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Neural and hormonal regulation of melanophores in fish, *Puntius species* (Ham.) melanophores

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Abstract
The present study was carried out to investigate the neural and hormonal regulation in fish species of *Puntius*. To understand the neural and hormonal regulation in fish, we analyzed chromatic and transparency effect of neuron and hormonal agent on melanophore in fish. The white-adapted fishes when placed on a black background attained darkening and the black-adapted fish when subjected to a white background, attained a paling. The response is biphasic *i.e.,* it was rapid initially and thereafter it was slow and gradual, suggesting to be co-ordinated both by nervous and endocrine or hormonal systems. Two peptide hormones, melanin-concentrating hormone and melanocyte stimulating hormone, having opposing actions, is associated with the color changes of fish. The observations pertaining to the effects of blinding and the effects of melatonin (a hormone from the pineal gland), do indicate that the gland functions as an extra-optic receptor for the colour changes of fish.

Keywords: Melanophores, neural and hormonal regulation, dispersion, aggregation, fish

1. Introduction
A well-known model used to study neuroendocrine communications in animals is the process called background adaptation. Lower vertebrates (e.g., some teleost and amphibians) adjust the colour of their skin in response to changes in background colour and/or reflectivity [1-3]. Pigment cells (chromatophores) of teleost fish have long been postulated to be under dual pituitary hormonal control [4-5]. One principle factor is, of course alpha melanophore-stimulating hormone (MSH) which disperses pigment granules within chromatophores. The other one, antagonistic in function, i.e. to aggregate pigment, was first proven by Enami [6] to exist in the pituitary, as well as in the hypothalamus, of the siluroid catfish *Silurus asotus*, and was termed 'melanophore-concentrating hormone (MCH). It was reported that 'melanin-concentrating hormone MCH is a small peptide of less than 2000 Dalton, and that most of its bioactivity appears in the hypothalamus [7]. Kawauchi succeeded in purifying MCH from chum salmon pituitaries and demonstrated its chemical structure [8]. The primary structure of MCH has since been determined for three additional fish species, and the hormone has been shown to be highly conserved: it is a cyclic heptadecapeptide with an identical structure for the chum salmon, the chinook salmon and the bonito [9-10]. MCH was shown to elicit pigment aggregation in melanophores of almost all teleost species when present at low concentrations [8, 11-14].

Fish colour change is accomplished by two distinct system, the physiological color change and morphological colour change. Physiological colour change in teleosts can be accomplished by two different mechanisms. The secondary colour responses intermediated by the eyes resulting into bidirectional movement of pigment in chromatophores being controlled by the neural and or endocrinal systems [15-17]. The other type is the 'primary colour response' where the chromatophores respond directly to light. The former takes place rapidly (min) and only involve a change in the dispersion of existing pigment within the chromatophores (*i.e.,* quantity of pigment does not alter, only its arrangement changes within the cell). Morphological colour changes on the other hand involved both altered rates of pigment synthesis within existing cells as well as the changes in total number of chromatophores present within an organism [18]. These type of colour changes are relatively slow (they occur over several days/weeks), which limits the use of this type of change in pharmacological testing. Such colour modulation may provide camouflage [19-20] or act in social signaling [20-22]. In the long term, these systems also influence survival or apoptosis of the chromatophores and contribute to morphological colour change [23].

Nervous mechanism seems to dominate in species eg. *Fundulus heteroclitus* [24], *Macropodus opercularis* [25] and *Oryzias latipes* [26], where the melanophores are controlled exclusively by nerves and the mechanism appears to have evolved for the rapid background adaptation of animals. Parker [27], studied the colour changes of the catfish, *Ameiurus nebulosus* (Leseur) and concluded that they are brought about not only through stimulation of chromatophore nerves but also through the cooperation of hormones and one of these hormones is that of the pituitary gland, which according to him, is secondary to the dominant nervous system.

