CHAPTER 2

Simultaneous melatonin administration to rat neonates fails to correct the negative impact of light induced functional pinealectomy on adult testis functions and hormonal profiles

Photoperiod is a potent environmental stimulus that affects reproduction in a number of species (Bronson, 1989). Reproductive functions in many species are known to be responsive to changes in environmental periodicity (Berndtson and Desbarjins, 1974; Wurtman, 1975; Lincoln and Short, 1980). Annual cycles and day-length impart a seasonal rhythm of reproduction in many mammalian species, especially those living in the temperate zone (Chapmann, 1970; Reiter, 1974; Hoffmann, 1978). The effects of photoperiod in adults are manifested by maintenance of reproductive function on long photoperiods and regression of reproductive structures on short photoperiods (Goldman and Nelson, 1993). The pineal gland and its hormone melatonin have been implicated in the photoperiodic regulation of reproduction in all mammals studied to date (Stetson *et al.*, 1984). Different laboratory strains of rat differ in their responses to photoperiod, with more strains tested either having no response or slight to

moderate reproductive inhibition and/or reduced growth rate (Cohen and Mann, 1979; Vanecek and Illnerova, 1982; Rivest *et al.*, 1986; Aubert *et al.*, 1989; Boon *et al.*, 1997). The weak photoperiodic responses of most strains with the very short critical photoperiods for their effect have led to the characterization of rats as being functionally non-photoperiodic (Reiter, 1980; Wallen and Turek, 1981; Nelson *et al.*, 1982; Bronson, 1989). However, reproductive responses to short day and/or melatonin treatment can be induced in adult male rats by several procedures (Reiter *et al.*, 1968; Reiter *et al.*, 1969, 1971; Sorrentino *et al.*, 1971; Wallen and Turek, 1981; Peiper *et al.*, 1990). In another study of short photoperiod, testis development of Wistar or Sprague-Dawley rats was either not affected (Vanecek and Illnerova, 1982; Heidman and Sylvester, 1997) or moderately inhibited (Rivest *et al.*, 1986). Since, there was no study on rat neonates with regard to photoperiodic manipulation, effect of exposure of neonates to continuous light inducing functional pinealectomy (LFPx) from postnatal day 0 to day 21, on histomorphology of adult testis and hormone profile had been studied previously (Chapter 1). The above study showed neither any alteration in adult body and testes weight nor any gross changes in testis histoarchitechture except for significantly less number of germ cells and greater degree of germ cell degeneration/apoptosis. There were also certain alterations in the hormonal *milieu*. So in the

present study, effect of melatonin (MT) replacement simultaneous to LFPx has been studied to see whether melatonin can reverse the changes brought about by exposure to continuous light.

MATERIALS AND METHODS:

Animals and Maintenance:

Healthy male laboratory rat neonates (Charles Foster strain) were used for the present study. The animals were maintained in Sarabhai Research Center, with a constant temperature range of 21 ±2°C and under a lighting regimen of LD 8:16 or LD 24:0 throughout the experimental period of study. The animals were fed with standard diet (Amrut Rat Feed) and water *ad libitum*. The treatment was initiated on day '0' (day of birth) and terminated on day 21 postpartum.

Experimental Protocol:

The experimental setup was divided into two groups of study consisting of 10 animals each

Group I: Control (C)

Male rat neonates were maintained under normal lighting regimen of LD 8:16 and were provided with food and water *ad libitum*.

Group II: Functional Pinealectomy (LFPx) and treated with Melatonin (MT) (LFPx+MT)

Rat neonates were functionally pinealectomised by exposing them to continuous light of 250 lx intensity (LFPx) from day '0' to day '21'. These neonates were simultaneously administered with melatonin *i.p.* (40μg /animal/day) at 16.30 hrs.

Lighting schedules

Controls rats were exposed to a photoschedule of LD 8:16 with lights on at 09.00 hrs and lights off at 17.00 hrs. The experimental rats were exposed to continuous light (LD 24:0) with lights on fully in a separate enclosure. Lighting was from white fluorescent tubes and with an intensity of 250 lx.

Parameters and Methods of Evaluation:

As in chapter one

Histology and Histometry:

As in chapter one

Hormone Assays:

As in chapter one

STATISTICAL ANALYSIS:

All data are expressed as mean ± SEM. The data were analysed by student's 't' test and analysis of variance (ANOVA) wherever applicable, at 95% confidence limit.

RESULTS:

Body and Testes Weight

The LFPx+MT rats showed higher body weight at 35 and 60 days, however at 90 days the weight was not significantly different from controls. Though the absolute testes weight in LFPx + MT animals was significantly higher at 45 days but lower at 60 and 90 days than the controls, the relative weight was significantly low in LFPx+MT rats compared to controls (Tables 2.1 & 2.2; Figs. 2.1a, 2.1b, 2.1c, 2.2a & 2.2b).

Table 2.1: Chronological alterations showing body weight (g) and absolute (g) and relative (g/100g) testes weight of control and functionally pinealectomized melatonin treated rats

Treatment	Body Weight				Absolute Testes Weight				Testes Relative Weight			
	Age in Days				Age in Days				Age in Days			
	35	45	60	90	35	45	60	90	35	45	60	90
C	96.00 ±3.804	123.00 ±3.677	197.00 ±6.396	361.00 ±6.280	0.860 ±0.050	1.350 ±0.058	2.600 ±0.085	3.301 ±0.10	0.900 ±0.033	1.10 ±0.027	1.33 ±0.033	0.92 ±0.030
LFPx+MT	105 ±6.49	167^{c} ±1.64	207 ±5.40	350 ±3.01	0.68 ±0.10	1.98^{c} 0.05	2.24^{b} ±0.02	3.02^{a} ±0.01	0.64^{b} ±0.07	1.26^{a} 0.05	1.10^{c} 0.01	0.86 ±0.00
LFPx*	108 ±6.396	157^{c} ±2.456	268^{c} ±7.496	348 ±16.73	0.94 ±0.051	1.36 ±0.030	2.67 ±0.085	3.11 ±0.14	0.87 ±0.038	0.87^{c} ±0.006	1.00^{c} ±0.017	0.90 ±0.024
MT@	110.3^{c} ±2.490	152.3^{c} ±2.490	233.7^{c} ±3.242	398.7^{c} ±9.898	0.938 ±0.073	1.779^{c} ±0.053	2.714^{a} ±0.102	3.375^{c} ±0.07	0.854 ±0.084	1.167 ±0.042	1.16 ±0.063	0.847 ±0.043

