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Effect of Citrus Peels Mingled Diets on *Carassius auratus* Coloration

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ABSTRACT

Present project was an endeavor to use natural carotenoid sources to enhance skin color of gold fish *Carassius auratus*. Citrus peels were collected from local market dried and grinded. Organic solvent extraction was carried out by hexane and acetone mixture (1:1 v/v). Carotenoid concentration was determined by thin layer chromatography (TLC) and found satisfactory. By mixing fish meal, sunflower meal and rice polish four treatment diets, T_1, T_2, T_3, were prepared and citrus peel powder was added @ 200g, 400g and 600g/ 1000g, respectively. While fourth one, control diet (T_o) was without citrus peels. These is nitrogenous diets (30% protein) were offered to *Carassius auratus* juvenile having body weight 20±7.54g in powder form for 92 days. Weight gain, length gain and FCR were calculated fortnightly. At completion of feeding trial, color intensity and pigment concentration was measured in *Carassius auratus* skin. Statistical analysis of results showed non-significant differences ($P<0.05$) among all treatments in weight gain and FCR. Maximum color intensity 1.52±0.08 and carotene concentration in lateral and tail region, 0.4±0.01 and 0.05 ±0.02 were recorded in T_3 without any harmful effect. It is concluded that citrus peels are good, natural, low price Carotene source for color enhancement.

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MSH and S Abbas planned the research and wrote the manuscript. FK conducted research work. AM, MB and S Ashraf helped in data analysis and paper-writing.

Key words
Carotene, Goldfish, TLC, Growth, Color.

INTRODUCTION

Now a days, ornamental fishes are rapidly gaining importance because of their aesthetic and commercial value in the export trade all over the world. Attractive colouration determines the commercial value of any ornamental fish. Pigmentation in the skin is responsible for colouration in the fish (James and Sampaths, 2003). Ornamental fish keeping is also a popular hobby, among young and old alike. It has been estimated that 1.5 to 2 million people worldwide keep marine ornamental fish aquaria (Mandal *et al*., 2010; Lim and Wong, 1997). Estimated value of marine ornamental trade is 200-330 million US$ per year (Degnai and Gur, 1992).

Carotenoids is the primary source of the pigmentation in fish skin. Fishes cannot synthesis carotenoid in natural environment, so they meet their carotenoid requirements by ingesting specific aquatic plants (Zhao *et al*., 2003). Dietary carotenoids play significant role in regulation of skin and muscle color in fish after absorption carotenoids are transformed into other carotenoids if necessary, and incorporated into tissues. Carotenoids are vital for healthy growth, metabolism, and reproduction, as well as color, in fish (Li *et al*., 2005). Astaxanthin is the main carotenoid pigment of red-pink coloured aquatic animals, being widely used in aqua-cultural processes due to its chemical stability (Madhupratap *et al*., 2001). The colour enhancing diets should contain additional natural pigments to enhance the colour of ornamental fish (Rickman *et al*., 2007).

Carotenoids in fish feed give yellow, red and pink color to fish skin, flesh and eggs. Some 600 species of plant and water organism contain these colorant substances (Irie and Seki, 2002; Furuita *et al*., 2003). Astaxanthin, which is the most effective coloring pigment exsists densely in water organisms such as gammarus, copepods etc and starfish (Ando *et al*., 1992).

Astaxanthin and canthaxanthin are widely prepared artificially and used as supplements in diets for goldfish and other ornamental fishes to induce the desired coloration (Kithara, 1983).

Goldfish (*Carassius auratus*) is a widely cultured and commonly traded ornamental fish in the global aquarium business (Shahidi *et al*., 1998). Color is a major factor that determines the quality and market value of any ornamental fish (Kitahara, 1983). Skin pigmentation of goldfish has been accomplished by supplementing their diets with synthetic or extracted carotenoids, such as zeaxanthin, lutein or astaxanthin (Royes *et al*., 2006; Seyedi *et al*.,

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2013). From natural sources containing Carotenoids, the Red Yeast, *Xanthophyllomyces dendrorhous* (Wang *et al*., 2006), *Spirulina* (Wallat *et al*., 2002), *Chlorella vulgaris*, *Haematococcus pluvialis* and *Arthrospira maxima* (Gouveia *et al*., 2003) have already been used for pigmentation of Goldfish. Citrus (*Citrus reticulate*) peels have been tested as a protein source in some ornamental fish species (Reitain *et al*., 1997; Passos *et al*., 2007). However, nothing has been reported on its use as a pigment source for goldfish or other aquatic animals, except in freshwater crayfish, *Cheraxquadri carinatus* (Paulo and Antonio, 2005). So, recent effort has focused on use of this natural carotenoid source citrus (*Citrus reticulate*) peels as alternative to synthetic carotenoids in goldfish diet for color enhancement.

