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Monica J
Research Assistant, Department of Biotechnology, Maharani Lakshmi Ammanni College for Women, Bangalore, Karnataka, India

Dr. Vyjayanthi Neelakantan
Former Associate Professor, Department of Biotechnology, Maharani Lakshmi Ammanni College for Women, Bangalore, Karnataka, India

Dr. Seenappa D
Former Chief Scientific Officer, Inland fisheries, University of Agricultural sciences, Bangalore, Karnataka, India

Correspondence
Dr. Vyjayanthi Neelakantan
Former Associate Professor, Department of Biotechnology, Maharani Lakshmi Ammanni College for Women, Bangalore, Karnataka, India

Effect of dietary incorporation of anthocyanin pigments on the coloration and growth of orange sword tail fish (*Xiphophorus helleri*)

Monica J, Dr. Vyjayanthi Neelakantan and Dr. Seenappa D

Abstract
The study evaluated effects of dietary incorporation of co-stabilized anthocyanin pigment extract of red cabbage and bio waste-beetroot peel (@ 400 mg/kg feed on enhancement of coloration, growth, feed utilization and general health of orange sword tail fish (*Xiphophorus helleri*). The feeding trial was carried out in in-door aquarium tanks (54 liters of water capacity) stocked with uniform sized (Initial average weight of the fish 0.8± 0.1g) orange sword tail fish @ 10 no's/tank.
The findings of study after 45 days of feeding indicated high red intense coloration (Red: 246.3 ±1.24; Green: 53.3 ±0.4 and Blue 31 ±0.47) in fish fed on anthocyanin incorporated feed compared to control fish fed on non-anthocyanin incorporated feed (Red: 147.5 ±1.5, Green: 117 ±1 and Blue: 84± 0.5). No significant variation in fish growth, Food Conversion Ratio (FCR) and water quality between control and treatment was observed.
The study indicated possibility of using anthocyanin extract (@ 400 mg/kg feed) as one of the biological sources of pigment for enhancing coloration in orange sword tail fish.

Keywords: Anthocyanin, co-stabilized pigments, red sword tail fish, RGB image J software

1. Introduction

Indian water possesses a rich diversity of ornamental fishes with over 300 varieties of indigenous species. However, the popularity and trade of ornamental fish in India is dominated by domesticated exotic species that are bred in captivity and are known for their wide diversity of colours and colour patterns. The present export of ornamental fishes from India is predominantly confined to indigenous freshwater species [1].

In ornamental fish, apart from body shape and finnages, colouration is one of the major traits which determine fish quality and pricing in the commercial trade. Pigments are responsible for the colouration and quality of most fishes. Principally, skin coloration of fishes is genetically determined and generally fishes have the capacity to absorb, metabolise from the food they eat and deposit pigments in their skin/flesh. Hence, colour expression of fishes is closely related to the nature of food it eats and its pigment content. As fishes cannot synthesize their own pigments [2], they need natural food or dietary supplementation of biological colour pigments to enhance their natural coloration. Additional supplementation of biological pigments through feeds are needed for enhancement of certain colour types *viz*., red, orange, yellow, green, and some blue in fishes as they don't produce these colour pigments [3]. Ornamental fishes raised in in-door aquariums/open outdoor ponds without pigment supplementation in their diet are tending to lose their vibrant colours. There are various methods of incorporating colours to fishes such as direct injection, dipping in solutions, tattooing, tagging etc., and these methods are known to stress fishes and the pigments may get faded over time. Colouring fish stresses them and makes them more susceptible to disease [4].

The best convenient method to enhance and intensify coloration in ornamental fishes is through their diet and pigment supplementation through feeds [5]. The red carotene pigments *viz*., astaxanthin and canthaxanthin, and the yellow xanthophyll pigments *viz*., lutein and zeaxanthin are the two major carotenoids most commonly fed to fishes to enhance their coloration [6, 7]. Utilization of plant sources of carotenoid pigments for food fishes *viz*., yeast (*Rhodotorula sunny*) [8], Chesnutt flowers [9], hippophae oil (*Hippophae rhamnoides*) [10] for rainbow trout; dried flowers for salmonids [11] and for ornamental fishes; paprika (*Capsicum annum*) [12], Spirulina [13] for gold fish to enhance coloration of flesh/skin are reported. Better pigmentation in gold fish fed on dietary incorporation level of carotenoid from marigold meal @200 mg/kg feed has been reported [14, 15]. Chapman (2000) [16] suggested use of combination of both synthetic and natural carotenoid pigments to enhance coloration in ornamental fish.

