CHAPTER VI

EVIDENCE SHOWING THAT THE TIME OF ADMINISTRATION OF THE INDOLEAMINE, MELATONIN, DETERMINES ITS PROREGENERATIVE OR ANTIREGENERATIVE EFFECT IN THE GEKKONID LIZARD, HEMIDACTYLUS FLAVIVIRIDIS.

A feature of every vertebrate pineal is its capacity to synthesize different indoleamines including melatonin (5-methoxy -N- acetyltryptamine) and its precursor serotonin (5-hydroxytryptamine). The daily rhythmic secretion of the pineal methoxyindole, melatonin, is able to synchronize seasonal and photoperiodic changes (for references see Tamarkin et al., 1985; Skene et al., 1987). Light and temperature are two important external stimuli that can affect pineal melatonin levels in vertebrates, and alterations in the length of light have been shown to alter the levels of nocturnal melatonin levels in some non-mammalian vertebrates, such as the quail (Underwood and Siopes, 1985), the rainbow trout (Duston and Bromage, 1987), the laying chicken (Liou et al., 1987), the box turtle (Vivien-Roels et al., 1988), and the anole lizard (Underwood, 1985).

Pineal melatonin levels are higher at night than during the day regardless of habits (diurnal or nocturnal) of the animal (see Underwood, 1985). The rhythm in pineal melatonin content is a true circadian rhythm (i.e. driven by an internal

biological clock), since the melatonin-serotonin rhythms will persist in constant conditions (Takahashi et al., Mahapatra et al., 1980; 1988). Light is the main enviornmental stimulus affecting pineal melatonin levels in homeotherms whereas both light and temperature can control pineal melatonin levels in poikilotherms (Birau and Schloot, 1981; Underwood, 1985).

In lizards, melatonin has been identified in the pineal organ, the parietal eye, and blood plasma, with its levels responding to changes in both light and temperature (Menaker and Wisner, 1983; Underwood, 1985; Firth and Kennaway, 1980, 1987). Levey (1973) in his extensive work on female Anolis carolinensis and Haldar (1977) on male and female Calotes versicolor showed that response of gonads to exogenous melatonin varies with seasons. Exogenous melatonin alters thermoregulatory behaviour in Crotaphytus collaris (Cothran and Hutchinson, 1979) and in the turtle, Terrapene carolina triunguis (Erskine and Hutchinson, 1981). Exogenous melatonin also causes gonadal regression in the lizard C. versicolor; the degree of response varies with the time of day at which the hormone is administered (Misra and Thapliyal, 1979). In mammals, melatonin can have either antigonadal or progonadal effects depending on species and photoperiodic conditions (Reiter, 1980; Carter and Goldman, 1983). Melatonin has been reported to inhibit the gonadal activity in mammals by inhibiting the synthesis and/or release of the

gonadotropic hormones (Reiter, 1981; Cardinali et al.,1983). Evidence exists that the antigonadotropic action of melatonin in mammals is dependent on the time of the hormone injection (Birau and Schloot, 1981; Pevet and Haldar-Misra, 1982; Cardinali et al., 1983). More recent studies have shown that morning administration of melatonin antagonizes the antigonadotropic effect of afternoon injections of melatonin, 5-methoxytryptophol and arginine vasotocin in intact mice (Ng,1987). However, melatonin did not influence the mitotic activity of regenerating adrenal cortex in the rat (Karasek, et al., 1987).

Our own recent observations have shown that pinealectomy or light deprivation to the pineal organ abolished the stimulatory influence of continuous illumination and significantly retarded the regeneration process (Ramachandran and Ndukuba, 1989a/Chapter 3). Further evidence suggests that parachlorophenylalanine (p-CPA) an inhibitor of tryptophan hydroxylase (Koe and Weisman, 1966; Walker, 1983), and an agent employed for chemical pinealectomy, significantly retarded tail regenersyion in animals exposed to continuous light (Ramachandran and Ndukuba, 1989b/Chapter 8). In our continuing investigations, the present study was designed to elucidate the role of the pineal methoxyindole, melatonin, on tail regeneration in lizards. The data presented here is probably the first report showing that the influence of exogenous melatonin, on lacertilian tail

regeneration is governed by the time of day at which the hormone is administered and that the melatonin precursor, serotonin, when injected intraperitoneally to intact lizard, Hemidactylus flaviviridis does not significantly affect either the total new growth or the total percentage replacement of the lost (autotomized) tails when compared with the saline-injected controls.

