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Effect of Dietary Supplementation of Vitamin C and Seeds of *Achyranthes aspera* on Growth, Digestive Enzyme Activities, Immune System and Lipid Peroxidation of Snow Trout *Schizothorax richardsonii*

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Abstract

Nutrition plays significant role in the survival, growth and physiology of fish. Snow trout *Schizothorax richardsonii* larvae were cultured under three feeding regimes: control diet (diet D1) and test diets, supplemented with vitamin C (800 mg/kg diet, diet D2) and *Achyranthes aspera* seeds (5 g/kg diet, diet D3). There was no significant ($P > 0.05$) difference in the survival rate of larvae cultured under three different feeding regimes. Significantly ($P < 0.05$) higher average weight and specific growth rate and lower food conversion ratio were found in D3 diet fed larvae compared to others. Amylase, total protease, trypsin and lipase activities were significantly ($P < 0.05$) higher in D3 diet fed larvae and chymotrypsin activity was significantly ($P < 0.05$) higher in D2 diet fed larvae compared to others. Dietary supplementation of vitamin C and *Achyranthes aspera* seeds improved immune system as myeloperoxidase and nitric oxide synthase levels were significantly ($P < 0.05$) higher in enriched diets fed larvae compared to the control one. Reduced lipid peroxidation was recorded in enriched diets fed larvae as thiobarbituric acid reactive substances level was significantly ($P < 0.05$) lower in D2 and D3 diets fed larvae compared to the control diet fed larvae.

Keywords: *Schizothorax richardsonii; Achyranthes aspera;* Vitamin C; Digestive enzymes; Myeloperoxidase; Lipid peroxidation.

Introduction

Schizothorax richardsonii (Gray) is an important food fish of the Himalayan region. So far fish were harvested from the natural resources. Development of proper husbandry is required for the sustainable aquaculture development in the hill region. Considerable emphasis has been given for the development of reproductive techniques *viz*. induced breeding, artificial fertilization, egg rearing, hatching and semen cryopreservation for snow trout species [1] [2] [3] [4] [5]. Though artificial propagation of snow trout through induced breeding has been successful [1] [3], rearing of snow trout larvae under controlled condition is still less explored. The major bottleneck in culturing snow trout has been reported to be slow growth rate and poor disease resistance capacity [6] along with shortage of current literature on snow trout nutritional aspect.

Inadequate nutritional and culture conditions attribute slow growth rate and poor disease resistance in many farmed fish. Hence, supply of proper diet is a necessity in

preserving animal's health and maintaining its ability to resist the disease [7]. Snow trout being a teleost, lacks the enzyme L-gulono-lactone oxidase which is responsible for the endogenous synthesis of ascorbic acid (AA) from L-gulonolacetone in liver and kidney [8]. Hence, dietary supplementation of vitamin C is necessary to improve immunomodulatory properties [9] [10], metabolic antioxidant function [11]. It serves as a cofactor in the hydroxylation of proline and lysine in collagen synthesis [12]. Vitamin C has been used in feed for improving fish immunity and growth [13] [14], reproduction and health [15] [16] and tissue protection against UV-B radiation [17].

Dietary supplementation of plant ingredients as immunostimulants to modulate the non-specific immune system of fish or prophylactic measure against disease [18] has become an important area in aquaculture to avoid the ill effect of use of antibiotics. *Achyranthes aspera* L. (family: Amaranthaceae) is considered as a medicinal plant, used traditionally in treating fever, especially malarial fever, dysentery, asthma, hypertension and diabetes [19] [20] in humans. Incorporation of plant ingredients in diets enhanced the survival rate, growth and disease resistance in carps *Catla catla*, catla [21], *Labeo rohita*, rohu [22] and *Cyprinus carpio*, common carp [23].

Most of the growth performance studies in snow trout have been conducted with grow-out stage [24] [25] [26] [27] and brooders [28]. Review of literature shows that there is dearth of information on the physiology of larval period, especially the digestive enzyme profile; information related to larval immune system is also not available. Small differences in early growth and survival rates can affect the number of recruits entering the adult stock [29] [30]. Haematological parameters are good indicators of health status of fish and therefore, are important in diagnosing the structural and functional status of fish exposed to toxicant [31]. Myeloperoxidase is an abundantly expressed lysosomal protein stored in neutrophil. Release of myeloperoxidase by neutrophils and monocytes during inflammation plays an important role in the innate immune response [32].