In the fishes, the pineal organ has been recognized as a photoreceptor Young [28] has demonstrated that the marked diurnal rhythms of colour changes in lampreys was abolished in ammocoetes and distributed in adult *Lampetra planeri* following pineal extirpation. Earlier investigation on fishes and lampreys has confirmed the strong melanophore stimulating effect of melatonin [29-37].

In the 3 species of Puntius under investigation attempt has been made to study the transitory colour change in the General body surface component by subjecting the fish to a white and a black background under constant illumination. The study will help in elucidating the nature of mechanism involved in the regulation of chromatophores and colour changes in these barbs. The aims of the present work are: (i) to establish whether this species is able to adapt skin colour to light conditions by background modifications (ii) to study the rate of paling and darkening in blinded fish (iii) to investigate the pharmacological characterization of chromatic nerves (sympathetic pigment aggregating adrenergic nerves) (iv) effect of hormones (Melanophores stimulating hormone/ Melanin-concentrating hormone) and effect of melatonin (circadian colour changes).

2. Materials and Methods

The fresh-water Indian teleosts, the *Puntius species* (*P. sophore*-the pool barb, *P. conchonius*-the rosy barb and *P. ticto*- the ticto barb) of either sex were used as the experimental material. The fish (*Puntius sophore* and *Puntius conchonius*) were collected from Tighra reservoir located at about 23 km from Gwalior Madhya Pradesh, India and the *Puntius ticto* were collected from Pilua dam, 40 km away from Gwalior Madhya Pradesh, India and kept in large glass aquaria (60x60x30cm) under natural day-night lighting, containing dechlorinated fresh water. These fishes are omnivorous; their diet includes worms, insects, crustaceans and plant matter.

2.1 Background–related colour changes (physiological/transitory or chromomotor colour change)

To study the colour changes of skin to background tones in light, the healthy fish from the stock tank placed in natural light condition, were taken out and placed in white/black background with overhead illumination. Five fish as experimental groups were placed for a period of 24 hrs in troughs (30x10x10cm) painted black/white and covered with black/white cotton net on the open surface of the trough so as to serve as the black/white background. To study the rate of paling these black adapted fish from the aquarium were gently transferred to white painted glass troughs. The pre-experimental shade was recorded using Munsell grey series colour standards. The colour changes were recorded at regular intervals of time until no further change was notices for a considerable time period. To study the rate of darkening, white –adapted fish were gently transferred to a black trough and the same procedures as mentioned above were followed.

2.2 The colour change upon blinding the fish: (*In vivo*)

For the purpose of this experiment the blinding of fish was achieved by anaesthetizing them in 0.5% paraldehyde solution. Both the eyes were removed by making an incision in the skin around the eye ball and then by transecting the optic nerve. After their recovery from the effects of anesthesia these blinded fish were placed under light and darkness for a period of 24 hr. the initial and subsequent observations were carried out at definite time intervals until the fish attained equilibrium and there was no more change in the shade.

2.3 Methods for measurement of melanophore responses (*In vitro)*

The effect of drugs on the response of melanophores were studied with light microscope and were evaluated according to melanophore index (MI) as originally used by Hogben and Slome (1931) for staging amphibian melanophores has been conveniently applied to the pigment cell of fish and sometimes even to chromatophores other then melanophores. This method includes and arbitory division of entire range of maximum dispersion and maximum aggregation into five units or stages, the stage 1 representing maximal dispersion, with each increasing number denoting increased dispersion and finally stage 5 corresponds to the fully dispersed stage (Fig.1).

