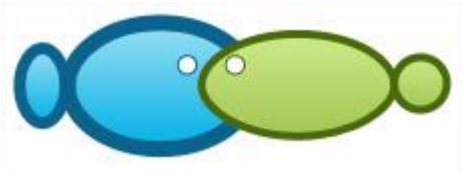

Impact of *Lantana camara*, a carotenoid source, on growth and pigmentation in Koi swordtail (*Xiphophorus helleri*)

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Abstract. The effects of dietary supplementation of the carotenoid pigment, β-carotene, from the flower of *Lantana camara,* incorporated in a formulated diet on the skin, muscle pigmentation and growth of koi swordtail (*Xiphophorus helleri*) ornamental fish was evaluated in the present study. The experimental fish with the initial weight of 0.8±0.02 g were fed with formulated diet incorporated with carotenoid pigment (50 mg/1000 g and 100 mg/1000 g), whereas the control group was fed a formulated diet without the pigment source for a period of 60 days. An increase in growth values, like the body weight gain, specific growth rate (SGR) and length was recorded in fish from the group fed with the diet with 100 mg of pigment. The feed conversion ratio (FCR) and condition factor (CF) values showed an increase in the fish fed with the diet with 50 mg of pigment, when compared with the values from the control group ($P<0.05$). A significant increase ($P<0.05$) was observed in the carotenoid content of skin and muscle after feeding the fish for 60 days. This is a pilot study analyzing the effect of a commonly found weed and a medicinal plant, *L. camara*, on the growth and coloration of ornamental fish, *X. helleri*. It is demonstrated that the pigment source from the flower had a positive effect, when added to the formulated diet.

Key Words: coloration, FCR, plant formulated diet, SGR, *Xiphophorus*.

Introduction. Ornamental fish are characterized by a wide range of color patterns and success in the ornamental fish trade is dependent on the lively color of the fish. India ranked second place after China in the world fishery production and it was placed sixth in Marine and inland capture fisheries, as reported in The State World Fisheries and Aquaculture, in 2008 (FAO 2010). The live-bearer, commercially important, tropical ornamental fish, *Xiphophorus helleri* (Heckel, 1848) also referred to as "koi swordtail", is native to North and Central America, in Rio Nantla, Veracruz, Mexico, northwestern Honduras. In Africa, populations were reported in Natal and eastern Transvaal, as well as in Lake Otjikoto, Namibia (Wischnath 1993). The characteristic color, body and fin shape play an important role in determining its market value. The koi swordtail male fish has an anal fin modified into a gonopodium and the tail fin has the aspect of a sword. These characteristics are absent in female fish. Fertilization is internal and it is accomplished by the insertion of the gonopodium with milt.

Carotenoids are organic pigments naturally occurring in chloroplasts and chromoplasts in plants and photosynthetic organisms. They provide the basis of pigmentation, and are incorporated in fish feeds by aquaculturists to induce a bright color in fish. Since the fish are unable to synthesize carotenoids *de novo* (Arulvasu et al 2013), they rely on dietary carotenoid content for their coloration, increasing their market value. Carotenoids are hydrophobic compounds that cannot be easily solubilized in the digestive tract of the fish. Therefore, to digest, absorb and transport carotenoids in the gasterointestinal tract, lipid emulsions are required. Carotenoids are solubilized in mixed bile salts and absorbed through the brush border of enterocytes (Ho et al 2014; Castenmiller & West 1998). Carotenoids are present in chromatophores, highly specialized cells present in the integument, classified according to the color pigment they

store: melanophores, xanthophores, erythrophores, iridophores and leucophores (Coliheuque 2010). Shahidi & Brown (1998) reported that color is the first characteristic perceived and a selection criterion of fish product, directly related to the subsequent acceptance or rejection.

