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**Research Article**

# Effects of Guanine-rich diet in coloration of *Colisa fasciata* (Bloch & Schneider, 1801)

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**Abstract**

**To truly grasp the mechanism behind colouration, it is important to study which genes are involved and how they are expressed. Iridophore, a specialized light-reflecting cell that is present inside the dermis of the skin tissue of fish has guanine crystals is responsible for the metallic sheen in fishes. For the current study, an attempt was made to study the impact of different guanine diets on colouration alterations as well as the expression of genes related to iridophore in *Colisa fasciata*. Four different types of diets were fed, with diet 1 (C) as control and the other diets T1, T2 and T3, guanine fortification of 2 mg, 4 mg and 8 mg per gram, respectively were performed. Expressions of iridophore related genes, *MITFa*, *TYR*, *ALK*, *GBX2*, *SOX10* and *TFEC* were also studied. The results demonstrate that a low dose, 2 mg of guanine fortification in feed could enhance skin colouration with *MITFa* and *TFEC* expressing highest on treatment 1, showing its relevancy to the overall feeding intensities and to the results of both colorimetric parameters and the organoleptic tests.**

**Keywords:** Fish pigmentation, Guanine, Iridophore, *Colisa fasciata*, Gene expression

**Highlights**

- Highest colour enhancement by guanine supplementation observed at 2 mg $g^{-1}$
- No adverse effects were observed on both growth and survival
- Enhances visual quality of the fish

## INTRODUCTION

The range of fish colours significantly influences the ornamental fish trade's success (Dey, 1997). The demand for ornamental fish has grown as maintaining them as home aquariums has grown in popularity in several regions of the world. The current export trade of ornamental fishes from India is primarily restricted to the freshwater kinds of indigenous fishes collected from the North-Eastern states. Fish skin colours are produced via the absorption of specific wavelengths of light by pigmentary molecules and the scattering and refraction of light by intracellular structures whose refractive indices differ from the cytoplasmic matrix (Fujii & Oshima, 1986; Fujii, 2000). The various types of chromatophores present in many ornamental fish species form the foundation for their distinctive colour shades and pattern (Lau et al., 2023).

The basic mechanisms underpinning these characteristics and how they manifest in later developments are poorly understood (Tata, 1993). The iridophore cells that generate the guanine crystals are found beneath the scales of fish that hold reflective materials like guanine, hypoxanthine, and adenine, which produce iridescence by reflecting light. Fish iridophore produces thin, elongated guanine crystal plates, preferentially expressing with a high refractive index (Denton, 1970; Liu et al., 2024). Guanine crystals are used by several organisms, particularly fish, to form structural colours. In specialized cells known as iridophores, guanine crystals are created inside vesicles (Gur et al., 2013). Due to crystalized anhydrous guanine's unusually high refractive index (n=1.83 in the direction of molecule stacking), guanine is well suited for the creation of structural colours (Herring, 1994). Light interacts with a structure made of alternating layers of high-refractive-index material (guanine) and low-refractive-index material (cytoplasm), where some wavelengths interfere constructively and others interfere destructively, to produce structural colours (Kinoshita & Yoshioka, 2005).

Banded gourami or striped gourami is a freshwater fish species, belongs to the family Osphronemidae of order Anabantiformes which is native to Eastern India, Bangladesh, Nepal and Pakistan. It dwells in freshwater ponds, ditches, streams, rice fields, etc. It is a resilient fish that can survive and reproduce in murky waters. Due to its bright colour pattern, peaceful demeanor

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and small size, it is a common aquarium fish species (Gupta, 2015).

This species is orange in colour with iridescent blue stripes, and its caudal fin has brilliant red spots near the end and blue spots at the base. The anal fin has an iridescent blue tint and crimson borders on the spine ends. It lives in slow-moving lakes, streams and rivulets and is found in regions with a variety of vegetation. In the present study, six genes for iridophore-based pigmentation that is *MITFa*, *GBX2*, *SOX10*, *ALK*, *TYR* and *TFEC* were used to study their expression patterns, using qPCR both in natural as well as experimental conditions by feeding guanine-rich diet.

