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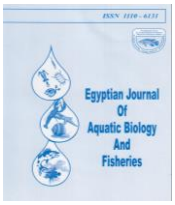

Effects of Ascorbic Acid Dietary Supplementation on the Red Tilapia (*Oreochromis* spp.); Broodstock Growth, Reproductive Efficiency, Immunity, and Gonadal Fatty Acids Profile

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ABSTRACT

Ascorbic acid (C) is needed for fish physiological functioning and growth. A feeding trial of 60 days was conducted to determine the optimal rational C inclusion level for the red tilapia juveniles based on growth, serum biochemical markers, antioxidative capacity, and ovarian fatty acid profile. Four diets were designed to have graded C concentrations of 0, 0.1, 0.2, and 0.3g/ kg diet (C0, C1, C2, & C3). The experimental period was followed by an additional 60 days to assess the spawning efficiency of the experimental fish groups. The results revealed that C did not improve the indicators of fish development performance or biochemical composition but did increase serum antioxidant activity. Furthermore, fish immunity was improved. The gonadosomatic index, hepatosomatic index, and relative fecundity were significantly increased with higher percentages of C, which also led to an increase in ovarian EPA and DHA contents. An increase in spawning efficiency was associated with increasing the C supplementation level based on the spawns' number for each female, the mean eggs number per spawn, and decreasing inter-spawn intervals. The statistical analysis revealed that the ideal C level in the diet was 0.3g/ kg for improving fish immunity, reducing oxidative stress, optimizing reproductive parameters for supporting the production of good egg and larval quality for the red tilapia broodstock profitability.

INTRODUCTION

In aquatic animals, vitamins are pivotal nutriment that their deficiency can lead to a variety of morphological and functional abnormalities **(Liang *et al.*, 2017)**. Ascorbic acid (C) is a key component in fish feed formulations that promotes fish development and maintains physiological equilibrium. Most aquatic species lack gluconolactone oxidase enzymes, hence according to **El Basuini *et al.* (2021)**, they cannot transform D-glucose to synthesis C. As a result, dietary inclusion is crucial to deliver the nutrients required for an optimal fish performance **(Ai *et al.*, 2004)**.

Haematological and plasma biochemical processes, as well as protein metabolism, are enhanced by vitamin C supplementation in fish diets **(Tewary & Patra, 2008)**. Additionally, according to **Zafar and Khan (2020)**, vitamin C facilitates iron absorption in the stomach by transforming iron salts into ferrous formula, which boosts hemoglobin role. Besides, C has a part in the construction of blood vessels, collagen, bone matrix, and connective tissue. Moreover, it is a co-factor enzyme in the modification of matrix proteins **(Harsij *et al.*, 2020)**. Furthermore, as acknowledged by **Yadav *et al.* (2015)**, C is an essential antioxidant mediator that scavenges reactive oxygen species (ROS) for instance superoxide, hydrogen peroxide, and hydroxyl radicals while maintaining the balance between antioxidants and ROS.

Extra ROS can cause oxidative stress, which can lead to oxidative impairment to proteins, lipids, and DNA **(Chowdhury & Saikia, 2020)**. Negative symptoms including reduced collagen production, anaemia, and growth retardation were brought on by a vitamin C deficiency **(Zehra & Khan, 2012)**. In addition, as a water-soluble vitamin, vitamin C is eliminated by the body through urine instead of being stored. As a result, **Tewary and Patra (2008)** mentioned that it is imperative to consistently supplement with vitamin C since excluding it from fish diets results in having negative consequences such as slower growth, exophthalmia, bleeding either internally or externally, deformation of the spinal column, fin erosion, and dark skin pigmentation.

Depending on their age, size, species, and circumstances during growing, fish have varying needs for vitamin C **(Chen *et al*., 2015; Xu *et al*., 2016)**. The recommended nutritive intakes of C varies from 10 to 10,000mg/ kg **(Webb & Villamor, 2007)**. The maximal growth rate of the Nile tilapia (*Oreochromis niloticus*), the Pacific bluefin tuna (*Thunnus orientalis*), and the kuruma prawns (*Marsupenaeus japonicas*) required 53–186mg/ kg of dietary C **(Schleicher *et al*., 2009)**. According to the previous researches, the ideal C level for the large yellow croakers (*Pseudosciaena crocea*) is 28.2mg/ kg, the grass carp (*Ctenopharyngodon idella*) ranges from 92.8 upto 129.8mg/ kg, the cobia (*Rachycentron canadum*) is 13.6mg/ kg; the yellow drum (*Nibea albiflora*) is 142.2mg/ kg, and the wild bream (*Megalobrama amblycephala*) is 700mg/ kg **(Ai *et al*., 2006; Ming *et al*., 2012; Zhou *et al*., 2012; Xu *et al*., 2016; Wang *et al*., 2017)**, respectively. Studying the C nutritional requirement for every aquaculture species is required to identify its optimal C requirement **(Baroi *et al*., 2019; Zou *et al*., 2020)**.

