www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 18:1303-1313(2018) DOI: 10.4194/1303-2712-v18_11_07

RESEARCH PAPER

Dietary Carotenoid Supplementation Improves Fillet Appearance, Antioxidant Status and Immuneresponses in Striped Catfish (*Pangasianodon hypophthalmus*) Neverthless the Growth Performance

Amrutha Gopan[1], Muralidhar P Ande[2,*], Tincy Varghese[1], Narottam Prasad Sahu[1], Syamlal Lalappan[1], P.P Srivastava[1], K.K. Jain[1]

[1] Division of Fish Nutrition, Biochemistry and Physiology, ICAR-Central Institute of Fisheries Education, Mumbai- 61, India.
[2] ICAR-Central Institute of Fisheries Education, Beach Road, Near Old Burmah Shell, Kakinada, Pin- 11 533 001, Andhra Pradesh, India.

* Corresponding Author: Tel.: +91.884 2379146; Fax: +91.884 2373602;
E-mail: ande.muralidhar@gmail.com

Received 24 June 2017
Accepted 15 January 2018

Abstract

A feeding trial of 45 days was conducted for delineating the effect of carotenoids on the fillet appearance, antioxidant status and nonspecific immune responses of *Pangasianodon hypophthalmus* fingerlings. Two hundred and fifty-two fingerlings were randomly distributed into seven different experimental groups in triplicates. Seven isonitrogenous and isolipidic diets were prepared with two levels (150 and 300 $mgkg^{-1}$) of astaxanthin (T2 & T3), beta-carotene (T4 & T5), canthaxanthin (T6 & T7) and a control diet (T1) without any carotenoids. The experimental fish were fed daily at the rate of 3% body weight in two instalments. At the end of the experiment, the fish were sampled to evaluate the colour and texture parameters, of fillets along with antioxidant and nonspecific immune status of fish. The fillet colour exhibited significantly higher ($P<0.05$) intensity of redness in the groups fed astaxanthin and a higher yellowish colouration in the beta-carotene fed groups. Significant ($P<0.05$) reduction in the activities of antioxidant enzymes and a significantly ($P<0.05$) higher PUFA/SFA ratio was also observed in astaxanthin fed groups. The total erythrocyte count, haemoglobin, respiratory burst, lysozyme activities were also enhanced in both astaxanthin fed groups (T2 & T3). The effect of carotenoids in modulating the texture of pangasiusfillets was diminutive and further, there was little influence of carotenoids on growth. Thus, from this study, it can be concluded that astaxanthin supplementation can improve fillet appearance, antioxidant status and enhance the nonspecific immune response.

Keywords: Carotenoids, fillet appearance, PUFA/SFA ratio, respiratory burst, lyzozyme, antioxidant enzymes.

Introduction

Striped catfish (*Pangasianodon hypophthalmus*) is a suitable candidate for aquaculture, particularly in Asia.Vietnam is the main exporter of this species; which produce 1.14 million tons with an estimated export income of US$ 1.4billion (De Silva & Phuong, 2011). Pangasius culture in India has flourished successfully though it got introduced only in the late 1990's. It is estimated that *P. hypophthalmus* is being farmed in an area of about 40,000 ha in India and contributes 0.7 million tons to world fishery (Singh & Lakra, 2012). Even though production of pangasius is at a faster rate, the export of this species is on a slower pace. The major factor which decides the consumer preference of pangasius is the appearance of the fillet where white fillets often considered as of superior quality and any deviation from this colour are found to be of inferior quality in the seafood industry. Light pink coloured fillets are considered to be second best to white coloured fillet, in quality. Thus, yellow discoloration of the fillets makes the fish lose its export value and find a place only in the domestic markets, fetching a lower price (Hu, Roy & Davis, 2013). This forms a major bottle neck to the pangasius farming in different countries, especially in India.

Changes in water temperature and intensification of the culture practices results in stress and then affect the growth, reproduction and survival of fish. Stressors can irritate the mucous coating of fish resulting in immune suppression which in turn cause disease outbreaks (Harikrishnan, Balasundaram & Heo, 2011). Use of antibiotics for disease management has a negative impact such as accumulation of antibiotic residues and antibiotic resistance (Schmidt, Bruun, Dalsgaard, Pedersen & Larsen, 2000). In this context, preventive measures by feeding immuno stimulant are a better practice than the antibiotic treatment.

Carotenoids are a class of 800 natural fat-soluble pigments with antioxidant properties, found in plants, algae, photosynthetic bacteria, non-photosynthetic bacteria and some of the members of animal kingdom

such as crustaceans, which is absorbable by the fish muscle. They are split into two classes, carotenes such as *β*-carotene and *α*-carotene, which are purely hydrocarbons, either linear or cyclized at one or both ends of the molecule, and contain no oxygen, whereas xanthophylls are the oxygenated derivatives of carotenes (Sajilata, Singhal, & Kamat,2008). Gupta, Jha, Pal & Venkateshwarlu (2007) used carotenoids from natural sources such as microalgal pigment, yeast extract, marigold, capsicum *etc*. in fish colour improvement. However, the major limitation in the use of carotenoids from natural sources is its low availability as they are embedded in the food matrix that must be digested before the carotenoid component can release and be made available (Zariphen & Erdman, 2002). Natural carotenoids are typically esterified with fatty acids that may negatively influence its absorption (Perez-Galvez & Minguez-Mosquera, 2005). Li, Robinson & Oberle (2007) reported that *Ictalurus punctatus* can accumulate yellow pigments lutein and zeaxanthin and pink pigments canthaxanthin and astaxanthin in the flesh. Fat-soluble carotenoids follow the same intestinal absorption pathways of dietary fats (Krinsky & Johnson, 2005).

Enhanced coloration through carotenoid deposition also protects health, as carotenoids have antioxidant functions (Baker & Gunther, 2004). Several authors (Amar, Kiron, Satoh, & Okamoto,2000; Amar, Kiron, Satoh& Watanabe, 2001; Amar, Kiron, Satoh & Watanabe, 2004) revealed effects of feeding carotenoids from both natural and synthetic sources on the non-specific immune responses of rainbow trout and found augmented non-specific responses along with protection from pathogenic challenge *in vivo*. The resistance of rainbow trout to infectious hematopoietic necrosis virus (IHNV) showed significant improvement after oral ingestion of natural and synthetic carotenoids, particularly astaxanthin (Amar, Kiron, Akutsu, Satoh & Watanabe, 2012). Thus, carotenoid incorporation in fish feeds can be considered as a viable option to improve the colouration, protection of the stored fat in muscle from peroxidation and enhancement of immune responses. With this background, the present study was designed to evaluate the effect of different carotenoids on pangasius flesh quality, antioxidant status and immune responses.

