CHAPTER

5

Time Dependent Influence of Melatonin on Tail Regeneration is Mediated Through Modulation of PRL Release : Neuropharmacological Evidence

Circadian and seasonal rhythms in vertebrates are essentially regulated by a central neuroendocrine organization. The pineal gland modulates the functioning of this organisation by conveying information regarding changes in photoperiod through its cyclic secretion of melatonin (Reiter, 1981; Binkley, 1988). An important role is attributed to the pineal gland in the circadian organisation of lower vertebrates (Foa *et al.*, 1992). In addition to photoperiod, ambient temperature is also important in regulating various seasonal adaptations in poikilotherms (Lofts, 1975). In some of the reptiles studied, temperature has been shown to influence melatonin levels (Vivien-Roels and Arendt, 1981, 1983; Underwood, 1985a; Vivien-Roels, 1985). Further studies established that temperature can increase the amplitude of circulating melatonin level without altering the phase of secretion (Menaker and Wisner, 1983; Underwood, 1985a; Vivien-Roels, 1985; Vivien-Roels *et al.,* 1988; Tilden and Hutchinson, 1993). Obviously in reptiles, pineal is a photo-thermal transducer.

Tail regeneration in lizards is a process tractable to environmental cues like photoperiod and temperature. In this respect, past studies have shown pineal mediated photoperiodic modulation of tail regeneration in *H. flaviviridis* (Ndukuba and Ramachandran, 1988; Ramachandran and Ndukuba, 1989a). Previous study had shown the importance of both light and temperature and a dominant influence of the latter in relation to the former (Chapter 1). Based on the observations on photic and thermal influences, it was assumed that altered profiles of melatonin secretion and the

resultant effect on the neuroendocrine system could be responsible for the observed influence on regeneration (Chapter 1). This assumption was tested by evaluating the influence of exogenously administered melatonin at different times of the day on the course of tail regeneration (Chapter 4). The evaluations showed a proregenerative effect of evening administration of melatonin and an antiregenerative effect by morning, morning and noon, or noon and evening and, even morning, noon and evening administrations. These effects were related to the altered secretion of prolactin (PRL) which is already established as a promoter of regenerative growth (Ramachandran and Ndukuba, 1989c; Ndukuba and Ramachandran, 1989a).

The present study was undertaken to garner confirmative evidence for the hitherto inferred modulatory effect of melatonin on PRL secretion. To this end, neuropharmacological approach has been made by using bromocriptine, a dopamine (DA) agonist, pimozide, a dopamine antagonist and, cyproheptidine, a serotonin (5-HT) antagonist as, DA and 5-HT have been identified as modulators of PRL secretion.

MATERIAL AND METHODS

Procurement, maintenance and acclimation of animals are as mentioned in Chapters 1 & 2. A total of 120 lizards divided into 12 groups of 10 each were used for the experiments. The experiment consists of three set-ups.

Set-up I : *Morning melatonin (Mm) and Pimozide* -

This experiment was carried out in the month of June and a total of 40 lizards was divided into 4 groups of 10 each. One group of lizards was administered melatonin (20 μg) in 0.1 ml of saline intraperitoneally (*ip*) at 07.00 hrs daily for 30 days starting from the day of autotomy. The second and third groups of lizards received same amount of melatonin at the same time and for the same duration but, was preceded by (15 min.) administration of 5 or 10 μg of pimozide respectively in 0.1ml saline. The fourth group of lizards served as control and received 0.1ml saline at 07.00 hrs daily for 30 days starting from the day of autotomy.

Set-up II : *Evening melatonin (Me) and morning cyproheptidine* -

This experiment was carried out during July-August and consisted of 40 lizards divided into 4 groups of 10 each. Group I lizards received intraperitoneal (ip) injections of 20 μg melatonin in 0.1ml of saline in the evening at 17.00 hrs. daily for 30 days starting from the day of autotomy. Group II lizards received the same amount of melatonin at the same time for the same duration but were also injected with cyproheptidine (10 μg/lizard) in 0.1ml saline in the morning at 07.00 hrs. Group III lizards received only cyproheptidine as above. Group IV lizards served as control and received two injections of 0.1 ml of saline, one in the morning and one in the evening for the same duration.

Set-up III : *Evening melatonin and bromocriptine* -

These experiments were carried out during August-September and consisted of 40 lizards divided into 4 groups of 10 each. One group of lizards was administered with bromocriptine (20μg/lizard) in 0.1ml of saline followed 15 minutes later by melatonin (20μg/lizard) in 0.1ml saline at 17.00 hrs for 30 days starting from the day of autotomy. Second and third groups of lizards received melatonin or bromocriptine respectively at the same time and for the same duration. Fourth group of lizards received two injections of saline (0.1ml each) corresponding to the schedules in group I and served as the control.

