CHAPTER II

OBSERVATIONS ON CYCLIC HISTOCHEMICAL VARIATIONS OF LIPIDS AND STEROID DEHYDROGENASES IN TESTES OF INDIAN FERAL BLUE ROCK PIGEON COLUMBA LIVIA G.

Extensive literature is available on the cyclic histophysiological variations on avian testes. As in the case of fishes, amphibians, reptiles and seasonal mammals, testicular regression and its subsequent recrudescence is by now an accepted fact for majority of birds. As mentioned earlier (Chapter I), most of the seasonal histophysiological observations pertaining to gonads have been reported in case of male birds viz. geese (Parton and Parton, 1922); fulmar petrel (Marshall, 1949); jackdaw, (Threadgold, 1956); California gull (Johnston, 1956); mallard duck (Johnson, 1961); brown-headed cowbird (Scott and Middleton, 1968); pied flycatcher (Siverin, 1973, 1975); eider (Gorman, 1974); white-crowned sparrow (Lewis, 1975a&b); eastern rosella (Smith et al., 1976); Indian house crow (Ambadkar and Chauhan, 1976) and Canada goose (Mori and George, 1978). A more or less continuous breeding activity in temperate and tropical climates has been reported in case of many Columbid species like the rock dove, wood pigeon, frugivorous rain forest & ground

feeding pigeon, domestic & feral pigeon and some pigeon species in arid and semiarid Australia. (Riddle, 1925; Less, 1946; Schein, 1954; Murton et al., 1963; Dunmore and Davies, 1963; Lofts et al., 1966; Ljunggren, 1969; Murton et al., 1973; Frith et al., 1974, 1976). Light is an important factor triggering gonadal growth but in the tropics where day length does not vary much, internal rhythms of reproduction may play a more significant role (Thapliyal, 1968).

Different workers have considered different criteria for assessment of the physiological state of gonads. Common among which are the characteristics of Leydig cells as reflected in their relative abundance, sudanophilic gradings, lipid histochemistry and, more recent one, the steroid dehydrogenase activity. Some other parameters taken into account are testicular size, weight, diameter, volume and, ofcourse, stages of spermatogenesis. Over and above the traditional criteria employed for the assessment of functional states of testes, demonstration of steroid dehydrogenases has gone a long way in elucidating the role chiefly played by the Leydig cells in the synthesis of sex steroid hormones. Pioneering contributions on Δ^5-3β-hydroxysteroid dehydrogenase, a key enzyme in steroid hormone biosynthesis, have been made by Samuels et al. (1951) employing biochemical techniques and by Wattenberg (1958)

employing histochemical means. Of course, one of the most reliable ways to assess the functional/non-functional state of gonads is to quantify the gonadal hormones or gonadotrophins in tissues or body fluids by the sensitive and precise radio-immuno assay technique (RIA method).

The present study was undertaken to substantiate the previous quantitative findings on feral pigeons (Chapter I) with a view to characterize the breeding and non-breeding phases more precisely. To investigate this aspect, histochemical localization of lipids and the activities of Δ^5-3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenases in testes was undertaken on a seasonal basis.

MATERIAL AND METHODS

Feral rock pigeons (Columba livia) inhabiting the University campus were used for the investigation. They were shot down with an air rifle between 8.00 a.m. and 10.00 a.m. in the year 1975-1976. The birds were then immediately brought to the laboratory. Testes were taken out, blotted free of the tissue fluids and weighed separately. Left testis was then fixed on a cryostat chuck maintained at -20°C, whereas the right one was fixed in Bouin's fixative for histology. 18μm thick sections were cut on the cryostat-microtome. Sections were taken

up on coverglass and employed for histochemical demonstrations. Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSDH) was demonstrated according to the method of Wattenberg (1958). NAD was used as the cofactor and both, dehydroepiandrosterone (DHEA) and pregnenolone were employed separately as substrates. 17β-hydroxysteroid dehydrogenase (17β-HSDH) was demonstrated as per the method of Kellogg and Glenner (1966) employing testosterone and estradiol-17β as substrates and NAD as co-factor. Fettrot 7 B and Sudan Black B were utilized in demonstrating neutral and total lipids respectively (Pearse, 1968). Control sections for enzymes were incubated without using steroid substrates.