C – Control; **LFPx+MT** – Functionally; Pinealectomized Melatonin treated; **LFPx** – Functionally Pinealectomized (Continuous Light); **MT** – Melatonin

Values expressed as Mean ± SEM of six animals. $^{a}p < 0.05$, $^{b}p < 0.005$, $^{c}p < 0.0005$

* - Values taken from Chapter 1

@ - Values taken from Lagu, (2001)

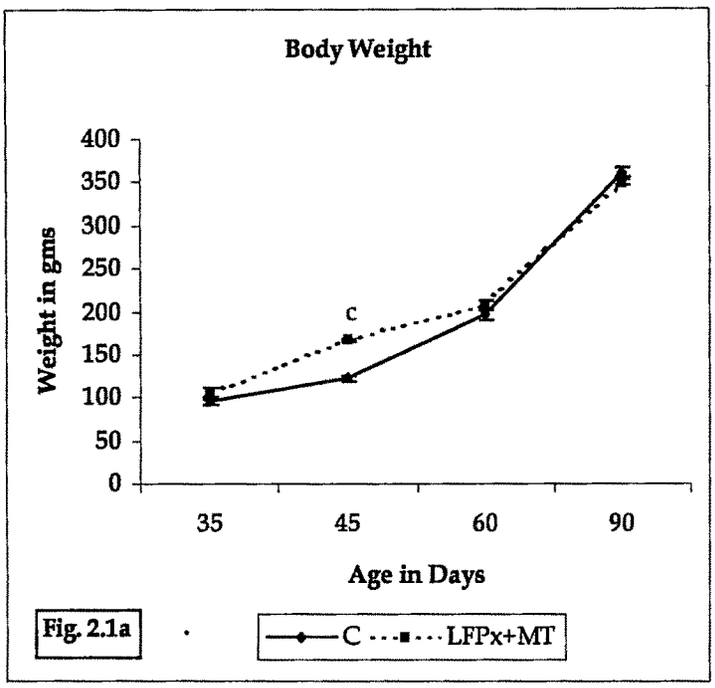
Table 2.2: Per day Body and Testes Growth Rate (g/day) in Control and Functionally pinealectomised Melatonin treated rats

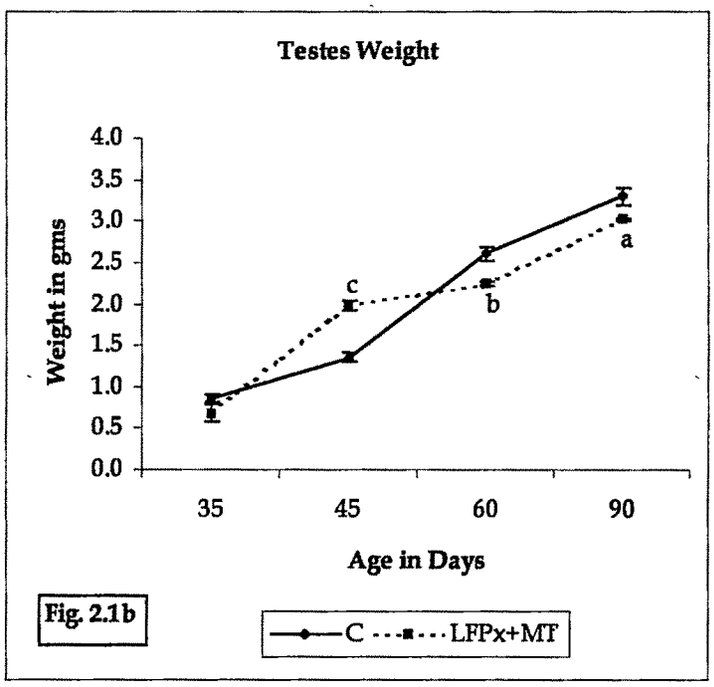
Treatment	Per Day Body Growth Rate				Per Day Testes Growth Rate			
	Age in days				Age in days			
	0-35	35-45	45-60	60-90	0-35	35-45	45-60	60-90
C	2.583	0.589	1.247	1.815	0.024	0.011	0.021	0.008
LFPx+MT	2.833	1.369	0.673	1.584	0.019	0.029	0.004	0.009
LFPx*	2.934	1.07	1.863	0.888	0.027	0.009	0.022	0.005
MT@	2.901	4.200	5.422	5.500	0.026	0.084	0.062	0.022

C – Control; LFPx+MT – Functionally Pinealectomized Melatonin treated; LFPx – Functionally Pinealectomized (Continuous Light); MT – Melatonin

