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Physiological Responses of Nile Tilapia, *Oreochromis niloticus*, Fed Vitamin C- and Lipid-Supplemented Diets and Submitted to Low-Temperature Stress*

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Abstract.—Water temperature alterations can determine harmful physiological modifications in fish, which should be prepared to cope with this, and nutrition strategies seem to be essential. This study evaluated the effects of different levels of vitamin C and lipids on physiological responses of Nile tilapia, *Oreochromis niloticus*, submitted to temperature stress. There were two phases: Phase I – preparing fish to store vitamin C and lipid at appropriate temperature, and Phase II – evaluating the contributions these reserves make to fish physiology under low-temperature stress. The experiment used a 3 × 2 factorial design with three vitamin C levels (300, 600, and 1200 mg/kg diet) and two lipid levels (8.0 and 12.0%), plus absence of nutrient test and a diet of 6.0% lipids and 125.0 mg/kg vitamin C. In Phase I, 192 fish were kept at 26.0 ± 1.0 C for 112 d, and in Phase II, 48 fish were kept at 18.0 ± 0.5 C for 32 d and at 15.0 ± 0.5 C for 11 d. Fish fed C_0L_0 diet showed lower erythrocytes values in both phases; higher vitamin C supplement determined higher red blood cell (RBC) number and higher hematocrit (Htc) (Phase II); Htc was significantly lower in Phase II; after temperature stress, fish fed C_0L_0 diet had higher mean corpuscular volume, lower hemoglobin corpuscular concentration, and significantly lower vitamin C concentration in the liver; and higher supplementation determined a higher concentration in the liver (Phases I and II). Higher plasmatic cortisol concentration was seen in fish fed C_0L_0 diet. In conclusion, our results show that the absence of vitamin C in diets impairs RBC formation and does not enable fish to cope with stress; excess vitamin C is efficient in mitigating stress and 600 mg/kg diet is economic and physiologically sufficient to prepare fish for coping with low-temperature stress. Lipid supplementation does not determine alterations in stress biochemical parameters.

In intensive culture systems, fish are continuously exposed to stress, which can cause temporary homeostasis modifications leading to physiological adjustments. These responses aimed at mobilizing energy by adrenergic system stimulation, release catecholamine and increase adrenocorticotropic hormone (ACTH) and plasmatic cortisol (Gamperl et al. 1994). These responses can be extended and cause chronic stress, which increases the imbalance.

Low resistance and high disease susceptibility during a stress period, such as winter, can cause fish death and, consequently, economic problems to producers. According to Sealey et al. (1997), the annual loss from disease problems caused mainly by *Edwardsiella ictaluri* bacteria is estimated to be millions of dollars in the American catfish industry.

Weight loss has also been observed during winter as a result of body fat reduction (Lovell and Sirikul 1974). According to Lemly (1996), lipid reserves have significant mobilization and can be totally used as an energy source, indicating the need for an appropriate reserve to maintain good body condition.

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The increase in metabolism during adverse situations also determines vitamin redistribution and higher demand, especially vitamin C (Wedemeyer 1969). Although some research showed that higher vitamin C doses than required for normal growth have been recommended for better resistance in channel catfish, *Ictalurus punctatus* (Li and Lovell 1985), others showed that high doses were not effective (Li et al. 1998).

Studies have shown that high ascorbic acid concentration in tissue determines higher tolerance to ambient pollution and better resistance to bacteria infection (Li and Lovell 1985). Jaffa (1989) also recommended that high levels of vitamin C supplementation minimize physiological stress in fish. Kitabchi (1967) stated that high ascorbic acid levels inhibit steroid synthesis, suggesting that an increased vitamin C reserve might prevent stress response severity.

This study evaluated the effects of different vitamin C and lipid levels and their interaction on physiological responses of Nile tilapia, *Oreochromis niloticus*, submitted to low-temperature stress.

Material and Methods

There were two phases: Phase I (112 d) prepared fish vitamin C and lipid reserves at an appropriate temperature and Phase II (36 d) evaluated the contributions these reserves made to fish physiological responses when submitted to low-temperature stress.