Preparation of solutions

Melatonin was prepared as detailed in Chapter 4 and cyproheptidine as detailed in Chapter 2. Bromocriptine (2-bromo-ergocriptine as Proctinal by Biddle Sawyer Pvt. Ltd., Bombay, India) was prepared and stored at 4°C for daily injections. The drug was weighed and dissolved in a few drops of ethanol and warm saline (0.6%) was then added to give the required concentration.

Experimental protocol

Caudal autotomy was performed by pinching off the tail at the 3rd segment from the vent and were maintained in a normal light : dark schedule of 12:12. The

length of tail autotomised was measured for calculating the total percentage replacement. These experiments being carried out in the monsoon months, the average cage temperature was 26°C.

Parameters evaluated

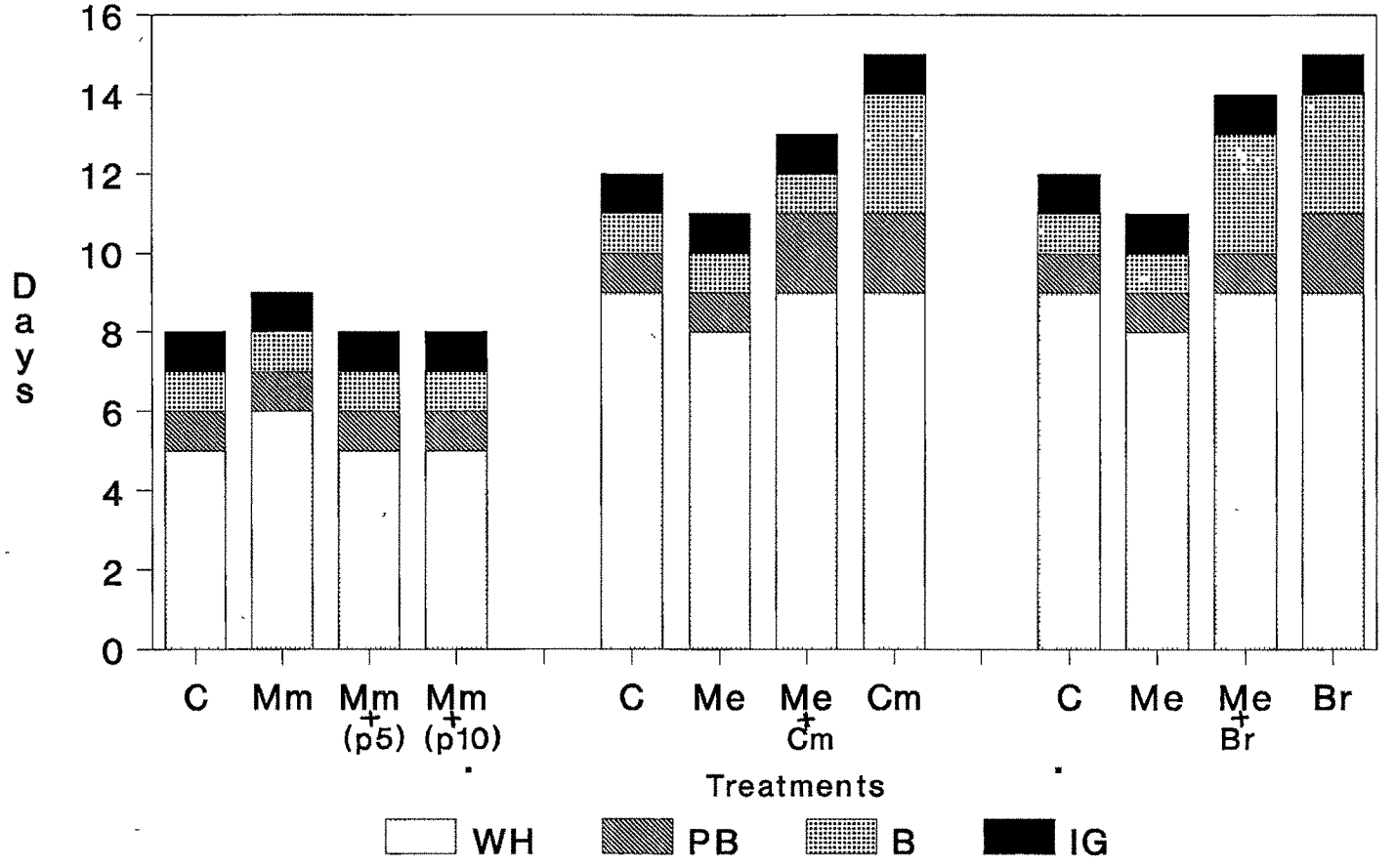
The various parameters evaluated were, the number of days taken to attain the various arbitrary stages of regeneration, the total length of tail regenerated, the percentage replacement at the end of 30 days and, the per day growth rate. The data were subjected to statistical analysis by Student's t-test and Duncan's multiple range test (Duncan, 1955).

