

Vitamin C combined with oregano essential oil, *Origanum vulgare*, reduced oxidative damage in *Astyanax aff. bimaculatus* exposed to air

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Abstract

The dietary impacts of water-soluble (vitamin C) and fat-soluble (oregano essential oil, OEO) antioxidants on the stress responses and redox status of *Astyanax aff. bimaculatus* exposed to the air were assessed. Five diets with vitamin C (VC 0, VC 40, VC 80, VC 120, and VC 160 mg kg⁻¹) with or without OEO inclusion (0 or 1,000 mg kg⁻¹) were evaluated. After 55 days of feeding, fish were exposed to air for 5 min. VC 120 and VC 160 reduced glycemia. In the gills, VC 120 and VC 160 reduced oxidative damage (malondialdehyde, MDA, and carbonylated proteins, PC) and superoxide dismutase (SOD) activity only in the presence of OEO. By contrast, in the absence of VC, OEO increased PC levels and SOD activity. In the liver, all VC concentrations, without OEO, reduced MDA, whereas, in the presence of OEO, vitamin C reduced MDA only at VC 40 e VC 160. All vitamin C concentrations, independently of OEO, reduced PC levels and SOD and catalase (CAT) activity. By contrast, OEO increased CAT and glutathione S-transferase (GST) activity regardless of VC level. Although VC 40 with 1,000 mg kg⁻¹ OEO was sufficient to reduce oxidative damage to lipids in the gills and liver, reduction in protein and lipid damage in the gills was only obtained with at least VC 120 + OEO. Therefore, we recommend simultaneous supplementation of these antioxidants, as their combined use is more efficient in reducing oxidative damage in *A. aff. bimaculatus* subjected to air.

1. Introduction

Farmed fish are often exposed to air during biometric measurement and grading (Pereira-da-Silva and Oliveira 2017), triggering stress responses, such as increased levels of cortisol, glucose, and plasma lactate (Lim and Hun 2018). Air exposure also causes oxidative stress resulting from a lack of oxygen supply to the gills and reoxygenation when fish are returned to the water and gill ventilation is reestablished (Lushchak et al. 2001). Oxidative stress is harmful to fish health in that it damages lipids, proteins, and nucleic acids (Lushchak and Bagnyukova 2007; Paital 2013) which can lead to lower feed intake, reduced growth, and increased disease susceptibility (Wilhelm Filho et al. 2005; Gao et al. 2013).

Oxidative stress occurs when the production of reactive oxygen species (ROS) and reactive nitrogen species overcomes the body's capacity for oxidant neutralization via enzymatic and non-enzymatic defenses (Machlin and Bendich 1987). Several enzymes participate in the defense against oxidative damage, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, glutathione reductase, and glutathione-S-transferase (GST). Non-enzymatic defenses involve the donation of electrons or neutralization of free radicals by antioxidants such as glutathione, lipoic acid, carotenoids, flavonoids, and vitamins C and E (Beutner et al. 2001; Mirończuk-Chodakowska et al. 2018).

Vitamin C, the main antioxidant in the aqueous compartments of cells, acts by neutralizing free radicals (Frei et al. 1989), increasing the activity of antioxidant enzymes (Chen et al. 2015), and regenerating fat-soluble antioxidants, such as vitamin E (Burton and Ingold 1986). Studies have shown that water-soluble antioxidants, such as vitamin C, and fat-soluble antioxidants, such as vitamin E, act in a complementary manner in different cell processes, producing additive effects on antioxidant capacity (Hamre et al. 1997). Therefore, it is likely that other fat-soluble antioxidants have an additive effect with vitamin C.