In the country, use of several natural ingredients *viz*., carrot, marigold petal, china rose petal as sources of pigments to enhance and intensify coloration in ornamental fishes are reported [17, 18]. Orange peel waste as source of carotenoid pigments was used in ornamental fish feed to enhance the effect of colour and growth of orange molly [20].

Anthocyanin extracts are also used to enhance coloration of fish. Beetroot peel and red cabbage are rich sources of anthocyanin pigments. The total anthocyanin content in red cabbage is estimated at 0.876mg/L and beetroot peel 0.635mg/l. [19]. Anthocyanins obtained from various sources *viz*., mango peel, curry leaves, beetroot, spirulina has been used for dietary incorporation to enhance coloration in blue morph, *Pseudotropheus lombardoi* [21]. Similarly, anthocyanin extracts of sweet potato leaves, cassava leaves, colocasia leaves has been successfully used for dietary supplementations to enhance coloration in gold fish and red cap oranda fish [22].

Considering the availability of plant material rich in anthocyanins in the region and new scope for use of this pigments for enhancing colour of ornamental fish, the present study was designed to evaluate the effects of dietary supplementation of anthocyanin pigments on coloration, growth, feed utilization and general health of orange sword tail fish (*Xiphophorus helleri).* The fish was selected for the study considering its popularity among the hobbyists for its varied body shape, fins and coloration, hardy nature, fast-growing and easy to breed, better market demand and high value.

2. Materials and Methods

2.1 Experimental fish and design

Feeding trial was carried out in glass aquarium tanks positioned indoor. Size graded orange sword tail of uniform size (average.wt.0.8± 0.1g) and common parentage obtained from the ornamental fish unit of Inland Fisheries Division, University of Agricultural Sciences, Hebbal, Bangalore, Karnataka, India was used for the study. Fish were maintained under quarantine conditions for three weeks before selecting them for experimental stocking. The fish was stocked @10 nos./tank (2 males and 8 females) in randomly allotted glass aquarium tanks each of 54 liters of water holding capacity and fitted with 3Watt white LED lighting (AC-90-260V) and sponge air filter. The stocked fish was initially acclimatized to experimental captive conditions for a period of five days by feeding with formulated feed (control feed). The treatment had three replicate tanks and the control had two replicate tanks.

2.2 Fish feed formulation

Formulated fish feed was prepared using the ingredients fish meal, ground nut cake, rice bran, wheat flour, fish oil and vitamin & mineral premix. The ingredients were ground into fine particles in electric mixer and strained before use to avoid clogging of die pores. Pre-weighed ingredients except oil and premix were mixed thoroughly in a plastic bowl with required amount of water to form dough. The dough was then kept for 30 minutes for proper conditioning followed by steaming for 25 minutes in a pressure cooker. The steamed dough was allowed to cool and oil and premix was added and mixed uniformly. Pellets were prepared by hand pelletizer having die with ~4 mm diameter pore size. Finally, the pellets were air dried for 24 hours and kept in oven for 3-4 hrs at 35^0C for drying to reduce moisture to <10%. The pellets were ground and passed through sieve to get even sized particles (< 500μm). The feed texture was coarse powder and the colour was light brown. Experimental feed was prepared by incorporating co-pigment anthocyanin powder (extracted from beetroot peel and red cabbage) @400mg/kg feed.

2.3 Fish rearing and sampling

Fishes were hand fed twice a day during morning and evening (at 9.30a.m and 5p.m) @10% of body weight day^1 during the experimental period of 45 days. The tanks were checked daily to observe any mortality of fish and to record water temperature. Uneaten feed and faeces were siphoned out regularly on a day to day basis and the water level was maintained by replacing with fresh water. All aquaria were maintained under a constant photoperiod (12 h light and 12 h dark) created by fluorescent lamps. Fish were sampled once a fortnight to assess enhanced coloration, growth/biomass gain and general health. During every sampling, quantity of feed required was recalibrated based on the increased biomass.

Water quality parameters *viz*., temperature, pH, dissolved oxygen level, alkalinity, hardness of water, nitrite level was measured using standard AOAC methods [23]. After each sampling, water replacement in aquarium tanks was carried out (about 2/3rd) to maintain acceptable water quality parameters.