MATERIALS AND METHODS

A total of 70 lizards was used for this investigation, and they were balanced for size and sex in order to eliminate any possible error in the final statistical analysis due to size and sex differences. The animals were then dividied into five groups, namely:

Group 1. Morning melatonin injections (MM)

The first group of 10 lizards was given once daily intraperitoneal injection of 2 mg/kg crystalline melatonin (Sigma) in the morning (07.00 hrs), 5 days prior to tail autotomy and 30 days post-caudal autotomy.

Group 2. Evening melatonin injections (EM)

A second group of 10 lizards received once daily intraperitoneal injection of 2 mg/kg crystalline melatonin (Sigma) in the evening (17.00 hrs), 5 days prior to tail autotomy and 30 days thereafter.

Group 3. Evening serotonin injections (ES)

The third group of 10 animals was given once daily intraperitoneal injection of 2mg/kg , [illegible] serotonin (Sigma) at 17.00 hrs, 5 days prior to tail autotomy and 30 days postcaudal autotomy.

Group 4. Pinealectomized lizard (PX)

The fourth group of ten lizards had their pineal organs surgically removed (pinealectomy-PX). Pinealectomy was performed by the method of Ramachandran and Ndukuba (1989a) Chapter 3. Microscopic examination of PX lizards showed that the pineal was completely removed and no damage was done to the brain. Pinealectomy in each lizard took about three minutes and PX animals were allowed 5 days recovery period in order to eliminate any traumatic side effect due to surgery. PX lizards were given once daily intraperitoneal injection of 2mg/kg crystalline melatonin (sigma) at 17.00 hrs, 5 days prior to tail autotomy and 30 days thereafter.

Group 5. Control lizards (0.6% saline)

The fifth group of lizards, made up of twenty normal (NL) and ten PX, served as controls and received only once daily intraperitoneal injection of 0.6% saline; 10 NL in the morning and 10-NL + 10-PX in the evening, 5 days prior to tail autotomy and 30 days post-caudal autotomy.

Preparation of melatonin

Melatonin, for the injections, was prepared fresh daily.

Crystalline melatonin (Sigma Chemical Co., St. Louis, U.S.A) was dissolved in a few drops of ethanol before being diluted to the required concentration with 0.6% saline.

Preparation of saline (0.6%)

0.6 gm of reagent grade sodium chloride (NaCl) was dissolved in 100 ml re-distilled water and stored in a refrigerator for daily use.

Experimental set up:

Tail autotomy was performed by pinching off the tail at the third segment from the vent and the animals exposed to the intermediate photoperiod of 12 hours of light and 12 hours of darkness (12L : 12D) throughout the entire period of experimentation.

The description of the light schedule and the dimensions of the cages that housed the animals have been well documented in our previous reports (Ndukuba and Ramachandran, 1988, 1989a; Chapter 2 & 3 Ramachandran and Ndukuba, 1989a). Food and water were provided ad libitum. This investigation was conducted during the monsoon month of August, and the average daily temperature at the level of the animals was 26°C.

Statistical analysis

The length of new growth (regenerate), in mm, was measured with a graduated meter rule and recorded at fixed time inter-

vals of 10, 15, 20, 25 and 30 days post-caudal autotomy. The measurements were later used for morphometric calculations. The data on the length of tail and total percentage replacement were subjected to Student's 't' test and Duncan's multiple range test (Duncan, 1955) for statistical significance. Values which were different at the $P < 0.05$ level were considered to be statistically significant.

RESULTS

The results are clearly shown in figures 1-3 and table 1.

Growth rate, total length of tail regenerated and total percentage replacement.

The regeneration blastema appeared in NL + Sal, NL + EM and NL + ES groups of animals by day 8 to day 10 and in NL + MM and PX groups of lizards by day 12 to day 14 post-caudal autotomy. The total length of tail regenerated by the 30th day in normal lizards injected with saline (NL + Saline; morning and evening), normal lizards with morning melatonin injections (NL + MM), normal lizards with evening melatonin injections (NL + EM), normal lizards with evening serotonin injections (NL + ES), pinealectomized lizards with evening melatonin injections (PX + EM) and pinealectomized lizards with saline injections (PX + Saline) was 18.0 mm, 17.5 mm, 25.4 mm, 17.0 mm, 11.5 mm and 12.1.mm, respectively which was a replacement of 35.0%, 34.0%, 24.2%, 49.3%, 33.0%, 22.3%

TABLE 1. APPROXIMATE NUMBER OF DAYS TAKEN TO REACH THE VARIOUS ARBITRARY STAGES OF TAIL REGENERATION IN MELATONIN AND SEROTONIN TREATED AND CONTROL LIZARDS, H. FLAVIVIRIDIS DURING THE MONSOON MONTH OF AUGUST.

