Towards regressive evolution: the periodic colour change behaviour of a troglophilic fish *Nemacheilus evezardi* (Day)

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SUMMARY

Present study is an attempt to know the existence of colour change physiology of the cave fish *Nemacheilus evezardi* (Day) along a circadian time scale. Though, due to subterranean mode of life, practically this function has no survival value. The study has been conducted simultaneously in two different photoperiodic conditions (LD 00: 24 and LD 12: 12 hr). The variation in different states of chromatophore have been computed with respect to different time points of the day. Results suggest that the phenomenon is lying completely disturbed in its *in situ* conditions. Interestingly, circadian rhythm in all the types of chromatophores were validated when the same fish was exposed under LD 12: 12 hr photoperiod.

INTRODUCTION

Cave environments are usually characterized by several relatively stable physical, chemical and biological parameters which directly impose their own effects on the cavernicoles. However, perpetual darkness alone is such a characteristic for any subterranean cave, that needs several adjustments to exist a successful life in it. Some of them have lower metabolic rates due to food scarcity (Poulson, 1964; Barr, 1968; Culver, 1982; Huppopp, 1985; Biswas, 1991), and show development of various types of extra sensory organs due to non-operation of visual system in the dark (Greenwood, 1967), including almost no temporal adjustments due to the lack of light/dark cycles (Erckens and Martin, 1979; Pradhan et al., 1989; Biswas, 1990, 1993; Biswas et al., 1990a, b, c) etc. Besides it, other light dependent physiological functions, present in some groups of organisms are very interesting to study.

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Chromatophore dependent colour change, is a physiological function according to which, in nature, some organisms e.g., fish, frog and lizard are capable of changing their body colour with reference to its background colouration and/or the intensity of sunlight. In teleost, chromatophore dependent colour change, is a well established phenomenon, due to which the fish becomes pale or dark by bringing about the movement of melanosomes (melanin pigments) to the centre of chromatophore sac; aggregation of melanophores and palling the animal or by dispersion of the pigments in the extended branches of the sac to darken it. Several stimuli are responsible for operating this physiology via neural and/or hormonal pathway (Bagnara and Hadley, 1973; Kavaliers et al., 1980; Reed, 1988; Young, 1935; Owens et al., 1978; Rance and Baker, 1979; Hafeez, 1970; Enam, 1955; Abbott, 1970; Abbott and Favreau, 1971). Nevertheless, light (stimuli) is the main and major factor to act behind this physiology either directly or through the background response on which the animal is placed. Further, skin colour changes can also be seen to be completed in few minutes due to some excitement or due to sudden changes in ambient temperature. However, in teleosts, the chromatophore physiology is either entirely under nervous (Abbott, 1970; Abbott and Favreau, 1971; Schwasmann, 1977) or entirely under hormonal control (Neill, 1940) or through an interplay of both the agencies in various proportions (Healey, 1940). According to Kavaliers and Abbott (1977) the two systems usually act synergistically, but under any required circumstances one may act independently.

Nemacheilus evezardi (Day) Cobittidae: Pisces, is a hill stream loach, abundantly found in the river and cave of Kanger Valley National Park. From a pilot study, it has been found that the epigeal form of this fish darkens very quickly with little excitement and it is not possible to study the colour change phenomenon of the same without any sophisticated method. However, in the present study a troglomorphic counterpart of the same fish *Nemacheilus evezardi* (Day) has been taken as an experimental model. It is a tiny loach with very faint pigmentation and regressed vision. Several comparative reports on morphological, behavioural and physiological parameters from its nearest epigeal counterpart *Nemacheilus evezardi* (Day) have placed it in a cavernicolous status (Biswas, 1990, 1993; Biswas et al., 1990a, b, c; Biswas and Pati, 1991). Here, an attempt has been made to study the chromatophore dependent colour

change phenomenon of this same dark adapted fish with respect to the circadian time scale. Attempt has also been made to know the influence of 12: 12 hr. LD (light/dark) photoperiod on synchronizing the rhythmic pattern of this phenomenon.