Materials and Methods

Experimental Fish and Diet

Seven purified isocaloric diets were prepared with same composition with graded level of different carotenoids (CD) like AX (Carophyll Pink, 10%AX), BC (Rovimix β-carotene, 5%BC) and CX (Carophyll Red,5% Canthaxanthin) (DSM Nutritional Products Ltd, Mumbai, India) and a control with no CD. The experimental diets used are T1-control, T2- AX, 150mgkg^{-1}, T3- AX, 300mgkg^{-1}, T4-BC 150mgkg^{-1}, T5-BC 300mgkg^{-1}, T6-CX, 150 mgkg^{-1} and T7- CX, 300 mgkg^{-1}. Purified diets were prepared by using fat-free casein and gelatin as the protein source, whereas sunflower oil and cod liver oil were used as a lipid source, Dextrin and starch soluble as a carbohydrate source (Table 1). All the ingredients were thoroughly mixed with water to make dough except vitamin-mineral mixture, carotenoids and betaine chloride followed by autoclaving. Butylated hydroxy toluene (BHT) dissolved in oil, vitamin mineral premix and solution of water soluble bead lets of each CDs were mixed properly with the dough after cooling. The feed was then pelletized by an automatic pelletizer (SB Panchal Company, India) using a 2.5 mm die. The pellets were dried manually crumbled, packed in airtight containers and stored at −20 °C to avoid oxidation of the CD until use.

Experimental Design

Fingerlings of pangasius with an average weight of 12-14g were procured from Hassan Muslai farm, Pali Village, Raigarh (M.H) and acclimatised to the experimental conditions for 15 days. During acclimatization in the Laboratory, fish were fed with control diet for two weeks and transferred to 21 tubs (80×57×42 cm, 150 L capacity), to receive their respective treatments (three replicates per treatment) at stocking density of 12 fish per tub following a completely randomised design. Round the clock aeration was provided to all the tubs from a compressed air pump and about 50% water was exchanged every other day. Uneaten feed and faecal matter were siphoned off every day. Dissolved oxygen concentration was monitored weekly, which ranged from 5.2- 6.8 mgkg^{-1} throughout the experimental period. Water temperature and pH ranged from 25.1^{0}C-28.4^{0}C and, 7.8 to 8.4 respectively. The feeding trial was conducted for 45 days. Feeding was done upto satiation level twice a day at 10:00 and 18:00 hours. At the end of the experiment, all fish were counted and weighed then used for analysis.

Growth Trial

During the feeding trial of 45 days, the fishes were batch weighed at every two weeks, after one-day feed deprivation. Three fish from each tank at the end of the experiment were sampled. Weight gain percentage, Specific growth rate (SGR), Feed conversion ratio (FCR) Protein efficiency ratio (PER) were determined according to the formulae given beneath Table 2. Difference in number of fish stocked at the beginning and end of the experimental trial were determined for calculation of survival.

Sample Preparation

Three fish from each replicate with a total of

Table 1. Composition of different Experimental Diets

Ingredients (g kg $^{-1}$)	T1	T2	T3	T4	T5	T6	T7
Casein[1](Hi Media, India)	350	350	350	350	350	350	350
Gelatine[2](Hi Media, India)	80	80	80	80	80	80	80
Starch(Hi Media, India)	200	200	200	200	200	200	200
Dextrin(Hi Media, India)	150	150	150	150	150	150	150
Cellulose(Hi Media, India)	82	81.85	81.7	81.85	81.7	81.85	81.7
codliver oil: Sunflower oil(1:1)	100	100	100	100	100	100	100
Vitamin-mineral mixture[3]	20	20	20	20	20	20	20
CMC(Hi Media, India)	15	15	15	15	15	15	15
BHT(Hi Media, India)	2	2	2	2	2	2	2
Betain chloride(Hi Media, India)	1	1	1	1	1	1	1
Astaxanthin	0	0.15	0	0	0	0	0
Astaxanthin	0	0	0.3	0	0	0	0
Beta carotene	0	0	0	0.15	0	0	0
Beta carotene	0	0	0	0	0.3	0	0
Canthaxanthin	0	0	0	0	0	0.15	0
Canthaxanthin	0	0	0	0	0	0	0.3
Total	1000	1000	1000	1000	1000	1000	1000
Proximate composition (g kg $^{-1}$DM)							
Dry matter	925.2 ±1.6	920.5 ±3.1	913.7 ±1.7	913.7 ±2.1	919.9 ±1.5	928.2 ±2.2	928.3 ±3.3
Ash	29.0±1.8	30.1±1.7	25.4±1.6	26.5±2.1	18.3±1.83	24.7±1.6	28.0±1.4
Crude protein	365.4±3.0	369.7±2.1	365.4±2.6	367.5±3.1	360.7±2.2	363.6±3.04	365.2±2.17
EE[4]	84.3±3.7	76.2±2.18	84.0±3.4	78.9±4.01	88.1±5.7	86.9±4.21	89.0 ± 6.05
CF[5]	102.0±5.71	99.6±4.44	99.4±5.21	100.8±9.5	97.4±8.3	109.0±7.5	104.3±3.1
NFE[6]	419.3±3.5	424.5±4.0	425.8±1.5	426.3±5.1	420.6±5.3	415.3±1.5	410.7±3.1
DE[7] (kJ g^{-1}Diet)	15.06±0.8	14.89±1.1	14.92±0.6	14.98±1.0	15.05±0.8	15.14±0.4	15.14±0.9

Data expressed as mean ± SE, n=3

[1] Casein fat free: 86% CP.

[2]Gelatin: 96% CP.

[3] Composition of vitamin mineral premix (AGRIMIN FORTE Powder) (quantity kg^{-1}). Vitamin A-7,00,000 IU; Vitamin D_3-70,000 IU; Vitamin E-250 mg; Nicotinamide-1000mg ; Cobalt-150 mg; Copper-1200 mg; Iodine-325 mg; Iron-1500 mg; Magnesium-6000 mg; Manganese-1500 mg; Potassium-100 mg; Sodium-5.9 mg; Sulphur-0.72%; Zinc-9600 mg; Calcium-25.5%; Phosphorus-12.75%

[4]EE- Ether Extract

[5]CF-Crude Fibre

[6]NFE (Nitrogen free extract) = 1000− (Crude protein + Crude lipid + crude fibre + ash).

[5]DE (Digestible energy) =(%CP×4) + (%EE×9) + (%NFE×4) (Halver, 1976

Table 2. Growth parameters and survival rate of *P. hypophthalmus* fingerling fed different experimental diets

Parameters	T1	T2	T3	T4	T5	T6	T7
Initial Body wt(gm)	14.38±0.50	13.95±0.21	14.71±0.49	14.67±0.62	14.52±0.42	14.26±0.48	14.48±0.25
Final Body wt(gm)	18.59±0.19	18.10±0.42	18.43±0.48	18.33±0.46	18.53±0.74	17.93±0.37	18.63±0.39
Wt Gain%[1]	29.55±2.19	29.73±2.37	25.35±1.36	25.18±2.14	27.52±1.45	25.86±1.77	28.76±2.70
SGR[2]	0.57± 0.07	0.58± 0.04	0.50± 0.02	0.50± 0.04	0.54± 0.03	0.51± 0.03	0.56±0.05
FCR[3]	2.52± 0.23	2.38± 0.19	2.72± 0.15	2.76± 0.21	2.53± 0.14	2.73± 0.13	2.46 ± 0.21
PER[4]	1.09± 0.11	1.15± 0.08	1.00 ± 0.05	0.99± 0.08	1.08± 0.06	0.99 ± 0.05	1.11 ± 0.09
Survival%	100.00	100.00	100.00	100.00	100.00	100.00	100.00
P value	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

Data expressed as mean ± SE, n=3

[1] Weight gain (%) =100 [(final body wt (g) ^ Initial body wt (g))/initial body wt (g)].