Some important examples of fat-soluble substances with antioxidant activity include flavonoids present in essential oils (Bozin et al. 2006), which have the ability to neutralize free radicals, enhance antioxidant enzyme

activity, and regenerate the reduced form of vitamin E (Cervato et al. 2000; Hirano et al. 2001; müş et al. 2017; Nazeri et al. 2017). The essential oil extracted from oregano is rich in flavonoids (Yan et al. 2016) and has high antioxidant capacity (Dorman et al. 2000; Yan et al. 2016). These characteristics suggest good potential for combined use with vitamin C against oxidative stress as oregano also has antimicrobial, anti-inflammatory, digestive, and stress-reducing properties (Bozin et al. 2006; Ocaña-Fuentes et al. 2010; Zhang et al. 2015; Ferreira et al. 2016). Therefore, the use of oregano in fish diets can reduce the adverse effects of oxidative stress and, consequently, minimize growth losses, improve nutrient use efficiency, and enhance the general health of farmed fish. However, there is no information in the literature on the combined use of vitamin C and oregano essential oil (OEO) in fish diets.

The cultivated freshwater species *Astyanax aff. bimaculatus* is an omnivorous fish with good acceptance of processed diets based on vegetable ingredients (Sussel et al. 2014). This species has a short life cycle and rapid growth, which is favorable for economic profitability, particularly in the production of fish snacks and live bait for sport fishing (Garutti 2003). Furthermore, *A. aff. bimaculatus* serves as an indicator of environmental quality (Santos et al. 2012) and is suitable for assessing stress responses and oxidative status under challenging conditions. In this study, we aimed to evaluate the interaction between vitamin C and oregano essential oil on the stress responses and redox status of *A. aff. bimaculatus* challenged by air exposure.

2. Materials And Methods

2.1. Fish, diets, and experimental conditions

This study was approved by the Ethics Commission on the Use of Farm Animals of the Federal University of Viçosa (CEUAP/UFV, protocol No. 46/2014). Experimental procedures comply with guidelines from the Brazilian National Council for the Control of Animal Experimentation (CONCEA, Brasília, Brazil) and current legislation.

A 5×2 factorial scheme was used in a completely randomized design, with five dietary levels of vitamin C (0, 40, 80, 120, and 160 mg kg^{-1}) combined or not with OEO (0 or $1,000 \text{ mg kg}^{-1}$) in three replicates per treatment. The basal diet was formulated to contain 360 g kg^{-1} crude protein and $3,000 \text{ kcal kg}^{-1}$ gross energy (Table 1). A vitamin and mineral supplement without vitamin C was used (Table 1). Vitamin C (Ascorsil Aqua®, Vansil, São Paulo, Brazil) and OEO (Laszlo®, Belo Horizonte, Brazil, Table 2) were added to the basal diet in replacement of inert (kaolin). Concentrations were chosen based on previous studies conducted by our research group on OEO supplementation (Ferreira et al. 2014; Carneiro et al. 2021) and investigations conducted by (Nasar et al. 2021) and (Yusuf et al. 2021) on vitamin C supplementation.

Feed ingredients were manually homogenized, and the mixture was pelleted, dried (30°C for 48 h), ground, and sieved. Gross energy, dry matter, mineral matter, crude protein, and ether extract contents were analyzed according to (AOAC 2006) and (Detmann et al. 2012).

Table 1
Ingredient and chemical composition of the basal diet.

Ingredient	Content (g kg ⁻¹)
Soybean meal	675.0
Corn meal	98.4
Wheat bran	120.0
Kaolin	1.5
L-Lysine	0.6
dl-Methionine	7.8
Soybean oil	50.0
Dicalcium phosphate	39.0
Common salt	2.5
Vitamin–mineral supplement without vitamin C ¹	5.0
Butylated hydroxytoluene (antioxidant)	0.2
Chemical composition	
Dry matter (%)	90.90
Gross energy (kcal kg ⁻¹)	2,959.43
Crude protein (% dry matter)	36.04
Ether extract (% dry matter)	7.08
Crude fiber ²	5.12
Mineral matter (% dry matter)	9.09
Total calcium ²	1.15
Available phosphorus ³	0.71

¹ Provided per kg of diet: folic acid, 50 mg; pantothenic acid, 1,000 mg; BHT, 20,000 mg; biotin, 15 mg; cobalt, 10 mg; copper, 1,600 mg; choline, 20,000 mg; iron, 10,000 mg; iodine, 60 mg; manganese, 1,900 mg; niacin, 3,000 mg; selenium, 18 mg; vitamin A, 4,000,000 IU; vitamin B1, 590 mg; vitamin B12, 1,200 mg; vitamin B2, 1,000 mg; vitamin B6, 590 mg; vitamin D3, 1,200,000 IU; vitamin E, 15,000 IU; vitamin K3, 625 mg; zinc, 5,000 mg.

