CHAPTER

2

Time Dependent Effect of 5-HT in Relation to Prolactin Secretion and *in loco* Events During Tail Regeneration.

Previous studies have shown photoperiodic influence on regeneration with long photic schedules hastening and short photic schedules dampening tail elongation (Ramachandran and Ndukuba, 1989a; Ndukuba and Ramachandran, 1991a,b;). Prolactin induced linear growth of tail was purported to be under the dual control of serotonin (5-HT) and Dopamine (DA), both being sensitive to modulation by photoperiodic regimes (Ndukuba and Ramachandran, 1989a,b; Ramachandran and Ndukuba, 1989c). In this respect, there is enough evidence for the inhibitory influence of DA and stimulatory influence of 5-HT on PRL release in birds and mammals (Weiner and Ganong, 1978; El Halawani *et al.*, 1980, 1988; Rabii, 1981). There is evidence, for similar control mechanisms for PRL release in lower vertebrates as well (Ball, 1981; Hall and Chadwick, 1984).

Previously it was reported that long photic schedules and exogenous administration of melatonin in the evening both had positive influence on tail regeneration by increasing hypothalamic 5-HT turnover leading to greater PRL release (Ramachandran and Ndukuba, 1989c; Ramachandran and Ndukuba, 1993; Kurup *et al.*, 1995).

Similarly, short photic schedules and exogenous administration of melatonin in the morning have both been purported to retard tail regeneration by increased dopaminergic tone leading to attenuated PRL release (Ndukuba and Ramachandran,

1989b; Ramachandran and Ndukuba, 1993; Kurup *et al.*, 1995). In this context, the inhibitory influence of dopamine on PRL release has been proved by neuropharmacological studies involving bromocriptine (a dopamine agonist) and pimozide (a dopamine antagonist) during tail regeneration in *H. flaviviridis* (Ndukuba and Ramachandran, 1989b; Ramachandran and Ndukuba, 1991). However, the involvement of 5-HT has not been experimentally confirmed. The present study is an attempt to elucidate the 5-HT related PRL release in the control of tail regeneration under different photoperiodic conditions. To this end, cyproheptidine, a 5-HT_2 receptor antagonist was administered systemically, either in the morning or in the evening under normal light and dark schedule as well as under continuous light and continuous darkness.

Many previous studies on pre-nervous embryos of various animals have demonstrated the presence of neurotransmitters. Studies involving administration of neurotransmitters, especially 5-HT, and their receptor antagonists in pre-nervous embryos have established the importance of neurotransmitters in many aspects of early developmental events such as cell proliferation, maintenance of membrane potential and morphogenetic processes (Buznikov, 1981). This provided impetus to test the possible involvement of 5-HT locally during tail regeneration. For this, cyproheptidine was injected locally either in the morning or in the evening post-caudal autotomy.

MATERIAL AND METHODS

Adult *Hemidactylus flaviviridis* of both sexes weighing 10±2grms and measuring 80±5 mm snout to vent length were obtained from a local animal supplier and maintained on a diet of cockroaches *ad libitum* for 7 days prior to experimentation for acclimation to the laboratory conditions. For the experiment, 112 lizards were used and they were divided into 16 groups of 7 each as detailed below.

Experiment I

Groups I-XII (Systemic Administration Of Cyproheptidine)

Four groups were maintained in a normal light-dark schedule (LD 12:12; NLD), four groups were maintained in continuous light (LD 24:0; LL) and the remaining four groups were maintained in continuous darkness (LD 0:24; DD). Two groups of lizards from each schedule received once daily intraperitoneal injection of 0.1ml saline (0.6%) either in the morning at 09.00 hrs or in the evening at 17.00 hrs for 30 days starting from the day of autotomy. Remaining two groups from each schedule received once daily intraperitoneal injection of 10µg cyproheptidine lizard, in 0.1ml saline either in the morning or in the evening and served as the experimentals.

Experiment II

Groups XIII-XVI (Local Administration Of Cyproheptidine)

Two groups of lizards were injected 0.1ml of saline locally at the cut end of the tail either in the morning or in the evening for 30 days starting from the day of autotomy. The remaining two groups of lizards were injected once daily with cyproheptidine (10 μg/lizard in 0.1ml of saline) locally at the cut end of the tail either in the morning or in the evening for 30 days.

Preparation of cyproheptidine

Commercially available cyproheptidine, in the trade name of Ciplactin (Cipla, Bombay) was purchased and was dissolved in appropriate amount of 0.6% saline to get a final concentration of 100μg/ml.