MATERIALS AND METHODS

Site and sample collection

Present experiment was conducted in Fish Hatchery and Laboratory, University of Veterinary and animal Sciences, Lahore, Pakistan. Goldfish of uniform size 20±7.54g were collected from ornamental fish market, Akbar Chowk, Lahore and acclimatized to laboratory conditions for one week before initiation of experiment. Complete randomized design (CRD) was used in this study. Citrus (*Citrus reticulate*) peels were collected from local market, dryed and grinded in powder form by electric grinder machine.

Pigment extraction from citrus peel

Veronica *et al*. (2010) was followed for pigment extraction from citrus peels. Citrus peel powder (200g) was weighed and immersed in 400 ml acetone solution and shaked well. The mixture was kept at room temperature, in darkness, for 12 h and continuously shaken by magnetic stirrer. Afterward mixture was filtered and evaporated on hot plate. Residues were extracted with petroleum ether and extract was left to saponify for 12 h at room temperature. Later, it was washed with 100 ml ether and diluted with 200 ml distilled water. Two phases were successively partitioned at this stage. The upper lipophilic phase contains the carotenoids. These carotenoids were concentrated on hot plate almost to dryness and stored till further use. The stored extract was dissolved in acetone and submitted to TLC on silica using petroleum ether and diethyl ether in same ratio.

The total carotenoids contents were calculated as µg per g weight of sample as follows:

$$\text{Total carotenoid content} = \frac{\text{absorption at maximum wave length}}{0.25 \text{ sample weight}} \times 10\ {}^{\mu g}/_{g}$$

Where, 10 is the dilution factor and 0.25 is the extinction coefficient.

Feeding trial

Fish feed was prepared by mixing traditional feed ingredients *i.e.* fish meal, sunflower meal and rice polish following Gupta *et al*. (2007) and were termed as T_1, T_2, T_3 and T_0. All diets were iso-nitrogenous with 30% protein level and were offered to fish in powdered form. Already ground citrus peels were mixed @200, 400, 600g/1000 g of basal diet in T_1, T_2 and T_3, respectively, while fourth treatment diet T_0 was control and prepared without citrus peels.

After preparation, proximate analysis of diets was performed for determination of total moisture, proteins, lipids and ash contents following AOAC (2000). When nutritional value of prepared diets was found satisfactory, prepared diets were offered to experimental fish in troughs @ 4% body weight for 90 days. The experiment was conducted in triplicate in 4 troughs having 70 liter volume capacity. In each experimental group for 90 days. Experimental groups were termed as G1, G2 and G3. In each group, fifteen fish approximately of equal size (20±7.54 g) were randomly selected and kept. Water quality parameters (dissolve oxygen level, temperature and pH) were recorded on daily basis while growth parameters like weight gain (WG), length gain (LG), feed conversion ratio (FCR), specific growth rate (SGR) and survival rate (SR) were calculated fortnightly. At the end of feeding trial, three fish were randomly selected from each treatment group and were subjected to pigment extraction procedure.

Fish growth parameters were calculated by following formulas:

$$\text{WG} = \text{Final wt.} - \text{Initial wt.}$$

$$\text{FCR} = \frac{\text{Duration in days consumed feed}}{\text{Final wt.} - \text{Initial wt.}}$$

$$\text{SGR}(\%) = \frac{\log_e(\text{Final wt.}) - \log_e(\text{Initial wt.})}{\text{Duration in days}} \times 100$$

$$\text{SR} = 100 \times \frac{\text{Final fish number}}{\text{Initial fish number}}$$

Pigment extraction from fish tissue

At the end of feeding trial, color pigment was extracted from fish tissue following Sant'Anna *et al*. (2005). One gram of fish body tissue was taken and mixed with 2.5 g of anhydrous sodium sulphate and gently meshed. Then 5ml of chloroform was added in it and left overnight at 0°C. Chloroform formed a clear 1-2 cm layer of carotenoids on upper surface of this mixture. The layer was removed and its optical density was recorded at ƛ470nm using spectrophotometer, taking 0.3 ml of chloroform diluted aliquots mixed with 3ml absolute ethanol. Blank tube

was used as standard for comparison. Wavelength at which maximum absorption was recorded was used for calculation of carotenoid concentration. Calculations were made by formula given below:

$$C = \text{Absorbance} \times \frac{10000}{2100}$$

Where, C is concentration (µg/g for tissue), 2100 is E (1%, 1 cm) or extinction coefficient of carotenoids in hexane at 472 nm and 10000 is the scale factor.