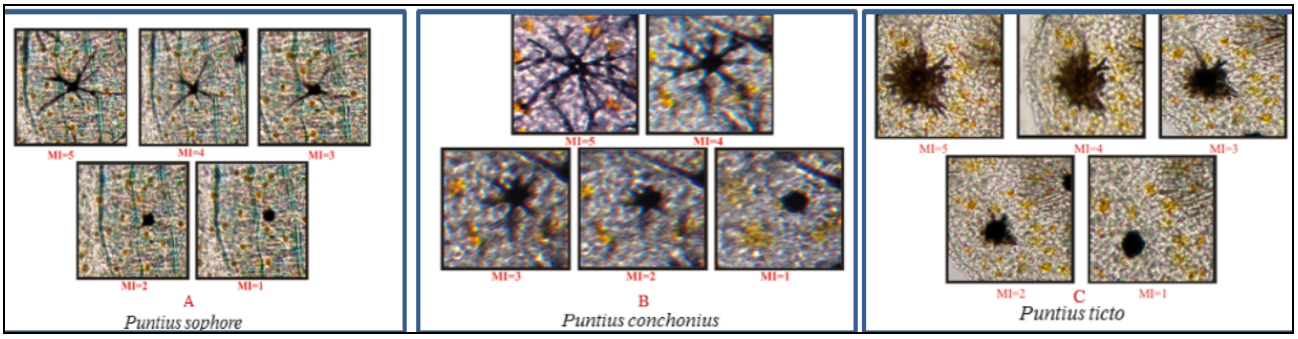

Fig 1: Melanophore indices (5-1) as were used for measurement of melanophore responses in the study.

2.4 Preparation and administration of dose

Stock solutions of drugs/hormones (10^{-2} to 10^{-3} M) were prepared using the physiological saline but in case of melatonin, it was first dissolved in ethanol and then diluted with Physiological saline or distilled water as considered appropriate. Reserpine was first dissolved in citric acid and then an appropriate amount of Physiological saline was added. All stock solutions were kept in refrigerator and

working solutions were freshly prepared just before the experiment. The physiological saline had the following composition (mM): NaCl -128.3, KCl - 2.68, $CaCl_2$-1.8, Glucose-5.6, Hepes NaOH-5.0. The pH value of the PS was 7.4. To prepare K^+-rich saline, NaCl was substituted by an equimolar solution of KCl in the PS

3. Results

3.1 Background related colour changes

3.1.1 Rate of paling and darkening

The fresh water teleosts, the species of *Puntius* are quite sensitive in their background-related chromatic responses and like many other teleosts, they become dark on a black-background and pale on a white one under overhead illumination.

The rate of paling and darkening was quite rapid initially for first 5 min *Puntius sophore*, *Puntius conchonius* and *Puntius ticto*. Thereafter, the rate of paling and darkening become slow and gradual. The maximum paling and darkening achieved by fishes were recorded on the next day at a stage of 24 h. when they were allowed to remain in the white and black background, respectively (Fig. 2).

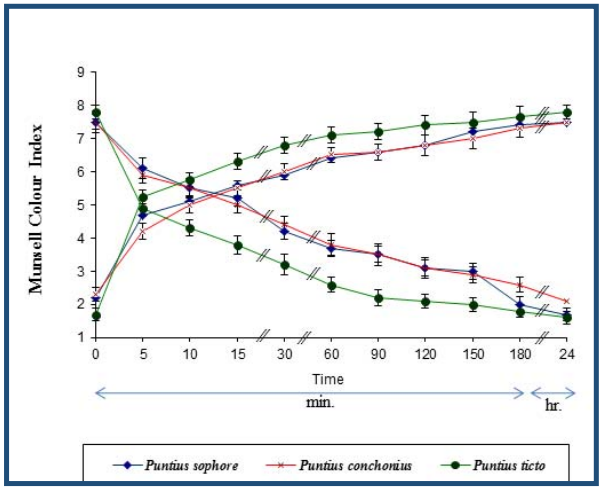

Fig 2: Change in body shade of black-and white-adapted fish as a result of adaptation to white and black background with overhead illumination. The values are expressed as mean (5 animals) ± S.D. (vertical lines) of the mean.

On both white and black backgrounds the chromatic responses were accomplished in two phases. The initial phase was rapid and lasted for 5 min. Further changes as a second phase occurred slowly and gradually until maximal adaptation to the respective background was attained.