* - Values taken from Chapter 1

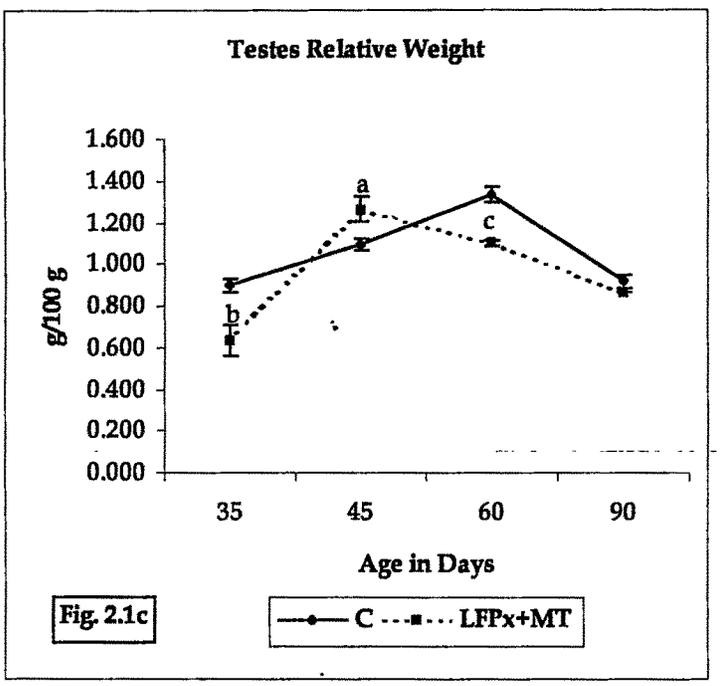
@ - Values taken from Lagu, (2001)





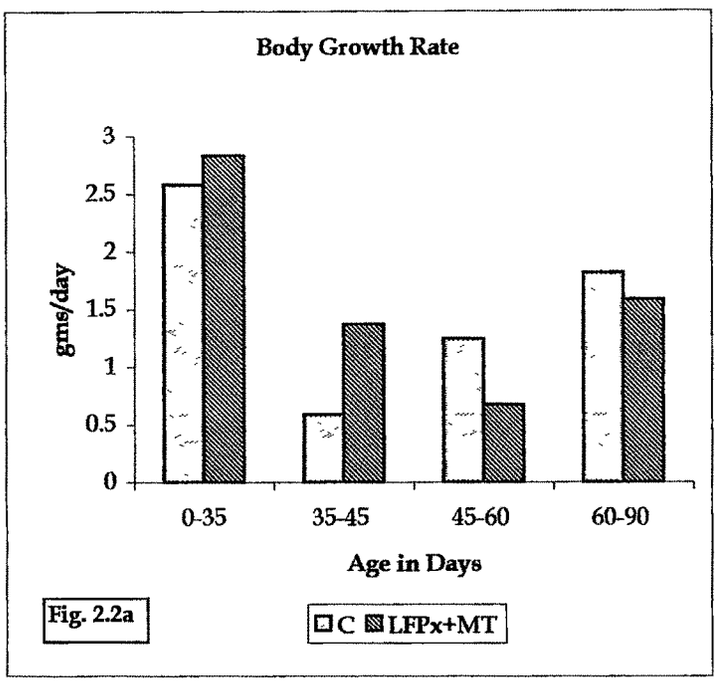
Figures 2.1a&b: Chronological alterations showing body and testes (g) weights in control (C) and functionally pinealectomised melatonin treated (LFPx+MT) rats

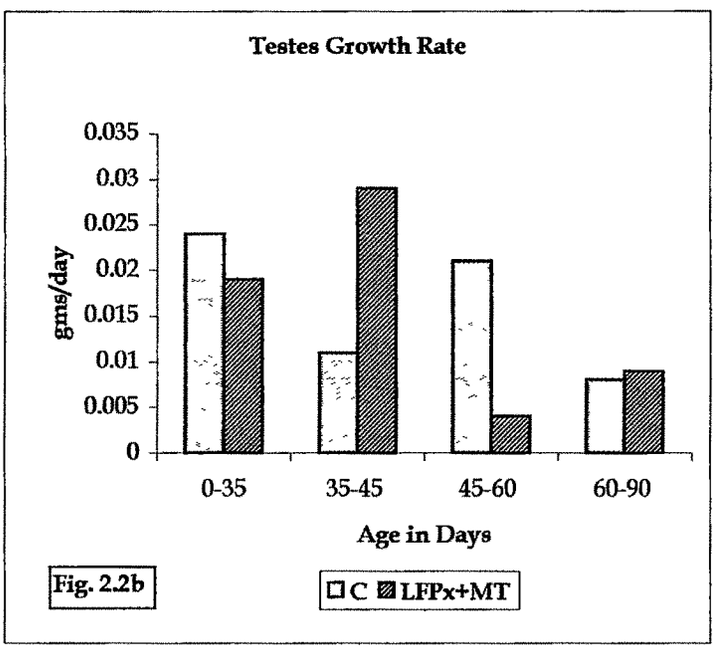
Values expressed as Mean ± SEM of six animals; $^{a}p<0.01$; $^{b}p<0.005$, $^{c}p<0.0005$



Figures 2.1c: Chronological alterations showing relative (g/100g) testes weight in control (C) and functionally pinealectomised melatonin treated (LFPx+MT) rats

Values expressed as Mean ± SEM of six animals; [a]$p<0.01$; [b] $p<0.005$, [c]$p<0.0005$





Figures 2.2a&b: Chronological alterations showing body and testes (g/day) growth rate in control (C) and functionally pinealectomised melatonin treated (LFPx+MT) rats

Values expressed as Mean ± SEM of six animals; [a]$p<0.01$; [b] $p<0.005$, [c]$p<0.0005$

Histology and Histometrics of Testis

Control:

At 35 days well formed seminiferous tubules with meiotic germ cells and well-formed interstitial cells could be seen. Spermatogenesis progressed to elongating spermatids by 45 days. By 60 days, tubules were well formed and spermatogenesis was fully established with sperms seen in most of the tubules. The Leydig cells were well formed. Fully established spermatogenesis in tubules and well formed Leydig cells were the features at 90 days (Plate IA).

LFPx+MT:

Though closely packed tubules with dense germ cells with meiotic cell types could be seen at 35 days, the Leydig cells were not well formed. Spermatogenesis advanced faster and sperms seen at 45 days. By 60 days, seminiferous tubules were closely packed with densely packed germ cells with decreased number of sperms. At 90 days, the tubules appeared smaller with very much decreased population of sperms (Plate II).