RESULTS

The number of days taken for the attainment of various arbitrary stages are shown in figure 1, tables 1-3.

As is evident, evening administration of melatonin showed an advancement by one day. The enhancement shown by evening administration of melatonin was nullified by either bromocriptine or cyproheptidine. Similarly the delay shown by morning melatonin was nullified by pimozide. Bromocriptine or cyproheptidine given alone produced greater delay by two to three days.

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



Mm - morning melatonin p5 - pimozid 5ug p10 - pimozid 10ug
Me-evening melatonin Cm-Cyproheptidine morning Br-bromocriptine

TABLE - 1

Showing the number of days taken to reach the arbitrary stages of regeneration, total length of tail regenerated and percentage replacement at the end of 30 days and per day rate of growth during blocks of 5 days in control and experimental lizards.

Groups	Number of days taken to attain various arbitrary stages				Total length of tail regen-erated	Percentage replacement	PER DAY RATE OF GROWTH DAYS				
	WH	PB	B	IG			5-10	10-15	15-20	20-25	25-30
CONTROL	5	6	7	8	23.00 ± 2.48	37.70 ± 3.10	0.40	1.00	0.88	1.20	1.12
Mm	6	7	8	9	15.15[c] ± 1.53	25.21[c] ± 2.58	0.30	1.12	0.76	0.56	0.27
Mm + Pimozide (5 μg)	5	6	7	8	20.12[a] ± 2.13	32.60[a] ± 2.71	0.28	0.88	0.76	1.08	1.00

WH - Wound healing ; PB - Pre-blastema; B - Blastema ; IG - Initiation of growth; Mm - morning melatonin.
a - $P < 0.005$, c - $P < 0.001$ compared to the control.

TABLE - 2

Showing the number of days taken to reach the arbitrary stages of regeneration, total length of tail regenerated and percentage replacement at the end of 30 days and per day rate of growth during blocks of 5 days in control and experimental lizards.

Groups	Number of days taken to attain various arbitrary stages				Total length of tail regen-erated	Percentage replacement	PER DAY RATE OF GROWTH DAYS				
	WH	PB	B	IG			5-10	10-15	15-20	20-25	25-30
CONTROL	9	10	11	12	14.00 ± 2.62	23.09 ± 2.01	-	0.62	0.96	0.63	0.58
CH(m)	9	11	14	15	9.00[b] ± 0.92	14.75[b] ± 1.51	-	0.30	0.35	0.50	0.64
Me	8	9	10	11	19.50[b] ± 2.96	31.96[b] ± 3.28	-	1.30	1.30	0.40	0.90
CH(m) + Me	9	11	12	13	9.50b ± 0.88	15.57[b] ± 1.16	-	0.34	0.53	0.65	0.36

WH - Wound healing ; PB - Pre-blastema; B- Blastema ; IG - Initiation of growth; CH - Cyproheptidine, Me - Evening melatonin, m - morning.

b - $P < 0.005$ compared to the control.

TABLE - 3

Showing the number of days taken to reach the arbitrary stages of regeneration, total length of tail regenerated and percentage replacement at the end of 30 days and per day rate of growth during blocks of 5 days in control and experimental lizards.

Groups	Number of days taken to attain various arbitrary stages				Total length of tail regen-erated	Percentage replacement	PER DAY RATE OF GROWTH DAYS				
	WH	PB	B	IG			5-10	10-15	15-20	20-25	25-30
CONTROL	8	9	11	12	16.12 ± 2.28	26.42 ± 3.80	-	0.43	0.73	1.00	1.12
Bromo-riptine	9	10	13	14	7.16[c] ± 0.74	11.68[c] ± 1.08	-	0.20	0.40	0.37	0.25
Me	8	9	10	11	20.78[c] ± 2.65	34.06[c] ± 3.67	-	1.36	1.36	0.46	0.96
Bromo-riptine + Me	9	11	14	15	8.76c ± 0.61	14.27[c] ± 1.12	-	0.28	0.34	0.49	0.62

WH - Wound healing ; PB - Pre-blastema; B- Blastema ; IG - Initiation of growth; Me - Evening melatonin.

c - $P < 0.001$ compared to the control.

Total length of tail regenerated, total percentage of tail replaced and per day growth rate are depicted in figures 2-4 and tables 1-6.