Experimental Diets and Design (Phases I and II)

The experiment used a 3 × 2 factorial design for both phases, with three vitamin C (mg/kg diet) and two lipid (%) levels: 300 mg, 8% ($C_{300}L_8$); 600 mg, 8% ($C_{600}L_8$); 1200 mg, 8% ($C_{1200}L_8$); 300 mg, 12% ($C_{300}L_{12}$); 600 mg, 12% ($C_{600}L_{12}$); and 1200 mg, 12% ($C_{1200}L_{12}$). Two other treatments were added: a nonsupplemented (C_0L_0) and a 125 mg, 6% ($C_{125}L_6$). Polyphosphated vitamin C with 35% ascorbic acid activity (Stay-C® Roche Socil, Brazil) was used with commercial soybean oil as the lipid source.

Eight practical diets were formulated containing approximately 32.0% digestible protein (Table 1). All ingredients were ground to pass through a 1-mm mesh screen and processed into 3-mm-diameter pellets. Vitamin and mineral supplements did not contain vitamin C. Diets were dried and frozen (−20.0 C) to avoid vitamin C loss as a result of oxidation. Crude protein, crude energy, and ether extract were chemically analyzed according to AOAC (2000) protocol. Dietary vitamin C levels were assessed by high-performance liquid chromatography (HPLC). Feed samples (1.0–2.0 g) were ground to a fine powder, extracted by ultrasound bath for 15 min, cooled, and then filtered in millex (0.45 μm). Chromatography conditions were as follows: mobile phase flux 2.0 mL/min, ODS column (C18 – 150 × 4.6 mm), 25 C, and 280-nm UV detector. Detection level was about 10 ppm, and L-ascorbyl-2-polyphosphate recovery efficiency was 94%.

Phase I – Fish and Feeding

In Phase I, 192 Nile tilapia fingerlings from a single spawn and sex reverted (methyltestosterone treated), with an average weight of 5.57 ± 0.50 g, were randomly distributed into 32 net cages (200 L each), four cages per treatment and six fish per cage, placed in eight 1000-L aquaria in a closed recirculation system. Aquaria were supplied with 6 L/min dechlorinated tap water passing through a biofilter to remove impurities and reduce ammonia concentration. The system was supplied with a heater and kept at 26.0 ± 1.0 C. Water temperature and dissolved oxygen were measured once a week in four randomly selected tanks using a DM4 oxygen meter (DIGIMED Adamo, Brazil); accumulated wastes were removed by siphoning.

Each of the eight experimental diets was randomly fed to fish in four net cages four times a day (0830, 1130, 1430, and 1730 h) for 112 d. At each feeding, diet was offered two or three times until apparent satiation was reached. A 12-h photoperiod was maintained with artificial fluorescent illumination.

TABLE 1. *Proximal and chemical composition of experimental diets.*

Ingredient	Diets[a]							
	C_0L_0	$C_{125}L_6$	$C_{300}L_8$	$C_{600}L_8$	$C_{1200}L_8$	$C_{300}L_{12}$	$C_{600}L_{12}$	$C_{1200}L_2$
Soybean meal	54.88	56.27	56.74	56.74	56.80	57.65	57.08	57.70
Corn gluten meal	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Fish meal	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Corn meal	24.10	16.67	14.15	14.07	13.84	9.24	9.13	8.94
Wheat middlings	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Soybean oil	—	6.00	8.00	8.00	8.00	12.00	12.00	12.00
Dicalcium phosphate	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Vitamin–mineral mix[b]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
BHT[c]	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Vitamin C[d]	—	0.04	0.09	0.17	0.34	0.09	0.17	0.34
Chemical composition (dry matter basis)								
CP (%)[e]	36.05	36.12	35.70	34.97	35.69	36.20	36.50	36.03
CE (kcal/kg)[e]	4640.10	4702.04	4809.71	4858.14	4853.41	5192.06	5184.51	5157.60
EE (%)[e]	2.79	8.88	10.89	10.81	10.68	14.59	14.44	14.68
CF (%)[f]	4.28	4.19	4.16	4.16	4.16	4.12	4.12	4.12
Vitamin C (mg/kg)[e]	ND	189.00	360.00	527.00	1247.00	331.00	493.00	1108.00

BHT = butylated hydroxytoluene; CP = crude protein; CE = crude energy; EE = ether extract; CF = crude fiber; ND = not determined.

