

## EFFECTS OF PINEAL INDOLES AND PARACHLOROPHENYLALANINE ON SEASONAL REPRODUCTION IN THE PIGEON

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### Summary

The effect of melatonin, methoxytryptophol (ML), methoxytryptamine (MT) and parachlorophenylalanine (p-CPA) on the mass and histology of the testes and the adrenal and thyroid glands and on serum thyroid hormone levels have been studied in the recrudescing phase of the feral pigeon *Columba livia*. All the pineal indoles showed a common anti-gonadal effect, resulting in the arrest of spermatogenesis and the degeneration of germ cells. Treatment with melatonin resulted in adreno-cortical enlargement and medullary hypertrophy, whereas ML, MT and p-CPA induced medullary enlargement and

hypertrophy without having much influence on the cortex. Treatment with melatonin and ML brought about colloid retention in the thyroid follicles, while MT and p-CPA induced colloid depletion and hypertrophy of the follicular epithelium. From the present study, it can be concluded that both pineal indoles and p-CPA induce testicular regression in the breeding phase but do so by different mechanisms.

Key words: pineal indoles, pigeon, parachlorophenylalanine, testis, adrenal, thyroid, *Columba livia*.

### Introduction

The pineal gland, through its hormone melatonin, has been implicated in the mediation of photoperiodically induced changes in pubertal development and in seasonal reproduction in mammals (Reiter, 1991). The essential link between melatonin and the occurrence of seasonal breeding has been identified as the hypothalamic gonadotropin-releasing hormone (GnRH) pulse generator (Silman, 1991). Attempts to show a similar relationship between the pineal gland and seasonal reproduction in birds has, however, met with little success. Even in cases where there appeared to be some pineal effects, these were transitory and marginal (Ralph, 1981; Gwinner *et al.* 1981), leading to the conclusion that the pineal gland may not be of primary importance in the control of avian reproduction. Subsequent studies on Japanese quail (Simpson *et al.* 1983), on the turkey (Siopes and El Halawani, 1986, 1989; Siopes and Underwood, 1987) and on the American tree sparrow (Wilson, 1991) have provided further evidence that the pineal gland is not an important component of seasonal reproduction in birds.

In contrast to these reports from temperate avian species, observations suggesting an involvement of the pineal gland in seasonal reproductive activity have been made in some tropical species of birds. Our previous studies on both domestic and feral pigeons have shown that the pineal gland has an influence on the gonads, since pinealectomy in both the recrudescing and breeding seasons resulted in testicular regression (Ramachandran *et al.* 1987; Ramachandran and Patel, 1986).

Similar results have been reported by Haldar and Ghosh (1990) in the Indian jungle bush quail *Perdica asiatica*. Concurrent with the pinealectomy-induced testicular regression, alterations in the functional status of both the adrenal and thyroid glands were seen, indicating definite pineal–adrenal and pineal–thyroid interactions (Patel *et al.* 1985; Ramachandran and Patel, 1986). As a corollary to the above observations, the influence of exogenous melatonin on reproductive function in tropical species needed evaluation since melatonin is the principal pineal indole hormone and mediates most of the effects of the pineal gland. In addition to melatonin, other methoxyindoles, including methoxytryptophol (ML) and methoxytryptamine (MT), may have a role in reproductive function (Reiter, 1981; Pevet, 1983, 1985). The influence of pineal indoles on reproductive function and their time-specific effects have largely been evaluated in seasonally breeding mammals, although various other submammalian species have been examined (Misra and Thapliyal, 1979; Pevet *et al.* 1986; Ng, 1987; Haldar and Ghosh, 1988; Haldar and Pandey, 1989; John *et al.* 1990). The comparative effects of methoxyindoles and of parachlorophenylalanine (p-CPA), a specific depletor of serotonin levels, on gonadal function have not been studied in birds. In this context, we have examined the effects of melatonin, ML, MT and p-CPA on the mass, structure and function of testicular, adrenal and thyroid tissue in male pigeons.