² Values were calculated according to the chemical composition of ingredients reported by Rostagno et al. (2011).

³ Values were calculated for Nile tilapia according to Miranda et al. (2000).

Table 2
Chemical composition of oregano essential oil,
as provided by the manufacturer (Laszlo®).

Constituent	Content (g 100 g ⁻¹)
α-Thujene	0.4
α-Pinene	1.6
Camphene	0.7
Sabinene	0.8
Myrcene	1.4
α-Terpinene	1.1
<i>p</i> -Cymene	12.8
γ-Terpinene	8.4
1,8-Cineole	0.3
<i>cis</i> -Sabinene hydrate	1.6
Thymol	4.7
Carvacrol	63.0
β-Caryophyllene	1.4

Essential oil was subjected to high-performance gas chromatography using an HP 5890 gas chromatograph equipped with an SGE BP1 column (25 m × 0.25 mm). The following operating conditions were applied: 60°C (3 min), heating ramp of 3°C min⁻¹ up to 200°C, injector temperature of 250°C, 1:200 split ratio, FID detector at 250°C, and injection volume of 1 µL (0.5% in chloroform).

Juveniles of *A. aff. bimaculatus* (n = 54 per treatment, body weight = 0.25 ± 0.007 g) were kept in 30 aquariums (experimental units) containing 60 L of water at a stocking density of 0.58 fish L⁻¹, in a recirculation system, with aeration, and filters (mechanical, biological, and ultraviolet). We use both males and females due to the impossibility of distinguishing between sexes at this stage of the species' development. Water temperature was controlled by heaters and thermostats (27°C). Fish were fed to apparent satiation four times a day for 55 days. Water quality variables were monitored throughout the experimental period. Temperature and dissolved oxygen were measured by using a digital oximeter (Traceable® Products, Texas, USA), pH by using a digital pH meter (Kasvi®, Paraná, Brazil), and nitrite and total ammonia by using colorimetric kits (Alcon®, Santa Catarina, Brazil). Unionized ammonia (UA) was calculated according Emerson et al. (1975):

$$UA = \frac{totalammonia}{1 + 10^{(0.0902 - pH) + \left[\frac{2730}{273,2 + watertemperature} \right]}}$$

All variables remained within the recommended limits for cultivation of tropical freshwater fish (Popma and Masser 1999). Mean values (± standard deviation) were as follows: water temperature, 27.14 ± 0.09°C; dissolved

oxygen, $6.34 \pm 0.50 \text{ mg L}^{-1}$; pH, 7.05 ± 0.21 ; nitrite, 0.00 mg L^{-1} ; and unionized ammonia, $0.007 \pm 0.004 \text{ mg L}^{-1}$.

2.2. Air exposure challenge

After 55 days of feeding, fish (mean weight of $1.56 \pm 0.58 \text{ g}$) were subjected to an air exposure challenge, based on the method of Luz and Portella (2005). Briefly, 18 fish per experimental unit (54 fish per treatment) were individually exposed to air for 5 min and transferred to recovery aquariums with controlled aeration and temperature (27°C) for 30 min. After that, fish were euthanized with clove oil (400 mg L^{-1} water) for sample collection.

2.3. Redox status

2.3.1. Sample preparation

After the air exposure challenge, gill and liver samples were collected until reaching 200 mg of gill (2–3 fish/replicate) and 100 mg of the liver (5–7 fish/replicate) to set up 3 replicates per treatment. Liver and gill samples were homogenized separately in 0.2 mol L^{-1} phosphate buffer and 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), pH 7.4, using a tissue homogenizer (Omni International, Georgia, USA) and centrifuged at $9,60 \times g$ and 4°C for 10 min. The supernatant was used for analysis of total proteins, SOD, CAT, GST, and lipid peroxidation (measured as malondialdehyde, MDA). The pellet was used for determination of carbonylated proteins (PC).

