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Effect of various drugs on isolated scale melanophores of fish, *Balantiochelios melanopterus* (Bleeker)"

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ABSTRACT: All organisms, ranging from simple invertebrates to highly complex human beings, exist in different color patterns, arising from unique allocation of pigments all over the body. Several drugs mediate their biological activities accelerated by different immunological and non immunological stimuli via differential expression of receptors on effector cells, including the melanophores. Melanophores of lower vertebrates are specialized type of smooth muscles cells. We here have examined the effects of several drugs on the isolated scale melanophores of fish *Balantiocheilos melanopterus* accentuating the potential physiomodulatory effects of these drugs on the melanophore pigments of *Balantiochelios melanopterus.* The results obtained where adenosine is able to enhance the rate of dispersion, probably by its own specific receptors and where caffeine is able to back such a response of adenosine is in favour of the mediation of melanophore dispersion by the cAMP pathway as a signal transduction mechanism operative in the fish.

INTRODUCTION

Balantiocheilos melanopterus, also known by names tricolor shark, silver shark, or shark minnow, is a fish species belonging to family Cyprinidae, is one among the two species in the genus *Balantiocheilos*. *Balantiocheilos melanopterus* is found naturally in slow running waters of south East Asia; Bornes, Thailand, Sumatra, Penninsular Malaysia and in Mekong and Chao Pyraya river basins. *B. melanopterus* possesses cycloid cloured scales that are distributed all over the body. The chromatic component in the skin remains attached to the scales. The dorso-lateral trunk region of the body is mostly comprised of basic chromatophore types namely melanophores and others like xanthophores and irridophores.

Melanophores display a radial array of microtubules extending from perinuclear area of the cell where microtubule organizing centre is located and projecting into cell extensions. Regulation of pigment transport involves changes in level of cAMP which regulate activity of microtubule motors (Nascimento *et al*., 2003). Under the histological studies it was demonstrated that a network of nerve fibers are associated with teleostean melanophores (Ballowitz, 1893 a, b; Eberth and Bunge, 1895). Parker (1948) claimed that teleost melanophores are inerverated by both pigment-aggregating fibres and pigment-dispersing fibres and postulated that pigment-aggregating fibres were sympathetic (adrenergic) and pigment – dispersing fibres were parasympathetic (cholinergic). Other workers, however, have disagreed with Parker's conclusions and have postulated that teleost melanophores are innervated by sympathetic pigment-aggregating fibres only (Healey, 1957; Waring, 1963; Bagnara and Hadley, 1973; Fujii and Oshima, 1986).

The two types of adrenoceptors that have been reported are designated as α and β adrenoceptors (Ahluqiust, 1948). Many workers have used pharmacological studies, to indicate that the peripheral nerve fibres controlling melanosome aggregation are adrenergic, that the transmitter involved may be norepinephrine (Fujii, 1961; Scheline, 1963; Grove, 1969a, b; Reed and Finnin, 1972; Fernando and Groove, 1974a, b; Fujii and Miyashita, 1975; Jain, 1976; Patil and Jain, 1989; Acharya and Ovais, 2007, Amiri, 2009). From all these studies it has naturally been supposed that adrenergic receptors mediating pigment aggregation in teleost melanophores are of alpha nature. On functional basis α-adrenoceptors have been divided into α_1 and α_2 subtype (Langer, 1974). Some of the researchers have demonstrated that receptors mediating the sympathetic melanosome aggregation are the α_2–adrenoceptors (Andersson *et al*, 1984; Karlsson *et al;* 1987, Morishita, 1987; Jain and Patil, 1992; Burton and Vockey, 2000a). Since α_2-agonist have been found to be more effective than α_1-agonist and transmission is more easily blocked by α_2 blockers than by α_1 blockers. The α_1–receptor activation results in the rise of cytosolic calcium concentration. The α_2 receptors are thought to lower intracellular cAMP by inhibiting adenylate cyclase via Gi stimulation.

The melanotropic peptides play a major role in the control of vertebral integumental pigmentation. The two physiologically important melanotropins are α-MSH (α-melanocyte stimulating hormone) and MCH (melanin concentrating hormone). In lower vertebrates like fishes, MSH is released from the intermediate lobe of pituitary gland. MCH was shown to elicit pigment aggregation in melanophores of almost all teleostean species when present at low concentrations (Wilkes *et al*., 1984; Oshima *et al*., 1985; 1986; Nagai *et al.,* 1986 Jain and Patil,1990), and was shown to act via MCH receptors on the cell membrane (Oshima *et al.*, 1985). On the other hand cyclic melanotropin (Cys4, Cys 10) α–MSH has been reported to be a potent agonist of tetrapod melanophores, leading to skin darkening (Sawyer *et al.,* 1982). Since MCH is also a cyclic peptide, Wilkes *et al. (*1984*),* thought that melanin dispersion in response to MCH at higher concentration might be caused through the activation of MSH receptors by MCH.

The ichthyofauna in the Chambal Division is dominated by the members of the family cyprinidae (Dubey *et al.,* 1980). Only 6 genus namely *Labeo, Catla, Cirrhinus, Garra, Rasbora* and *Puntius* have so far been studied with respect to their pigment cell system and the regulatory mechanisms. Looking to a diversity in its various aspects such as the chromatophore types, colour pattern, physiological responses etc, it was thought worthwhile to study the rate of colour change, nature of chromatic fibres, occurrence of receptors on the dominant of the pigment cell types *i.e.*, melanophores from the fresh water fish, *Balantiocheilos melanopterus*, belonging to family cyprinidae.

MATERIALS AND METHODS

A. Fish Used

Balantiocheilos melanopterus, a tropical fish belonging to family cyprinidae was used for the present study. It is commonly known as Bala shark, tricolor shark, silver shark or shark minnow. They are named sharks because of appearance and shape of their dorsal fin.

The Fish *Balantiocheilos melanopterus* of either sex were used for the present study having body length 8 to 11 cm. The fish originated from wild or cultured populations in Thailand and were obtained from a local dealer of ornamental fish and maintained in the laboratory.

Balantiocheilos melanopterus have a silver body with black margins on their dorsal, caudal, anal and pelvic fins. It grows to a maximum length of 15 inches but takes a long time to do so.

B. Care and Maintenance

Fishes collected from the commercial source were brought to laboratory and were stocked in sized transparent glass aquarium (60 × 30 × 30 cm), containing fresh aerated water. As these fishes are omnivorous, they were fed with commercial fish diet, chopped earthworms and planktons. During the experiments feeding was avoided. Water in aquaria was changed by siphoning process that also helps in removal of feacal material and uneaten food. This was done on every third day. Before the experiments the animals were acclimatized to laboratory conditions for at least 15 days. No attempt was made to control the photoperiod and temperature (ranged from 25 to 32°C).

C. Adaptation to different backgrounds. (measuring in vivo responses)

To study the chromatic response as adaptation to contrasting backgrounds, the healthy stocked fish placed in natural light condition were taken out and placed in white/black backgrounds with overhead illumination (400 Lux). The experimental fish were weighed and placed for a period of 24 hours over a black painted trough (30 × 10 × 10 cm) and covered at top with black cotton net tied on it. To study the rate of paling these black adapted fishes were gently transferred to white-painted glass troughs. Munsell grey series color standards were used to record the pre-experimental shade of fish body. The color changes are recorded at regular intervals of time until no further change could be noticed for a considerable time period. Similarly to study the rate of darkening the white adapted fishes are gently and carefully transferred to black painted trough for considerable time period until no further change could be noticed and changes in the body shade was recorded similarly. Macroscopic changes in the body shade to background adaptation were recorded by a nine-point scale prepared from colour charts beloning to Munsell grey series. It involves matching of general colour of dorsol–lateral surface of fish as seen with naked eye against serially graded 9 colour standards. These are mounted on a wooden plate in the form of small rectangular strips. All grades 1-9 are known for their power of reflectance out of total light which fall on them. This series permits graphical representation of data for experimentally produced paling or darkening in fish.