Experimental Protocol

Appropriate groups of animals were exposed to continuous light (LD 24:0) of 1200 lux intensity or 12 hrs of light and 12 hrs of darkness (LD 12:12) or continuous darkness (LD 0:24) throughout the entire period of experimentation, starting five days prior to autotomy. The description of lighting conditions and the dimensions of the cages housing the animals have been well documented in earlier reports (Ndukuba and

Ramachandran, 1988; Ramachandran and Ndukuba, 1989a). The investigations under Experiment I were carried out in the month of March (late winter) and the average cage temperature was 28°C. Investigations under Experiment II were carried out in September-October and the average cage temperature was 31°C. Tail autotomy was performed by pinching off the tail at the 3rd segment from the vent. The length of tail autotomized varied between 60±2mm. The length of new growth (regenerate), in mm was measured daily with a meter rule and recorded at fixed intervals of 5, 10, 15, 20, 25 and 30 days after caudal autotomy. The recorded measurements were later used for morphometric calculations and, Student's t-test was used to determine the statistical significance.

RESULTS

Attainment of arbitrary stages : The time taken for the attainment of various arbitrary stages like wound healing (WH), preblastema (PB), blastema (B) and initiation of growth (IG) is represented in figure 1 and Table 1. Lizards in NLD and LL conditions formed a blastema by day 11 and growth was initiated by the 12th day. Lizards in DD showed a delay by one day. Treatment with cyproheptidine in the evening did not show any change in the number of days taken for formation of blastema or for initiation of growth in NLD and LL lizards.

Fig.1 Number of days taken to attain the arbitrary stages in control(C) and experimental lizards.

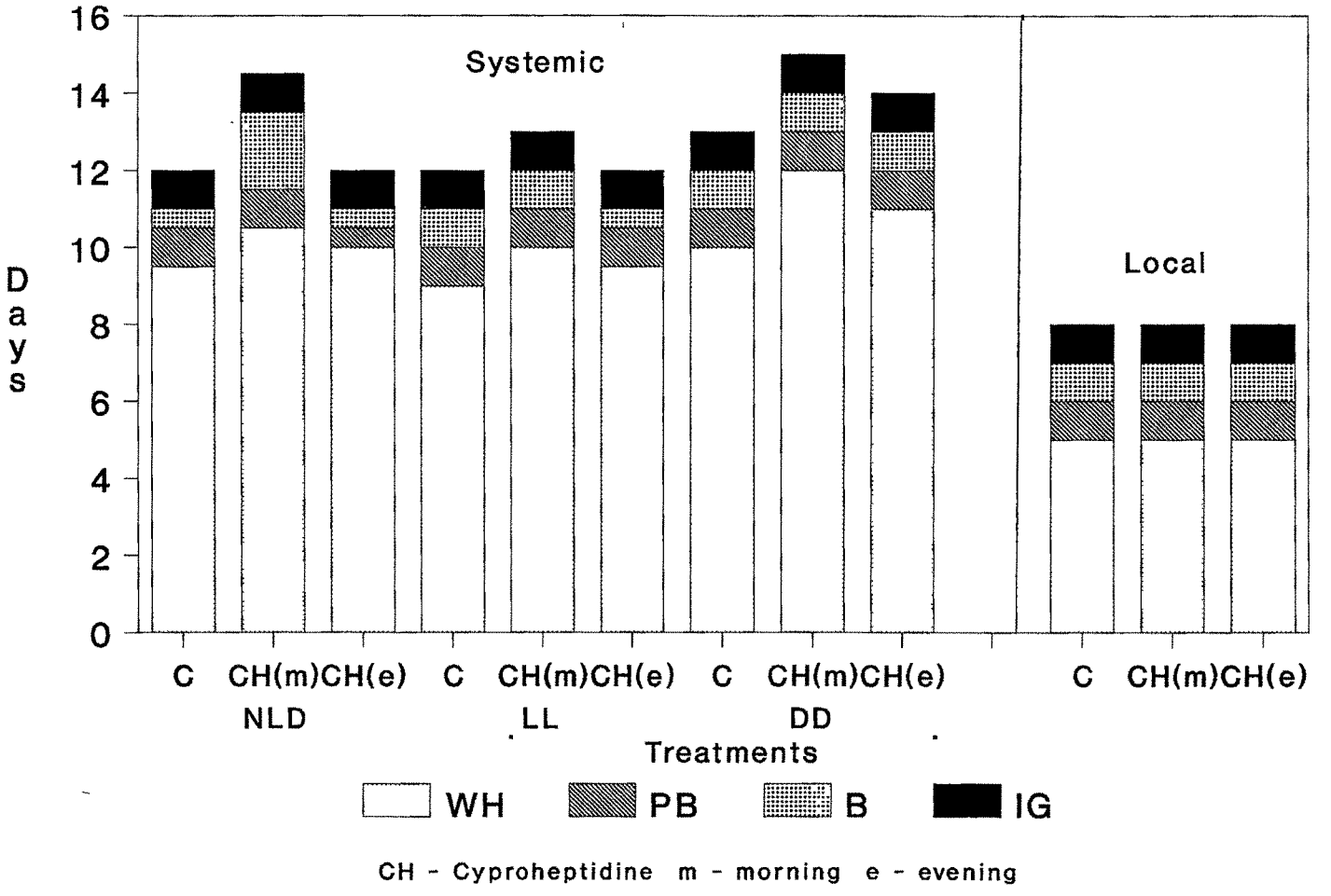


TABLE - 1

Showing the length of tail regenerated, and the total percentage replacement at the end of 30 days and the number of days taken to attain the various arbitrary stages in control and cyproheptidine (systemic) treated lizards under different light schedules.