Total protein concentration was determined according to the method proposed by Bradford (1976), using a microplate scanning spectrophotometer (Multiskan GO, Thermo Scientific) at 595 nm and bovine serum albumin as standard.

2.3.2. SOD activity

SOD activity was determined based on the ability of the enzyme to scavenge superoxide radicals (O_2^-) and thus decrease pyrogallol autoxidation (Dieterich et al. 2000). Pyrogallol autoxidation was measured using a microplate scanning spectrophotometer at 570 nm. SOD activity is expressed as units (U) per milligram of protein, where one unit of SOD is defined as the amount of enzyme needed to inhibit the rate of pyrogallol autoxidation by 50%.

2.3.3. CAT activity

CAT activity was measured by the method described by Aebi (1984), which uses 30% hydrogen peroxide (H_2O_2) as substrate. The decrease in H_2O_2 level was determined using a spectrophotometer (UV-Mini 1240, Shimadzu) at 240 nm for 60 s at 30 s intervals. One unit (U) of CAT is defined as the amount of enzyme that breaks down 1 mmol of H_2O_2 in 1 min. CAT activity is expressed as U mg^{-1} protein.

2.3.4. GST activity

GST activity was determined based on the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) (Habig et al. 1974). The molar extinction coefficient used for CDNB was $\epsilon_{340} = 9.6 \text{ mmol L}^{-1} \text{ cm}^{-1}$. One unit of GST was defined as the amount of enzyme required for the formation of 1 μmol of product per minute per

milliliter. GST activity was measured spectrophotometrically (UV-Mini 1240, SHIMADZU) at 340 nm for 90 s at 30 s intervals and is expressed as U mg^{-1} protein.

2.3.5. MDA determination

MDA, the main product of lipid peroxidation, was measured according Buege and Aust (1978). MDA concentration was determined using a microplate scanning spectrophotometer at 535 nm, with 1,1,3,3-tetramethoxypropane (TMPO) as standard. Results are expressed as $\mu\text{mol mg}^{-1}$ protein.

2.3.6. PC determination

PC content was determined based on the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) (Levine et al. 1994). PC content was measured using a microplate scanning spectrophotometer at 370nm. Results are expressed as nmol mg^{-1} protein.

2.4. Blood glucose analysis

Blood glucose analysis was performed using reagent strips and an Accu-Chek Active® digital monitor device (Roche, Brazil). For blood collection, a scalpel cut was performed next to the caudal fin peduncle in 3 fish per experimental unit (9 fish per treatment). A drop of blood was placed directly on the reagent strip.

2.5. Statistical analysis

Main and interaction effects of OEO and vitamin C were assessed using two-way analysis of variance (ANOVA). Residuals were tested by Shapiro–Wilk and Bartlett's tests to confirm the normality of distribution and homogeneity of variances, respectively. Then, significant differences among means were determined by Duncan's test. All analyses were performed using the ExpDes.pt package (Ferreira et al. 2021) of R® software version 4.2.1 (R Core Team 2022) at the 5% significance level. Three replicates per treatment were used. Each aquarium was considered as an experimental unit (the number of fish per experimental unit varied depending on the variable analyzed).

3. Results

3.1. Blood glucose

There was no significant interaction between vitamin C and OEO supplementation on blood glucose. Fish supplemented with 120 and 160 mg kg^{-1} vitamin C showed lower blood glucose levels after air exposure (Fig. 1). There was no significant effect of OEO on blood glucose (Fig. 1).

3.2. Gills redox status

There were significant interaction effects of vitamin C and OEO supplementation on MDA, PC, and SOD in gills after the air exposure challenge (Table 3). For these variables, vitamin C supplementation had an effect only when combined with OEO. Fish fed 40, 120, and 160 mg kg^{-1} vitamin C + 1,000 mg kg^{-1} OEO had lower MDA levels in gills compared with fish supplemented with OEO only (Table 3). Fish supplemented with 120 and 160 mg kg^{-1} vitamin C + 1,000 mg kg^{-1} OEO showed lower PC levels and SOD activity than fish treated with OEO only (Table 3). In the absence of vitamin C, OEO supplementation caused an increase in PC values and SOD