The percentage of light reflected from each colour standard or each index number has been standardized and values are given as under:

Munsell grey series Standard Quality
Index Number of light reflected

1. (Maximum darkening) 1.5%
2. 3.0%
3. 6.5%
4. 12.1%
5. 19.1%
6. 30.3%

7. 44.3%
8. 57.5%
9. (Maximum paling) 72.5%

D. Procedure for in vitro study using scale slips

The scale slips isolated from the dorsal sites (AD/MD) of the skin are exclusively used for *in vitro* study. These scale slips are carefully fixed with its epidermis side down on the perpex trough and held by a fine glass needle glued to the coverglass which is placed on the perfusion chamber containing the physiological saline. The anterior unpigmented part of the scale remains under the glass needle and the posterior pigmentary part remains free for observation. This device ensures the contact of the integument with experimental solution in the trough and also protects the scale from any mechanical disturbance during the exchange of external solutions and thus allows a particular area permanently under observation.

The isolated scale slips are immediately perfused with the physiological saline, and when required it is replaced by experimental (drug/hormone) solution. The experimental solution is drained completely through an outflow suction, either by Pasteur pipette or by soaking with the filter paper and the chamber was filled by another inflow pipette.

At least five preparations from different fishes are used to see the effect of various drugs during the particular experiment for each individual experiment. 25 melanophores from 5 scales belonging to different animals were thus observed. All experiments were performed at room temperature (25±2.4°C).

E. Composition of physiological saline solution

The isotonic physiological saline solution used for maintaining the melanophore in a dispersed state has the following composition (mM):

NaCl 12.8
KCl 2.68
$CaCl_2$ 1.8
Glucose 5.6
Hepes (NaOH) buffer 5.0
with a pH value of 7.4.

The isotonic K^+-rich saline was also used where equimolar concentration of KCl was substituted for NaCl in the physiological saline.

F. Measuring melanophore responses: (In vitro)

Hogben and Slome (1931) developed a "Melanophore Index" response for staging amphibian melanophores. In this microscopic assay, the degree of melanosome dispersion is determined according to a 5 point scale to yield a 'melanophore index' that ranges between 1 and 5. When melanosomes are dispersed throughout the cytoplasm of the cell, skin appears darker while the aggregated melanosomes in the cytoplasm of the cell results in the paling of the skin.

In this method five distinct stages represents the different degrees of pigment dispersal within melanophores. Melanophore Index 1 (M.I. = 1) designates most aggregated state and most dispersed states are designated by M.I. 5. Intermediate states are designated by M.I. 2, 3 and 4. (Fig. 2).

G. Drugs used

1. Adrenaline tartrate (M.I. Pharmaceutical Works Pvt Ltd., Kolkata)
2. Noradrenaline bitartrate (Somrath Life Sciences Pvt. Ltd. Mumbai).
3. Clonidine hydrochloride (R.B.I., USA)
4. Prazosin hydrochloride (Sun Pharmaceutical Ind. Ltd., Mumbai).
5. Yohimbine hydrochloride (Poul Neeuoundrof, Germany)
6. Propranolol (Ranbaxy laboratories Ltd. India)
7. Atropine sulphate (Neon Laboratories Ltd., Thane)
8. Isoxsuprine hydrochloride (Salvay pharma India Ltd., Mumbai
9. Adenosine (Zuvius Life Sciences Pvt. Ltd. India)
10. Caffeine (Sun Pharmaceutical Ind. Ltd., Mumbai).

Stock solutions of the drugs were prepared by dissolving them in physiological saline or distilled water. In case of adrenaline injection fluid is used that was diluted with physiological saline. All stock solutions were kept refrigerated and freshly diluted before experiments.

OBSERVATIONS AND RESULTS

A. Chromatophore characteristics

The fish, *Balantiocheilos melanopterus* under the present study is a scaly fish which possesses cycloid scales distributed all over the body. The scales can be very easily slipped out from the scale pockets by gently pulling through their posterior projected surfaces, separating readily at a fibrous septum. This was done by a very fine forcep without any injury to any component of the skin over the scales. The scale on its epidermis side down was perfused with PS on being kept into perfusion chamber. The chromatophore characteristics and their distribution were studied by light microscopic techniques.

The dermal melanophores occur in various shapes and sizes with branched and unbranched dendrites emerging from centre of the cells. In the present study dermal melanophores distributed in the middle layers in the pigmentory part of the scale were utilized for study.

Sometimes these fishes have yellow colour on their fins between the silver and black band, revealing the presence of xanthophores in them. In the scales of the fish xanthophores are also present.

Iridophores are peculiar chromatophores, usually non-dendritic and without coloured organalles. Thin crystals of guanine are generally present as stacks and are called reflecting platelets, as they are strongly light-reflecting because of their very high refractive index of no less than 1.83 as mentioned by Fujii, (2000). These cells are principally responsible for silvery glitters and whiteness of the side and belly skin. Thus the cells are non-motile. These cells are densely distributed as compactly packed structures in the intermediate region between the peripheral and central part of the scale.

B. Physiological colour change/background-related chromatic response (Rate of paling and darkening)

When white-or black-adapted *Balantiocheilos melanopterus* which were kept had previously on these respective backgrounds for 24 hours were subjected to background reversal, they showed a quick response by changing their body shade according to their background (Fig.1).

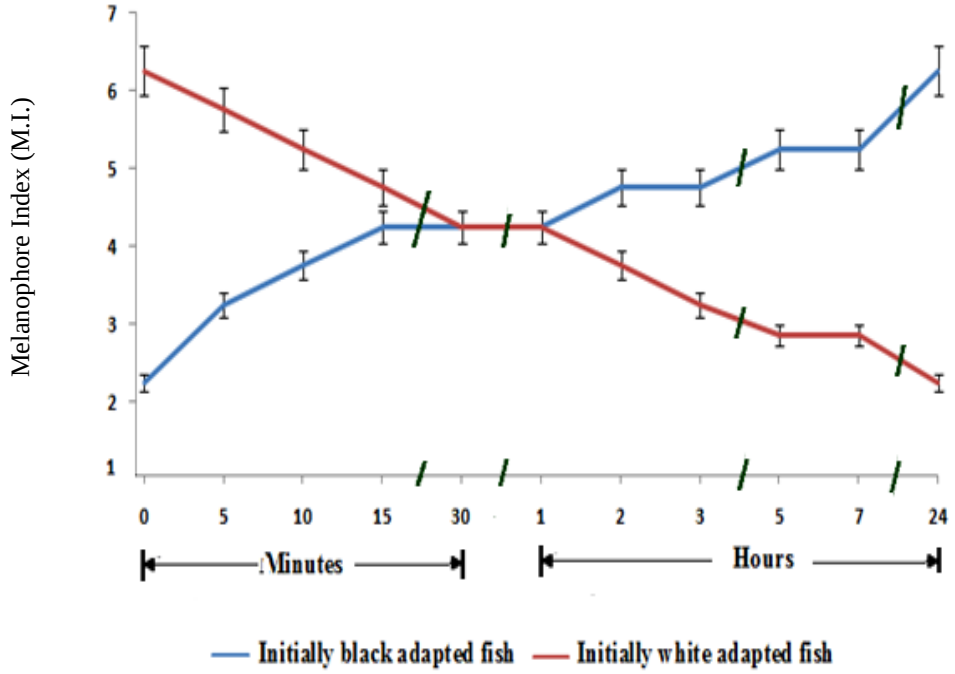

Fig. 1. Changes in brightness of dorsolateral trunk area during the adaptation process. A fish kept in a dark or white background for 24 hrs was transferred to a white background or a black one.
(a) Changes in body brightness of fish transferred from a white background to a black one.
(b) Changes in body brightness of fish transferred from a black to a white background.
The degree of brightness is expressed as the Index Number in the Munsell grey series in which black and white corresponds to 1 and 9, respectively. Each value ± standard error (vertical lines) of the mean of the responses (body shade) of the animals at the times noted.