Manipulation	Total length (mm)	Percentage replacement	No. of days taken to attain various arbitrary stages			
			WH	PB	B	IG
CONTROL NLD	20.66 ± 1.63	33.87 ± 3.51	9.5	10.5	11	12
Cyproheptidine (m) NLD	13.40[b] ± 1.12	21.96[b] ± 2.81	10.5	11.5	13.5	14.5
Cyproheptidine (e) NLD	13.80[b] ± 1.28	22.62[b] ± 1.78	10	10.5	11	12
CONTROL LL	25.33 ± 1.98	41.52 ± 3.63	9	10	11	12
Cyprohetidine (m) LL	17.20[c] ± 1.19	28.19[c] ± 2.42	10	11	12	13
Cyproheptidine (e) LL	22.60[b] ± 1.30	37.04[b] ± 2.94	9.5	10.5	11	12
CONTROL DD	17.20[c] ± 1.80	28.19 ± 2.16	10	11	12	13
Cyproheptidine (m) DD	7.80 ± 1.5	12.78 ± 1.80	12	13	14	15
Cyproheptidine (e) DD	14.00[b] ± 1.69	22.95[b] ± 2.61	11	12	13	14

NLD - LD 12:12; LL-LD 24:0; DD - LDO:24, m - morning; e - evening; WH - wound healing; PB - Preblastema; B - Blastema; IG - Initiation of growth. b - $P < 0.005$, c -$P < 0.001$.

Treatment with cyproheptidine in the morning induced a delay by one day in both the lighting schedules. Cyproheptidine injections in DD lizards induced a delay of one day in the evening and a delay of two days in the morning. Local administration of cyproheptidine did not influence the number of days taken for attainment of various arbitrary stages. (Table-4).

Growth rate, total length of tail regenerated and percentage replacement : (Fig. 2-4, tables 1-6)

Since the experiments involving the systemic administration of cyproheptidine was carried out in the month of March; the first half of the month having an ambient temperature range of 25-28°C, the initiation of growth in these groups of animals occurred later. Accordingly, expressible growth rate occurred only after 10-15 days post-autotomy.

The NLD lizards showed an initial growth rate of 0.8mm between 10-15 days and peaked to a growth rate of 1.4mm between 20-25 days and with a slightly reduced growth rate of 1.22 mm between 25-30 days. Treatment with cyproheptidine induced significant retardation in growth rate with both the morning and evening schedules, more pronounced in the former. The maximum growth rate in evening schedule, of 0.96mm, occurred between 20-25 days while, in the morning schedule the maximum growth rate of 0.88mm occurred only between 25-30 days. The LL lizards showed consistent significantly greater growth rates throughout. Treatment with

