CHAPTER

4

Influence of Exogenous Melatonin Administered at Different Times of The Day on Tail Regeneration

The pineal gland has been considered as a major pacemaker in the circadian organization of ectotherms. By the rhythmic synthesis and secretion of melatonin, it communicates with other central circadian neuroendocrine oscillators (Reiter, 1981; Binkley, 1988; Underwood, 1989). In poikilotherms, both light and temperature can entrain the pineal melatonin rhythm and so it functions as a photo-thermo-neuroendocrine transducer which enables the animals to synchronize its internal cycles with the cyclic variations in the environmental cues. Though these functions are extensively investigated in birds and mammals, very little is known about such aspects and their control by pineal in reptiles (Underwood, 1992). The effect of pinealectomy or exogenous melatonin administration on circadian locomotor activity or thermoregulation are the ones generally studied (Ralph *et al.,* 1979, Ralph, 1983, Vivien-Roels, 1985). Studies conducted on reptiles have demonstrated the ability of exogenous melatonin to alter thermoregulatory behaviour in the lizard, *Crotaphytus collaris* (Cothran and Hutchison, 1979) and in the turtle *Terrapene carolina triunguis* (Erskin and Hutchison, 1981). The role of the pineal and of exogenous melatonin on gonadal condition has also been investigated in seasonally breeding lizards (Misra and Thapliyal, 1979, Thapliyal and Haldar, 1979, Underwood, 1985b) and in a snake (Halder and Pandey, 1989a,b). The effects varied from progonadal to antigonadal, just as in homeothermic vertebrates, depending on the time of year pinealectomy was done or melatonin was administrated, and the species involved.

Though the above parameters of animal functions influenced by the pineal are essentially with a circadian or circannual rhythmicity, a phenomenon exhibited by some of the lizards, which though shows a seasonal variation, but cannot truly be included under rhythmic functions, and yet influenced by the pineal, is tail regeneration. Previous studies on tail regeneration in *Hemidactylus flaviviridis,* has demonstrated the stimulatory influence of longer duration of light and, retardary influence of longer durations of darkness (Ndukuba and Ramachandran, 1991a). The favourable influence of photoperiod on regeneration was shown to be essentially mediated by the pineal rather than by the eyes (Ndukuba and Ramachandran, 1988, Ramachandran and Ndukuba, 1989b). The principal pineal hormone in all vertebrates, is melatonin and, its rhythmic secretion has been shown to synchronize circadian and seasonal rhythms (Reiter, 1981; Binkley, 1988). A ubiquitous feature of melatonin secretion is that high levels are recorded in the scotophase and low levels in the photophase of a daily light-dark cycle (see Underwood, 1989). Light is an important environmental factor that can reduce pineal melatonin level as, variations in the duration of light have been shown to alter the nocturnal melatonin level in lower vertebrates as well. (see Underwood, 1989).

In the context of these reports, and the observations from this laboratory, that photic schedules and pinealectomy influence regenerative growth in *H. flaviviridis*, it

was thought pertinent to test the influence of exogenous melatonin on tail regeneration. Considering the fact that the effect of exogenous melatonin in both mammals and lizards is time dependent (Reiter *et al.,* 1976; Reiter, 1981; Misra and Thapliyal, 1979), in the present study, different schedules of timed melatonin administration have been carried out.

MATERIAL AND METHODS

Procurement, maintenance and acclimation of the animals are as outlined in Chapters 1 and 2.

A total of 90 lizards divided into seven groups with 30 in group I and 10 each in the other groups were used for the investigation.

Group I : This group comprised of control lizards injected with 0.1ml of vehicle for 30 days starting from the day of autotomy with 5 each receiving the injection as per the schedules in groups II to VII.

Group II : *Morning Melatonin* (Mm). These lizards received 20 µg melatonin in 0.1ml of (0.6%) saline intraperitoneally (*ip*) at 07.00 hrs daily for 30 days starting from the day of autotomy.

Group III : *Evening Melatonin* (Me). This group of lizards was administered with 20 µg melatonin in 0.1ml saline *ip.* at 17.00 hrs for 30 days starting from the day of autotomy.