2.4 Image analysis & Visual scores

Fishes were analysed through RGB software for comparison of pigmentation/coloration between control replicates and treatment at fortnightly interval. During the experimental period, images of the fish were taken by using digital camera. Later these images were cropped and colour pattern was made with predefined points using web application. The colour patterns were analysed for RGB with help of image J (Computer application). Additionally, orange colour index was prepared and compared with actual colour of orange sword tail to give scores 1-6 where score of one is minimum and six as maximum. Colour comparison of fish reared in control and treatment tanks during each sampling phase was done using RGB Image.

Fig 1: *Aquarium set up*

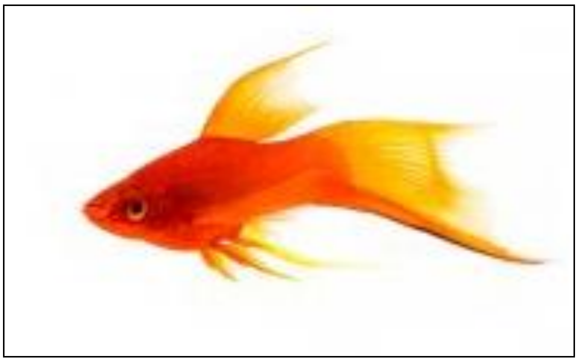

Fig 2: *Orange sword tail*

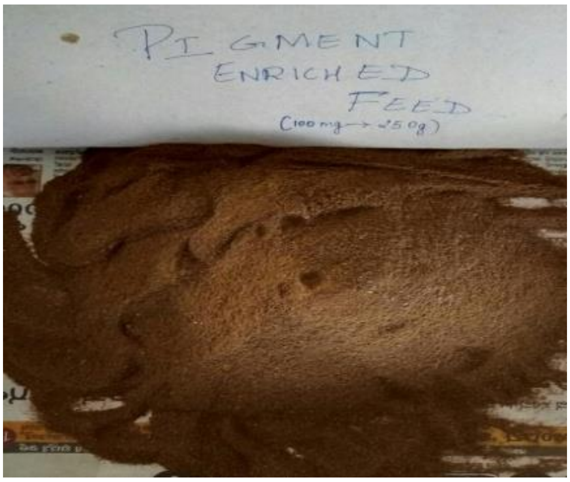

Fig 3: *Anthocyanin feed mix*

2.5 Data analysis

Growth performance was evaluated based on body weight gain during each sampling and overall after the termination of study. A total of 20 fishes for control and 30 fishes for treatment were considered for study analysis. Feed conversion rate and efficiency was calculated based on fish biomass gain and fish feed fed. The weight gain (%) was calculated based on (final weight-initial weight (g)/initial weight (g)) x100. Feed Conversion Ratio (FCR) and Feed Conversion Efficiency (FCE) was calculated based on total quantity of feed fed and final fish biomass gain.

At the end of experiment all the data were statistically analysed by statistical package SPSS where comparison between treatments at different intervals of time was done by Duncan's Multiple Range Test (DMRT). Comparison among all the treatment was done by one-way ANOVA. The level of significance employed was 0.05.

3. Results and Discussions

3.1 Effect of feeding on coloration

3.1.1 Visual scores based on color index chart

Initially at the time of stocking, the colour of fish was light or pale orange (Scale-3). With the feeding of anthocyanin incorporated feed (@400 mg/kg feed), the coloration of fish in treatment group has enhanced compared to control group that received only formulated feed. Over the time of the study, the coloration of fish was bright orange in treatment tanks and was closely matching to Scale-6 of the colour index card. While in control tanks, the fish remained pale orange throughout the study (Figure 4).