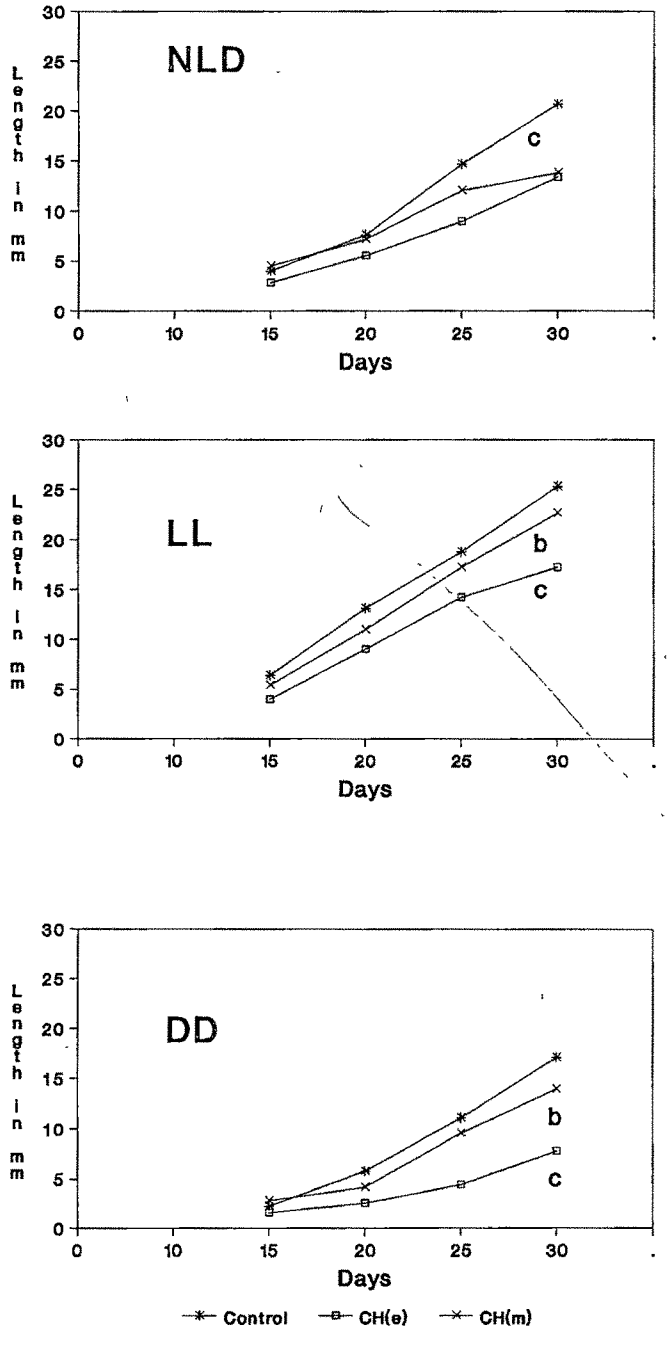
TABLE - 4

Showing the length of tail regenerated and the percentage replacement at the end of 30 days and the number of days taken to attain the various arbitrary stages in control and cyproheptidine (local) treated lizards, under LD 12 : 12.

Manipulation	Total length (mm)	Percentage replacement	No of days taken to attain various arbitrary stages			
			WH	PB	B	IG
CONTROL	24.57 ± 2.51	40.27 ± 2.85	5	6	7	8
Cyproheptidine (m)	25.50 ± 2.83	41.80 ± 3.56	5	6	7	8
Cyproheptidine (e)	15.20^{c} ± 1.11	25.00^{c} ± 2.12	5	6	7	8

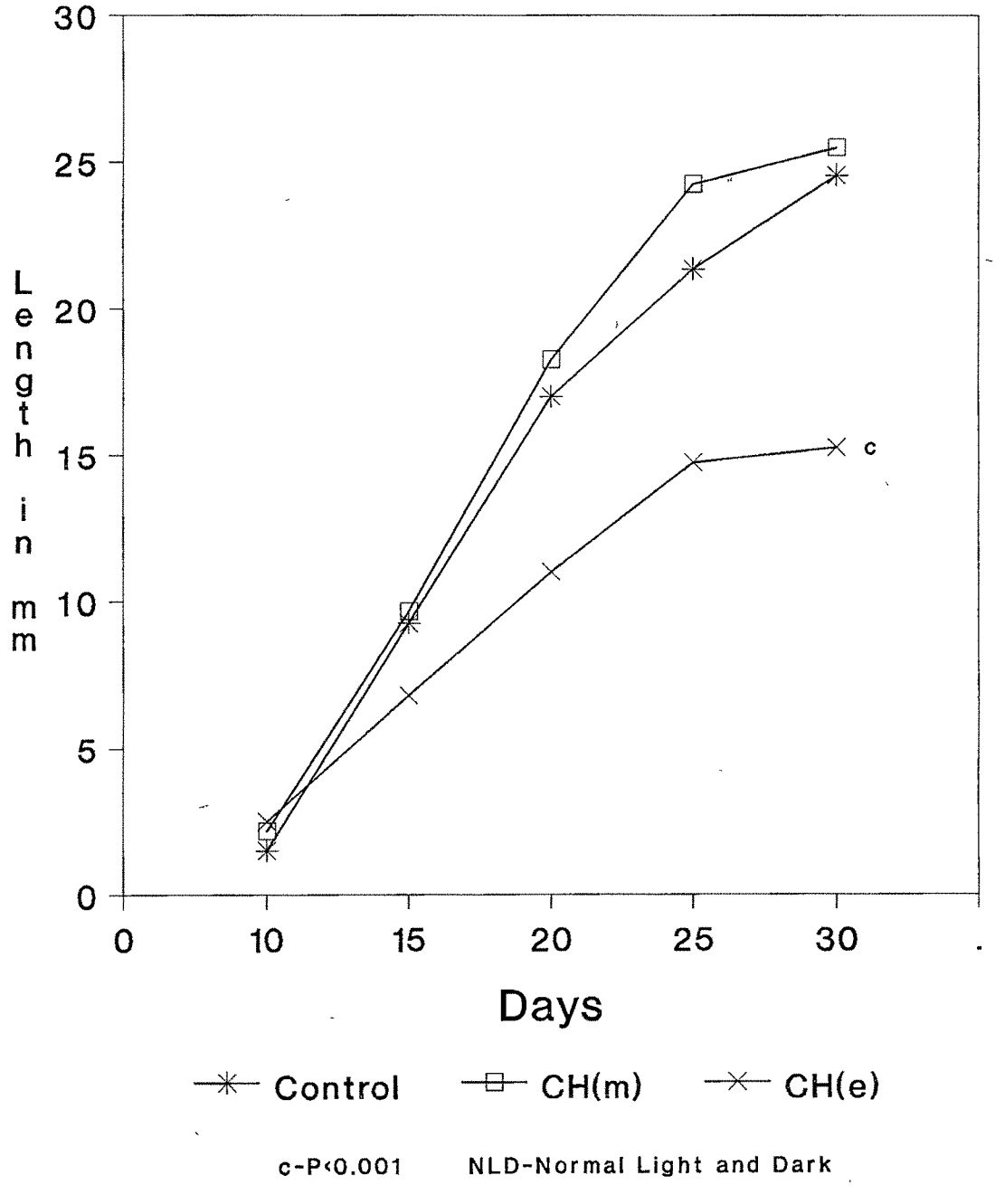
WH - wound healing; PB - Preblastema; B - Blastema; IG - Initiation of growth; m - morning; e - evening, c - $P < 0.001$.