The amino transferases, aspartate aminotransferase (SGOT) and alanine aminotransferase (SGPT) are usually found in different tissues *viz*. liver, muscles, kidney etc. Elevated amount of these amino transferases are indicators of tissue damage. Nitric oxide synthase are a family of enzymes that catalyze the production of cellular signalling molecule nitric oxide. This nitric oxide plays vital role in many biological processes. Oxidation of lipid shows the free radical-induced damaged to aquatic organisms. Elevated amount of thiobarbituric acid reactive substances (TBARS) is an indicator of lipid peroxidation in tissues. The knowledge of digestive enzyme profile (*viz*. amylase, total protease, trypsin, chymotrypsin and lipase) and immune system of larvae is required for the development of proper culture technique of a new aquaculture species. This will enhance the survival rate, promote growth and help in the production of healthy, disease free stocks. Therefore, the present study aimed to evaluate the effect of vitamin C and *Achyranthes aspera* seeds enriched diets on the digestive enzyme profile, immune system and lipid peroxidation of snow trout *Schizothorax richardsonii* (family: Cyprinidae) larvae.

Materials and Methods

Source and culture of larvae

Snow trout *Schizothorax richardsonii* larvae were collected from Gandi River, Champawat, Uttrakhand (29°20'44" N and 80°6'19" E) and transported to Delhi in oxygenated plastic packets. Larvae were acclimated in glass aquaria and fed with live zooplankton and prepared diet (40% protein) *ad libitum* twice a day. The composition of zooplankton was as follows: *Brachionus calyciflorous* (32.5%), *Ceriodaphnia cornuta* (20.7%), *Asplanchna* sp. (8.1%) and nauplii (38.5%). The culture units were fitted with chilling (Hailea 300, China) and a filtration unit (Sera bioactive, Germany) for the maintenance of optimum temperature (18 - 20°C) and to reduce ammonia level in the culture unit. After 25 days larvae (0.54 ± 0.03 g) were distributed randomly in glass aquaria (10 l). The stocking density was 15 larvae/ aquarium.

Three diets are formulated - control diet (diet 1, D1) without seeds and vitamin C and two test diets (Table 1). In diet 2 (D2), vitamin C (L-ascorbate-2-triphosphate calcium salt, Himedia Laboratory Pvt. Ltd., Mumbai, India) was incorporated at the rate of 800 mg/kg diet; diet 3 (D3) was enriched with *Achyranthes aspera* seeds (5 g/kg diet). Larvae were fed with one of the three diets. Three replicates were maintained for each feeding regime. The dose of vitamin C and *Achyranthes aspera* seeds were selected based on the previous study [33] [17] [34]. Larvae were fed twice daily at the rate of 5% of body weight. The whole amount of diet was divided into two equal amounts and was fed at 0900 hours and 1700 hours. Duration of experiment was 72 days. Survival rate, length and weight of individual larva were recorded.

Table1: Proximate composition of control and test diets of the *Schizothorax richardsonii* larvae.

Ingredient (g/kg)	Diets		
	D1	D2	D3
Dry fish powder	583.3	583.3	583.3
Wheat flour[a]	402.7	401.9	397.7
Cod liver oil[b]	10.0	10.0	10.0
Vitamin-mineral premixes[c]	4.0	4.0	4.0
Vitamin C[d]	-	0.8	-
Achyranthes aspera seeds	-	-	5.0
Proximate analysis (% dry matter basis)			
Crude protein	44.63	42.1	44.63
Crude fat	7.1	6.48	7.62
Energy value (cal/g)	3.60	3.49	3.68

[a]Local market, [b]SEACOD©, BP Universal Medicare Pvt. Ltd., Mumbai, India. [c]Supradyn, Bayer Consumer Care AG, Basel Switzerland. [d]HiMedia Laboratories Pvt., Mumbai, India.

D1= Control diet; D2= Vitamin C incorporated diet; D3= *Achyranthes aspera* seeds incorporated diet.

Water temperature, pH, conductivity, total dissolved solids (TDS) and salinity of the culture units were measured using Eutech probe (PCSTestr 35, USA) and dissolved oxygen

level through HACH (HQ 40d, USA). The ammonia (NH_3^+) and Nitrate (NO_3^-) concentrations in the water of the culture unit was estimated with Orion™ Versastar Probe (Thermo Scientific, USA) and nitrite (NO_2^-) was measured following the method of APHA [35].

Biochemical assays

Digestive enzymes

The digestive system from individual larva was collected and tissue from five larvae was pooled to make one replicate; pooled sample was weighed and homogenized in 1 ml of chilled distilled water (pH 7.0). Three replicates were used for each parameter. The homogenate was centrifuged at 10000 x *g* for 30 min at 4°C. The supernatant was collected for the enzyme assays. Total soluble protein was measured according to Bradford [36] with bovine serum albumin (BSA) as standard (1 mg/ ml).