Fish feed consists of important macronutrients, trace elements and vitamins, among others, for the maintenance of good health and development. Carotenoids play a significant role in the growth, reproductive performance, vitamin A production, antioxidant functions and immuno-regulation (Nakano et al 1999). Guroy et al (2012) reported that the pigmentation and growth performance, as well as cost and quality of the yellow tail cichlid (*Pseudotropheus acei*) showed improvements when fish were fed *Spirulina* sp. meal. Gupta et al (2007) observed an increase in body pigmentation of the tiger barb (*Barbus tetrazoa*) when fed with diets containing carotenoids from shrimp, marigold petals and annatto seed extracts. Corresponding results were observed by Joseph et al (2011) when *X. helleri* were fed with diets containing flowers of *Hibiscus rosa-sinensis, Rosa indica, Ixora coccinea* and *Crossandra infundibuliformis*.

Lantana camara is a notorious weed, known to cure several diseases and used in herbal medicine formulas. Thus, there is a strong scientific interest in its therapeutic potential in modern medicine, being a possible candidate for new medicinal drugs. This species can be a source of new livelihood opportunities by creating a market niche in herbal medicine (Priyanka & Joshi 2013). The chemical composition and biological-pharmacological activities of *L. camara* have been studied extensively. The chemical analyses of flower pigments revealed the presence of β-carotene (Ram & Mathur 1984). This plant possesses various medicinal properties, such as anti-microbial, fungicidal and insecticidal properties. Different parts of the plant may be used as herbal medicine for treating skin ailments, asthma, chicken pox, measles, and other affections (Mello et al 2005; Priyanka & Joshi 2013)

The present study was carried out to examine the efficiency and potential of two concentrations (50 mg/1000 g and 100 mg/1000 g) of *L. camara* flowers incorporated in formulated diets for exotic fish in affecting the coloration and growth of *X. helleri* (Koi swordtail).

Material and Method

Experimental fish and design. The test fish, *X. helleri* juveniles, one and a half months old, with an average initial weight of 0.8±0.02 g and a mean length of 3.5±0.01 cm were obtained from brood stock from the Ornamental Fish Research Center, Hebbal, Bangalore, India. The collected juveniles were transported to the laboratory in bags with oxygenated water. The fish were quarantined in tanks with potassium permanganate (1:1000 ppt) (2-3 dips for one minute each). The fish were stocked in a glass tank measuring 30x30x55 cm, full of water. Water was aerated by a constant supply of compressed air to maintain the dissolved oxygen (DO) level. 25% of the water in each tank was renewed daily with fresh dechlorinated water, to remove the feces and uneaten feed. The physico-chemical characteristics of water sampled from the experimental tanks were monitored every week.

Experimental procedures. Each experimental tank was stocked with 14 fish (4 males and 10 females). The experiment was carried out for 60 days in three replicates. The fish were acclimatized for 7 days prior to the beginning of the experiment. Prior to the experiment start day, fish were starved for 24 h and the total length and weight were measured. The fish were fed formulated diets containing 22.2% crude protein and 5.02% crude fat. The optimal daily feeding rates for *X. helleri* are sometimes contradictory, but the feeding rates (% body weight/day) were determined based on the recommendations of different researchers (El Sayed et al 2015; Rad et al 2006). Thus, fish were fed at the rate of 15% (28 days), 10% (20 days) and 5% (12 days) of body weight per day for 60 days. Daily rations were adjusted every two weeks according to fish body weight in each of the tanks. The physico-chemical characteristics of water sampled from all the tanks throughout the experimental period were analyzed weekly. The DO, free ammonia,

hardness, total alkalinity, ammonia nitrogen and pH were determined by the standard methods of APHA (2005).

Collection and identification of L. camara. Samples of *L. camara* flowers were collected from the nursery garden of Bangalore university campus, Bangalore. Plant material was identified and authenticated by the Department of Botany, Bangalore University. *L. camara* belongs to the phylum Spermatophyta, subphylum Angiospermae, class Dicotyledonae, order Lamiales, family *Verbenaceae*. The powdered flower of *L. camara* was used as a feed additive containing β-carotene and fed to the experimental fish.