### Materials and methods

Adult feral blue rock pigeons *Columba livia* (Gmelin) (250–300 g) obtained from a local animal dealer were used in the present study during January and February. Although tropical pigeons appear to have an extended period of breeding, observations in our laboratory have established that most of the feral pigeons used in our experiments have an active breeding period extending from March to May, followed by gonadal regression and a recrudescence phase between January and March. A few individuals have a secondary breeding season from September to November (Ambadkar and Kotak, 1976a,b; Ramachandran *et al.* 1987; Asnani and Ramachandran, 1993). In addition to these findings, other reports have suggested that many tropical species have discrete breeding seasons and that, even in species that appear to reproduce at all times of year, individuals may have distinct cycles of gonad maturation and regression (Baker, 1938; Miller, 1962). The birds used for the present study were housed in a well-ventilated aviary and provided with food and water *ad libitum* under a natural photoperiod of 11 h:13 h light:dark. After a week of acclimation, the birds were sexed using a laparoscope, and male birds of similar testicular condition were selected for experimentation.

### Experimental design

In the recrudescence phase (December–February), 36 male pigeons were randomly divided into six groups of six each. *Group i* (control). These birds were given daily injections of saline with a few drops of ethanol at 17:00 h. *Group ii* (melatonin morning, MM). These birds were given daily injections of 50 µg of melatonin in 0.1 ml at 09:00 h. *Group iii* (melatonin evening, ME). These birds were given daily injections of 50 µg of melatonin in 0.1 ml at 17:00 h. *Group iv*

(methoxytryptophol, ML). These birds were given daily injections of 25 µg of ML in 0.1 ml at 17:00 h. *Group v* (methoxytryptamine, MT). These birds were given daily injections of 25 µg of MT in 0.1 ml at 17:00 h. *Group vi* (parachlorophenylalanine, p-CPA). These birds were given daily injections of 1 mg of p-CPA in 0.1 ml at 17:00 h. All the treatments were given intraperitoneally for 15 days, and on the sixteenth day the birds were weighed and killed by decapitation under mild ether anaesthesia using an ether cone, taking maximum care to avoid any stress during handling.

### Solutions

Pineal indoles and p-CPA were obtained from Sigma Chemical Company, St Louis, USA. 5 mg of melatonin, 2.5 mg of ML, 2.5 mg of MT or 100 mg of p-CPA was first dissolved in a few drops of 100% ethanol and diluted to 10 ml with 0.9% phosphate-buffered saline. Parachlorophenylalanine was dissolved in 0.9% phosphate-buffered saline, brought to pH 6.0 by the addition of 5 mmol l<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub> and stored at 4 °C for daily use.

### Parameters and methods of evaluation

#### Mass

Immediately after decapitation, the viscera were exposed and the testes, adrenals and thyroid were excised and individually weighed on a digital Mettler balance. The absolute masses thus obtained were converted to relative masses expressed as a percentage of body mass.

#### Histology

Tissues were fixed (immediately after decapitation) in Bouin's fluid for 24 h, dehydrated in alcohol grades and cleared