Black-adapted fish having pre-experimental shade i.e. grade 2.2 of M.C.I, when placed to adapt on an illuminated white-background attained a grade of 3.2 in first five minutes. In 30 minutes they attained the grade of 3.2. They paled further slowly to attain a grade of 5.2 at 7 hours stage. Subsequently they attained the maximum grade of 6.2 when observed the day next at a stage of 24 hours.

White-adapted fish having the experimental shade i.e., grade 6.2 when transferred to black-background reached to a grade of 5.7 in first 5 minutes. In 30 minutes they attained the grade of 4.2. The fish darkened further slowly and gradually to attain a grade of 2.8 at 7 hours stage. The maximum darkening i.e., grade 2.2 was attained by them when observed at a stage of 24 hours. Thus, the grades within which the fish changes its shade ranges from 6.2 to 2.2 when subjected to adapt to a white- and a black-background (Fig. 2).

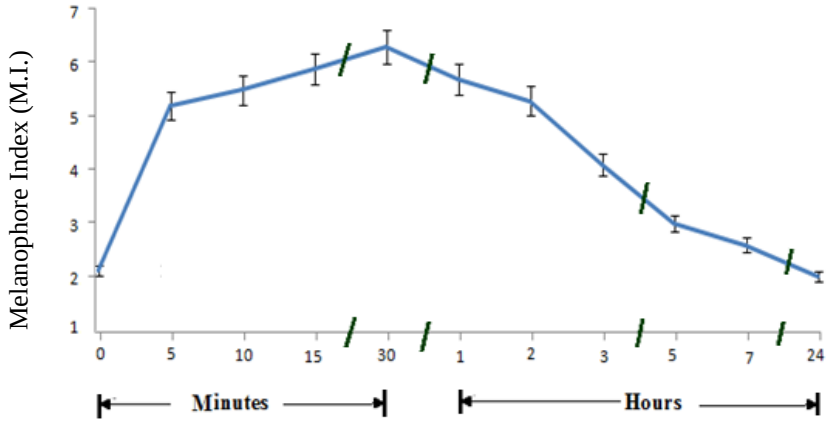

Fig. 2. Chromatic response (paling) in black-adapted fish kept over a black background on being injected with MCH (100 ng/g). The data are shown as means (5 fishes) ± SEM.

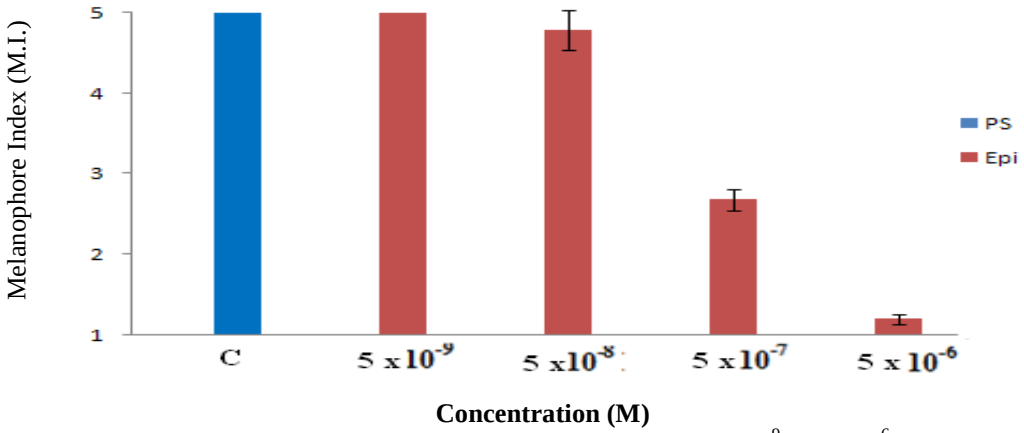

Fig. 3. Dose–dependent melanosome aggregatory effects of epinephrine (E) ($5 \times 10^{-9} – 5 \times 10^{-6}$ M) on melanophores of the fish. The solutions of various concentrations of the drug were applied for 10 min. The results (data) are shown as means ± SEM from five measurements on scales from five different animals.

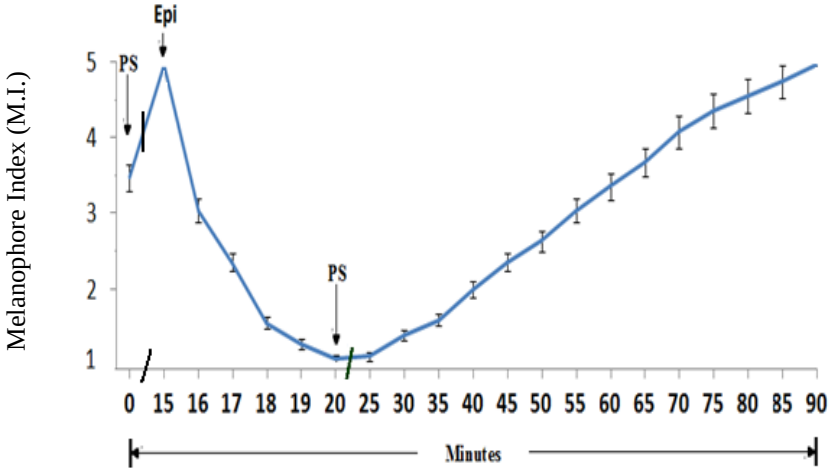

Fig. 4. Aggregation of pigment in melanophores by treatment with epinephrine (5×10^{-6}M) and their recovery in P.S. The results (data) are shown as means ± SEM from five measurements on scales from five different animals.

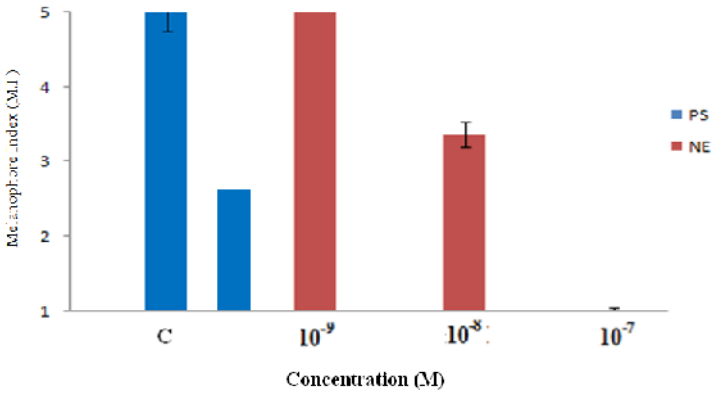

Fig. 5. Dose–dependent melanosome aggregatory effects of Norepinephrine (NE) (10^{-9} - 10^{-7} M) on melanophores of the fish. The solutions of various concentrations of the drug were applied for 20 min. The results (data) are shown as means ± SEM from five measurements on scales from five different animals.

