Influence of Vitamin C on Hematology of Pangasianodon hypophthalmus (Sauvage, 1878) Juveniles during pre and post-challenge with Aeromonas hydrophila (Chester, 1901)

Daniel Dani
Central Institute of Fisheries Education

Ande Muralidhar
Central Institute of Fisheries Education

Prem Srivastava
Central Institute of Fisheries Education

Kurcheti Pani Prasad
Central Institute of Fisheries Education

Fishery Technology 55 (2018) : 120 - 127

Influence of Vitamin C on Hematology of *Pangasianodon hypophthalmus* (Sauvage, 1878) Juveniles during pre and post-challenge with *Aeromonas hydrophila* (Chester, 1901)

N. Daniel[1]*, A. P. Muralidhar[1], P. P. Srivastava[1], K. K. Jain[1], K. Pani Prasad[2], R. Anandan[2] and J. Manish[1]

[1] *Biochemistry and Physiology Division, ICAR-Central Institute of Fisheries Education, Mumbai - 400 061, India*

[2] *ICAR-Central Institute of Fisheries Technology, P. O., Cochin - 682 029, India*

Abstract

A 60-day feeding trial was conducted to know the effect of vitamin C on the hematological responses of *Pangasianodon hypophthalmus* juveniles during pre- and post-challenge conditions with *Aeromonas hydrophila*. A total of seven purified diets were prepared with 0 (control), 17.5 (T1), 35 (T2), 70 (T3), 175 (T4), 350 (T5) and 700 (T6) mg ascorbic acid (AA) equivalent per kg of diet, supplied as L-ascorbyl-2-polyphosphate (LAPP) and fed to triplicate group of *P. hypophthalmus* juveniles (mean initial body weight: 3.23±0.01 g to 3.38±0.01 g) for 60 days twice daily. After the feeding and after challenging with *A. hydrophila*, the haematological parameters were measured in *P. hypophthalmus* juveniles. All haematological parameter values (except albumin-globulin ratio) *viz.,* serum glucose, total protein, albumin, globulin, hemoglobin (Hb), total erythrocyte count (TEC), total white blood cells count (WBC), hematocrit value (HCT) increased significantly ($p<0.05$) in treatment groups than control (vitamin C depleted diet). Further, the increased hematological values for most of the parameters were observed in the range of 175 to 350 mg AA kg^{-1}. Therefore, it is suggested that dietary supplementation of 175 to 350 mg AA kg^{-1} diet would be beneficial for the optimal hematological responses for *P. hypophthalmus* juveniles.

Keywords: Vitamin C, L-ascorbyl 2-polyphosphate, *Pangasius*, hematology, immunity, health, challenge study

Received 08 September 2016; Revised 20 February 2018; Accepted 21 March 2018

**E-mail: danielfnft@gmail.com*

Introduction

Extreme changes in the water quality, environmental and rearing conditions can cause a variety of disease outbreak in fishes (Snieszko, 1974). Fish lives in the aquatic environment, which contains a variety of pathogenic and opportunistic bacteria that attack the animals when they get a favourable condition (Woo & Bruno, 1998). *Aeromonas hydrophila* is one of such opportunistic bacteria. The invasion of *A. hydrophila* is recorded to be widespread in the freshwater ponds as they are ubiquitous in the ecosystems. The negative impacts of *A. hydrophila* have been recorded in the fish farming with varying gross signs to the animals starting from the common infections to death and affecting profitability (Austin & Austin, 2007).

Antibiotic treatment plays a significant role in controlling the disease, but in the recent decades due to its negative impacts on fish consumers and antibiotic resistance, some of the antibiotics were recently banned (Rico et al., 2013). As an alternative to the antibiotics, immunostimulants in the feed has received great attention (Sugita et al., 1996; Esiobu et al., 2002). But, an addition of immunostimulants raises the feed costs, which in turn marginally affects the profitability of the fish farming. It is observed that proper nutrition has significant influence in the immune system of aquatic organisms (Chandra & Chandra, 1985). Fish feed contains many ingredients which is shown to have an immunostimulatory activity on the fish which strengthen the activity of non-specific defense mechanism and confer protection against many diseases (Jeney & Anderson, 1993). Many nutrients have been found to increase the immunostimulatory activity on the animals, including vitamin C (Klasing, 1998). Vitamin C is needed for proper metabolism, growth, and disease resistance (Lim et al., 2000). However, most of the farmed fishes cannot synthesize vitamin C *de novo*, as they lack the enzyme called L-gulonolactone oxidase needed for its synthesis (Eo et al., 2008) from either glucose or galactose (Halver & Hardy, 2002) and hence it need to be supplied through feed (Chatterrjee et al., 1975). Many nutrition related diseases were reported in fishes when they fed diets deficient in vitamin C (Sandnes, 1991).