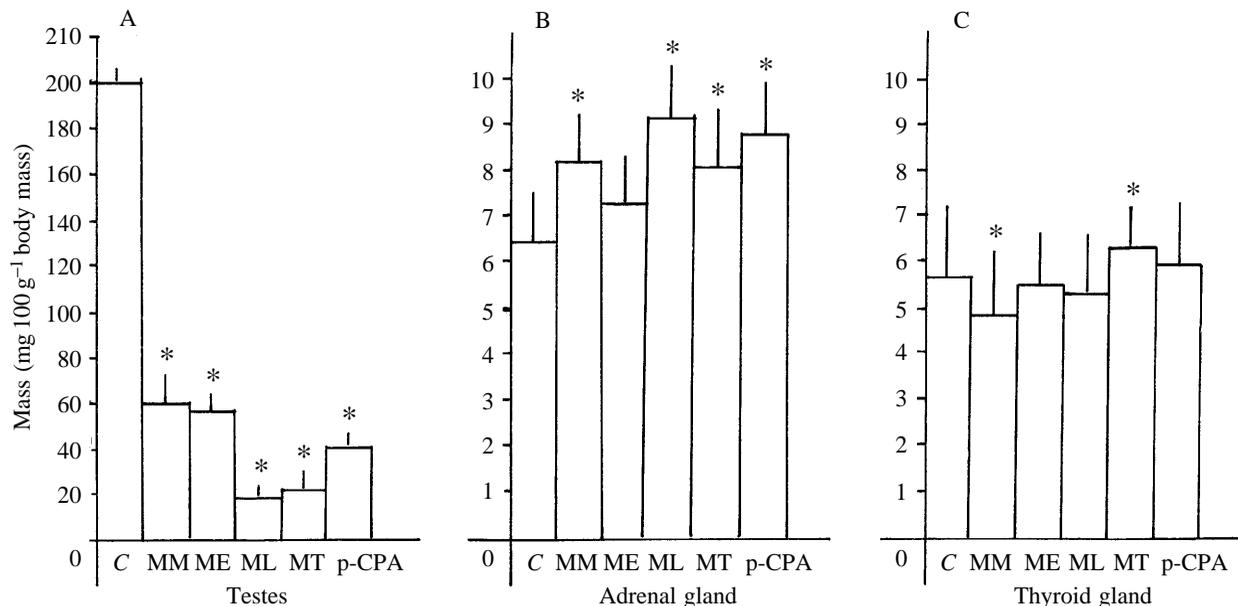


Fig. 1. Changes in mass of (A) testes, (B) adrenal glands and (C) thyroid glands in pigeons treated with a variety of methoxyindoles or p-CPA during the recrudescence phase. \*Significantly different from the control value at  $P < 0.05$ ; values are given as means + S.D.,  $N = 6$ . MM, melatonin morning; ME, melatonin evening; ML, methoxytryptophol; MT, methoxytryptamine; p-CPA, parachlorophenylalanine; C, control.

in toluene prior to embedding in paraffin wax. Sections 5  $\mu\text{m}$  thick were cut on a microtome, stained with Haematoxylin and Eosin and mounted in diesterase phthalate xylene.

#### Collection of serum

Prior to decapitation, blood was collected from the jugular vein of the anaesthetised animal. The skin in the neck region was cut to expose the jugular vein and blood was collected in vials rinsed with a saturated solution of EDTA by puncturing the vein. The blood was then centrifuged at 4000 revs  $\text{min}^{-1}$ , and serum thus obtained was stored at  $-20^\circ\text{C}$  until assayed for thyroid hormones.

#### Measurement of thyroid hormone ( $T_3$ and $T_4$ ) levels in serum

Circulating levels of thyroid hormones  $T_3$  and  $T_4$  were assayed by solid-phase radioimmunoassay (RIA) using commercial kits RIAK4/4A for  $T_3$  and RIAK 5/5A for  $T_4$  from the radiopharmaceutical division of Bhabha Atomic Research Centre, Bombay. The assays were carried out following the protocols provided with the kits. The sensitivity of the assay based on the 90% B/Bo intercept (bound/free) is 0.24  $\text{ng ml}^{-1}$  for  $T_3$  and 0.5  $\mu\text{g } 100 \text{ ml}^{-1}$  for  $T_4$  and the specificity is 100%. The levels of  $T_3$  and  $T_4$  were expressed as  $\text{ng ml}^{-1}$ .

#### Data analysis

In the present study, the statistical significance of all the quantitative data was determined using both Student's *t*-test and analysis of variance (ANOVA). Significance was defined as  $P < 0.05$ .

