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Potential role of L-ascorbic acid with Field cricket (*Gryllus bimaculatus*) meal in diets of Nile Tilapia (*Oreochromis niloticus*) during sex reversal and nursing

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Abstract

A synthetic androgen 17-α methyl testosterone (MT) commonly used in the production of mono-sex fry of Nile tilapia (*Oreochromis niloticus*) during the first 21 days for sex reversal has been considered to suppress immunity thereby reduce survival. Present trial was conducted to evaluate whether vitamin C (L-ascorbic acid, AA) supplementation would benefit in terms of survival, growth, and stress resistance. Nile tilapia fry were fed with isonitrogenous (57.8±0.2% CP) diets formulated using Field cricket (*Gryllus bimaculatus*) meal (approx. 79%) and fish meal (approx. 20%) to use as control, and five other diets were prepared by supplementing 10, 20, 30 40 and 50 g of vitamin C (L-ascorbic acid per kg diet). Eighteen aquaria or glass tanks (100 L) were used having three replicates per treatment. Each aquarium was stocked with 300 fish (0.01±0.00 g). Fry were nursed for another 91 days to check their sex-ratio. Gonad histology showed increased number of spermatogonia when L-ascorbic dose was 30g/kg diet. At the end of the feeding trial results indicated significant increase ($P<0.05$) in growth, feed utilization and survival when fed with vitamin C at the dose of 10 g/kg diet during sex-reversal and nursing periods as compared to the control. Similarly, hematological information also showed 10 g vitamin C dose per kg diet. Polynomial regression showed that the optimum dietary ascorbic acid doses were calculated at 15.9, 10.0 and 12.0 g AA per kg diet for highest survival (83.5% max), weight gain and SGR, respectively, but the doses higher than 20 g of L-ascorbic acid/kg diet was not beneficial. Highest apparent digestibility (AD%) of protein (84.1%) was at 15.37g and AD% of lipid (91.4% max) was 17.8g of vitamin C/kg diet. Salinity challenge test also showed highest survival can be achieved at 15.8 g AA per kg diet. As the survival of fry is the most important parameter at these stages, the dose which resulted highest survival i.e., 15 g/kg diet is recommended.

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Introduction

All-male tilapia farming rapidly expanded worldwide as profitable business due to its high growth, hardiness, and human nutritional benefits which contributes food and nutrition security globally (Bhujel, 2014). Fishmeal (FM) is used as the main protein source and 60 mg 17-α methyl-testosterone steroid (MT) hormone used per kg feed for sex reversal to feed for 21 days (Little et al., 2003; Bhujel, 2009, 2014), and 10 mg MT per kg feed (Ron et al., 1995). Due to sustainability issue of fishmeal, its use in aquaculture has been questioned and various attempts have been made to reduce or replace it completely. Fishmeal is used as sole ingredient of the standard feed for hormonal sex-reversal in tilapia. Therefore, nowadays, insect meals are getting attraction amongst researchers and feed producers due to their potential as fishmeal replacement (FAO, 2013).

Field cricket meal (FCM) is a good source of protein, amino acid, lipids (Wang et al., 2005), minerals (Na, K, P and Zn) and vitamins (B1, B12, E, K) (Khan, 2018). An earlier report has showed that Field cricket successfully replaced soya bean in broiler diet (Wang et al., 2005) and suitable source for alternative protein resources for fish diet. FCM could replace up to 100 % of fishmeal in *Clarias gariepinus* (Taufek et al., 2016a; 2017). Field cricket can be found in abundance in the tropics and locally available in Thailand (Van Huis et al., 2013). They can be easily cultured, and mass harvested in controlled environments with the benefit of low cost of production. However, there is no good alternative found so far to replace fishmeal partially or fully for Nile tilapia swim-up fry during sex reversal. Some literature has indicated the immunity problem might be due to the side effect of MT steroid hormone causing histopathological changes on gill, liver, kidneys, and intestine (Abo-Al-Ela, 2018; Suseno et al., 2020). Due to poor immunity, fish may get negative effects during overcrowding, periodic handling, sudden rise and fall in temperature, poor water quality and poor nutritional status leading to stress or immunosuppressant causing increased susceptibility to diseases (Bhujel, 2014). However, both fish meal and FCM are deficient in vitamin C. In an earlier report it has been suggested that males showed lower lysozyme activity than females (Saha et al., 2010); therefore, attention has been given on sex reversal tilapia feed. Initial feeding of early life stages is more sensitive to vitamin C deficiency (Dabrowski et al., 1996) as they grow faster than adults (Kolkovski et al., 2000).

Vitamin C or L-Ascorbic acid is water soluble health-promoting agent in aquaculture (Eo and Lee, 2008). However, fish cannot synthesize vitamin C (ascorbic acid, AA) in their body due to lack of L-gulonolactone oxidase enzyme (Eo and Lee, 2008). Therefore, an exogenous source of vitamin C is very essential in fish diets (Wang et al. 2003). Early larvae stage feeding with vitamin C reduces fish stress and antioxidation status (Jiménez-Fernández et al., 2015). An earlier report has showed that vitamin C regulates the immune-toxic effect such as higher phagocytic activity, phagocytic index and lysozyme activity of MT in tilapia fry and that help to increase fish survival (Abo-Al-Ela et al., 2017). In addition, vitamin C helps to minimize toxicity by water contaminants (Eo and Lee, 2008), survival and disease resistant (Suwanmanee et al., 2012). Shahkar et al. (2015) reported that changes of testes colour and proliferation of spermatogonia while increasing ascorbic acid dose in brood stock Japanese eel. However, there was no publication on tilapia fry testes. Inadequate supply of ascorbic acid causes poor growth, poor immunity, higher mortality, poor muscle quality, and impaired collagen formation (Shahkar et al., 2015; Wei et al., 2020). On the other hand, higher dose of vitamin C showed spinal deformities for channel catfish (Naggar and Lovell, 1991) and limited reported on higher dose effects on fish. Therefore, Vitamin C is essential nutrient for all the cultured fish species including tilapia (Toyama et al., 2000; Nsonga et al., 2010; Saha et al., 2010; Suwanmanee et al., 2012; Martins et al., 2016; Vieira et al., 2018). Quantitative requirements of vitamin C depends on the fish species, age, size, stages of development, and culture conditions (Nsonga et al., 2010).

Publications are limited regarding the benefits of vitamin C for SRT and nursing fry (Toyama et al., 2000; Suwanmanee et al., 2012). Although, there were no reports on vitamin C dose used for supplementation above 5g AA/kg diet used for sex reversal treatment and the effect on testes, 10 g/kg was recommended based on the existing practice. Fry often die at high rate during sex reversal putting challenges in terms of survival due to compromised immunity caused by steroid hormone i.e., MT. These indicated that some research on vitamin C has been done for standard feeding protocol; however, research is limited when inclusion of FCM as the main source of protein instead of commonly used fishmeal. Present study uses the FCM replacing almost 80% of the fish meal as per our earlier outcome of trial. However, whether it had any impact on immunity was not known. Therefore, present experiment was conducted to assess the effects of vitamin C which is considered to play a role on the survival, growth, feed utilization and immunity / stress resistance of Nile tilapia fry.