activity in gills (Table 3). Combined with 40 mg kg⁻¹ vitamin C, OEO supplementation reduced MDA values, whereas, when combined with 80 mg kg⁻¹ vitamin C, OEO supplementation increased MDA and SOD activity in gills (Table 3). For fish supplemented with 120 and 160 mg kg⁻¹ vitamin C, there were no significant differences in MDA, PC, and SOD between OEO supplementation (Table 3). For GST activity, there were no significant interaction effects between vitamin C and OEO (Table 3). Vitamin C supplementation reduced GST activity compared with non-supplementation of vitamin C, regardless of OEO (Table 3).

3.3. Liver redox status

Following the air exposure challenge, there was a significant interaction of vitamin C and OEO supplementation on liver MDA levels (Table 4). In the absence of OEO, the liver of fish supplemented with vitamin C showed lower MDA levels than that of not supplemented (Table 4). In OEO treatments, MDA levels decreased with 40 and 160 mg kg⁻¹ vitamin C supplementation (Table 4). OEO supplementation reduced MDA levels in the absence of vitamin C but increased MDA levels when combined with 80 mg kg⁻¹ vitamin C (Table 4). Liver PC, SOD, and CAT were significantly influenced only by vitamin C supplementation (Table 4). Fish fed vitamin C showed lower PC, SOD, and CAT than fish not supplemented with vitamin C, regardless of OEO (Table 4). OEO supplementation led to an increase in CAT and GST activities, regardless of vitamin C levels (Table 4).

Table 3

Biomarkers of redox status in the gills of *Astyanax* aff. *bimaculatus* fed diets supplemented with different levels of vitamin C and oregano essential oil.

	OEO (mg kg ⁻¹)	Vitamin C (mg kg ⁻¹)					Mean**	CV (%)	P-value		
		0	40	80	120	160			Vitamin C	OEO	Vitamin C*OEO
MDA	0	0.13 Aa	0.18 Aa	0.11 Ba	0.09 Aa	0.10 Aa	0.12	33.31	0.021	0.161	0.014
	1000	0.20 Aa	0.10 Bb	0.22 Aa	0.10 Ab	0.10 Ab	0.15				
	Mean*	0.17	0.14	0.17	0.09	0.10					
CP	0	1.06 Ba	1.25 Aa	1.32 Aa	1.39 Aa	1.82 Aa	1.37	37.94	0.647	0.243	0.041
	1000	2.41 Aa	1.43 Aab	1.96 Aab	1.27 Ab	1.02 Ab	1.62				
	Mean*	1.73	1.34	1.64	1.33	1.42					
SOD	0	0.48 Ba	0.71 Aa	0.47 Ba	0.52 Aa	0.57 Aa	0.55	27.05	0.158	0.327	0.028
	1000	0.80 Aa	0.60 Aab	0.77 Aa	0.43 Ab	0.41 Ab	0.60				
	Mean*	0.64	0.65	0.62	0.47	0.49					
CAT	0	0.06	0.08	0.06	0.06	0.08	0.07	32.62	0.499	0.247	0.322
	1000	0.11	0.08	0.07	0.07	0.06	0.08				
	Mean*	0.09	0.08	0.06	0.06	0.07					
GST	0	1.19	0.54	0.74	0.46	0.51	0.69	37.50	0.019	0.391	0.323
	1000	1.02	0.78	0.58	0.59	0.92	0.78				
	Mean*	1.11 a	0.66 b	0.66 b	0.52 b	0.72 b					

CV, coefficient of variation; OEO, oregano essential oil; MDA, malondialdehyde ($\mu\text{mol mg}^{-1}$ protein); CP, carbonylated proteins (nmol mg^{-1} protein); SOD, superoxide dismutase (U mg^{-1} protein); CAT, catalase (U mg^{-1} protein); GST, glutathione S-transferase (U mg^{-1} protein); *, overall

mean for each vitamin C level; **, overall mean for each OEO level. Means in a row followed by different lowercase letters (vitamin C effect) and means in a column followed by different uppercase letters (OEO effect) differ significantly at $p < 0.05$ by Duncan's test.