## Results

### Changes in mass

Treatment with methoxyindoles and p-CPA reduced the mass of the testes significantly by, on average, 80% and 90%, respectively (Fig. 1A). The mass of the adrenal glands was significantly increased by 15–35% except for the ME group (Fig. 1B), while the changes in mass of the thyroid gland were generally insignificant except for the MM group, in which there was a significant decrease, and the MT group, in which there was a significant increase (Fig. 1C).

### Histological changes

#### Testis

The testis of control birds in the recrudescence phase had enlarged seminiferous tubules lined with many layers of germ cells. Spermatogenesis had started in most of the tubules and developmentally advanced stages including spermatocytes and round spermatids were present (Fig. 2A). The interstitium showed regional differences; hypertrophied cells were present adjacent to tubules containing early stages of germ cells and small reduced cells were found in the neighbourhood of tubules containing mature stages of germ cells.

In general, all treatments induced regressive changes. Both MM and ME arrested spermatogenesis and the diameter of the seminiferous tubules was reduced. The changes were more pronounced after ME treatment (Fig. 2C) than after

MM treatment (Fig. 2B), with the appearance of prominent intertubular spaces (Fig. 2C). Germ cells were confined to one or two layers and most were hypertrophied and exhibited nuclear pyknosis. The interstitial cells were mostly small and regressed with a fibroblast-like appearance. Treatment with both ML (not illustrated) and MT (Fig. 2D) also induced spermatogenic arrest and tubular regression, with the regressive effects being more apparent after ML treatment. However, hypertrophy of the germ cells was more apparent