Materials and Methods

Experimental fish and system

An experiment was conducted at the Aquaculture laboratory facility of the Asian Institute of Technology (AIT) (14.1° N and 100.6° E), Thailand using the standard protocol of hormonal treatment of 21 days followed by nursing for 91 days i.e., a total of 112 days during March-June 2020. Free swimming fry of Nile tilapia (*Oreochromis niloticus*) were collected from the Department of Fisheries (DOF), Pathum Thani province in central Thailand. Initial fry were sampled for proximate analysis. Swim-up fry (0.01 ±0.00 g, mean ± SE,) were transferred randomly distributed to 18 glass tanks (300 fish/tank, 6 treatment with three replicates) with 100 L volume of dechlorinated freshwater. During the experiment, compressed air diffused was supplied in each glass tank through air-stones to ensure dissolved oxygen (DO) remains adequately high. During hormonal treatment, the fry were fed with test-diets five times per day for 21 days. They were further nursed for 91 days feeding with the diets which contained the same ratio of FM and FCM but with the addition of rice bran and other ingredients. At the beginning, the individual weight and length of each fish was recorded, then the fish from each replicate tank were weighed in batches. Three fish were sampled: one per replicate, for each treatment. They were pooled and stored in a freezer (-20°C) for proximate analysis. Fish mortality and feed intakes were recorded daily during the experimental period. Settle fish waste and uneaten feed were siphoned daily and water volume was kept same level for each tank. The proximate composition (AOAC, 2003) and amino acid profile (In-house method WI-TMC-06) of fishmeal (FM) and FCM are shown in **Table 1**.

Table 1 Proximate composition and amino acid profile of Fishmeal and FCM compared to dietary amino acid requirements of *Oreochromis niloticus.*

Components	*Fishmeal*	*FCM*	*Dietary requirements*[2]
Dry matter (%)	89.2[a] ± 0.0	91.6[b] ± 0.1	-
Protein (%)	56.0[a] ± 0.2	56.6[a] ± 0.2	40-45
Lipid (%)	10.0[a] ± 0.3	23.4[b] ± 0.5	10-15
Fiber (%)	1.1[a] ± 0.1	7.0[b] ± 0.1	-
Ash (%)	22.5[a] ± 0.2	11.6[b] ± 0.2	-
GE[1] (kcal/kg)	4533[a] ± 19	5475[b] ± 33	-
Amino acid profile			
Arginine	3.96	4.29	2.82
Histidine	5.98	4.51	1.05
Isoleucine	2.61	2.37	2.01
Leucine	3.96	3.55	3.40
Lysine	5.45	3.57	3.78
Methionine	1.94	1.45	0.99
Phenylalanine	3.64	4.17	2.50
Threonine	4.19	3.47	2.93
Tryptophan	3.33	2.86	0.43
Tyrosine	1.86	2.48	1.80
Alanine	4.07	3.26	
Aspartic acid	5.22	4.14	
Cystine	1.33	1.52	
Glutamic acid	7.09	5.28	
Glycine	4.13	2.92	
Proline	2.04	1.58	
Serine	2.58	2.64	
Valine	2.54	2.63	

All values are Mean ± SE, calculate from three replicates. SE=Standard error.
[1]Gross energy (GE) was calculated according to NRC (1993) as 5.65, 9.45, and 4.11 kcal/g for protein, lipid, and carbohydrate, respectively.
[2]Dietary requirements of *Oreochromis niloticus*; Essential amino acid (Santiago and Lovell, 1988); Protein (El-Sayed and Teshima, 1992); Fat (Ng and Chong, 2004)

Experimental diet and design

Adult live Field crickets were collected from Talat Thai market located in Klong Luang, Pathum Thani approx. 42 km north of Bangkok where these Field crickets are sold as human food. They were dried in an oven at 60°C for 2 days. The dried Field cricket was then ground to make powder with the help of a grinder and stored in a cold condition (-20°C). The experiment was set up accordingly to a completely randomized design with six treatment of L-ascorbic acid, AA (99%, Sigma-Aldrich, Germany). Supplementation was prepared to contain 0, 10, 20, 30, 40 and 50 g AA kg^{-1} diet (dry matter basis, DM) with three replications. The composition of the main protein sources and experimental feed was confirmed by following the standard procedure for fish feed analysis (AOAC, 2003). Prepared feed samples were kept inside sealed bags and stored at -20°C until used. The feed was formulated to contain iso-protein feed of average 57.8±0.2% protein and 4,999±10 kcal kg^{-1} gross energy diets with include 60 mg MT hormone (97% (HPLC), Sigma-Aldrich, Germany) per kg feed and conducted for 21 days, fed five times per day. All experimental diets (T1 to T6) which contained the same ratio of fish meal (FM) and FCM. Formulation and chemical composition of all the experimental SRT diets are shown in **Table 2**.

Table 2 Formulation and chemical composition of the SRT diets (21 days)

Increasing L-ascorbic acid amount (g/kg)						
Ingredients	*0 (T1)*	*10 (T2)*	*20 (T3)*	*30 (T4)*	*40 (T5)*	*50 (T6)*
FCM (g)	78.96	78.96	78.96	78.96	78.96	78.96
FM (g)	19.74	19.74	19.74	19.74	19.74	19.74
Vit C (g/kg)	0	10.0	20.0	30.0	40.0	50.0
Vit mix (g) [2]	0.3	0.3	0.3	0.3	0.3	0.3
Mineral (g) [1]	1.0	1.0	1.0	1.0	1.0	1.0
Alcohol (mL)	12.0	12.0	12.0	12.0	12.0	12.0
MT (mL)	12.0	12.0	12.0	12.0	12.0	12.0
Total	100.0	100.0	100.0	100.0	100.0	100.0
Nutrient level determined by as is basis (% dry matter basis)						
DM (%)	90.6±0.0^{a}	90.5±0.0^{a}	90.0±0.0^{bc}	90.1±0.0^{b}	89.9 ±0.0^{c}	89.9±0.1^{bc}
Moist. (%)	9.4±0.0^{a}	9.5±0.1^{a}	10.0±0.0^{bc}	9.9±0.0^{b}	10.1 ±0.0^{c}	10.1±0.1^{bc}
CP (%)	57.8±0.1^{a}	57.7±0.2^{a}	57.8 ±0.8^{a}	57.6 ±0.1^{a}	57.8 ±1.0^{a}	57.8±0.3^{a}
CL (%)	16.3 ±0.1^{a}	16.4 ±0.0^{a}	16.4 ±0.5^{a}	16.3±0.4^{a}	16.4±0.1^{a}	16.3 ±0.3^{a}
CF (%)	5.5±0.1^{a}	5.5±0.2^{a}	5.4 ±0.1^{a}	5.4 ±0.0^{a}	5.4 ±0.1^{a}	5.2±0.0^{a}
NFE [3]	3.9±0.2^{a}	4.1±0.8^{a}	4.5±2.3^{a}	5.3±1.4	5.0±0.2	5.2±1.0^{a}
CA (%)	16.5 ±0.1^{c}	16.2±0.2^{bc}	15.9±0.1^{ab}	15.6±0.2^{a}	15.3±0.1^{a}	15.5 ±0.1^{a}
GE[4] (kJ /kg)	4963±5^{a}	4978±6^{a}	4997.1±44^{a}	5015±37^{a}	5024±7^{a}	5017±11^{a}