[2] Specific growth rate (SGR, %/day) =100×(ln final body weight−ln initial body weight)/experimental duration in days

3 Feed conversion ratio (FCR) = total dry feed given (g)/wet weight gain (g).

4 Protein efficiency ratio (PER) = Net weight gain (g)/protein fed (g).

nine from each treatment group were anaesthetized with clove oil (50µl of clove oil per litre of water). Fillets without skin were prepared for flesh quality analysis and for enzyme assays, tissues (liver and gill) were homogenized with chilled 0.25 M sucrose solution using a mechanical tissue homogenizer. The homogenized samples were centrifuged (7,000 g, 4 ^{0}C for 10 min) and supernatants were collected and stored at -20 ^{0}C for subsequent enzyme assays.

Proximate Composition of Diets

Experimental diets were analysed using standard AOAC (1995) methods (Table 1) for dry matter, crude protein, ether extract, ash, crude fibre. Nitrogen free extract (NFE) was calculated by difference of the total of all the nutrients from 100 (Hastings, 1969) and approximate digestible energy content was calculated according to Halver (1976).

Flesh Quality Analysis

Texture Profile Analysis (TPA)

Textural measurements were done by instrumental analysis with texture analyzer (Perten instrument, TVT 6700, Sweden). The texture is commonly measured and presented as some mechanical properties including hardness/firmness, gumminess, chewiness, springiness, (Hagen Solberg, Sirnes & Johnston, 2007). Three measurements were taken along the lateral line on both sides of the fish at the anterior part, middle part and posterior part of the body of fish. Analysis of sample was done by using software named Easy Match QC version 4.77 [Database EZQC].

Colour Analysis

Instrumental colorimetric analyses were performed with a Lab scan XE – spectro- colorimeter (Hunter Associates Laboratory Inc. Reston VA, USA). Fillet from each treatment group was pooled, minced and densely packed into the glass sample cup. Measurements were recorded using the tri-stimulus L* a* b* scale (Hunter & Harold1987). The L* variable represents lightness (L*=0 for black, L*=100 for white),a* scale represents the red/green (+a* intensity in red and –a* intensity in green) and the b* scale represents the yellow/blue (+b* intensity in yellow and –b* intensity in blue).Further, ratios such as Redness/yellowness (a*/b*) value and yellowness/lightness (b*/ L*) has been calculated to get more clarification on the obtained colour variables.

Total Carotenoid Content

The total carotenoid content was measured by the modified method (Olson, 1979). To 1g muscle taken in a 10 ml screw capped glass vial and added 2.5g of anhydrous sodium sulphate then the mixture was mashed gently with glass rod with adding 5ml of chloroform and sealed, which was kept at 0^0 C for overnight. A clear layer of chloroform above the caked residue was taken for measuring absorbance using as pectrophotometer. A blank was also prepared without the sample in a similar way.

Antioxidant Enzymes

Superoxide dismutase **(**SOD) was assayed according to Misra & Fridovich (1972) based on the oxidation of epinepherine–adrenochrome transition by the enzyme. The reaction mixture consisted of 50 ml of sample, 1.5 ml phosphate buffer and 0.5 ml epinephrine. The solution was mixed well and optical density (OD) was immediately read at 480nm. SOD activity is expressed as unit activity (amount of protein required to give 50% inhibition of epinephrine auto-oxidation). Catalase (CAT) was assayed according to the method described by Takahara, Hamiton, Neel, Kobara, Ogura, & Nishimura (1960) using 50 mM phosphate buffer (pH 7.0). The reaction was initiated by adding 30% H_2O_2 as the substrate and OD were recorded at 240 nm. Enzyme activity was expressed as nanomoles H_2O_2 decomposed /min /mg protein.

Estimation of Lipid Peroxidation

Fatty Acid Profiling and Estimation of PUFA/SFA Ratio

Crude lipid was extracted as described by the method of Folch, Lees, &Sloane-Stanley (1957). The extracted lipids were used for fatty acid analysis. Fatty acid methyl esters were prepared from the total lipids by saponification and methyl esterification (AOAC, 1995). The component fatty acids were then identified by a Gas Liquid Chromatography system. GC-MS measurements were performed in a Shimadzu QP2010 (Japan) quadrupole mass spectrometer, with ionization energy of 70 eV operating in positive electronic impact set to 100 µA, connected to a GC 8060 gas chromatograph (Shimadzu) equipped with a Carbowax (25 m x 0.25 mm; 0.25- µm film thickness) column (Cromlab S.A.) with helium as a carrier gas. The injection was performed in split mode at 250^0C. Fatty acid methyl esters were separated at constant pressure (23.1kPa) with the following oven program: (a) 50^0C for 2 min; (b) increase at a rate of 10^0C/min up to 230^0C. The mass spectrometer was tuned to get the relative abundances of m/z ranging from 40.00 to 550.00. The percentage area of total PUFA and total SFA was calculated separately. The lipid peroxidation levels were determined by computing the ratio of percentage composition of total PUFA (Polyunsaturated Fatty Acid) to SFA (Saturated Fatty Acids).

Peroxide Value

A 10% tissue extract in 25mM sodium phosphate (pH 7.4) buffer was used for lipid peroxidation assay. The homogenate was centrifuged at 3000 rpm for 15 min, and the supernatant was taken. The modified IDF (International Dairy Federation, 1991) method (spectrophotometric) for peroxide determination is based on the ability of the peroxides to oxidise ferrous ions to ferric ions. To a 10 ml screw-capped test tube, 0.1ml of homogenate was taken. Then Chloroform/Methanol mixture was added to make it 10 ml and mixed well. Ammonium thiocyanate solution and Fe (ll) solution was added 50µl each to the mixture without any time delay. Then the tube was kept in the dark for 5 min at room temperature. The absorbance reading was taken against a blank at 500 nm. The peroxide value of the sample was expressed as milli-equivalents of oxygen

per kilogram sample.

Immuno-Haematological Parameters

Collection of Blood and Serum

At the end of the experiment, two fish per replicate, a total of six fish per treatment were anaesthetised with clove oil at 50 µl L^{-1} and blood samples were drawn from the caudal peduncle using a medical syringe with EDTA as an anticoagulant. The blood was used immediately for analysis of respiratory burst activity, haemoglobin and total erythrocyte count (RBC). Another set of blood collected as above was transferred immediately to dry microfuge tubes. The tubes were allowed to stand in a tilted position at room temperature for one h to collect the serum, which was analysed for lysozyme activity and myeloperoxidase activity.

Nitrobluetetrazolium (NBT), Hemoglobin (Hb), Total Erythrocyte Count (RBC)

Nitrobluetetrazolium assay was done by the method modified by Stasiack & Baumann (1996). The Hb level of blood was analysed following the cyanmethemoglobin method using Drabkin's Fluid (Qualigens Diagnostics, India). RBCcounts were determined using improved Neubauer haemocytometer. A total of 5 squares amounting total volume of 0.02µl were counted and calculated by the method of Blaxhall & Daisley (1973).