[a] Treatments: C_0L_0 (0 mg/kg vitamin C and 0% lipid); $C_{125}L_6$ (125 mg/kg vitamin C and 6% lipid); $C_{300}L_8$ (300 mg/kg vitamin C and 8% lipid); $C_{600}L_8$ (600 mg/kg vitamin C and 8% lipid); $C_{1200}L_8$ (1200 mg/kg vitamin C and 8% lipid); $C_{300}L_{12}$ (300 mg/kg vitamin C and 12% lipid); $C_{600}L_{12}$ (600 mg/kg vitamin C and 12% lipid); and $C_{1200}L_{12}$ (1200 mg/kg vitamin C and 12% lipid).

[b] Vitamin and mineral mix (Socil Guyomarc'H®): vitamin A, 1,200,000 UI; vitamin D_3, 200,000 UI; vitamin E, 1200 mg; vitamin K_3, 2400 mg; vitamin B_1, 4800 mg; vitamin B_2, 4800 mg; vitamin B_{12}, 4800 mcg; vitamin B_6, 4800 mg; vitamin C, 0; calcium D-pantothenate, 12,000 mg; niacin, 24,000 mg; folic acid, 1200 mg; biotin, 48 mg; choline chlorate, 108 g; cobalt, 10 mg; cooper, 3000 mg; iron sulfate heptahydrate, 50,000 mg; iodine, 100 mg; manganese, 20,000 mg; selenium, 100 mg; zinc sulfate, 30,000 mg; carrier q.s.p., 1000 g.

[c] Antioxidant.

[d] L-ascorbyl-2-polyphosphate, 35.0% vitamin C.

[e] Determined value.

[f] Calculated value.

Phase I – Hematological Assay

Blood samples were obtained after 112 d. Six fish per treatment were randomly chosen and anesthetized with benzocaine at 1.0 g/15 L. Blood samples were collected from the caudal vein using tuberculin syringes rinsed with ethylenediaminetetraacetic acid (dipotassium salt PA 2-hydrate 3.0%) to determine red blood cell (RBC) count, hematocrit (Htc), and hemoglobin (Hb).

RBC counts were obtained in a hemocytometer with diluted whole blood. Htc was determined by the microhematocrit method and Hb by the cianometahemoglobin method using a standard kit (Labtest Diagnostica S.A.mega, Brazil). Mean corpuscular volume (MCV) and mean corpuscular Hb concentration (MCHC) were calculated. Blood analyses methodology was as per Jain (1986).

The same fish were bled again for glucose and cortisol analyses using heparin as anticoagulant. Plasma samples were collected following centrifugation of whole blood from six fish at 2000 *g*. Plasma from two fish from the same treatment were pooled to obtain one composite sample. Glucose was determined using King and Garner's (1947) methodology and cortisol by DPC radioimmunoassay kit – Diagnostic Products Corporation (coat-a-count, solid-phase DPC, Brazil).

Phase I – Measurement of Liver Content of Vitamin C

Liver samples were obtained after 112 d. Six fish per treatment were randomly chosen and

sacrificed with high-dose anesthesia (benzocaine USP Synth). Livers were collected and immediately immersed in liquid nitrogen to avoid vitamin C oxidation and then stored frozen at −80 C for subsequent vitamin C concentration determination by HPLC – reverse phase as per Wang et al. (1988). Vitamin C concentration in the liver was determined in an HPLC equipped with diode array detector (SPD-M10AVP; Shimadzu Labtec, Brazil), through a Supelcosil LC-NH_2 column with 1.5 mL/min constant flux and using a mobile phase 10 mM acetonitril/sodium phosphate buffer, pH 2.6 (75/25) at 40 C.

Phase II – Procedure

After the above analyses, 48 fish were redistributed, in the same experimental structure, into three aquariums per treatment at a density of two fish per aquarium, six fish per treatment, with average weight of 105.13 ± 19.71 g. The water temperature was gradually dropped from 26.0 to 18.0 C over 7 d.