Pangasianodon hypophthalmus is a catfish, commonly known as iridescent shark, belongs to the family Pangasiidae. It is endemic to the Mekong and Chao-Phraya river systems of South-East Asia and it was introduced to India clandestinely via Bangladesh (Singh & Lakra, 2012). Compared to commonly cultured freshwater fish species in India, *P. hypophthalmus* has faster growth rate and takes less time to reach marketable size, which has made it popular for culture. Now, it is one of the key freshwater cultivable species of India with the annual production of about 2,00,000 t (Lakra & Singh, 2010). However, at present, the culture of *P. hypophthalmus* is affected with many bacterial diseases at the farm level. Many micro nutrients including vitamin C stimulates the immune system of fish and can be delivered through feed (Klasing, 1998). The hematological parameters are very useful for assessing the health status of the animals. It has been evidenced from research that vitamin C influences the hematology of fishes. It has been demonstrated that immunostimulants in the diet enhanced the serum protein content in fishes (Siwicki, 1989). Although, the vitamin C requirement for immune and hematological responses is available for many fish species, no reports are available for *P. hypophthalmus.* The prior available information for any species cannot be used for another species as requirements are species specific and utilization of each nutrient is not same. Keeping in view of these aspects, the present study was undertaken to study the effects of dietary ascorbic acid on hematological responses in *P. hypophthalmus* juveniles.

Materials and Methods

Five hundred numbers of fishes (*P. hypophthalmus*) with an average weight range of (3.08±0.02 g) were procured from a fish farm in Kolkata in the oxygenated polythene bags and were transferred to acclimatization tanks (500 l capacity). They were acclimatized under normal conditions for 15 days and feeding was done with commercial feed containing 35% of crude protein.

After acclimatization, a total of 315 juveniles of *P. hypophthalmus* (average weight of 3.23±0.01 g to 3.38±0.01 g) were randomly distributed to 7 distinct experimental groups. Seven experimental groups were carried out in triplicate (total 21 tanks with 150 l capacity each) following a completely randomized design (CRD). Experimental tanks were manually siphoned to remove faecal matter throughout the experimental period of 60 days.

The purified ingredients such as casein (vitamin-free), gelatin, dextrin, starch, cellulose, carboxymethyl cellulose (CMC), choline chloride, butylated hydroxytoluene (BHT), cod liver oil, sunflower oil, vitamin mix (vitamin C free) and mineral mixture were properly mixed. After mixing, seven different graded levels of vitamin C diets were prepared with the addition of L-ascorbyl 2-polyphosphate to contain specific ascorbic acid (AA) levels in the diet (0 mg kg^{-1}), T1 (17.5 mg kg^{-1}), T2 (35 mg kg^{-1}), T3 (70 mg kg^{-1}), T4 (175 mg kg^{-1}), T5 (350 mg kg^{-1}) and T6 (700 mg kg^{-1}) by adjusting the cellulose content in the diet. L-ascorbyl 2-polyphosphate was procured from Biostadt India Limited. The ingredients of the diet for the study is given in Table 1. Feeding was done at 2% of body weight initially and adjusted accordingly over the 60 days of culture. The daily ration was divided into two equal parts and was fed in the morning and in the evening.

Fishes were anesthetized using clove oil (50 μl of clove oil per litre of water) before taking blood from the fish. For blood collection, blood was taken from vena caudalis using a medical syringe which was previously rinsed with 2.7% EDTA solution and then transferred immediately to a test tube containing a thin layer of EDTA powder (as an anticoagulant) and shaken well to prevent haemolysis. For the collection of serum, blood was taken from vena caudalis using a medical syringe without using anticoagulant and then they were transferred immediately to a dried microcentrifuge tube. The tubes were then allowed to stand in tilting position at room temperature for an hour which allowed the blood to clot. After clotting, the yellow straw colour serum was carefully collected and transferred to an eppendorf tube.

Blood glucose was estimated by following Nelson (1944) and expressed as mg dl^{-1}. Serum total protein

Table 1. Ingredients and proximate composition of the experimental diets

Ingredients	Control	T_1	T_2	T_3	T_4	T_5	T_6
	Ingredients composition of the diet (g kg^{-1} dry matter basis)						
Casein	320	320	320	320	320	320	320
Starch	220	220	220	220	220	220	220
Dextrin	150	150	150	150	150	150	150
Cellulose	98.8	98.75	98.7	98.6	98.3	97.8	96.8
CMC	30	30	30	30	30	30	30
Gelatin	60	60	60	60	60	60	60
Cod liver oil	50.5	50.5	50.5	50.5	50.5	50.5	50.5
Sun flower oil	50.5	50.5	50.5	50.5	50.5	50.5	50.5
[2]BHT	0.2	0.2	0.2	0.2	0.2	0.2	0.2
[1]LAPP	0	0.05	0.1	0.2	0.5	1	2
*Vit-mineral mix	20	20	20	20	20	20	20
GE (MJ.Kg^{-1})	19.63	19.61	19.63	19.63	19.62	19.61	19.6