MATERIALS AND METHODS

Cave fish, *Nemacheilus evezardi* (Day) were collected from subterranean cave (Kotumsar), situated at the bank of river 'Kanger' in Kanger Valley National Park, India (Lat.: 18°52'09" N; Long.: 81°56'05" E). It is more than 100 meters downwards from the land surface and remains completely dark all along with constant temperature $26 \pm 1^\circ\text{C}$. Inside the cave, flooding occurs during the monsoon, otherwise the water ditches of this cave are fed by seepage water throughout the year. Fish from such water ditches were transported to our laboratory at Raipur (Lat.: 21°14' N, Long.: 81°38' E) in a light proof container and maintained in a stock aquaria 75×30×30 cm, inside a completely dark double doored room for more than 3 months as an acclimation period. Before starting the study, total twenty four fishes of approximately same body weight (580 ± 5 mg) and snout to vent length (4.00 ± 0.5 cm) were randomly removed from the stock aquarium to a separate jar and at each of the following time points viz: 0900; 1300; 1700; 2100; 0100 and 0500 hr four fishes were killed by decapitation. The process for catching, killing and fixing the skin for study was completed in 30 seconds time period. Small pieces of skin from five different places of the dorsal portion of each fish were peeled and glycerine mounts were prepared and sealed with the help of nail polish. Chromatophore states (Hogben, 1936) per 0.5 mm^2 from the most gathered area of each slide were examined and numerically scored regarding the state of the chromatophores, such as punctate (state-I), punctostellate (state-II), stellate (state-III), reticulostellate (state-IV) and reticulate (state-V). These states are indeed the structure forms which appear during the dispersion/contraction of melanin pigments in the chromatophore sacs. Averages of such chromatophore states for each fish were calculated and final averages for each group of fish at different time points were tabulated. The spread of variance of data was expressed as standard error for each group.

In the IInd set of this experiment twenty four fishes were again

chosen randomly from the stock aquarium and were transferred to another similar aquarium, exposed to LD photoperiodic schedule with 12 hours of light alternating with 12 hours of darkness (light switched off at 2000 hr and on at 0800 hr) continuously for 21 days. On 22nd day, that is, after 21 days of such acclimatization, fishes were killed and skins peeled and processed for determining the number of chromatophore types. The methods and timing of sampling were essentially the same as described earlier (set-I).

Statistical Methods: Means ($\pm 1SE$) for each chromatophore type were computed and tabulated as a function of circadian time scale. Data were subjected to one way analysis of variance (Brunner and Kintz, 1977) to prove the time effect. Between groups, comparisons were made with the help of Duncan's multiple range test. Cosinor rhythmometry was employed to determine rhythm parameters (Nelson et al., 1979). In addition, the cosinor parameters (details in Table 1) were also compared between the two treatments (LD 00:24 and LD 12:12) for each state of chromatophore separately.

RESULTS

Results are summarized in Table 1 & 2, Figure 1.

Under this section the chromatophore states of the cave fish, per 0.5 mm² skin area, has been described numerically both under continuous darkness schedule (LD 00:24) and after exposing the fish to a photoperiod (LD 12:12) consisting of 12 hours light alternating with 12 hours of darkness.

- 1) ANOVA followed by Duncan's multiple range test.
 - a. LD — 00:24 photoperiod — Results of ANOVA didn't reveal statistically significant time effects on numbers of chromatophore for any observed state (State I - V). Nevertheless, the two different time points where the chromatophore states were observed in its peak and nadir were seen to be differed only in first three states (I-III) at 5% level (from DMR tests).
 - b. LD — 12:12 photoperiod — Statistically significant time effects on the number of chromatophores occurrence were validated

Fig. 1 - Circadian acrophase charts for five different states of chromatophore studied under complete darkness (hatched area) and LD 12:12 hr photoperiod (Light and dark area). Dark dots represent the acrophase time points, vertical bars around the dots define 95% CL, where the rhythm detected was found to be statistically significant ($N = 4 \times 6 = 24$).

only on reticulostellate and reticulate states. However, excluding the chromatophore state-III (stellate) DMR tests revealed a significant difference between the time points when the number of chromatophores were found to be at its highest and lowest concentration.

2) Cosinor analysis

- a. LD — 00:24 photoperiod — Statistically significant circadian rhythm was only valid in the occurrence of state-I chromatophore (punctate), with an acrophase timing of -102° (0648 hr) (95% CL - 64° & -140°).
- b. LD — 12:12 photoperiod — Excluding for the state III chromatophores, a highly significant circadian rhythm was observed in the other states: state I ($\phi = -101^\circ$; 95% CL = -60° & -140°), state II ($\phi = -79^\circ$; 95% CL = -42° & -116°), state IV ($\phi = -264^\circ$; 95% CL - 235° & -293°) and state V ($\phi = -262^\circ$; 5% CL = -237° & -287°).