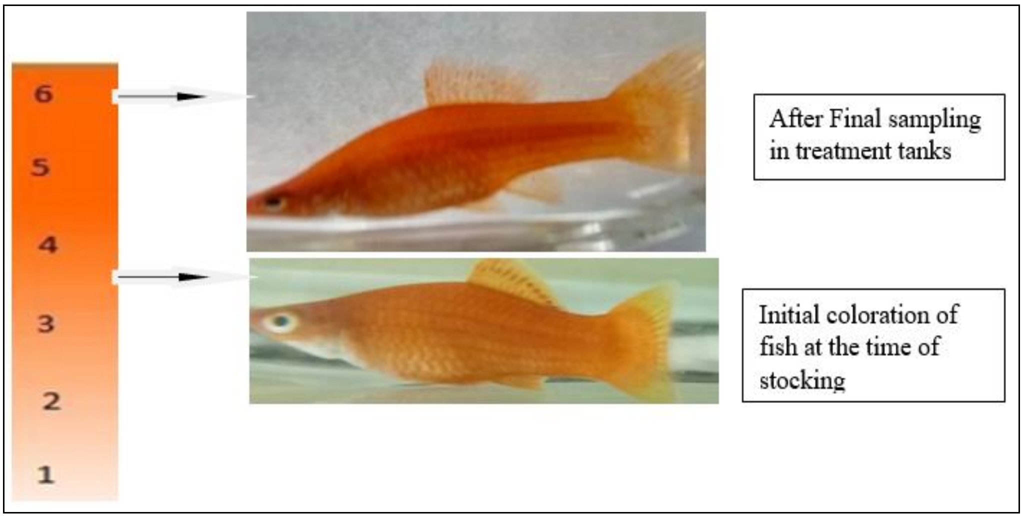

Fig 4: Visual scores based on colour index – Depending on intensity of colour

3.2 Image analysis

The RGB analysis of fish photos using Image J software both of treatment and control tanks taken during different intervals of feeding trial indicated lower average values of 147.5± 1.5 for red, and increased values for green (117 ±1) and blue (84 ±0.5) in control tanks compared to treatment tanks where fish were fed on anthocyanin incorporated feed (Table 1).

The intensity of red coloration of fish in control group showed no significant increase over the time of the study and the average values was 147.5± 1.5 (on 15th day), 155± 1 (on 30th day) and 165± 1 (45th day) respectively(p<.05). With regard to intensity of green, the value slightly decreased with the rearing period (p<.05) and was 117± 1(15th day), 113.5 ±0.5 (30th day) and 105.5 ±0.5 (45th day) respectively. Similarly, the intensity of blue also decreased very little over a period of time (p<.05) and was 84 ±0.5 (15th day), 81.5 ±0.5 (30th day) and 74.5 ±0.5 (45th day) respectively.

The intensity of red coloration of fish in treatment group increased significantly over the time of the study and the average values was 175.33± 2.62 (on 15th day), 213.6± 2.05 (on 30th day) and 246.3± 1.24 (45th day) respectively(p<.05). With regard to intensity of green, the value decreased significantly with the rearing period (p<.05) and was 98.3± 2.49(15th day), 73.6 ±2.05 (30th day) and 53.3 ±0.47 (45th day) respectively. Similarly, the intensity of blue also decreased significantly over a period of time ($p<.05$) and was 77.3 ±2.05 (15th day), 50 ±2.74 (30th day) and 31 ±0.47 (45th day) respectively.

Table 1: Detailed data of treatment and control average RGB values± Standard deviation at different time interval (initial day, 15th day, 30th day and 45th day).

Fishes reared in Treatment tank (average RGB values± S.D)				
TIME	Initial day	15th day	30th day	45th day
Red	147.5± 1.5	175.33± 2.62	213.6± 2.05	246.3± 1.24
Green	117± 1	98.3± 2.49	73.6± 2.05	53.3± 0.47
Blue	84± 0.5	77.3± 2.05	50± 0.74	31± 0.47
Fishes reared in Control tank (average RGB values ±S. D)				
TIME	0th day	15th day	30th day	45th day
Red	147.5± 1.5	147.5± 1.5	155± 1	165± 1
Green	117± 1	117± 1	113.5± 0.5	105.5± 0.5
Blue	84± 0.5	84± 0.5	81.5± 0.5	74.5± 0.5

3.3 Fish growth, feed conversion rate and efficiency

The total weight gain in fish fed on anthocyanin incorporated feed was marginally better than control group. Both FCR and FCE (%) was apparently better in fish fed on anthocyanin incorporated feed compared to control (Table-2). FCR in treatment was marginally better (1.92) compared to control group (2.08). Similarly, feed conversion efficiency (%) was also better in treatment groups (0.52%) compared to control (0.48%). The absence of fish mortality in both control and treated tanks indicate the non-toxic nature of pigment incorporation in feed.

Table 2: Growth performance, feed conversion and survival:

Analysis Parameter	Control @10 fish/tank +2tanks=20 fish	Treatment @10 fish/tank +3tanks=30 fish
Mean initial weight of the fish (g)	0.8	0.8
Mean final weight of the fish(g)	3.2	3.4
Total initial weight of fish (g)	16	24
Total final weight of the fish (g)	64	102
Wt. Gain (%)	300	325
Survival (%)	100	100
Total quantity of feed fed (g)	100	150
Feed conversion ratio	2.08	1.92
Feed conversion efficiency (%)	0.48	0.52

3.1. Water quality parameters

During the feeding trial, the water quality parameters *viz*., temperature was between 26.6 and 28.2 °C, pH ranged 7.9 – 8.3; dissolved oxygen 7.6-8.4 mg/l, water hardness 200-250 mg/l and alkalinity 50-54 mg/l (Table 3). The values between control and treatment tanks, within replications and during sampling period were similar. Throughout the experimental period, the water quality parameters were well within the optimal values as recommended. All the fishes were healthy with no signs of stress or external infection.