*Composition of vitamin mineral mix (quantity kg^{-1}) (Vitamin C free):

Vitamin A, 55,00,000 IU; Vitamin D3, 11,00,000 IU; Vitamin B2, 2,000 mg; Vitamin E, 750 mg; Vitamin K, 1,000 mg; Vitamin B6, 1,000 mg; Vitamin B12, 6 mg; Calcium Pantothenate, 2,500 mg; Nicotinamide, 10 g; Choline Chloride, 150 g; Mn, 27,000 mg; I, 1,000 mg; Fe, 7,500 mg; Zn, 5,000 mg; Cu, 2,000 mg; Co, 450 L- lysine, 10 g; DL- Methionine, 10 g; Selenium, 125 mg.

[1]LAPP % L-ascorbyl-2-polyphosphate, [2]BHT % Butylated hydroxytoluene.

was assessed by biuret method (Reinhold, 1953) using a kit (Erba® Diagnostic Mannheim; Transasia Bio-medicals Ltd., Solan, HP, India). The albumin concentration (gram %) was determined by using Bromocresol green binding method described by Doumas et al., 1997 and expressed as gram %. Globulin content was calculated by subtracting albumin from total protein and A/G ratio was calculated as follows,

$$\text{A/G ratio} = \frac{\text{Albumin in (gram \%)}}{\text{Globulin in (gram \%)}}$$

The blood hemoglobin content was determined following Cyanomethemoglobin method (Van Kampen & Zijlstra, 1961) using Drabkins Fluid (Qualigens, India, TM). Total erythrocyte count (TEC) was taken using haemocytometer (Hendrick, 1952). Total leucocyte count (TLC) was determined following Shaw (1930). Hematocrit value (HCT) was determined by drawing blood by capillary action into microhematocrit tubes and centrifuged in a microhematocrit centrifuge for 5 min at 10,500 rpm and measurement was made by microhaemotocrit reader and expressed as percentage.

After experimental trials with vitamin C for 60 days, the fishes were challenged with the pathogenic isolates of *A. hydrophila*, grown on nutrient agar for 24 h at 30°C in a Biochemical oxygen demand (BOD) incubator. The cells were harvested and washed thrice in sterile phosphate-buffered saline (PBS) and then re-suspended in PBS at concentration of 10^5 cells ml^{-1}. The fishes in each experimental group were injected intra-peritoneally with 0.2 ml of this suspension. After a week, serum and blood samples of the different challenged groups were collected to study the hematological parameters.

The data were statistically analyzed by using statistical package SPSS version 20.0 in which data were subjected to one-way ANOVA (Cuevas et al., 2004) and Duncan's multiple range test (Duncan, 1955) was used to determine the significant differences between the means. Comparisons were made at the 5% probability level.

Results and Discussion

Result of the present study revealed that after 60 days of immunomodulatory trial, *P. hypophthalmus* juveniles that received dietary vitamin C showed a significant improvement in the hematology than that of control ($p<0.05$) animals. The results were in agreement with the results of earlier studies in the fishes such as tilapia (Al-Amoudi et al., 1992), bagrid catfish (Anbarasu & Chandran, 2001), parrot fish (Wang et al., 2003), soft-shelled turtles (Zhou et al., 2003), Japanese seabass (Ai et al., 2004); golden shiner (Chen et al., 2004), grouper (Lin & Shiau, 2005), yellow croaker (Ai et al., 2006), milkfish (Azad et al., 2007), rohu (Misra et al., 2007) and Japanese eel (Shankar et al., 2015).

Blood glucose, total protein, albumin, globulin content and A:G ratio were analyzed in both pre- and post-challenged conditions with *A. hydrophila* and found significant ($p<0.05$) among different treatments (Table 2).

In pre and post-challenge studies, the dietary vitamin C improved the blood glucose levels of *P. hypophthalmus* juveniles. In both conditions, higher glucose level was recorded in fishes fed with 700 mg AA kg^{-1} and lowest glucose level was found in fishes fed with control diet (vitamin C deficient). It has been reported that the measurement of blood glucose level is considered as an effective method to evaluate the stress effect in fish (Mommsen et al., 1999). However, differences in the physiological and metabolic status of fish possibly result in the variation of glucose concentration in fish blood. Moreover, the blood parameters of fish vary with seasons (Fange, 1994). According to Zhou et al. (2006), an increase in glucose content of the fish shows the healthy physiology; therefore, the increased glucose content of fish reared in the dietary vitamin C groups may be attributed to the effect of vitamin C on healthy physiology of *P. hypophthalmus* juveniles. Our results were in accordance with the earlier findings in Mekong giant catfish (Pimpimol et al., 2012), cobia (Zhou et al., 2012) and Japanese eel (Shahkar et al., 2015) in response to dietary vitamin C.