Preparation of the formulated diet. In the present study, the formulated basal diet serving as control was prepared with the ingredients mentioned in Table 1, procured from the local market. The chemical composition of the feed is mentioned in Table 2. The ingredients were ground into fine particles and sieved before use to avoid large particles. Pre-weighed ingredients, except the premix and oils, were mixed thoroughly in a plastic container with water to form a dough. The dough was left for 30 minutes for conditioning and was then steamed for 25 minutes. The steamed dough was allowed to cool and vitamin mineral mixtures (Maxirichforte capsules) and plant oil were added. Pellets were prepared by a hand pelletizer. They were air dried for 24 hours and kept in an oven for 3-4 hours at 35^0C for further drying using the square method (Hardy 1980).

The formulated diet was analyzed for proximate composition prior to formulation of the test diets employing standard methods (AOAC 2005) (Table 2). The determination of the proximate composition was conducted with the following methods: crude protein - Kjeldahl method (2100-Auto-analyzer, Foss, Hillerod, Denmark); crude fat - ether extraction using Soxtec System HT (Soxtec System HT6, Foss, Hillerod, Denmark); moisture - oven drying at 105℃ for 24 hours; ash - combustion at 550℃ for 12 hours.

The proximate composition of the *L. camara* on a dry matter basis (%) is mentioned in Table 3. Experimental feed was prepared by the incorporation of dried and powdered petals of flowers of *L. camara* at 50 mg in 1000 g of formulated diet (0.01 µg carotenoid pigment) in Diet 1 and 100 mg in 1000 g of formulated diet (0.02 µg carotenoid pigment) in Diet 2.

Table 1

Ingredients of formulated diet (Basal diet), Diet 1 and Diet 2

No	*Diet Ingredients (g/100 g feed)*	*Control (Basal Diet)*	*Experimental Diet 1*	*Experimental Diet 2*
1	Fish meal	27.5	27.5	27.5
2	Groundnut oil cake	10	10	10
3	Wheat flour	20	20	20
4	Rice bran	20	20	20
5	Vegetable oil (mL)	2	2	2
6	Vitamin and mineral mix	4	4	4
7	*Lantana camara* powdered flower (mg)		50	100

Note: vitamin and mineral mix (mg/100 g feed): vitamin A 1600 IU; vitamin D3 100 IU; vitamin E acetate 5 IU; energy 7.457 Kcal; protein 0.00253 g; fat 0.824 g; carbohydrates 0.01.4 g; calcium 75 mg; phosphorous 58 mg; vitamin C 25 mg; nicotinamide 15 mg; magnesium 3 mg; potassium 2 mg; vitamin B1 1 mg; vitamin B2 1 mg; calcium pantothenate 1 mg; vitamin B6 0.5 mg; manganese 0.5 mg; zinc 0.5 mg; folic acid 50 mcg; vitamin B12 0.5 mcg; copper 0.45 mg; molybdenum 0.1 mg; iodine 0.075 mg.

Analysis of growth parameters. After 60 days, all the fish were harvested and their length and weight was recorded. Body weight gain (WG), length gain (LG), specific growth rate (SGR) and survival rate were analyzed based on standard formulas (Zhu et al 2014). The feed conversion ratio (FCR) was determined (Bailey et al 2003). The condition factor (CF) was also determined (Ai et al 2006). The formulas used to determine the aforementioned parameters are:

BWG (%) = final body weight - initial body weight

LG (%) = final body length - initial body length

SGR (% body weight/day) = 100 × [Log(W_2)-Log (W_1)]/time (days)

Where: W_1 and W_2 - initial and final wet weight (g), respectively.

FCR = Feed delivered to group/Live biomass gain of that group

CF = 100 x (W/L^3)

Where: W - wet body weight (g); L - standard body length (cm).