Table 3: Details of water quality parameters

Sampling Water parameters	Initial		15th day		30th day		45th day	
	C	T	C	T	C	T	C	T
Temperature (^{0}C)	27.0	27.0	27.0	27.0	27.0	28.2	27.0	26.6
pH	8.3	8.3	8.3	8.0	8.3	7.9	8.3	8.3
Dissolved oxygen(mg/l)	8.0	8.0	8.0	7.6	8.2	8.4	8.0	8.0
Hardness of water (mg/l)	250	250	240	229	208	200	240	242
Alkalinity (mg/l)	53	54	53	50	53	50	53	53

C: Control; T: Treatment

4. Discussion

As fish cannot synthesize pigments on their own, they rely on dietary supplementation to enhance natural fish pigmentation [4, 15]. Several studies have demonstrated enhanced coloration and significant positive effect of dietary pigments in fishes. Use of natural pigments from plant sources to enhance the skin coloration in ornamental fishes has been reported. In the present study, anthocyanin pigments extracted from the peel of Beetroot (*B. vulgaris*), a waste material which generally goes to garbage was used to evaluate its potential application in enhancing the coloration of ornamental fish. Anthocyanins are known to be water soluble, and unstable on exposure to light, heat, and chemicals like any other natural pigments extracted from plant sources. The major concern in the anthocyanin's incorporation in feed was its stability. This was achieved by the authors through the process of co-pigmentation. The co-stabilized anthocyanins used for feed incorporation showed stability during the study period and was non-toxic to fish (Table-2).

Better growth (net weight gain) and colour enhancement is reported in red swordtail fish fed on feed incorporated with beetroot extract @10% as a source of carotenoids pigment (Singh and Kumar, 2016). Similar observations were reported in sword tail fed on natural carotenoid pigments extracted from marigold and ixora flowers [11]; turmeric powder, marigold, spirulina, drumstick leaves, pomegranates peel, orange peel, curry leaves [14] and in goldfish fed on natural carotenoid extracts of spirulina [12], microalgal biomass [21], red yeast (*Xanthophyllomyces dendrorhous)* [24] and alfalfa (*Medicago sativa*). Better coloration was reported in marine ornamental fish *Amphiprion ocellaris* when fed with natural carotenoids extracted from five different plant sources [17]. Spectrophotometric analysis of colour pigment deposition/ concentration showed enhanced coloration even in the flesh of the treated fishes as compared to the untreated ones. However studies related to use of anthocyanins extracts for enhancement of coloration in ornamental fishes are limited. Effect of incorporation of anthocyanin pigment extracted from beetroot peel and red cabbage as colour enhancer in ornamental and food fishes has not been reported. The present findings of better growth and skin color pigmentation in orange sword tail fish associated to feeding co-stabilized anthocyanin extracts (@400 mg/kg feed) obtained from beet root peels and red cabbage waste supports earlier reports of Tsushima, 1998 [11] and Kiriratnikom, 2005 [12] in gold fish relates to feeding anthocyanin extracts obtained from leaves of sweet potato, cassava and colocasia, and of dietary incorporation of carotenoid extracts of marigold, turmeric powder, spirulina, drumstick leaves, pomegranates peel, orange peel, curry leaves [14, 16].

5. Conclusion

The findings of present study indicate significant increase in red intensity on skin colouration of sword tail fish suggesting that the fish is ably utilize co-stabilized anthocyanin pigment extract of red cabbage and bio waste-beetroot peel.

The dietary incorporation level @400 mg/kg feed is found to be effective in bringing desirable colour intensification on skin pigmentation of orange sword tail fish with no negative effect on growth and survival of fish.

The study throws up new opportunities for the industry to commercially explore extraction of anthocyanin pigments from red cabbage and bio waste-beetroot peel which is mainly going as waste /used for uneconomical activities

The possible use of anthocyanin extract for dietary incorporation to enhance flesh coloration in edible fishes is a new opportunity that could be further explored. Such value-added fishes are known be much healthier with therapeutic value and have high commercial propositions.

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