The dietary vitamin C improved the serum total protein level in *P. hypophthalmus* juveniles in pre- and post-challenged conditions. The highest and lowest values were recorded in T4 (175 mg AA kg^{-1}) and control group, respectively. The results indicated that dietary vitamin C had influenced the serum protein content of *P. hypophthalmus* juveniles. Also, serum protein has a positive influence in the regulation and transport of the ionic molecules involved in the defence against pathogens and often they are used as a clinical indicator for fish (Rudneva & Kowrshina, 2011). So, total protein content depends on the physiological and pathological conditions of the fish (Georgiev & Kamenov, 1980). In fish, it is generally accepted that animals with an improved immune system will have more protein content in the serum (Talpur & Ikhwanuddin, 2013). The results from our study supports the assertion that dietary vitamin C enhances the serum protein content in fishes as shown in tilapia (Nsonga et al., 2009), pirarucu fish (Andrade et al.,

Table 2. Hematological parameters (Glucose, Protein, Albumin, Globulin and A:G Ratio) of *P. hypophthalmus* juveniles during pre and post challenged conditions

Parameters	Conditions	Control	T1	T2	T3	T4	T5	T6
Glucose	Pre-challenge	$48.28^{a}\pm1.45$	$75.86^{c}\pm2.14$	$44.83^{a}\pm3.12$	$58.62^{b}\pm0.98$	$73.56^{c}\pm2.48$	$110.34^{d}\pm3.21$	$134.48^{e}\pm1.56$
	Post-challenge	$65.52^{a}\pm1.99$	$81.61^{b}\pm3.04$	$93.10^{c}\pm1.99$	$124.14^{d}\pm0.22$	$75.86^{b}\pm0.43$	$134.48^{e}\pm1.12$	$147.13^{f}\pm2.44$
Protein	Pre-challenge	$1.74^{a}\pm0.06$	$1.80^{a}\pm0.06$	$2.30^{b}\pm0.14$	$2.74^{d}\pm0.04$	$2.83^{d}\pm0.02$	$2.59^{d}\pm0.04$	$2.48^{c}\pm0.04$
	Post-challenge	$1.54^{a}\pm0.06$	$1.63^{a}\pm0.15$	$1.70^{a}\pm0.19$	$2.73^{b}\pm0.10$	$2.80^{b}\pm0.04$	$2.59^{b}\pm0.19$	$2.50^{b}\pm0.08$
Albumin	Pre-challenge	$0.59^{a}\pm0.01$	$0.60^{a}\pm0.01$	$0.66^{b}\pm0.02$	$0.88^{d}\pm0.01$	$0.91^{d}\pm0.01$	$0.89^{d}\pm0.03$	$0.81^{c}\pm0.01$
	Post-challenge	$0.71^{a}\pm0.01$	$0.73^{a}\pm0.01$	$0.83^{a}\pm0.01$	$0.81^{b}\pm0.03$	$1.17^{e}\pm0.01$	$1.10^{d}\pm0.01$	$1.06^{c}\pm0.01$
Globulin	Pre-challenge	$1.15^{a}\pm0.05$	$1.20^{a}\pm0.05$	$1.65^{b}\pm0.16$	$1.86^{bc}\pm0.04$	$1.92^{c}\pm0.01$	$1.70^{bc}\pm0.01$	$1.67^{b}\pm0.01$
	Post-challenge	$0.84^{a}\pm0.06$	$0.92^{a}\pm0.15$	$0.96^{a}\pm0.19$	$1.93^{c}\pm0.10$	$1.63^{bc}\pm0.04$	$1.48^{b}\pm0.19$	$1.44^{b}\pm0.08$
A:G Ratio	Pre-challenge	$0.54^{b}\pm0.02$	$0.50^{b}\pm0.02$	$0.41^{a}\pm0.05$	$0.47^{ab}\pm0.02$	$0.47^{ab}\pm0.01$	$0.52^{b}\pm0.02$	$0.48^{ab}\pm0.01$
	Post-challenge	$0.85^{b}\pm0.07$	$0.83^{b}\pm0.05$	$0.83^{b}\pm0.07$	$0.42^{a}\pm0.02$	$0.72^{ab}\pm0.03$	$0.78^{ab}\pm0.07$	$0.75^{ab}\pm0.05$

Serum glucose (mg/dl), Protein (g %), Albumin (g %), Globulin (g %) and A: G ratio.