## MATERIALS AND METHOD

**Ethical statement:** All the fish of *C. fasciata* used in this study, procured from ponds of ICAR Research Complex, Lembucherra, Tripura. Ethical approval, specimen collection and maintenance were performed in strict accordance with the recommendations of the ethical standards of the Institute of Animal Ethics Committee, College of Fisheries, Central Agricultural University, Imphal, India.

**Experimental design and diet:** Live *C. fasciata*, with an average weight of 7.0±9.0 g, were acclimatized for 14 days in the aquarium with the control diet. Fish were maintained in a 100 L capacity 2.5×1×1.5f glass aquarium with aerated and filtered dechlorinated water. After acclimatizing, the fishes were randomly stocked following a completely randomized design into 4 groups (3 replicate tanks per group, 10 fish per tank). The tanks or fish groups were named C, T1, T2 and T3 with their replicates according to the fortification of the experimental diets. The fish were fed two times (09:00 and 14:00 h) a day for 60 days at 3% of body weight. The tanks were provided with aquarium stones. Fifty percent of the total water was exchanged every 2 weeks to remove excess feed.

Four iso-nitrogenous diets viz. control (C), 2 mg (T1), 4 mg (T2), 8 mg (T3) of 35% crude protein were formulated by using locally available feed ingredients including wheat bran, fish meal, soybean meal, corn flour and guanine and the details are mentioned in Table 1. The control (C) was prepared without fortification of guanine. The composition of basal diets remained the same except for the different levels of guanine. To prepare, the required amount of finely ground ingredients were weighed as formulated and kept in an autoclaved at 121ºC for 15 min. After cooling, the required amount of Carboxymethyl Cellulose (CMC) and Vit C were added and mixed thoroughly. An extruder machine manufactured by NEC Motors Pvt. Ltd., India was used to ensure uniform pellet formation (2 mm dia). The pellet was then allowed to dry under shade and stored in an air-tight condition.

**Table 1. Composition of formulated feeds (g per 100 g ingredient, oil in mL)**

| Ingredients | C | T1 | T2 | T3 |
|---|---|---|---|---|
| Soybean meal[1] | 15 | 15 | 15 | 15 |
| Wheat Bran[1] | 29 | 29 | 29 | 29 |
| Fish meal[1] | 14 | 14 | 14 | 14 |
| Corn flour[1] | 30 | 30 | 30 | 30 |
| Sunflower Oil[2] | 4 | 4 | 4 | 4 |
| Vit-Min premix[3] | 3 | 3 | 3 | 3 |
| CMC[4] | 5 | 4.8 | 4.6 | 4.2 |
| Guanine[5] | **0** | **0.2** | **0.4** | **0.8** |
| **Proximate composition (dry matter basis)** | | | | |
| Dry matter | 92.84±0.20 | 92.74±0.15 | 92.62±0.10 | 92.87±0.21 |
| Crude protein (%) | 34.07±0.12 | 34.45±0.12 | 34.16±0.26 | 34.18±0.12 |
| Ether extract (%) | $6.32^{a}$±0.07 | $6.56^{b}$±0.06 | $6.76^{c}$±0.05 | $6.37^{ab}$±0.04 |
| Ash (%) | 8.45±0.16 | 8.17±0.33 | 7.94±0.36 | 8.71±0.14 |
| NFE and fiber (%) | 51.16±0.22 | 50.82±0.27 | 51.14±0.54 | 50.74±0.11 |
| *Gross energy (kcal/100 g) | $397.77^{a}$±0.95 | $400.12^{ab}$±1.31 | $402.07^{b}$±1.70 | $397.02^{a}$±0.37 |

[1]Obtained from feed mill of College of Fisheries, Lembucherra, Tripura, India. [2]Saffola sunflower oil, Mumbai. [3]Vit-Min premix manufactured by Anand International Biopharma, New Delhi. [4]Carboxymethylcellulose (CMC), manufactured by Himedia Laboratories Pvt. Ltd., Mumbai. [5]Guanine, manufactured by Research Lab Fine Chem Industries, Mumbai

*Gross energy=Multiply grams of protein by 4 kcal/g+Multiply grams of ether extract by 9 kcal/g+Multiply grams of NFE and fiber by 4 kcal/g.