Table 4

Biomarkers redox status in the liver of *Astyanax aff. bimaculatus* fed diets supplemented with different levels of vitamin C and oregano essential oil.

	OEO (mg kg ⁻¹)	Vitamin C (mg kg ⁻¹)					Mean**	CV (%)	p-value		
		0	40	80	120	160			Vitamin C	OEO	Vitamin C × OEO
MDA	0	0.12 Aa	0.06 Ab	0.05 Bb	0.05 Ab	0.03 Ab	0.06	29.85	< 0.001	0.828	0.017
	1,000	0.08 Ba	0.05 Ab	0.09 Aa	0.06 Aab	0.04 Ab	0.06				
	Mean*	0.10	0.06	0.07	0.06	0.03					
CP	0	-	0.50	1.03	0.61	2.14	1.07	63.58	< 0.001	0.2816	0.877
	1,000	10.07	1.09	2.51	1.19	2.21	3.41				
	Mean*	10.07 a	0.79 b	1.77 b	0.90 b	2.17 b					
SOD	0	0.65	0.43	0.50	0.35	0.39	0.46	16.85	< 0.001	0.348	0.905
	1,000	0.69	0.44	0.51	0.43	0.39	0.49				
	Mean*	0.67 a	0.43 bc	0.51 b	0.39 c	0.39 c					
CAT	0	3.56	2.72	2.67	1.96	2.00	2.58 B	18.05	< 0.001	0.041	0.411
	1,000	4.35	2.52	3.47	2.34	2.22	2.98 A				
	Mean*	3.96 a	2.62 bc	3.07 b	2.15 c	2.11 c					
GST	0	1.75	1.39	2.07	1.94	1.31	1.69 B	18.76	0.112	0.002	0.092
	1,000	2.47	2.49	2.10	2.01	1.83	2.18 A				
	Mean*	2.11	1.94	2.08	1.98	1.57					

CV, coefficient of variation; OEO, oregano essential oil; MDA, malondialdehyde ($\mu\text{mol mg}^{-1}$ protein); CP, carbonylated proteins (nmol mg^{-1} protein); SOD, superoxide dismutase (U mg^{-1} protein); CAT, catalase (U mg^{-1} protein); GST, glutathione S-transferase (U mg^{-1} protein); *, overall mean for each vitamin C level; **, overall mean for each OEO level.

Means in a row followed by different lowercase letters (vitamin C effect) and means in a column followed by different uppercase letters (OEO

effect) differ significantly at $p < 0.05$ by Duncan's test.

4. Discussion

This was the first study to assess the association of a water-soluble (vitamin C) and a fat-soluble (OEO) antioxidant in the diet of fish subjected to stress (air exposure challenge). The results showed that antioxidants have complementary actions and, consequently, additive effects against oxidative damage in *A. aff. bimaculatus*.

Exposure of fish to air triggers stress responses and causes an imbalance in redox status (Lushchak et al. 2001; Lim and Hun 2018). As a primary response to stress, fish increase the secretion of the hormones cortisol and adrenaline, which, in turn, causes an increase in glucose, lactic acid (Lim and Hun 2018), and metabolic rates, leading to increased mitochondrial activity (Barton 2002). Given that mitochondrial activity is the main source of ROS generation, exposure to stressors also causes oxidative damage (Costantini et al. 2012).

Supplementation with 120 and 160 mg kg⁻¹ vitamin C promoted a reduction in stress responses in *A. aff. bimaculatus* and, therefore, represents an important tool to reduce the impacts caused by fish handling, such as capture, containment, grading, and transport, which inevitably exposes fish to air (El Basuini et al. 2021; Wu et al. 2022). Thus, vitamin C can reduce impacts on the redox status of fish, not only for its ability to neutralize ROS (Frei et al. 1989) but also for its capacity to minimize stress responses, preventing elevation of cortisol and glucose levels (De la Fuente et al. 1998; Peters et al. 2001; Ortuño et al. 2003) and, consequently, avoiding the increase in metabolic rate and mitochondrial activity. Wu et al. (2022) observed a reduction in the plasmatic levels of glucose, lactic acid, and cortisol, as well as an increase in the activity of the antioxidant enzymes CAT, SOD, and GPx in *Carassius gibelio*, while Chagas and Val (2006) observed a reduction in the glucose and MDA content in the plasma of *Colossoma macropomum* exposed to hypoxic stress.

