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Effect of natural β-carotene from-carrot (*Daucus carota*) and Spinach (*Spinacia oleracea*) on colouration of an ornamental fish - swordtail (*Xiphophorus hellerii*)

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Abstract

The present experiment was conducted to evaluate the impact of two natural β-carotene vegetable sources *viz*., carrot (*Daucus carota*) and spinach (*Spinacia oleracea*) for inducing colouration in an ornamental fish swordtail (*Xiphophorus hellerii*). The Experimental fish were cultured for 35 days in aquarium tanks. The experimental diets were prepared with two natural vegetable sources *viz.* carrot (*Daucus carota*) and spinach (*Spinacia oleracea*) and fed to the fish once a day at 3 percent of their body weight to supply 20, 25 and 30 mg of β-carotene individually per 100 g of diet. There were 6 experimental diets three for each β-carotene sources and 1 common control diet (devoid of β-carotene). The water quality in the entire aquarium was maintained periodically. The proximate analysis of the experimental diet was also carried out but doesn't depict any significant changes in their composition. The colour intensity and prominence of orange colour increased in the fish with diets enriched with natural β-carotene sources *viz*., carrot (T_1, T_2 and T_3) and spinach (T_4, T_5 and T_6). Red-orange colour pigmentation was highest in fish fed with a carrot. The hue angle (h°) and yellow-orange colour intensity was found significantly high ($P<0.05$) in fish fed with spinach in T_4 (h° = 58.33). In the case of carrot h° value was found highest in T_1 (h° = 43.81) where the orange colour intensity was found higher. The maximum luminosity (L*) was found in T_2 (20.81). The highest red colour intensity red (a*) value was found in T_6 (4.22) whereas, the maximum yellow colour intensity (b*) value was noted in T_4 (5.34). The maximum colour saturation i.e. chroma (C*) was observed in T_4 (6.29) in case of spinach and in case of carrot it was maximum in T_1 (5.81). The result revealed that the fish fed with spinach have developed more yellow colour and fish fed with carrot developed red colour. From this study, it could be concluded that the easily available natural β-carotene sources incorporated feed as a colour enhancer can be prepared at a lower cost.

Keywords: β-carotene, colour, orange, carrot, spinach, aquarium

1. Introduction

Attractive colouration of ornamental fishes determines its commercial value. Pigmentation in the skin is responsible for colouration in the fish. Carotenoids are the key source of the pigmentation of the skin of fishes. In the natural environment, the fishes meet their carotenoid requirements by ingesting aquatic plants or through their food chains. Colouration in fishes has a great importance in camouflaging as well as during breeding.

Fish do not possess the ability to synthesize carotenoid [13]. The carotenoid pigmentation of fish results from the pigment present in the diet [19, 32]. Pigments are responsible for the wide spectrum of colours in fishes which is essential prerequisite for their quality as brilliantly colored fishes fetch higher price in the commercial market. As fishes cannot synthesize their own colouring pigments *de novo*, the colouring agents which are synthesized by plants, algae and microorganisms, need to be incorporated in their diet [23, 9]. Carotenoids are responsible for many of the red, orange and yellow hues of plant legumes, fruits and flowers. Thus, pigmentation is an important criteria for the fishes, since their colour affect commercial acceptability. The growing interest in aquarium fishes has resulted in a steady rise in the aquarium fish trade globally. Ornamental fishes are nowadays rapidly gaining importance in aquaculture because of their aesthetic and immense commercial value in the export trade world over. The ornamental fish trade with a turnover of US $ 6 Billion and an annual growth rate of

8 percent offers a lot of scope for development in India [27]. Many studies have proved that the fish can be pigmented by including processing wastes and plant sources in their diet [22, 6, 1]. Carotenoid are the class of 800 natural fat-soluble pigments found principally in plants, algae, photosynthetic bacteria and some non-photosynthetic bacteria, and they play critical role in the photosynthetic process. They also occur in yeast and moulds where they carry out a protective function against damage by light and oxygen. Carotenoid also plays other important functions as pro-vitamin A, antioxidant, immunoregulators and they are mobilized from muscle to ovaries in salmonids, which suggest a function in reproduction [30, 5]. It has also been observed that fishes with a high level of carotenoid are more resistant to bacterial and fungal diseases [30]. The commercial value of these fishes reflects this requirement; hence, the ornamental fish growers are constantly exploring methods of enhancing skin colouration. Carotenoid, beta-carotenoid pigments are widely used to produce red, yellow, orange, and pink coloration, especially in fishes.