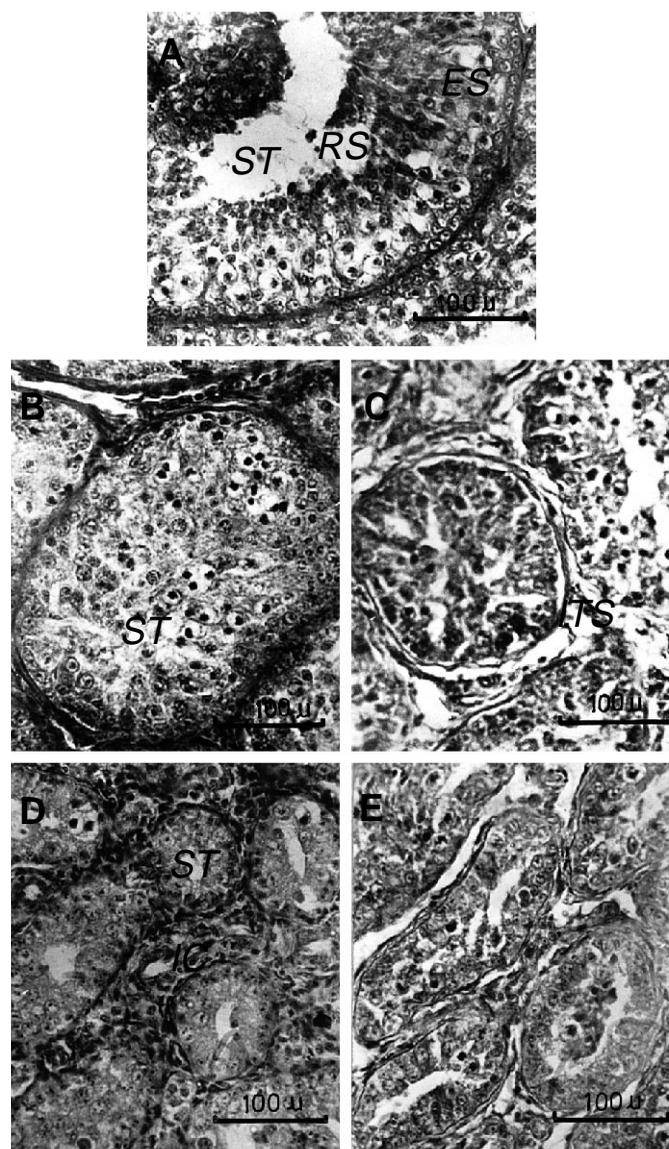


Fig. 2. Photomicrographs of testis from a control animal and from birds treated with methoxyindoles and p-CPA. (A) Control showing spermatogenic stages; (B) after melatonin (morning) treatment showing degenerating germ cells; (C) after melatonin (evening) treatment showing shrunken tubules containing degenerated germ cells; (D) after methoxytryptamine treatment showing several highly regressed tubules with a single basal layer of germ cells surrounded by the prominent interstitium; and (E) after p-CPA treatment showing regressed tubules with degenerating germ cells. RS, round spermatids; ES, elongating spermatids; ST, seminiferous tubules; IC, interstitium; ITS, intertubular space. Scale bars, 100  $\mu\text{m}$ .