Administration of melatonin in the evening showed significantly greater length of regenerate with greater percentage replacement. The peak growth rates were found between 10 and 20 days as against 20-25 days in the control animals. Lizards treated with cyproheptidine or bromocriptine, irrespective of whether they were coadministered with melatonin or not, showed similar degree of retardation and, near identical percentage replacement. Whereas the cyproheptidine plus melatonin treated lizards showed peak growth rate between 20-25 days, the cyproheptidine, bromocriptine and, bromocriptine plus melatonin treated lizards showed peak growth rates between 25-30 days. Lizards treated with 5 μg pimozide plus melatonin in the morning showed only marginal increase in regenerative growth compared to melatonin administered lizards. In both melatonin morning and melatonin morning plus 5μg pimozide treated lizards, growth rate started decreasing from the fifth day onwards. Lizards treated with 10 μg pimozide alongwith melatonin nearly nullified the retardation and, the growth rates were also identical to those of controls.

DISCUSSION

Previous studied had revealed an antiregenerative effect of morning melatonin injections and a proregenerative effect of evening melatonin injections. These effects

Fig.2 The length of tail regenerated in control(C) and experimental lizards at the end of 30 days.

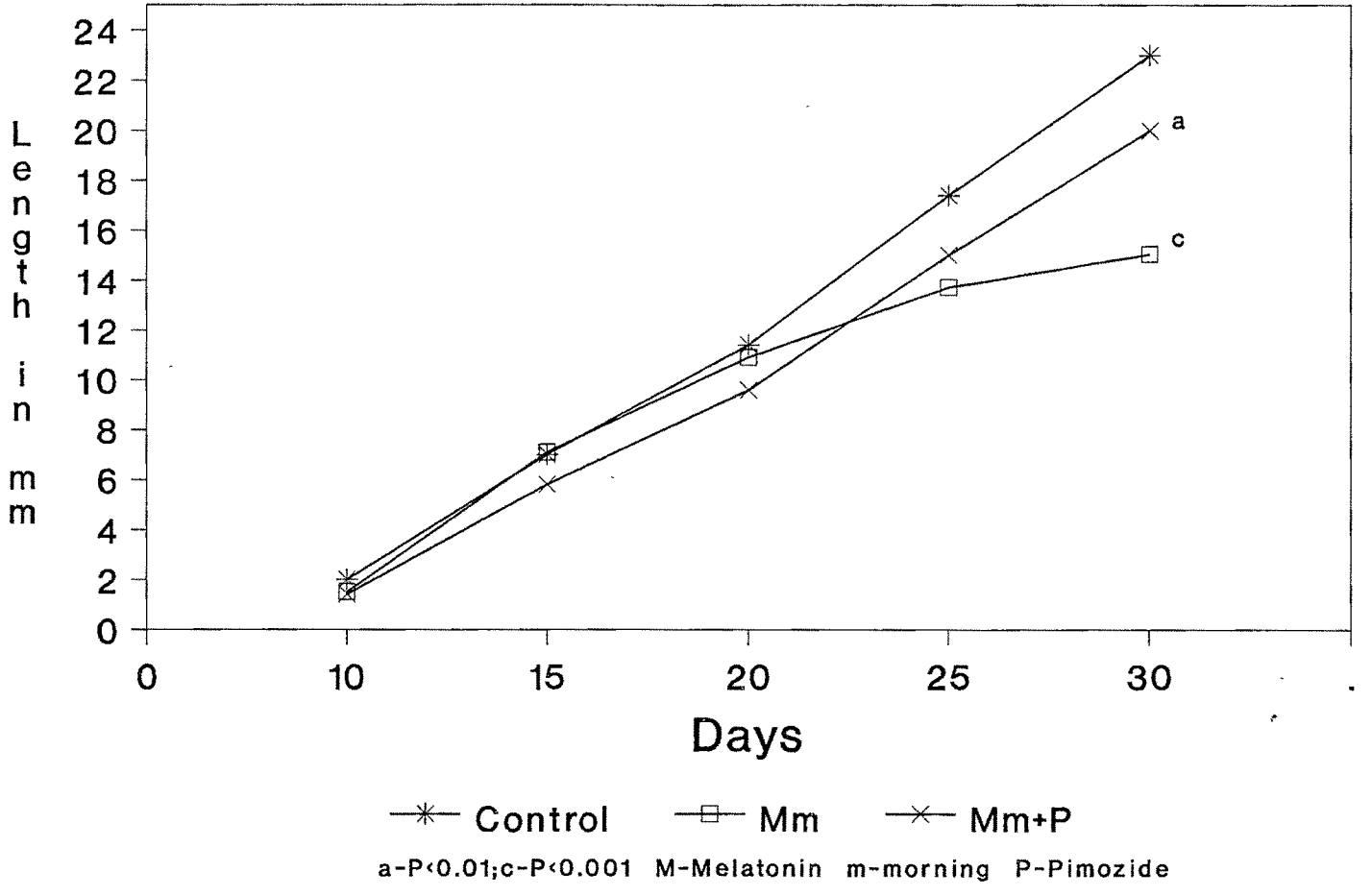
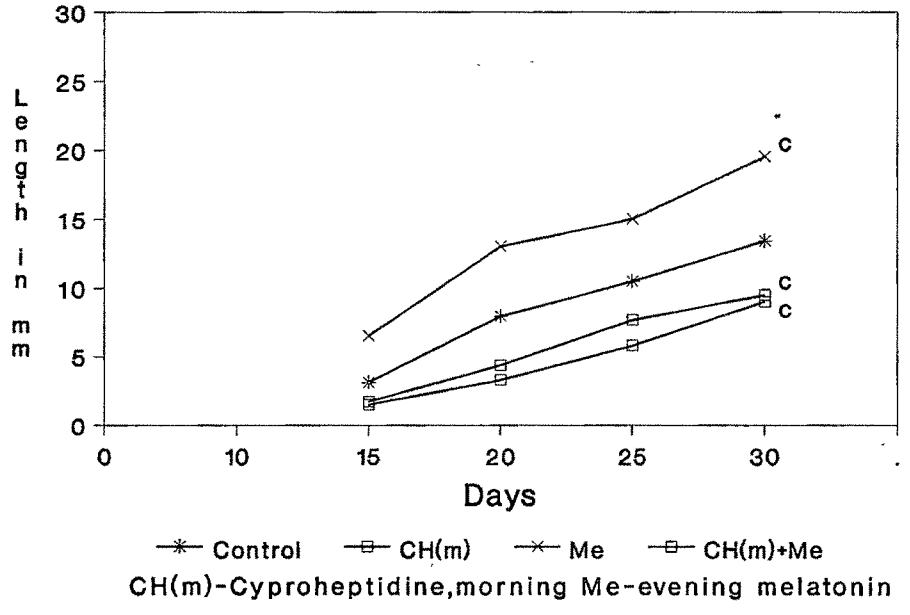
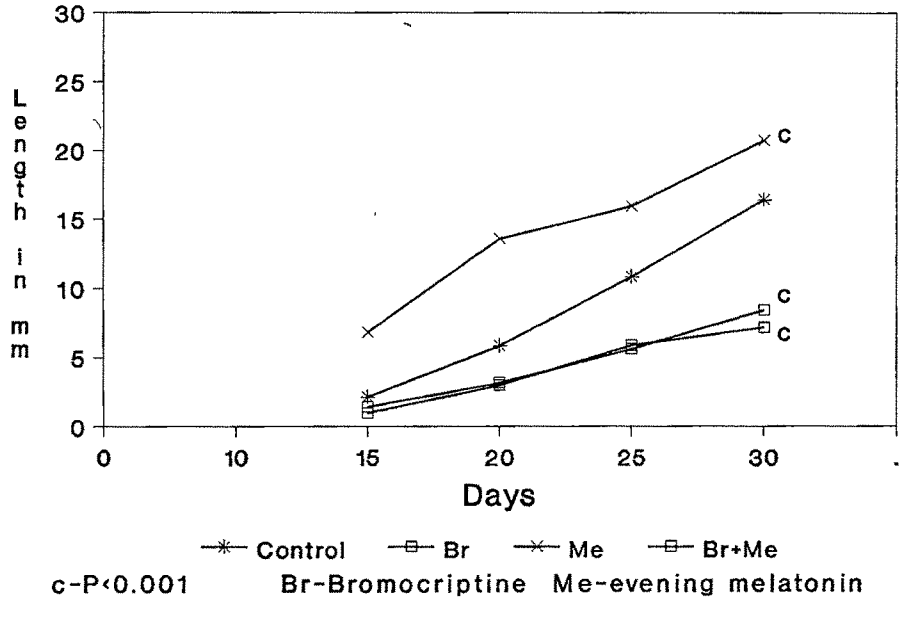


Fig.3 The length of tail regenerated in control(C) and experimental lizards.