DM=Dry matters; SRT = Sex reversal treatment; Moist. =Moisture; CP=Crude protein; CL=Crude lipid; CF=Crude fiber; CA=Crude ash.

[1] Mineral premix (g/kg of diet): calcium biphosphate, 20 g; sodium chloride, 2.6 g; potassium chloride, 5 g; magnesium sulphate, 2 g; ferrous sulphate, 0.9 g; zinc sulphate, 0.06 g; cupric sulphate, 0.02 g; manganese sulphate, 0.03 g; sodium selenite, 0.02 g; cobalt chloride, 0.05 g; potassium iodide, 0.004 g.

[2]Vitamin mixture (IU or mg/kg of diet): vitamin A, 500,000 IU; vitamin D3, 100,000 IU; vitamin E, 10,000 IU; vitamin K,800 mg; vitamin B1,250 mg; vitamin B2, 1200 mg; vitamin B6, 750 mg; vitamin B12, 5 mg; vitamin B5, 3000 mg; vitamin B3, 2150 mg; biotin, 25 mg; folic acid, 300 mg; inositol, 25,000 mg; Selenium, 30 mg; Iron, 20,000 mg; Zinc, 32,000 mg; Copper, 2000 mg. Values for each experiment group in the same row followed by different superscripts are significantly ($P<0.05$) different.

[3]Nitrogen free extract (NFE) = 100 – (crude protein % + crude lipid% +crude fiber %+ total ash %).

[4]Gross energy (GE) was calculated according to NRC (1993) as 5.65, 9.45, and 4.11 kcal/g for protein, lipid, and carbohydrate, respectively.

After 21 days of hormone feeding, fish were nursed for another 91 days fed with diets containing 40.3±0.1% protein and 5,122±16 kJ/kg gross energy formulated adding mainly rice bran with the same ratio of fish meal (FM) and FCM. Chromic oxide was also included to assess the digestibility. The diets were prepared by mixing dry ingredients and proximate composition of each diet are given in **Table 3**. Experimental diets were prepared in the lab using simple mincer machine and dried at 50°C for 48 hours in an electric oven. Final pellets were kept in sealed bags and stored at -20°C until they were used.

Table 3 Formulation and chemical composition of the nursery diets

	Increasing vitamin C amount (g/kg)					
Ingredients	*0 (T1)*	*10 (T2)*	*20 (T3)*	*30 (T4)*	*40 (T5)*	*50 (T6)*
FCM (g)	30.6	30.6	30.6	30.6	30.6	30.6
FM (g)	7.6	7.6	7.6	7.6	7.6	7.6
Rice bran (g)	60.0	60.0	60.0	60.0	60.0	60.0
Vit C (g/kg)	0	10.0	20.0	30.0	40.0	50.0
Vit. mix (g)	0.3	0.3	0.3	0.3	0.3	0.3
Mineral (g)	1.0	1.0	1.0	1.0	1.0	1.0
Cr_2O_3(g)	0.5	0.5	0.5	0.5	0.5	0.5
Total	100.0	100.0	100.0	100.0	100.0	100.0
	Nutrient level determined by as is basis (% dry matter basis)					
Moist. (%)	94.8±0.0^{c}	93.9±0.0^{d}	93.7±0.0^{e}	96.3±0.1^{a}	95.0±0.0^{c}	95.3±0.0^{b}
DM (%)	5.2±0.0^{c}	6.1±0.0^{b}	6.3±0.0^{a}	3.6±0.1^{e}	5.0±0.0^{c}	4.7±0.0^{d}
CP (%)	40.3±0.4^{ab}	40.7±0.1^{b}	40.9±0.2^{b}	39.7±0.2^{a}	40.0±0.1^{ab}	40.1±0.1^{ab}
CL (%)	19.2 ±0.1^{b}	18.3±0.1^{b}	18.4±0.3^{b}	18.9±0.3^{b}	16.8±0.3^{a}	16.5 ±0.3^{a}
C (%)	6.2±0.1^{ab}	6.0±0.1^{a}	6.1±0.0^{ab}	6.0±0.1^{ab}	6.2±0.0^{ab}	6.2±0.0^{b}
NFE	24.2±0.4^{a}	24.4±0.3^{ab}	24.1±0.5^{a}	26.0±0.2^{bc}	27.2±0.3^{c}	27.6±0.2^{c}
CA (%)	9.1±0.5^{a}	9.5±0.1^{a}	9.3±0.4^{a}	8.6±0.1^{a}	8.9±0.1^{a}	8.8±0.1^{a}
GE (kJ/kg)	5184±22^{b}	5138 ±8^{b}	5161±29^{b}	5164±13^{b}	5048±14^{a}	5034±17^{a}

Sex determination

Nursing period was continued another 91 days until they reached 10±2 g of fish which were dissected and isolated male (testes) and female (ovary) gonads (male: testes and Female: ovary). A small piece of gonad was placed on a slide and poured a drop of indigo-carmine stain (Guerrero and Shelton, 1974) and allowed to develop the color. Then observed under simple microscope (Huma Scope Advanced LED 40X power with 0.5X CCD adapter)

Histological analysis

From each treatment three fish were sampled, dissected, their testes were isolated, immediately fixed in Bouin's solution for 48 hours and then transferred to 70% alcohol solution. Afterwards, the samples were dehydrated in a series of alcohol solutions, placed in xylene and then embedded in paraffin. Tissue blocks were sectioned and stained with hematoxylin and eosin. Tissue sections were observed under CX 31 Olympus (Japan) microscope.

Fish growth and feed utilization

At the end of each phase of both the experiments, fish were counted and weighted in each tank to calculate fish survival rate, final weight (FW), weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), feed conversion efficiency (FCE), and protein efficiency ratio (PER). All the values were average of the triplicates for each treatment.