Survival rate %= (Final fish number - Initial fish number) × 100/Initial fish number

Analysis of total carotenoid content (TCC). The total carotenoid content was analyzed in the skin and muscle tissue of the fish, immediately after the 60 days of rearing, by following the pigment extraction method as described by Olson (1979). One gram of koi swordtail skin and muscle tissue was sampled. The tissue was gently mashed in a glass homogenizer. 2.5 g of anhydrous sodium sulphate and 5 mL of chloroform were added and it was kept overnight at 0°C. 0.3 mL of aliquot of separated chloroform was then diluted with 3 mL of absolute ethanol. The optical density was read at 380, 450, 470 and 500 nm, in a Systronic spectrophotometer Model no. 104 and maximum absorption was recorded at 470 nm wavelength. A blank was prepared in a similar manner without using the fish body tissue.

Total carotenoid concentration (µg) = [absorbance at maximum wave length/(0.25 x sample weight in grams)] x 10

Where: 10 - dilution factor; 0.25 - extinction coefficient.

Statistical analysis. The statistical analysis of the data was performed and tabulated by using One-way analysis of variance (ANOVA) and Tukey's multiple comparison post-hoc test. The linear relationship was assessed by using linear regression and the Pearson correlation coefficient. All statistical analyses were performed using GraphPad Prism version 6.

Results and Discussion

Water parameters of experimental tanks. The water analysis showed values within the recommended range for ornamental fish culture. The DO levels were between 6.13±0.08 and 7.4±0.13 mg L^{-1}, the free ammonia mean level was 0.73±0.02 mg L^{-1}, the hardness ranged from 240±0.07 to 300±0.12 ppm. The alkalinity ranged from 120±0.32 to 213.5±0.02 mg L^{-1}, ammonia nitrogen had a mean value of 0.063±0.02 ppm and pH values ranged between 7.5±0.04 to 8.5±0.09, in all the tanks throughout the experiment period of 60 days. The values of all water parameters were within the range of those recommended for the culture of ornamental fish (OATA 2008).

The chemical composition of feed ingredients and experimental diets. The chemical composition of the formulated diet showed the highest quantity of dry matter (89.76±0.34%) and the lowest quantity of crude fat (3.62±0.01%). Nitrogen-free extract was 60.25±0.01. Crude protein was present in adequate quantities of 22.18±0.01%. The information is presented in Table 2.

Table 2

Chemical composition (%) of the formulated diet on a dry matter basis (mean ± SE)

No	*Chemical Composition*	*Formulated Diet*
1	Crude protein	22.18±0.01
2	Dry matter	89.76±0.34
3	Moisture	10.24±0.01
4	Crude lipid	3.62±0.01
5	Total ash	16.19±0.02
6	Nitrogen-free extract	60.25±0.01

The chemical composition of L. camara. The chemical composition of *L. camara* is presented in Table 3. The chemical composition of *L. camara* showed a quantity of moisture of 51.72±0.23%. *L. camara* also contains high levels of ascorbic acid (25.34±0.76%), crude fiber (20.73±6.35%) and β-carotene (20±0.01%). Minimum amounts of crude protein, zinc and total ash (Table 3) were also detected.

Table 3

Proximate composition of *L. camara* on dry matter basis (%) (mean ± SE)

No	*Proximate composition*	*L. camara (%)*
1	Moisture	51.72±0.23
2	Crude protein	1.62±0.31
3	Crude fiber	20.73±6.35
4	Total ash	0.84±0.04
5	β-carotene	20±0.01
6	Ascorbic acid	25.34±0.76
7	Zinc	1.12±0.32