**Table 2. Primers used in PCR amplification**

| Gene | Forward primer 5'→3' | Reverse primer 3'→5' | Product Length | Annealing Temperature (p C) |
|---|---|---|---|---|
| **18s r RNA** | CTGTCAATCCTTTCCGTGTCC | AAAGGAATTGACGGAAGGGC | 101bp | 54 ºC |
| **Mitfá** | AGCATCAGGTGGAGGTTGG | CCGTGGACTACATCAGGAAG | 100bp | 54 ºC |
| **Gbx2** | GTTCGGCCCGTCCTTTATG | GGTAGCCGGTGTAGACGAAG | 122bp | 54 ºC |
| **Tyr** | TCCTTCTGACTAACTCTGAGCC | CGATCCTATGCGATGCTTTGT | 107bp | 54 ºC |
| **Sox10** | GCTGCTCGTCCGTCAAATC | CTCGTGCTTCCGGAGTTCA | 136bp | 56 ºC |
| **Alk** | CTTCAGCAACACCAGCAGC | GGATGGAACGGCGAAATCC | 139bp | 56 ºC |
| **Tfec** | ACGACCATCACTAGACTGCT | TTCCTGGTCTTCTGCTGTGG | 140bp | 50 ºC |

**Tissue collection:** The samples were collected for analysis from freshly collected fish that is, nature and at two different feeding days of the experiment that is 30th and 60th day. Tissues like skin, muscle and brain were collected and preserved in RNA ladder (Sigma-Aldrich, USA) and kept at "80°C for RNA extraction.

**RNA isolation and cDNA synthesis:** Total RNA were isolated from the skin, brain and muscle of *C. fasciata* by the TRIzol reagent method (Marcedo & Ferreira, 2014). 30-40 mg of tissue sample was taken directly into the 1.5 mL centrifuge tube and homogenized the sample with 50 µL of TRIzol reagent (Invitrogen, USA) and the homogenate was vortexed for 1 min followed by 5 min of incubation at room temperature for proper lysis. Twenty microlitre of chloroform: isoamyl alcohol (24:1) was added and mixed vigorously till a white pinkish colour appeared and then followed by 5 min of incubation at room temperature. The tube was centrifuged at 12,000 rpm for 15 min at 4ºC. Three layers were formed. The supernatant was collected without disturbing the interface and transferred to the 1.5 mL fresh tube. The RNA was precipitated by adding 25 µL of ultra-pure isopropanol, mixed well, and kept at room temperature for 15 min for precipitation. The total RNA was then centrifuged at 12,000 rpm for 10 min at 4ºC. The precipitated RNA pellet was washed with 50 µL of 75% ethanol and kept for 2 min at room temperature followed by centrifuging at 7500 rpm for 5 min at 4ºC, after which the ethanol is discarded. The RNA pellet was air dried for 10-15 min and dissolved in 20 µL of DEPC-treated (Thermo Scientific, USA) and stored at -80°C for further use.

cDNA synthesis was done using the Thermo-Scientific Verso cDNA Synthesis kit (Fermentas, USA) as per the manufacturer's protocol.

**PCR amplification of target genes:** Primers were designed from the conserved area of the reported sequences of the gene from closely related species through the NCBI-primer blast (Ye et al., 2012). The primers used in this study for PCR amplification is shown in Table 2.