Proximate analysis of diets and fish

The feed ingredients formulated diets and whole fish body samples from each treatment at the initial and final days were analyzed for proximate composition (moisture, crude protein, total lipid, total ash, and total fiber) using the standard method of AOAC (2003). For instance, moisture content was estimated as the loss in weight after drying the samples at 105^{0}C in a dry air oven. Total ash was determined by incineration at 550^{0}C for 12 hours. Total nitrogen was determined by Kjeldahl apparatus and crude protein was estimated by multiplying the nitrogen content by 6.25 after. Crude lipid content was determined by the Soxhlet extraction. Crude fiber was analyzed under the Weende method using the Fibertec™ system.

Blood sampling

Six fish from each replicate group (45 fish/aquarium) were randomly selected after 90 days of feeding and anesthetized using 60 mg/L of MS222 (Ethyl 3-aminobenzoate methane sulfonate). Hematological studies were done collecting blood samples in a sterile 1 mL syringe from the caudal vein of each fish and inserted to EDTA coated tubes. Serum was separated from the bloods centrifuging at 3500x g for 15 min and stored at -20^{0}C until analysis. Another set of blood sample was added into Eppendorf tube and kept on slanting position for 12-18 hours in room temperature. Serum samples were taken from top layer by using pipette. All blood and serum samples were sent to Thai Vet Lab Co. Ltd to check CBC (cells blood count) and total protein amount. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) were calculated.

Apparent digestibility (AD)

Fecal collection was done 2 weeks after the stocking of fish and continued daily for 14 days. Tanks were fully cleaned 30 min after the afternoon meal. Feces were collected daily, before each morning meal. Uncontaminated fecal matters were gently siphoned off from the tanks onto a filter with bolting silk cloth, then it gently rinsed in distilled water, dried on a foil and collected and dried at 50°C. Random samples of feed and feces from each replicate were subjected to chemical analysis of crude protein, crude lipid and chromic oxide content (AOAC, 2003) in triplicate. Acid digestion method was used to analyze chromic oxide content in feces and diets and absorbance was measured using spectrophotometer at wavelength of 350 nm. Apparent Digestibility Coefficient (ADC) of protein and ADC of lipid were computed as follows.

% Chromic oxide content = $X/W_0 \times 100$
Where, X = Weight of chromic oxide in the sample (mg), W_0= Weight of the sample (mg)

$$X = \frac{Y - 0.0032}{0.2089}$$

Where, Y = Absorbance (at 350 nm)

$$\text{ADC of Protein } \% = 100 - 100 \times \frac{(\% \text{ chromic oxide in diet} \times \% \text{ protein in faeces})}{(\% \text{ chromic oxide in faeces} \times \% \text{ protein in diet})}$$

$$\text{ADC of Lipid } \% = 100 - 100 \times \frac{(\% \text{ chromic oxide in diet} \times \% \text{ lipid in faeces})}{(\% \text{ chromic oxide in faeces} \times \% \text{ lipid in diet})}$$

Salinity challenge test

LC_{50} value was determined as 24 ppt, with dipping 2-hour period with 10 fishes with different salinity concentration as 0, 6, 12, 18, 24, and 30ppt with triplicates. Challenge test was carried out taking 24 ppt (LC_{50} value) and run for 3 hours with continuous monitoring and counting of dead fish.

Water quality analysis

Water pH, temperature, and dissolved oxygen (DO) were monitored daily at 08:30h. Dissolved oxygen was measured with a dissolved oxygen meter (Cyberscan DO, 110 RS232 model). Temperature and pH were measured using a pH meter (Cyberscan pH11 model). Water samples were collected to analyze ammonia concentration using the phenate method and nitrite using spectrophotometer (APHA, 2003). Experimental site was controlled to have 12-12 light–dark photoperiod cycle using fluorescent tubes.

Data analysis

The SPSS 22.0 statistical package for social sciences (SPSS Inc.) was used for multiple comparisons among several means, all at 5% significance level. All means are presented with ± standard error (SE). One-way analysis of variance (ANOVA) was carried out to see the effects of factor/vitamin C dose followed by Tukey HSD for comparisons. Regression analysis was used to see the relationship between the variables and the vitamin C doses, thereby to determine dietary vitamin C dose which resulted in maximum levels in response variables.

Results

Growth and survival

Results of the first trial are presented in **Table 4** which showed that vitamin C improved survival and growth parameters when fed 10 and 20 g/kg of diet. Beyond that level, negative effects were seen leading to abnormal swimming patterns and spinal deformity of in fry were observed at the highest dose of vitamin C. Results from the feeding trial during the 21 days, initial fish weight (0.01±0.0 g) and protein intake (range 21.76-21.82) were not significantly ($P>0.05$) different, but final body weight (ranged 0.11-0.15 g) of fish significantly ($P<0.05$) affected by vitamin C dose. Effects of vitamin C on the growth and feed utilization (SRT period) are presented in **Table 4**.

Table 4 Growth performance of fish fed with experiment during the SRT period (21 days).

SRT	*Increasing levels of L-ascorbic acid (g/kg of feed)*						
	0 (T1)	*10 (T2)*	*20 (T3)*	*30 (T4)*	*40 (T5)*	*50 (T6)*	*P-value*
IW (mg/fish)	0.01 ±0.0^{a}	0.01 ±0.0^{a}	0.01 ±0.0^{a}	0.01 ±0.0^{a}	0.01 ±0.0^{a}	0.01 ±0.0^{a}	>0.05
FW (g/fish)	0.12 ±0.0^{a}	0.15±0.0^{ab}	0.13±0.0^{a}	0.12±0.0^{a}	0.11 ±0.0^{a}	0.11 ±0.0^{a}	<0.001
Survival (%)[1]	67.1±3.1^{a}	85.4±0.9^{c}	78.4±2.6^{bc}	79.6±0.6^{bc}	73.3±2.2^{ab}	73.1±1.8^{ab}	0.001
WG (g/fish) [2]	0.10±0.4^{ab}	0.13±1.8^{c}	0.11±3.3^{b}	0.10±0.0^{ab}	0.10±0.0^{a}	0.10±0.0^{a}	<0.001
SGR (%) [3]	10.4±0.0^{ab}	11.4±0.0^{c}	10.7±0.1^{b}	10.3±0.1^{a}	10.2±0.1^{a}	10.2±0.1^{a}	<0.001
FCR[4]	1.73±0.1^{d}	1.02±0.0^{a}	1.32±0.1^{b}	1.45±0.1^{bc}	1.63±0.1^{cd}	1.62±0.0^{cd}	<0.001
FCE[5]	0.58±0.0^{a}	0.98±0.0^{c}	0.76±0.0^{b}	0.69±0.0^{ab}	0.62±0.0^{a}	0.62±0.0^{a}	<0.001
PI [6]	21.8±0.0^{a}	21.8±0.1^{a}	21.8±0.3^{a}	21.8±0.2^{a}	21.8±0.4^{a}	21.8±0.2^{a}	>0.05
PER[7]	0.91±0.0^{a}	1.53±0.0^{c}	1.19±0.1^{b}	1.08±0.0^{ab}	0.96±0.0^{a}	0.96±0.0^{a}	<0.001

