al., 2016), which partly supports our findings. The FQ of the histopathological changes indices were influenced by the n6/n3 = 3.85 diet given the high nuclear displacement value. This may be associated with lipid deposition as the glycogen values were similar in hepatocytes. Linseed oil and sunflower oil promoted the greatest PUFA deposition in the whole body of the tilapia exposed to 22 °C and 28 °C, and promoted growth at 22 °C (Corrêa et al., 2018). Conversely, a bigger amount of dietary n3 led to low lipid deposition in the livers of tilapia (Boonanuntanasarn et al., 2019) which could, in turn, influence nuclear displacement less. In addition to the variable results found in the literature, we suggest that these results do not reflect hepatic degeneration or lipid infiltration, but reveal a fish body condition upon homeostasis, which falls in line with our low nuclear degeneration and necrosis results. Caballero et al. (1999) showed irregular nuclei displaced to the periphery of hepatocytes in sea bream (*Sparus aurata*) when fed only at a high dietary lipid level, but this was not related to a pathological situation. Lipid droplets in tilapia liver are normally involved in maintaining the homeostasis of lipid synthesis, transport, storage and metabolism (Boonanuntanasarn et al.,

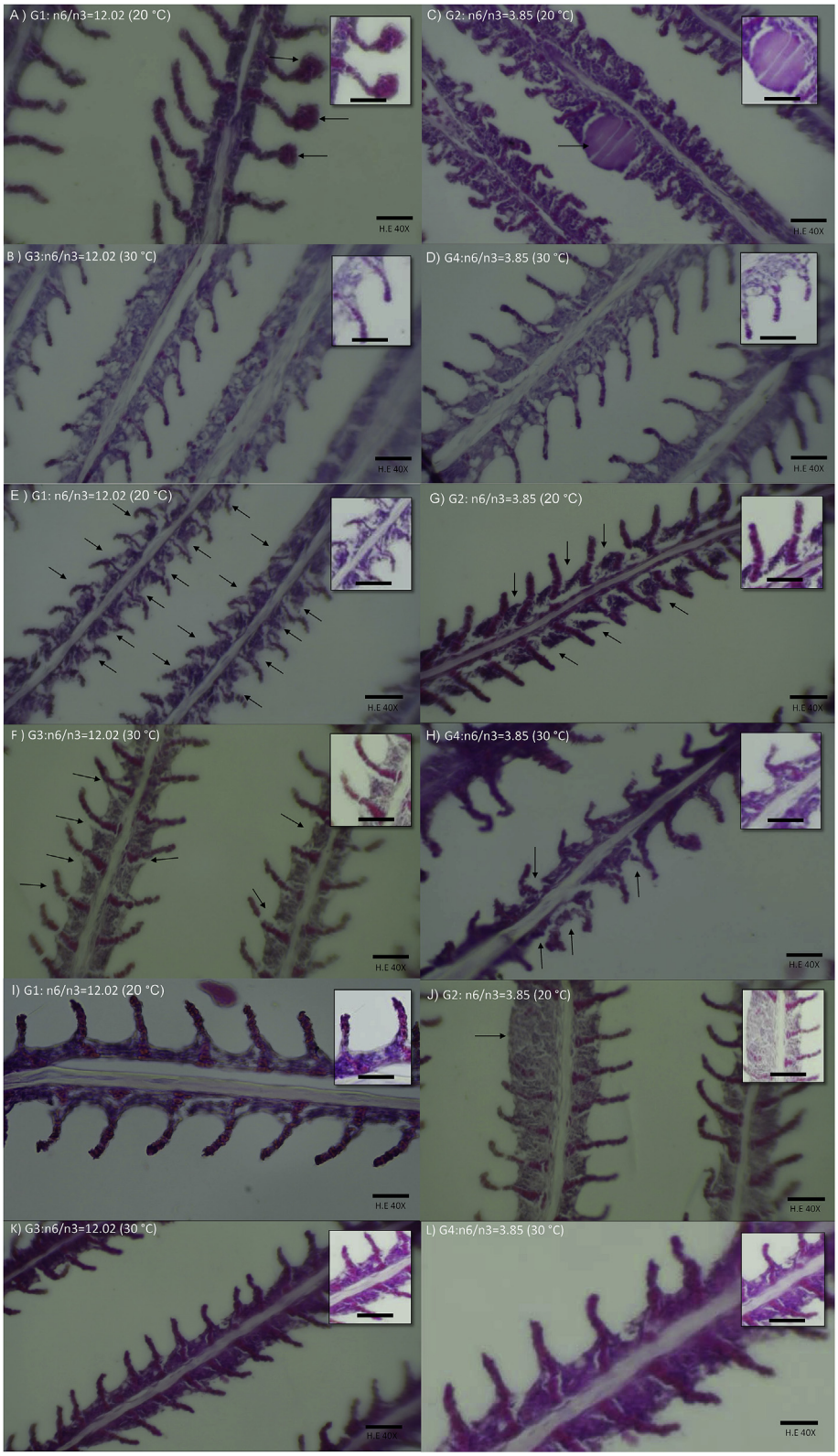

Fig. 2. The gill alterations assigned to each category in juvenile tilapias. Aneurysm: A "G1: n6/n3 = 12.02 (20 °C)", B "G3 n6/n3 = 12.02 (30 °C)", C "G2: n6/n3 = 3.85 (20 °C)", D "G4: n6/n3 = 3.85 (30 °C)"; Epithelial lifting of lamellae: E "G1: n6/n3 = 12.02 (20 °C)", F "G3: n6/n3 = 12.02 (30 °C)", G "G2: n6/n3 = 3.85 (20 °C)", H "G4: n6/n3 = 3.85 (30 °C)"; Lamellar hyperplasia: I "G1: n6/n3 = 12.02 (20 °C)", J "G3: n6/n3 = 12.02 (30 °C)", K "G2: n6/n3 = 3.85 (20 °C)", L "G4: n6/n3 = 3.85 (30 °C)". Black arrows represent alterations.