This work is a comprehensive study that investigated the effects of supplementing diets of the red tilapia (*Oreochromis* sp.) with ascending levels of C on fish spawning efficiency, growth, health, serum biomarkers, immunity, oxidation status, and the female reproductive performance. Furthermore, this was the first study to look into how C dietary supplementation affects the ovarian fatty acids composition.

MATERIALS AND METHODS

1. Experimental procedure and preparing diets

The red tilapia were purchased from the 21-km hatchery and delivered to the aquaculture the marine hatchery (National Institute of Oceanography and Fisheries, Alexandria). The fish were given a commercial food for a week after acclimating to the experimental setup. Moreover, they were initially housed in 12 tanks, each with a capacity of 100 litres, and had an initial body weight of approximately 5g. Each tank had 25 fish in it at the establishment of the trial. The tanks were simultaneously supplied with filtered brackish water (20ppt, 26.4 ± 1.2^{o}C), and the third water renewal was set per day. Throughout the trial period, water quality elements were verified using the **APHA (2017)** typical technique. The feeding experiment was conducted with a natural photoperiod of 11 hours of light and 13 hours of dark.

Four diets were created to meet the fish's nutritive needs, and the test meals were gradually administered three times per day at a 4% daily ratio at 08:00, 13:00, and 17:00. The testing diets were developed in accordance with **Saleh *et al*. (2018)**. Pellets were stored at -20°C for future use. The experimental diets were designed as follows (Table 1): a vitamin C-free complemented diet (control, C0) and three diets with increasing doses of C (ROVIMIX® STAY-C® 35): 0.1 (C1), 0.2 (C2), and 0.3g/ kg diet (C3). The four diets were evaluated in triplicate. The C- content in the formulated diets was evaluated by the high-performance liquid chromatography (HPLC).

2. Growth and feed utility

Weight gain (WG, g) and specific growth rate (SGR, % day^{-1}) were the metrics used to evaluate fish growth. Feed conversion ratio (FCR) and protein efficiency ratio (PER) were considered to express feed exploitation. The equations applied were as follows:

WG = fish weight at the end (W_E) - fish weight at the start (W_s), SGR = [(ln W_E) - (ln W_S)] / feeding trial days × 100, FCR = utilized feed /WG, PER = WG /protein consumed.

3. Proximate analyses

Fish feeding was discontinued 24 hours prior sampling, upon the completion of the experiment. After anesthesia with clove oil (20mg/ L), the fish count and weight were registered per tank. Ten fish were collected at random and pooled from each tank then kept at -20°C for the analysis of biochemical configuration. Moisture, lipid, protein, and ash contents were analyzed using the **AOAC (2012)** technique. Feed and fish were analyzed on triplicate basis (Tables 1, 2). Throughout the feeding test, fish in every single tank were weighed and scored biweekly to assess their growth and survival changing feeds accordingly. Upon the completion of the experiment, fish were retrieved out of every tank, briefly anaesthetized with 20ppm clove oil for three minutes, and their final weights were noted.

Table 1. Construction and proximate analysis (% dry weight) of the experimental diets fed to the red tilapia (*Oreochromis* sp.) for 60 days

g/kg	C0	C1	C2	C3
Fish meal[1] (FM)	130	130	130	130
Soybean meal[2] (SBM)	350	350	350	350
Yellow corn	200	200	200	200
Wheat bran	100	100	100	100
Wheat flour	150	149.9	149.8	149.7
Sunflower oil	50	50	50	50
Vitamin premix-vitamin C free[3]	10	10	10	10
Mineral premix[4]	10	10	10	10
Vitamin C[5]	0	0.1	0.2	0.3
Proximate composition (%)				
Dry matter (DM)	90.40	90.00	90.40	90.40
Crude protein (CP)	28.99	29.02	29.00	29.73
Total lipids (L)	8.88	8.58	8.98	8.07
Ash	5.27	5.36	5.80	5.57
Fiber	3.25	3.66	3.89	3.80
Nitrogen free extract[6] (NFE)	53.91	53.38	52.33	53.13
Gross energy[7] (GE, MJ/ Kg)	19.84	19.63	19.60	19.55
Vitamin C mg/ kg	350.40	403.45	468.41	510.30

[1]FM laboratory prepared (65% CP)

[2] SBM (44% CP) was provided by Research Institute of Oil Crops, Agricultural Research Center Cairo

[3, 4] Purshased from AGRI-VET, Egypt

[5] Vitamin C: ascorbate-2-poly-phosphate, ROVIMIX® STAY-C® 35

[6] NFE = 100 − (crude protein + crude lipid + ash + Fiber).

[7] GE, considered based on 23.64, 39.54 and 17.57 MJ/Kg for protein, lipids and carbohydrates, in turn.

Table 2. Growth performance, feed utilization efficiency of the red tilapia (*Oreochromis* sp.) fed the experimental diets for 60 days (mean ± SE)

	C0	C1	C2	C3
IW	5.57±0.07	5.07±0.41	5.19±0.26	4.95±0.51
FW	25.25±0.55	22.10±0.41	22.79±1.62	23.05±0.88
WG	19.68±0.49	17.03±0.37	17.60±1.54	18.11±0.88
SGR	1.78±0.35	1.73±0.42	1.74±0.40	1.81±0.46
FI	26.51±0.35	24.60±0.83	26.64±0.40	23.64±0.06
FCR	1.35±0.03	1.44±0.05	1.54±0.14	1.31±0.07
PER	2.46±0.36	2.43±0.17	2.33±0.37	2.47±0.19

Means in the same row with dissimilar letters are significantly different ($P < 0.05$).