EXPERIMENTAL ANIMALS	WOUND HEALING	BLASTEMA	EARLY DIFFEREN-TIATION	MID DIFFEREN-TIATION	LATE DIFFEREN-TIATION
NL + SALINE	5	8 - 10	12 - 14	20	30*
NL + MM	7	12 - 14	18 - 20	25	30
NL + EM	5	8 - 10	12 - 14	20	30
NL + ES	5	8 - 10	12 - 14	20	30
PX + EM	7	12 - 14	18 - 20	25	30
PX + SALINE	7	12 - 14	18 - 20	25	30

NL + SALINE - NORMAL LIZARDS INJECTED WITH SALINE
NL + MM - NORMAL LIZARDS WITH MORNING MELATONIN INJECTIONS
NL + EM - NORMAL LIZARDS WITH EVENING MELATONIN INJECTIONS
NL + ES - NORMAL LIZARDS WITH EVENING SEROTONIN INJECTIONS
PX + EM - PINEALECTOMIZED LIZARDS WITH EVENING MELATONIN INJECTIONS
PX + SALINE - PINELECTOMIZED LIZARDS INJECTED WITH SALINE
* - DAYS POST-CAUDAL AUTOTOMY

TABLE 2. LENGTH OF TAIL REGENERATED AND TOTAL PERCENTAGE REPLACEMENT IN NORMAL AND PINEALECTOMIZED H. FLAVIVIRIDIS EXPOSED TO 12L : 12D EXPERIMENTAL PHOTOPERIODIC CONDITION AND TREATED WITH SEROTONIN AND MELATONIN IN THE MORNING AND EVENING.

EXPERIMENTAL ANIMALS AND HORMONES INJECTED	DAYS POST - CAUDAL AUTOTOMY					TOTAL % TAIL REPLACEMENT
	DAY 10	DAY 15	DAY 20	DAY 25	DAY 30	
NL + SALINE (10)	3.7 ± 1.4	6.7 ± 1.7	10.7 ± 1.7	15.1 ± 1.7	18.5 ± 2.3	35.9%
NL + MM (10)	1.6 ± 0.4	4.6 ± 1.6	8.8 ± 2.9	11.2 ± 3.8	12.5 ± 3.7	24.2%
NL + EM (10)	4.4 ± 1.8	9.0 ± 3.1	13.8 ± 3.0	19.8 ± 2.7	25.4 ± 2.6	49.3%
NL + ES (10)	3.6 ± 0.8	6.4 ± 1.2	9.5 ± 1.5	14.9 ± 1.9	18.3 ± 2.0	34.5%
PX + EM (10)	1.4 ± 0.4	3.6 ± 1.2	7.3 ± 1.6	9.9 ± 1.5	11.5 ± 1.2	22.3%
PX + SALINE (10)	1.5 ± 0.5	4.2 ± 1.0	7.7 ± 1.5	10.4 ± 1.4	12.1 ± 1.2	23.4%

NL + SALINE - NORMAL LIZARDS INJECTED WITH SALINE
NL + MM - NORMAL LIZARDS TREATED WITH MELATONIN IN THE MORNING
NL + EM - NORMAL ANIMALS INJECTED WITH MELATONIN IN THE EVENING
NL + ES - NORMAL ANIMALS TREATED WITH SEROTONIN IN THE EVENING
PX + EM - PINEALECTOMIZED LIZARDS TREATED WITH MELATONIN IN THE EVENING
PX + SALINE - PINEALECTOMIZED ANIMALS INJECTED WITH SALINE
(N) - TOTAL NUMBER OF EXPERIMENTAL ANIMALS