Skin colour analysis

For color analysis skin samples were taken from from lateral and tail regions. Reflectance spectroscopy and Portable Minolta Chroma Meter CR-300 (Konica USA) was used for color analysis following (Storebakken, 1987).

Statistical analysis

For statistical analysis SAS Version 9.4 was used. One way analysis of variance (ANOVA) was applied on data to find out the significant differences among means. Results were considered significant at ($p< 0.05$).

RESULTS AND DISCUSSIONS

Proximate analysis

Four treatment diets were prepared by mixing fish meal, sun flower meal and rice bran. Citrus peel (*Citrus reticulate*) powder was incorporated in diets at different ratios. According to Govind (2013) nutrients required for commercial and ornamental fish are same as per other animals.

Table I.- Nutritive evaluation of different treatment diets based on different ratio of citrus peel powder.

Tr.	Moisture	Protein	Fat	Ash	Orange peel g/kg
T_1	4.89±0.57^b	31.88±0.52^a	7.53±1.32^b	6.58±0.60^b	200
T_2	6.69±0.43^a	32.03±0.15^a	9.40±0.63^a	8.41±0.76^a	400
T_3	5.08±0.83^b	32.10±0.29^a	6.69±0.52^b	8.79±0.58^a	600
T_o	6.05±0.66^{ab}	31.81±0.21^a	4.75±0.84^c	5.70±0.50^b	-

Means with similar letters in a column are statistically non-significant ($p<0.05$).

According to Wang *et al*. (2006) various nutrients vary in proportion according to fish type. Protein concentrations vary from 30-32% in diets. Proximate analysis of four treatment diets was performed following AOAC (2000). All treatment diets were isonitrogenous and protein level was maintained at 30%. While moisture, fat and ash ratio vary in all diets, the values are presented in Table I.

According to Ho *et al*. (2014) protein is most crucial nutrient in fish feed and its requirements vary according to ornamental fish species, size, feeding rate and water quality parameters. According to Sim *et al*. (2005) protein requirement of guppy (*C. reticulate*) is 30-35%%, goldfish (*C. auratus*) is 30%. By observing these arguments it can be said that our prepared feed is suitable for ornamental fish. According to Wilson (1994) and Halver and Hardy (2002) fish growth rate mostly solely depends upon protein utilization rate. Remaining parameters like moisture, protein, fat and ash contents were also within range as described by different authors (Sargent *et al*., 1995; Li and Galtin, 2008). However, the concentrations of orange peel vary in all treatment diets.

Rf value is ratio between distance travelled by a pigment, solute and the solvent. In other words Rf = (distance moved by solute) / (distance moved by solvent) (Hartely and Kennedy, 2006).

Calculated Rf values of extracted pigments are presented in Table II. According to our results T_1 showed lycopene and xanthophyl with RF value 0.88 and 0.86, respectively. In T_2 leutine and pheophytin were found with Rf value 0.68 and 0.71. While in T_3 xanthophyl, α and ß-carotene were found having Rf value of 0.89 and 0.90. These values are in line with values as described by Gouveia *et al*. (2003) and Donato *et al*. (2003). They described Rf values for xanthophyl, chlorophyl b and a and carotene, as 0.16, 0.32, 0.44, and 0.91, respectively. So our selected orange peel powder has enough concentration of carotenoids and can be used as color enhancer in ornamental fish diet.

Table II.- Rf value and colors pattern of different plates showed under UV lamp.