In *A. aff. bimaculatus* gills, vitamin C reduced MDA and PC levels when combined with OEO, indicating an additive effect between water-soluble (vitamin C) and fat-soluble (OEO) antioxidants against oxidative damage. Vitamin C, being water-soluble, protects cell membranes, neutralizing reactive species present in the cytoplasm, thereby preventing peroxidation (Frei et al. 1989; Hamre et al. 1997). OEO, given its solubility in fat, complements the action of vitamin C, neutralizing reactive species during the chain reaction phase (Cervato et al. 2000), an important cycle affecting redox status (Yoshikawa and Naito 2002). The chain reaction stage begins when a hydrogen atom is removed from a phospholipid unsaturated fatty acid in the cell membrane, forming a lipid radical (L[•]), which is highly reactive. Lipid radical reacts with O₂ to form a peroxy radical (LOO[•]), which, when not eliminated by antioxidant defenses, reacts with other lipids in cell membranes, forming more L[•] and accumulating throughout chain reactions (Fridovich 1999; Valko et al. 2007).

Protein carbonylation is one of the main markers of ROS-induced oxidative stress in biomolecules (Dalle-Donne et al. 2006; Yao and Rahman 2011). It is an irreversible process (Fedorova et al. 2014) that modifies the tertiary structure of proteins and, consequently, compromises their properties, which may lead to cell apoptosis and death (Curtis et al. 2012). PCs are generated from direct oxidation by ROS (Fedorova et al. 2014), action of lipid peroxidation products (Esterbauer et al. 1991; Orioli et al. 2007; Niki 2009; Guo et al. 2011), and metal-catalyzed oxidation (Stadtman and Oliver 1991). Here, it was found that vitamin C at a minimum concentration of 120 mg kg⁻¹ + 1,000 mg kg⁻¹ OEO can protect cellular proteins, minimizing the need for protein turnover, thereby decreasing energy expenditure and increasing the availability of amino acids for growth.

The reduction of SOD activity with vitamin C supplementation at 120 and 160 mg kg⁻¹ in the presence of OEO indicates that antioxidants were effective in neutralizing ROS in fish gills and, therefore, there was no need to

activate the enzyme. SOD acts in the conversion of $O_2^{\cdot-}$ to H_2O_2 , which is then converted to water and molecular oxygen by CAT. A reduction in SOD activity is likely an indirect effect of ROS neutralization by vitamin C. Given that GST degrades products such as MDA, hydroperoxides, carbonyl-containing metabolites, peroxides, and epoxides (Hayes and Pulford 1995; Eaton and Bammler 1999; Huber et al. 2008), the reduction in GST activity in all vitamin C treatments is indicative of the indirect action of vitamin C in neutralizing ROS, thereby avoiding peroxidation of lipids and proteins.

Vitamin C showed similar effects in the liver and gills, being, however, also effective in the absence of OEO in the former organ. All vitamin C concentrations reduced protein damage (PC level) and SOD and CAT activities, indicating that even the lowest vitamin C concentration (40 mg kg^{-1}) was sufficient to neutralize ROS (Frei et al. 1989) without requiring an increase in enzyme action. In the liver, OEO showed more promising results than in the gills, as it increased antioxidant enzyme activity and reduced lipid oxidative damage. Magouz et al. (2022) observed that OEO attenuated heat stress in Nile tilapia supplemented with $1,000\text{ mg kg}^{-1}$ OEO, causing an important reduction in lipid oxidative damage and an increase in antioxidant enzyme activity. Similarly, Rashidian et al. (2021) found that OEO reduced MDA levels and increased SOD and CAT activities in *Danio rerio* challenged with *Aeromonas hydrophila*. Therefore, OEO has been shown to exert protective effects against different types of challenges in different fish species.