IW: Initial weight (g), FW: Final weight (g), WG: Weight gain (g), SGR: Specific growth rate (%/day), FI: Feed intake (g), FCR: Food conversion ratio, PER: Protein efficiency ratio.

4. Haemato-immunological assays

Upon the completion of the experiment, five fish (15 fish per treatment) were removed from each tank to determine the blood composition. Using heparinized syringes, blood was drawn from the fish caudal vein, gathered into microtubes, and combined for haematological examination. Fish haematological profiles were measured using the accepted methods of Dacie and Lewis **(Bain *et al*., 2016)**, and blood smears stained with Wright-Giemsa were used to do the differential leucocyte count.

Additional blood specimens were obtained, left to clot at 4°C, centrifuged for 10 minutes at 3500g, and the sera were stored at -20° C, in preparation for use in the serum biomarkers and immunological tests.

4.1. Indicators of serum biochemistry

Total protein (TP) was evaluated using the Biuret reaction **(Doumas *et al*., 1981)**. Additionally, serum albumin (Alb) was quantified as per the method of **Reinhold (1953)** using Chemroy and Pasteur Lab kits. Serum globulin (Glb) was calculated by deducting the serum albumin from total serum protein **(Kumar *et al*., 2005)**.

The **Pan and Wang (1997)** approach was used to measure lipase activity. BioMerieux-France test kits were utilized to quantify aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) per the method of **Reitman and Frankel (1957)**.

4.2. Antioxidant enzymes assay

As directed by the manufacturer and using the procedures outlined by **McCord and Fridovich (1969)** and **Aebi (1984)**, the diagnostic kits used to assess tcatalase (CAT) and superoxide dismutase (SOD) activities were purchased from MyBioSource Inc. (San Diego, California, USA). Moreover, the peroxidative destruction of lipids was assessed using the **Utley *et al*. (1967)** method, by means of a few adjustments suggested by **Fatima *et al*. (2000)**, in order to determine the lipid peroxidase (LPO).

4.3. Assessment of immunological parameters

Kawahara *et al*. (1991) method was accepted to calculate the phagocytic activity and index. The proportion of phagocytic cells that contain yeast cells is referred to as phagocytic activity (PA). The phagocytic index (PI) is defined as the quantity of phagocytized yeast cells / number of phagocytic cells. The nitro blue tetrazolium (NBT) test was conducted to evaluate respiratory burst activity **(Anderson *et al*., 1992)**.

Serum lysozyme activity (LYS) was assessed using the **Ellis (1990)** method after lysis of *Micrococcus lysodeikticus*. As per **Rainger and Rowley (1993)**, serum bactericidal activity (SBA) counter against the *A. hydrophila* strain was determined. Turbidimetric immunoassay was used to measure the amounts of serum reactive protein (CRP) **(Igor & Stanislav, 2000)**. Conferring the protocol of **Lim *et al*. (2009)**, the level

of immunoglobulin M (IgM) was assessed by an ELISA assay utilizing a commercialized kit (Cusabio, Wuhan, Hubei, China).

5. Ovarian growth and fecundity

By the end of the experiment, 4% buffered formalin was used to store the ovaries of the sampled fish to determine fecundity. Sub samples of mature ovaries were weighed and the mature oocytes with big diameter counted three times in each sample.

The Relative fecundity/g = Number of whole mature eggs /g total weight of the fish. Gonado-somatic index (GSI) = *g*onad weight/gutted weight ×100 and the hepato-somatic index (HSI) = liver weight/gutted weight ×100

6. Female spawning performance

After sampling, fully mature fish from each dietary group were transferred to separate tanks with sex ratio 1 male: 2 female; each fish group had 4 females and 2 males in triplicate, the experiment was directed for 60 days to study the spawning performance, and fish were fed the same diet as previous.

Every other day, the females in each tank had their eggs checked. Brooding females were caught and the eggs were carefully taken out from their mouths. Dates of spawning were noted along with the number of eggs that were collected. The following were found to be the spawning factors:

1. Average number of spawnings/female = Total number of spawnings/number of females.

2. Inter-spawn intervals (ISI) by days = Time between one spawn to the next of repeat spawning in the same tank

3. Average number of eggs / spawn = Total number of eggs per tank/number of spawnings.

7. Composition of fatty acids in ovaries

Fish ovary fatty acid content was ascertained in two stages: lipid esterification and lipid extraction from the ovarian tissues using **Folch (1957)** and **Kenari and Naderi (2016)** methods, correspondingly. Gas chromatography (GC-17A Shimadzu) was used to determine the fatty acid content of the samples.

8. Statistical analysis

Results were presented as mean ± standard error. The statistical significance of the data was determined using the one-way analysis of variance (ANOVA) with statistical package for social sciences (SPSS)® (Ver. 17.0 for Windows). The data were analyzed post hoc using the Duncan test to compare means. *P*-values < 0.05 specified statistical significance.