After this, fish were transferred to another experimental structure and randomly stocked into twenty-four 40 L aquariums at two fish per aquarium. Each aquarium was supplied with individual aeration and biofiltration. Room temperature was controlled at 18.0 C. The experimental design was the same as Phase I.

Fish were kept at this temperature for 25 d and then at 11.0 C for another 11 d. After this 36-d low-temperature stress period, the same blood parameters and vitamin C liver content as at the end of Phase I were evaluated.

Statistical Analysis

The statistical analysis used a two-way ANOVA technique for a 2 × 3 factorial experiment in a completely randomized design, with two additional treatments. The ANOVA was complemented with both the Scheffé multiple contrasts and the Tukey test for comparisons within the factorial structure (Zar 1999). Differences between means were reported as significant if $P < 0.05$. Variables in both phases were studied by repeated measurement to compare the mean profile of each treatment, with the construction of simultaneous confidence intervals (Johnson and Wichern 1992).

Results

Fish fed basal diet (C_0L_0) had significantly ($P < 0.05$) lower erythrocytes and Htc values than the average Phase I factorial treatments. Hb was lower, although not significantly different from factorial averages. After temperature stress, C_0L_0 fish had lower blood values than at Phase I (Table 2). Fish fed vitamin C-supplemented diets showed a significant effect of this vitamin on erythrocyte number and Htc with a slight increase in Hb. There was a linear increase for vitamin C supplementation, although 600 and 1200 mg/kg diet vitamin C was not significantly different.

There was a significant effect after low-temperature stress for mean values of MCV and MCHC between blood from fish fed basal diet and those from the factorial groups. This was not seen between fish fed supplemented diet (factorial) and $C_{125}L_6$. Vitamin C and lipid supplementation did not affect any of these hematimetric values, irrespective of supplementation level.

Table 3 shows the mean vitamin C liver concentration, plasmatic cortisol, and glucose levels in fish fed diets with different levels of vitamin C supplement and lipids submitted to low-temperature stress. Levels were significantly higher ($P < 0.05$) in fish fed vitamin C supplemented diets than in fish fed basal diet (C_0L_0) before stress. The 125 mg/kg vitamin C-supplemented diet also had a lower liver vitamin C concentration than other supplemented groups. Higher supplementation levels produced an average increase of 35.8% in liver concentration. Similar to blood values, increased liver vitamin C was related to diet vitamin C, although there was no significant difference between 600 and 1200 mg/kg diets. Comparing the before and after stress values, there was a similar tendency in fish on no vitamin C supplementation and fish on 125-mg vitamin C supplementation.

There was a significant poststress effect on plasmatic cortisol on fish fed basal diet compared with factorial supplemented fed fish; no supplementation produced a similar plasmatic cortisol concentration before and after. However,

TABLE 2. *Mean values of erythrocytes (Erit), hematocrit (Htc), and hemoglobin (Hb) for Nile tilapia juveniles fed diets supplemented with different levels of vitamin C and lipids and submitted to temperature stress.*[1]