Amylase activity was estimated using EnzChek ® Ultra Amylase Assay Kit (E33651) of Molecular Probes™ Invitrogen (Oregon, USA) at 485 nm (excitation) and 520 nm (emission) in fluorometer (Biotek Synergy H1, USA). Amylase activity was expressed as U/mg protein/min. Molecular Probes EnzChek® Protease Assay Kits (E6638) - green fluorescence (Oregon, USA) was used to estimate total protease activity. The change in fluorescence at excitation (485 nm) and emission (530 nm) was recorded and activity was expressed as fluorescence change/µl/min. Trypsin activity was estimated using BZ-L-Arg-MCA (4-methylcoumarinyl-7-amide) as substrate [37]. The change in fluorescence was recorded after 2, 4, 6 and 8 min at excitation (380 nm) and emission (440 nm) using the fluorometer at 37°C. Trypsin activity was expressed as the amount of AMC (nM)/mg protein/min. Chymotrypsin activity was estimated with substrate Suc-Leu-Leu-Val-Tyr-MCA (4-methylcoumarinyl-7-amide) following the method of Cao et al [38]. The fluorescence intensity from 7-amino-4-methylcoumarin (AMC) liberated in the hydrolysis of substrate was measured at excitation (380 nm) and emission (450 nm). The activity was expressed as the amount of AMC (nM)/mg protein/min. Activity of neutral lipase was determined using 4-methylumbelliferyl butyrate (4-MUB), a non-fluorescent substrate [39]. The change in fluorescence was measured at excitation (365 nm) and emission (450 nm). The lipase activity was expressed as the amount of 4-MU (nM) liberated/mg protein/min.

Immunological assays

Myeloperoxidase activity (MPO) activity in serum of larvae was estimated following the method of Quade and Roth [40]. Optical density of the reaction mixture was recorded at 450 nm using Microplate reader (Synergy HT, Biotek, Loveland Colorado, USA). Nitric oxide synthase (NOS) activity in the myotomes of larvae was estimated following the method of Lee et al [41]. The absorbance was recorded at 540 nm. The nitrite concentration was expressed as mol/mg tissue.

Lipid peroxidation and stress indicators

The concentration of thiobarbituric acid reactive substances (TBARS) in the myotomes was estimated [42]. The absorbance was recorded at 532 nm. and the concentration was expressed as µM MDA/mg protein. Serum glutamic oxaloacetic transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) were estimated using kits (Bayer Diagnstics, Baroda, India). The absorbance was recorded at 340 nm and level was expressed as IU/l.

Length-weight relationship

The length-weight relationship [43] was determined by measuring the total length (cm) and weight of individual larva and using the log transformation equation:

$$W = aL^b$$

Where, W = weight of larva in g, L = total length in cm, a = exponent describing the rate of change of weight with length (intercept of the regression line on the Y axis) and b = slope of the regression line or allometric coefficient.

Specific growth rate, food conversion ratio and protein efficiency ratio

Specific growth rate was calculated using the following formula:

$$SGR\ (\%) = \frac{(\ln \text{ final weight of fish} - \ln \text{ initial weight of fish}) \times 100}{\text{Duration of experiment}}$$

Food conversion ratio (FCR) was calculated using the formula:

$$FCR = \frac{\text{Total weight of the feed consumed (g)}}{\text{Total weight gain of the fish (g)}}$$

The protein efficiency ratio (PER) was evaluated to quantify the nutritional value of diets using the formula:

$$PER = \frac{\text{Total wet weight gain (g)}}{\text{Dry weight of protein in diet (g)}}$$

Statistical analysis

Results were given as mean ± SE One-way analysis of variance (ANOVA) and regression analyses were performed for various parameters. Statistical significance was accepted at $P < 0.05$ level.

Results

Water quality parameters

Water quality parameters *viz*. temperature, pH, dissolved oxygen, ammonia, and nitrites were not significantly ($P > 0.05$) different among treatments throughout the study period. Water temperature ranged from 18.7 - 21.8°C in various days of culture in three different feeding regimes. pH ranged from 7.6 - 7.9, 7.67 - 7.9 and 7.56 - 7.87 in D1, D2 and D3 treatments, respectively during the study period. Dissolved oxygen levels were 7.8 ± 0.05, 7.7 ± 0.14 and 7.71 ± 0.06 mg/l in D1, D2 and D3, respectively. Ammonia concentrations were 0.14 ± 0.01, 0.10 ± 0.06 and 0.10 ± 0.02 mg/l in D1, D2 and

D3, respectively during the study period. Nitrite levels were 0.61 ± 0.05, 0.60 ± 0.04, 0.62 ± 0.20 mg/l and nitrate levels were 0.11 ± 0.02, 0.10 ± .01, 0.09 ± 0.01 mg/l in D1, D2 and D3, respectively during the culture period.