Histometrics

There was no significant difference in testis and seminiferous tubular volume, diameter or, total Sertoli cell count between control and experimental rats at 90 days. However, germinal epithelial thickness, tubular length and area of basement membrane were all significantly greater in the experimental rats. Total number of germ cells per testis was more but number of germ cells per meter length of tubules was significantly less in experimental rats (Table 2.3).

Table 2.3: Histometric enumeration of seminiferous tubules of Control and functionally pinealectomised melatonin treated rats at 90 days

Treatment	T_V In cc	S_D in cm	GE in cm	S_V in cc	S_L in cm	bm in cm^2	SC_N $\times 10^6$	TGC_T $\times 10^6$	AGC_T $\times 10^6$	TGC_M $\times 10^6$	AGC_M $\times 10^6$	% Loss
C	1.503 ±0.030	0.0279 ±0.0006	0.0074 ±0.0003	1.427 ±0.050	2321.03 ±94.200	204.045 ±5.230	32.49 ±1.800	311.00 ±6.300	280.84 ±5.600	13.39 ±0.260	12.1 ±0.150	10.00 ±0.0002
LFPx+MT	1.378^c ±0.009	0.0234^c ±0.0004	0.0128^c ±0.0005	1.3098 ±0.035	3039.1^c ±98.675	223.72 ±7.835	33.45 ±1.67	302.09 ±2.829	295.74 ±1.559	11.02^c ±0.360	9.71^c ±0.277	18.69^c ±1.843
LFPx*	1.419 ±0.055	0.0269 ±0.004	0.0192^c ±0.0001	1.348 ±0.065	2357.96 ±58.260	199.81 ±4.350	35.22^c ±2.60	330.8^a ±3.405	267.88 ±1.995	14.05 ±0.135	11.79 ±0.172	14.18^c ±0.119
MT@	1.541 ±0.070	0.033^c ±0.001	0.0098 ±0.002	1.448 ±0.060	1725.16^c ±45.30	177.205^c ±4.690	24.15^b ±1.600	340.00^b ±5.600	279.45 ±2.800	19.70^c ±0.350	16.20^c ±0.210	19.00^c ±0.200

C – Control; **LFPx+MT** – Functionally Pinealectomized Melatonin treated; **LFPx** – Functionally Pinealectomized (Continuous Light); **MT** – Melatonin

Values expressed as Mean ± SEM of minimum fifteen observations. $^a p < 0.05$, $^b p < 0.005$, $^c p < 0.0005$

* - Values taken from Chapter 1; @ - Values taken from Lagu, (2001)

T_V - Volume of Testis, S_D - Seminiferous tubule diameter, GE - Germinal epithelial thickness, S_V - Volume of Seminiferous tubule, S_L - Length of seminiferous tubule, bm - basement membrane area of the seminiferous tubule, SC_N - Total Sertoli cell number in testis, TGC_T - Theoretical germ cell number per testis, AGC_T - Actual germ cell number per testis, TGC_M - Theoretical germ cell number per meter of seminiferous tubule, AGC_M - Actual germ cell number per meter of seminiferous tubule.

PLATE – II

Figures 1 – 8: **Photomicrographs of sections of testis of LFPx rats treated with melatonin.**

Figures 1 and 2: Sections of testis of 35 day old rats showing closely packed tubules with higher density of germ cells and interstitium not very well formed.

Figures 3 and 4: Section of testis of 45 day old rats of showing well established spermatogenesis and sperms in many tubules.

Figures 5 and 6: Section of testis of 60 day old rats showing closely packed tubules with reduced sperm production.

Figures 7 and 8: 90 day old testis section showing more number of reduced sperms and more degeneration.