**qPCR analysis for gene expression studies in different genes:** Real-time PCR based on SYBR green chemistry was done to study the expression analysis of genes using a specific primer designed from its sequence amplified across the collected cDNA samples. As an internal control, the housekeeping gene i.e., beta-actin was utilized for expression level normalization. Three technical replicates were created for each sample during real-time PCR. Real-time PCR was carried out in a total volume of 10 µL containing 5 µL of SYBR Green Master Mix (Thermo Scientific, USA), 0.5 µL each of forward and reverse primers (1 pM µL-1), 1 µL of a template (cDNA, 1 µg µL-1) and nuclease-free water to make up the volume to 10 µL. The reactions were carried out in the Step One Real-Time PCR system (Applied Biosystems, USA). The standardized thermal profile used for PCR amplification consisted of 95p C for 10 min, 40 cycles for 95p C for 15 sec and 60p C for 11 min; with a melt curve starting from 95p C for 15 sec, 60p C for 1 min and 95p C for 15 sec. the threshold cycle (Ct) value was determined using the automatic setting on the Step One Real-time PCR system. The relative expression level of the target gene was obtained using the 2-ΔΔCT method (Pfaffl, 2007).

**Determination of colour by Colorimeter:** Colour measurement was done using Hunter Lab Colorimeter (Hunter Lab Scan XE, USA) and it was calibrated to white and black standard sites of a sample. Illuminant C was used as a light source measurement. The tristimulus L*a*b* measurement mode was used as it relates to the human eye's response to colour. The L* variable represents lightness (L*=0 for black, L*=100 for white), the a* scale represents the red/green

(+a*=intensity in red, -a*=intensity in green) and the b* scale represents the yellow/blue (+b*=intensity in yellow, -b*=intensity in blue). Whiteness, redness and yellowness were calculated using the following equation (National Fisheries Institute [NFI], 1991):

Whiteness = 100-[(100-L*) 2+a*2+b*2]1/2
Redness = Cos (160)a*+ Sin(160)b*
Yellowness = -0.7[-Sin (160) a*+ Cos(160)b*]

**Sensory Evaluation test:** Test panels consisting of teachers and PhD scholars were recruited randomly to judge the colour. The treatments were not revealed to the individuals who were asked to judge and rank the fish according to the intensity of colour based on eye estimation. Colour ranking was done by a score of 1-9 points on a hedonic scale with one being the lowest for each treatment group.

**Statistical analysis**

The data obtained were analyzed statistically using Statistical Package for Social Sciences (SPSS, version 23.0). Analysis of variance (one-way - ANOVA) was performed to determine the differences between the mean values of different treatments. Differences in means were compared by Duncan's New Multiple Range test (multiple range test) (Duncan, 1955) at the $p<0.05$ level.

**RESULTS**

**RNA isolation:** Total RNA, isolated from various tissues, were tested for its quantity, purity and integrity. The concentration of the isolated RNA sample was in the range of 700-1800 ng µL-1 and the A260/A280 ratio was in the range of 1.9-2.0, which indicated that the purity of the isolated RNA was good. The integrity of the extracted RNA was verified by running through 2% agarose gel electrophoresis, which showed a clear separation of 28s rRNA and 18s rRNA bands.

**qPCR analysis:** Differential gene expression was noted in the genes related to iridophores due to dietary addition of guanine. The light reflectivity was determined to be more favourable in T1.