Performance of larva

There was no significant (P > 0.05) difference in the survival rate of larvae cultured under three different feeding regimes. Survival rates were 91 ± 1, 93 ± 1 and 93 ± 1% in D1, D2 and D3, respectively. The average weight of larvae was significantly (P < 0.05) higher in fish feed with *Achyranthes aspera* seeds supplemented diet (D3) compared to others (Table 2). The length and weight relationship of snow trout larvae cultured in three different feeding regimes showed close R^2 values (D1: 0.959, D2: 0.961 and D3: 0.971). The allometric coefficient value "b" was 2.872, 2.67 and 2.74 for D1, D2 and D3, respectively. Significantly (P < 0.05) higher specific growth rate and protein efficiency ratio were found in larvae fed with diet D3 compared to others. FCR value was significantly (P < 0.05) lower in larvae fed with diet D3 compared to other two feeding regimes (Table 2).

Table 2: Growth and feed efficiency parameters of *Schizothorax richardsonii* larvae. Values are represented as Mean ± SE (n=3).

Parameters	Diets		
	D1	D2	D3
Final length (cm)	5.06 ± 0.10	5.18 ± 0.32	5.72 ±0.51
Final weight (g)	1.035 ± 0.03	1.318 ± 0.16	1.57 ± 0.02
R^2	0.959	0.961	0.971
b	2.872	2.699	2.738
K (g/cm)	0.983 ± 0.02	1.634 ± 0.26	1.324 ± 0.04
PER (%)	0.508 ± 0.01	0.948 ± 0.12	1.25 ± 0.16
SGR (%/d)	0.828 ± 0.04	1.23 ± 0.2	1.53 ± 0.5
FCR	3.12 ± 1.04	1.9 ± 0.10	1.44 ± 0.12

R^2= Coefficient of determination; b= Slope; K= Condition factor; PER= Protein efficiency ratio; SGR= Specific growth rate; FCR= Feed conversion ratio; D1= Control diet; D2= Vitamin C incorporated diet; D3= *Achyranthes aspera* seeds incorporated diet.

Biochemical assays

Amylase, total protease, trypsin and lipase activities were significantly (P < 0.05) higher in larvae fed with D3 diet compared to other feeding regimes. Chymotrypsin activity was significantly (P < 0.05) higher in fish fed with vitamin C supplemented diet compared to others. This group was followed by D3 diet fed larvae (Table 3).

Myeloperoxidase and nitric oxide synthase levels were significantly (P < 0.05) higher in larvae fed with vitamin C and *Achyranthes aspera* seeds supplemented diets compared to the control diet fed larvae. There was no significant (P > 0.05) difference in nitric oxide synthase level in larvae fed with D2 and D3 diets. Thiobarbituric acid reactive substances level was significantly (P < 0.05) lower in larvae fed with diet D3 compared to others. SGOT level was minimum in D2 diet fed larvae (Table 3). There was no significant (P > 0.05) difference in SGOT level in larvae fed with D1 and D3 diets. There was no significant (P > 0.05) difference in SGPT level in different diets fed larvae.

Table 3: Digestive enzyme activities, immunological, lipid peroxidation, SGOT and SGPT levels of *Schizothorax richardsonii* larvae cultured in three different feeding regimes. Values are represented as Mean ± SE (n=3).

Parameters	Diets		
	D1	D2	D3
Digestive enzymes			
Amylase (U/mg protein/min)	17.25±0.04	17.73±0.04	27.36±0.06
Total protease (Fluorescence change/µl/min)	706.26±34.23	731.06±29.4	848.25±29.34
Trypsin (nM AMC/mg protein/min)	3600.86±13.16	4345.33±40.5	7714.61±46.1
Chymotrypsin (nM AMC/mg protein/min)	4691.68±80.03	6315.6±41.4	5353.28±43.9
Neutral lipase (nM MUB/mg protein/min)	965.82±57.8	1338.1±33.3	1398.58±93.8
Immunological parameters			
Myloperoxidase (OD at 450 nm)	1.616±0.38	2.419±0.01	2.808±0.08
Nitric oxide synthase (mol/mg tissue)	72.128±3.36	107.13±7.95	104.003±4.57
Lipid peroxidation and stress indicators			
Thiobarbituric reactive substances (µM MDA/mg protein)	23.54±1.8	13.85±0.85	5.82±1.44
Serum glutamate oxalate transaminase (SGOT, IU/l)	64.78±0.05	57.93±0.02	62.71±0.13
Serum glutamate pyruvate transaminase (SGPT, IU/l)	59.99±0.18	58.01±0.43	58.63±0.14