IW=Initial weight; FW= Final weight; DWG= Daily weight gain; SGR= specific growth rate; FCR=Feed conversion ratio; FCE= feed conversion efficiency; PER= protein efficiency ratio; SGR= specific growth rate; WG=weight gain

1. Survival of fish = (Final number / Initial number) × 100
2. Body weight gain = Final weight (g) – Initial weight (g)
3. Specific growth rate (SGR, %/day) = (Ln Weight at harvest – Ln Weight at stocking) / (number of days) ×100
4. Feed conversion ratio (FCR) = Feed intake in dry matter/Wet weight gain
5. Feed efficiency ratio (FCE) = Wet weight gain/ Feed intake in dry matter
6. Protein intake (PI) = Protein % of diet × amount of diet (g)
7. Protein Efficiency ratio (PER) = Wet weight gain / Protein intake in feed

Improvements were also seen in weight gain (WG), specific growth (SGR), protein efficiency ratio (PER) and feed conversion efficiency (FCE) with the increase in the vitamin C dose when supplemented at 10 g/kg diet as compared to the control. However, with the increasing dose, the relationship was polynomial quadratic in nature, which vitamin C dose ranged as 10-20 g L-ascorbic acid during the SRT period. Average survival of tilapia ranged from 67.1 to 85.4% during the SRT period, significant ($P<0.05$) differences were seen only between the treatments with the doses of 10 and 20 g/kg diet with the control. During the SRT, relationships were polynomial for survival ($Y = 0.001x^3-0.0926x^2+2.1853x+68.138$, $R^2=0.82$) and SGR ($Y = 0.00009x^3-0.0069x^2+0.1268x+10.476$, $R^2=0.84$). Survival and SGR peaked at the dietary supplementation of vitamin C at 15.86g and 12.02g/kg diet, respectively. However, the highest ($P<0.05$) protein efficiency ratio (PER) and lowest FCR were at 10 g vitamin C/kg diet (T2).

Table 5 Growth performance of fish fed with experiment during nursing period (30 days).

Nursing	*Increasing levels of L-ascorbic acid (g/kg of feed)*						
	0 (T1)	*10 (T2)*	*20 (T3)*	*30 (T4)*	*40 (T5)*	*50 (T6)*	*P-value*
IW (mg/fish)	0.14 ±0.0^{a}	0.14 ±0.0^{a}	0.14 ±0.0^{a}	0.14 ±0.0^{a}	0.14 ±0.0^{a}	0.14 ±0.0^{a}	0.734
FW (g/fish)	0.39 ±0.0^{b}	0.45±0.0^{c}	0.41±0.0^{b}	0.39±0.0^{ab}	0.39 ±0.0^{ab}	0.37 ±0.0^{a}	<0.001
Survival (%)	67.6±1.6^{a}	82.6±3.5^{b}	79.3±3.2^{b}	73.0±0.8^{ab}	72.±2.0^{ab}	73.5±1.8^{ab}	<0.05
WG (g/fish)	0.25±0.0^{a}	0.31±0.0^{b}	0.27±0.0^{a}	0.24±0.0^{a}	0.25±0.0^{a}	0.23±0.0^{a}	<0.001
SGR (%)	4.65±0.0^{a}	4.47±0.2^{a}	4.29±0.2^{a}	4.36±0.1^{a}	4.57±0.2^{a}	4.24±0.3^{a}	0.645
FCR	2.87±0.2^{c}	1.63±0.0^{a}	2.14±0.1^{ab}	2.68±0.1^{bc}	2.46±0.0^{bc}	2.66±0.2^{bc}	0.001
FCE	0.35±0.0^{a}	0.61±0.0^{c}	0.47±0.0^{b}	0.37±0.0^{ab}	0.41±0.0^{ab}	0.38±0.0^{ab}	<0.001
PER	0.83±0.1^{a}	1.42±0.0^{c}	1.08±0.1^{b}	0.91±0.0^{ab}	0.97±0.0^{ab}	0.91±0.1^{ab}	<0.001

Fish growth performance of Nile tilapia during 30 days of nursing are shown in **Table 5**. During the 30 days of experimental period, average survival of tilapia ranged from 67–83%, the value for 10 g/kg diet was significantly (P<0.05) higher than that of control. Survival showed polynomial relationship with the peak at the doses between 10 and 20 g/kg diet. Fish weights gain increased polynomial, which showed significantly higher weight gain in for 10 g/kg diet (0.31±0.0 g) than other treatments when compared using Tukey's HSD test. Similar trends were also seen in case of protein efficiency ratio (PER) and feed conversion ratio (FCR).

Proximate analysis of fish

Proximate composition of the whole-body moisture, dry matter, and ash contents were not significant difference among the dietary treatments. But crude lipid, crude protein, Nitrogen free extract (NFE) and gross energy (GE) were significantly difference (P<0.05) among the treatments. Carcass proximate composition of different feeding groups is presented in **Table 6**.

Table 6 Proximate composition (% on dry matter basis) of the experimental fish (Mean ± SE)

	At the end of sex reversal – increasing vitamin C (g/kg of feed)						*P-value*
	0 (T1)	*10 (T2)*	*20 (T3)*	*30 (T4)*	*40 (T5)*	*50 (T6)*	
DM	86.25±0.4^{a}	86.84±0.3^{a}	86.10±0.3^{a}	86.31±0.6^{a}	86.14±0.5^{a}	86.52±0.7^{a}	0.895
Ash	17.59±0.2^{a}	17.27±0.3^{a}	16.66±0.3^{a}	16.81±0.3^{a}	16.46±0.3^{a}	16.45±0.2^{a}	0.056
Lipid	14.87±0.2^{b}	14.84±0.5^{b}	14.65±0.4^{b}	14.63±0.5^{b}	14.07±0.0^{ab}	13.39±0.3^{a}	0.002
Protein	56.39±0.0^{abc}	57.72±0.0^{bc}	57.79±0.1^{c}	56.6±0.1^{ab}	55.80±0.6^{a}	55.37±1.3^{a}	0.001
NFE[1]	11.15±0.1^{a}	0.17±0.1^{a}	10.91±1.0^{a}	12.4±0.5^{ab}	13.67±0.3^{bc}	14.79±1.2^{c}	<0.001
GE[2]	5050±8^{ab}	5082±10^{b}	5097±0.7^{b}	5065±13^{b}	5044±21^{ab}	5001±39^{a}	0.004

Values for each experiment group in the same row followed by different superscripts are significantly (P<0.05) different.
[1]Nitrogen free extract (NFE) = 100 – (crude protein % + crude lipid% +crude fiber %+ total ash %).
[2]Gross energy (GE) (kJ/kg) was calculated according to NRC (1993) as 5.65, 9.45, and 4.11 kcal/g for protein, lipid, and carbohydrate, respectively.
DM=Dry matters

The crude lipid content of fed T1-T4 diet (ranged 14.63-14.87 %) were significantly higher than T6 diet (13.39±0.3 %). Similarly, gross energy was higher in diet T2-T4 (ranged 5,065-5,097 kJ/kg) compared with diet T6 (5001±39). But the crude protein was higher in diet T3 (57.79±0.1 g) than diet T4-T6 (ranged 55.37-56.6 %).