Table 1 — Circadian variations in the number of chromatophore types (Mean $\pm$ 1SE) of cave fish *N. evezardi* maintained under continuous darkness (LD 00:24) and LD 12:12 photoperiod.

State of chromatophore	Light schedule	Time of day					
		09.00	13.00	17.00	21.00	01.00	05.00
I	LD 0:24	18.66 $\pm$ 1.87	09.66 $\pm$ 2.96 ^a	08.80 $\pm$ 4.10 ^a	11.50 $\pm$ 1.60	09.70 $\pm$ 1.93 ^a	17.16 $\pm$ 2.04
	LD 12:12	15.28 $\pm$ 1.77	10.54 $\pm$ 1.73	11.02 $\pm$ 0.96	10.06 $\pm$ 0.73	12.57 $\pm$ 2.51	13.95 $\pm$ 1.85
II	LD 0:24	08.13 $\pm$ 1.49 ^c	09.56 $\pm$ 2.31	15.00 $\pm$ 2.08	06.03 $\pm$ 1.33 ^c	11.00 $\pm$ 1.97	09.33 $\pm$ 0.88
	LD 12:12	11.87 $\pm$ 1.16	09.30 $\pm$ 0.88	07.23 $\pm$ 0.97 ^a	09.86 $\pm$ 1.44	10.83 $\pm$ 1.06	11.32 $\pm$ 0.34
III	LD 0:24	11.43 $\pm$ 1.88	10.06 $\pm$ 2.83	08.33 $\pm$ 1.45 ^d	13.00 $\pm$ 2.08	10.18 $\pm$ 1.40	05.60 $\pm$ 1.40 ^d
	LD 12:12	06.88 $\pm$ 0.31	07.27 $\pm$ 0.49	08.33 $\pm$ 0.66	07.96 $\pm$ 1.42	07.71 $\pm$ 1.26	07.57 $\pm$ 1.63
IV	LD 0:24	03.33 $\pm$ 0.88	05.50 $\pm$ 0.28	04.83 $\pm$ 1.36	05.66 $\pm$ 1.85	05.33 $\pm$ 0.88	03.40 $\pm$ 0.87
	LD 12:12	04.00 $\pm$ 0.41 ^c	06.80 $\pm$ 1.22	07.80 $\pm$ 0.53	06.42 $\pm$ 1.47	05.45 $\pm$ 0.44	04.38 $\pm$ 0.43 ^c
V	LD 0:24	01.86 $\pm$ 1.16	03.00 $\pm$ 1.52	02.33 $\pm$ 1.33	02.16 $\pm$ 0.60	03.06 $\pm$ 1.09	02.83 $\pm$ 0.92
	LD 12:12	02.14 $\pm$ 0.29 ^{b c}	03.67 $\pm$ 0.58	04.25 $\pm$ 0.40	03.32 $\pm$ 0.63	2.94 $\pm$ 0.11 ^c	02.17 $\pm$ 0.37 ^{b c}

n=4

^a Differs from mean value obtained at 09.00 h : P < 0.05

^b Differs from mean value obtained at 13.00 h : P < 0.05

^c Differs from mean value obtained at 17.00 h : P < 0.05

^d Differs from mean value obtained at 23.00 h : P < 0.05

Results from DMR Tests.

Table 2 — Rhythmometry summary based on Least-Square fitting of 24 hr Cosine Function (24 hr=360°) to data illustrated in table 1.

Chro- matophore States	Light Schedule	Pr ^a	p ^b	Mesor ^c +1SE ^d	Am- plitude ^e	Acrophase ^f (Ø) in degree 360°=24 hr	in hours
I Punctate	LD 00:24	36	<0.033	12.58+1.08	4.49	-102	0648
	LD 12:12	27	<0.036	12.24+0.65	2.57	-101	0645
II Punctostellate	LD 00:24	06	<0.616	09.84+0.93	1.31	-247	1628
	LD 12:12	31	<0.020	10.07+0.45	1.96	-079	0516
III Stellate	LD 00:24	06	<0.623	09.71+0.89	1.25	-278	1832
	LD 12:12	04	<0.622	07.62+0.17	0.57	-305	2020
IV Reticulostellate	LD 00:24	08	<0.501	04.68+0.46	0.78	-298	1952
	LD 12:12	40	<0.004	05.81+0.34	1.81	-264	1736
V Reticulate	LD 00:24	01	<0.919	02.54+0.05	0.25	-056	0344
	LD 12:12	45	<0.001	03.08+0.17	1.02	-262	1728

^a Percent of total variability contribution by the fitted curve (PR).