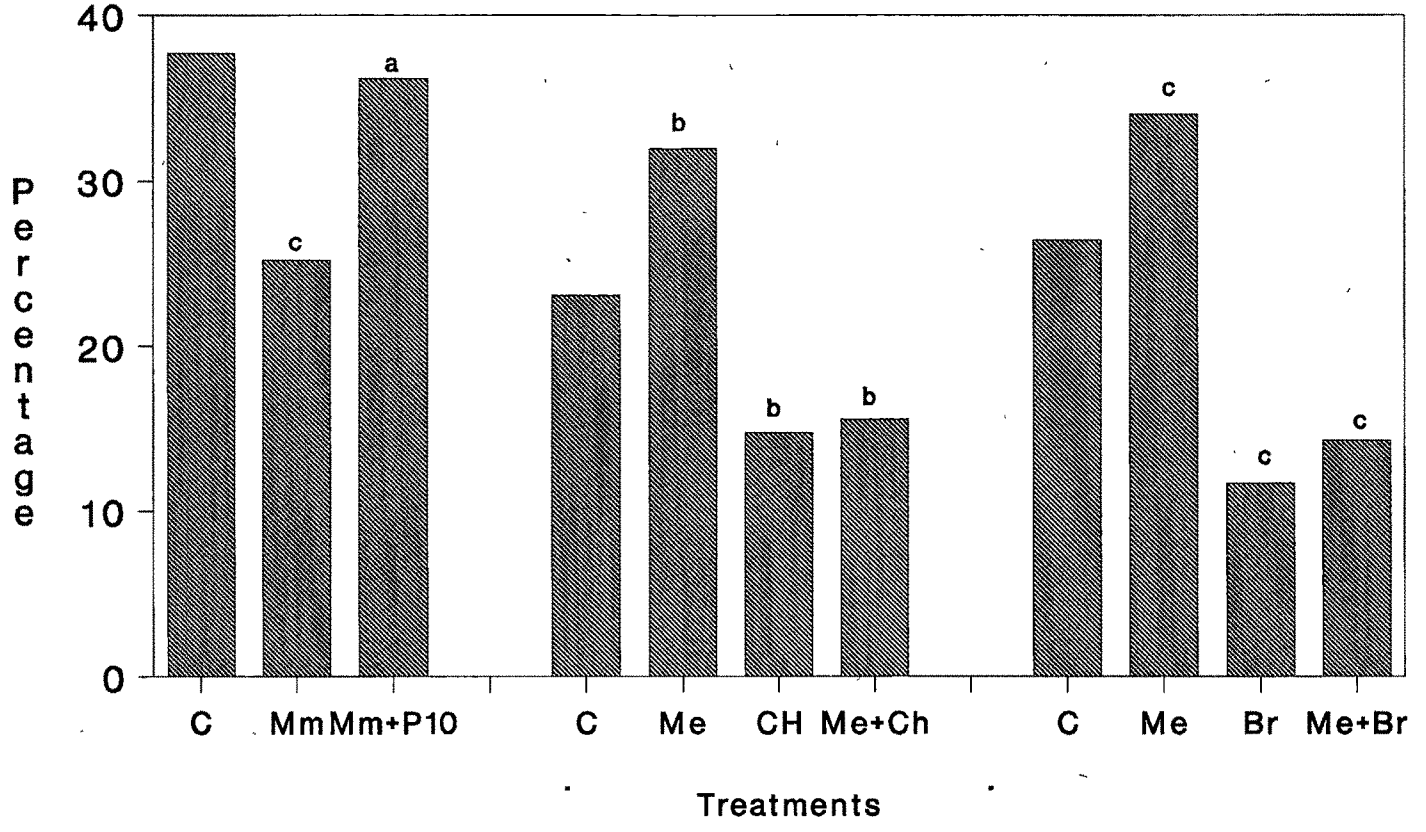


CH(m)-Cyproheptidine,morning Me-evening melatonin



c-P<0.001 Br-Bromocriptine Me-evening melatonin

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



M-melatonin e-evening m-morning P10-Pimozide 10ug CH-Cyproheptidine Br-Bromocriptine

a- P<0.01, b-P<0.005, c-P<0.001

TABLE - 4
Length of tail regenerated at different time period (days) in control and experimental lizards.

Manipulations	DAYS					
	5	10	15	20	25	30
CONTROL	-	2.00 ± 0.12	7.00 ± 0.86	11.40 ± 1.21	17.40 ± 2.50	23.00 ± 2.64
Mm	-	1.5* ± 0.08	7.10 ± 0.72	10.90 ± 0.98	13.70[b] ± 2.17	15.05[c] ± 2.14
Mm+ Pimozide (5 µg)	-	1.4* ± 0.13	5.8[a] ± 0.61	9.6[b] ± 1.26	15.0[a] ± 1.44	20.00[a] ± 2.86

Mm - morning melatonin

* - $P < 0.05$, a - $P < 0.01$, b - $P < 0.005$, c - $P < 0.001$ compared to the corresponding control values.

TABLE - 5
Length of tail regenerated at different time period (days) in control and experimental lizards.