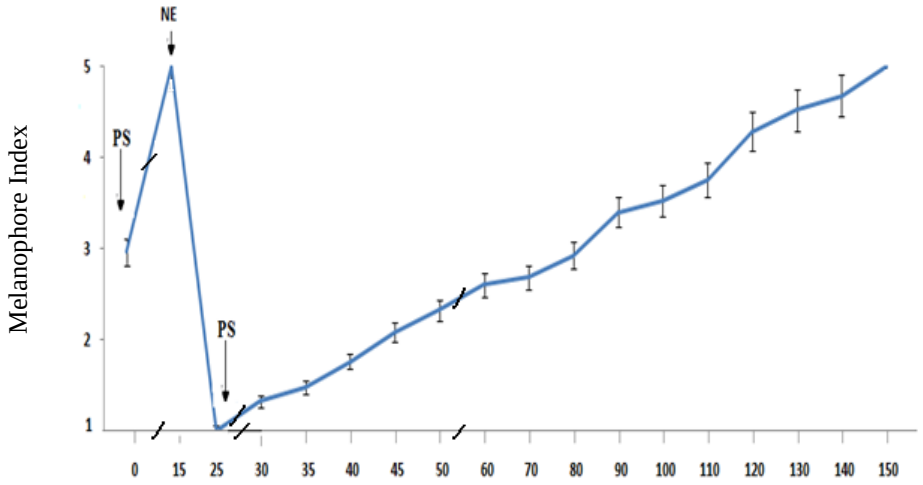

Fig. 6. Aggregation of pigment in melanophores by treatment with Norepinephrine (NE) (10^{-7} M) and their recovery in P.S. The results (data) are shown as means ± SEM from five measurements on scales from five different animals.

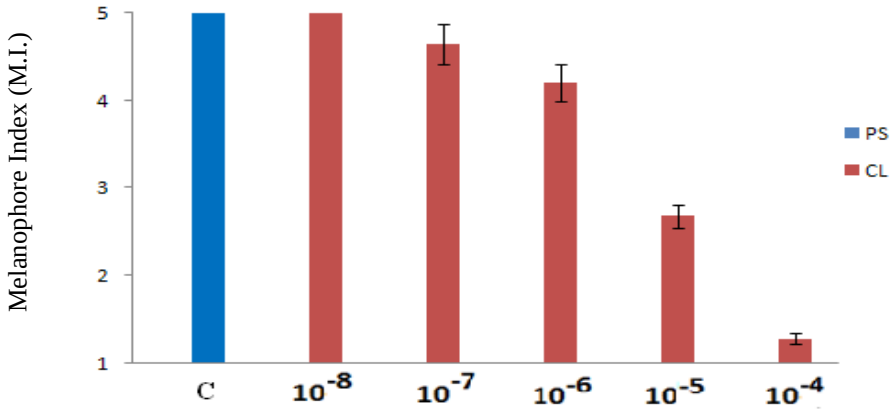

Fig. 7. Effect of clonidine at varying concentrations on the melanophores of the fish. Solutions were applied for 30 minute. The results (data) are shown as means ± SEM from five measurements on scales from five different animals.

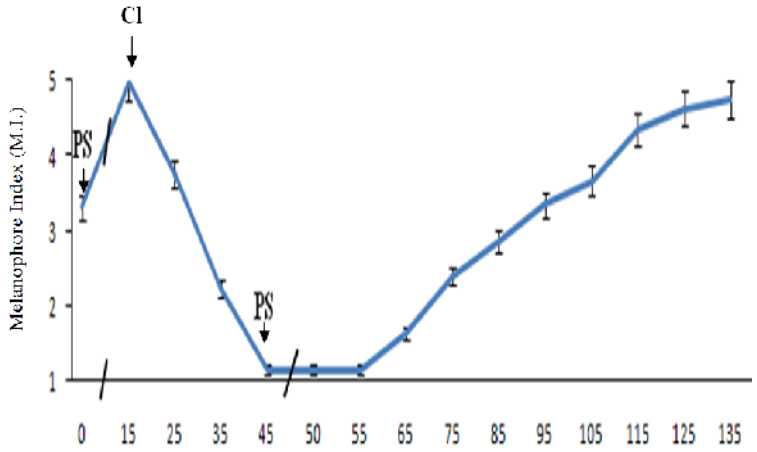

Fig. 8. Aggregation of pigment in melanophores by treatment with clonidine (10^{-4}M) and their recovery in P.S. The results (data) are shown as means ± SEM from five measurements on scales from five different animals.

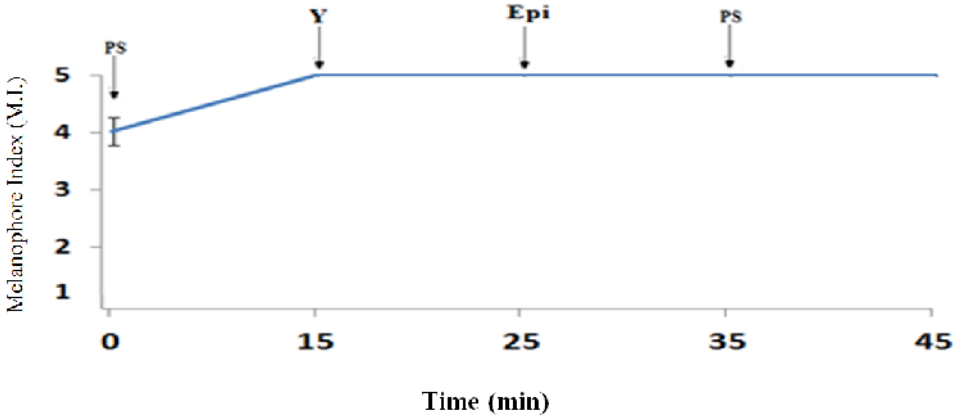

Fig. 9. Complete blockade of epinephrine (5×10^{-6} M) induced melanophore aggregation by pretreatment with α_2–adrenoceptor blocker, yohimbine (10^{-4} M). The results (data) are shown as means ± SEM from five measurements on scales from five different animals.

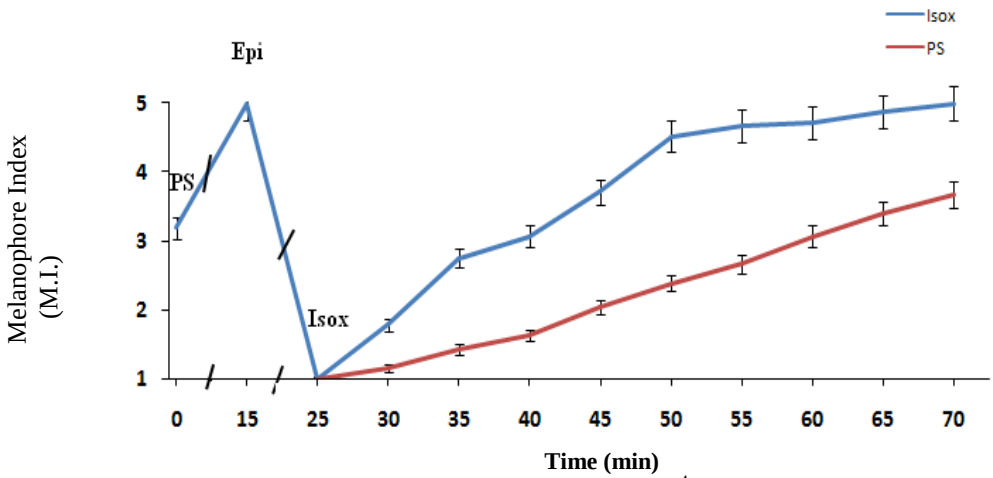

Fig. 10. Effect of pretreatment with α_1–adrenoceptor antagonist-prazosin (10^{-4} M) on the response of epinephrine (5×10^{-6} M) in melanophores on isolated scale preparation of the fish. The results (data) are shown as means ± SEM from five measurements on scales from five different animals.