Group IV : *Morning and noon melatonin* (Mmn). These lizards received twice daily intraperitoneal injections of 20μg melatonin in 0.1ml saline at 07.00 hrs and 12.00 hrs, for 30 days from the day of autotomy.

Group V : *Morning and evening melatonin* (Mme). This group received two injections of 20 μg melatonin in 0.1ml saline at 07.00 hrs and 17.00 hrs daily for 30 days starting from the day of autotomy.

Group VI : *Noon and evening Melatonin* (Mne). This group of lizards received 20 μg of melatonin (*ip*) both at 12.00 hrs and 17.00 hrs daily for 30 days starting from the day of autotomy.

Group VII : *Morning, noon and evening melatonin* (Mmne). These lizards received three *ip*. injections of 20 μg melatonin at 07.00 hrs, 12.00 hrs and 17.00 hrs daily for 30 days starting from the day of autotomy.

Preparation of melatonin

Melatonin (Sigma Chemical Company, St. Louis, USA) was prepared fresh daily before injection. Melatonin was dissolved in a few drops of ethanol before being diluted to the required concentration with 0.6% saline.

The tail of all lizards was autotomized by pinching off the tail at the third segment from the vent and were maintained in a normal light : dark schedule of 12 hrs

of light and 12 hrs of dark. The length of tail autotomized was measured for calculating the total percentage replacement. These experiments were conducted in the months of May and the average temperature at the level of the animals was 30°C with an amplitude of 36°C maximum and 24°C minimum.

Parameters and Statistical Analysis

The number of days taken to reach the various arbitrary stages of regeneration like wound healing (WH), preblastema (PB) blastema (B) and initiation of growth (IG) was recorded. The length of the regenerate was measured with a pair of compasses and scored against a meter scale. The measurement was recorded every alternate day and used for recording the tail length at fixed time intervals of 5, 10, 15, 20, 25 and 30 days post-autotomy. The per day growth rate and total percentage replacement of the tail at the end of 30 days were also calculated. The data was subjected to Anova and Duncan's multiple range test (Duncan, 1955) with an alpha level of both 0.05 and 0.01.

RESULTS

Number of days taken to reach the various arbitrary stages of regeneration :

The control and Me lizards completed wound healing by the 5th day, formed a blastema by the 7th day and growth was initiated by the 8th day. In all the other

experimental groups, there was a delay by one day which persisted for all the stages and, a new growth was initiated by the 9th day (Table 1).

Length of tail regenerated : (Fig. 1 and Tables 2 and 3)

Compared to the control, the length of the tail regenerated in all the experimental lizards except Me, was significantly less ($p < 0.001$). The least growth was shown by Mmne, group of lizards. This was followed by Mne, Mmn and Mme groups of lizards. The minimal retardation was shown by the Mm group. The Me group of lizards regenerated a significantly longer length of tail as compared to the controls.

Growth rate and total percentage replacement : (Fig. 2, Tables 3 and 4)

As can be seen from the table, the per day growth rate in the control as well as all the experimental groups showed a peak between 10-15 days. However, all the experimental groups, other than Me showed overall reduced growth rate at all time periods, more pronouncedly between 5-10 and after 15 days. The growth rate in the control and the Me groups of lizard was distinctly greater throughout, more markedly in the latter. The total percentage replacement of tail at the end of 30 days was significantly greater in Me group and significantly lesser in all other groups compared to control.