Manipulations	DAYS					
	5	10	15	20	25	30
CONTROL	-	-	3.13[c] ± 0.67	7.95 ± 0.86	10.47 ± 1.22	13.41 ± 1.61
CH (m)	-	-	1.50[b] ± 0.23	3.28[c] ± 0.33	5.80[c] ± 0.81	9.00[c] ± 0.88
Me	-	-	6.50[b] ± 0.98	13.00[c] ± 1.48	15.00[c] ± 1.44	19.50[c] ± 1.48
CH (m) + Me	-	-	1.74[b] ± 0.11	4.39[c] ± 0.52	7.65[c] ± 0.63	9.49[c] ± 0.96

m -morning; CH - Cyproheptidine, Me - Evening melatonin;. Mm - Morning melatonin.
b - $P < 0.005$, c - $P < 0.001$ compared to corresponding control values.

TABLE - 6
Length of tail regenerated (mm) at different time period (days) in control and experimental lizards.

Manipulations	DAYS					
	5	10	15	20	25	30
CONTROL	-	-	2.153 ± 0.22	5.83 ± 0.83	10.83 ± 0.94	16.44 ± 1.51
Bromocriptine	-	-	1.00[b] ± 0.02	3.00[b] ± 0.41	5.88[c] ± 0.32	7.166[c] ± 0.56
Me	-	-	6.8[c] ± 0.88	13.60[c] ± 1.45	15.94[c] ± 1.61	20.78[c] ± 1.96
Bromocriptine + Me	-	-	1.445[a] ± 0.73	3.17[b] ± 0.82	5.63[c] ± 0.99	8.44[c] ± 1.02

Me - evening melatonin.
a - $P < 0.01$, b - $P < 0.005$, c - $P < 0.001$ compared to the corresponding control values.

were reasoned to be due to a duration effect of melatonin signal in the former and an amplitude effect of the melatonin signal in the latter. The consequences of these altered melatonin signals were the suggested decrease in PRL secretion due to the long duration of melatonin signal (melatonin morning) and increased PRL secretion due to an amplitude effect of melatonin (evening melatonin) mediated by increased dopaminergic and serotonergic activities respectively (Chapters 3 and 4). Evidences abound regarding the role of DA as a major physiological inhibitor of PRL secretion and 5-HT as a potent inducer of PRL release in both mammals and birds (Rabii *et al.*, 1981, Murai *et al.,* 1989; Pan and Teo, 1989; Alier *et al.,* 1990; Lamberts and Macleod, 1990; Arey and Freeman, 1992; El Halawani *et al.,* 1995). The present study in this context is a neuropharmacological approach to garner confirmative evidence for the purported dopaminergic and serotonergic modulation by the duration v/s amplitude nature of melatonin signal. The antiregenerative effect of Mm which makes the animals to read it as an extended dark phase is similar to the earlier observed decreasing regenerative performance under decreasing photic schedules (Ndukuba and Ramachandran, 1991a;Chapter 2). Compared to our previous study on regenerative performance under different photic schedules in different seasons, the presently recorded length of regenerate at 30 days is similar to the length regenerated between LD 6:18 and LD 8:16 photoschedules (Ndukuba and Ramachandran, 1991b). This provides validity to the contention that morning melatonin administration mimics

short photoperiod induced duration effect. Darkness induced melatonin content and resultant decrease in PRL release as inferred presently as well as previously (Ndukuba and Ramachandran, 1989a; 1991a,b; Ramachandran and Ndukuba, 1989a) are substantiated by the report that increased pituitary PRL mRNA levels in light deprived male hamsters is mediated through the pineal (Massa and Blask, 1989). The herein inferred longer duration melatonin induced increase in DA content/tone and decreased PRL secretion finds justification in the observation of reduced PRL release and increased DA content in hamsters transferred from long to short photoperiod and also increased sensitivity to DA (see Curlewis, 1992; Steger *et al.,* 1995). The contention that Mm increases dopaminergic activity and decreases PRL secretion thereby retarding tail regeneration is fully validated by the present observation of pimozide, a potent DA antagonist, to nullify the antiregenerative effect of Mm. Whereas 5 μg pimozide was ineffective, 10μg pimozide nearly nullified the effect thereby suggesting the need for an appropriate dosage to nullify the increased dopaminergic activity. Corroborative evidences are available in this context to show that DA agonist as well as DA antagonist can affect PRL gene transcription and PRL release (Giusle *et al.,* 1989; Shull and Gorski, 1990; Swearingen *et al.,* 1990; Webster *et al.,* 1992).