However, recent efforts were focused on naturally available two vegetables sources of β-carotene i.e. Carrot (*Daucus carota*) and Spinach (*Spinacia oleracea*) in powdered form in diet to work out their effect on the colouration of commonly cultured live bearer aquarium fish swordtail (*Xiphophorus hellerii*) as an alternative because of concerns about the use of synthetic additives and their high cost.

2. Materials and methods

2.1 Experimental Fish: Experimental ornamental fish swordtails (*Xiphophorus hellerii*) were procured from the local ornamental fish trader and acclimatized to laboratory condition in glass aquaria. The experiment was run with three hundred sixty swordtail with body length and weight range between 3.6 to 2.8 cm and 0.55g to 0.46g respectively. The fish were conditioned and fed with control diet (devoid of β-carotene source) for 3 weeks to equalize their body carotenoid content in indoor conditions. The water exchange and aeration were given sufficiently. Three FRP tanks of capacity 200 litres were used for conditioning of swordtail and 120 fish were placed in each tank before administrating the experimental diets.

2.2 Carotenoid Analysis in natural sources: The natural β-carotene sources i.e. carrot and spinach were analyzed for carotenoid content [33]. Dried ground powdered form of 200 mg samples were transferred into 10 ml pre-weight glass tube. The samples were mixed thoroughly in about 5 ml acetone containing 1.5 gm of a hydrous sodium sulphate with the help of homogenizer. The extraction quantity was made up to 10 ml of acetone. The samples were stored in refrigerator for 3 days at 4°C. They were extracted 3 to 4 times until no more colour would be obtained. The solution was centrifuged at 5000 rpm for 5 minutes and then absorption was measured in a spectrophotometer (wavelength (λ) 470, 662 and 645). Once detecting β-carotene content in carrot and spinach i.e. 1.10 and 1.58 percent respectively, proportional amount of dried and powdered natural β-carotene source was mixed in basal diet, replacing equivalent quantity of rice bran (Table. 1), for achieving 20, 25 and 30 mg/100 g β-carotene in experimental diet for both the selected sources.

2.3 Experimental Feed Preparation: The experimental feed were prepared with basic ingredients such as wheat flour, rice bran flour, tapioca flour, soya bean flour, fish flour, groundnut oil cake and vitamin mixture [29]. All ingredients were split into 7 equal parts. The two vegetable sources of β-carotene i.e. carrot (*Daucus carota*) and spinach (*Spinacia oleracea*) were collected, air-dried in dark room to avoid denaturing of carotenoids. The natural sources were oven dried at 45°C for 48 hours, and then the contents were powdered and sieved in particle size of 0.1 to 0.2 mm, afterward stored in the refrigerator at 4°C. The experimental diets were prepared using carrot and spinach powder mixed in the basal diet to supply 20, 25 and 30 mg of β-carotene per 100 g of diet. For the purpose dried carrot powder was added in the diet at the rate of 1.82, 1.27 and 1.72 g per 100 g of diet replacing the rice bran. Similarly, Spinach powder was added in the diet at the rate of 1.33, 1.67 and 1.99 g per 100 g of diet (Table 1).

Table 1: Formation of Experimental Diets, (Ingredients in gm/100gm)

Ingredients	Treatments/ in gram						
	T_1	T_2	T_3	T_4	T_5	T_6	T_7 (Control)
Soya bean	17	17	17	17	17	17	17
Fish meal	15	15	15	15	15	15	15
Groundnut oil cake	15	15	15	15	15	15	15
Wheat flour	14.5	14.5	14.5	14.5	14.5	14.5	14.5
Tapioca flour	14	14	14	14	14	14	14
Rice bran*	12.68	12.23	11.77	13.17	12.83	12.51	14.5
Vegetable oil	5%	5%	5%	5%	5%	5%	5%
Vitamins	5%	5%	5%	5%	5%	5%	5%
Dried Carrot powder **	1.82	2.27	2.73	---	---	---	---
Dried Spinach powder **	---	---	---	1.33	1.67	1.99	---
Total	100	100	100	100	100	100	100
Final β-carotene content mg/100 gm	20	25	30	20	25	30	0.00

*****Adjusted according to the quantity of powdered carrot/spinach.

****** The quantity of natural carotenoid will be proportionate to supply 20, 25 and 30 mg β carotene/100 gm diet. The dried Carrot powder used contains 1.1033% β- carotene and Dried Spinach powder contains 1.5837% β- carotene.