RESULTS

1. Growth performance and fish biochemical composition

Table (2) shows the indices values of growth performance and feed use. Results indicated insignificant variations among all dietary tested groups (P>0.05). Furthermore, analyses of the investigational fish groups biochemical composition indicated also insignificant variations (P> 0.05) (Table 3).

Table 3. Carcass biochemical composition of the red tilapia (*Oreochromis* sp.) fed experimental diets for 60 days (mean± SE)

Biochemical parameter (%)	C0	C1	C2	C3
Moisture	72.47±2.23	72.79±2.44	72.57±3.11	72.32±3.49
Protein	16.03±0.19	15.48±0.04	16.57±0.42	15.89±0.82
Lipid	5.52±0.20	6.10±0.08	6.43±0.24	7.43±0.23
Ash	4.39±013	4.63±0.49	4.13±0.34	4.71±0.01

Means in the same row with dissimilar letters are significantly different (P < 0.05).

2. Hematological parameters

The complete blood picture for the experimental fish groups is summarized in Table (4). Results revealed a significant elevation in counts of the red blood cell (RBC), white blood cell (WBC), haemoglobin (Hb), and values of packed cell volume (PCV) in fish fed C2 and C3 diets, relating to other fish groups.

Furthermore, the serum biomarkers concentration is given in Table (5). The concentrations of TP and Alp indicated the superiority of supplementing C at both 0.2 and 0.3g/ Kg diet supplementation levels. Lipase level was gradually increased simultaneously with increasing the C level in the fish diet. In contrast, ALT and AST levels were gradually decreased with increasing C level in diets. All serum biochemical indices, measured in the present study, indicated an enhancement in the fish general health.

3. Oxidation enzymes activity

Fish fed C0 diet had the lowest SOD and CAT concentrations, which increased when the C level increased in the diets used for experiments (Table 5). In contrast, LPO level was gradually decreased with increasing C supplementation level.

4. Immune response

LYS, PA, PI, NBT and Igm values showed a gradual significant increase with increasing C level in fish rations, and fish fed C0 diet recorded the lowest values (Table 5) that matched with other diets (P< 0.05). Opposing, CRP values were significantly and gradually diminished with increasing the C level in fish diets.

Table 4. The complete blood count analysis of the red tilapia (*Oreochromis* sp.) fed the experimental diets for 60 days (mean± SE)

Parameter	C0	C1	C2	C3
WBCs ((×10^4/UL)	21.80±0.78^d	25.25±0.50^c	35.82±1.62^a	29.76±0.88^b
RBCs (10^6/mm^3)	0.98±0.45^c	1.62±0.52^b	2.17±0.03^a	2.11±0.07^a
Hb (g/100ml)	10.01±0.29^c	10.84±0.24^b	13.02±0.07^a	13.08±0.18^a
PCV (%)	21.92±0.4^b	23.46±0.46^b	27.74±0.67^a	27.80±1.02^a
Leucocytes differentiation (10^3/µl)				
Neutrophil	15.67± 0.33^a	14.67±0.33^a	12.33±0.32^b	15.00±1.00^a
Lymphocytes	56.67± 0.33^b	66.0±0.58^a	68.0±0.58^a	63.33±0.67^a
Eosinophils	12.33±0.67^a	9.33±0.33^b	9.33±0.88^b	9.00±0.58^b
Basophils	13.00±0.58^a	10.00±0.01^b	9.00±0.58^b	9.33±0.33^b
Monocytes	2.33±0 .33^{ab}	3.00±0.00^a	1.33±0.33^b	3.33±0.67^a

Means in the same row with dissimilar letters are significantly different (P< 0.05).

Table 5. Serum biochemical biomarkers and Immunological indicators analysis of the red tilapia (*Oreochromis* sp.) fed on experimental diets for 60 days (mean± SE)

Biochemical parameter	C0	C1	C2	C3
TP (g/L)	3.49±0.17^c	5.94±0.39^b	9.87±0.37^a	10.07±0.19^a
Alb (g/L)	2.09±1.36^c	3.23±0.08^b	3.37±0.11^b	3.90±0.13^a
Glb (g/L)	140.±0.15^c	3.08±0.16^b	6.17±0.23^a	6.16±0.14^a
Lipase (U/mg protein)	11.90±0.13^d	17.38±0.30^c	27.09±0.96^b	33.53±0.54^a
AST (IU/L)	68.90±1.35^c	48.32±0.58^b	36.86±0.81^a	37.77±1.06^a
ALT (IU/L)	27.37±0.66^d	21.17±0.27^c	17.42±0.29^b	15.79±0.58^a
Non-specific immunity parameter				
PA	19.41±0.49^d	28.33±0.57^c	40.22±0.8^b	42.09±0.24^a
PI	8.50± 0.47^d	13.83±0.64^c	19.96±0.61^b	21.86±0.04^a
NBT	2.77±0.12^c	8.32±0.58^b	15.56±0.56^a	15.76±0.40^a
LYS (IU/ml)	0.50±0.03^d	2.83±0.07^c	6.09±0.30^b	6.79±0.11^a
IgM (U/mL)	2.36±0.23^c	7.01±0.34^b	8.87±0.21^a	8.94±0.24^a
SBA	2.48±0.11^c	6.75±0.30^b	10.06±0.32^a	9.75±0.24^a
SRP	49.32±1.19^a	37.60±0.62^b	24.97±0.35^c	24.13±0.39^c
Oxidative stress bio-marker				
CAT (U/mg protein)	2.38±0.09^c	5.32±0.23^b	8.73±0.65^a	8.81±0.30^a
SOD (U/mg protein)	0.28±0.004^d	0.49±0.02^c	0.82±0.03^b	0.91±0.01^a
LPO (µM MDA/mg protein)	16.09±0.17^a	10.88±0.24^b	8.20±0.34^c	8.72±0.32^c