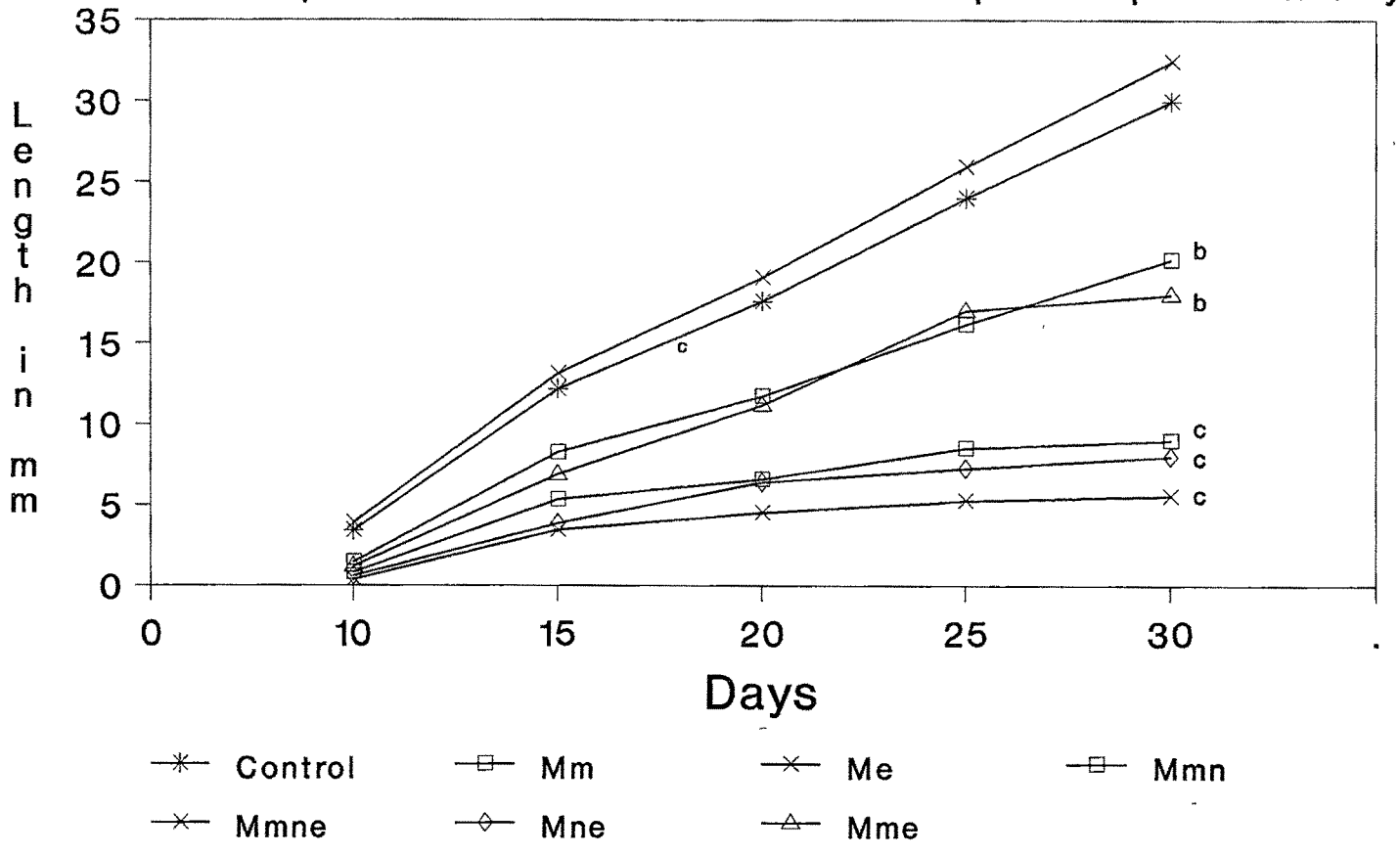
TABLE - 1

Number of days taken to attain various arbitrary stages of regeneration in control and experimental lizards receiving melatonin at different times of the day.

Manipulations	WH	PB	B	IG
Control	5	6	7	8
Mm	6	7	8	9
Me	5	6	7	8
Mmn	6	7	8	9
Mmne	6	7	8	9
Mne	6	7	8	9
Mme	6	7	8	9

Mm - Morning Melatonin, Me - Evening Melatonin, Mmn - Morning and noon Melatonin, Mmne - Morning, noon and evening Melatonin, Mne - Noon and evening Melatonin, Mme - Morning and evening Melatonin, WH - wound healing; PB - Pre-blastema; B - Blastema; IG - Initiation of growth.

Fig.1 Length of tail regenerated in control(C) and experimental lizards at different time periods post-autotomy



b-P<0.005;c-P<0.001 M-Melatonin m-morning n-noon e-evening

TABLE - 2

Length of tail regenerated at different time periods (days) post-autotomy in control and experimental lizards.