2.4 Feeding Experimental diet: This experiment was carried out for one control and six treatments in twenty one experimental glass aquaria in the indoor system. There were three replication for each experimental diet. All fish were fed at the rate of 3 percent of their body weight once a day with the experimental diet. Throughout the experiment, physico-chemical parameters were maintained. The experiment was carried out for 35 days. The observations for colour enhancement of the fish were measured initially and then at every 7 days interval and accordingly the diets were readjusted every week in each aquaria. The water quality in aquaria was maintained by aeration, removal of faecal matter and replacement of at least 20 percent of water every week.

2.5 Analytical Methodology: Water quality parameter *viz*., air and water temperature, pH, electrical conductivity, dissolved oxygen, free carbon dioxide, total alkalinity, N-nitrogen, orthophosphate and total hardness were analyzed initially and weekly [4, 34]. The experimental diets were analyzed for the proximate composition viz., moisture, crude protein, lipid, and carbohydrate and ash contents as per standard methods [3]. Colour intensity was analyzed by using colour difference meter equipment (Hunter colour lab, USA). The instrument measures colour parameters in terms of CIE L* (Luminosity), a* (red to green), b* (yellow to blue), h° (hue angle) and C* (chroma).

The lightness coefficient (CIE L*), ranges from black = 0 to white = 100, a* and b* are on the horizontal and vertical axis respectively. (+ a*) indicates a hue of red colour and (- a*) indicate a hue of green colour on x-axis. On vertical axis i.e. y-axis, (+ b*) indicate yellow and (- b*) indicates blue colour values. a* and b* are merely co-ordinates that indirectly reflects hue and chroma but are difficult to interpret separately. These co-ordinates are not independent variables [11]. Chroma (C*) indicates degree of departure from grey toward pure chromatic colour and hue angle (h°) indicates red, orange, yellow, green etc. a colour from the spectrum. The proper quantification of tristimulus colorimetric data is based upon trigonometric function [21]. A colour wheel subtends 360° with red traditionally placed at an angle of 0°, yellow at an angle of 90°, green at an angle of 180° and blue at an angle of 270°.

The data obtained for colouration characteristics of swordtail fed with β-carotene fortified diets were statistically analyzed by applying complete randomized design (CRD) and analysis of variance (ANOVA) for checking the significance (P<0.05) level of treatments [31].

3. Results and discussion

No considerable fluctuation in water temperature was observed (22.94°C to 23.60°C). The pH of the experimental water was in the range 7.6 to 8.2, whereas, electrical conductivity was between 900 to 1040 µS/cm. A slight fluctuation was recorded in dissolved oxygen and the value ranged between 5.4 to 7.1 mg/l. The free carbon dioxide was absent in all the treatments. The total alkalinity ranged between 300 to 340 mg/l and hardness between 460 to 490 mg/l. The value of orthophosphate and nitrate-N was recorded between 0.03 to 0.05 mg/l and 0.42 to 0.60 mg/l respectively in experimental water.

The proximate composition of vegetable sources i.e. spinach and carrot mixed diet and control diet used in present study with their maximum values were carbohydrates (45.20 to 45.90 percent), lipid (14.39 to 14.70 percent), protein (21.00 to 22.22 percent), ash (13.10 to 13.22 percent) and moisture (4.60 to 4.72 percent).

Carotenoid are the primary source of pigmentation in ornamental tropical fish, responsible for various colours like yellow, orange, red and other related colours. Normally, these are obtained through organisms rich in carotenoid content organisms in the aquatic food chain. Other carotenoid-rich ingredients used are marigold meal (lutein), red pepper (*Capsicum sp.*) extract (capsanthin) and krill or crustacean meals (astaxanthin) [7, 8].