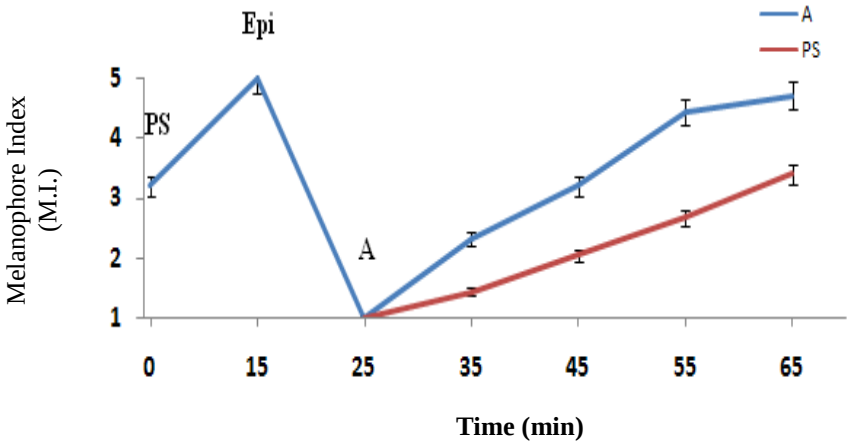

Fig. 11. Acceleration of melanophore dispersion by treatment of atropine (2 x 10^{-4} M) as observed in epinephrine (5 x 10^{-6} M)-treated melanophores of the fish. The results (data) are shown as means ± SEM from five measurements on scales from five different animals.

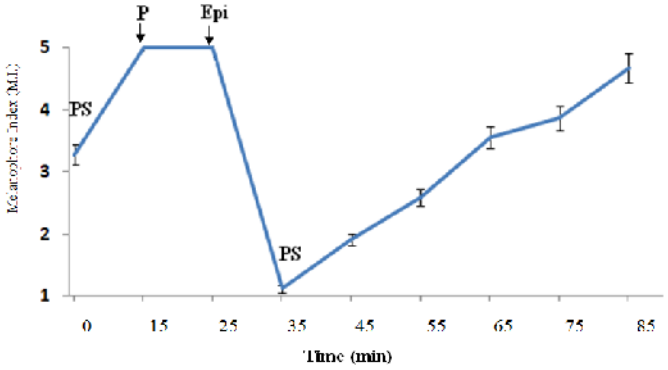

Fig. 12. Effect of propranolol (10^{-4} M), a β – adrenoceptor blocker on the melanosome aggregatory action of epinephrine (5 x 10^{-6} M) on the fish melanophores. The results (data) are shown as means ± SEM from five measurements on scales from five different animals**.**

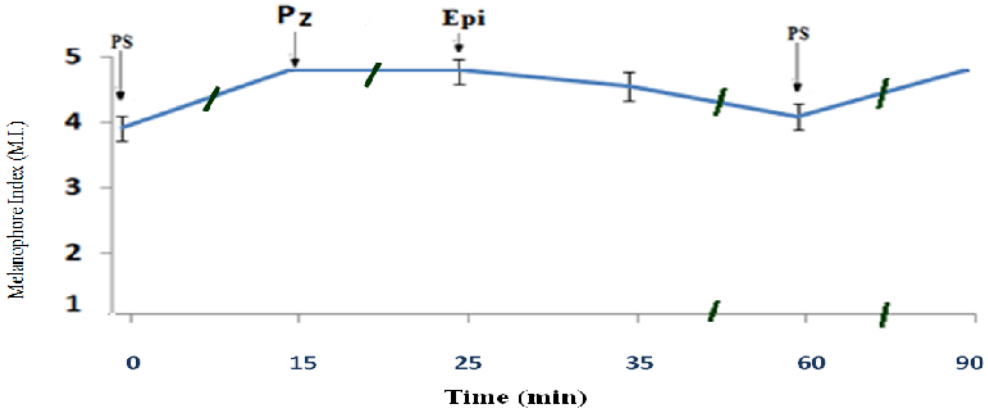

Fig. 13. Acceleration of melanophore dispersion by treatment of isoxsuprine (2 x 10^{-4} M) as observed in epinephrine (5 × 10^{-6} M)-treated melanophores of the fish. The results (data) are shown as means ± SEM from five measurements on scales from five different animals.

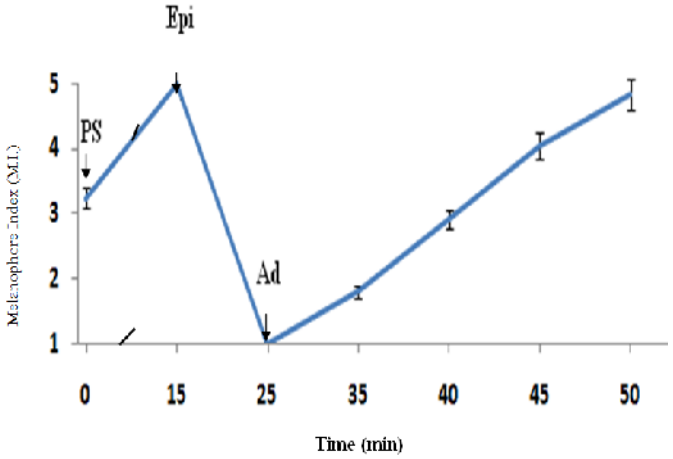

Fig. 14. Acceleration of melanophore dispersion by treatment of adenosine (10^{-4} M) as observed in epinephrine (5 x 10^{-6} M)-treated aggregated melanophores of the fish. The results (data) are shown as means ± SEM from five measurements on scales from five different animals.

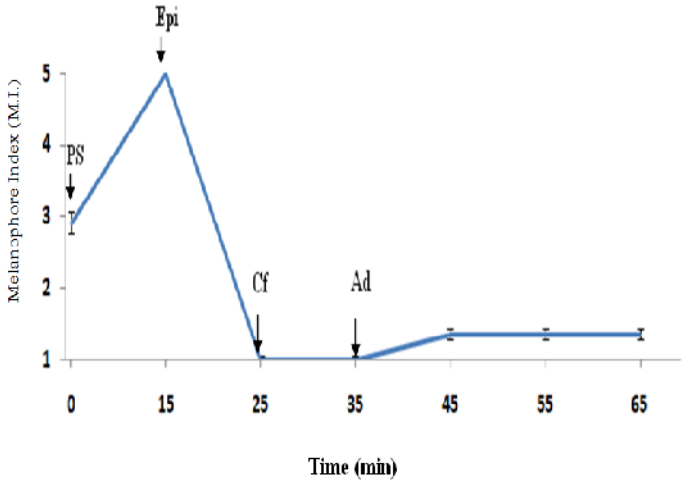

Fig. 15. Blockade of effect of Adenosine (10^{-4} M) on melanophores pre-treated with caffeine (10^{-4} M). The results (data) are shown as means ± SEM from five measurements on scales from five different animals.

C. Effect of MCH (melanin concentrating hormone) on melanophores

Melanin concentrating hormone (MCH) is a cyclic 17-amino acid hypothalmic peptide originally isolated from pituitary gland of teleost fish, and it aggregates the melanosomes within melanophores in majority of teleosts. In mammals it is involved in the regulation of feeding behaviour and energy balance.

Effect of MCH (100 ng/g) on the melanophores of the fish *Balantiocheilos melanophores* was studied by injecting the hormone to the black-adapted fish over a black background. The pre-experimental shade i.e. grade 2.1, of the fully black adapted fish reached rapidly to a grade of 5.2 in first 5 minutes by a rapid paling in the fish. In 30 minutes it reached the peak value to be a grade of 6.3 and then after the shade starts declining, reaching the maximum grade of 2.0 when observed at 24 hours. Thus the grades within which the fish changes its shade in response to MCH (100 ng/g) ranges from 2.1 to a maximum of 6.3 and then declining to 2.0 (Fig. 2).