Diets	Erit Phase I ($10^6/\mu L$)	Erit Phase II ($10^6/\mu L$)	Htc Phase I (%)	Htc Phase II (%)	Hb Phase I (g/dL)	Hb Phase II (g/dL)
C_0L_0	1.67 (±0.17)	1.52 (±0.04)	27.10 (±1.20)	26.50 (±1.45)	7.75 (±0.75)	7.01 (±1.35)
$C_{125}L_6$	1.92α (±0.11)	1.72β (±0.11)	29.40α (±0.92)	26.20β (±1.21)	8.33 (±0.58)	8.54 (±1.16)
$C_{300}L_8$	1.94 (±0.14)	1.80b (±0.05)	28.26α (±0.17)	25.10bβ (±1.02)	8.17 (±0.56)	7.50 (±1.02)
$C_{600}L_8$	2.09 (±0.20)	1.95a (±0.07)	29.20α (±0.29)	27.60aβ (±0.80)	7.65 (±0.33)	7.70 (±1.49)
$C_{1200}L_8$	1.99 (±0.10)	1.95a (±0.06)	30.27α (±2.01)	27.75aβ (±0.67)	7.96 (±0.34)	8.39 (±0.40)
$C_{300}L_{12}$	1.98 (±0.19)	1.79b (±0.09)	29.50α (±0.82)	25.00bβ (±1.45)	8.22 (±0.80)	7.68 (±0.91)
$C_{600}L_{12}$	1.98 (±0.17)	1.80ab (±0.08)	29.00α (±0.77)	27.08aβ (±0.88)	8.00 (±0.49)	8.02 (±2.37)
$C_{1200}L_{12}$	1.97 (±0.17)	1.94a (±0.05)	29.17α (±0.83)	27.00aβ (±0.57)	8.01 (±0.41)	8.32 (±1.38)
Cont. I × fat.[2]	0.01	0.05	0.05	ns	ns	ns
Cont. II × fat.[3]	ns	ns	ns	ns	ns	ns
Vitamin C effect						
(*P* level)	ns	0.05	ns	0.05	ns	ns
300	1.96	1.80	28.88	25.05	8.20	7.59
600	2.04	1.88	29.10	27.34	7.83	7.86
1200	1.98	1.95	29.72	27.38	7.99	8.36
Lipid effect						
(*P* level)	ns	ns	ns	ns	ns	ns
	2.01	1.90	29.24	26.82	7.92	7.86
	1.98	1.84	29.22	26.36	8.08	8.01
Vitamin C × lipid						
(*P* level)	ns	ns	ns	ns	ns	ns

ns = not significant.

[1] Different lowercase letters indicate differences between vitamin C levels, different uppercase letters indicate differences between lipid levels, and different Greek letters indicate differences between Phase I and Phase II.

[2] Control I (C_0L_0) × mean of factorial treatments.

[3] Control II ($C_{125}L_6$) × mean of factorial treatments.

[4] *P* level = statistic significance.

the other treatments showed a 65.0% increase in plasmatic cortisol after stress. The 600 and 1200 mg vitamin C supplemented diets showed a significant reduction in plasmatic cortisol. Vitamin C supplementation had no effect on plasmatic glucose concentration.

Discussion

Nile tilapia fed vitamin C- and lipid-deficient diet had lower blood values as previously reported by Feldman et al. (2000). This author stated that erythrocyte synthesis requires adequate concentrations of many nutrients including lipids, proteins, carbohydrates, minerals, and vitamins. Abnormal erythrocyte synthesis can be caused by deficiencies in these nutrients that are critical for RBC production. They also emphasized that, at the beginning, pituitary, adrenal and thyroid regulate erythrocyte synthesis by (ACTH), growth hormone (GH), epinephrine, and norepinephrine which lead increased erythropoietin concentration stimulating RBCs production.

Decreased Htc in both nutrient absence and deficiency reported in channel catfish by Lim and Lovell (1978); rainbow trout, *Oncorhynchus mykiss*, Albrektsen et al. (1988) and hybrid tilapia, *Oreochromis niloticus* × *Oreochromis aureus*; Shiau and Jan (1992), was also seen in this study. Lim et al. (2000), however, reported that Htc was not affected by vitamin C levels.

Either vitamin C absence or deficiency can impair iron metabolism, affecting its distribution in the organism, which may lead to decreased RBC production (Albrektsen et al. 1988). According to Devlin (1997) vitamin C also facilitates the iron absorption by the intestine and its redistribution to different tissues, including Hb synthesis.

TABLE 3. *Mean vitamin C liver concentration, plasmatic cortisol, and glucose concentrations for Nile tilapia juveniles fed diets supplemented with different levels of vitamin C and lipids and submitted to temperature stress.*[1]