RESULTS

Lipids:

In the months of January and February, both neutral and total lipids were localized intensely in the Leydig cells, whereas lipids were not observable in the seminiferous tubules. In March, April and May (early May), lipids in the interstitial tissue diminished remarkably. Neutral as well as sudanophilic lipids were undoubtedly less and were seen as small patches in the interstitium (Fig. 1). Fine droplets of lipids were recorded in the seminiferous tubules. From late May onwards upto the

middle of June lipids gradually accumulated in the Leydig cells. After the middle of June, for the first time, a drastic change in the localization of lipids was noticed. Both the neutral as well the sudanophilic lipids, more so the latter, were localized in the seminiferous tubules (Fig.2), though it was noted that no massive tubular steatogenesis occurred. In other words, seminiferous tubules loaded heavily with lipids were not encountered. Practically no lipids were seen in the interstitium. But by July, tubules were found to contain less of lipids whereas Leydig cells were once again loaded with lipids (Fig. 3 & 4) evidently more than that observed in March, April and early May. August revealed decreasing localization of lipids in the interstitial cells, which however, increased slightly in September, but decreased significantly in October. On the whole, late August, September and October were noted for comparatively negligible amounts of lipids in the interstitial tissue. Fine particles of lipids appeared to be present towards the luminal side. In November, lipids could be seen to show accumulation in the seminiferous tubules of many birds, a state similar to that observed in the later part of June. Though there was increase in intratubular lipids, the intensity was comparatively lower than that noted in the second half of June. In other cases, the entire lining of the seminiferous tubules revealed lipids. Nevertheless, some

EXPLANATION TO FIGURES

Photomicrographs of T.S. of pigeon testes.

Fig. 1. Breeding stage (March) showing almost lipid-free interstitial cells and seminiferous tubules. X 75.

Fig. 2. Section of regressed testis (June) depicting lipids loaded in the seminiferous tubules : Sudan Black B. X 75.

Fig. 3. Lipids stained with Fettrot 7 B during non-breeding season (July); interstitial tissue showing heavy localization. X 100.

Fig. 4. Lipids stained with Sudan Black B in July; note lipid rich interstitium. X 100.

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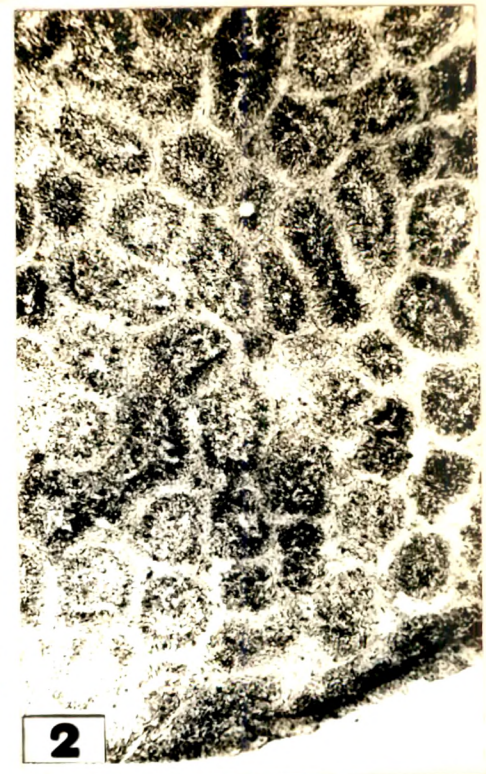