Blood hematological parameters

No significant difference was noticed in the RBC, WBC and MCH level among the experimental group (p>0.05). Conversely, the Hct, Hb, MCV, MCHC, and total protein (TP) were significant difference among the treatment (P<0.05). Data of blood hematological parameters and serum protein were illustrated (**Table 7**). Hemoglobin (Hb) was significant

between T1 either T2 diet compared with control. But Hct was significant at diet T2-T4 (ranged 32.0 -32.3) compared with diet T1 (29.17±0.44) and T6 (29.00±0.58) and overdose of vitamin C not beneficial for fish immunity. Dietary vitamin C at T1-T3 diets lead to significant (P<0.05) increased of total protein in blood serum sample and it was significant lower at diet T1 (2.63±0.09 g/dl) compared with diet T2-T3 (ranged 3.27-3.30 g/dl). Overall, dietary supplementation with T2 and T3 diets lead to significant increased blood hematological and serum biochemical measurements compared to other treatments.

Table 7 Hematological blood parameters of Nile tilapia fry fed dietary 0 -50 g vitamin C/kg diet.

	Dietary vitamin C level (g/kg diet)						*P-value*
	0 (T1)	*10 (T2)*	*20 (T3)*	*30 (T4)*	*40 (T5)*	*50 (T6)*	
RBC[1]	2.49±0.0^{a}	2.55±0.0^{a}	2.44±0.0^{a}	2.43±0.0^{a}	2.44±0.0^{a}	2.42±0.0^{a}	<0.05
WBC[2]	6900±100^{a}	6883±16.7^{a}	6900±57.7^{a}	6867±33.3^{a}	6900±57.7^{a}	6700±100^{a}	0.315
Hb[3]	10.6±0.1^{ab}	11.3±0.1^{c}	11.3±0.2^{c}	11.2±0.1^{bc}	11.0±0.1^{abc}	10.5±0.2^{a}	0.002
Hct[4]	29.2±0.4^{a}	32.3±0.3^{b}	32.3±0.9^{b}	32.0±0.6^{b}	30.7±0.3^{ab}	29.0±0.6^{a}	0.002
MCV[5]	117.2±3.6^{a}	127±2.3^{ab}	132.4±4.9^{b}	131.8±3.2^{ab}	125.6±2.3^{ab}	120.0±1.5^{ab}	<0.05
MCH[6]	42.7±1.2^{a}	44.5±0.6^{a}	46.3±1.1^{a}	46.1±0.8^{a}	44.9±0.7^{a}	43.6±0.3^{a}	>0.05
MCHC[7]	36.5±0.2^{c}	35.1±0.1^{ab}	35.0±0.5^{a}	35.0±0.3^{a}	35.8±0.2^{abc}	36.3±0.2^{bc}	0.05
TP[8]	2.63±0.1^{a}	3.27±0.1^{b}	3.30±0.2^{b}	3.13±0.1^{ab}	2.93±0.1^{ab}	2.93±0.1^{ab}	<0.05

Values are mean± Standard error (n=3). Values for each experiment group in the same row followed by different superscripts are significantly (P<0.05) different.
[1]RBC (×10^{6} cell µL): Red blood cell.
[2]WBC (×10^{6} cell µL): White blood cell.
[3]Hb (g/dl): Hemoglobin.
[4]Hct (%); Hematocrit.
[5]MCV (Mean corpuscular volume): Hematocrit / Red blood cell.
[6]MCH (Mean corpuscular hemoglobin): Hemoglobin content/ Red blood cell.
[7]MCHC (Mean corpuscular hemoglobin concentration): Hemoglobin content / hematocrit.
[8]TP (g/dl): Total protein.

Apparent Digestibility (AD)

The apparent digestibility (AD %) of the protein and lipid are illustrated in **Figure 1**(a) and 1(b), respectively. Polynomial relationship between treatment and apparent digestibility of protein as well as lipids were observed for all the parameters evaluated (P<0.05). Polynomial regression analysis showed these values were highest between 10 and 20 g/kg diet. ADC for protein was estimated around 84 % at 15.36g vitamin C/ kg diet and highest lipid was estimated around 91% at 17.75g vitamin C per kg diet.

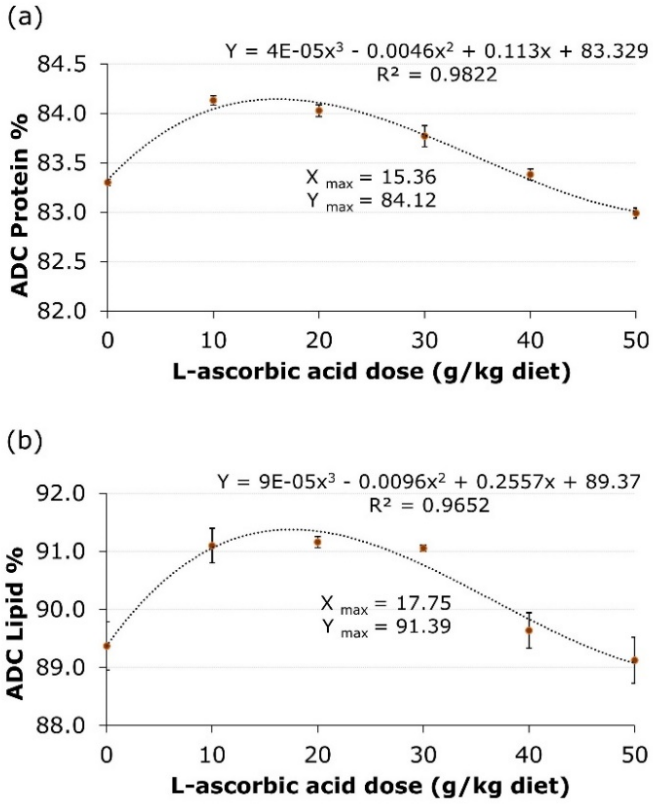


Figure 1 Apparent digestibility coefficient (%) of protein (a) and lipid (b) with dietary ascorbic acid dose of the six diet just after sex-reversal treatment.