Growth performance of X. helleri. Growth performance recorded during the 60 days of experimental period is presented in Table 4. During the present study, the fish were healthy and active, and no mortality was observed. 100% survivability was recorded in the control and experimental fish groups fed the three diets. We primarily laid out the inclusion of *L. camara* as a feed additive for *X. helleri*, without negatively affecting their health status. Jain (2015) reported 100% survivability of koi carp fed with supplementary diets with carrot meal 3%, 5% and 7%, whereas 80% survival rate was observed in the control group when fed with diets without carrot meal. In the present study, the fish group fed with Diet 2 (100 mg/1000 g) showed a significant increase in growth (body weight and body length) when compared to those fed with Diet 1 and control diet. The fish BWG, BLG and SGR showed significantly higher values for the fish fed with Diet 2 ($P<0.05$) when compared to those fed with Diet 1 and control diet. The values of the FCR showed a reduction for fish fed with Diet 2 (4.5±0.089) (one-way ANOVA; $P<0.0001$) when compared with those fed with Diet 1 (5.8±0.015) and control group (7.3±0.018). This indicated that Diet 2 incorporated with 100 mg/1000 g of natural pigment *L. camara* showed better feed utilization compared to Diet 1 and control. This can be attributed to the higher fiber content in Diet 1 and control, which reduced the digestibility of the diet. Thereby, increase in fish body weight and growth was recorded in fish fed with Diet 2. A correlation test between the growth parameters (body weight and body length) resulted as illustrated in Figure 1. Body weight was positively related with body length of *X. helleri* showing a slope of $Y=0.7283$ and $R^2=0.9464$.

CF values in experimental groups, when compared to the control groups, were insignificantly different ($P>0.05$), but fish fed with diet 1 showed higher values, when compared to those fed with Diet 2 and control group.

Table 4

Growth parameters of *Xiphophorus helleri* fed with experimental Diet 1, Diet 2 and control diet for 60 days

No	*Growth parameters*	*Control*	*50 mg/1000 g (Diet 1)*	*100 mg/1000 g (Diet 2)*
1	Initial body weight (g)	0.8±0.02	0.8±0.02	0.8±0.02
2	Final body weight (g)	2.1±0.16^{a}	2.4±0.03^{b}	2.9±0^{c}
3	Body Weight Gain (g)	1.3±0.16^{a}	1.6±0.03^{b}	2±0^{c}
4	Specific Growth Rate weight (% bw/day)	0.69±0.04^{a}	0.79±0.02^{b}	0.93±0.02^{b}
5	Initial Length (cm)	3.5±0	3.5±0	3.5±0
6	Final Length (cm)	5.7±0.05^{b}	5.9±0.05^{b}	6.2±0.05^{b}
7	Body length gain (cm)	1.2±0.33^{a}	1.4±0.33^{b}	1.7±0.33^{c}
8	Specific Growth Rate length (% tl/day)	0.25±0.058	0.2±0.012	2.23±0.012
9	Condition factor (CF)	1.135±0.002	1.208±0.04	1.183±0.017
10	Food conversion ratio (FCR)	7.3±0.018^{a}	5.8±0.015^{b}	4.5±0.089^{c}

Note: values are presented as the mean ± standard error (SEM) of the three replicates. Significance was calculated by one-way ANOVA and post-hoc test was done with Tukey's multiple comparison using GraphPad Prism 6.0. Significant differences (P<0.05) between carotenoid treatments are indicated by different superscript letters.

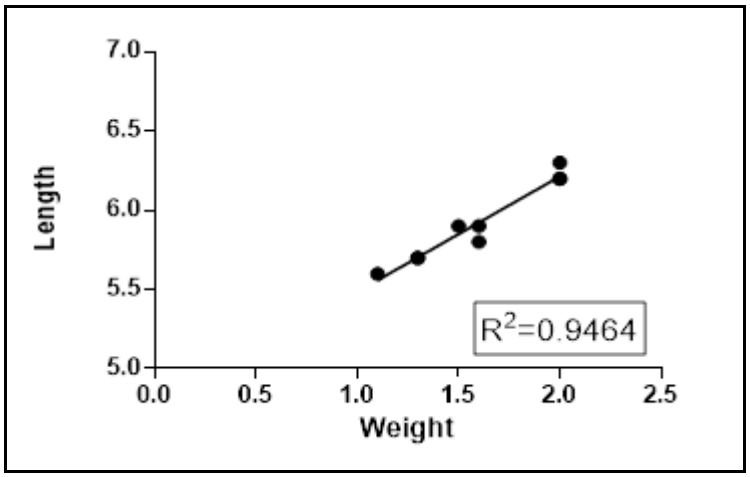

Figure 1. The linear relationship assed by linear regression and Pearson correlation coefficient, between the body weight and total length of *Xiphophorus helleri*, using GraphPad Prism ver. 6.0.