Figures: 1, 3, 5, & 7 – 250 x
Figures: 2, 4, 6, & 8 – 400 x

L – Lumen, S – Sperms, St – Spermatids, I - Interstitium

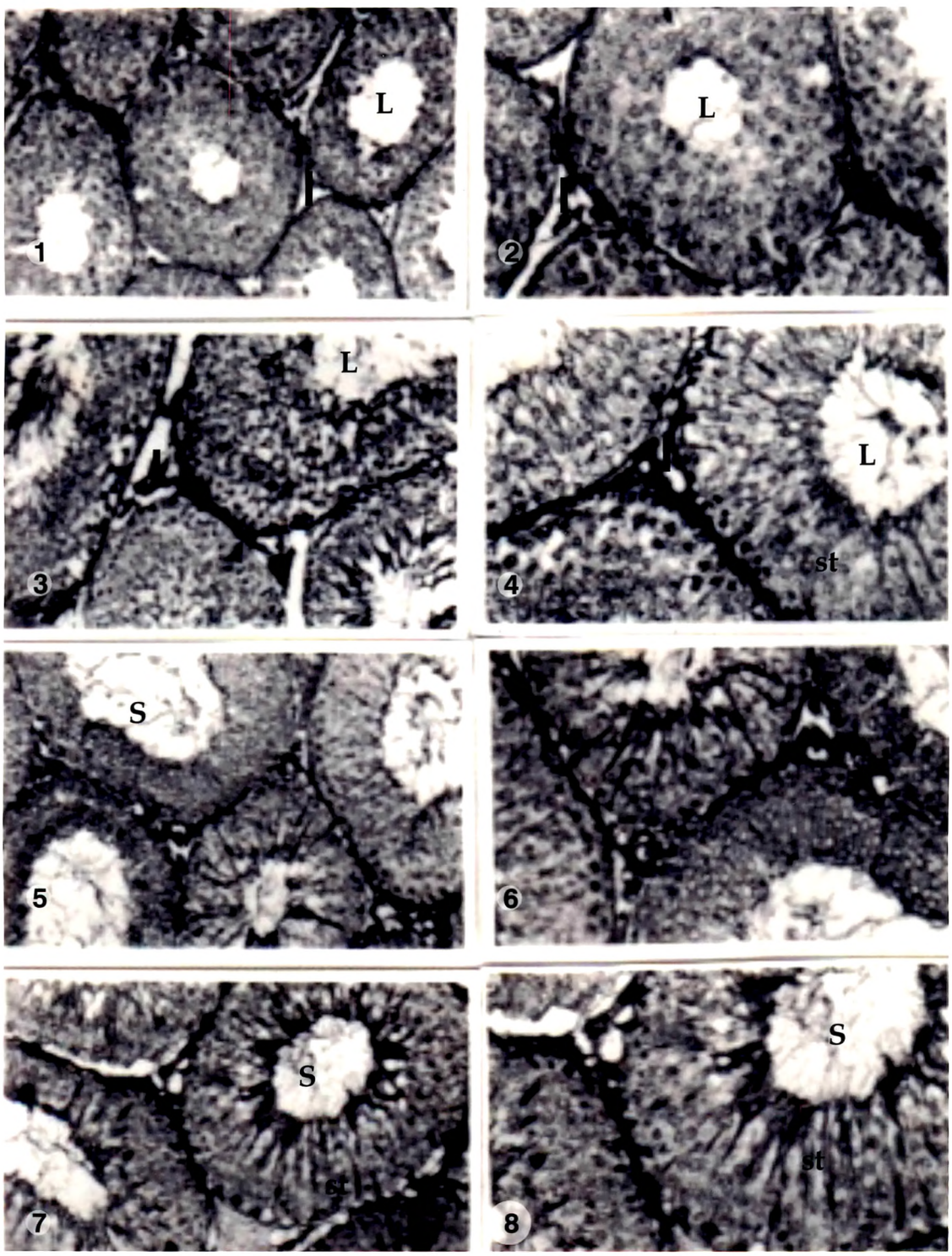


PLATE - II

Photomicrographs of Testis of Control & Functionally Pinealectomised Melatonin Treated Rats

Serum Hormone Profile

TSH and T4 levels were higher at 35 and 45 days and significantly lower at 90 days in LFPx+MT. However, T3 levels were significantly lower throughout from 35-90 days. Serum corticosterone levels were higher at all ages in experimental rats while LH and testosterone levels were significantly lower, though testosterone level at 35 days was higher significantly (Tables 2.4 & 2.5; Figs. 2.4a, 2.4b, 2.4c, 2.5a, 2.5b & 2.5c).

Table 2.4: Serum TSH, T_4 and T_3 (ng/ml) levels of Control and functionally pinealectomised melatonin treated rats at 90 days

Treatment	TSH				T_3				T_4			
	Age in days				Age in days				Age in days			
	35	45	60	90	35	45	60	90	35	45	60	90
C	6.60 ±0.12	6.87 ±0.11	7.49 ±0.14	5.44 ±0.06	0.45 ±0.01	0.30 ±0.10	0.60 ±0.08	0.65 ±0.05	0.58 ±0.08	1.17 ±0.06	2.56 ±0.02	2.36 ±0.22
LFPx+MT	8.33^{c} ±0.094	10.61^{c} ±0.098	13.54^{c} ±0.137	7.69^{c} ±0.054	0.26^{c} ±0.013	0.20 ±0.097	0.63 ±0.074	0.30^{b} ±0.069	0.85^{b} ±0.064	1.57^{b} ±0.057	3.48^{c} ±0.032	1.87^{a} ±0.162
LFPx*	13.3^{c} ±0.06	11.6^{c} ±0.06	9.2^{c} ±0.13	6.81^{c} ±0.04	0.91^{c} ±0.01	0.55^{a} ±0.02	0.71 ±0.02	0.79^{b} ±0.01	3.00^{c} ±0.07	2.72^{c} ±0.02	3.09^{c} ±0.01	2.90^{a} ±0.02
MT@	26.01^{c} ±1.577	9.050^{a} ±0.804	7.804 ±0.652	9.001^{c} ±0.365	0.484 ±0.056	0.800^{c} ±0.074	1.051^{c} ±0.084	0.910^{c} ±0.095	1.700^{c} ±0.053	2.805^{c} ±0.015	2.212^{b} ±0.068	1.742^{a} ±0.018

C – Control; **LFPx+MT** – Functionally Pinealectomized Melatonin treated; **LFPx** – Functionally Pinealectomized
(Continuous Light); **MT** – Melatonin
Values expressed as Mean ± SEM of four samples. $^{a}p < 0.05$, $^{b}p < 0.005$, $^{c}p < 0.0005$

* - Values taken from Chapter 1
@ - Values taken from Lagu, (2001)

Table 2.5: Serum LH, Corticosterone and Testosterone (ng/ml) levels of Control and Functionally pinealectomised melatonin treated rats at 90 days

Treatment	LH				Corticosterone				Testosterone			
	Age in days				Age in days				Age in days			
	35	45	60	90	35	45	60	90	35	45	60	90
C	16.45 ±0.634	21.75 ±0.854	48.12 ±1.235	53.25 ±1.031	0.80 ±0.618	1.00 ±0.155	4.80 ±0.705	4.50 ±1.699	0.56 ±0.166	2.23 ±0.278	2.62 ±0.217	4.37 ±0.265
LFPx+MT	14.03^{b} ±0.145	12.3^{c} ±0.743	10.9^{c} ±1.112	8.2^{c} ±0.028	7.52^{c} ±0.0866	8.90^{c} ±0.124	10.15^{b} ±0.493	25.50^{c} ±1.552	1.50^{b} ±0.194	1.60^{a} ±0.143	1.10^{b} ±0.116	1.10^{c} ±0.219
LFPx*	5.48^{c} ±0.02	20.69 ±0.01	39.51^{b} ±0.01	23.02^{c} ±0.07	2.08^{a} ±0.02	3.50^{c} ±0.03	2.17^{b} ±0.01	1.48 ±0.01	6.60^{c} ±0.01	6.20^{c} ±0.02	5.90^{c} ±0.04	5.60^{b} ±0.03
MT@	17.12 ±1.024	16.39^{b} ±1.008	17.77^{c} ±1.025	20.99^{c} ±2.025	24.70^{c} ±2.075	13.40 ±1.80	44.01 ±4.732	43.01 ±4.856	1.501^{c} ±0.048	1.503^{a} ±0.054	1.300^{c} ±0.058	2.002^{b} ±0.088