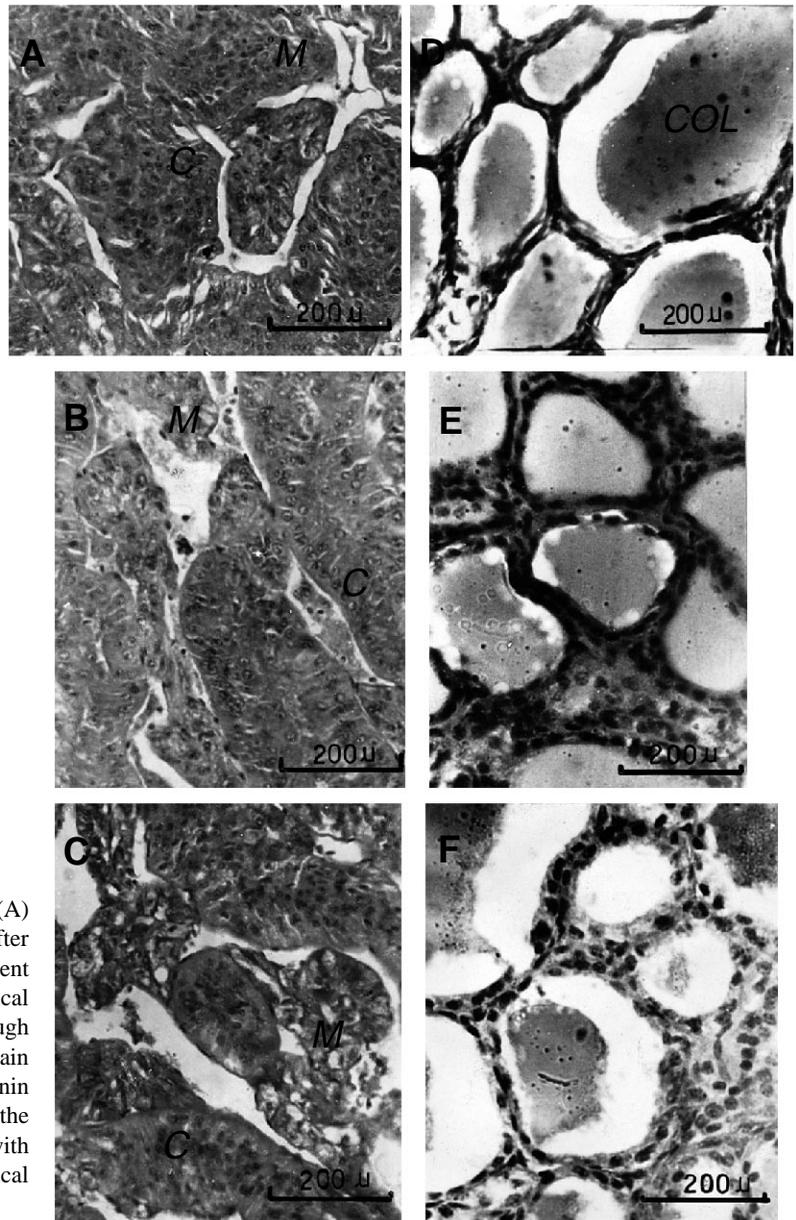


Fig. 3. (A–C) Light microscopy section of adrenal tissue. (A) Control showing cortical and medullary strands; (B) after melatonin (morning) treatment showing cortical enlargement and activation; (C) after p-CPA treatment showing cortical regression and medullary hypertrophy. (D–F) Section through thyroid tissue. (D) Control in which the follicles contain substantial amounts of colloidal material; (E) after melatonin (evening) treatment showing full colloidal content in all the follicles; and (F) after p-CPA treatment showing follicles with depleted colloid content. M, medullary chords; C, cortical chords; COL, colloid. Scale bars, 200  $\mu$ m.

in MT-treated birds than in ML-treated individuals. The interstitial cells were active and well-formed in some sections, and small and regressed in others. Treatment with p-CPA also induced tubular regression and an arrest of spermatogenesis (Fig. 2E). This was accompanied by a thickening of the basement membrane and considerable nuclear pyknosis of the hypertrophied germ cells. The interstitium showed a mixture of hypertrophied and regressed cells.

#### Adrenal gland

The adrenal glands of control birds were marked by well-formed cortical cords with prominent medulla scattered in between (Fig. 3A). Melatonin treatments (MM and ME) (Fig. 3B) induced cortical enlargement accompanied by medullary regression. Treatments with ML, MT and p-CPA

induced medullary hypertrophy and secretory exhaustion as observed histologically without having much influence on the cortical structure (Fig. 3C).

#### Thyroid

The thyroid gland of control birds showed medium to large follicles lined by a flattened epithelium (Fig. 3D). The amount of colloid contained in the follicles varied to give a mixture of fully filled, half-filled and empty follicles. Treatment with MM, ME and ML brought about colloid retention in the follicles, so that most follicles were filled with colloid but showed slight enlargement of the follicular epithelium (Fig. 3E). In contrast, treatment with MT or p-CPA induced epithelial cell hypertrophy and colloid depletion, so most of the follicles appeared empty. Treatment with MT also induced some degree of epithelial cell hyperplasia (Fig. 3F).

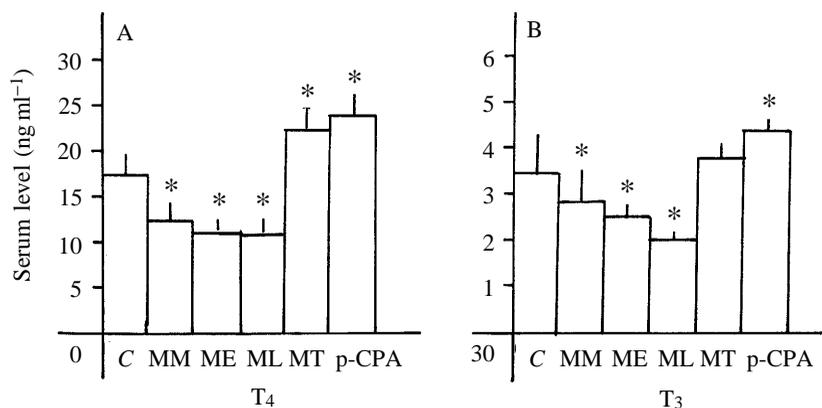


Fig. 4. Changes in serum levels of (A) T<sub>4</sub> and (B) T<sub>3</sub> in pigeons treated with methoxyindoles or p-CPA during the recrudescence phase. \*Significantly different from the control value ( $P < 0.05$ ); values are means + s.d.,  $N = 6$ . C, control; MM, melatonin morning; ME, melatonin evening; ML, methoxytryptophol; MT, methoxytryptamine; p-CPA, parachlorophenylalanine.

#### Serum T<sub>4</sub> and T<sub>3</sub> levels

Serum of control pigeons contained  $17.12 \pm 1.13$  ng ml<sup>-1</sup> of T<sub>4</sub> and  $3.46 \pm 0.13$  ng ml<sup>-1</sup> of T<sub>3</sub> in the recrudescence phase. Birds treated with MT and p-CPA showed significant increases in T<sub>4</sub> and T<sub>3</sub> levels (Fig. 4). Concurrently, MM-, ME- and ML-treated pigeons showed significant decreases in T<sub>4</sub> and T<sub>3</sub> levels.