Fig.2 Length of tail regenerated in control(C) and cyproheptidine(CH) treated lizards under NLD, LL and DD.



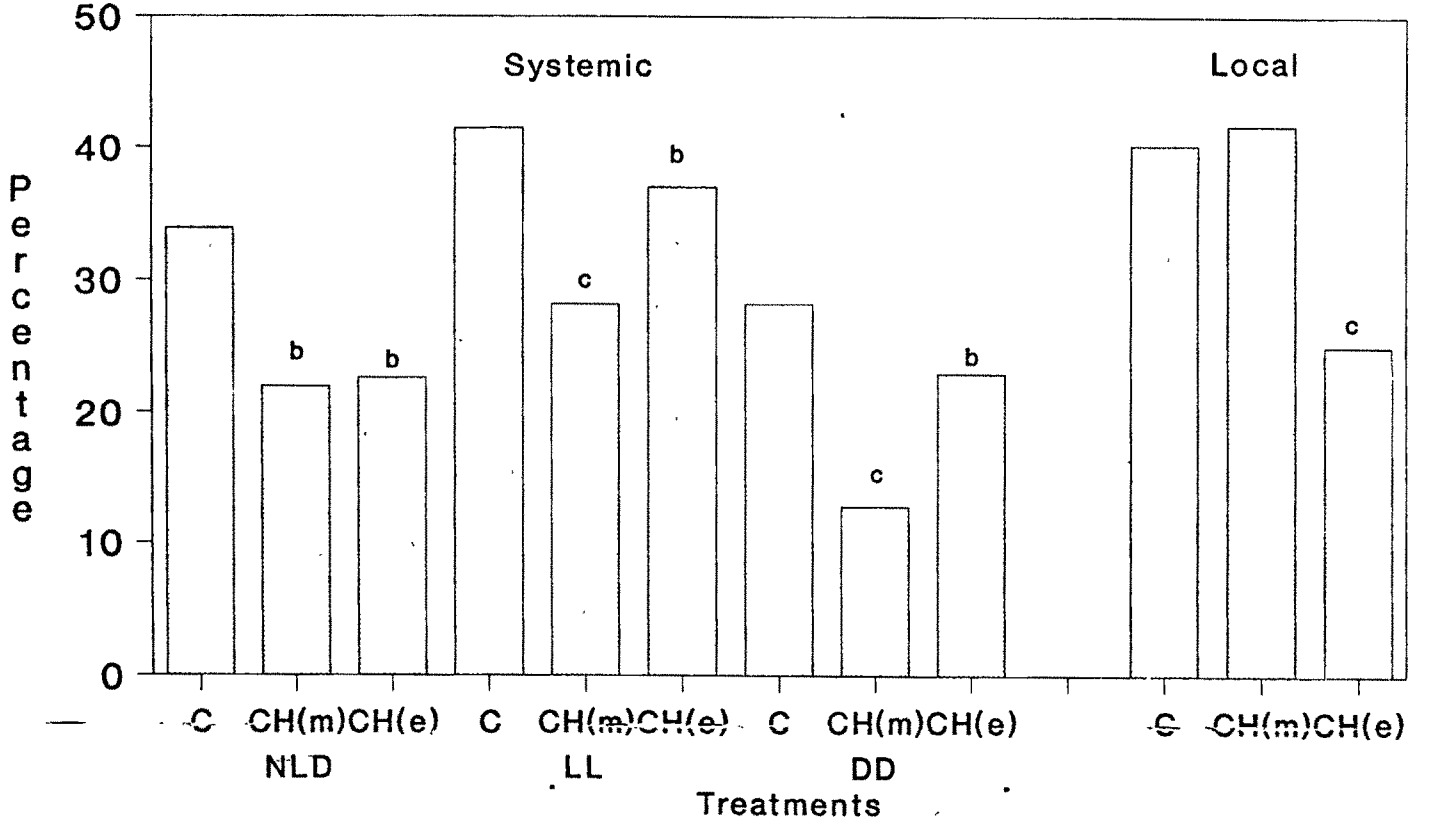
b-P<0.005, c-P<0.001 m-morning e-evening

Fig.3 Length of tail regenerated in control(C) and cyproheptidine(CH;local) treated lizards under NLD.



c-P<0.001 NLD-Normal Light and Dark

Fig.4 Percentage of tail replaced at the end of 30 days in control(C) and experimental lizards.