C – Control; **LFPx+MT** – Functionally Pinealectomized Melatonin treated; **LFPx** – Functionally Pinealectomized (Continuous Light); **MT** – Melatonin

Values expressed as Mean ± SEM of four samples. $^{a}p < 0.05$, $^{b}p < 0.005$, $^{c}p < 0.0005$

* - Values taken from Chapter 1

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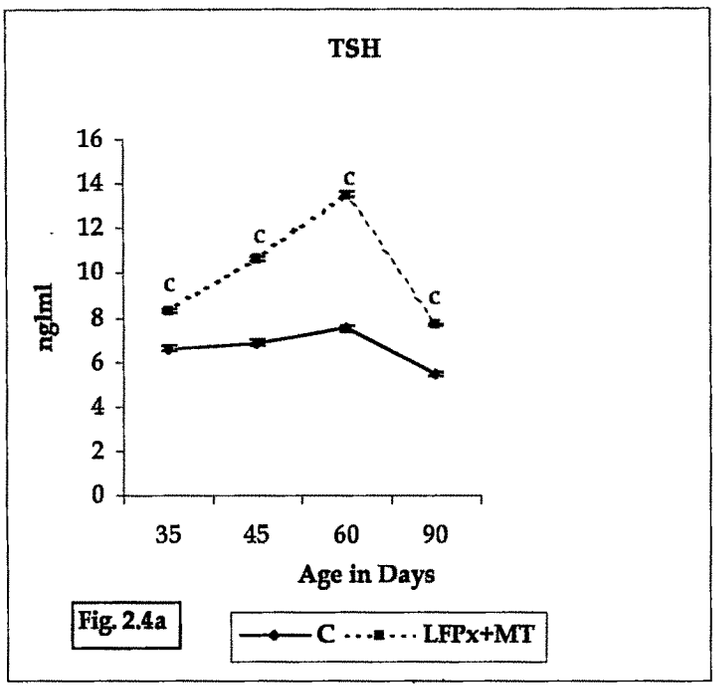
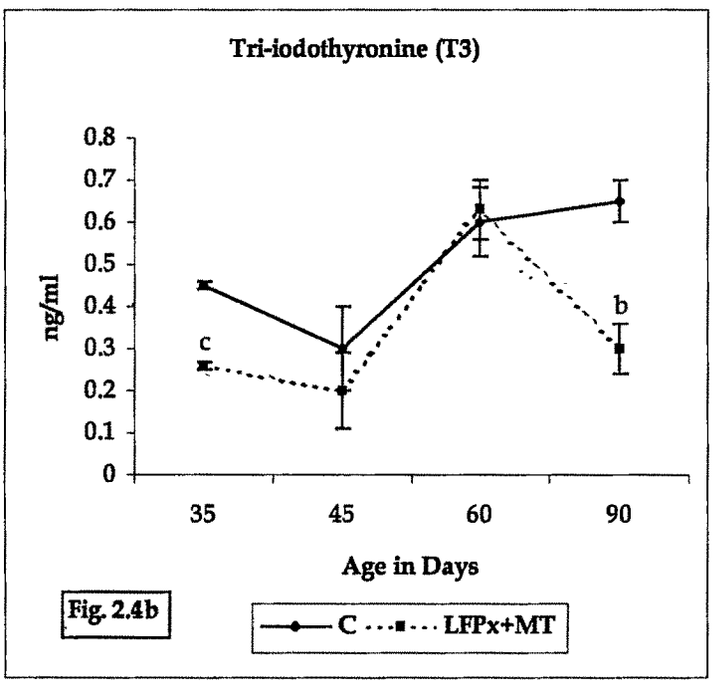




Figures 2.4a&b: Chronological alterations showing serum TSH and T3 (ng/ml) levels in control (C) and functionally pinealectomised melatonin treated (LFPx+MT) rats

Values expressed as Mean ± SEM of four samples;