3.1.2 Change in body shade in the blinded fish on exposure to light and darkness

Blinded fish failed to respond to changes in the body shade in relation to their background. They remained dark on a black as well as on a white background in light. The darkening was observed in all studied fish at 15 min after the recovery, when the fish were kept in light. The darkening then slows down in all the studied species of fish *Puntius* at 1hr post blinding stage. It took 7 hr to attain the maximum darkening. Paling was recorded in blinded fishes when they were allowed to remain in darkness for 24 hr in all the species (Fig. 3)

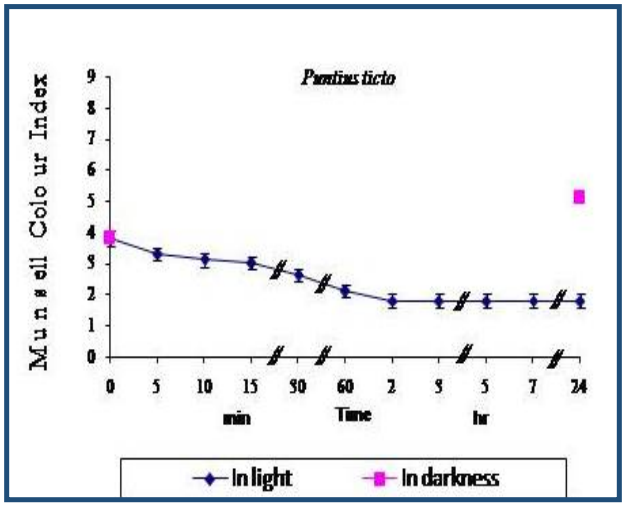

Fig 3: Effect of light and darkness on the body shade of the blinded fish. Ordinates, Munsell Colour Index showing body shade and abscissa, time after placing the fish in light or darkness. The values are expressed as mean (5 animals) ± S.D. (vertical lines) of the mean.

3.2 Pharmacological characterization of chromatic nerves:

3.2.1 Effect of K^+-ions

The effect of K^+-rich saline was then tested at different concentrations ranging from 50-90 mM the strength-response relationship in the melanosome aggregation response of innervated melanophores. The extant of response increased with the increase of the K^+ concentration in the test solution. The minimal effective concentration obtained was 50 mM and the maximum response *i.e.,* 100% aggregation was obtained at 90 mM concentration in all the 3 species under the study.

On 15 min perfusion with Physiological saline (PS), the melanophores in freshly isolated scale preparations achieve the state of full dispersion (M.I=5) in all the 3 species studied. When PS was substituted with K^+-rich saline, a quick aggregation of the pigment was recorded due to centripetal movement of melanosomes within the melanophores and the state of full aggregation (M.I=1) of the pigment was achieved within 4 min. After 5 min treatment with K^+-rich saline, it was replaced with PS which evoked complete re-dispersion of melanosomes (M.I=5) within 20 min of incubation (Fig. 4).

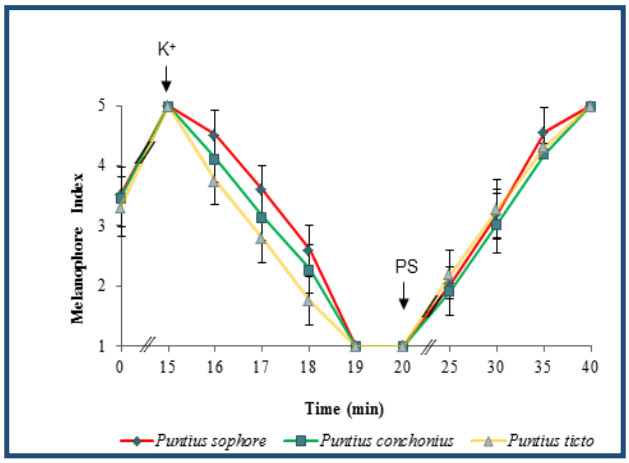

Fig 4: Melanophore aggregation in response of K^+-rich saline (5 min) in PS equilibrated dispersed melanophores (15 min) and redispersion in physiological saline after withdrawl of K^+ ions, in three species of the fish.