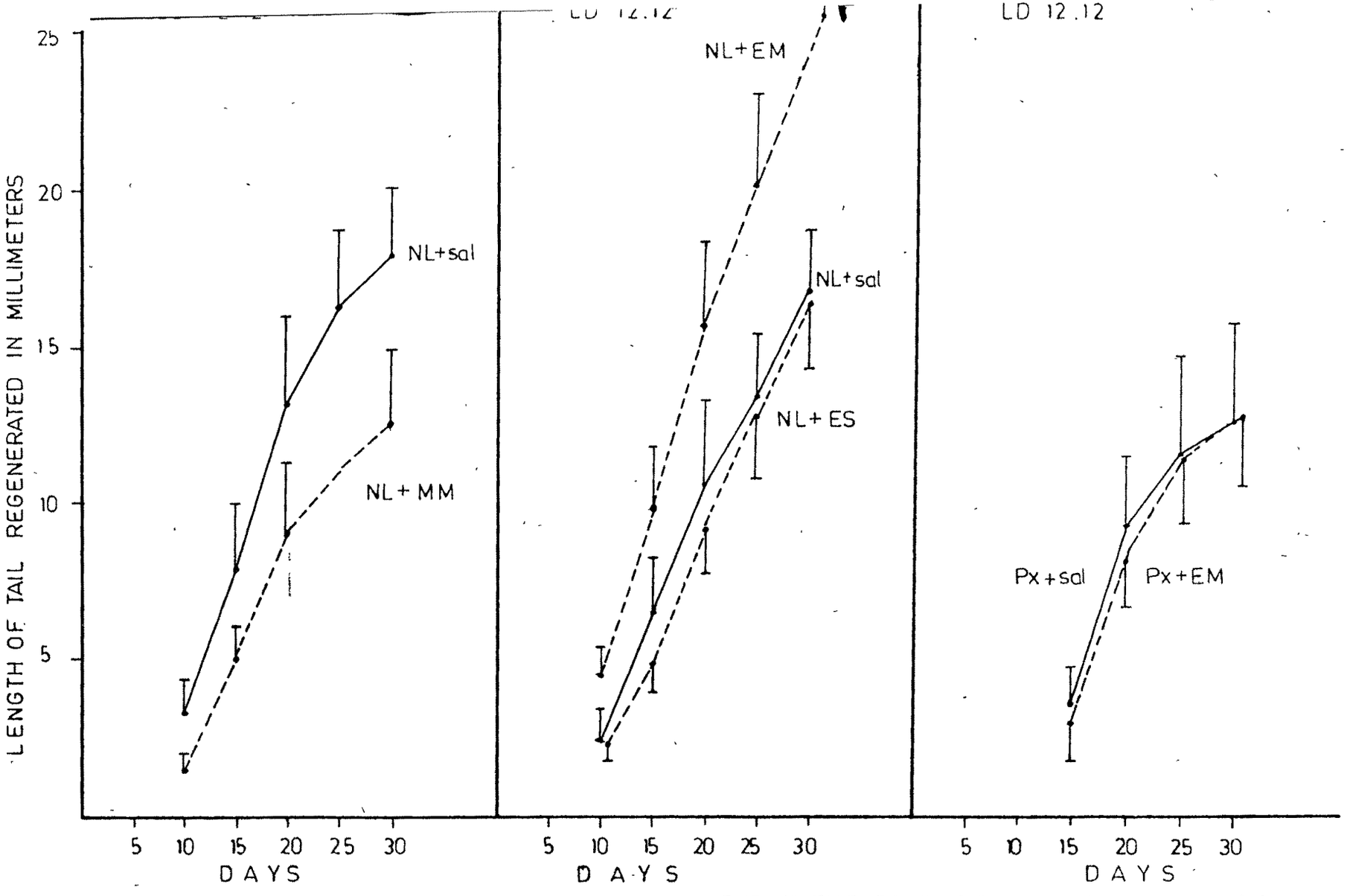

FIG.1 LENGTH OF TAIL REGENERATED IN H. FLAVIVIRIDIS DURING THE FIRST 30 DAYS POST-CAUDAL AUTOTOMY (± standard deviation, shown by vertical bars)
NL+SAL – NORMAL LIZARDS ADMINISTERED SALINE, NL+MM – NORMAL LIZARDS ADMINISTERED MELATONIN IN THE MORNING, NL+EM – NORMAL LIZARDS ADMINISTERED MELATONIN IN THE EVENING, NL+ES – NORMAL LIZARDS ADMINISTERED SEROTONIN IN THE EVENING, PX+EM – PINEALECTOMIZED LIZARDS ADMINISTERED MELATONIN IN THE EVENING, PX+SAL – PINELECTOMIZED LIZARDS ADMINISTERED SALINE.