The proregenerative effect manifested by evening administration of melatonin has been accredited to an amplitude effect of melatonin. Apparently an evening injection of melatonin by coinciding with the endogenous nocturnal elevation, results

in a much greater scotophase level. This high amplitude scotophase melatonin signal has been purported to induce increased PRL release due to increased hypothalamic 5-HT content/tone. Importance of 5-HT in inducing enhanced PRL release is well documented (Lawson and Gala, 1976, 1978; Clemens *et al.,* 1977; James and Wigham, 1984; Lamberts and Macleod, 1990; Pan, 1991; Arey and Freeman, 1992; Rozell and Mead, 1993; Signs *et al.,* 1994; Ramalbo *et al.,* 1995). Support to the contention that Me induces greater 5-HT production in the hypothalamus is provided by the reported increase of hypothalamic 5-HT content and induction of increased serotonergic activity some 16-20 hrs. after melatonin administration to intact goldfish (Olcese *et al.,* 1981). Additional confirmative evidence for increased PRL release under a high amplitude melatonin signal comes from the observation of elevated PRL level in rats subsequent to melatonin administration in the evening (Vanage and Sheth, 1991). The increased regenerative tail elongation with increase in temperature in lizards maintained under a constant photoperiod could be attributed to the amplitude effect of melatonin as temperature has been shown to be responsible for greater melatonin amplitude in reptiles (Chapter 1). The present observations of reduced regenerative growth with both bromocriptine and cyproheptidine as well as their nullifying influence on Me induced enhancement in tail elongation confirms the involvement of both DA and 5-HT in the control of PRL release related to tail regeneration.

A dissected view of the present results shows that, while both cyproheptidine and bromocriptine could effectively nullify the Me effect, the retardation induced by bromocriptine is of a greater magnitude (55%) than that induced by cyproheptidine (36%). Though the cyproheptidine induced nullifying influence on the pro-regenerative effect of Me is understandable in the context of presumed increased 5-HT content/tone under the increased amplitude of melatonin, the similar nullifying influence exerted by bromocriptine is apparently paradoxical and needs a detailed treatment. A dominant effect of DA relative to 5-HT is readily inferable from these observations. In fact, there are experimental evidences to show that anatgonizing the DA action leads to enhancement of the stimulatory effect of 5-HT and that 5-HT induced increase in PRL release may actually occur by inhibiting DA release (Pan and Teo, 1989; Flores *et al.,* 1992). It was inferred earlier that in *H. flaviviridis*, the melatonin rhythm and the neural rhythms (DA and 5-HT) controlling PRL release are driven by separate oscillators and that the melatonin rhythm may influence the neural rhythms (Chapter 1). The observations obtained herein also tend to indicate independent photothermal influence on melatonin rhythm and the PRL related neural rhythms. Since bromocriptine could nullify the favourable influence of Me, it is surmisable that the amplitude effect of melatonin is manifested best when DA content/tone is less. In this context, on a comparative basis, the increased regenerative growth obtained with Me is identical to what is obtained at 29°C (Chapter 1). Since

the present experimental animals were experiencing an average temperature of 26°C, the Me induced improvement compares well with that obtained with a 3°C increment in temperature. The regenerative growth is much greater in the summer month when the average temperature was 33°C. This maximal regenerative performance is a cumulative photothermal influence brought about by an increased 5-HT action induced by long photoperiod as well as, the greater amplitude of melatonin and decreased DA activity, a temperature effect (Brownstein, 1975; Matthiej and Swarts, 1978; Leining *et al.*, 1979; Tucker *et al.,* 1991). These influences are confirmed by the earlier observation of decreased effectiveness of Me during summer months (Chapter 4) and an almost absent Me response in the winter months (unpublished observations). The valid explanation is that, since the amplitude of the melatonin surge is already maximal during the summer months, the effect of Me is minimal and, in the winter months, as the lower temperatures increase DA activity, Me is not able to counteract the DA activity as discussed earlier.

Overall, the presently reviewed results confirm that Mm has a duration effect leading to dampened PRL release while Me has an amplitude effect leading to an increase in 5-HT activity; both of which could be nullified by DA antagonism or 5-HT antagonism respectively. It also becomes apparent that lower temperature and short photoperiod together decrease PRL secretion to the minimum (combined effect on DA activity) while, high temperature and long photoperiod increase PRL release maximally (by decreasing DA tone and increasing 5-HT activity respectively).

SUMMARY

Previous investigations showed that exogenous melatonin (M) has a time-dependent influence on regenerative growth and, was inferred to be due to altered PRL secretion. The present study in this context, tests the validity of the above concept by way of neuropharmacological agents. For this, lizards administered M either in the morning (Mm) or evening (Me) have been treated with pimozide or cyproheptidine and bromocriptine respectively. The results show that pimozide can nullify the antiregenerative effect of Mm while the proregenerative influence of Me was nullified by cyproheptidine and bromocriptine. These have been taken to prove the operation of both dopaminergic PRL inhibiting and serotonergic PRL release inducing mechanisms in lizards with, duration or amplitude of M influencing the same.