Means in the same row with dissimilar letters are significantly different (P< 0.05).

5. Gonadal growth and fecundity

Gonado-somatic index

The female GSI% results displayed significant elevation ($P< 0.05$) in C3 fish group (7.46± 0.44) relative to the control and C1 fish groups, while it was insignificant with C2 fish group ($P< 0.05$) (Table 6).

Hepato-somatic index

The HSI % results exhibited a significant elevation ($P< 0.05$) in the fish group fed on C3 diet (3.92± 0.14) relative to the control and C1 fish groups, while it was insignificant with C2 fish group (Table 6).

Relative fecundity

Relative fecundity of the red tilapia females fed on C3 diet was significantly elevated (18.55± 1.9 egg/g of fish weight) compared to the control group and that fed on C1 diet (9.05± 0.4 and 11.97± 0.9 egg/g of fish weight), respectively ($P< 0.05$). Moreover, fecundity of fish group fed on C2 diet (14.76± 0.9 egg/g of fish weight) showed a significant increase relative to C0 group ($P< 0.05$). The fecundity was augmented with the increase of an added percentage of vitamin C, respectively (Table 6).

Spawning performance

The number of spawning per female reported a significant increase ($P< 0.05$) for the two groups fed C2 and C3 diets to be 3.5± 0.10 and 3.7± 0.20 spawning times, respectively, during 60 days and with the maximum mean number of eggs per each spawn (468.6± 47.3 and 472.0± 23.8 egg/ spawn, respectively) (Table 7). While days elapsed between spawning times, inter-spawn intervals (ISI) was significantly ($P< 0.05$) less than those of the control for the three treatment groups in a dose- dependent mode (Table 7).

Table 6. Effects of vitamin C on the gonadosomatic index (GSI%), hepatosomatic index (HSI%) and fecundity of the red tilapia for 60 days (n=6) (mean± SE)

Somatic index	C0	C1	C2	C3
GSI%	5.61 ± 0.45^{b}	5.95 ± 0.39^{b}	6.62 ± 0.36^{ab}	7.46 ± 0.44^{a}
HSI%	3.40 ± 0.13^{b}	3.41 ± 0.09^{b}	3.78 ± 0.18^{ab}	3.92 ± 0.14^{a}
Fecundity Egg/g	9.05 ± 0.4^{c}	11.97 ± 0.9^{bc}	14.76 ± 0.9^{ab}	18.55 ± 1.9^{a}

Means in the same row with dissimilar letters are significantly different ($P< 0.05$).

Table 7. Effects of vitamin C on the spawning performance of the red tilapia broodstock, total spawning number per each female, inter-spawn intervals (ISI), and total egg number/spawn for 60 days (n=6) (mean± SE)

	C0	C1	C2	C3
Total spawning /female	2.3±0.19^{c}	2.7±0.11^{b}	3.5±0.10^{a}	3.7±0.20^{a}
ISI (days)	26.07±2.17^{a}	21.66±0.83^{b}	16.90±0.54^{c}	15.90±0.45^{c}
Egg/spawn	331.6±20.5^{b}	461.3±14.6^{ab}	468.6±47.3^{a}	472.0±23.8^{a}

Means in the same row with dissimilar letters are significantly different ($P< 0.05$).

6. Ovarian fatty acids composition

Table (8) shows the ovarian fatty acid profile in all the experimental fish groups. Palmitic acid (PA, 16:0) and oleic acid (OA, 18:1) were the major saturated (SFA) and mono-unsaturated (MUFA) fatty acids, respectively. OA acid and linoleic acid (LA, 18:2) proportion showed negative correlation with the dietary C- concentration in fish diets up to 0.2g/ kg diet supplementation level, and then started to increase again. The contents of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), n-3 PUFA, arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) in the ovary were positively affected by the dietary C- level ($P< 0.05$). In contrast, adding C to fish diets dramatically reduced SFA and MUFA levels. Fish given C1 diet had the highest ArA, EPA, DHA, n-3 / n-6, and n-3 PUFA levels. However, they had the lowest levels of OA ($P< 0.05$).