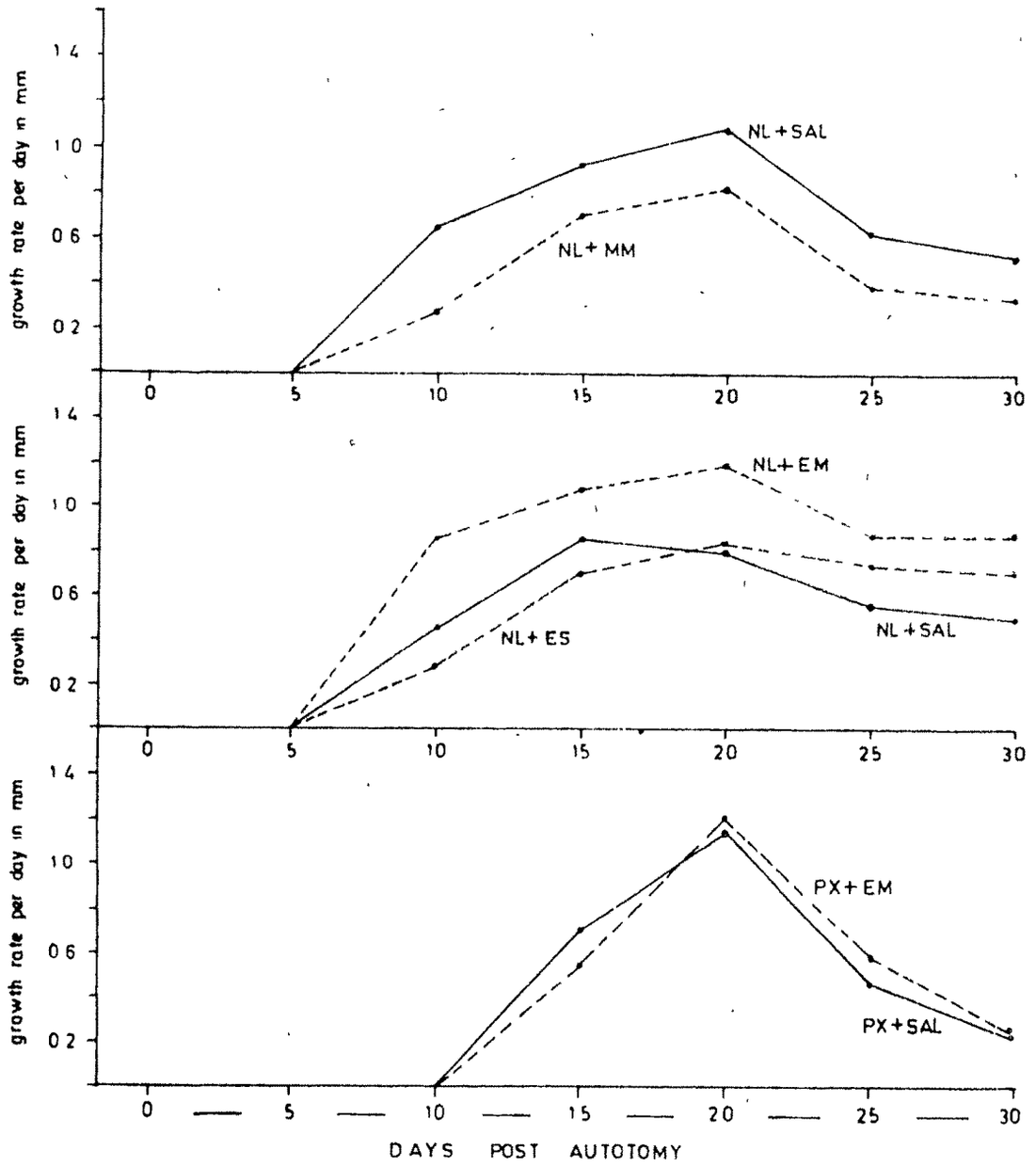

FIG 2 PER DAY GROWTH RATE IN BLOCKS OF 5 DAYS FOR 30 DAYS POST-CAUDAL AUTOTOMY IN H FLAVIVIRIDIS NL+SAL – NORMAL LIZARDS INJECTED WITH SALINE NL+MM – NORMAL LIZARDS INJECTED WITH MELATONIN IN THE MORNING NL+EM – NORMAL LIZARDS INJECTED WITH MELATONIN IN THE EVENING NL+ES – NORMAL LIZARDS INJECTED WITH SEROTONIN IN THE EVENING PX+EM – PINEALECTOMIZED LIZARDS INJECTED WITH MELATONIN IN EVENING PX+SAL – PINEALECTOMIZED LIZARDS INJECTED WITH SALINE.