#### Discussion

The results of the present study on the influence of exogenous melatonin administration are suggestive of an antigonadal effect. Some previous studies have shown that melatonin has no significant effect on the gonadal growth of juvenile chickens (Singh and Turner, 1967) or juvenile quails (Saylor and Wolfson, 1968). It was also reported that, in quails and chickens, the pineal gland does not play an important role in the photoperiodic response since neither gonadal activity nor locomotor activity was influenced by pinealectomy or melatonin administration (Homma *et al.* 1969; Menaker and Zimmerman, 1976; Simpson and Follett, 1981). However, some recent observations on tropical/subtropical species such as the lal munia *Estrilda amandava* and the parakeet *Psittacula krameri* show that exogenous melatonin has an inhibitory effect on gonadal activity (Gupta *et al.* 1987; Maitra and Dey, 1992). These observations are in agreement with the results we have obtained from the pigeon. Histological observations made in the present study show there is an arrest of spermatogenesis accompanied by cell degeneration in the regressing seminiferous tubules of melatonin-treated birds. Even the interstitial cells appear to be inactive. Similar effects on testis histology have also been reported by Maitra and Dey (1992) in the parakeet *Psittacula krameri*. These changes indicate that melatonin may have an inhibitory effect on the hypothalamo-hypophyseal axis regulating gonadal functions. It has been shown that, in the Japanese quail, melatonin applied directly to the pre-optic area located in the anterior hypothalamus causes inhibitory input to the infundibular nuclear complex (INC), which contains both photoreceptive and GnRH-containing neurones (Maruyama *et al.* 1984; Ohta and Homma, 1987, 1988).

The other methoxyindoles used, ML and MT, also induced testicular regression and spermatogenic arrest. This suggests a common antigonadal effect for all pineal indoles in the pigeon. This apparent similarity in the effects of all three pineal indoles is paradoxical when we consider that levels of ML follow a different circadian rhythmicity to melatonin and MT levels. ML levels are high during the photophase and low during the scotophase, while levels of melatonin and MT are high during the scotophase and low during the photophase (Reiter, 1981, 1984; Skene *et al.* 1986, 1991). In this context, it is noteworthy that the gonado-inhibitory effect of melatonin is not dependent on the time of administration; both the injections gave the same degree of inhibition. This time-independent action of melatonin has also been noted in the lal munia *Estrilda amandava*. The fact that the three indoles all have a similar effect despite their different circadian rhythmicity can be explained by the continuous sensitivity of the hypothalamo-hypophyseal-gonadal (HHG) axis to pineal indoles on the circadian time scale. Again, this differs from the situation in mammals, where the sensitivity of the HHG axis to melatonin shows a circadian variation, with a sensitive phase in the evening and a non-sensitive phase in the morning. Moreover, morning injections of melatonin are known to negate its inhibitory influence in the evening (sensitive phase) owing to down-regulation of the melatonin receptors (Reiter, 1981; Cardinali *et al.* 1983). The two essential points to emerge from our work on avian species are (i) that the central melatonin receptors purported to be in the anterior hypothalamus (Ohta *et al.* 1989) are sensitive to this indole throughout the circadian cycle and (ii) that both the duration of exposure to pineal indoles (morning administration) (Carter and Goldman, 1983a,b; Bittman and Karsch, 1984; Bittman *et al.* 1983) and the concentration applied (evening administration) (Reiter, 1991) are equally potent in their inhibitory effects on the HHG axis. The time-independent suppressive influence of pineal indoles on testicular function during the breeding phase has also been observed in the Indian jungle bush quail *Perdicula asiatica* (Halder and Ghosh, 1988).