Sex-reversal percent

We used MT fed mixing with the diet for 21 days to produce mono sex tilapia fry. Gonad histology was observed (6 replicates /treatment) at the end of the trial i.e., 112 day (**Figure 3**) and remaining all tilapia gonad analyzed using gonad squash method at 112 days gives results in **Table 8**.

Table 8 Percentage of male assessed by using gonad squash method after 112 days.

% male	*Increasing levels of vitamin C (g/kg of feed)*					
	0 (T1)	*10 (T2)*	20 (T3)	30 (T4)	40 (T5)	50 (T6)
Sample 1	100.0	99.3	99.2	99.1	99.1	99.2
Sample 2	98.3	98.4	99.2	98.3	100.0	99.0
Sample 3	99.0	99.3	100.0	98.2	99.1	98.2
Average	99 ±0.5[a]	99±0.3[a]	99±0.3[a]	98±0.3[a]	99±0.3[a]	99±0.3[a]

The average male percentage was 99.05±0.14 % which is acceptable by the farmers. The sex-ratio of tilapia fry fed without vitamin C supplement was around 99% male which was not significantly ($P>0.05$) different with other treatment. However, there was no significant variations in results among the treatments. Results illustrates that all treatments can be used for mono-sex tilapia production without considering the fish growth performance.

Concentration of spermatogonia increased while increasing in the vitamin C doses up to 30 g AA/ kg diet as well as changes in testis color. However, at higher doses of vitamin C diets concentrated spermatogonia were seen along with little spermatogonia around (**Figure 2**).

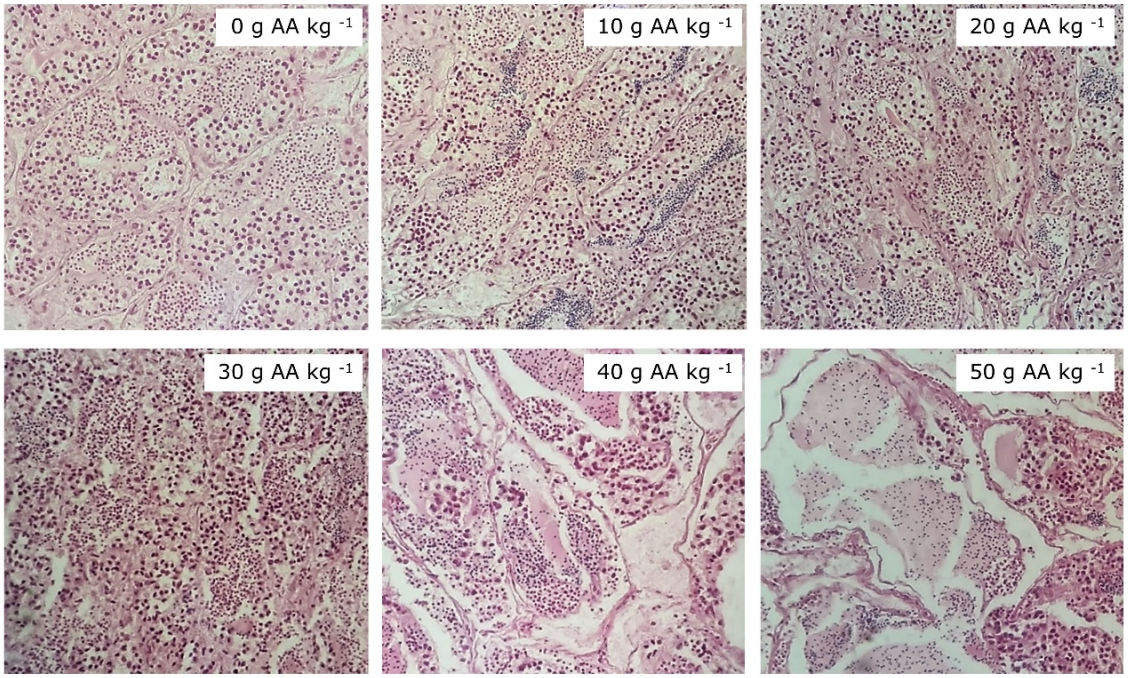
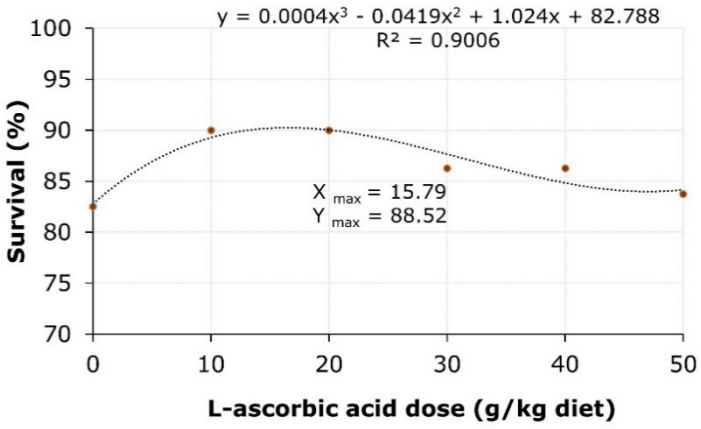


Figure 2 Representative histological pictures of Nile tilapia testes fed different dietary ascorbic acid level seen on the gonad sampled at 112th day.

Salinity stress test

Survival when fry were exposed to high salinity i.e., 24 ppt as determined as the 50% LC. Mortality occurred right from 3rd hour showing the signs of bleaching of skin near operculum and abdomen area L-ascorbic acid dose showed very clear effects on survival. However, highest survival reached average 86.4±1.7% survival at the dose between 10 and 20 g/kg diet (**Figure 3**).



Polynomial regression analysis showed that the highest survival was obtained when the L-ascorbic acid dose was at 15.79 g AA/kg diet. Higher doses showed decline in survival.

Figure 3 Average survival after challenge with 24 ppt salinity treatments for Nile tilapia fry.

Water quality

Average water temperature was 27.7°C ±0.08 °C with 12-12 light–dark photoperiod cycle using fluorescent tubes as a light source. Dissolved oxygen concentration was average 6.09±0.02 mg/L, the pH range 7.28±0.01, ammonia concentration was 0.38±0.04 mg/L and nitrite concentration was 0.02±0.01mg/L. There was no significant difference in water quality parameters among the treatment. Therefore, all the water quality parameters acceptable ranges for Nile tilapia fry growth.