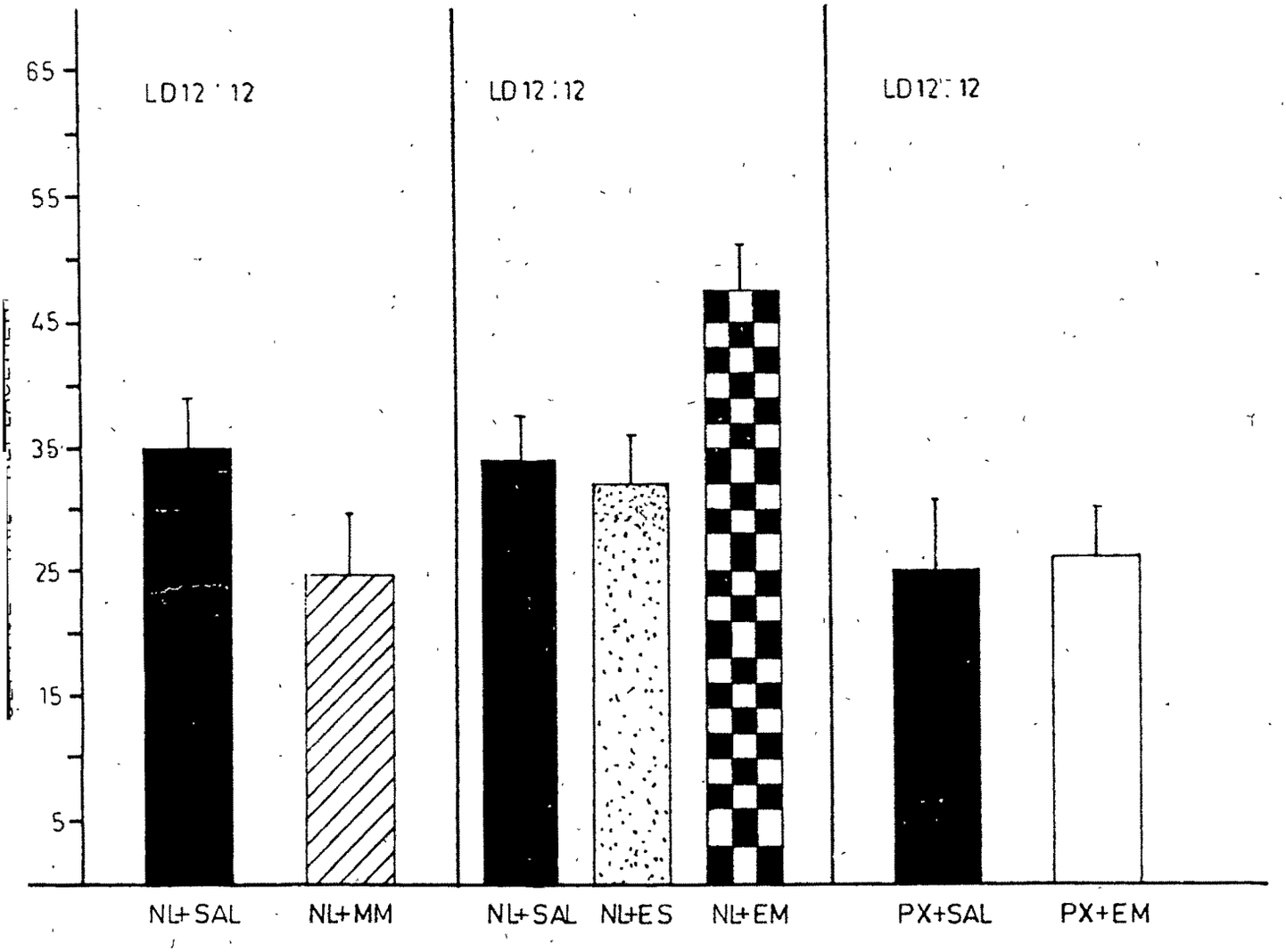

FIG.3 PERCENTAGE OF TAIL REPLACED AT THE END OF 30 DAYS POST-CAUDAL AUTOTOMY IN H FLAVIVIRIDIS. (±STANDARD DEVIATION, SHOWN BY VERTICAL BARS) NL+SAL – NORMAL LIZARDS ADMINISTERED SALINE, NL+MM – NORMAL LIZARDS ADMINISTERED MELATONIN IN THE MORNING, NL+EM – NORMAL LIZARDS ADMINISTERED MELATONIN IN THE EVENING, NL+ES – NORMAL LIZARDS ADMINISTERED SEROTONIN IN THE EVENING, PX+EM – PINEALECTOMIZED LIZARDS ADMINISTERED MELATONIN IN THE EVENING, PX+SAL – PINEALECTOMIZED LIZARDS ADMINISTERED SALINE.