Table 8. Ovaries fatty acid profile of the female red tilapia (*Oreochromis* sp.) fed on experimental diets for 60 days (mean± SE). (% of total fatty acids, n=3) ($P< 0.05$)

Fatty acid	C0	C1	C2	C3
C14:0	2.22 ± 0.02^{b}	3.57 ± 0.15^{a}	2.42 ± 0.03^{b}	2.27 ± 0.03^{b}
C15:0	0.23 ± 0.02^{c}	0.39 ± 0.04^{b}	0.47 ± 0.02^{a}	0.15 ± 0.02^{d}
C16:0	21.43 ± 0.52^{a}	18.70 ± 0.73^{b}	18.85 ± 0.12^{b}	21.12 ± 0.04^{a}
C17:0	0.44 ± 0.02^{b}	0.50 ± 0.10^{b}	0.74 ± 0.03^{a}	0.53 ± 0.03^{b}
C18:0	13.97 ± 0.71^{a}	13.20 ± 0.30^{a}	12.43 ± 0.04^{ab}	11.60 ± 0.06^{b}
C19:0	0.13 ± 0.02^{b}	0.28 ± 0.10^{a}	0.26 ± 0.03^{a}	0.14 ± 0.02^{b}
C20:0	0.39 ± 0.02^{ab}	0.47 ± 0.06^{b}	0.56 ± 0.02^{a}	0.33 ± 0.01^{c}
C22:0	0.06 ± 0.01^{c}	0.14 ± 0.02^{b}	0.18 ± 0.01^{a}	0.13 ± 0.02^{b}
Total SFA	38.86 ± 1.20^{a}	37.24 ± 0.50^{ab}	35.90 ± 0.13^{b}	36.28 ± 0.06^{ab}
C16:1	4.00 ± 0.04^{c}	5.24 ± 0.06^{a}	4.58 ± 0.04^{b}	4.68 ± 0.03^{b}
C18:1	27.58 ± 0.41^{a}	16.54 ± 0.25^{d}	20.92 ± 0.52^{c}	22.88 ± 0.04^{b}
C20:1	1.56 ± 0.04^{b}	2.13 ± 0.16^{a}	1.56 ± 0.04^{b}	1.28 ± 0.02^{c}
C22:1	0.28 ± 0.02^{c}	0.46 ± 0.04^{b}	0.57 ± 0.06^{a}	0.15 ± 0.01^{d}
Total MUFA	33.42 ± 0.34^{a}	24.37 ± 0.13^{d}	27.63 ± 0.49^{c}	28.99 ± 0.03^{b}
C18:2	20.84 ± 0.37^{a}	17.81 ± 0.50^{c}	19.13 ± 0.75^{b}	21.06 ± 0.11^{a}

C18:3	0.34 ± 0.03^{d}	0.89 ± 0.02^{b}	0.95 ± 0.04^{a}	0.72 ± 0.03^{c}
C20:2	1.10 ± 0.04^{c}	1.63 ± 0.11^{b}	2.06 ± 0.13^{a}	1.42 ± 0.03^{b}
C20:3	0.66 ± 0.04^{d}	2.12 ± 0.06^{a}	1.47 ± 0.02^{b}	1.16 ± 0.01^{c}
C20:4	0.55 ± 0.04^{d}	2.51 ± 0.15^{a}	1.58 ± 0.03^{b}	1.14 ± 0.01^{c}
C20:5	0.24 ± 0.02^{a}	0.26 ± 0.03^{a}	0.25 ± 0.02^{a}	0.14 ± 0.01^{b}
C22:5	0.15 ± 0.01^{d}	1.69 ± 0.10^{a}	1.19 ± 0.05^{b}	0.90 ± 0.03^{c}
C22:6	0.66 ± 0.04^{c}	5.19 ± 0.10^{a}	5.57 ± 0.03^{a}	4.91 ± 0.05^{b}
Total n-3 PUFA	2.66 ± 0.05^{d}	8.20 ± 0.18^{a}	7.99 ± 0.02^{b}	6.80 ± 0.02^{c}
Total n-6 PUFA	22.88 ± 0.43^{b}	24.28 ± 0.80^{a}	24.19 ± 0.57^{a}	24.65 ± 0.12^{a}
n-3 / n-6	0.07 ± 0.00^{c}	0.34 ± 0.01^{a}	0.33 ± 0.01^{a}	0.28 ± 0.00^{b}

Means in the same row with dissimilar letters are significantly different ($P< 0.05$).

SFAs: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids.

DISCUSSION AND CONCLUSION

Vitamins are one of the most significant elements utilized in fish diet formulation, and they are vital for all aquatic animals. Vitamin insufficiency induces numerous functional and morphological problems in various species **(Liang *et al*., 2017)**. The lack of C- supplementation resulted in a slow growth, low survival rate, or aberrant pigmentation as distinguished in the cobia **(Zhou *et al*., 2012)** and the wild bream (**Wan *et al*., 2014)**. Numerous studies have suggested that dietary C can improve survival rates, growth, immunological function, and stress resistance **(Combs & McClung, 2016; Liang *et al*., 2017; Dawood *et al*., 2018; Ibrahim *et al*., 2020)**.