D1= Control diet; D2= Vitamin C incorporated diet; D3= *Achyranthes aspera* seeds incorporated diet

Discussion

In the present study, supplementation of vitamin C and *Achyranthes aspera* seeds resulted in better performance of larvae compared to the control diet fed larvae. Vitamin C has been identified as a very essential nutrient for fish since the only source of vitamin C in the fish's body is through the diet [44]. The essentiality of vitamin C as nutrient for optimum growth and maintenance [45] [46] and its role in certain aspects of protein metabolism [47] are also reflected in FCR, PER and SGR values in present study. Supplementation of vitamin C in fish feed resulted in better survival and growth of tilapia *Oreochromis spirulus* [48], yellow croaker *Pseudociaena crocea* [49] and parrot fish *Oplegnathus fasciatus* [13]. The overall well being of fish fed with different diets was assessed through the length-weight relationship. Goel et al [50] ascribed that the ideal b value of *Schizothorax richardsonii* as 2.68. In the present study, b value ranged from 2.67-2.87. This showed the optimum culture conditions were maintained for this species.

Supplementation of seeds of *Achyranthus aspera* in diets enhanced survival, growth and feed utilization in rohu [17] [34] and common carp [23]. Higher growth rate in D3 diet fed snow trout was due to the presence of highly nutritive value of ingredients and presence of bioactive molecules like oleanolic acid, bis-desmosidic-triterpinoid-based saponins, ecdysterone and various amino acids in the seeds [51] [52].

Ecdysterone is reported to have pronounced growth-promoting effect due to high rate of protein synthesis [53]. In common carp, enhanced growth rate was recorded due to the presence of ecdysterone in the diet compared to the control diet fed fish [23].

The efficiency of food absorption and conversion also depend on the availability of digestive enzymes or on the capacity for trans-epithelial transport in the digestive tract [54]. A positive correlation was found between trypsin and chymotrypsin activities and FCR in striped bass *Morone saxatilis* [55], Atlantic cod *Gadus morhua* [56] and in Atlantic salmon *Salmon salar* [57]. Similar relationship was also found between the trypsin activity and FCR in the present study. Trypsin activity was reported to relate with growth rate of larvae of goldfish *Carassius auratus* L [58] and sea bass *Dicentrarchus labrax* [59] fed with high quality diets. In addition, the difference in trypsin activity level may have indirect effect on the immune system as it affects variations in nutrient influx [60], and dietary nutrients play important roles in relation to the immune function of fish [61].

Immunostimulants activate non-specific defence mechanisms to protect the fish against pathogens [62]. Snow trout larvae fed with vitamin C and *Achyranthus aspera* seeds supplemented diets showed significantly higher nitric oxide synthase and myeloperoxidase levels compared to the control diet fed larvae. Elevated NOS and MPO levels indicated prophylactic measure of the larvae against possible pathogen attack. Feeding of curcumin *Curcuma longa* to rohu [63] and levamisole to catla [64] and catfish *Clarias batrachus* [65] resulted in enhanced MPO level. This was an indication of positive non-specific immune response. According to Anderson and Siwicki [66], reduction of MPO activity was considered as stress in fish. The inducible isoform iNOS of nitric oxide synthase catalyze the production of cellular signalling molecule nitric oxide [67]. Higher level of nitric oxide synthase in catla [21], rohu [23] and common carp [22] was indicator of better immune system.

The source of dietary energy modulates lipid oxidation in muscle homogenates of rainbow trout and sea bass [68] [69] [70]. Supplementation of vitamin C and seeds of *Achyranthes aspera* in diets showed less lipid peroxidation compared to the control diet fed snow trout. Insignificant difference in SGOT and SGPT levels in snow trout larvae fed with different diets suggested the absence of challenge/ stress in the culture system. Higher levels of these enzymes indicated poor physiological status of the organism.

Conclusions

In conclusions, supplementation of vitamin C and seeds of *Achyranthes aspera* in diet enhanced the survival, growth and improved the digestive physiology of snow trout larvae. It also boosted the immune system of larvae.

Conflict of interest

The authors have declared no conflict of interest.

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