23.4% respectively (Figures 1 and 3, and table 1). The pattern of growth rate (figure 2) indicates a linear increase peaking at 15-20 days post-caudal autotomy in all the groups of animals. All possible comparisons between the six groups of animals revealed no statistically significant difference between NL + Saline and NL + ES on one hand and between NL + MM, PX + EM and PX + Saline on the other. However, all other comparisons other than these were statistically significant at the 5% level (Duncan, 1955).

DISCUSSION

The results of the present investigation demonstrate that the time of administration of the indoleamine, melatonin determines its proregenerative or antiregenerative effect in the gekkonid lizard, Hemidactylus flaviviridis. Melatonin, when administered daily to NL lizards in the early portion of the light phase of the diurnal cycle produced an antiregenerative effect, but a proregenerative effect in the early hours of the dark phase. However, melatonin when administered to PX lizards in the early hours of the dark phase produced no effect. Serotonin, a precursor of melatonin, when administered intraperitoneally to intact Hemidactylus in the early hours of the dark phase of the diurnal cycle also did not significantly alter either the length of new growth (regenerate) or the percentage replacement of the lost (autotomized) tails. Our observations show that the initiation of regeneration, the daily growth rate, the length of tail regenerated

by day 30 and the percentage replacement of the lost tails at the end of 30 days were all significantly enhanced in lizards treated with melatonin at dusk and depressed in animals injected at dawn when compared with their respective saline-injected controls. These results may indicate that the diurnal rhythm in sensitivity to melatonin in the intact Hemidactylus results from some aspect of pineal function. Further, it is presumed that, in lizards as in mammals (Douglass, 1971), exogenous serotonin does not readily pass through the blood-brain barrier and, thus, cannot participate in the pineal mediated neuroendocrine mechanism that leads to sustained stimulatory tail elongation.

In view of the pivotal position occupied by reptiles in the evolution of homeotherms, a comparison of the pineal organs of lizards and mammals is probably appropriate here. The main secretory cells, the pineal parenchymal cells or pinealocytes are present in mammals whereas the lizard pineal contains photoreceptive cells, though they are also capable of synthesizing indolemaines including melatonin (Collin, 1979). Evidence suggests that the mammalian pinealocyte is evolved from the pineal photoreceptive cells of the lower vertebrates (Collin, 1979).

Experimental studies have revealed that one of the important enviornmental factors that stimulates regeneration in the newt (Maier and Singer, 1981) and lizards (Ndukuba and

Ramachandran, 1989a) is continuous light, while continuous darkness depresses it. However, this light effect is not mediated via the lateral eyes, or retinae, as tail regeneration progressed similarly in lizards with, or without, the lateral eyes (Ndukuba and Ramachandran, 1988). The pineal organ does mediate the photoperiodic messages through a possible neuroendocrine mechanism(s) since the regeneration process in PX lizards as well as in lizards completely deprived of light to the pineal is retarded by 50% (Ramachandran and Ndukuba, 1989a). There is now comprehensive evidence showing that p-CPA, an inhibitor of serotonin (5-HT) synthesis, at the level of the enzyme tryptophan hydroxylase (Koe and Weisman, 1966, Walker, 1983) and an agent employed for chemical pinealectomy, produced similar retardation effects as did complete pineal ablation, indicating that lizards with physically intact pineal organs but deprived of their ability to synthesize 5-HT do not exhibit the favourable influences of light on tail regeneration in lacertilians (Ramachandran and Ndukuba, 1989b). Moreover, the pineal organ is involved in the stimulatory influence of PRL on tail regeneration since exogenous PRL stimulated both the length of tail regenerated and the total percentage replacement of the lost tail in NL, but not PX, lizards maintained in continuous darkness (Ndukuba and Ramachandran, 1989a). And exogenous melatonin produced an antiregenerative effect at dawn and a proregenerative effect at dusk in NL, but not PX, lizards exposed to the intermediate photoperiodic regimen

of 12 hours of light and 12 hours of darkness (LD 12 : 12) (Ramachandran and Ndukuba, this chapter)