Manipulations	DAYS				
	10	15	20	25	30
Control	3.40 ± 0.51	12.16 ± 1.20	17.62 ± 1.80	24.00 ± 3.00	30.00 ± 3.80
Mm	1.45[a] ± 0.09	8.25[a] ± 0.99	11.74[a] ± 1.89	16.15[b] ± 2.15	20.19[b] ± 2.83
Me	3.89 ± 0.96	13.14 ± 2.11	19.09 ± 2.30	25.94 ± 3.10	32.44 ± 3.80
Mmn	0.815[b] ± 0.03	5.32[b] ± 0.78	6.59[c] ± 1.10	8.49[c] ± 2.50	8.99[c] ± 2.90
Mmne	0.37[c] ± 0.02	3.42[c] ± 0.55	4.5[c] ± 0.88	5.25[c] ± 1.00	5.5[c] ± 0.90
Mne	0.571[c] ± 0.06	3.85[c] ± 0.85	6.4[c] ± 1.10	7.25[c] ± 1.20	8.0[c] ± 1.18
Mme	1.18[b] ± 0.07	6.87[b] ± 0.99	11.16[b] ± 1.14	17.0[b] ± 1.80	18.0[b] ± 2.11

a - $P < 0.01$, b - $P < 0.005$, c - $P < 0.001$ compared to corresponding controls, Mm - Morning melatonin, Me - Evening melatonin, Mmn - Morning and noon melatonin, Mmne - Morning, noon and evening melatonin, Mne - Noon and evening melatonin, Mme - Morning and evening melatonin.