Serum Lysozyme Activity, Myeloperoxidase Activity (MPO)

Serum lysozyme activity was determined by using kit (Bangalore Genei, Bangalore, India). Total Myeloperoxidase content present in serum was measured according to Quade & Roth (1997) with slight modification by Sahoo, Kumari, & Mishra, (2005).

Statistical Analysis

The various parameters were subjected to one-way analysis of variance (ANOVA) and 'F' test at P< 0.05 for comparisonamong different dietary treatments (Snedecor & Cochran 1967). Duncan's multiple range tests was used to determine the significant differences between the means.

Results

Growth and Feed Utilization

There were no significant differences (P>0.05) among the weight gain of *P. hypophthalmus* fingerling fed diets containing different levels of CDs (Table 2). The percentage weight gain, specific growth rate, protein efficiency ratio and feed conversion ratio of the different treatments were found to be statistically not different (P<0.05) from each other.

Flesh Quality Analysis

There were no significant effects of carotenoids on texture (Table 3) of *P. hypophthalmus* whereas colour (Table 4) differed significantly (P<0.05). The major texture parameters like hardness, chewiness and springiness values of the fillets were not significantly different (Table 3).L*values did not vary significantly (P>0.05) among the treatments but a*was significantly higher in the T2 group which was fed with 150 mg kg^{-1} astaxanthin and lowest value was observed in T1 group (Table 4) while b* value was significantly higher in the T4 and T5 group fed with BC (Table 4). Redness/yellowness (a*/b*) value was significantly higher in the T2 group although yellowness/lightness (b*/L*) value was significantly higher in the T5 group, which was fed with a higher dose of BC. The total carotenoid content of the fish muscle was analysed from all the treatments and given in the figure 1A. The higher carotenoid content was observed in the T3 group followed T2 group fed with higher and low level of AX. The lowest content was found in the control group fed with no CD (figure 1A).

Antioxidant Enzyme Analysis

The SOD and catalase activities of liver and gill were significantly affected (P<0.05) by carotenoid

Table 3. Different texture parameters of experimental groups fed with different experimental diets

Treatments	Hardness	Chewiness	Springiness
T1	28.16 ± 3.53	7.80 ± 1.49	0.66 ± 0.06
T2	27.03 ± 2.81	7.78 ± 1.73	0.80 ± 0.19
T3	24.34 ± 4.81	4.91 ± 0.16	0.52 ± 0.04
T4	26.61 ± 3.85	5.33 ± 0.57	0.53 ± 0.02
T5	18.76 ± 3.08	6.25 ± 2.96	0.66 ± 0.17
T6	26.39 ± 3.56	5.32 ± 1.24	0.48 ± 0.05
T7	34.00 ± 0.96	12.01 ± 6.53	0.67 ± 0.15
P value	0.168	0.659	0.456

Data expressed as (mean ± S.E.) (n = 3)

feeding (figure 1B&1C respectively). Higher dose AX fed group (T3) reported lowest SOD activities of liver and gill. Catalase activity of liver also showed a similar trend as the lowest value was recorded in T3 group whereas, the lowest value of catalase activity in gill was observed at both low and high dose of AX compared to other groups.

Estimation of Lipid Peroxidation

PUFA/SFA ratio (figure 1D) significantly ($P<0.05$) increased in higher dose AX fed group (T3) compared to all other treatment groups. The ratios were significantly lower ($P<0.05$) in T1, T4 and T6 groups, which were (control, low levels of BC and CX) respectively. Highest value ($P<0.05$) of peroxide (Table 5) was observed in the control group whereas the lowest value was found in both the AX fed groups (T2 and T3) compared to the other groups.

Immuno-Haematological Parameters

Significant effect of carotenoids ($P<0.05$) onimmune-heamatological parameters likes NBT, Hb, RBC, lysozyme activity and MPO (Table 5) wasseen at the end of 45 days of feeding. Lower dose of AX

Table 4. Chroma values of different experimental groups fed different experimental diets

Treatment	L*	a*	b*	a*/b*	b*/L*
T1	43.81± 1.27	$4.54^{a} \pm 0.18$	$16.85^{ab} \pm 0.76$	$0.27^{a} \pm 0.00$	$0.38^{ab} \pm 0.00$
T2	42.28± 1.39	$8.63^{f} \pm 0.37$	$18.25^{bc} \pm 0.72$	$0.48^{d} \pm 0.04$	$0.44^{bcd} \pm 0.03$
T3	43.35± 1.91	$6.74^{cd} \pm 0.25$	$18.97^{bc} \pm 0.77$	$0.36^{abc} \pm 0.00$	$0.42^{abc} \pm 0.02$
T4	45.32± 2.14	$6.84^{cd} \pm 0.27$	$23.79^{d} \pm 1.14$	$0.29^{a} \pm 0.00$	$0.50^{cd} \pm 0.03$
T5	45.27± 1.62	$7.35^{de} \pm 0.33$	$22.51^{d} \pm 1.32$	$0.33^{ab} \pm 0.03$	$0.54^{d} \pm 0.03$
T6	44.09± 1.44	$5.81^{b} \pm 0.22$	$18.08^{bc} \pm 0.78$	$0.32^{ab} \pm 0.00$	$0.43^{abc} \pm 0.02$
T7	43.11± 1.85	$8.20^{ef} \pm 0.36$	$20.72^{cd} \pm 1.17$	$0.40^{bcd} \pm 0.04$	$0.47^{bcd} \pm 0.06$
P value	0.894	0.001	0.001	0.002	0.010

L*(-): blackness, (+): lightness., a*(-): greenness, (+): redness, b* (-): blueness, (+): yellowness a*/b*- Redness/Yellowness b*/L*- Yellowness/Lightness

Different superscripts in the same column signify statistical differences ($P<0.05$) (mean ± S.E.) (n = 3)