**Expression of different genes in different tissues:** As shown in Figs 1-6, the expression of the *MITFa* gene was relatively higher on the 30$^{th}$ day on T1 of both skin and muscle tissue with a significant difference of $p<0.05$. While on muscle tissue, T2 shows the highest expression. However, when comparing the feeding interval of 30 days showed the highest expression in all three tissues with a significant difference ($p<0.05$) among the tissues on the 60$^{th}$ day of the feeding interval. *GBX2* gene showed higher expression in T1 of skin tissue on the 30$^{th}$ day with a significant difference of $p<0.05$, while muscle and brain tissue showed the highest expression of *GBX2* gene in control followed by T1 showing a significant difference of $p<0.05$. Similarly, with that of *MITFa*, *GBX2* also showed its expression in all three tissues compared with 60 days. No significant difference was observed in 60 days among the treatments. The expression of the *SOX10* gene showed higher expression in C (control) of skin on the 60$^{th}$ day of the feeding interval. The expression pattern was only observed in skin tissue of the 60th day. In the other two tissues, muscle and brain, there is no significant difference and among the treatments, muscle tissue of T1 showed higher expression with a significant difference of $p<0.05$ *TYR* gene showed relatively higher expression in T1 of both skin and muscle tissue of the 30$^{th}$day with a significant difference of $p<0.05$ followed by C, T3 and T2 in both the tissues. In brain, C showed the highest expression showing a significant difference of $p<0.05$, followed by T2, T1 and T3. Yet, while comparing the tissues on the 30$^{th}$ day, T1 of the skin tissue showed the highest expression. There is low expression on the 60$^{th}$ day of feeding interval compared to the 30$^{th}$ day without any significant difference among the treatments of the tissues. The expression of the *ALK* gene was comparatively higher in T1 of all the tissues on the 30$^{th}$ day of feeding interval showing a significant difference of $p<0.05$ but while comparing among the tissues, muscle showed the higher expression followed by skin and brain. However, on the 60$^{th}$ day, the expression of *ALK* gene was comparatively lesser in all the tissues of all the treatments without any significant difference. *TFEC* gene showed higher expression in the muscle of T1 of 30 and 60 days of feeding interval with a difference of $p<0.05$. But while comparing between them, the muscle tissue of the 30$^{th}$ day is higher. In skin and brain tissue of the 30$^{th}$ day, T1 also shows higher expression but comparatively lesser than muscle tissue of the 30$^{th}$ day but higher than the 60$^{th}$ day respectively.

**Colourimetric analysis**

**Blackness/Whiteness (L*):** The blackness level gradually increased in T2 and T3 but in T1, the colour went towards whiteness when compared with the control. On the other hand, on the 60$^{th}$ day C and T1, the graph falls towards whiteness with a significance difference of ($p<0.05$) as shown below in Fig 7. When