^b From a 'F' test of null amplitude rejection hypothesis (P).

^c Rhythm adjusted mean of cosine function (Mesor).

^d Standard error.

^e Half of the total predictable change (Amplitude).

^f Time of maximum in fitted cosine function, with local mid-night as reference (Acrophase).

Comparison of rhythm parameters: for punctate chromatophores neither circadian mesor nor amplitude and/or acrophase differed from each other when rhythm parameters of LD 00:24 and LD 12:12 were compared. However, circadian mesor values of only stellate and reticulate states of chromatophores were found to be different between two light treatments (LD 00:24 and LD 12:12 hr) at 5% level. Rest amplitude and/or acrophase could not be compared due to non-rejection of zero amplitude hypothesis under either 00:24 schedule or both (state-III).

DISCUSSION

It is well known that a given species adapted to dissimilar ecosystem behaves differently and exhibits dissimilar physiological activities. The degree of such divergences is directly dependent on the level of difference(s) between/among the ecosystem. And such differences often develop a completely new organism during the course of evolution. As stated earlier, subterranean mode of life requires several adjustments for existence and due to which various examples of regressive/constructive evolution could be observed in those organisms. In the present study, the chromatophore dependent colour change phenomenon is found to be functional with respect to the given photoperiods (12:12 hr) in troglophilic *Nemacheilus evezardi* (Day). Although in *in situ* conditions of this fish, all the five states of chromatophores have been observed, but due to the lack of light this function seems not at all dependent on melatonin (pineal hormone), serotonin, dopamine, or any other photo related hormones as it could be in the epigeal ones (Bagnara and Hadley, 1973). Perhaps, some other hormonal/neural mechanism(s) are operating in this fish in DD (completely dark) conditions. However, in the present situation no explanation can be given in this regard.

Further, following exposure of the fishes to LD 12:12 photoperiods, it can be seen that in all the states of chromatophores a systematic pace with respect to the time could be observed barring for the stellate type. A maximum dispersion of pigments was noticed during later half of the light phase of LD 12:12 photoperiod. Infact, a gradual change in the chromatophores dispersion states from I to V (punctate to reticulate) with respect to the circadian time scale under 12:12 hr photoperiod could be easily detectable. According to the cosinor analysis this relationship finds corroboration in the

detection of acrophases of reticulostellate (state IV) and reticulate (state V) chromatophores, respectively at -264° (1706 hr) and -262° (1747 hr), and likewise detection of acrophase of punctate (state I) and punctostellate (state V) type chromatophores, respectively at -101° (0673 hr) and -79° (0527 hr).

The importance of time relation in the study of chromatophore dependent colour change phenomenon in teleost has long been recognized (Hogben, 1924). Though the late studies in number of teleost in this regard evidenced that the required duration for complete physiological change from state I to state V or vice versa varies from few seconds to several days (refer Parker, 1943). Maximum pigment concentration was noticed during the dark span of the LD 12:12 hr photoperiod. Thus the phenomenon of dark-time pallor could be stated to be still functioning, though it is operating in a very slow fashion, unlike to its epigeal one. However, pigment development, including the colour change phenomenon is a very long process, exhibited by a wide norm of reactions. On the other hand, energy economy is known to be the most important physiological factor for any cavernicole. In such circumstances, the divergence in chromatophore dependent colour change phenomenon between hypogeal and epigeal population of any species is quite obvious. Does it not show a process of regressive evolution?

Arrhythmicity and/or extinction of a rhythm in any constant conditions does not support/oppose the theory of endogenous circadian system (Aschoff, 1960; Bunning, 1973). Perhaps, the underlying oscillator(s) is in damping state, which could again be restored under the presence of proper 'zeitgeber' (synchronizing factor). Results of the present study clearly indicate that in natural continuous dark conditions, the chromatophore physiology of this cave fish is operating at the basal level. The appearance of state I chromatophore (punctate) in a statistically significant circadian fashion and statistically no difference between the other circadian parameters suggest it. In addition, the given LD 12:12 photoperiod also failed to show any statistically difference in the total numbers of punctostellate (state II) from its value, observed under complete dark conditions. Nevertheless, marked differences have been observed in the concentration of later states of chromatophore (state III to V) under the influence of 12:12 hr light/dark schedules.

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