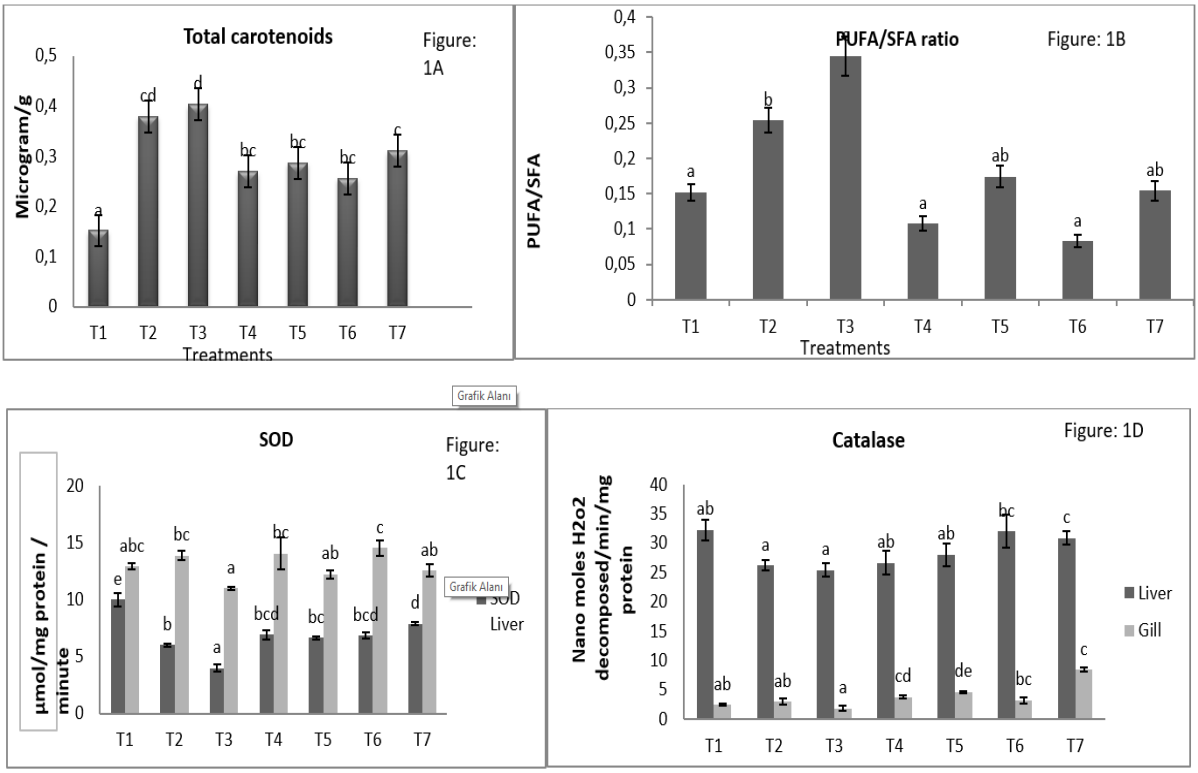

Figure 1. Figure 1A: Total carotenoid values different experimental groups fed different experimental diets, Figure 1B: Superoxide dismutase activities in the liver and gill of different experimental groups fed different experimental diets, Figure1C: Catalase activities in the gill of *Pangasianodon hypophthalmus* fingerlings fed with different experimental diets, Figure 1D: PUFA/SFA ratio of muscle in different treatment.

Table 5. RBC values, serum lysozyme activity, Serum myeloperoxidase activity of different experimental groups fed with the experimental diets

Treatment	Peroxide value	NBT activity	Total Haemogobin content	Total RBC (million cells/mm^3)	Lysozyme activity(U/mg protein)	Myeloperoxidase activity (OD @ 450nm)
T1	22.98^{f}±2.75	0.106^{b}±.01	9.20^{cd} ±0.09	2.73^{bc}±0.09	47.40^{ab}± 7.07	0.36^{a}±0.015
T2	4.39^{a}± 0.39	0.138^{d}±.04	12.95^{g}±0.43	3.64^{e}±0.04	82.21^{d} ±9.45	1.65^{e}±0.094
T3	5.44^{a}± 0.03	$0.111^{b,c}$±.03	10.79^{f}± 0.18	3.41^{de}±0.03	78.88^{cd} ±4.44	1.28^{d}±0.054
T4	11.66^{bc}±1.72	0.086^{a}±.03	9.73^{de}±0.08	2.90^{c}±0.11	69.99^{cd} ±8.89	0.80^{b}±0.013
T5	8.04^{ab}± 1.12	0.104^{b}±.04	8.82^{c}± 0.06	2.66^{ab}±0.05	59.62^{bc} ±6.49	1.02^{c}±0.014
T6	18.11^{e}± 1.37	0.118^{c}±.01	7.81^{b}± 0.14	2.61^{ab}±0.10	35.89^{a} ±4.98	0.74^{b}±0.020
T7	13.13^{cd}±0.56	0.092^{a}±.01	6.46^{a}± 0.43	2.26^{a}±0.10	41.07^{ab} ±2.28	0.53^{a}±0.031
P value	0.001	0.001	0.001	0.001	0.001	0.001

Different superscripts in the same column signify statistical differences ($p<0.05$)
(mean ± S.E.) (n = 3)

fed groups recorded higher NBTvalues, Hb level, RBC counts, serum lysozyme and MPO activity compared to other treatments.

Discussion

The present study evaluated the effect of dietary carotenoids ongrowth, flesh quality and immunity of pangasius fingerlings. Our results indicated that the dietary supplementation of carotenoids did not affect growth (Weight gain %, SGR) and feed utilization (FCR, PER) of pangasius fingerlings. Similar results were reportedin another study where AX supplementation (70 mgkg^{-1}) had little effect on the growth performance of Atlantic salmon, *Salmosalar* (Bell, McEvoy, Tocher& Sargent 2000). Amar *et al*. (2001) also found that there were no differences in feeding rates and growth among rainbow trout fed AX and BC. Our results are in agreement with the findings of several other studies such as (Wang, Chien, & Pan, 2006; Chatzifotis, *et al.*,2005; Kalinowski, Robaina, Fernández-Palacios, Schuchardt, & Izquierdo,2005; Tejera *et al.*,2007; Li *et al*., 2007). By contrast, a study by Kim, Kim, Cho, & Jo (1999) in Korean rose bitterling (*Rhodeusuyekii*) revealed that dietary AX supplementation resulted in improved growth performance when compared with lutein or BC supplementation or diet without carotenoids. Growth performance of the pangasius is mainly influenced by the digestibility and utilisation of major nutrients such as amino acids and the major feed ingredients (DA TA, 2013), which could be attributed to the non-significant effect of carotenoids.

This study revealed that carotenoid pigments supplied through diet is absorbed and deposited in the flesh of *P. hypophthalmus.* Visual yellow colour intensity score was highest for fish fed BC (T4 and T5) and lowest for the non supplemented group (T1). There were no significant differences in fillet whiteness among fish fed with various carotenoids. Yellowness was significantly higher in BC fed groups (T4 and T5) whereas fish fed AX had significantly higher redness values than fish fed the control and BC. Fillets of fish fed with CX exhibited a grade of coloration which will fall in a middle way between the colour of AX and BC fed fillets. Results of total flesh carotenoid analysis in this study also revealed that all the carotenoids were present in the muscle. These results are consistent with Li *et al.*(2007) who reported that channel catfish could accumulate yellow pigments, lutein and zeaxanthin and red or pink pigments, CX and AX in the flesh. However, our study revealed that BC gives maximum yellow colouration by way of getting deposited in the muscle. Foss *et al.* (1984) and Torrissen (1986) interpreted that rainbow trout can utilise AX more efficiently than CX. The current study also shows that AX can be absorbed more efficiently than other pigments in the fillet as revealed by a higher fractional yield of AX in the pigment extracted from the fillets with the chloroform. This has shown that muscle colouration of catfish fed CX was less than that fed AX. These findings are similar to those of Bjerkeng, Storebakken, &Liaaen-Jensen (1990) who reported that CX uptake was less efficient than AX uptake in rainbow trout.

Our study demonstrated that there were no effects of different dietary carotenoids on fillet texture of *P. hypophthalmus.* There are hardly any reports available on the effect of carotenoids on the muscle texture of fish. A different study reported that the deposition of carotenoids in the muscle is correlated with the lipid content of the muscle which can significantly affect the texture of muscle (Ytrestoyl, Coral-Hinostroza & Hatlen,2004), which is however not supported by the texture characteristics of the experimental groups of our study.