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

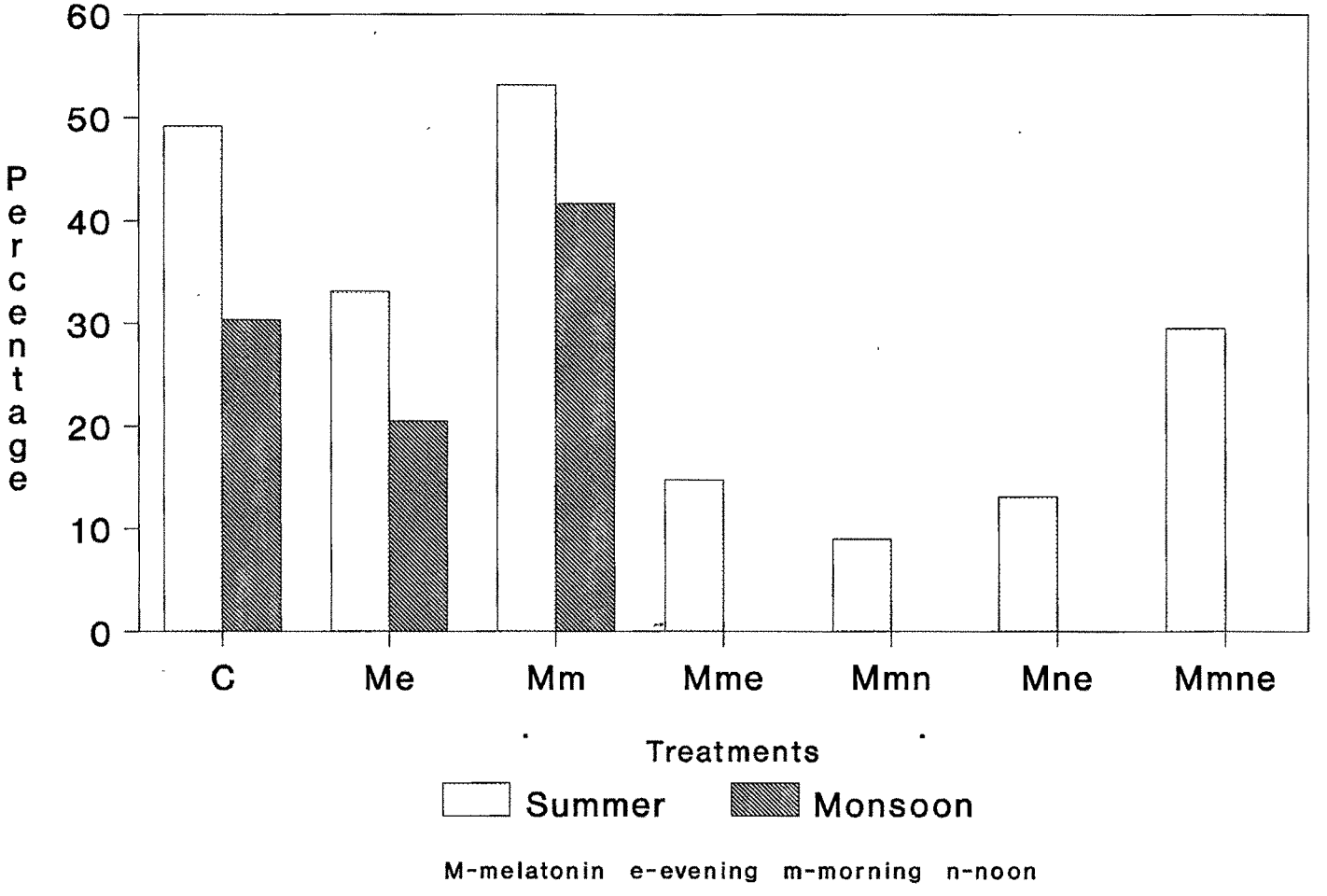


TABLE - 3

Length of tail regenerated (mm) and total percentage replacement at the end of 30 days in control and experimental lizards.

Manipulations	Total length	% Replacement
Control	30.00 ± 2.65	49.18 ± 4.29
Mm	20.19^{a} ± 1.96	33.09^{a} ± 3.14
Me	32.46^{b} ± 3.28	53.21 ± 5.18
Mmn	9.00^{b} ± 1.28	14.75^{b} ± 1.48
Mmne	5.50^{c} ± 0.99	9.01^{c} ± 0.92
Mne	8.00^{b} ± 1.08	13.10^{b} ± 1.21
Mme	18.00^{a} ± 2.01	29.5^{a} ± 2.64

a - $P < 0.01$, b - $P < 0.005$, c - $P < 0.001$ compared to controls, Mm - Morning melatonin, Me - Evening melatonin, Mmn - Morning and noon melatonin, Mmne - Morning, noon and evening melatonin, Mne - Noon and evening melatonin, Mme - Morning and evening melatonin.

TABLE - 4

Per day rate of growth (in mm) during blocks of 5 days in control and experimental lizards.

Manipulations	DAYS				
	5-10	10-15	15-20	20-25	25-30
Control	0.68 ± 0.17	1.75 ± 0.30	1.09 ± 0.48	1.27 ± 0.48	1.20 ± 0.46
Mm	0.29 ± 0.08	1.361 ± 0.28	0.69 ± 0.14	0.88 ± 0.29	0.80 ± 0.25
Me	0.77 ± 0.13	1.85 ± 0.44	1.19 ± 0.34	1.37 ± 0.36	1.30 ± 0.31
Mmn	0.16 ± 0.04	0.90 ± 0.18	0.25 ± 0.06	0.38 ± 0.09	0.10 ± 0.40
Mmne	0.70 ± 0.01	0.618 ± 0.13	0.21 ± 0.08	0.15 ± 0.06	0.05 ± 0.008
Mne	0.11 ± 0.009	0.65 ± 0.15	0.50 ± 0.13	0.17 ± 0.03	0.15 ± 0.05
Mme	0.23 ± 0.02	1.13 ± 0.29	0.85 ± 0.13	1.16 ± 0.29	0.20 ± 0.04

Mm - Morning melatonin, Me - Evening melatonin, Mmn - Morning and noon melatonin, Mmne - Morning, noon and evening melatonin, Mne - Noon and evening melatonin, Mme - Morning and evening melatonin.