Although vitamin C at 40 mg kg^{-1} combined with $1,000\text{ mg kg}^{-1}$ OEO was sufficient to reduce lipid oxidative damage in the liver and gills, a reduction in protein damage in gills was only possible with a minimum vitamin C concentration of 120 mg kg^{-1} combined with $1,000\text{ mg kg}^{-1}$ OEO. Protection against oxidative damage and attenuation of stress responses provides fish with greater ability to withstand the effects of handling, thereby reducing impacts on growth and disease susceptibility.

Abbreviations

CAT, catalase; PC, carbonylated proteins; GST, glutathione *S*-transferase; MDA, malondialdehyde; OEO, oregano essential oil; ROS, reactive oxygen species; SOD, superoxide dismutase.

Declarations

Statements & Declarations

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Competing Interests

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Figures

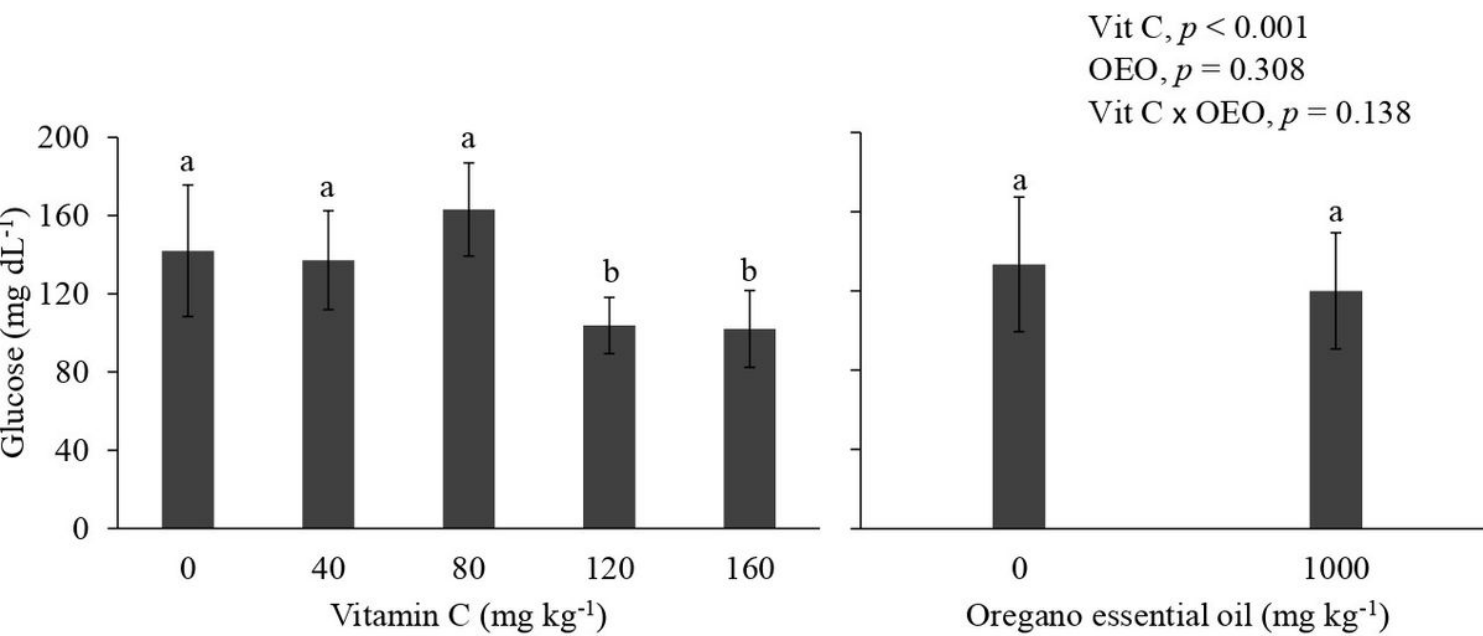


Figure 1

Mean blood glucose level of *Astyanax aff. bimaculatus* fed diets supplemented with different concentrations of vitamin C and oregano essential oil. Different letters indicate significant differences by Duncan's test ($p < 0.05$). Vit C, effect of vitamin C; OEO, effect of oregano essential oil; Vit C x OEO, interaction effect.