The current findings revealed no significant improvement in fish growth, feed utilization efficiency, or fish biochemical composition in fish fed rations containing C compared to the C0 group. In the rainbow trout, *Oncorhynchus mykiss*, growth did not vary significantly when fed diets containing graduated C- levels **(Dabrowski *et al*., 1996)**. Although dietary C- supplementation did not improve fish growth in the gibel carp (*Carassius gibelio*), it did have a substantial effect on antioxidant ability and gonad development in the on-growing fish **(Shao *et al*., 2018)**. In contrast, numerous fish species' growth performance was positively influenced. According to **Sandnes *et al*. (1992)**, C- supplementation greatly increased the SGR of the Atlantic salmon (*Salmo salar*). This disagreement demonstrated that the effects of C on fish can be altered by fish species, size, developmental stage, variation in experimental circumstances, and cultivation environment **(NRC, 2011)**.

Hematology biochemical indices are essential bio-indicators to evaluate the status of health and nutrition of living organisms **(Arnold, 2022)**. In this work, although the red tilapia growth performance was not significantly improved, however, referring to the blood picture (Table 4), the RBC, WBC, Hb and PCV values and WBC differentiation count revealed an enhancement in fish health when fed either C2 or C3 diets. The same

observations were registered by **Aboseif *et al*. (2022)**, where they recorded a significant increment in WBC, RBC, Hb, PCV, lymphocyte, platelets count and IgM concentrations when the common carp, *Cyprinus carpio*, was fed diet containing 0.2g C/ Kg diet relative to the control diet. This enhancement in fish health may be interpreted by that C has a fundamental role in all the fish physiological activities **(Zou *et al*., 2020)**.

Serum biochemical indices analysis (Table 5) showed a pronounced elevation in TP value in C2 and C3 fish groups and also an increment in lipase serum level in the same groups.

Furthermore, it has already been revealed that the increased TP, Alb and Glb levels imply a key immunocompetent capacity **(Abdel-Latif *et al*., 2022; Li *et al*., 2022)**.

ALT and AST are transaminases combined in protein metabolism **(Li *et al*., 2022)**. In aquatic animals, elevated AST and ALT enzyme activities indicate dysfunction, necrosis, and tissue deterioration **(Khalil *et al*., 2022, 2023)**. The current findings show a significant decrease in both ALT and AST activities in fish fed C as a food addition, indicating enhanced liver function and overall fish welfare compared to the control group.

C is a vital vitamin with numerous applications, the chief of which is to increase immunological response **(Dawood & Koshio, 2018)**. In the present work, results revealed that LYS activity was boosted by dietary C, in addition to NBT, PA, and SBA relative to those fed on C0 diet. In contrast, values of CRP significantly decreased. The decrease in SRP with increasing C inclusion level demonstrated C's usefulness as a modulatory agent during rearing conditions and in reducing fish stress. These findings indicate C's involvement in strengthening the red tilapia immune defense, which have previously been acknowledged in other species **(Verlhac *et al*., 1998; Liang *et al*., 2017; Aboseif *et al*., 2022)**. Furthermore, C improves aquatic species' immune systems and minimizes the stress negative effects **(Sarma *et al*., 2009; Khalil *et al*., 2023)**. Briefly, certain micronutrients' antioxidant properties may boost immunity by keeping immune cells functioning and structurally intact **(Innocent *et al*., 2011)**.

SOD and CAT are powerful antioxidant enzymatic defense systems against free radicals that can protect organisms from oxidative stress damage **(Winston *et al*., 1991; Santos-Sanchez *et al*., 2019)**. The current study found that CAT and SOD activities in fish given C-supplied diets were augmented in response to dietary C- supplementation levels. In contrast, LPO concentrations declined as dietary C- supplementation levels increased. These findings imply that dietary C can increase the antioxidant capacity of the experimental fish, indicating that C has powerful antioxidant characteristics **(Arrigoni & De Tullio, 2002)**. As a result, C can be regarded as a powerful antioxidant and strong free radical scavenger, defending against free radicals produced by normal cellular function and other stress forms **(Chew, 1995)**. Previous study has demonstrated that dietary C improves the antioxidant capacity of numerous fish species, such as large mouth bass, (*Micropterus salmoides*) **(Chen *et al*., 2015)**, and the Nile tilapia **(Ibrahim *et al*., 2020)**.

The current results showed that diet improved with 300mg/ kg ascorbic acid induced a significant elevation in female GSI% and HIS % compared to the control group and lower vitamin C- treatments. The elevation in GSI value can be attributed to the development of oocytes, as the process of vitellogenesis leads to an increase in both the number and size of yolk granules, resulting in an increase in the oocyte volume. Additionally, metabolic products are directed toward the progress of the gonads **(Darwisito *et al*., 2008)**. The increase in GSI% and HSI% values in response to vitamin C- treatment was reported in different species as in the perch broodstock, *Anabas testudineus* **(Fitriliyani *et al*., 2022)**.

The elevation of both GSI% and HSI% is induced by increasing the need for egg yolk required for oocyte growth **(Barbieri *et al*., 2000)**. The red tilapia is a species that undergoes simultaneous spawning with brief intervals of vitellogenesis. The gonadal growth in those species is directly influenced by nutrients **(Izquierdo *et al*., 2001)**. Furthermore, the increment in GSI% and HSI% might be attributed to a higher buildup of energy, particularly during the vitellogenic phase-mobilization, which is used for embryos' growth **(Babin *et al*., 2007)**. Inadequate or insufficient diets containing C have an impact on the weight of fish gonads, potentially caused by the absence of ascorbate, which enhances the quality of eggs and the rate of hatching **(Furuita *et al*., 2009)**.