CH - Cyproheptidine m - morning e - evening

NLD - Normal light & dark, LL – Continuous light, DD - Continuous darkness.
b - $P < 0.005$, c - $P < 0.001$

TABLE - 2

Length of tail regenerated at different time period (days) in control and cyproheptidine (systemic) treated experimental groups under different photic schedules.

Manipulation	DAYS				
	10	15	20	25	30
CONTROL NLD	-	4.00 ± 0.84	7.60 ± 1.02	14.65 ± 1.85	20.65 ± 2.03
Cyproheptidine (m) NLD	-	2.85[b] ± 0.56	5.55[b] ± 0.63	8.95[b] ± 1.21	13.35[c] ± 1.34
Cyproheptidine (e) NLD	-	4.50 ± 0.32	7.20 ± 0.87	12.00[a] ± 1.08	13.80[c] ± 1.25
CONTROL LL	-	6.35 ± 1.00	13.10 ± 1.07	18.75 ± 1.23	25.25 ± 2.12
Cyproheptidine (m) LL	-	4.00[b] ± 0.24	9.00[b] ± 1.04	14.25[b] ± 1.42	17.20[c] ± 1.88
Cyproheptidine (e) LL	-	5.40[a] ± 0.76	11.00[a] ± 1.05	17.20[a] ± 1.86	22.60[a] ± 2.38
CONTROL DD	-	2.28 ± 0.12	5.78 ± 0.44	11.08 ± 1.03	17.13 ± 2.14
Cyproheptidine (m) DD	-	1.60[a] ± 0.04	2.60[b] ± 0.28	4.40[c] ± 0.84	7.80[c] ± 0.96
Cyproheptidine (e) DD	-	2.85 ± 0.38	4.15[a] ± 0.58	9.58[b] ± 1.02	13.98[b] ± 1.53

NLD - LD 12 : 12; LL - LD 24 ; 0; DD - LD 0 : 24; m - morning, e - evening, a - $P < 0.001$, b - $P < 0.005$, c - $P < 0.001$.

TABLE - 3

Showing the per day rate of growth (in mm) in blocks of 5 days during tail regeneration in control and cyproheptidine (systemic) treated lizards under different light schedules.

Manipulation	PER DAY RATE OF GROWTH				
	DAYS				
	5-10	10-15	15-20	20-25	25-30
CONTROL NLD	-	0.80	0.72	1.41	1.20
Cyproheptidine (m) NLD	-	0.57	0.54	0.68	0.88
Cyproheptidine (e) NLD	-	0.90	0.54	0.96	0.36
CONTROL LL	-	1.27	1.35	1.13	1.30
Cyproheptidine (m) LL	-	0.80	1.00	1.05	0.59
Cyproheptidine (e) LL	-	1.08	1.12	1.24	1.21
CONTROL DD	-	0.45	0.70	1.06	1.21
Cyproheptidine (m) DD	-	0.32	0.20	0.36	0.68
Cyproheptidine (e) DD	-	0.57	0.26	1.08	0.88

NLD - LD 12 : 12; LL - LD 24 : 0; DD - LD 0:24; m - morning, e - evening.

TABLE - 5

Length of tail regenerated at different time periods post-autotomy in control and cyproheptidine (local) treated lizards under LD 12 : 12.

Manipulation	DAYS				
	10	15	20	25	30
CONTROL	1.50 ± 0.13	9.25 ± 0.72	17.00 ± 0.98	21.35 ± 1.12	24.55 ± 2.26
Cyproheptidine (m)	2.16[a] ± 0.24	9.66 ± 0.93	18.25 ± 1.10	24.25[b] ± 1.36	25.50 ± 2.80
Cyproheptidine (e)	2.50[a] ± 0.30	6.80[b] ± 0.56	11.00[c] ± 0.75	14.75[c] ± 1.99	15.25[c] ± 1.88

m - morning, e - evening, a - $P < 0.01$, b - $P < 0.005$, c - $P < 0.001$.

TABLE - 6

Showing the per day rate of growth in mm in control and cyproheptidine (local) treated lizards under LD 12 : 12.

Manipulation	PER DAY RATE OF GROWTH				
	DAYS				
	5-10	15-20	15-20	20-25	25-30
CONTROL	0.30	1.55	1.55	0.87	0.64
Cyproheptidine (m)	0.43	1.50	1.71	1.20	0.25
Cyproheptidine (e)	0.50	0.86	0.84	0.75	0.10