The results pertaining to the comparative colour characteristics of swordtail fed with natural β-carotene enriched diet i.e. carrot and spinach (20, 25 and 30 mg/100 g diet) respectively given in Table 3 and revealed that compared to the control fish the maximum luminosity (L*) was 20.81 in T_2 in fish fed with carrot mixed diet. The maximum red (a*) value was found in T_6 i.e. 4.22 on X- axis whereas, the maximum yellow colour intensity (b*) value on Y-axis, hue angle (h°) and chroma (C*) was found in T_4 i.e.5.34, 58.33° and 6.29 respectively in fish fed with spinach mixed diet (Table 3 and figure 2). In case of carrot mixed diet the maximum red (a*) value on X-axis, yellow (b*) value on Y-axis, hue angle (h°) and chroma (C*) were found in T_1 i.e. 4.19, 4.02, 43.81° and 5.81 respectively (Table 3 and figure 1). The values of a* and b* decides the hue angle (h°) of swordtail. As the hue angle (h°) in Table 3 increases the yellow colour intensity of swordtail increases and as the value of hue angle (h°) decreases the red colour intensity increases. The results revealed that fish fed with spinach mixed diet have developed more yellow colour and fed with carrot, developed more of red colour. Higher doses of β-carotene was found to increase the orange colour intensity in fish. The result showed that compared to the control fish the colour intensity and prominence of orange colour intensity increased in the fish fed with Carrot (T_1, T_2 and T_3) (Table 3 and figure 1) and Spinach (T_4, T_5 and T_6) (Table 3 and figure 2).

These results showed that the fish fed with spinach have developed better yellow colour compared to the fish fed with carrot in other words the fish fed with carrot have shown more of red colour. Secondly the higher doses of β-carotene as carrot and spinach in diet increased the orange colour in fish. These findings confirmed that higher the doses of β-carotene as a carrot, the fish indicated prominent red-orange colouration followed by the results of spinach fed fish which indicated yellow-orange colouration. As such the ranking of treatments of orange colour prominence incorporated with natural β-carotene can be arranged in their efficiency to develop prominence of orange colour in test fish can be written as T_3 (29.89°) > T_2 (41.70°) > T_1 (43.81°) and T_6 (45.06°) > T_5 (55.98°) > T_4 (58.33°) and > T_7 (Control-13.41°) (Table 3 and figure 1-2).

Colouration in fishes is influenced by several external and internal factors including dilation of blood capillaries, reflective irridocytes, chromatophores, genetic and environmental factors, stress and array of natural and artificial colour enhancing compounds and fish diets has also been tried by several researchers. The skin colour in fishes could be changed by altering surrounding conditions, light intensity and dietary conditions [35]. It was found that blue green algae and spirulina as feed greatly influenced colour development of fish. It was further, reported that in absence of this colour enhancing algae, the plankton feed as control diet was failed to produce the natural red colour in cherry barbs. The natural microalgae *Chlorella vulgaries*, *Haematococcus pluvialis* and Cyanobacteria – *Arthrospira maxima* and *Spirulina platensis* can be used as a carotenoid source for enhancing skin pigmentation and prominent colouration in ornamental fishes [2, 15, 14].

Colour enhancing agent as feed additive from plant origin have been successfully tried in ornamental fishes. The present study was supported by the several researchers who found enhancement of pink colour in the fish and flesh of tilapia by feeding red colour extracted from sandalwood [26]. Some observed the effect of astaxanthin on the pigmentation of gold fish (*Carassius auratus*) and measured a significant increase in skin pigmentation and survival of fish fed with diets containing astaxanthin 36-37 mg/kg [28]. Colour intensity enhancement by paprika as feed additives in gold fish and koi carp using a computer – assisted image analysis. The 'r' value has a tendency to increase due to paprika feeding but significant difference was achieved only after four weeks of feeding [18].