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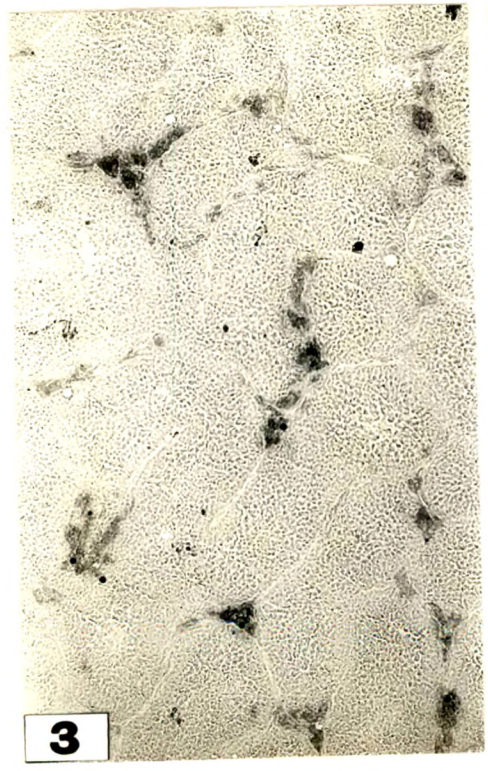

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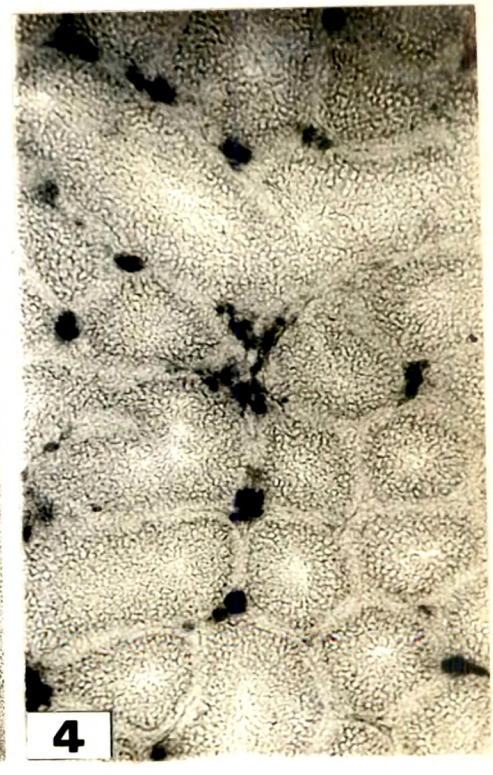

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birds showed lipids loaded in the Leydig cells with very little of lipids inside the seminiferous tubules. Testicular lipids decreased in December, yet the decrease was not as much as during the periods March to May and late August to October.

Δ^5-3β-hydroxysteroid dehydrogenase:

Localization of this enzyme activity was chiefly confined to the Leydig cells with a very moderate activity in the lining of the seminiferous tubules. Usually, the enzyme activity with DHEA as a substrate was more strong, particularly when lipids were less. Intensity of the enzyme activity with pregnenolone as the substrate, was stronger when lipids were accumulated in either the interstitium or seminiferous tubules, that is, particularly during non-breeding phases.

In the months of January and February, overall enzyme activity was moderate. At the time when lipids were decreasing ie. during March, April and early May, enzyme activity was intensely localized in the Leydig cells with moderate activity in the entire lining of the seminiferous tubules (Figs. 5 & 6). During this period, enzyme activity was stronger with DHEA than with pregnenolone (Figs 5 & 7). In late May and especially in June, intensity became very weak in the interstitial cells (Fig. 9). Through July