D. Effects of Drugs

Ten drugs were selected for the *In vitro* study of receptor mechanism of fish melanophores. The effects were indicated in terms of their responses shown by melanophores.

Effect of Epinephrine (Adrenergic agonist): Epinephrine is a hormone and neurotransmitter produced by adrenal medulla and chromaffin cells. It is a catecholamine, a sympathomimetic monoamine that acts directly on effector cells. Its actions are mediated through adrenergic receptors. It activates α_1, α_2, β_1 and β_2 receptors to different extents. The threshed for activation of β-receptors is lower than that of α-receptors (Goodman and Gilman, 1996).

In the present study, epinephrine at concentration of 5×10^{-6} M induces rapid and potent aggregation of pigment in PS equilibrated melanophores. Minimal concentration of epinephrine required for producing discernable melanosome aggregation was found to be 5×10^{-8} M. With increase in concentration, magnitude of response increases (Fig. 3).

Melanophores on freshly isolated scale were at intermediate state of pigment dispersion (M.I.=3.5) on isolation. After incubated in physiological saline for 15 minutes they attained the full dispersion state (M.I.=5). After application of epinephrine at a concentration of 5×10^{-6} M, these melanophores undergo rapid aggregation of melanosomes. It was seen that full aggregation (M.I.=1) was achieved after 5 minutes treatment. The epinephrine treated aggregated melanophores were then perfused by physiological saline that resulted in slow and gradual dispersion of pigmented granules within melanophores. A high dispersion (M.I. = 5) was recorded at 80 minute post incubation in the physiological saline (Fig. 4).

Effect of norepinephrine on melanophores: Norepinephrine is a catecholamine with dual roles as a hormone and a neurotransmitter. It is released from the adrenal medulla in the blood as a hormone. As a neurotransmitter in the central nervous system and sympathetic nervous system, it is released from non adrenergic neurons. The actions of norepinephrine are carried out via binding to adrenergic receptors.It differs from the epinephrine only by methyl substitution in amino group. Thus it directly acts as an adrenomimetic drug. It has high affinity for α and β adrenoceptors and low affinity for β_2-adrenoceptors.

Melanophores on freshly isolated scale preparations were treated with different concentrations of norepinephrine. It was found that norepinephrine (10^{-8} and 10^{-7}M) induces concentration related aggregation. The maximum i.e. 100% aggregation was recorded at 10^{-7} M (Fig. 5).

Melanophores were seen at intermediate state of pigment dispersion (M.I.=2.9) on isolation. When perfused in physiological saline for 20 minute, they attained full dispersion (M.I.=5). The norepinephrine (10^{-7} M) when applied to these melanophores, they undergo rapid aggregation of melanosomes, and the full aggregation (M.I.=1) was achieved after 10 minute treatment. Then norepinephrine treated melanophores were perfused with physiological saline. This resulted in slow and gradual dispersion of pigment granules within the melanophores. Full dispersion (M.I.=5) was recorded after 2 hours post incubation in the physiological saline (Fig. 6).

Effect of clonidine: Clonidine is an imidazoline derivative synthesized in early 1960s used to treat several medical conditions. It is centrally acting α-adronergic agonist with more affinity for α_2 than α_1 receptores. It has specificity towards the presynaptic α_2 receptors in vasomotor centre in the brain stem.

Clonidine as α_2–adrenergic agonist was tested at various concentrations ranging from 10^{-8} M to 10^{-4}M. At 10^{-7}M the melanophores show slight aggregation (M.I=4.6) and at 10^{-4} M concentration nearly maximum aggregation (MI=1.2) response was achieved after 30 minute treatment (Fig. 7).

These clonidine treated aggregated melanophores when perfused with physiological saline, the pigment granules within them showed a slow and gradual dispersion and nearly full dispersion (M.I.=4.7) was achieved after 90 minute incubation in physiological saline (Fig. 8).

Effect of Yohimbine: Yohimbine is an alkaloid with stimulant and aphrodisiac effects found naturally in Pausinystalia yohimbe. It is also found naturally in *Rauwolfia* serpentina. Yohimbine has high affinity for the α_2 adrenergic receptors, moderate affinity for 5-HT and D_2 receptors, and weak affinity for D_3 receptors. Yohimbine behaves as an antagomst to all receptors except for 5-HT_{1A} where it acts as a weak partial agonist. It blocks α_2–receptors selectively and thereby enhances release of noradrenaline for a short period. The drug is an important tool in the investigation of presynaptic α-receptors.

The blocking behvaiour of Yohimbine was detected by first treating the dispersed melanophores (M.I.=5) equilibrated in the physiological saline with Yohimbine (10^{-4} M) for 10 minutes. In this solution the melanophores remain dispersed retaining the M.I. value of 5. When these melanophores were treated with epinephrine (5×10^{-6}M), a complete blockade of melanosome aggregating action was well observed. Further, these melanophores maintained their dispersed state in physiological saline even after hours of incubation (Fig. 9).

Effect of Prazosin: Prazosin is a sympatholytic drug used as antihypertensive agent that has α_1-adrenoceptor blocker properties. Specifically, prazosin is selective for the α_1- receptors. Prazosin at concentration (10^{-4}M) was found to block the effect of epinephrine (5×10^{-6} M).

Very slight aggregatory response (M.I. = 4.6) was observed on treatment with epinephrine (5×10^{-6}M) (Fig. 10).

Effect of Atropine: Atropine is a tropane alkaloid extracted from deadly night shade (*Atropa belladonna*), Jimsonweed (*Datura stramonium*), mandrake (*mandiagora officinarum*) and other parts of plant family solanacea. It is a competitive antagonist for the muscarinic acetylcholine receptor and is thus referred as muscarinic blocking or antimuscarinic agent. In mammals atropine blocks the influence of post-ganglionic cholinergic fibers. This occurs because atropine is a competitive antagonist of the muscarinic acetylcholine receptors (Acetylchohine is the main neurotransmitter used by the parasympathetic nervous system).

Atropine (2×10^{-4} M) when applied to the previously aggregated melanophores (M.I.=1) through the action of epinephrine (5×10^{-6} M), the melanosomes within the cells dispersed actively to a great extent. While the dispersion attained in physiological saline without the drug atropine showing a lower magnitude of response (serving as a control). This shows the acceleration of melanophore dispersion by the action of drug atropine significantly (Fig.11).

Effect of Propranolol: Propranolol is a non-selective beta blocker that blocks the action of epinephrine and norepinephrine on both β_1- and β_2-adrenergic receptors and mainly used in the treatment of hypertension. It was the first successful beta blocker developed.

When propranolol (10^{-4}M) was applied on the dispersed melanopores (M.I. = 5) previously equilibrated in the physiological saline for 10 minute the melanophores remained in the dispersed state (M.I=5). Then on the application of epinephrine (5×10^{-6}M), the melanosome aggregating action was seen, and melanophores attained the full aggregation (M.I=1) within 10 minute of incubation (Fig. 12).

Effect of Isoxuprine Hydrochloride: Isoxuprine is used as a vasodilator in humans and equines. Chemically related to isoprenaline (β_1 and β_2 and agonist). It acts by stimulation of β_2-receptor resulting in relaxation of smooth muscles.

When isoxuprine (1.6×10^{-4}M) was applied to the melanophores whose pigment had been previously aggregated (MI=1) through treatment with epinephrine (5×10^{-6}M), melanosomes within the cells dispersed gradually to a great extent (Fig. 13). Epinephrine (5×10^{-6} M) treated melanophores dispersed passively and slowly in the physiological saline without isoxsuprine, served as the control. This comparison shows clearly that the β-adrenergic agonist, isoxsuprine accelerated dispersion of melanosomes within the melanophores by activating the β-receptors.