It is difficult to give a sound interpretative explanation for the observed dual effect of exogenous melatonin in intact Hemidactylus. However, two attractive hypotheses have been advanced earlier on the rhythmic changes of melatonin in vertebrate reproduction. Several lines of evidence support the view that, atleast in mammals, by extending circulating melatonin levels associated with dusk, the rise in PRL secretion is controlled by mechanisms not mediated by the pineal gland (Barrell and Lapwood, 1979; Munro et al., 1980; Symons et al., 1983). They believed it to be occurring through the hypothalamus or other higher center rather than at the level of the anterior pituitary gland. Alternatively, the indoleamines are known to produce their influence by way of the hypothalamo-hypophysial complex. In mammals and birds, it acts as anti-LH (Tamarkin et al., 1977). Daily melatonin injections given to the mouse, Peromyscus leucopus , during the early scotophase have no effect on the reproductive tract weight (Margolis and Lynch, 1981). A similar nocturnal effect was reported earlier in the Syrian hamster (Tamarkin et al., 1977). Such a daily effect supports Reiter's hypothesis (Reiter, 1980) that during the scotophase, when endogenous melatonin levels are high, there is down-regulation of the melatonin receptors. An increase in receptor sensitivity eventually occurs during the photophase. This increase is realised as a

greater responsiveness to the melatonin injections. Recent results, emanating from studies with ewes, provide convincing evidence in support of a third hypothesis; that the duration, not the circadian phase position, of melatonin is critical to the induction of photoperiodic effects (English et al., 1988; Wayne et al., 1988).

It has been reported here that exogenous melatonin injected to lizards at dawn produced an antiregenerative effect but a proregenerative effect at dusk. We will like to interpret these findings to suggest that the injection of melatonin at dawn produced its antiregenerative effect in Hemidactylus by prolonging the night time melatonin levels (English et al., 1988; Wayne, et al., 1988) to the photophase when melatonin receptors are sensitive (Reiter, 1980) and melatonin is a known mitotic inhibitor (Banerjee and Margulis, 1973). It may be suggested, however, that the proregenerative effect in lizards injected with melatonin at dusk may be under photoperiodic influence via the pineal organ leading to a serum PRL surge associated with dusk, possibly employing the serotonergic mechanism of PRL release. Apparently, exogenous melatonin had no effect in regenerating lacertilians at dusk rather the elevated serum PRL levels, as in mammals (Barell and Lapwood, 1979; Munro et al., 1980; Symons et al., 1983), may be responsible for the enhancement in the regenerative performance of NL lizards at this time of the day, since PRL is a known growth promoter in regenerating systems (Maier and Singer, 1981; Ndukuba and Ramachandran, 1989a). Similarly, PX lizards failed to respond to exogenous

melatonin at dusk. This finding, together with the above report (Ndukuba and Ramachandran, 1989a, chapter 7), support our contention that an intact pineal is somehow linked to the favourable influence of PRL on tail regeneration in lizards. Further, it has earlier been reported (Ramachandran and Ndukuba, 1989a, chapter 3; Ndukuba and Ramachandran, 1989a, chapter 1) that regenerating lizards maintain basal PRL levels which may account for the observed 50% regenerative ability in PX animals as well as in lizards exposed to continuous darkness. It can, therefore, be tentatively surmized that exogenous melatonin injected at dusk could not increase or decrease the basal PRL level in PX lizards and, thus, could not alter the regeneration process in this group of experimental animals. However, the possible influence of higher doses of melatonin in PX lizards needs to be ascertained and that forms the subject matter of an on-going investigation in our laboratory.

In conclusion, it is worth speculating the possibility of exogenous melatonin at dusk sparing or reducing the endogenous conversion of serotonin to melatonin, thereby providing a protracted mitogenic action of PRL release induced by a serotogenic mechanism. Similarly, exogenous melatonin in the morning may produce its antiregenerative effect either by its direct antimitotic effect (Banerjee and Margulis, 1973) or indirectly by dampening the increasing release in the light phase as both mitotic potential and PRL level have

been reported to peak during the light phase (Litwiller, 1940; Crim, 1975). As a sequel, the possible direct *in loco* effect of melatonin on tail regeneration is currently being looked into. However, other subtle modes of melatonin action either at higher centers of the hypothalamo-hypophysial axis on a differential basis in relation to the diurnal cycle, thereby affecting the ultimate neuroendocrine mechanism operative during tail regeneration in lizards cannot be overlooked.