Some naturally available micro algal pigments, yeast extract, marigold, capsicum etc. as a carotenoid rich ingredients were studied and discussed their utility for enhancement of pigmentation in fishes and recommended that 125 ppm carotene which gave excellent pigmentation and higher doses 200-300 ppm further improved pigmentation [16]. Skin colouration of angel fish (*Pterophyllum scalare*) improved due to the increased levels of dietary carotenoid-rich microalgae biomass [25]. However in the present investigation the higher doses of β-carotene in diet as carrot gave better red-orange colouration and in spinach mixed diet it gave orange colouration in *Xiphophorus hellerii* and lower doses gave better yellow colouration. Several authors examined the influence of carrot (*Daucus carota*) and red pepper (*Capsicum annum*) as natural pigment material on colouration of cichlid (*Cichlasoma severum* sp. Heckel, 1840) and investigated carotenoid amount in the fish samples fed with red pepper and carrot diets were 5.25 ± 0.90 and 5.60 ± 0.29 mg g^{-1}, respectively. Consequently a significant difference was found between individuals fed by natural pigment material and those by unpigmented feeds ($P<0.05$) [24]. Likewise few estimated prominent composition and carotenoid content of natural carotenoid sources and their impact on marine ornamental fish (*Amphiprion ocellaris* Cuveir, 1880) for colour enhancement and found that the pigmentation in fish was the highest in diets added with carrot, followed by marigold petal, china rose petal and rose petal [29]. These findings also connote the results of present study where prominent colouration was noted with Carrot supplemented diet; however it was not coefficient as spinach in diet. The application of microbial carotenoids as a source of colouration of *Xiphophorus hellerii* improved the pigmentation of ornamental fish effectively further author's indicated that the fish fed with carotenoid enriched feed showed faster recovery of carotenoids in the skin of the fish when compared to the control ($P<0.01$) [10]. Red pepper (*Capsicum annum*) meal at 50 g/kg diet can be appropriate as an alternative natural carotenoid source to ensure good pigmentation, suitable growth, and feed utilization of juvenile blue streak hap (*Labidochromis caeruleus*) [36]. The carotenoid content microalgae and spirulina are well utilized to enhance the skin colouration in fishes. Natural carotenoid source *Spirulina* meal has the potential to enhance the coloration on yellow tail cichlid *(Pseudotropheus acei.)* [17]. There is a general tendency amongst aquatic animals to preferentially accumulate xanthophylls rather than carotene pigments. Xanthophylls are one of the types of carotene which include; Astaxanthin (red), canthaxanthin (pink), zeaxanthine (orange) and lutein (yellow). The colour intensity in cichilid (*Cichlasoma myrnae)* was diminished when fish were stressed during a feeding trial with top coated algae in the diet [2]. The gold fish *(Carassius auratus)* belonging to cyprinidae family is able to biosynthesize astaxanthin from lutein sources [20]. However, in the present investigation the skin colouration of *Xiphophorus hellerii* has indicated an increasing pattern with prolonged feeding of β-carotene enriched diet. From these observations, it can be inferred that for maintaining appropriate colour in ornamental fish, suitable pigment enhancing agent should invariably form part of fish diet on a prolonged basis.

Table 2: Proximate Composition of Experimental Diets Fed to *Xiphophorus hellerii*

Sr. No.		Carrot mixed diet in%			Spinach mixed diet in%			Basal diet in% (Control) T_7
		T_1	T_2	T_3	T_4	T_5	T_6	
1.	Carbohydrate	45.20	45.21	45.22	45.61	45.62	45.63	45.90
2.	Fat	14.50	14.61	14.70	14.39	14.60	14.61	14.63
3.	Protein	21.20	21.21	22.22	21.10	22.11	21.12	21.00
4.	Ash	13.20	13.21	13.22	13.15	13.21	13.20	13.10
5.	Moisture	4.70	4.66	4.60	4.72	4.66	4.62	4.72

Table 3: Colouration parameter showing combine values of L*, a*, b*, h° and C* fed with natural β-carotene mixed diet

S. No.	Treatments	Source	β-carotene in diet (mg/100g)	Colour Characteristics				
				L*	a*	b*	h°	C*
1	T_1	Carrot (1.82)	20	19.91	4.19	4.02	43.81	5.81
2	T_2	Carrot (2.272)	25	20.81	3.94	3.51	41.70	5.28
3	T_3	Carrot (2.727)	30	16.93	3.88	2.23	29.89	4.48
4	T_4	Spinach (1.333)	20	15.33	3.30	5.34	58.33	6.29
5	T_5	Spinach (1.666)	25	13.98	3.47	5.14	55.98	6.20
6	T_6	Spinach (1.999)	30	11.69	4.22	4.23	45.06	5.97
7	T_7 (Control)	Nil	Nil	11.84	4.11	0.98	13.41	4.22
	CD			2.4619	3.16	2.7567	13.8792	3.8057
	CV			5.16	27	25	10.94	22.56
	SEm±			0.8498	1.0914	0.9516	4.7911	1.3137