$^{a}p<0.01$; $^{b}p<0.005$, $^{c}p<0.0005$

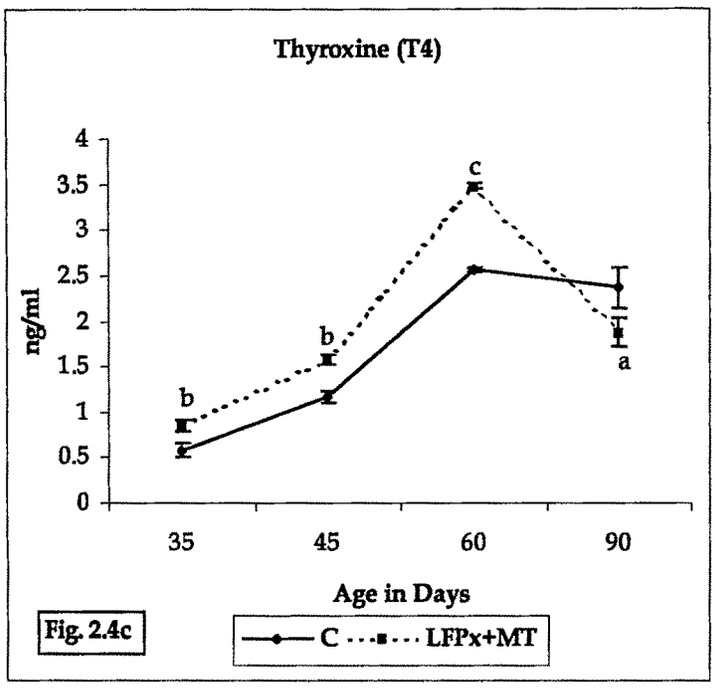


Fig. 2.4c

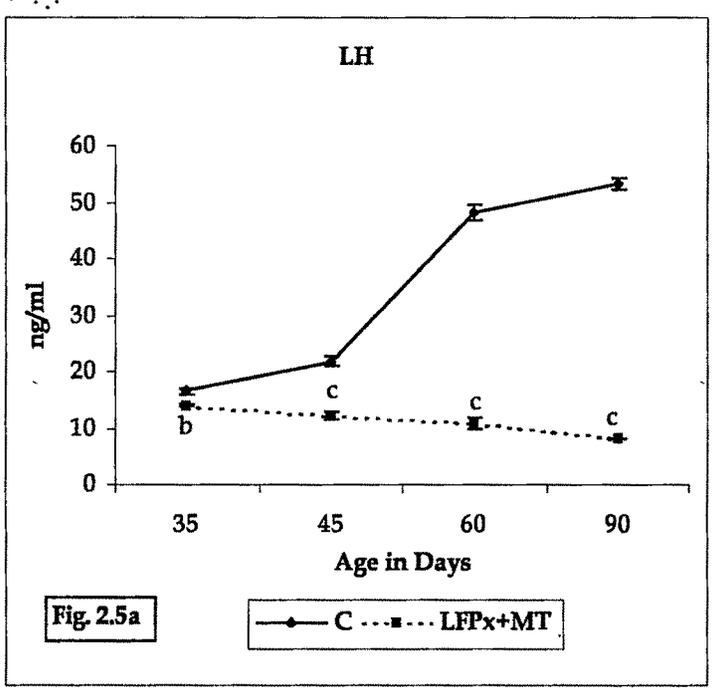
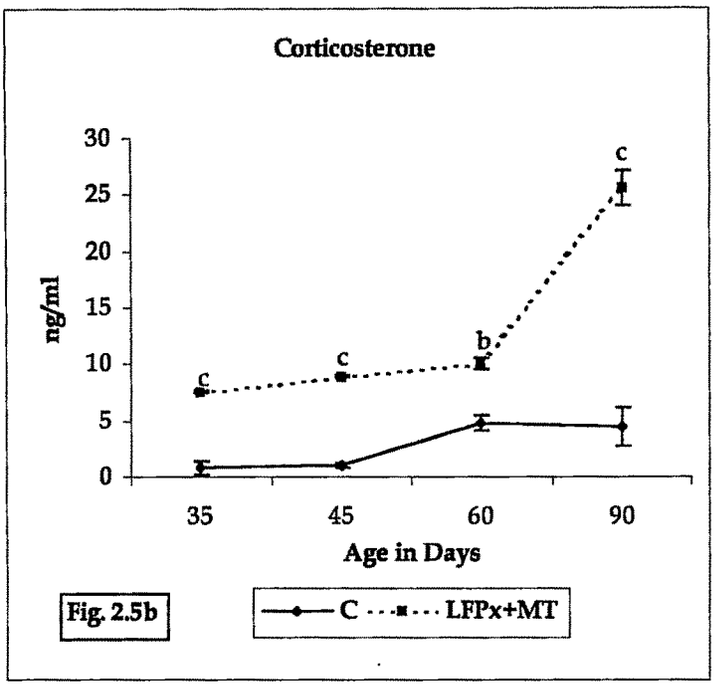


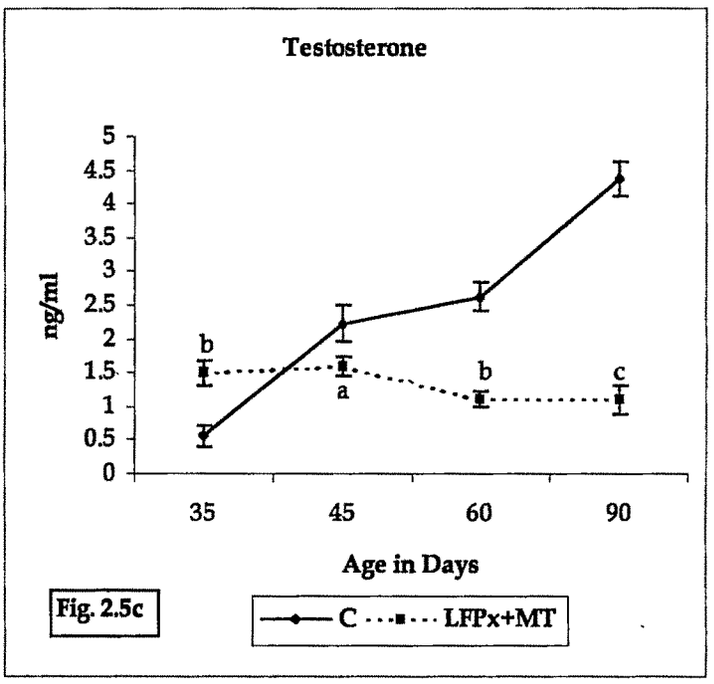
Fig. 2.5a

Figures 2.4c& 2.5a: Chronological alterations showing serum T4 and LH (ng/ml) levels in control (C) and functionally pinealectomised melatonin treated (LFPx+MT) rats

Values expressed as Mean ± SEM of four samples;

$^{a}p<0.01$; $^{b}p<0.005$, $^{c}p<0.0005$





Figures 2.5b&c: Chronological alterations showing serum Corticosterone and Testosterone (ng/ml) levels in control (C) and functionally pinealectomised melatonin treated (LFPx+MT) rats