Data expressed as mean ± SE (n=3). Mean values in the same column with different superscript differ significantly ($p<0.05$).

2007), Mekong giant catfish (Pimpimol et al., 2012) and Japanese eel (Shahkar et al., 2015) when fed with vitamin C diets.

In the present study, serum albumin and globulin level increased in response to dietary vitamin C. During pre-challenge condition, the highest serum albumin and globulin level was found in T4 (175 mg AA kg^{-1}) group. However, in post-challenged condition, highest serum albumin and globulin level were found in T4 (175 mg AA kg^{-1}) and T3 (70 mg AA kg^{-1}) group respectively whereas, lowest level in control group. The results are in agreement with the findings of previous studies in tilapia (Nsonga et al., 2009) and Japanese eel (Shahkar et al., 2015). Wiegertjes et al. (1996) reported that an increase in the serum proteins levels *viz.,* albumin and globulin are associated with a stronger innate immune response in fish. Therefore, the improved serum albumin and serum globulin content might be due to the effect of dietary vitamin C on eliciting the immune response in *P. hypophthalmus* juveniles.

Highest value of A:G ratio was observed in control group during pre and post-challenged conditions. But, the least value of A:G ratio was noticed in T2 (35 mg AA kg^{-1}) and T3 (70 mg AA kg^{-1}) group during the pre and post-challenge conditions respectively. The lower values of A/G ratio in fish is the indication of overproduction of globulins which indicates that fishes are immunologically competant (Wiegertjes et al., 1996; Nsonga et al., 2009; Shahkar et al., 2015). The results of the present study are in confirmation with literature that shows that low A/G ratio obtained in this study can be the effect of dietary vitamin C to improve the globulin content in *P. hypophthalmus* juveniles.

Blood parameters *viz.,* blood hemoglobin (Hb), total erythrocyte count (TEC), total leucocyte count (TLC) and hematocrit value (HCT) were analysed in both pre and post-challenged conditions with *A. hydrophila* and the results were found to vary significantly ($p<0.05$) among treatments during both conditions (Table 3).

The hemoglobin content improved in *P. hypophthalmus* juveniles with response to vitamin C diets and the highest and lowest values were observed in the T4 (175 mg AA kg^{-1}) and control group respectively during the pre and post-challenge condition. Adham et al., 2000 reported that animals fed with vitamin C deficient diet lead to macrocytic anaemia, which can be the result of reduced hemoglobin. It has also been reported that the absorption and synthesis of iron require vitamin C and diets deficient in the vitamin C, can lower the hemoglobin content in fish as iron is the part of the hemoglobin (Shiau & Jan, 1992; Banerjee et al., 2002). The results of the present study are in agreement with previous research findings in African catfish (Olumeyi et al., 2008) and tiger puffer (Eo et al., 2008) fed with vitamin C diets. In the present study, the highest hematological responses were found in vitamin C fed treatment groups for TEC, TLC and HCT parameters. Before and after challenge, the trends were same for the following parameters. The highest TEC and TLC count were observed in the T4 (175 mg AA kg^{-1}) groups and the maximum HCT values were recorded in T3 (70 mg AA kg^{-1}) group.

Table 3. Hematological parameters (Hb, TEC, TLC and HCT value) of *P. hypophthalmus* juveniles during pre and post challenged conditions

Parameters	Conditions	Control	T1	T2	T3	T4	T5	T6
Hb	Pre-challenge	3.4^{a}±0.10	4.1^{b}±0.06	4.4^{c}±0.07	5.2^{d}±0.09	6.3^{d}±0.06	5.6^{e}±0.06	5.3^{f}±0.09
	Post-challenge	3.2^{b}±0.06	4.3^{a}±0.06	4.5^{a}±0.03	5.4^{a}±0.06	6.1^{a}±0.18	5.7^{a}±0.09	5.4^{a}±0.07
TEC	Pre-challenge	1.05^{a}±0.01	1.12^{b}±0.01	1.23^{c}±0.01	1.35^{d}±0.01	1.67^{g}±0.01	1.58^{f}±0.01	1.54^{e}±0.01
	Post-challenge	0.76^{c}±0.01	0.94^{a}±0.01	0.95^{a}±0.01	1.16^{b}±0.01	1.56^{b}±0.01	1.53^{b}±0.01	1.49^{b}±0.01
TLC	Pre-challenge	57.97^{a}±0.01	61.73^{b}±0.09	69.17^{c}±0.26	74.93^{d}±0.26	109.73^{g}±1.03	98.47^{f}±0.66	93.10^{e}±0.35
	Post-challenge	32.8^{c}±0.17	52.2^{b}±0.15	63.93^{a}±0.09	66.6^{d}±0.24	101.7^{d}±0.33	92.5^{d}±0.47	87.9^{d}±0.23
HCT	Pre-challenge	11.40^{a}±0.17	11.90^{a}±0.06	12.13^{a}±0.09	16.33^{b}±0.28	15.67^{b}±1.90	15.13^{b}±0.28	14.90^{b}±0.06
	Post-challenge	8.7^{c}±0.06	9.4^{b}±0.06	9.6^{a}±0.03	15.3^{b}±0.28	14.8^{b}±0.03	14.8^{b}±0.06	12.3^{b}±0.19

Hb (g%), TEC ($10^6/mm^3$), TLC ($10^3/mm^3$) and HCT (%).