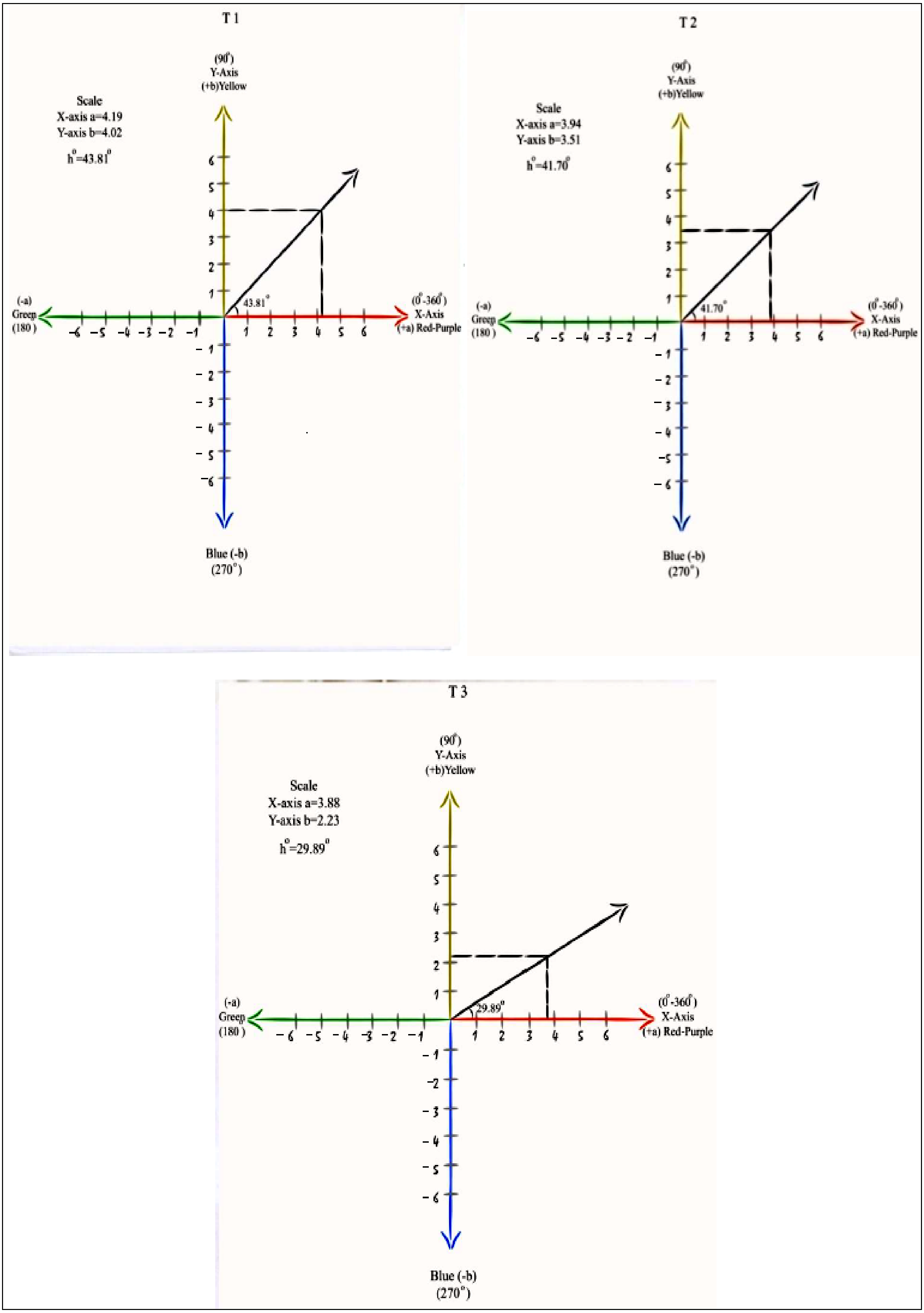

Fig 1: value of A*, h* after feeding carrot (T1, T2 and T3) mixed diet

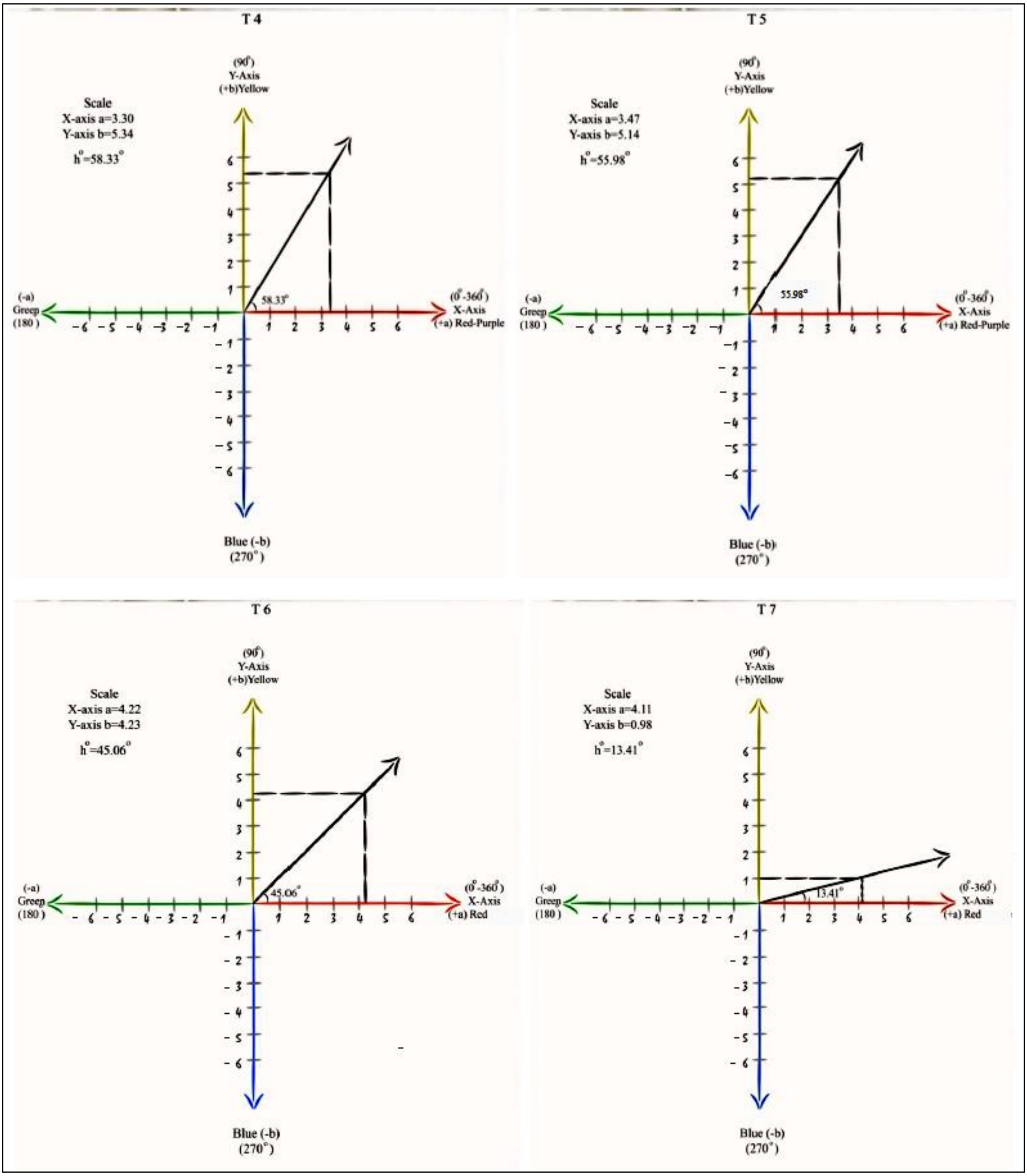

Fig 2: Showing the value of A*, B* after feeding spinach (t4, t5 and t6, mixed ddiet and control (t7) diet

4. Conclusion

From the present experiment it could be concluded that dietary supplementation of carrot at the rate 1.82 g/100 g of diet and spinach at the rate of 1.33 g/100 g of diet can be used for enhancing the orange colouration in swordtail (*Xiphophorus hellerii*). Since synthetic carotenoids are pricey, cheap and readily accessible natural carotenoid sources such as carrot and spinach can be incorporated into the diet to enhance better colouration in swordtail. This will help ornamental fish sellers to get superior cost in trading of this fish.

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