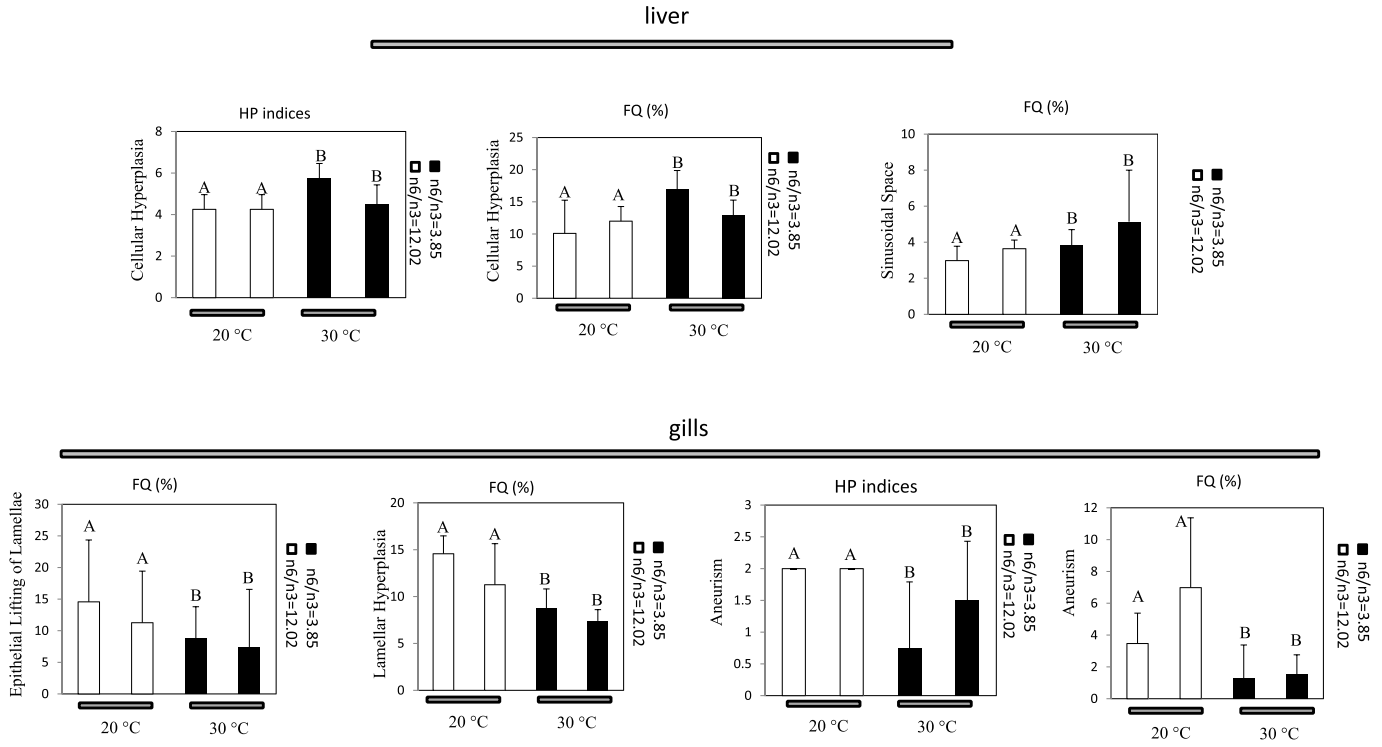

Fig. 3. Summary of the temperature effect in the liver and gill histopathological alterations. Different capital letters represents difference between temperatures (20 °C and 30 °C) by Tukey test ($p < 0.05$).

2019). This might suggest a protective effect of n6/n3 = 3.85. Temperature had no effect on cytoplasmic vacuolization, but dietary n6/n3 = 3.85 provided a low value for this parameter. A higher percentage of dietary linseed oil was related to the effect of linolenic acid on reducing cytoplasmic vacuolization in the liver of seawater sea bream, *Sparus aurata* (Caballero et al., 2004). Even though dietary n6/n3 = 3.85 has favoured nuclear displacement, which is associated to some extent with hepatic reserve, these vacuolization results could be associated with the dynamics of using these substrates. The vacuoles in the cytoplasm of hepatocytes contain lipids and glycogen, which are related to the liver's metabolic function (Camargo and Martinez, 2007). Linseed oil that is rich in 18: 3n-3, and a precursor for n-3 HUFA, was related to favouring the substrate for β-oxidation in fish (Bell et al., 2004), which may lead to hepatic reserves being rapidly used up.

When a pathological process was contemplated, the under fasting condition produced by low temperature, the liver of sea bream displayed a significant increase in the total lipid content caused by n-3 fatty acids, mainly 22: 6n-3, which led to physical changes in the liver associated with winter disease (Ibarz et al., 2005). In turn, our results revealed that at 20 °C, tilapia showed no nuclear hypertrophy, but low hepatocyte hyperplasia, regardless of diet. Considering the effect of fatty acids on liver hyperplasia, our results indicated no clear effect of dietary source on growth tissue under the thermal condition. Conversely at 22 °C, the highest daily weight gain occurred in the tilapia fed linseed, sunflower and fish oil (Corrêa et al., 2017), which revealed a body hyperplasia condition. In addition, this progressive alteration could be related with food intake reduction at suboptimal temperatures. Although some contradictions may exist, the importance of hypertrophy seems to increases as fish age. Hyperplasia is also an important variable for juvenile fish and its tissue growth (Mommsen, 2001). Obviously, growing fish as in the present study under optimum temperature conditions exhibited evident tissue hyperplasia.