Effect of Adenosine and Caffeine: Adenosine is a nucleoside composed of a molecule of adenine attached to ribose sugar molecule. It plays an important role in biochemical processes, such as energy transfer–as ATP and ADP as well as in signal transduction as cAMP. It is also an inhibitory neurotransmitter, beloved to play a role in promoting sleep and suppressing arousal.

Cellular signaling by adenosine occurs through four adenosine receptor subtypes (A1, A2A, A2B and A3). The different adenosine receptor subtypes (A1, A2A, A2B and A3) are all seven transmembrane spanning G-protein coupled receptors.

Adenosine (10^{-4}M) when applied to the melanophores whose pigment had been previously aggregated (M.I=1) through treatment with epinephrine (5×10^{-6}M), melanosomes within the cells dispersed fully (M.I=4.84) within 25 minute (Fig. 14). It was observed that adenosine (10^{-4}M) accelerates the melanosome dispersion as observed in epinephrine (5×10^{-6} M) treated melanophores of the fish.

Adenosine action was further confirmed by the application of caffeine–a methylxanthene which effectively inhibited the dispersing action of adenine derivative. Caffeine (10^{-4}M) was applied on the previously aggregated epinephrine treated melanophores, which completely inhibited the dispersing action of adenosine on melanophores (Fig. 15).

DISCUSSION

Majority of teleostean fishes display colour change as a result of background response giving evidence that in them both the hormonal and neural mechanisms are involved and the physiological mechanisms associated with the change of colour is the result of combined effects of hormones and neurotransmitters operating synergistically in these animals.

Fujii and Oshima (1986) highlighted the advantages of sympathetic–chromatophore systems in analyzing physiologically or pharmacologically the properties of the conductive and the transmitter releasing portions of peripheral fibres over smooth muscle preparations with which so many studies have been performed on autonomic neural transmission.

In the background reversal experiments, a biphasic chromatic response was found. In the first phase the response was rapid covering a larger part of colour change exhibited by the fish. This was followed by the second phase of slow and gradual colour change.

Neill (1940) while working on three different teleostean species viz, *Anguilla anguilla*, *Lebistes reticulatus* and *Salmo salar*, was able to draw a relationship between the time and the co-ordination of chromatic response under different experimental conditions. Thus he analysed the relative influence of nervous and hormonal (endocrine) systems on physiological (chromomotor) colour changes in these species.

Highlighting the significance of time Neill (1940) stated that for a co-ordianted response, where eyes act as a receptor, the time taken for the stimulus to act on them, is at most a matter of seconds. Further, the time taken for the distribution of a disturbance through the co-ordinating mechanism (if it is a neural one), which involves, (a) the transmission of nervous impulse along the nerve trunks concerned (b) delay at synopsis and (c) delay at the nerve effector junction time taken together is also at most a matter of seconds. Thus the total time taken for these events together may therefore be taken as under 1 min. and any excess within the period of complete cycle must be due to the time taken for the effector (the chromatophores in this case) to execute its response *i.e.,* to complete the colour change.

With this interpretation Neill (1940) concluded that if, therefore, it is found that the total time for complete colour change *i.e.,* for the complete cycle is of the same order as the effector–reaction time, then there is nothing to suggest that the co-ordinating mechanism is other than the nervous reflex. If, however, the total time for the colour change is markedly greater than the effector time, then same other co-ordinating mechanism must be involved e.g. the reflex liberation of hormone & its gradual accumulation in the blood stream to a certain limiting value.

Thus he proposed that animals exhibiting complete background adaptation in 10 min or less have melanophores entirely or predominantly under nervous control. Those requiring more than 10 min for this have melanophores primarily under the influence of hormones.

Abbott (1973) supporting, Neill's observations in his review on endocrine regulation of pigmentation in fish has clearly stated that control of the melanophores in fishes, ranges from fully nervous to fully hormonal with an interplay of these agencies in varying proportions.

Thus according to him, where the control of melanophore is mixed, the two systems usually act synergistically, but under same circumstances even one can act independently.

Parker (1948) and Waring (1963) by the time course of colour change in all the Elasmobranch species tested, which was a matter of hours suggested a hormonal control of colour change in these fishes. Hyphophysectomy in *Scyllinm canicula* (which takes 100 hr for completion of adaptation to background) abolishes the response to black background and the blood transfusion from a darkfish inducing darkening in a recipient fish, supports for a role of darkening hormone (intermedin) in these animals. Intermedin is apparently functionally identical to MSH. Evidence concerning a paling hormone was in conclusive.

As electrical stimulation and injected adrenaline could aggregate the melanophores and hypophyseal hormones failed to elicit any response to melanophores, Ando (1960) concluded that the colour change in *Oryzias latipes* is mainly controlled by nerves and the hypophyseal hormone has little effect, similar to what was reported for *Fundulus heteroclitus* by Kleinholz (1935). It was unlike *Anguilla* on one hand where colour changes are reported to be mainly controlled by hypophyseal hormone (Neill, 1940), and Ameiurus on the other where both nerves & hypophyseal hormones were implicated to have roles in the colour changes.

The control of colour changes on the baiss of time course of background responses in the fish, *Balantocheilus melanopterus* appears to be regulated both by nerves and the hypophyseal hormone (possibly MCH) but it does not match to that of *Ameiurus.*

The initial rapid colour changes taking place during the rate of paling or the darkening clearly shows the involvement of neural control in colour change mechanism of the fish. The comparatively slower changes in the later stages of adaptation could be interpreted as due to release of pigment-aggregating hormone (the MCH) from the pituitary gland of the fish. There is certainly a strong correlation between rapid chromatic responses and neural control (Fujii and Oshima, 1986). It was earlier clearly established that in many fishes the rapid pigment-aggregating response of integumental chromatophores are primarily controlled by sympathetic post ganglionic fibres (Iwata and Fukuda, 1973; Fujii and Oshima, 1986).

Bhargava *et al.,* (1993) while working on *Trichogaster fasciatus* reported on the basis of time relations with reference to two contrasting backgrounds that colour change mechanism is controlled predominantly, if not exclusively, by the nervous system.

In the fish *Balantiochelios melanopterus* under the present study, the nerves innervating the melanophores are adrenergic pigment aggregating fibres and the aggregation is mediated through α-adrenoceptors, since yohimbine an α_2–antagonist and prazosin an α–antagonist antagonised effectively the melanosome aggregation effect of K^+- rich saline and epinephrine.

The melanosome aggregation resulting from the action of epinephrine is certainly one of the most remarkable phenomenon in the chromatic physiology of teleost fishes. This drug consistently induces pigment aggregation. Aggregation of melanophores as they behave during adaptation of the animal to a white background has long been considered to be associated with the release of an epinephrine like substance. von Frisch's finding of pigmento-motor fibres and their course in *Phoxinus* are generally accepted for various other teleost species. The action of epinephrine has always been stated to be that of causing aggregation of melanophores, whether injected to the living animal (*in vivo*) or applied to the isolated scales (*in vitro*). Epinephrine is known to act on the nerve ending as well as on the melanophores themselves directly (Fujii, 1969). Since action is peripheral and thus this brings the problem within the range of nervous control of colour change.

In the present study, concentration dependent response of epinephrine was recorded. The concentration 5 x 10^{-8} M was found to be the minimal effective concentration to arouse a discernible melanosome aggregation within melanophores. Complete melanophore aggregation (M I=1) was however recorded at 5x 10^{-6} M. Recovery to the dispersion of melanosomes, here takes approximately 70 minutes after the treatment of epinephrine 5 x 10^{-6}M .