The effect of K^+-rich saline caused a graded aggregation response which was quantitated according to the melanophore index and have also been shown by successive photomicrographs (Fig. 5).

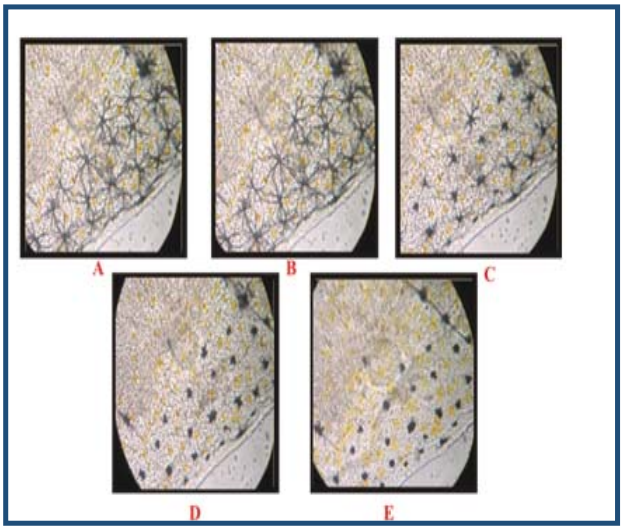

Fig 5: Typical serial photomicrographs showing responses of a group of innervated melanophores in an isolated scale preparation of the fish, *Puntius conchonius* to K^+-rich saline

3.2.2 Effect of epinephrine on the melanophores:

The concentration of epinephrine (10^{-9}M), non selective α, β agonist causes minimum aggregation of pigment as 29%, 11% and 21% respectively in studied species of the fish *Puntius* (*Puntius sophore, Puntius conchonius and Puntius ticto*). At 10^{-8} and 10^{-7} M the magnitude of aggregation response was increased and the maximum pigment aggregation (100%) was observed at the concentration of 10^{-6} M in all the 3 fish species. (Fig. 6)

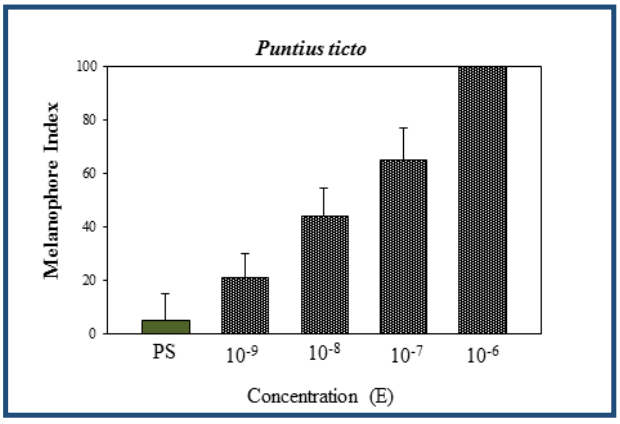

Fig 6: Melanin-aggregating activity of epinephrine as determined *in vitro* in isolated scale preparations of the fish. Each bar represents the mean ± S.D. of the mean of the responses (melanosome aggregation) of the scale (N=5) melanophores to epinephrine.

In the present study, epinephrine at a concentration of 10^{-6} M induces rapid and quite potent aggregation of pigment in Physiological saline equilibrated melanophores. The effect starts within a minute and the full aggregation (M.I. =1) was achieved within 5 min. On being reverted to PS the melanophores gradually returned to the maximum dispersed state (M.I. =5.0) in about 50 min incubation in all the species studied.

3.3 Effect of Hormones

3.3.1 Effect of MSH (Melanocyte-stimulating hormone):

Fig.7 demonstrates this effect of E in the absence of MSH and after it was replaced by MSH. Rapid and profound pigment dispersion can be clearly seen to set about in response to the hormone. Similar measurements with different concentrations of MSH showed that discernible melanosome dispersion took place in response to the hormone at about 10^{-12} M and that the rate and degree of responses increased upon increase in the strength of MSH. A positive correlation between hormonal concentration and the rate as well as the degree of the response is evident.