Antioxidant enzyme defences include SOD, catalase and GPx, which contribute to the removal of reactive oxygen species from the body (Noctor& Foyer, 1998). Consequent upon the action of these enzymes, lipid peroxidation of the body is well balanced and kept under control. In the present study, SOD activity was diminished significantly in the higher level AX fed group both in the liver and gill tissues. A similar result was also reported by Wang *et*

al. (2006) that fish supplemented with AX had shown lower SOD activity compared to other carotenoid fed fish. SOD, a specific cytosolic enzyme that is responsible for scavenging superoxide radicals, is involved in protective mechanisms within tissue injury following oxidative process and phagocytosis. The higher the SOD activity, the more superoxide radicals need to be reacted. In liver maximum SOD activity was in control group which was fed with the purified diet without any CDs. In this study, the decrease of SOD with increasing dietary AX concentration indicated that dietary AX effectively reduced SOD levels. Catalase activity also reduced significantly in the AX fed groups irrespective of the dose compared to other carotenoid fed groups. Similar results are also observed by Lygren, Hamre & Waagboe (1999) who reported when there are high levels of fat-soluble antioxidants, such as AX and vitamin E, there was a reduced need for endogenous antioxidant enzymes, such as catalase and SOD. Another study by (Martin *et al.*,1999) also supported that SOD, catalase and glutathione peroxidase activities decreased with the increase of dietary AX.

PUFA/SFA ratio is a critical indicator of lipid peroxidation as the fraction of PUFA decreases with increase in lipid peroxidation (Kang, Shin, Park & Lee, 2005). PUFA/SFA ratio of all the treatment groups have significantly differed among the treatments and a high PUFA/SFA ratio were found in the higher level AX fed group and followed by the lower level AX fed group. This is because of the strong antioxidant activity of astaxanthin protect PUFA from oxidation compared to other carotenoids. PUFA, in particular the highly unsaturated fatty acids (HUFA); eicosapentaenoic acid (EPA) [20:5(n-3)] and docosahexaenoic acid (DHA) [22:6(n-3)] are fundamental cellular membranes components susceptible to attack by reactive oxygen species. Our study showed that AX protects oxidation of essential polyunsaturated fatty acids (PUFA), which is also supported by Guerin, Huntley & Olaizola (2003), who reported that AX preserves the essential (PUFA) and give protection against UV light effects. Carotenoids are potent antioxidants which act synergistically with non-enzymatic antioxidants such as vitamin E (Bell *et al*., 2000). Astaxanthin possesses good singlet oxygen quenching properties and may serve as an antioxidant in systems containing unsaturated fatty acids by quenching free radicals (Martin *et al*., 1999). The good antioxidant properties of astaxanthin may relieve oxidative stress and have beneficial health effects (Higuera-Ciapara, Félix-Valenzuela, & Goycoolea, 2006; Hussein Sankawa, Goto, Matsumoto & Watanabe, 2006).

The antioxidant activity of astaxanthin attracts particular attraction as fish tissues are rich in PUFA. The extent of lipid oxidation can be determined by measuring amounts of primary peroxidation products, losses of unsaturated fatty acids, and concentrations of secondary products, such as carbonyls and hydrocarbon gases (Halliwell & Chirico, 1993). In the present study, the peroxide value of fish muscle of different treatments differed significantly with lowest values were observed for the diet supplemented with astaxanthin and highest values in the fishes fed control diet without any added carotenoids. This suggests that dietary astaxanthin may serve to protect the PUFA in fish tissues from the detrimental effects of oxidation.

Increased NBT can be correlated with the increased bacterial pathogen killing activity of phagocytes. AX in the present study was observed to increase the NBT values of the experimental groups significantly. Kim, Song, Kim & Lee (2012) also reported increased neutrophil activation by NBT in fish fed with the 2% and 3% AX. Highest NBT value was observed by lower level AX (T2) fed group, suggesting the immune protective effect of the AX in the fish. However, higher level of AX in the diet could not produce the same type of phagocyte response. The RBC count of pangasius in the present study fell in the same range which was also reported by Yaghobi Dorafshan, Paykan-Heyrati, & Mahmoudi (2013). Highest RBC count was found in the low-level AX supplemented group. Hb levels also followed the same trend as that of RBC. Maximum values were observed in the low AX fed group followed by high AX fed counterpart. Immuno stimulants have the general capacity to improve the RBC and the haemoglobin level, which might have caused due to AX supplementation. The present study also observed that RBC and Hb levels were lower in higher dose CD supplemented groups compared to their lower dose counterparts. Lysozyme plays an important role in immunity by lysis of the bacterial cell wall and thus stimulates phagocytosis of the bacteria. In the present study, AX was found to cause an increase in the serum lysozyme activity of the fish; Li *et al*. (2014) also reported increased lysozyme activity with the increasing AX. Jha, Pal, Sahu, Kumar & Mukherjee (2007) reported that immunostimulants such as BC, ω-3 PUFA and yeast RNA can enhance the lysozyme activity in the serum. MPO is a hemoprotein, which plays a key role in the non-specific immune response of the organism, which is secreted during activation of neutrophils. It utilizes hydrogen peroxide during the respiratory burst to produce hypochlorous acid (Kumar *et al.*,2013). The treatment groups with AX supplementation (T2 &T3) showed higher MPO activity. In a different study, Kumari & Sahoo (2006) reported raised MPO activity with immunostimulants. In the present study, the increased serum lysozyme and MPO activity in the AX supplemented groups is an indication of the activation of the non-specific immune system in the presence of this CDs. Thus the study demonstrated that AX supplementation not only improves the colouration of flesh but enhance the immune response as well.

Conclusion

The present study suggests that AX supplementation in the feed at dose of 150mg/kg can improve the pinkish colouration of the fillet of pangasius. This is especially helpful for farmers of many countries who experience the issue of yellow discolouration in their farmed sutchi catfish. Further, our study revealed that most of the yellow discolouration problem in the fillet occurs due to the accumulation of carotenoids like beta-carotene, which can be minimised by avoiding the use of those ingredients which indispensably rich in yellow pigments for feed formulation. However, further studies with manipulation of the level of carotenoid and feeding duration are required before recommending carotenoids to pangasius farmers.

Acknowledgements

The authors thank the Director, Central Institute of Fisheries Education, for providingfunds and facilities for carrying out the present study. The first author also thankfultoDr.Jayakumari, Central Institute of FisheriesTechnology Mumbai for providing facilities for texture and colour analysis. The authors also thank the Vilas, Managing Director, DSM Nutrition, Indiafor providing the carotenoids for the present study.