m - morning ; e - evening

cyproheptidine affected less significantly lizards in the evening schedule as compared to those in the morning schedule. Lizards in DD showed a reduced initial growth rate which gradually increased to a peak of 1.2 mm between 25-30 days. Administration of cyproheptidine in the morning dampened the growth rate significantly and maximum growth rate recorded was 0.68mm between 25-30 days. The total length of tail regenerated at the end of 30 days in control lizards under NLD was 20.66 mm representing a percentage replacement of 33.87. Lizards receiving cyproheptidine either in the morning or in the evening showed a much reduced length of tail regenerate and a percentage replacement which was lesser by 33 to 35%. Control lizards under LL replaced relatively longer length of tail compared to NLD with a percentage replacement of 41.52. Though treatment with cyproheptidine in the evening did not show significant difference, treatment in the morning induced a 32% retardation. Control lizards under DD regenerated lesser length of tail at the end of 30 days representing a percentage replacement of only 28.19. Once again treatment with cyproheptidine in the evening did not induce that significant difference, while treatment in the morning brought about significant retardation of 54.6% (see figs. 2,3). Since the experiments involving local administration of cyproheptidine was carried out in the month of September, with an approximate average temperature of 31°C, the control lizards showed peak growth rates of 1.55mm between 10-20 days and thereafter there was a gradual decline. Administration of cyproheptidine in the

morning showed a similar growth rate, while administration in the evening showed reduced growth rate. The control lizards regenerated a much longer length of tail representing 40.27% replacement. Local administration of cyproheptidine in the morning did not have any influence while administration in the evening retarded tail regeneration by 38%. The total length of tail regenerated at end of the 30 days, the per day rate of growth in blocks of 5 days and, the total percentage replacement at the end of 30 days are depicted in Table 1-6, Figs 3 and 4.

DISCUSSION

The present experimental design utilizing a 5-HT_2 receptor antagonist (cyproheptidine) provides evidence for 5-HT mediated PRL release during tail regeneration in *Hemidactylus flaviviridis*. The results also suggest that blockage of 5-HT action can retard tail regeneration under normal diurnal LD cycle as well as under photoperiodic extremes. The results also indicate that apart from the central action of 5-HT mediating PRL release, it may also have a local influence in the regenerating tail prior to histo-differentiation and establishment of neural innervation. The potent role of 5-HT in neuroendocrine control of PRL release has been well established in mammals (Cowen *et al.*, 1990; Toivonen *et al.*, 1990; Jorgensen *et al.*, 1992a,b).

Previously it was shown that long photic schedules hasten tail regeneration and short photic schedules retard tail regeneration (Ramachandran and Ndukuba, 1989a; Ndukuba and Ramachandran 1991a,b). These effects of photoperiod were related to increased PRL release under long photic schedules and decreased PRL release under short photic schedules. Further, it was shown that exogenous PRL stimulated the regeneration process in *Hemidactylus* exposed to LD 0:24 but had no apparent effect in lizards exposed to LD 12:12 when injected during the photophase (Ndukuba and Ramachandran, 1989a). The above observations together with certain neuro-pharmacological evidence such as retardation of tail regeneration in lizards exposed to LD 12:12 but not in those exposed to LD 24:0 by bromocriptine administration and, the ability of pimozide to completely nullify the retardary influence of LD 0:24, have led to the proposal of a model involving both stimulatory serotonergic and inhibitory dopaminergic regulatory systems of PRL release operating at par under a LD 12:12 schedule (Ndukuba and Ramachandran, 1989b, Ndukuba and Ramachandran, unpublished).

The present study is intended to understand the involvement of serotonergic component in PRL release related to tail regeneration and to that end cyproheptidine a 5-HT_2 receptor antagonist has been administered to lizards exposed to LD 0:24, LD 12:12, and LD 24:0 schedules either in the morning or in the evening.

Interestingly, cyproheptidine caused an identical delay in regenerative tail elongation when administered in the morning or in the evening in lizards exposed to LD 12:12 regime, while a more significant delay was evident only when administered in the morning in lizards exposed to photoperiodic extremes. Enough evidences are available for 5-HT as a potent secretogogue of PRL acting through 5-HT_2 receptor (Di-Renzo, *et al.*, 1989; Gartside and Cowen, 1990; Jorgensen, *et al.*, 1992a,b;). In this context, the presently observed retardation in regeneration by the administration of cyproheptidine, a 5-HT_2 antagonist is self explanatory. Long photic schedules are known to increase PRL release essentially by increasing the hypothalamic 5-HT content (Ramachandran and Ndukuba, 1989c). These observations as well as the previous suggestion that in *H. flaviviridis*, the stimulatory serotonergic and inhibitory dopaminergic mechanisms of PRL release are in operation at par under normal light and dark cycle, tend to suggest a daily rhythm of 5-HT and DA turnover. This rhythm is marked by higher 5-HT content during the light phase and decreased content in the dark phase and conversely lower DA content during the light phase and higher content during the dark phase. Existence of such a circadian rhythm of 5-HT and DA in the hypothalamus and other brain centres has been well documented in mammals (Cohen and Wise, 1988; Pearce-Kelly *et al.*, 1992; Mai *et al.*, 1994; Rao *et al.*, 1994). Viewed in this context, the retardation in regeneration caused by morning injection of cyproheptidine is essentially due to the blockage of 5-HT induced diurnal PRL