X. helleri fed the formulated diet incorporated with carotenoid pigment from flowers of *L. camara* showed significant increase (P<0.05) in growth and coloration in both experimental groups. The group fed with high carotenoid Diet 2 presented higher values in terms of BWG, BLG, SGR and CF when compared with the fish fed the control diet. This can be attributed to the proper utilization of the highly adequate dietary nutrients, ascorbic acid and growth enhancing capacity present in the feed, because *L. camara* is a suitable ingredient for animal feeds due to its high protein content, essential vitamins, minerals, macro and micronutrients. It can be used to improve food security (Mugera et al 2015). Liang et al (2012) observed a significant increase in weight gain in red white koi carp fed with 150, 200, and 250 mg of astacin/kg of diet. Similar results were also observed by James et al (2006) regarding the body length, body weight and SGR of *X. helleri* fed with a dietary inclusion of 8% *Spirulina* spp. Amar et al (2010) suggested that carotenoid pigments play a positive role in the enhancement of fish metabolism, which in turn is responsible for improved nutrient utilization and, thereby, an acceleration in growth. Yanar et al (2008) established that alfalfa (*Medicago sativa)* is an alternative

natural carotenoid source compared with the synthetic ones and Aderolu & Sogbesan (2010) suggested that graded levels of cocoyam *(Colocasia esculenta)* (0, 25, 50, 75 and 100%) added to maize meal can ensure good pigmentation, growth and feed utilization in goldfish (*Carassius auratus)* and *Clarius gariepinus*. In the present investigation, the decrease in FCR values for fish fed with experimental Diet 1 and 2 clearly indicated a better utilization of the diet due to the presence of ascorbic acid in the powdered flower of *L. camara.* This limitation in its digestibility may be attributed to presence of high crude fiber levels in the diet, since carnivorous/omnivorous fish lack cellulase activities in the gut for effective carbohydrate digestibility. In the present study, the positive correlation between BW and BL in fish fed with Diet 1 and 2 suggested that diets with carotenoids improved the growth performance of *X. helleri* (Figure 1).

Carotenoid pigment in the skin and muscles of fish. After 60 days of treatment, carotenoid pigment values in the skin and muscles of the fish were analyzed (Figure 2). The results clearly showed a presence of high carotenoid content (1.716 µg/g) in the skin and muscles of the fish fed with Diet 2, followed by its decline in fish fed with Diet 1, throughout the experiment. The results indicated that the efficiency of carotenoid assimilation by *X. helleri* is not age dependent, but it depends on the concentration of carotenoid pigments incorporated in the diet. One-way ANOVA analysis revealed significant effects of pigment incorporated formulated diets, ($P<0.0001$). Through multiple comparison tests it was determined that the differences between control and Diet 1, control and Diet 2 as well as Diet 1 and Diet 2 were extremely significant ($P<0.0001$), in terms of carotenoid content in fish.