Exposure to p-CPA also brought about testicular regression and spermatogenic arrest in accordance with our previous findings from pinealectomized birds and indicated that the

pineal gland has a role in promoting gonad function during the reproductively active phase of pigeons (Ramachandran and Patel, 1986; Ramachandran *et al.* 1987). Such a role for the pineal body in tropical and subtropical birds is further supported by the observations of Haldar and Chosh (1989a,b) demonstrating that pinealectomy has an inhibitory effect on testicular growth in *Perdicula asiatica*. The fact that both pineal indoles and pinealectomy cause regression of the testis indicates that two separate mechanism are influencing this aspect of the reproductive cycle.

One explanation of this dual effect of the pineal gland may be the observation that in pinealectomized birds there is a decrease in adreno-cortical activity and an increase in thyroid activity, suggesting that the pineal gland increases adrenal function and reduces thyroid activity during the breeding season in the pigeon (Patel *et al.* 1985; Ramachandran and Patel, 1986). This pattern of activity is supported by the finding that serum T<sub>4</sub> and T<sub>3</sub> levels increase and corticosterone levels decrease in pinealectomized pigeons in the breeding season (Patel, 1993). The present study adds further support by showing that injection of melatonin induced adreno-cortical activation and inhibited thyroid activity, as indicated by the retention of colloid within the follicles and the decrease in the levels of serum T<sub>4</sub> and T<sub>3</sub>. A similar decrease in thyroid activity occurs in *Estrilda amandava* in response to administration of melatonin (Gupta *et al.* 1987). The effect of ML on the thyroid was identical to that of melatonin, suggesting a similarity in action. In contrast, MT and p-CPA treatments increased thyroid activity, as shown by the depleted colloid content of the follicles, the increase in epithelial cell height and the rise in levels of serum T<sub>4</sub> and T<sub>3</sub> (Figs 3F, 4). This effect of p-CPA was similar to that of pinealectomy (Patel *et al.* 1985; Ramachandran and Patel, 1986). In addition, our studies provide evidence for an inverse relationship between thyroid activity and testis function as indicated by the testicular regression that occurs when exogenous T<sub>4</sub> is administered during the breeding season. Previous studies have provided similar evidence for the antagonistic role of the thyroid in many birds (see Pathak and Chandola, 1983). On the basis of thyroidectomy studies (Chandola and Thapliyal, 1978), T<sub>4</sub> administration (Chandola and Bhatt, 1982; Chandola *et al.* 1982) and the circannual variations in serum levels of T<sub>4</sub> and T<sub>3</sub> in the spotted munia *Lonchura punctulata*, it has been concluded that T<sub>4</sub> is the factor responsible for the role of the thyroid in reproduction and that levels of T<sub>4</sub> are inversely related to gonadal activity (Pathak and Chandola, 1983). The present findings suggest that increased thyroid activity leading to high levels of T<sub>4</sub> results in testicular regression in the pigeon and that pinealectomy or MT treatment induces hyperactivity of the thyroid. Both these treatments seem to enhance HHT activity and the resultant surge of T<sub>4</sub> may further suppress HHG activity and/or antagonize testosterone action (Murton and Westwood, 1977; Jallageas *et al.* 1978).

In contrast, the testicular regression induced by M and ML, which reduce thyroid activity, seems to involve an entirely different mechanism. Melatonin and ML may directly suppress

the HHG axis and there is probably some local effect involving a reduced responsiveness of the testicular tissue to gonadotropins (Tamarkin *et al.* 1976; Amador *et al.* 1988; Ohta *et al.* 1989).

In conclusion, our observations suggest that pinealectomy and pineal indoles can induce testicular regression in the breeding phase by different mechanisms. Pinealectomy and MT appear to cause gonadal regression by increasing thyroid hormone secretion, while melatonin and ML appear to have an inhibitory effect on the HHG axis. It is also likely that the pineal body has a definite phase relationship with the thyroid and the adrenal gland in the blue rock pigeon.

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