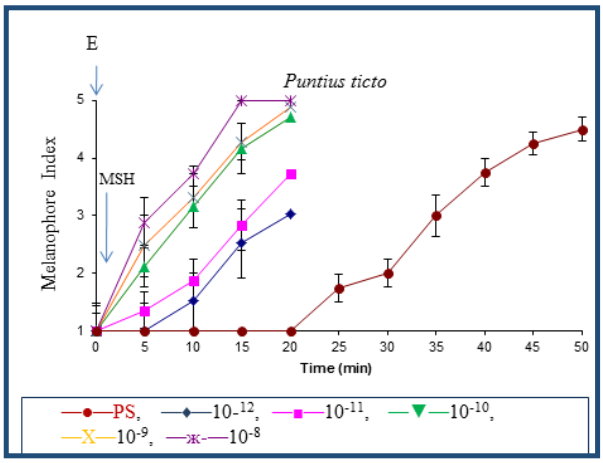

Fig 7: Concentration response curve of MSH at varying concentration (10^{-12} to 10^{-8} M)) in melanophores of the fish, Puntius. Vertical lines represent the standard deviation.

3.3.2 Effect of MCH (Melanin-concentrating hormone) on the melanophores

The threshold concentration of MCH to induce pigment aggregation was found to be 10^{-13} M. Stronger concentration of MCH (10^{-9} M) caused full aggregation. The effect of MCH was reversible as the recovery in reintroduced physiological saline was well recorded and it took about 40-45 min for complete restoration of the pre-experimental melanosome dispersion (Fig. 8).

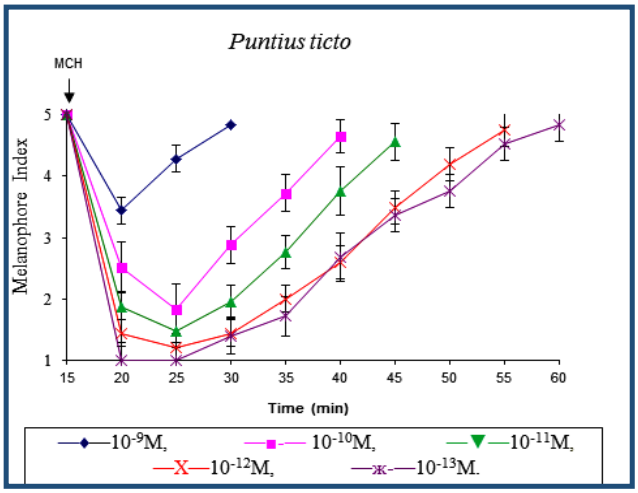

Fig 8: Concentration response relationship of melanosome aggregating action of MCH (10^{-13} to 10^{-9} M) on isolated scale melanophores of the fish. Vertical lines represent the standard deviation

3.3.3 Effect of melatonin

When melatonin, the pineal hormone was tested at a dose of 10mg/kg, the black-adapted fish over a black background paled notably within 5 min. The paling was maintained by the fish for 30 min after the treatment. They returned to their original body shade in 3 hr post-injection stage. When observed the subsequent day at 24 hr stage they were maximally dark (Fig. 9).

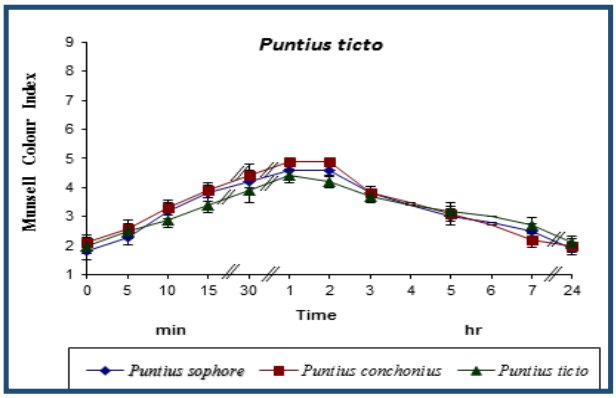

Fig 9: Effect of melatonin (10 mg/kg) on blinded fish kept over a white background. The vertical lines represent the S.D. of the mean.