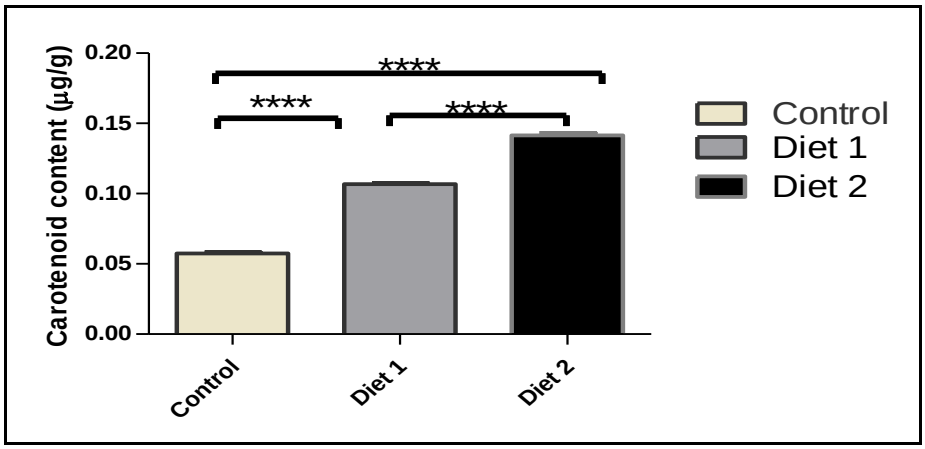

Figure 2. Spectrophotometer analysis (ug/g wet weight) of *Lantana camara* as a carotenoid pigment in skin and muscle of *Xiphophorus helleri*. Extremely significant differences among treatments are denoted by **** ($P<0.0001$).

Koi swordtails have a patchy orange-red color on their dorsal side, head and caudal region and fins. In the present experiment, Diets 1 and 2 incorporated with pigment successfully enhanced the carotenoid pigment level in fish skin and muscle. This was confirmed by visual observations of the darkening of the skin color (dark orange-red). The appropriate level of carotenoid varied in different studies, due to the different effectiveness of the species in depositing carotenoids (Ha et al 1993). Lee et al (2010) suggested that the pigmentation of fish is affected by the source, chemical structure and concentration of carotenoids, dietary fats, fish species, feeding period and environmental conditions. Ramamoorthy et al (2010) observed an enhanced coloration in marine ornamental fish *Amphiprion ocellaris* when fed with diets with different types of carotenoid pigments (carrot meal, China rose-petal meal, rose petal meal and marigold meal). Carrot meal resulted in higher carotenoid contents in fish skin and muscle when compared with the results of the other flower meals and the non-pigmented meal. Similar results were observed in the skin carotenoid content of the jewel cichlid, *Hemichromis bimaculatus*, and in *Cichlasoma severum* fed with carrot meal as compared with red

pepper meal (Mirzaee et al 2013; Kop et al 2010). The results are in line with the findings of Yedier et al (2014) for Zebra cichlid (*Maylandia estherae*) fed with *Spirulina* spp. The fish showed a decrease ($P<0.05$) in L values (lightness) along with increased 'a' (red) and 'b' (yellow) values, which led to increased chroma (C). The increase in carotenoid content in the muscle, skin and fins was directly proportional to the increase in carotenoid content in the diet of *X. helleri*. According to Swain et al (2014), plant additives can be used as cheap sources of pigment for different ornamental fish species. These results were in conformity with the ones reported by Soler-Vila et al (2009), who observed a dark orange pigmentation in *Onchorhynchus mykiss* fed red algae *Porphyra* spp. diets. Ezhil (2008) observed the same effects in *X. helleri* fed with 15 g of marigold pellet meal (6 g/100 g).

The formulated diet used in this study limits the effects of dietary deficiencies or energy difference that may occur in commercial diet formulations. The results indicate that the efficiency of carotenoid assimilation by *X. helleri* is not age dependent, but dependent on the carotenoid concentration in the diet. These types of studies would help researchers and feed manufacturers to further refine a diet formulation that will produce a better level of orange-red skin coloration in the fish.

Conclusions. To conclude, this study presents insights to a low cost pigment application, from a natural product incorporated in a formulated diet for healthy fish, which is a pre-requisite for potential aquaculture practices. In this study, growth and coloration showed marked improvements due to carotenoids from the flowers of *L. camara*.

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