Most of the studies done on temperature effect on changes in gills have been associated with pollutants and heavy metal effects as gills are potential biomarkers of polluted environments. Gills have a large external contact surface that favours sensitivity to physical changes in aquatic environments. Our results revealed that the fish maintained at 20 °C showed no vasodilation effect, but this thermal condition influenced aneurysm, probably due to having to cope with low temperature. The stressor agent could lead increase the blood flow inside lamellae by causing dilation to the marginal channel, aneurysm, and even ruptured gill epithelium, which can associated with the seasonal changes effect (Camargo and Martinez, 2007). The FQ of the epithelial lifting of lamellae tends to increase at suboptimal temperatures, but n6/n3 = 3.85 seemed to favour few lamellae lesions. As a result of epithelial lifting, the distance between water and blood increases and impairs oxygen uptake (Flores-Lopes and Thomaz, 2011). So the use of n6/n3 = 3.85 in Nile tilapia feeds seems to enhance their cold tolerance. Despite having a lower lamellar fusion trend in tilapia gills at 30 °C, the statistical analysis revealed a clear effect of dietary n6/n3 = 3.85 on reducing this pathology. Increased polyunsaturated fatty acids in gill tissues help protect cell membranes in tilapia (Cengiz et al., 2012). 22: 6 (n-3) diminished gill damaged as inter-lamellar spaces that could help chloride cells to disappear, and could compromise both respiration and osmoregulation in turbot (*Scophthalmus maximus*) (Bell et al., 1985). The 30 °C temperature provided high lamellar hyperplasia, which would not represent a disease status, but was also associated with fish growth at optimal temperature. Fasting periods can affect tissue growth mechanisms in tropical fish at suboptimal temperature. The hyperplasia and hypertrophy of gill tissue were more evident in the fish at 35 °C compared to 18 °C in tambaqui (*Colossoma macropomum*) (Salazar-Lugo et al., 2011). Protruding gill lamellae would increase the amount of energy used for osmoregulation in winter (Sollid and Nilsson, 2006), which would not be necessary in fish at optimal temperatures.

Tilapia showed high glycogen stored in tissue at suboptimal temperatures and the n6/n3 = 3.85 ratio favoured glycogen storage in both muscle and gills. Body glycogen is essentially the sole source of fuel in winter months (Sollid and Nilsson, 2006). Cold shock can modify the lipid metabolism in Nile tilapia by lowering total body saturated fatty acids and raising n-6 and n-3 UFA to protect cell membranes (Abdel-