Data expressed as mean ±SE (n=3). Mean values in the same column with different superscript differ significantly ($p<0.05$).

The results of this study is well supported by earlier findings in tilapia (Nsonga et al., 2009), channel catfish (Yildirim-Aksoy et al., 2008), tiger puffer (Eo et al., 2008) and African catfish (Olumeyi et al., 2008) when fed with vitamin C diets. The increased levels of TEC, TLC and HCT noticed in the present study would be attributed to the effect of vitamin C in protecting the cells from oxidation (Sahoo & Mukherjee, 2003), blood to attain more leukocytes (Verlhac et al., 1996) and prevent anaemic condition results in less hematocrit content (Talpur & Ikhwanuddin, 2013).

In conclusion, the dietary vitamin C positively increased the hematological values of *P. hypophthalmus* juveniles; but the higher values for most of the hematological parameters were observed between 175 to 350 mg AA kg^{-1} in the diet. Based on the results, the incorporation of aforesaid level of vitamin C in the diet of *P. hypophthalmus* juveniles would be economically beneficial for farmers.

Acknowledgements

The authors are very grateful to the Director, ICAR-Central Institute of Fisheries Education, Mumbai (India) for providing the necessary facilities, funds, support and encouragement to complete the research work.

References

Adham, K. G., Hashem, H. O., Abu-Shabana, M. B. and Kamel, A. H. (2000) Vitamin C deficiency in the catfish *Clarias gariepinus*. Aquacult. Nutr. 6(2), 129 p

Ai, Q., Mai, K., Tan, B., Xu, W., Zhang, W., Ma, H. and Liufu, Z. (2006) Effects of dietary vitamin C on survival, growth, and immunity of large yellow croaker, *Pseudosciaena crocea*. Aquacult. 261(1): 327-336

Ai, Q., Mai, K., Zhang, C., Xu, W., Duan, Q., Tan, B. and Liufu, Z. (2004) Effects of dietary vitamin C on growth and immune response of Japanese seabass, *Lateolabrax japonicus*. Aquacult. 242(1): 489-500

Al-Amoudi, M. M., El-Nakkadi, A. M. N. and El-Nouman, B. M. (1992) Evaluation of optimum dietary requirement of vitamin C for the growth of *Oreochromis spilurus* fingerlings in water from the Red Sea. Aquacult. 105(2): 165-173

Anbarasu, K. and Chandran, M. R. (2001) Effect of ascorbic acid on the immune response of the catfish, *Mystus gulio* (Hamilton), to different bacterins of *Aeromonas hydrophila*. Fish Shellfish Immunol. 11(4): 347-355

Andrade, J. I. A., Ono, E. A., de Menezes, G. C., Brasil, E. M., Roubach, R., Urbinati, E. C., Tavares-Dias, M., Marcon, J. L. and Affonso, E. G. (2007) Influence of diets supplemented with vitamins C and E on pirarucu (Arapaima gigas) blood parameters. Comp. Biochem. Physiol. A Mol Integr. Physiol. 146(4): 576-580

Austin, B. and Austin, D. A. (2007) Bacterial fish pathogens: disease of farmed and wild fish. Springer Science and Business Media

Azad, I. S., Dayal, J. S., Poornima, M. and Ali, S. A. (2007) Supra dietary levels of vitamins C and E enhance antibody production and immune memory in juvenile milkfish, *Chanos chanos* (Forsskal) to formalin-killed *Vibrio vulnificus*. Fish Shellfish Immunol. 23(1): 154-163

Barnerjee, S. K., Patra, B. C., Bandoyopahyay, P. and Teway, A. (2002) Changes of the blood parameters in Carp, *Catla catla*. J Aquat. Biol. 17: 79-84

Chandra, S. K. and Chandra, R. K. (1985) Nutrition, immune response, and outcome. Prog. Food Nutr. Sci. 10(1-2): 1-65

Chatterjee, I. B., Majumder, A. K., Nandi, B. K. and Subramanian, N. (1975) Synthesis and some major functions of vitamin C in animals. Ann. N. Y. Acad. Sci. 258(1): 24-47