Tr. No	Replicate No	R_f Value	Colors	Pigment name
1	1	0.88	Red-Orange	ß-carotene
	2	0.86	Yellow	Zeaxanthin
	3	0.87	Yellow	Astaxanthin
2	1	0.88	Yellow – brown	ß-carotene
	2	0.89	Yellow – brown	α−carotene
	3	0.81	Olive Green	Zeaxanthin
3	1	0.89	Yellow – Orange	−carotene
	2	0.90	Yellow – orange	Xanthophyll
	3	0.88	Yellow – orange	ß-carotene

Growth parameters

Fish growth parameters like weight gain, length gain, FCR, SGR and survival rate were recorded fortnightly (Table III). All treatments showed non-significant variation

for weight gain among all treatment diets, however in case of FCR T_1 and T_3 showed significant difference ($p<0.05$) from other two treatments T_2 and T_4. Total weight gain was recorded as 39.76±1.12, 40.63±1.43, 34.85±1.61and 42.34±1.21 among T_1, T_2, T_3 and T_0. While FCR was recorded as 3.31±1.74, 2.93±0.85, 3.87±1.07 and 2.43±1.37 among T_1, T_2, T_3 and T_0. This variation in weight gain can be justified by Macartney (1996) and Sales and Janssens (2003), who described that in captivity ornamental fish may show variation in protein digestibility. The difference in FCR value among four treatments can be justified by Pannevis (1993) and Lim *et al*. (2001). They stated that along with protein other components like lipids, carbohydrates and minerals also effect growth rate of fish. As there is variation in feed proximate analysis results so there is also variation in FCR values in our treatment diet results.

Table III.- Weight gain, length gain, FCR, SGR, and survival rate of *Carassius auratus* fed on different treatment diets.

Tr.	Total weight gain	Total length gain	FCR	SGR	Survival %
T_1	39.76±1.12^{a}	12.51±2.56^{c}	3.31±1.74^{a}	0.95^{a}	100%
T_2	40.63±1.43^{a}	13.37±3.18^{b}	2.93±0.85^{b}	1.27^{b}	100%
T_3	34.85±1.61^{ab}	13.81±1.12^{b}	3.87±1.07^{a}	0.91^{a}	100%
T_0	42.34±1.21^{a}	15.50±1.25^{a}	2.43±1.37^{b}	1.29^{b}	100%

Means with similar letters in a column are statistically non-significant ($p<0.05$).

Table IV.- Carotenoid concentration in lateral, tail region (µg/g) and color intensity in muscle region of *Carassius auratus* in different treatment groups of fish tissue.

Sample	T_0	T_1	T_2	T_3
Lateral region	0.03±0.03^{a}	0.3±0.04^{b}	0.4±0.01^{b}	0.8±0.01^{c}
Tail region	0.02±0.01^{a}	0.03±0.02^{a}	0.05±0.02^{b}	0.06±0.01^{b}
Muscle color	1.23±0.15^{a}	1.32±0.17^{ab}	1.52±0.08^{b}	1.72±0.11^{c}

Muscle color was observed by comparing muscle sample to Salmonids Roche TM Color Card. Means with similar letters in a column are statistically non-significant ($p<0.05$).

Color assessment

Recoded color assessment and total number of carotenoid calculated are presented in Table IV. During experiment all groups were refractive to color enhancer. Skin color was estimated by comparing muscle sample with Salmonids Roche TM Color Card (Lim *et al*. 2001). In lateral region, carotenoid contents were measured as 0.3±0.04 0.4±0.01 0.8±0.01 and 0.03±0.03 in T_1, T_2, T_3 and T_0, while in tail region the carotenoid concentration was recoded as 0.03±0.02, 0.05±0.02, 0.06±0.01 and 0.02±0.01 for T_1, T_2, T_3 and T_0, respectively. Carotenoids are categorized as micronutrient and attractant for fish. For ornamental fish addition of carotenoids in fish diet enhance color pigment concentration in fish muscle (Hardy and Barrows, 2002). As the concentration of carotenoids increase in fish diet its color also enhanced.

Kruger *et al*. (2001) reported that when swordtail fish was fed with 40-80 mg kg^{-1} astaxanthin equivalent of orange peel carotenoids for eight weeks, it scored the highest for customer preferences and its muscle color had no difference with those treated with astaxanthin (Carophyll Pink TM) at same concentration. All groups showed enhancement in muscle color. Muscle color concentration was recorded as 1.32±0.17, 1.52±0.08, 1.72±0.11 and 1.23±0.15 for T_1, T_2, T_3 and T_0, respectively. These results are in agreement with results of Woods (2003), who reported similar findings when rainbow trout fed with from *H. roretzis* extract.

CONCLUSIONS

It can be concluded from above experiment that citrus (*Citrus reticulate*) peel is a good source of carotenoids and when given in feed positively effect color pattern in goldfish (*Carrassius auratus*).

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Statement of conflict of interest

The authors declare no conflict of interest.

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