EXPLA NATION TO FIGURES

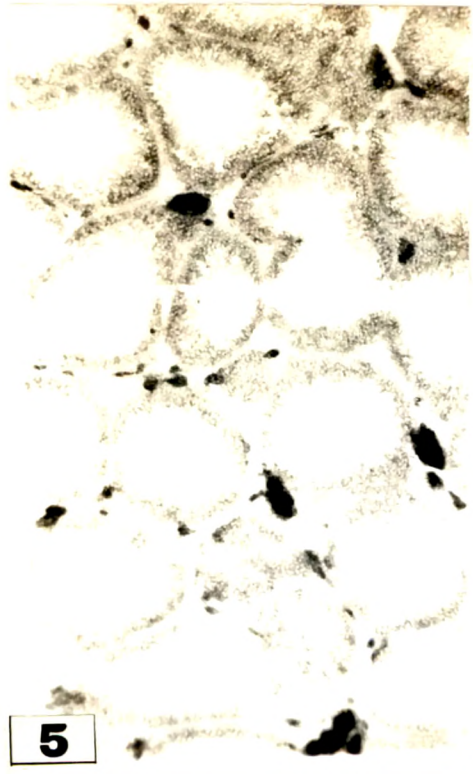
Photomicrographs of T.S. of pigeon testes.

Fig. 5. 3β-HSDH (DHEA) activity in full breeding phase. X 75.

Fig. 6. do. X 200.

Fig. 7. Distribution pattern of 3β-HSDH activity with pregnenolone (P) as substrate in the breeding phase. Note relatively less intensity as compared to that shown in Figs. 5 & 6. X 75.

Fig. 8. Same as Fig. 7. X 200.



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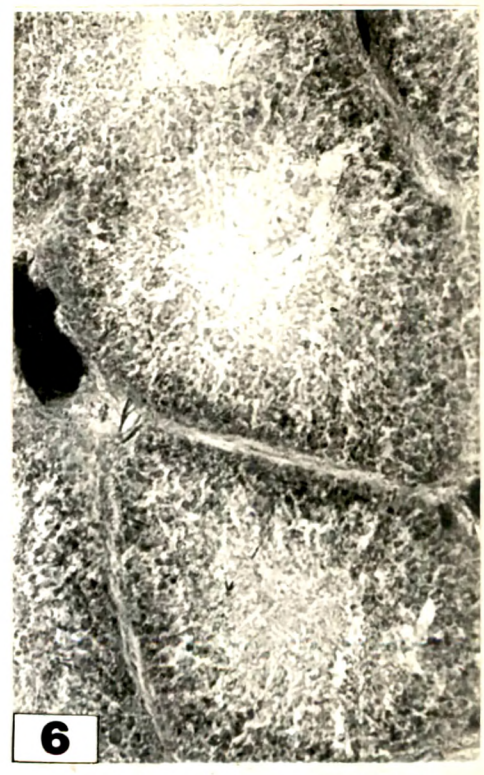

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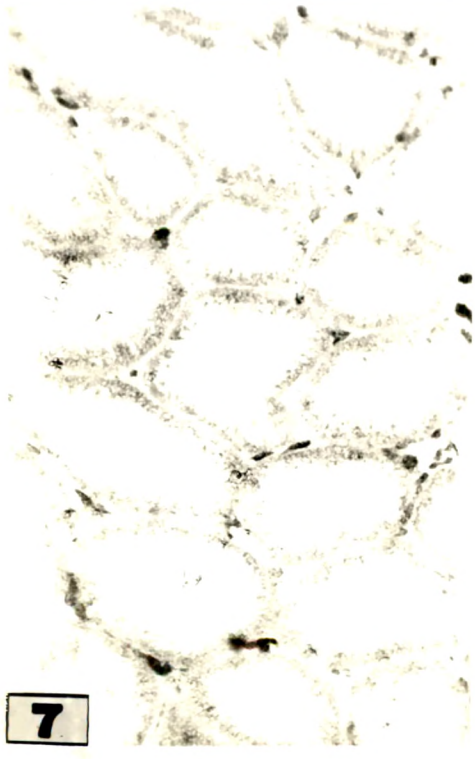