**Effect of Guanine-rich diet in coloration of *Colisa fasciata***

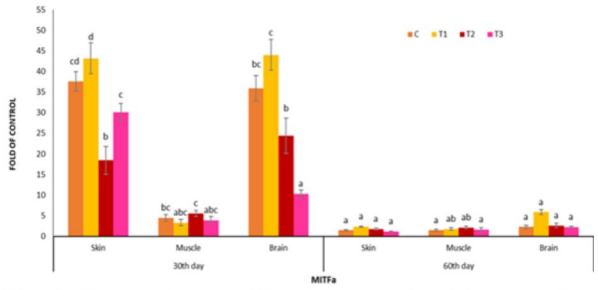


**Fig. 1. Expression profile of *MITFa* in skin, muscle and brain on 30th and 60th day**

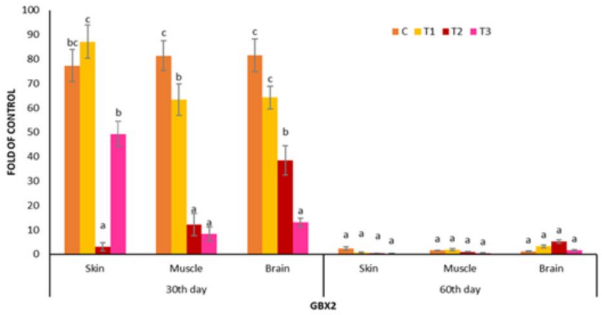


**Fig. 2. Expression profile of *GBX2* in skin, muscle and brain on 30th and 60th day**

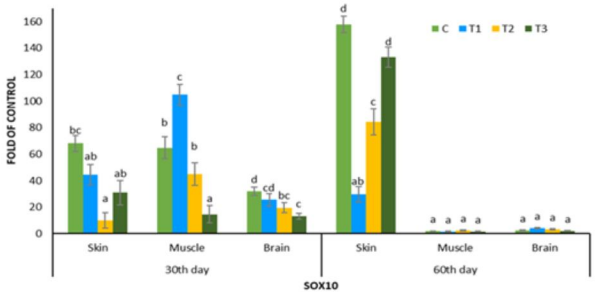


**Fig. 3. Expression profile of *SOX10* in skin, muscle and brain on 30th and 60th day**

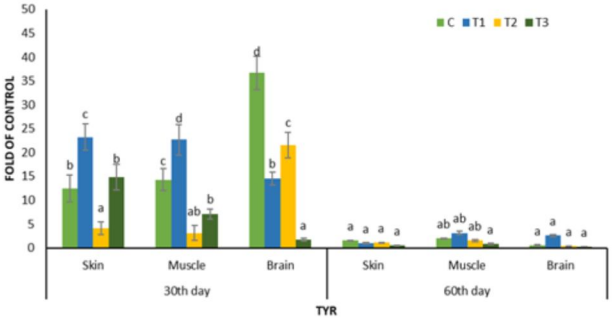


**Fig. 4. Expression profile of *TYR* in skin, muscle and brain on 30th and 60th day**

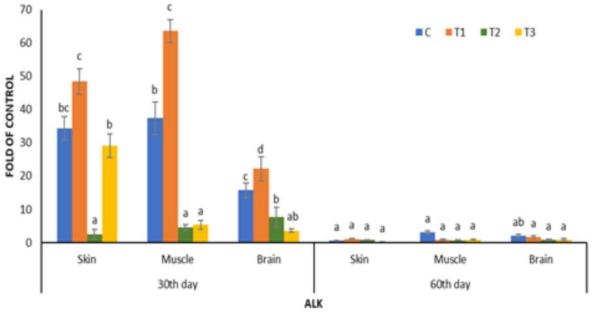


**Fig. 5. Expression profile of ALK in skin, muscle and brain on 30th and 60th day**

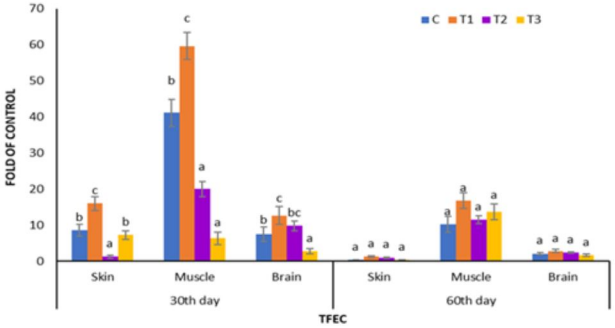


**Fig. 6. Expression profile of *TFEC* in skin, muscle and brain on 30th and 60th day**

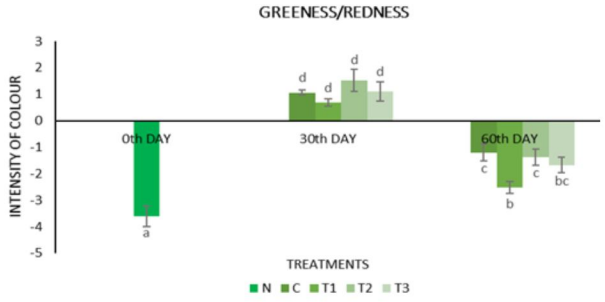


**Fig. 8. Temporal comparison of a* value (Redness/ Greenness) in Hunter Lab Colorimeter during 0th day, 30th day and 60th day sampling between the treatments, with data representing significance at p<0.05**

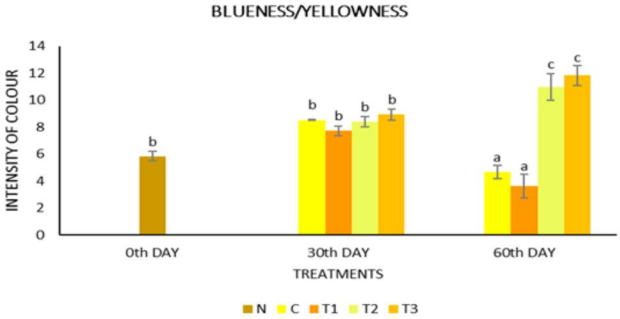


**Fig. 9. Temporal comparison of b* value (Blueness/ Yellowness) in Hunter Lab Colorimeter during 0th day, 30th day and 60th day sampling between the treatments, with data representing significance at p<0.05**

comparing the wild fish with the experimental fish, except for T1 of both the days along with C of 60th day, the other treatments showed darker pigment.