DISCUSSION

The present results show that exogenous administration of melatonin to intact lizard exerts a generalized retardary influence at all time periods studied, except in the evening. All the schedules i.e., Mm, Mmn, Mne and Mmne retarded regenerative growth significantly with maximal retardation being observed in Mmne. In contrast, Me significantly enhanced regenerative growth. Differential time dependent effects of melatonin were demonstrated in mammals with reference to gonadal functions (Carter *et al.,* 1982). Tamarkin *et al.* (1976) demonstrated that single daily injections of melatonin administered in the afternoon to pineal intact Syrian hamsters induced gonadal regression, while single daily injection given in the morning was ineffective. In intact hamster kept on long days, melatonin injected in the evening caused gonadal regression, while when administered at other times were not effective. (Tamarkin *et al.,* 1976, 77; Rollag and Stetson, 1982). Margolis and Lynch (1981) reported that single daily injection of melatonin in the afternoon can induce gonadal regression in the white-footed mice, *Peromyscus leucopus*. However, in this animal, injection in the morning before lights on, also induced gonadal regression. Similar experimental paradigm involving administration at different times of the day in the Indian garden lizard, *Calotes versicolor,* induced testicular regression at all times, though more pronouncedly at 17.00 hrs and 05.00 hrs. This may suggest differential sensitivity to

melatonin in mammals and reptiles. The present study demonstrates continuous responsiveness to melatonin throughout the day though manifested either in the form of an antiregenerative or a proregenerative effect.

Of the dual effects obtained herein, the antiregenerative effect of melatonin given once in the morning or twice daily (in the morning and noon or morning and evening or noon and evening) or thrice daily (i.e., in the morning, noon and evening) seems more easy to explain. Earlier studies had shown decreasing regenerative tail elongation with decreasing LD regimes resulting in the lowest regenerative performance being exhibited in LD 0:24 (Ndukuba and Ramachandran, 1991a). The presently recorded regenerative growth in Mm lizards is quite comparable with that shown by lizards maintained in LD 8:16 in the above study. Apparently, administration of melatonin in the morning leads to a prolonged melatonin signal which makes the animal to read it as an extended dark phase. This is clearly akin to a duration effect of melatonin shown in Djungarian hamsters and ewes. In both these animals, melatonin was inferred to be important for photoperiodic time measurement and a critical duration of the melatonin level was found to be essential for the induction of photoperiodic effects on the reproductive system (Maywood *et al.*, 1990; Goldman, 1991; Bartness *et al.*, 1993).

Based on the previous inference that tail regeneration in Hemidactylus is mediated by the growth promoting influence of prolactin (PRL) and that, longer

photic schedules lead to increased PRL release and vice versa (Ramachandran and Ndukuba, 1989a; Ndukuba and Ramachandran, 1991a,b), the presently observed antiregenerative effect of Mm could be related to the reduced PRL secretion occuring due to the duration effect of melatonin. This is supported by the earlier observation that exogenous PRL can completely nullify the retarding influence of constant darkness (Ndukuba and Ramachandran, 1989a). Unequivocal evidences are available in mammals to show that PRL secretion is positively related with the increase in light regime (Matthiej and Swarts, 1978; Barrell and Lapwood, 1979; Brown and Forbes, 1980; Munro *et al.*, 1980) and that melatonin can interfere with PRL secretion (Kennaway *et al.*, 1982; Martinet *et al.*, 1983; 1985; Symons *et al.*, 1983; Murphy *et al.*, 1990). Further, Murphy *et al.* (1990) showed that PRL administration can overcome the effect of melatonin in the mink, *Mustella vision.* Considering the inhibitory effects of melatonin on thyroid hormones in mammals and brids (Linda, 1981; Krotewicz and Lewinski, 1992; Ramachandran *et al.*, 1996), it is also likely that the currently observed retardation in regeneration by exogenous melatonin could be also due to the reduced thyroid hormone levels. The presently presumed short photoperiodic effects of Mmne and, Mmn and Mne are corroborated when the length of tail regenerated in these groups is compared with that observed in the previous studies involving photoperiods (Ramachandran and Ndukuba, 1989a; Ndukuba and Ramachandran, 1991a). The length obtained in the Mmne, is comparable with the

length regenerated in LD 8:16 group in the winter, while the length regenerated by the Mmn and Mne is comparable with that obtained in LD 6:18 groups of lizards in the monsoon. It is apparent from the above comparison, that, either two or three consecutive melatonin administrations (given five hrs. apart in this study) during the photophase, convey a signal similar to that imparted by a short photoperiod, resulting in slower regenerative growth. There are evidences in this connection to relate both short photoperiod and injections of melatonin that mimic short photoperiod, induced reduction in PRL secretion (Steger *et al.,* 1982, 1985; Benson, 1987; Massa and Blask, 1989; Maywood *et al.,* 1990, 1991, 1992; Steger and Bartke, 1991; Bartke and Steger, 1992; Alexiuk and Vriend, 1993; Grosse *et al.,* 1993; Asher *et al.,* 1994; Maywood and Hastings, 1995; Steger *et al.,* 1995).