Diets	Vitamin C Phase I (μg/g)	Vitamin C Phase II (μg/g)	Cortisol Phase I (mg/dL)	Cortisol Phase II (mg/dL)	Glucose Phase I (mg/dL)	Glucose Phase II (mg/dL)
C_0L_0	120.85α (±13.60)	18.90β (±1.63)	30.65 (±3.19)	29.07 (±7.94)	33.19 (±4.27)	32.46 (±3.68)
$C_{125}L_6$	313.18α (±19.60)	20.93β (±4.49)	15.91 (±7.80)	10.27 (±6.75)	32.48 (±7.28)	33.41 (±4.37)
$C_{300}L_8$	280.19bα (±24.30)	27.77bβ (±1.18)	15.97 (±17.79)	11.56 (±1.99)	40.70 (±5.82)	69.59 (±38.15)
$C_{600}L_8$	480.39aα (±46.90)	33.65aβ (±4.87)	22.43α (±5.86)	9.62β (±3.26)	34.09 (±4.31)	65.51 (±37.20)
$C_{1200}L_8$	510.00aα (±32.90)	30.19aβ (±1.41)	34.20α (±8.83)	8.49β (±3.94)	35.94 (±8.47)	32.57 (±2.67)
$C_{300}L_{12}$	340.43bα (±63.70)	27.05bβ (±1.81)	20.91 (±20.26)	13.80 (±17.73)	33.85 (±6.50)	32.35 (±5.11)
$C_{600}L_{12}$	450.52aα (±70.18)	32.80aβ (±2.52)	30.06α (±6.48)	10.81β (±3.08)	37.19 (±4.48)	33.03 (±4.76)
$C_{1200}L_{12}$	490.47aα (±60.18)	34.55aβ (±4.53)	21.63α (±9.80)	6.75β (±6.09)	43.14 (±6.80)	43.89 (±16.06)
Cont. I × fat.[2]	0.05	0.05	ns	0.05	ns	ns
Cont. II × fat.[3]	0.05	0.05	ns	ns	ns	ns
Vitamin C effect						
(*P* level)	0.05	0.05	ns	ns	ns	ns
300	310.31	27.41	18.44	12.68	37.28	50.97
600	465.46	33.23	26.25	10.12	35.64	49.27
1200	500.24	32.37	27.92	7.62	39.54	38.23
Lipid effect						
(*P* level)	ns	ns	ns	ns	ns	ns
	423.53	30.54	24.20	9.89	36.91	55.89
	427.14	31.47	24.20	10.45	38.06	36.42
Vitamin C × lipid						
(*P* level)	ns	ns	ns	ns	ns	ns

ns = not significant.

[1] Different lowercase letters indicate differences between vitamin C levels; different uppercase letters indicate differences between lipid levels; and different Greek letters indicate differences between Phase I and Phase II.

[2] Control I (C_0L_0) × mean of factorial treatments.

[3] Control II ($C_{125}L_6$) × mean of factorial treatments.

[4] *P* level = statistic significance.

Although Hb concentration was not affected by vitamin C supplementation before or after temperature stress, Hb levels were lower (Table 2) in fish fed basal diet than those supplemented showing that they could be depleting their organic reserves, which could lead to anemia. The lack of significance in initial Hb values could be because of an insufficient feeding period (Phase I) and also sufficient diet iron and hepatic vitamin C concentration (Table 3). Soliman et al. (1994) reported that the absence of vitamin C in diets for hybrid tilapia led to low Htc, low liver vitamin C concentration, decreased growth, poor feed conversion ratio, and low protein efficiency.

After low-temperature stress fish fed vitamin C-deficient diet had difficulty maintaining erythrocyte synthesis, as in Phase I, revealed by an 18.7% decrease in erythrocytes and 11.7% decrease in Hb. Pickering (1981) and Wedemeyer et al. (1990) describe physiological changes during stress due to catecholamines and adrenocorticotropic hormones including energy reserve mobilization and an increase in blood pressure and oxygen concentration. Based on results in Table 2, it can be seen that vitamin C supplementation facilitates iron absorption, the most important Hb component, mitigating the effect of stress, and keeping the cellular membrane intact because of its antioxidant action. This allowed vitamin E to prevent the oxygen reactive species action on unsaturated fatty acids, thus avoiding reduced erythrocyte membrane resistance with possible hemolysis. Also, Waagbo et al. (1993) observed a significant increase in Hb concentration related to increased vitamin C supplement in Atlantic salmon, *Salmo salar.*

Analyzing blood parameters both before and after low-temperature stress (Table 2), vitamin C supplementation maintained them at appropriate levels after stress, although a significant decrease in Htc could have been caused by low Hb and erythrocytes. Hattingh and Van Pletzen

(1974) also observed decreased Htc in *Labeo umbratus* after 3 d of capture stress.