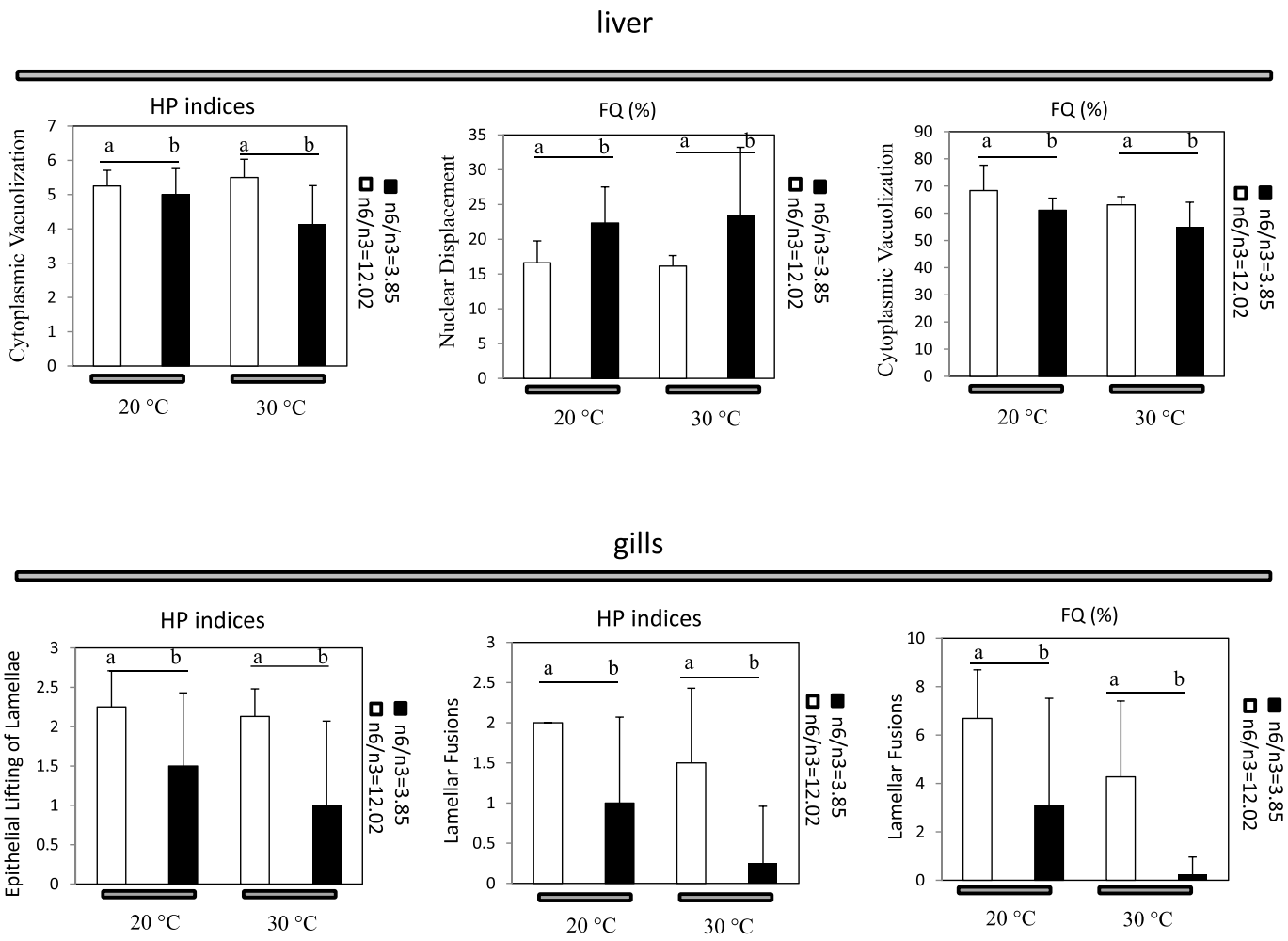

Fig. 4. Summary of the dietary linoleic/α-linolenic effect in the liver and gill histopathological alterations. Different lowercase letters represents difference between diets (n6/n3 = 12.02 and n6/n3 = 3.85) by Tukey test ($p < 0.05$).

Ghany et al., 2019). However, this metabolic conversion could lead to high energy costs. In turn, the tilapia fed n6/n3 = 3.85 ratio could use this energy to store glycogen. This result suggests tilapia's adaptive capability to subtropical aquaculture areas. In winter, fish prolong the utilization of stored glycogen, which can be postponed, and glycogen is more likely to last long enough to assure cell homeostasis and survival (Sollid and Nilsson, 2006).

5. Conclusions

Our findings showed that suboptimal temperature influenced the incidence of histological alterations in tilapia. In addition, the dietary fatty acids ratio (n6/n3 = 3.85) suggested a protective effect of liver regressive alterations, such as nuclear displacement or cytoplasmic vacuolization, over the surface epithelia of gills (lifting and fusions of lamellae). Circulatory and progressive alterations were associated only with the influence of temperature. Our findings herein reinforce the importance of starting histopathologic practices on farms and the use of plant oil sources to develop modern subtropical aquaculture.

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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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jtherbio.2019.07.028.

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