Discussion

Vitamin C (L-ascorbic acid, AA) is an essential micronutrient to improve immunity, physiological functions (Ibrahem et al., 2010). The positive growth response (FW, WG, SGR, survival) and nutrient utilization (FCR, PER, ADC% protein), salinity challenge, hematological responses (Hb, Hct, MCV, MCHC) and serum biochemical measurement (Total protein) indicators of the fish fed 10 and 20 g vitamin C per kg feed were significantly higher than those fed control diet. Hence, the increased growth observed in the current study can be attributed to the multiple roles of ascorbic acid as reported for fish growth, nutrient utilization, protein metabolism and collagen formation (Kumari and Sahoo, 2005; Shahkar et al., 2015). It is not clear why the higher doses of vitamin C were detrimental. It is likely that excessive amount might have caused binding of other nutrients affecting the digestion and absorption of protein, lipids, and minerals. thereby negatively affecting the survival and growth, other parameters.

The amino acids profile and proximate composition of FCM show that it has adequate nutrients for fish growth, which are close to the values of earlier reports (Wang et al., 2005; Taufek et al., 2016a; 2017; Khan, 2018). Fish survival and growth, hematological indicators, and salinity challenge were reflection of the health status. During the sex reversal period, MT was used as a steroid hormone to produce mono-sex male tilapia. Survival rate of tilapia fry are considerably higher than earlier reports (Toyama et al., 2000; Suwanmanee et al., 2012). The improved survival rate of fry were indicated that the use of the FCM might have beneficial effects on immunity. This type of positive scenario represents due to the combinations of Vitamin C and various nutrients and other factors. Among the nutrients, the insect meal may have high level of Vitamin D3 as they are exposed to sunlight. Moreover, the nutrient composition with the rich crude protein, the amino acid profile, and lipids, confirm the high-performance capacity of the FCM than the FM alone.

An earlier research has also reported that increment of ascorbic acid dose increased spermatogonia as well as color intensity (Shahkar et al., 2015), but higher dose of vitamin C showed difference. Nevertheless, the percentage of male in all the treatments very high (>98%) and are at acceptable levels for the industry which produce and supply mono-sex tilapia. The present study has also indicated that lower dose (<30 g/kg AA diet) might have positive effects on proliferation of spermatogonia, as well as higher doses (>30 g/kg AA diet) might have negative effects. Importantly, the histological observation of this study revealed that L-ascorbic acid (AA) is mainly involved in gonadal development of male Nile tilapia during Sex-reversal treatment.

However, in case of SRT fish, alternative solution is needed to improve fish survival during the culture period that MT steroid might have any negative effect (Little et al., 2003; Ibrahem et al., 2010; Abo-Al-Ela et al., 2017). Present study showed higher dose i.e., 15.86 g of vitamin C per kg diet results in a higher survival, than that of earlier report. A dose of 4.9g AA/kg diet was found to result in 75% fish survival (Suwanmanee et al., 2012). For larger tilapia, lower level of vitamin C diet (150 mg vitamin C/kg diet) has been recommended i.e., for 800 g initial weight of Nile tilapia (Martins et al., 2016). Similarly, some of the authors have reported higher specific growth rate when given up to 1g vit C/kg diet compared with control fish (Tewary and Patra, 2007), 1.5-2 g/kg diet in common carp (Labh et al., 2017; Faramarzi, 2016), and 3g vitamin C/kg was enhanced the antibody production in channel catfish (Li and Lovell, 1985). By analyzing previously highlighted literature results and present research findings, clearly suggest that the small size of Nile tilapia needs higher doses of vitamin C than bigger size tilapia. Although, present study used 17-α MT to convert all-male tilapia (99% or more); hence, the present study demonstrated that vitamin C in SRT diet might be a suitable solution to maintain immunity which might have disrupted from steroid hormone.

Fish carcass composition especially moisture, ash, NFE and GE did not significantly differ in the present study. All though, it was not analyzed in the present study, an earlier report has shown elevated vitamin C in the tissue while increasing vitamin C in diet, a trend which is also observed in other species too (Wang et al., 2003; Shahakr et al., 2015). Salinity challenges indicate that ability to adapt to stress conditions, higher survival percentage of fish more tolerance to salinity, as well as stress condition (MacNiven and Little, 2001). Stress challenge occurs when predisposing factors are present such as abrupt temperature changes, and stress due to handling, crowding, and inadequate feed and oxygen (Yardimci and Aydin, 2011). An earlier report has suggested that leaching of vitamin C was increased with high water temperature and long immersion time (Soliman et al., 1987). Therefore, the water temperature has maintained between 27-29°C throughout this experiment, and other water quality parameters were controlled to present the optimum conditions. Due to this fact, farmers are suggested to maintain stable water quality and smooth handling of fish to maintain optimum level of vitamin C in sex reversal tilapia production.

In the present study reported low level of RBC and WBC. Similar finding results of reported by Sayed and Moneeb (2015) with use of MT steroid hormone. Hemoglobin results also associate with fish health, higher hemoglobin value was shown in good health fish; Hemoglobin (Hb), Hct, MCV and MCHC were significantly low in control treatment in present study and it was significantly higher with vitamin C mixed diet. These results have indicated that vitamin C leaded to modulates the immunotoxin effect of 17-α MT in Nile tilapia (Abo-Al-Ela et al., 2017). Nevertheless, care needs to be taken or more research needs to be done as AA has been shown associated with anemic condition due to poor absorption and redistribution of iron, therefore, iron supplementation also most important to improve blood Hb (Shahkar et al., 2015). Hct values reflect the health status of fish that indicator for oxygen transportation capacity of fish, hence nutritional deficiency and diseases are always associated with low levels of Hct (El-Asely et al., 2014). Significantly higher hematocrit value and superoxide dismutase activity was found in fish fed higher vitamin C/kg diet (Shahkar et al., 2015). In the present study, higher amount of serum total proteins was detected compared to control treatment, due to the significant positive effect of 10-20 g vitamin C/kg diet. This scenario reflects the role of vitamin C included FCM diet, in enhancement of fish immunity response. Additionally, this helps to assess their potential benefits in Nile tilapia (*Orechromis niloticus*) production, during sex-reversal period.

There were limited publications on higher than 5g dose of vitamin C with sex reversal treatment on tilapia. Based on the present study results, the dose higher than 5g/kg vitamin C serves as a nutrient supplementation that can help enhance survival, and improve growth, feed utilization, stress resistance and sex differentiation that are major key indicators of mono-sex Nile tilapia fry. Research on the fishmeal replacement with insect meals or other products have been popular for other freshwater fish species. However, there were very limited information is available for tilapia. The results of the present study on the use of FCM, has proved that it is a potential source of animal protein that contains high level of nutrients comparable to fishmeal. Therefore, FCM with the supplementation of vitamin C diets of all male tilapia appears to be practical and viable provided that it is cultured on mass scale.

In conclusion, fish survival, growth and other parameters improved when vitamin C was supplemented at 10-20 g/kg. The doses higher than 20 g/kg diet were not beneficial as per thought before. Regression analysis showed 15 g vitamin C/kg is recommended when 80% fishmeal is replaced with FCM. More research is needed to see whether other vitamins, minerals or other factors could help enhance survival of tilapia fry further.

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