This may demonstrate the body's attempt to maintain blood cell synthesis, releasing immature cells with higher corpuscular volume, and consequently lower Hb concentration. Barros et al. (2002) observed that high vitamin C supplementation in diet resulted in higher Htc. However, this increase was also a result of higher corpuscular volume. Hrubec et al. (2000) determined normal values (115.0 and 183.0 fL) of MCV for hybrid tilapia. Although our results seem to be appropriate, the poststress results in vitamin C-deficient fish (MCV = 174.34 fL) were close to the highest value determined by this author.

Although absence of vitamin C supplementation had determined a significantly lower vitamin C concentration in the liver (Table 3), it could still be considered high as none was added by diet. As fish liver results from the vitamin C-deficient diet do not correspond to the diet analysis, it could not be explained. The importance of vitamin C supplement concentrations higher than necessary to avoid deficiency and to determine a good reserve was described by Durve and Lovell (1982). Similarity between the 600 and 1200 mg vitamin C results could be explained by possible liver saturation, demonstrating that 600 mg/kg vitamin C in diet is the cost-effective level for maintaining organic reserves and that 125 mg/kg vitamin C in diet and below was not sufficient to maintain liver concentrations after low-temperature stress. The minimum liver concentrations of vitamin C for rainbow trout (Hilton et al. 1978) and channel catfish (Lim and Lovell 1978) were 20.0 and 30.0 μg/g, respectively. Although there was no fish mortality after low-temperature stress, in intensive culture systems where homeostasis is continually threatened and vitamin C reserves were redistributed, fish may not resist cumulative stress as a result of low vitamin C reserve, thus leading to mortality. The possible decrease in tissue vitamin C concentration after long periods of stress and the possible impairment in immune responses were observed by Li et al. (1994, 1998).

Poststress decreased cortisol concentrations in fish fed vitamin C-supplemented diets (Table 3) could be explained by Kitabchi (1967) who proposed that high levels of ascorbic acid have an inhibitory role in steroid synthesis, by preventing the conversion of unsaturated fatty acid into cholesterol esters, which are incorporated into steroids. Consequently, vitamin C supplementation could mitigate the effect of stress. Similar results were obtained by Ortuno et al. (2002) with *Sparus aurata*. Studies with common carp, *Cyprinus carpio* (Dabrowska et al. 1991), channel catfish (Li et al. 1998), and Atlantic salmon (Davies et al. 1998) failed to detect the positive effects of vitamin C supplementation in plasmatic cortisol levels after stress.

Although this study showed no significant effect from lipids on cortisol and glucose levels (Table 3) before or after stress, Barton (1997) observed that increased fatty acids gave fish better resistance to stress because of high energetic mobilization. Barton et al. (1988) showed that *Oncorhynchus tshawytscha* fed a diet supplemented with high levels of energy had the highest glucose response to handling, suggesting better resistance. From this we can infer that in our study fish fed of diets supplemented with high lipid levels could be more prepared to cope with low-temperature stress because of their organic reserves. There was a similarity between glucose values before and after temperature stress. Schreck (2000) emphasized that fish were able to compensate and acclimatize to stress conditions as a result of behavioral and physiological changes. However, there is a metabolic cost linked to this compensation that can impair growth, reproduction, and resistance.

The physiological responses can not be considered as only resulting from low-temperature stress. There was additional adaptation as fish were transferred from one experimental facility to another. Although the situation describes a cumulative stress, the extended period could facilitate homeostasis with different stress hormone concentrations. Certainly, in these challenging situations, glycogen reserves may not be sufficient to maintain energy demand, which could require the use of organic reserves mainly because of cortisol action. Survival results suggest good physiological conditions attained by

supplemented vitamin C and lipid diet. The physiological results from fish fed a vitamin C- and lipid-deficient diet indicated that an extended period of stress would certainly result in mortality.

Conclusion

Results from this study show that the absence of vitamin C supplementation impairs RBC formation and does not enable fish to cope with stress, vitamin C supplement above species requirement is an efficient way to mitigate stress and 600 mg of vitamin C supplement is an economic and physiologically sufficient way to prepare fish to cope with low-temperature stress, and lipid supplementation does not impact stress biochemical parameters.

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