References

Amar, E. C., Kiron, V., Akutsu, T., Satoh, S., & Watanabe, T. (2012). Resistance of rainbow trout *Oncorhynchusmykiss* to infectious hematopoietic necrosis virus (IHNV) experimental infection following ingestion of natural and synthetic carotenoids. *Aquaculture*, 330,148-155. https://dx.doi.org/10.1016/j.aquaculture.2011.12.007

Amar, E. C., Kiron, V., Satoh, S., Okamoto, N., & Watanabe, T. (2000). Effects of dietary β- carotene on the immune response of rainbow trout *Oncorhynchusmykiss*. *Fisheries Science*, *66*(6), 1068-1075. https://dx.doi.org/10.1046/j.1444-2906.2000.00170.x

Amar, E. C., Kiron, V., Satoh, S., & Watanabe, T. (2004). Enhancement of innate immunity in rainbow trout (*Oncorhynchus mykiss*, Walbaum) associated with dietary intake of carotenoids from natural products. *Fish and shellfish immunology*, *16*(4), 527-537. http://dx.doi.org/10.1016/j.fsi.2003.09.004.

Amar, E. C., Kiron, V., Satoh, S., & Watanabe, T. (2001). Influence of various dietary synthetic carotenoids on bio- defence mechanisms in rainbow trout, *Oncorhynchusmykiss* (Walbaum). *Aquaculture research*, *32*(s1), 162-173. http://dx.doi.org/10.1046/j.1355-557x.2001.00051.x

AOAC, (1995). Official methods of Analysis of AOAC International, Vol. 1, 16thedn. (ed. By P. A. Cunnif,),1, pp. AOAC International, Arlington, USA.

Baker, R., &Günther, C. (2004). The role of carotenoids in consumer choice and the likely benefits from their inclusion into products for human consumption. *Trends in Food Science and Technology*, *15*(10), 484-488. https://dx.doi.org/10.1016/j.tifs.2004.04.0094

Bell, J. G., McEvoy, J., Tocher, D. R., & Sargent, J. R. (2000). Depletion of α-tocopherol and astaxanthin in Atlantic salmon (*Salmosalar*) affects autoxidativedefense and fatty acid metabolism. *The Journal of nutrition*, *130*(7), 1800-1808.

Bjerkeng, B., Storebakken, T., & Liaaen-Jensen, S. (1990). Response to carotenoids by rainbow trout in the sea: resorption and metabolism of dietary astaxanthin and canthaxanthin. *Aquaculture*, *91*(1-2),153-162. https://dx.doi.org/10.1016/0044-8486(90)90184-O

Blaxhall, P. C. &Daisely, K. W. (1973). Routine haematological methods for use with fish blood. *Journal of Fish Biology*, 5,771-781. https://dx.doi.org/10.1111/j.1095-8649.1973.tb04510.x

Chatzifotis, S., Pavlidis, M., Jimeno, C. D., Vardanis, G., Sterioti, A., &Divanach, P. (2005). The effect of different carotenoid sources on skin coloration of cultured red porgy (*Pagruspagrus*). *Aquaculture Research*, *36*(15), 1517-1525. http://dx.doi.org/10.1111/j.1365-2109.2005.01374.x

De Silva, S. S., & Phuong, N. T. (2011). Striped catfish farming in the Mekong Delta, Vietnam: a tumultuous path to a global success. *Reviews in Aquaculture*, *3*(2), 45-73. https://dx.doi.org 10.1111/j.1753-5131.2011.01046.x

Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *The Journal of biological Chemistry*, *226*(1), 497-509.

Foss, P., Storebakken, T., Schiedt, K., Liaaen-Jensen, S., Austreng, E., &Streiff, K. (1984). Carotenoids in diets for salmonids: I. Pigmentation of rainbow trout with the individual optical isomers of astaxanthin in comparison with canthaxanthin. *Aquaculture*, *41*(3), 213-226. https://dx.doi.org/10.1016/0044-8486(84)90284-9

Guerin, M., Huntley, M. E., & Olaizola, M. (2003). Haematococcusastaxanthin: applications for human health and nutrition. *TRENDS in Biotechnology*, *21*(5), 210-216. http://dx.doi.org/10.1016/S0167-7799(03)00078-7

Gupta, S. K., Jha, A. K., Pal, A. K., &Venkateshwarlu, G. (2007). Use of natural carotenoids for pigmentation in fishes. *Indian Journal of Natural Products and Resources*.6, 46-49.

Hagen, Ø., Solberg, C., Sirnes, E., & Johnston, I. A. (2007). Biochemical and structural factors contributing to seasonal variation in the texture of farmed Atlantic halibut (*Hippoglossushippoglossus* L.) flesh. *Journal of agricultural and food chemistry*, *55*(14), 5803-5808. https://dx.doi.org/10.1021/jf063614h

Halliwell, B., & Chirico, S. (1993). Lipid peroxidation: its mechanism, measurement, and significance. *The American journal of clinical nutrition*, *57*(5), 715S-724S.

Halver, J. E. (1976). The nutritional requirements of cultivated warmwater and coldwater fish species. In *FAO Technical Conference on Aquaculture, Kyoto (Japan), 26 May 1976*.

Harikrishnan, R., Balasundaram, C., &Heo, M. S. (2011). Impact of plant products on innate and adaptive

immune system of cultured finfish and shellfish. *Aquaculture*, *317*(1), 1-15. https://dx.doi.org/10.1016/j.aquaculture.2011.03.039

Hastings, W.H. (1969). Nutritional score. In: Fish in Research (Newhaus, O.W. &Halver, J.E. eds), pp. 263–292. Academic Press, New York.

Higuera-Ciapara, I., Felix-Valenzuela, L., &Goycoolea, F. M. (2006). Astaxanthin: a review of its chemistry and applications. *Critical reviews in food science and nutrition*, *46*(2), 185-196. https://dx.doi.org/10.1080/10408690590957188

Hu, B., Roy, L. A., & Davis, D. A. (2013). Correlations of xanthophylls in catfish fillets, plankton, shad and snails in catfish production ponds in west Alabama. *Aquaculture*, *402*, 46-49. https://dx.doi.org/10.1016/j.aquaculture.2013.03.022

Hussein, G., Sankawa, U., Goto, H., Matsumoto, K., & Watanabe, H. (2006). Astaxanthin, a carotenoid with potential in human health and nutrition. *Journal of natural products*, *69*(3), 443-449.http://dx.doi.org/10.1080/10408690590957188

International IDF Standards International Dairy Federation. 1991. IDF-Square Vergote 41, Brussels, Sec. 74A:

Jha, A. K., Pal, A. K., Sahu, N. P., Kumar, S., & Mukherjee, S. C. (2007). Haemato-immunological responses to dietary yeast RNA, ω-3 fatty acid and β-carotene in Catlacatla juveniles. *Fish and shellfish immunology*, *23*(5), 917-927. https://dx.doi.org/10.1016/j.fsi.2007.01.011

Kalinowski, C. T., Robaina, L. E., Fernandez-Palacios, H., Schuchardt, D., &Izquierdo, M. S. (2005). Effect of different carotenoid sources and their dietary levels on red porgy (*Pagruspagrus*) growth and skin colour. *Aquaculture*, *244*(1), 223-231. https://dx.doi.org/10.1016/j.aquaculture.2004.11.001

Kang, M.J., Shin, M.S., Park, J.N. & Lee, S.S. (2005). The effects of polyunsaturated: saturated fatty acids ratios and peroxidisability index values of dietary fats on serum lipid profiles and hepatic enzyme activities in rats. *British journal of nutrition*, *94*(04), .526-532.https://dx.doi.org/10.1079/BJN20051523

Kim, H. S., Kim, Y., Cho, S. H., & Jo, J. Y. (1999). Effects of dietary carotenoids on the nuptial color of the bitterling (*Rhodeusuyekii*). *Korean Journal of Fisheries and Aquatic Sciences*, *32*(3), 276-279.