Chen, R., Lochmann, R., Goodwin, A., Praveen, K., Dabrowski, K. and Lee, K. J. (2004) Effects of dietary vitamins C and E on alternative complement activity, hematology, tissue composition, vitamin concentrations and response to heat stress in juvenile golden shiner (*Notemigonus crysoleucas*). Aquacult. 242(1): 553-569

Cuevas, A., Febrero, M. and Fraiman, R. (2004) An anova test for functional data. Comput. Stat. Data Anal. 47(1): 111-122

Doumas, B. T., Watson, W. A. and Biggs, H. G. (1997) Albumin standards and the measurement of serum albumin with bromcresol green. Clin. Chim. Acta. 258(1): 21-30

Duncan, D. B. (1955) Multiple range and multiple F-tests. Biometrics. 11: 1-42

Eo, J. and Lee, K. J. (2008) Effect of dietary ascorbic acid on growth and non-specific immune responses of tiger puffer, *Takifugu rubripes*. Fish Shellfish Immunol. 25(5): 611-616

Esiobu, N., Armenta, L. and Ike, J. (2002) Antibiotic resistance in soil and water environments. Int. J. Environ. Health Res. 12(2): 133-144

Fange, R. (1994) Blood cells, haemopoiesis and lymphomyeloid tissues in fish. Fish Shellfish Immunol. 4(6): 405-411

Georgiev, G. S and Kamenov, I. (1980) Serum proteins of rainbow trout in viral hemorrhagic septicemia. J. Vet. Med. 17(1): 52-57

Halver, J. E. and Hardy, R. W. (2002) Fish nutrition. 3rd edn., Academic press, 824p

Hendrick, L. J. (1952) Erythrocytes counts and haemoglobin determinations for two species of sucker, genus *Catostomus* from Colorado. Copeia 4: 265-266

Jeney, G. and Anderson, D. P. (1993) Enhanced immune response and protection in rainbow trout to *Aeromonas salmonicida* bacterin following prior. Fish Shellfish Immunol. 3: 51-58

Klasing, K. C. (1998) Nutritional modulation of resistance to infectious diseases. Poult Sci. 77(8): 1119-1125

Lakra, W. S. and Singh, A. K. (2010) Risk analysis and sustainability of *Pangasianodon hypophthalmus* culture in India. Aquacult. Asia. 15(1): 34-37

Lim, C., Klesius, P. H., Li, M. H. and Robinson, E. H. (2000) Interaction between dietary levels of iron and vitamin C on growth, hematology, immune response and resistance of channel catfish (*Ictalurus punctatus*) to *Edwardsiella ictaluri* challenge. Aquacult. 185(3): 313-327

Lin, M. F. and Shiau, S. Y. (2005) Requirements of vitamin C (L ascorbyl 2 sulphate and L ascorbyl 2 polyphosphate) and its effects on non specific immune responses of grouper, *Epinephelus malabaricus*. Aquacult. Nutr. 11(3): 183-189

Misra, C. K., Das, B. K., Mukherjee, S. C. and Pradhan, J. (2007) Effects of dietary vitamin C on immunity, growth and survival of Indian major carp, *Labeo rohita*, fingerlings. Aquacult. Nutr. 13(1): 35-44

Mommsen, T. P., Vijayan, M. M. and Moon, T. W. (1999) Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fish. 9: 211-268

Nelson, N. (1944) Photometric adaptation of the somogyi method for determination of glucose. J. Biol. Chem. 153: 375-380

Nsonga, A. R., Kang'Ombe, J., Mfitilodze, W., Soko, C. K. and Mtethiwa, A. H. (2009) Effect of varying levels of dietary vitamin C (ascorbic acid) on growth, survival and hematology of juvenile tilapia, *Oreochromis karongae* (Trewavas, 1941) reared in aquaria. Braz. J. Aquat. Sci. Technol. 13(2): 17-23

Oluyemi, K. G., Adeparusi, E. A. and Olanrewaju, J. (2008) Basic haematogical parameters in African catfish, *Ciarías gariepinus* (Burchell, 1822) fed ascorbic acid supplemented diets. Res. J. Anim. Sci. 2: 17-21

Woo, P. T. K and Bruno, D. W. (1998) Fish diseases and disorders: viral, bacterial and fungal infections. CABI. 3: 479-511

Pimpimol, T., Phoonsamran, K. and Chitmanat, C. (2012) Effect of Dietary Vitamin C Supplementation on the Blood Parameters of Mekong Giant Catfish (*Pangasianodon gigas*). Int. J. Agric. Biol. 14(2): 256-260

Reinhold, J. G. (1953) Total protein, albumin and globulin. Stand. methods Clin. Chem. 1: 88-97