The observations pertaining to the effects of blinding and the effects of melatonin, a hormone from the pineal gland on the colour changes in the intact and the blinded fish do indicate that the gland functions as an extra-optic receptor for the entrainment of dark-to-pale colour changes of animals to the light-dark cycle in the environment.

The effect of injected hormone is not as potent as that of epinephrine (Fig. 9). The fish either intact (black-adapted) or blinded (white-adapted) undergo paling due to pigment-aggregating action of the melatonin on melanophores. This is the direct effect of hormone *via* specific receptors on the melanophore membrane.

4. Discussion

The ability of *Puntius (Puntius sophore and Puntius conchonius)* to match its background has earlier been reported by Sharma *et al* [38] and Dubey and Jain [39] who have described the appearance of the fish on different backgrounds.

The initial faster background response over the two contrasting backgrounds; the pale and the dark one, as has been recorded in this study in three species of the barbs, *Puntius,* clearly appear to implicate a predominant neural co-ordination responsible for initiation and regulation of early phase of background-related chromatic response in the fish. The data recorded (Fig. 2) reveal that superimposed on endocrine mechanisms, neural regulation of colour change, which can be virtually instantaneous, is well marked and operative in these fishes. The biphasic response, an initial faster one and later slow, gradual and prolonged as recorded, do suggest that the process of colour change to be completed do depend on both neural as well as endocrine mechanisms both operative synergistically in the studied fishes, until equilibrium is reached.

Thus in comparison with other species, the time relations for these background adaptations as recorded here, point clearly that the responses are slower than reported for predominantly nervous reaction of fishes like, *Phoxinus* [40], but faster than reported for the predominantly hormonal reaction of *Anguilla* [41].

Working on the crucian carp, *Carassius carassius*, Iwata and Fukuda [42] in their study conclude that the systems controlling the motility of melanophores are mononeuronic in the peripheral system, their function being solely the paling of the integument and dineuronic in the central system, one division being excitatory and the other inhibitory in their final connections with the preganglionic efferent neurons. A fish adapts a dark background by suppressing the spontaneous discharge of the motor neurons in the medulla and the spinal cord, while one adapting a lighter background becomes pale due to augmentation of the spontaneous discharge form the same motor neurons. Thus contrary to earlier suggested that the by Parker [43], the pigment-dispersing fibres belonging to parasympathetic division of ANS (theory of double innervation) has not been shown to take part in the regulation of chromatophores. Instead evidences are pouring in for adenosine, to function as a co-transmitter and acting to disperse chromatosomes rapidly, thereby enabling the fish to change their colouration very quickly. With this, singly innervated chromatophores of teleost have been shown to acquire the ability to react very quickly, resembling in this regard reciprocally innervated effectors, as interpreted nicely [44-45].

The visual chromatic responses are known to be co-ordinated either neurally or hormonally and more often by co-operation of both these homeostatic mechanisms. While neurohumors released from chromatic nerve terminals represent the biomolecules to regulate the activities of chromatophores neurally implicating the ANS; the hormonal control with elaboration of hormones (MSH and MCH) from the pituitary is probably a more archaic of the control mechanisms found even in primitive fishes and almost universally operative in vertebrates. The other hormones that are known to regulate chromatophore movements are melatonin from the pineal gland and epinephrine, which may possibly originate in the chromaffin cells correspond to the adrenal medullary cells of higher vertebrates.