release. However, the similar retarding influence caused by the evening injection of cyproheptidine may be hypothesized as due to dampened PRL release brought about by increased DA tone due to 5-HT blockage. Some support to this hypothesis is provided by the reported suggestion of Flores *et al.* (1992) that the *in vivo* secretion of PRL mediated by the 5-HT pathway may ultimately occur through the inhibition of DA release. Distinct from the dual effects under normal LD cycle, cyproheptidine under photoperiodic extremes produced a retardative influence only in the morning. The identical response produced by cyproheptidine in both continuous light and continuous dark tends to indicate the persistence of the daily rhythm of 5-HT content, *albeit* with greater amplitude in continuous light and with lesser amplitude in continuous darkness. Obviously, blockage of 5-HT action in the morning leads to reduced PRL release under both photoperiodic extremes and the consequent retardation in regeneration. The reduced response, by antagonizing 5-HT action in the evening can be due to the fact that under LD 24:0 the DA tone is very much suppressed (probably because of the higher 5-HT content) and as cited earlier the nocturnal modulation of PRL release may be essentially due to altered DA tone. This contention is strengthened by the report of Mistry and Voogt (1990) of inhibition of nocturnal PRL release in pregnant rats by 5-HT blockage. In this perspective, the lesser responsiveness of evening injection of cyproheptidine under LD 0:24 is due to the higher DA content and tone. The increased DA tone in continuous darkness is well

reflected in the attenuated regenerative response shown by lizards maintained in continuous darkness. Further retardation in regeneration occurs by the blockage of 5-HT in the subjective morning due to the persistence of 5-HT rythms, *albeit* with a lesser amplitude, as discussed earlier. Darkness induced increase in melatonin content and the consequent decrease in PRL release as inferred by previous works from this laboratory (Ndukuba and Ramachandran 1989a, 1991b; Ramachandran and Ndukuba, 1989a,b) are corroborated by the report of Massa and Blask (1989), that increased pituitary PRL mRNA levels in light-deprived male hamsters is mediated through the pineal. The pineal mediated decrease in PRL secretion could be related to a longer melatonin signal inducing increased DA content/tone. Justification for both increased DA content and tone is provided by the observations of Steger *et al.* (1995) of reduced PRL release and increased DA content in hamsters transferred from long photoperiod while there is also evidence for increased pituitary sensitivity to DA under short day lengths without an actual increase in the DA content (see Curlewis, 1992).

Presently, it is shown that cyproheptidine when injected locally could also retard tail regeneration, though this effect was specific for only evening administration. This would be quite distinct from its action when administered systemically and implicates 5-HT in the control in some local events integral to regenerative development. Relevant to this are the reported presence of neurotransmitters such as acetylcholine, catecholamines and serotonin in both

invertebrate and vertebrate pre-nervous embryos, including mammals (Buznikov, 1973; 1979). Based on experiments involving administration of serotonin as well as its antagonist to pre-nervous embryos, some functional involvement for this neurotransmitter in such events as degree of tubulin polymerization in microtubules, regulating the contractile activity of micro-filament and regulation of intracellular macromolecular transport have been indicated (see Buznikov, 1981). The presently observed retardation in regeneration caused due to 5-HT antagonism in the context of the above reports tend to suggest the involvement of 5-HT in developmental mechanics associated with regenerative growth. Pertinent to this connection are the reports showing involvement of serotonin in regeneration of cilia in *Tetrahymena thermophilia* and during regeneration in planaria (Castrodad *et al.*, 1988; Csaba, 1993). The observation of a time specific effect (in the evening but not in the morning) is a novel feature of the present study. This will suggest the possible existence of the circadian periodicity in various cellular and sub-cellular events during development and growth. Presumably, in the early part of the diurnal phase, various cellular events related to developmental process are in relative quienscence while they are quite active in the later part of the diurnal phase.

Overall, the present observations give evidence for the involvement of 5-HT in the control of PRL release during regeneration as well as its participation in local molecular events during regenerative growth.

SUMMARY

Previous studies have established the importance of prolactin (PRL) as a growth promoter during tail regeneration in the lizard, *Hemidactylus flaviviridis*. Probable involvement of both dopamine (DA) and serotonin (5-HT) in PRL release was also suspected. In the present study, cyproheptidine (CH), a 5-HT antagonist has been used under different experimental conditions to elucidate the role of 5-HT and its relation with PRL release during tail regeneration in *H. flaviviridis*. In one set of experiments, lizards exposed to different photoperiodic schedules have been injected with CH either in the morning or evening systemically. In another set of experiments, lizards exposed to LD 12:12 photoperiodic schedule were administered CH either in the morning or evening locally. Both morning and evening administrations systemically retarded tail regeneration, while retardation with *in loco* administration occurred only by the evening schedule under LD 12:12 regime. Cyproheptidine administered in the morning, in lizards exposed to photoperiodic extremes (LD 24:0, LL and LD 0:24, DD), retarded tail regeneration, while, retardation was less significant with the evening injections. These results are discussed in terms of the involvement and turn over of 5-HT and its receptor and, the modulatory influence of extremes of photoperiod and melatonin on the same, coupled to PRL release, as related to tail regeneration in *Hemidactylus flaviviridis*.