**Greenness/Redness (a*):** It was seen that greenness gradually increased on the 60th day from T1 to T3. Among the treatments, the redness level was comparatively higher on the 30th day with the highest value on T2 followed by T3 of the 30th day with a significance difference of ($p<0.05$) as shown in Fig 8.

**Blueness/Yellowness (b*):** As shown in Fig. 9, among all the treatments, the yellowness level showed a comparatively higher value on T2 and T3 of the 60th day without any significant difference but has a significant difference of $p<0.05$ with the wild fish. No significant difference was observed among the treatments of the 30th day as well as with that of wild fish.

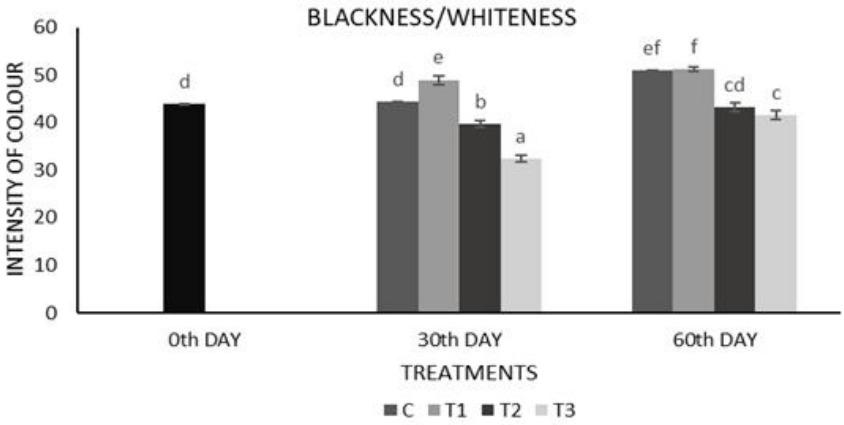


**Fig. 7. Temporal comparison of L* value (Blackness/Whiteness) in Hunter Lab Colorimeter during 0th day, 30th day and 60th day sampling between the treatments, with data representing significance at $p<0.05$**

**Table 3. Sensory evaluation test ratings of 60th day experimental fish expressed in Mean±SE**

| Parameters | Rating by panelist on 1-9 point hedonic scale | | | |
|---|---|---|---|---|
| Experimental groups | C | T1 | T2 | T3 |
| Brightness | 5.33±0.28 | 7.00±0.00 | 7.09±0.28 | 7.16±0.38 |
| Colour pattern | 4.66±0.28 | 7.26±0.28 | 7.00±0.50 | 7.00±0.50 |
| Fin pattern | 5.00±0.50 | 7.33±0.28 | 6.09±0.86 | 6.66±0.76 |
| Body shape | 5.33±0.28 | 6.33±0.76 | 6.33±0.76 | 6.46±0.76 |

**Sensory evaluation test:** The T1 group exhibited a greater number in colour pattern as well as fin pattern, while the T3 group had shown intensified colour which ultimately led to lesser light reflectivity. According to the 1–9 point hedonic scale, the panellists indicated that the fish from the T1 group displayed the most vibrant overall reflectivity which is shown in Table 3.

## DISCUSSION

*MITFa* is a key regulator for the expression of melanin production responsible genes like *TYR*, *TYRP1* and *DCT* via binding to the conserved M-box motif in their promoter regions for promoting the production of melanin (Bentley et al., 1994; Ganss et al., 1994). In the present study, the mRNA transcript of *MITFa* showed higher expression on T1 of brain tissue on 30th day and showed lower expression in all the treatments on all the tissues of 60th day, thus, indicating that melanocyte production was significantly reduced on the 60th day. Similarly, in *Carassius auratus*, the expression of the *MITFa* gene dropped when the skin turned from grey to red (Zhang et al., 2017). When tissue-specific expression was studied, upregulation of *MITFa* was observed in skin tissue.