Norepinephrine, which is also known to influence α_1 and α_2-adrenoceptors, induces a discernible aggregation at 10^{-8} M and full aggregation at 10^{-7} M, thereby showing that it is more potent than epinephrine in aggregating the pigment.

In the present study, potassium depolarisation of the melanophores induced a rapid aggregation of melanosomes (M.1=1.48). Further the effect was completely inhibited by yohimbine and prazosin, the adrenergic antagonists, which clearly indicates that K^+ acts by liberating the adrenergic transmitter from the chromatic nerve terminals. Such antagonising effect of yohmbine, an α_2–adrenoceptor blocking agent was also observed, in a variety of teleosts (*Phoxinus phoxinus*, Healey and Ross, 1966; *Nandus nandus*, Jain 1976; *Labrus ossifagus*, Andersson *et al.*, 1984, *Channa punctatus*, Shrotriya, 1989; *Puntius conchonius*, Khare, 1990; *Labeo rohita*, Jain and Patil, 1992; Bhargava *et al*., 1993).

It has earlier been demonstrated that the prazosin, an α_1–adreceptor blocking agent have failed to elicit any response (darkening) in the white adapted teleosts fish placed over a white background (Bhargava *et al.*, 1993).

On the contrary in the present study prazosin shows a remarkable effect. It effectively antagonized the melanosome aggregation induced by Epinephrine. This clearly points the involvement of α_1-adenoreceptors in the aggregation of the melanophores.

In the present study the specific α_2–agonist, clonidine was observed to induce concentration dependent aggregation of melanosomes within the melanophores (paling). The action of drug was inhibited by yohimbine the α_2 antagonist as well. Thus it is likely that both α_1 and α_2 adenoreceptors might be present on the melanophores of *Balantochelios melanopterus* as was reported for Labeo *rohita* (Jain and Patil, 1992) on the basis of both *in vivo* and *in vitro* experiments. The findings are in accordance with some other earlier studies as well (Andersson *et al*., 1984; Karlsson *et al*., 1987, Moroshita, 1987; Katayama *et al.*, 1999).

In the present study it has been found that the atropine accelerates the rate of melanosome dispersion within fish melanophores in comparison to physiological saline induced recovery from the effect of epinephrine. This is in accordance with the earlier findings (Fujii, 1960; Nagaishi *et al.*, 1992). The effect a direct one on the melanophores might have caused an enhancement of the activity of adenylate cyclase resulting in the increase of intracellular cAmp (Nagaishi, *et al.*, 1992).

The administration of nonselective, β- adrenergic blocking agent propranolol, failed to elicit any response in white–adapted fish, thereby indicating the lack of any role of the β–adenoreptors in the dispersion of melanosomes within the melanophores of the fish.

Adenosine and adenine nucleotides were very potent in dispersing the pigment in catfish and guppy melanophores (Miyashita *et al.*, 1980). Methylxanthenes as phosphodiesterase inhibitor are known to antagonize the action of adenine nucleotides and adenosine. It has been demonstrated that they interfere with adenosine receptors specifically (Sattin and Rall, 1970; Fain and Malbon, 1979). In our study involving adenosine and caffeine, it seems to be evident that the pigment dispersing action of adenosine may have mediated through the activation of specific A_1–receptors. This was further supported by the antagonizing action of the caffeine where adenosine failed to elicit its response. Our present study is in accordance with the earlier findings (Miyashita *et al.*, 1984).

It has repeatedly been asserted that in many lower vertebrates including teleosts, colour changes are under dual pituitary hormonal control (Pickford and Artz, 1957; Baker and Ball, 1975). Nagai *et al*., (1986) studied the action of melanin concentrating hormone (MCH) on melanophores from 27 teleost species. The hormone induced melanosome aggregation in all the teleosts studied, including two siluroid catfishes in which melanin-aggregating nerves are known to be cholinergic instead of usual adrenergic.

Further, the mode of action of the peptide has been found to be identical in either adrenergicially or cholinergically innervated melanophores (Nagai *et al*., 1986). Their results suggest that MCH may be a biologically active hormone common to teleosts. The other reports that also implicate MCH as a systemic hormone related to colour change in a variety of teleosts includes those of Kent (1959), Baker and Ball (1975), Rance and Baker (1979), Baker and Rance (1983), Kawauchi *et al*., (1983) Gilham and Baker (1984), Wilkes *et al*., (1984), Baker *et al.,* (1985), Oshima *et al*., (1985, 1986), Barber *et al*., (1987), Castrucci *et al*., (1988), Hadley *et al*., (1988), Lebl (1980) and Jain and Patil (1990).

Our present study also agrees with the above as a single intraperitoneal injection at a dose of 100 ng/g of the body weight turned the body colour of a black adapted silver shark to inducing a considerable paling within first 5 minutes after the injection.

Thus, it seems that when fish is kept in a white background, MCH may be released into the circulation to induce the melanin aggregation, thereby reducing the pressure on the firing rate of neurons innervating the melanophores. When they are transferred to black background, the hormone (MCH) release may be suppressed more rapidly than hormone production as pointed out by Barber *et al*., (1987) and the same results in accumulation of MCH in nerve terminals in the pituitary and also in the hyphothalamic fibres.

Pigment aggregation induced by MCH has been linked with a decrease in cAMP level via inactivation of adenylate cyclase by Gi protein. (Oshima and Wannitikul, 1996). Oshima *et al.,* (2001), demonstrated that intracellular concentration of cAMP in *Corydoras, Oreochronmis* and *Oryzias* melanophores was decreased due to the treatment of MCH. Further, the effect was found to be concentration dependent. MCH at higher concentration causes increase in the cAMP level significantly thus suggesting that re-dispersion of pigment granules is caused by an increase in cAMP content and that β–MCH receptors have a role in mediating pigment dispersion that may be coupled with Gs protein (Oshima *et al.,* 2001).

CONCLUSIONS

The present overall finding seem to conclude that colour changes in the fish, *Balantiocheilos melanopterus* are dependent on the 3 types of chromatophores possessed by the fish on the skin attached to their scales on the body. Whether the scale packets or deep dermis also contains the melanophores has not been investigated in this study. The species of chromatophores found include the dominant of the pigment cell types *i.e.,* the melanophores, the xanthophores and the iridophores, on examining the isolated scale preparations under light transmission microscopy the. The time course taken by the fish to adapt the either a light (pale) or a dark (black) background do suggest a neural regulation of colour change which initiates the rapid background response with support of hormonal regulation at later slow and gradual response to complete the near adaptation in about 7 hrs. The melanophores appear to have a direct innervation by sympathetic pigment-aggregating (chromatic) nerve fibres. The pharmacological nature of these nerves has been identified to be an orthodox one *i.e.,* they are adrenergic in character. Further, the neural response appears to be mediated by α_{2-} adrenorecptors, dominantly possessed by the melanophores of these species. α_1 & β_2 -adrenoceptors are the other species of receptors that are likely to be present on the melanophores, though more experiments are required before a claim like this can be made in the present on the melanophores, in the present material. The results obtained where adenosine is able to enhance the rate of dispersion, probably by its own specific receptors and where caffeine is able to back such a response of adenosine is in favour of the mediation of melanophore dispersion by the cAMP pathway as a signal transduction mechanism operative in the fish. The activation of α_2–adreneptor is also likely to use this pathway for neural control of colour change in the fish. The hypothesis and the actual release of co-transmitter ATP along with the principal transmitter (NE) released from the adrenergic nerve terminals, as demonstrated by Kumazawa *et al.,* (1984) appears to be consisted with these studies on melanophore regulation in this teleost, the *Bantiocheilos melanopterus.*

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