Kim, S., Song, J., Kim, K., &Lee, K. (2012). Effects of dietary astaxanthin on innate immunity and disease resistance against *Edwardsiellatarda* in olive flounder *Paralichthysolivaceus*.http://hdl.handle.net/10524/23182

Krinsky, N. I., & Johnson, E. J. (2005). Carotenoid actions and their relation to health and disease. *Molecular aspects of medicine*, *26*(6), 459-516. https://dx.doi.org/10.1016/j.mam.2005.10.001

Kumar, S., Raman, R. P., Pandey, P. K., Mohanty, S., Kumar, A., & Kumar, K. (2013). Effect of orally administered azadirachtin on non-specific immune parameters of goldfish *Carassiusauratus* (Linn. 1758) and resistance against *Aeromonashydrophila*. *Fish and shellfish immunology*, *34*(2), 564-573. https://dx.doi.org/10.1016/j.fsi.2012.11.038

Kumari, J., &Sahoo, P. K. (2006). Non-specific immune response of healthy and immunocompromised Asian catfish (*Clariasbatrachus*) to several immunostimulants. *Aquaculture*, *255*(1),133-141. https://dx.doi.org/10.1016/j.aquaculture.2005.12.012

Li, M. H., Robinson, E. H., Oberle, D. F., & Zimba, P. V. (2007). Effects of various dietary carotenoid pigments on fillet appearance and pigment absorption in channel catfish, *Ictaluruspunctatus*. *Journal of the World Aquaculture Society*, *38*(4), 557-563. https://dx.doi.org/10.1111/j.1749-7345.2007.00130.x

Li, M., Wu, W., Zhou, P., Xie, F., Zhou, Q., & Mai, K. (2014). Comparison effect of dietary astaxanthin and *Haematococcus pluvialis* on growth performance, antioxidant status and immune response of large yellow croaker *Pseudosciaena crocea*. *Aquaculture*, *434*, 227-232. https://dx.doi.org/10.1016/j.aquaculture.2014.08.022

Lygren, B., Hamre, K., & Waagbø, R. (1999). Effects of dietary pro-and antioxidants on some protective mechanisms and health parameters in Atlantic salmon. *Journal of Aquatic Animal Health*, *11*(3),211-221. http://dx.doi.org/10.1577/1548-8667(1999)011%3C0211:EODPAA%3E2.0.CO;2

Martin, H. D., Jäger, C., Ruck, C., Schmidt, M., Walsh, R., &Paust, J. (1999). Anti- and Prooxidant Properties of Carotenoids. *Advanced Synthesis and Catalysis*, *341*(3), 302-308.

Misra, H. P., & Fridovich, I. (1972). The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *Journal of Biological chemistry*, *247*(10), 3170-3175.

Noctor, G., & Foyer, C. H. (1998). Ascorbate and glutathione: keeping active oxygen under control. *Annual review of plant biology*, *49*(1), 249-279.

Olson, J. A. (1979). A simple dual assay for vitamin A and carotenoids in human liver. *Nutrition Reports International (USA)*.

Sajilata, M. G., Singhal, R. S. & Kamat, M. Y. (2008). The Carotenoid Pigment Zeaxanthin a Review. *Comprehensive Reviews In Food Science And Food Safety* **7**, 29-49. https://dx.doi.org/10.1111/j.1541-4337.2007.00028.x

Quade, M. J., & Roth, J. A. (1997). A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. *Veterinary Immunology and Immunopathology*, *58*(3), 239-248. https://dx.doi.org/10.1016/S0165-2427(97)00048-2

Sahoo, P. K., Kumari, J., & Mishra, B. K. (2005). Non- specific immune responses in juveniles of Indian major carps. *Journal of Applied Ichthyology*, *21*(2), 151-155. https://dx.doi.org/10.1111/j.1439-0426.2004.00606.x

Schmidt, A. S., Bruun, M. S., Dalsgaard, I., Pedersen, K., & Larsen, J. L. (2000). Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. *Applied and environmental microbiology*, *66*(11), 4908-4915. https://dx.doi.org/10.1128/AEM.66.11.4908-4915.2000

Singh, A. K., & Lakra, W. S. (2012). Culture of *Pangasianodonhypophthalmus* into India: impacts and present scenario. *Pakistan Journal of Biological Sciences*, *15*(1), 19. 10.3923/pjbs.2012.19.26

Snedecor, G.W. & Cochran, (1967). Statistical Methods. *6th(edn) Iowa State University Press, Ames*

Stasiack, A. S. & Bauman, C. P., (1996). Neutrophil activity as a potent indicator of concomitant analysis. *Fish and*

Shellfish Immunology, 37, 539-546.

Takahara, S., Hamilton, H. B., Neel, J. V., Kobara, T. Y., Ogura, Y., & Nishimura, E. T. (1960). Hypocatalasemia: a new genetic carrier state. *Journal of Clinical Investigation*, *39*(4), 610. https://dx.doi.org/10.1172/JCI104075

Tejera, N., Cejas, J. R., Rodríguez, C., Bjerkeng, B., Jerez, S., Bolaños, A., & Lorenzo, A. (2007). Pigmentation, carotenoids, lipid peroxides and lipid composition of skin of red porgy (*Pagruspagrus*) fed diets supplemented with different astaxanthin sources. *Aquaculture*, *270*(1), 218-230. https://dx.doi.org/10.1016/j.aquaculture.2007.01.019

Torrissen, O. J. (1986). Pigmentation of salmonids—a comparison of astaxanthin and canthaxanthin as pigment sources for rainbow trout. *Aquaculture*, *53*(3-4), 271-278.

Wang, Y. J., Chien, Y. H., & Pan, C. H. (2006). Effects of dietary supplementation of carotenoids on survival, growth, pigmentation, and antioxidant capacity of characins, *Hyphessobrycon callistus*. *Aquaculture*, *261*(2), 641-648. https://dx.doi.org/10.1016/j.aquaculture.2006.08.040

Yaghobi, M., Dorafshan, S., PaykanHeyrati, F., & Mahmoudi, N. (2014). Growth performance and some haematological parameters of ornamental striped catfish (*Pangasianodon hypophthalmus*) fed on dietary nucleotide. *Iranian Journal of Veterinary Research*, *15*(3), 262-265. http://dx.doi.org/10.22099/ijvr.2014.2537

Ytrestøyl, T., Coral-Hinostroza, G., Hatlen, B., Robb, D. H. F., & Bjerkeng, B. (2004). Carotenoid and lipid content in muscle of Atlantic salmon, *Salmosalar*, transferred to seawater as 0+ or 1+ smolts. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *138*(1), 29-40. https://dx.doi.org/10.1016/j.cbpc.2004.01.011

Zariphen, S. & ErdmanJr, J.W. (2002). Factors that influence the bioavalability of xanthophylls. *The Journal of Nutrition*, 132,531-534.