In a similar fashion, the *TYR* gene was also downregulated in all the treatments of 60th day compared to the treatments of 30th day. *TYR* showed a comparatively higher expression in T1 of both skin and muscle and was highest on the C of brain tissue of 30th day. Low activity of *TYR* is associated with loss in melanocyte production (Camp & Lardelli, 2001; Makpol et al., 2014). Since *TYR* is regulated by the *MITFa* gene (Bentley et al., 1994; Ganss et al., 1994), with the decrease in expression of *MITFa*, the expression of *TYR* is also decreased.

The upregulation of the *SOX10* gene in all the treatments of the skin tissue on the 60th day had a positive result in decreasing melanin synthesis. This result was supported by a previous study conducted on zebrafish where iridophores subtypes allowed the zebrafish to alter its skin patterning to make it more or less distinctive (Gur et al., 2020).

The expression of the *TFEC* gene showed the highest expression in T1 of muscle tissue on the 60th day when comparing between the treatments. Higdon et al. (2013), found *TFEC* to be one of several transcription factor genes with enriched expression, especially in the iridophore lineage and low levels of

expression in purified melanocytes. The expression of *TFEC* was found to be higher than that of the *MITFa* gene, which resonates with the findings of Higdon et al. (2013), in which they also observed the inverse relation between *TFEC* and *MITFa* genes (Bharti et al., 2012).

In T1 where 2 mg of guanine was included in the feed, there was an upregulation of *GBX2* genes on the 30th day. It is already studied that *GBX2* is required for the migration and positioning of neural crest (NC) in the mature vertebrate hindbrain (Roeseler et al., 2020). The upregulation of this gene was observed in the brain.

*ALK*, which regulates important functions in the central nervous system is activated by the *ALK*1 and *ALK2* ligands. *ALK*1 and *ALK2* are essential to drive embryonic iridophore development and adult stripe development but interestingly, the processes are mediated entirely by *ITK* and not by *ALK* (Mo et al., 2017). In the current experiment, it was observed that the expressions of *ALK* in T1 of all three tissues were higher on the 30th day. Similarly, *ALK* (Zebrafish *ITK* homolog) showed co-expression with melanocytes in multipotent progenitor cells and becomes restricted when the pigment pattern is stabilized and progenitor activity ceases (Fadeev et al., 2018; Subkhankulova et al., 2023).

This overall statement on guanine diet treatment is also satisfied with the organoleptic test as well as the colorimeter parameters. The highest value for L* was recorded on the fish fed with diet T1 on the 60th day. Whiteness intensity gradually increased in C and T1 on the 0th, 30th, and 60th day showing a similar pattern. Treatment 2 and treatment 3 also showed a similar pattern of decreasing gradually. It may be the effect of melanosome aggregations and dispersion. The intensity of blackness on the diet T2 and T3 showed an increased pattern which implied that the treatments had an effect on melanin pigmentation in comparison to the control group ($p<0.05$) indicating that C and T1 were remarkably effective in enhancing whiteness and T2 and T3 in enhancing blackness i.e., melanin pigmentation in all the experimental fishes. Overall, we might draw the conclusion that lower quantities of guanine treatments tend to increase whiteness.

## Conclusion

The present study concludes that for the production of pigmentation in fish, several genes interaction is responsible. Different genes work together directly or indirectly to create different pigments. Guanine diet with 2 mg g-1 showed the best results on the 30th day of feedinginterval. But in some aspects, control diet also showed good results. Therefore, enhancement of colouration in fishes is genetical and it depends upon not only the diet but also the duration of feeding. Further studies regarding the gene regulatory network for iridophore differentiation needs to be explored along with the interaction between the three chromatophore related genes and factors responsible for this mechanism are required to be studied.

**Conflict of interest:** The authors declare no conflict of interestamong them.

**Author's contributions:** CC: Field, laboratory, and molecular work and wrote the manuscript; SCM, JP, NCD, SKS: Study design, analytical tools and data analysis and feed formulation; YM, SY: Sampling, Primer designing.

**Data availability statement:** Data will be provided as per request.

**Ethical approval:** Ethical approval was taken from the institutional animal ethics committee (IAEC) of the college before conducting the experiment.

**Use of artificial intelligence tools:** No artificial intelligence tools were used for the study. The authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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