Rico, A., Phu, T. M., Satapornvanit, K., Min, J., Shahabuddin, A. M., Henriksson, P. J., Murray, F. J., Little, D. C., Dalsgaard, A. and Van den Brink, P. J. (2013) Use of veterinary medicines, feed additives and probiotics in four major internationally traded aquaculture species farmed in Asia. Aquacult. 412: 231-243

Rudneva, I. I. and Kovyrshina, T. B. (2011) Comparative study of electrophoretic characteristic characteristics of serum albumin of round goby *Neogobius melanostomus* from Black Sea and Azov Sea. IJABBR. 1(1): 131-136

Sahoo, P. K. and Mukherjee, S. C. (2003) Immunomodulation by dietary vitamin C in healthy and aflatoxin B 1-induced immunocompromised rohu (*Labeo rohita*). Comp Immunol Microbiol Infect Dis. 26(1): 65-76

Sandnes, K. (1991) Vitamin C in fish nutrition-a review. Fisk. Dir. Skr. Ser. Ernering. Vol. IV, No 1: 3-32

Shahkar, E., Yun, H., Kim, D. J., Kim, S. K., Lee, B. I. and Bai, S. C. (2015) Effects of dietary vitamin C levels on tissue ascorbic acid concentration, hematology, non-specific immune response and gonad histology in broodstock Japanese eel, *Anguilla japonica*. Aquacult. 438: 115-121

Shaw AF (1930) A direct method for counting the leucocytes, thrombocytes and erythrocytes of birds blood. J. Path. Bacteriol. 33: 833-835

Shiau, S. Y. and Jan, F. L. (1992) Dietary Ascorbic Acid Requirement of Juvenile Tilapia, *Oreochromis niloticus* X *O. aureus*. B Jpn Soc Sci Fish. 58(4): 671-675

Singh, A.K. and Lakra, W.S. (2012) Culture of *Pangasianodon hypophthalmus* into India: impacts and present scenario. Pak. J. Biol. Sci. 15(1),19 p

Siwicki, A. K. (1989) Immunostimulating influence of levamisole on nonspecific immunity in carp (*Cyprinus carpio*). Dev. Comp. Immunol. 13(1): 87-91

Snieszko, S. F. (1974) The effects of environmental stress on outbreaks of infectious diseases of fishes. J. Fish Biol. 6(2): 197-208

Sugita, H., Shibuya, K., Shimooka, H. and Deguchi, Y. (1996) Antibacterial abilities of intestinal bacteria in freshwater cultured fish. Aquacult. 145(1): 195-203

Talpur, A. D. and Ikhwanuddin, M. (2013) *Azadirachta indica* (neem) leaf dietary effects on the immunity response and disease resistance of Asian seabass, *Lates calcarifer* challenged with *Vibrio harveyi*. Fish Shellfish Immunol. 34(1): 254-264

Van Kampen, E. J and Zijlstra, W. G. (1961) Recommendations for haemoglobinometry in human blood. Bri. J. Haematol. 13: 71

Verlhac, V., Gabaudan, J., Obach, A., Schüep, W. and Hole, R. (1996) Influence of dietary glucan and vitamin C on non-specific and specific immune responses of rainbow trout (*Oncorhynchus mykiss*). Aquacult. 143(2): 123-133

Wang, X., Kim, K. W., Bai, S. C., Huh, M. D. and Cho, B. Y. (2003) Effects of the different levels of dietary vitamin C on growth and tissue ascorbic acid changes in parrot fish (*Oplegnathus fasciatus*). Aquacult. 215(1): 203-211

Wiegertjes, G. F., Stet, R. M., Parmentier, H. K. and van Muiswinkel, W. B. (1996) Immunogenetics of disease resistance in fish: a comparative approach. Dev. Comp. Immunol. 20(6): 365-381

Yildirim Aksoy, M., Lim, C., Li, M. H. and Klesius, P. H. (2008) Interaction between dietary levels of vitamins C and E on growth and immune responses in channel catfish, *Ictalurus punctatus* (Rafinesque). Aquacult. Res. 39(11): 1198-1209

Zhou, Q., Wang, L., Wang, H., Xie, F. and Wang, T. (2012) Effect of dietary vitamin C on the growth performance and innate immunity of juvenile cobia (*Rachycentron canadum*). Fish Shellfish Immunol. 32(6): 969-975

Zhou, Q. C., Wu, Z. H., Tan, B. P., Chi, S. Y. and Yang, Q. H. (2006) Optimal dietary methionine requirement for juvenile cobia (*Rachycentron canadum*). Aquacult. 258(1): 551-557

Zhou, X., Xie, M., Niu, C. and Sun, R. (2003) The effects of dietary vitamin C on growth, liver vitamin C and serum cortisol in stressed and unstressed juvenile soft-shelled turtles (*Pelodiscus sinensis*). Comp. Biochem. Physiol. A Mol Integr. Physiol. 135(2): 263-270