The three fresh water fish species of *Puntius* are considerably sensitive in their background (secondary) responses as can be seen by initial phase of the response (15 min stage) which is quite rapid. However, thereafter the colour change as a result of background response becomes slow and gradual and in 3 hrs of time the fish adapts to either of the backgrounds almost fully

The fishes show a rapid colour change during first 15 min which suggests a nervous co-ordination. This initial phase in both the experiments *i.e.,* the paling as well as the darkening is fast. In later phase, the rates become greatly subdued and the colour change process continues at slow rate to attain almost the full response in about 3 hrs. However, on being left in the appropriate backgrounds overnight, the fishes on a white-background and those on a black-background, paled and darkened a little further, respectively.

The results pertaining to this study of transitory or physiological colour change in the fish as a result of background response do suggest that co-ordination of colour change process seems to be not only nervous (to initiate the responses) but also humoral (to supplement the responses) working synergistically.

The observations in *Puntius* species as recorded here do resemble those described in *Phoxinus laevis* [18], *Gasterosteus aculeatus* [46], *Lebistes reticulatus* [41], *Macropodus opercularis* [25], *Rasbora daniconius* [47], *Nandus nandus* [48], *Labeo rohita* and *Cyprinus carpio* [49], *Labeo gonius* [50], *Catla catla* [51], and *Trichogaster trichopterus* [52]. These are species

where colour changes to a large extent are predominantly controlled neutrally (rapid initial phase) but melanotropic peptides are also required to regulate longer chromatic adaptations (slow second phase and longer) to achieve the equilibrium.

The receptors for the adaptation to background are unequivocally the eyes, as bilateral blinding virtually abolishes the rapid, background-related responses and likewise fish attain a darker shade irrespective of the background. Experiments involving evaluation of rates of paling and darkening in the fish due to a pale and dark illuminated backgrounds (Fig. 2) and those wherein chromatic responses are recorded as a result of bilateral blinding coupled with "primary" responses on account of activation of extra-optic receptors (Fig. 3) and MCH induced melanosome aggregation in the fish (Figs. 8) do support the view that in *Puntius* species studied, the chromatic responses are initiated by neural agency at a faster pace which then later appear to be supplemented by endocrine means (*i.e.,* MCH released by pituitary) to complete the responses in the fish.

The rapid and complete melanosome aggregations as recorded in isolated scale melanophores (innervated) from the dorsal trunk region of all the 3 species of the fish, *Puntius*, in response to K^+-ions stimulation do demonstrate that they are controlled nervously with involvement of sympathetic nervous system. The K^+-ions very effectively induced melanosome aggregation in dispersed melanophores in physiological saline solution and they get soon redispersed in the latter solution after with-drawl of K^+-stimulation. This clearly indicates that melanophores in these teleostean species are directly innervated and the peripheral pre-synaptic and post-synaptic mechanisms controlling bi-directional movements of melanosomes within scale melanophores are highly effective. Further the K^+-ions act on the melanophores in a concentration-related manner, which also supports the contention [53].

Conclusion: The initial faster background response over the two contrasting backgrounds; the pale and the dark one, as has been recorded in this study in three species of the barbs, *Puntius,* clearly appear to implicate a predominant neural co-ordination responsible for initiation and regulation of early phase of background-related chromatic response in the fish. The data recorded (Fig. 24) reveal that superimposed on endocrine mechanisms, neural regulation of colour change, which can be virtually instantaneous, is well marked and operative in these fishes. The biphasic response, an initial faster one and later slow, gradual and prolonged as recorded, do suggest that the process of colour change to be completed do depend on both neural as well as endocrine mechanisms both operative synergistically in the studied fishes, until equilibrium is reached. The rapid and complete melanosome aggregations as recorded in isolated scale melanophores (innervated) from the dorsal trunk region of all the 3 species of the fish, *Puntius*, in response to K^+-ions stimulation do demonstrate that they are controlled nervously with involvement of sympathetic nervous system. Epinephrine could serve as a neurotransmitter in the adrenergic chromatic nerve terminals innervating melanophores on isolated scales of the fish, *Puntius*. The two peptide hormone, *i.e.,* melanophore stimulating hormone and melanin concentrating hormone both are indicate the hormonal regulation in fish species of *Puntius.*

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