Current results displayed that the highest total egg production for the female red tilapia was recorded in 200 and 300mg C/kg supplemented groups, also minimum days that elapsed between successive spawning times and the highest relative fecundity were detected in C2 and C3 groups. In agreement with the obtained results, the case of the Nile tilapia broodstock fed diet enhanced with 400mg C/kg; this indicated the highest total seed productions, absolute fecundity and relative fecundity with the highest system productivity **(Suloma *et al*., 2017)**, furthermore the reproductive performance of the female Nile tilapia is negatively affected when they are fed diets lacking or containing low amounts of C. Conversely, C at higher levels in their diet are directly associated with increased hatching rates and greater production of eggs and larvae per female **(Sarmento *et al*., 2018)**. Research has demonstrated that adding C as a supplement has beneficial impacts on the performance of reproduction of different fish species. For example, studies have shown positive effects in the rainbow trout **(Waagbo *et al*., 1989)** and the milkfish (*Chanos chanos*) **(Emata *et al*., 2000)**. In the rainbow trout, C is a key player in vitellogenesis and semen quality **(Ciereszko & Dabrowaki, 2000)**. According to **Gammanpila *et al*. (2007)**, C- supplementation improved the hatching rate and survival rate of the Nile tilapia larvae, but did not have an effect on egg production. C need varies across different fish species. In the case of the Cod, *Cadus morhua*, a dose of 500mg/ kg of C did not result in any improvement in fertilization rate, egg production, or offspring survival, as reported by **Mangor-Jesen *et al*. (1994)**.

The correlation between C- requirements and various aspects of fish development, such as gonadal maturation and larval metamorphosis, reproduction completion, gamete

quality, and fertility, has been suggested **(Dabrowaki & Ciereszko, 2001)**. Additionally, ascorbic acid is involved in steroid synthesis and vitellogenesis in fish **(Hernandez de-Dios *et al*., 2022)**. The ascorbic content present in brooders is conveyed to the eggs, where it undergoes sorting to provide nourishment for the larvae until their initial feed intake **(Soloman *et al*., 1986)**. Nutrients transfer to gametes is critical for ensuring the proper development of the embryos **(Navarro *et al*., 2010)**.

It was uncovered that the content of gonadal ascorbic acid increased during gonadal maturation and incorporated in successful breeding as in the carp **(Seymour, 1981)**. Vitamin C is an effective antioxidant that is proficient in counterbalancing reactive oxygen species and regenerating anti-oxidantive molecules. It is well-known for its conserving effects against free radicals, which helps prevent instability of lipid membranes **(Lee & Dabrowski, 2004)**. C is believed to affect the ovaries of the tilapia by carrying out metabolic and antioxidant functions during the development of eggs and expansion of ovarian follicles. This eventually leads to ovulation via the enzyme glutathione peroxidase **(Pasa, 2010)**. In addition, a deficiency of ascorbic acid might cause damage during mitotic division in germ cells **(Cabrita *et al*., 2014)**.

In this research, the ovarian fatty acid profile (Table 8) differed between treatments with varying doses of C- supplementation. C0 fish group showed the highest OA and LA, and their concentration was diminished by adding C to fish diet and then started to increase in C3 group. Matching result was verified by **Han *et al*. (2019)** in the juvenile grass carp (*Ctenopharyngodon idella*) liver. Fish fed 0.1g C/kg diet had the peak levels of n-3 and n-6 PUFAs, approving that keeping high PUFA levels is linked to the existence of C, which is supplementary to feeds to boost the nutritional value, but also to struggle and neutralize the free radicals before they oxidize the fat in the cells' membrane **(Pita *et al*., 2004)**. **Gonçalves *et al*. (2010)** mentioned that dietary C enhances the PUFAs protection in the fish tissues, inducing an improvement in the product quality.

Moreira *et al*. (2001) observed comparable results in three Brazilian freshwater fish species, as did **Rahman *et al*. (1995)** and **Justi *et al*. (2003)** in the Nile tilapia. According to **Mourente *et al*. (2005)**, C may play a role in the stimulation of desaturase and elongase enzymes, which take a part in the transition of LA into ArA. Furthermore, the C0 fish group had the highest concentrations of LOP and SFAs, demonstrating that an optimal C- supplementation is a limiting factor in lipid oxidation. The current finding suggests that fatty acid unsaturation is a major component in determining sensitivity to the recorded LPO activities, which is consistent with **Rajas *et al*. (1993)** findings.

To conclude, adding vitamin C in the red tilapia diet at 0.2-0.3g/ Kg improves the fish health, serum biomarkers, immunity, oxidation status, and the female reproductive performance. Furthermore, the ArA, EPA, and DHA concentrations in the mature fish ovaries are increased relative to the control fish group. Additionally, supplementation of diet with C supports the feed proficiency toward gonadal growth and egg quality enhancement.

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