Values expressed as Mean ± SEM of four samples;

$^{a}p<0.01$; $^{b}p<0.005$, $^{c}p<0.0005$

DISCUSSION:

Some past studies have demonstrated neuroanatomical connections that mediate reproductive sensitivity to photoperiod in laboratory rats (Wallen and Turek, 1981; Nelson *et al.*, 1982). These connections have been considered to be non-functional in adults to, functional in young rats of some strains (Sizonenko *et al.*, 1985; Heideman and Sylvester, 1997). Peri-pubertal rodents are considered to be theoretically more likely to be responsive to photoperiod than are adults (Donham *et al.*, 1989; Horton and Rowsemitt, 1992). Higher sensitivity of younger animals to photoperiods has been reported in rats and some other rodents (Sorrentino *et al.*, 1971; Johnston and Zucker, 1979; Donham *et al.*, 1989; Stanfield and Horton, 1996). In the previous study, neonates were subjected to LFPx from day 0 to day 21and assessed at 35, 45, 60 and 90 days of age, for their body and testis weight and histoarchitecture of testis. This exposure to continuous light amounting to a state of functional pinealectomy did not show any significant change in body weight or absolute testis weight at 90 days compared to controls though there were higher growth rates initially (Chapter 1). However, another study involving surgical pinealectomy had shown increased adult body weight (Sharma and Ramachandran, Unpublished). Apparently, surgical pinealectomy has a differential effect on long- term body and testis weight as, complete lack of pineal abolishes pineal rhythm

permanently while, in LFPx, melatonin rhythm gets re-established on return back to ambient photoperiod. In the present study on simultaneous replacement of melatonin to neonates subjected to LFPx, there is no significant change in body weight while; the absolute and relative weights of testes are significantly reduced. Obviously, LFPx combined with MT treatment has the potential to decrease testis weight. Early onset of spermatogenesis with appearance of sperms by 45 days seen in the present study as against 60 days in control animals is similar to the effect seen in rats treated with MT alone in an earlier study (Ramachandran *et al.*, 2004). This early appearance of sperms can be correlated with the increased T4 and T3 levels in the pre-pubertal period. However, with reference to the pituitary-thyroid axis, LFPx+MT tended to reverse the increase in TSH and T4 levels seen in LFPx rats till puberty, to intermediate levels between control and LFPx levels. Similarly, the TSH and T4 levels, which were less than controls at 90 days in LFPx, were reversed towards control levels in LFPx+MT rats. In contrast, LFPx+MT seems to have an independent effect of reducing T3 levels significantly below control levels throughout, while LFPx alone had a significant elevating influence (Chapter 1). It is now clearly established that higher thyroid hormone levels in the neonatal to weanling periods induce early differentiation of Sertoli cells (Maran and Aruldhas, 2002). Based on the present

observations it is obvious that even an increase in T4 alone in the neonates during the pre-weanling period without an elevation in T3 is sufficient to induce early Sertoli cell differentiation and hence early appearance of sperms in LFPx+MT rats.

Comparison of the histometric enumeration of seminiferous tubules of control and LFPx+MT rats reveals that apart from an increased germinal epithelial thickness (in LFPx alone, it is lower), there is also a significant increase in tubular length, basement membrane area and total number of germ cells. This is in contrast to the changes observed in LFPx or melatonin treated rats (Chapter 1; Ramachandran *et al.*, 2004). Apparently, LFPx+MT have effects on the above parameters of testis, distinct to those induced by LFPx or MT alone. Though there is a generalized increase in the number of germ cells, it is also obvious that late spermatids and spermatozoa are sloughed off into the lumen with more degenerative/apoptotic changes. This effect of increased loss of advanced germ cells seems to be similar to that seen in MT treated rats which was related to the increased apoptotic changes induced by the higher corticosterone levels seen in these rats (Ramachandran *et al.*, 2004). In the LFPx+MT rats too, the corticosterone levels are elevated significantly if not as high as in MT rats (Ramachandran *et al.*, 2004) but more than that seen in LFPx rats (Chapter 1). The pre-pubertal increase in corticosterone seems to induce degeneration/apoptotic

Further, an earlier study from this laboratory has also reported decreased LH and T levels in adult rats subjected to neonatal melatonin excess by exogenous administration (Ramachandran *et al.*, 2004). The herein observed poorly developed Leydig cells also attests to the lower T titres. However, the increased T level in the early period (35 days) could be the probable reason for the noted early onset of spermatogenesis and appearance of sperms. The continued lower T level thereafter could also be related with the loss of advanced germ cells from the tubules.

Finally, it can be concluded from the present observations that simultaneous melatonin administration in LFPx neonates is not able to reverse all the effects of LFPx but show effects which are either of LFPx, melatonin, intermediate between the two, or even, unique and independent.

SUMMARY:

The objective of the present study was to see effect of simultaneous melatonin administration to LFPx neonates on histomorphology of adult testis and hormone profiles. The rat neonates were exposed to continuous light and simultaneously administered with 40µg melatonin/animal/day at 16.30 hrs from day 0 to day 21. There was no much difference in body weight of LFPx+MT rats compared to controls. But absolute testis and relative weights were lower in experimental animals than the controls. Histology of testis showed that spermatogenesis was advanced relative to controls and sperms could be seen at 45 days in LFPx+MT rats and, tubules appeared to be smaller with much decreased population of sperms at 90 days. The total number of germ cells per testis was more but the number of germ cells per length of the tubule was significantly less in the experimental rats. Serum corticosterone levels were higher whereas the T3 levels were significantly lower at all age groups in LFPx+MT rats. The TSH and T4 titres were higher at 35 & 45 days and lower at 90 days and reverse was the case with LH and T levels. Finally it can be concluded that simultaneous MT administration to LFPx neonates is unable to reverse all the effects of LFPx and has shown intermediate changes between those of LFPx and MT or even unique and independent effects.