In contrast to single daily injection in the morning or, two or three consecutive injections during the day, single injection of melatonin daily in the evening depicted a proregenerative effect marked by a longer tail regenerate. This proregenerative effect of Me is as inferred earlier (Chapter 3; Kurup *et al.,* 1995), an amplitude effect of melatonin, which leads to increased PRL release through the serotonergic mechanism. Administration of melatonin in the evening seems to have a distinct positive effect compared to injection at any other time of the day. This schedule coincides with the endogenous elevation of dark phase melatonin, leading to a greater amplitude of the nocturnal melatonin signal. Interestingly, Watson-Whitmyre and Stetson (1983) also

obtained a melatonin response in hamster in the form of gonadal regression when exogenous melatonin was administered in the evening prior to the normal endogenous rise. How this amplitude signal is translated into increased regenerative growth as seen in the present study can only be speculated. It is presumed that higher melatonin levels during the scotophase increases serotonin (5-HT) production in the hypothalamus which initiates mechanisms leading to enhanced PRL release (see Chapter 3; Kurup, *et al.,* 1995).

In a previous study, importance of both photoperiod and temperature on regeneration in terms of melatonin cycle and, the dominant influence of temperature were highlighted (Chapter 1). Therein, it was assumed that, greater amplitude in temperature cycle as during the summer months, induces enhanced melatonin release at night while, increasing darkness leads to a longer phase of melatonin signal. The present results are in conformity with these inferences. The amplitude effect of melatonin leading to augmented PRL release mediated by 5-HT, is clearly exemplified by the presently observed difference in the degree of regenerative growth between monsoon and summer temperatures in the control animals and that between control and Me animals in monsoon and summer (Table 5). A difference of 5°C between the average monsoon temperature of 25°C and the average summer temperature of 30°C, results in 62% increment in regenerative growth. This can be related to the temperature mediated high amplitude melatonin release and the attendant PRL release.

TABLE - 5

Showing the total length of tail regenerated and the percentage replacemen at the end of 30 days in control and melatonin treated lizards in the monsoon season.

Manipulations	Total length	% Replacement
Control	18.50 ± 2.08	30.32 ± 3.51
Mm	12.50 ± 1.26^{c}	20.49 ± 2.60^{c}
Me	25.40 ± 2.52^{c}	41.63 ± 4.32^{c}

c - $P < 0.001$ compared to control, Mm - Morning melatonin, Me - Evening melatonin.

On the other hand, the proregenerative effect of Me was significantly evident in the monsoon months (lower temperature) while it was attenuated to an insignificant level in the summer months (higher temperature). Obviously, the amplitude of night time melatonin being already high in the summer, evening administration of melatonin is unable to cause any further increase in the amplitude. However, administration of melatonin in the morning prolongs the duration of melatonin signal in either of the existing entrained cycle (monsoon or summer) resulting in identical retardation in regenerative growth in both the seasons.

Overall, the present results indicate that sensitivity towards melatonin affecting regenerative growth is expressed throughout the day in lizards but the degree of responses can be modulated by the ambient photothermal cues.

SUMMARY

Since photothermal cues are known to influence melatonin (M) rhythms and as pinealectomy has been shown to retard tail regeneration, influence of exogenous M on tail regeneration in *H. flaviviridis* was deemed appropriate. As M is reported to have time-dependent influence in mammals, M administration was carried out at different times during the day, morning (Mm), evening (Me), morning and noon (Mmn), noon and evening (Mne), morning and evening (Mme) and morning, noon and evening (Mmne). The experiments revealed a proregenerative effect of Me and antiregenerative effect of all other schedules, with the maximal retardation in Mmne. These observations have been related with an amplitude or duration effect of M, akin to that of higher temperature or short photo-period respectively, resulting in altered PRL secretion with the attendant effect on regenerative performance. The present results also show that sensitivity towards M is manifest on 24 hrs. circadian cycle.