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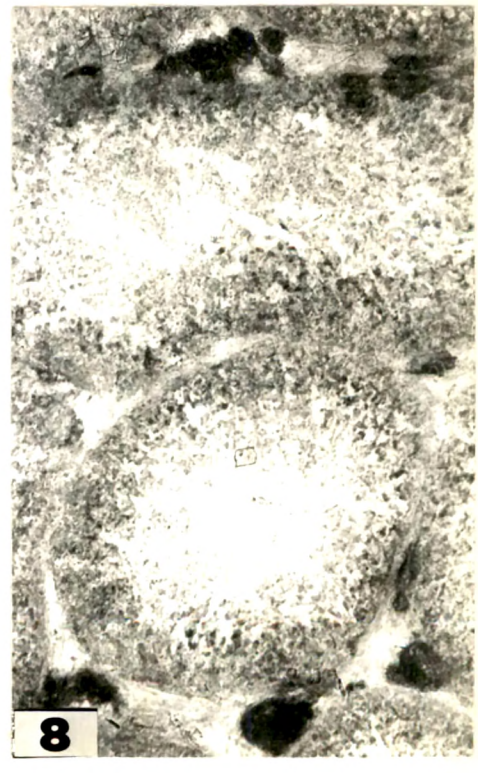

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EXPLA NATION TO FIGURES

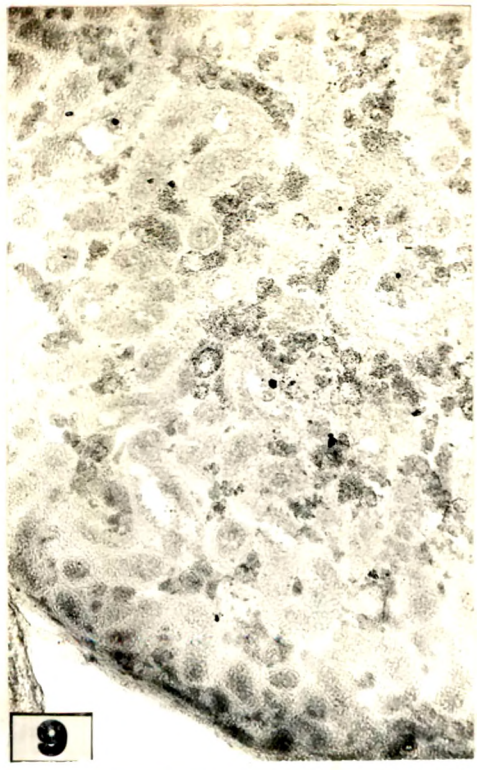
Photomicrographs of T.S. of pigeon testes.

Fig. 9. Feeble intensity of 3β-HSDH (DHEA) in the non-breeding phase. X 75.

Fig. 10. 17β-HSDH (E) activity during breeding stage. X 200.

Fig. 11. Localization of 3β-HSDH (P), activity is more than that shown in Fig. 9. X 100.

Fig. 12. 17β-HSDH activity with testosterone as substrate during breeding phase revealing stronger intensity than 17β-HSDH (E) Fig. 10 but relatively less than 3β-HSDH (Fig. 6). X 200.



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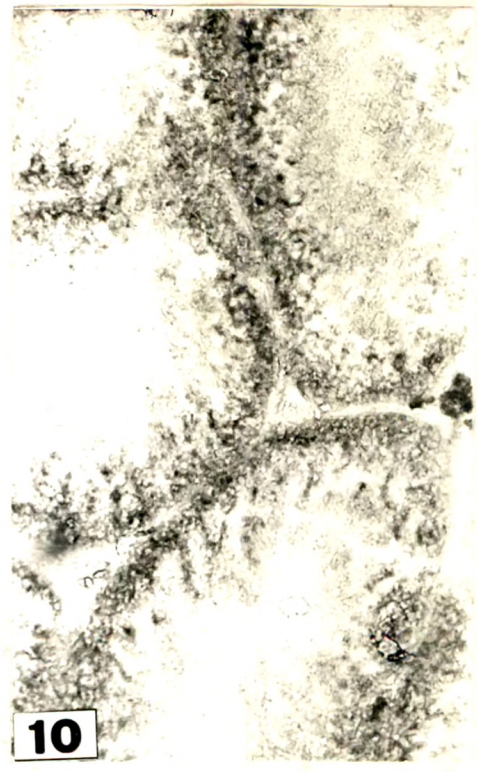

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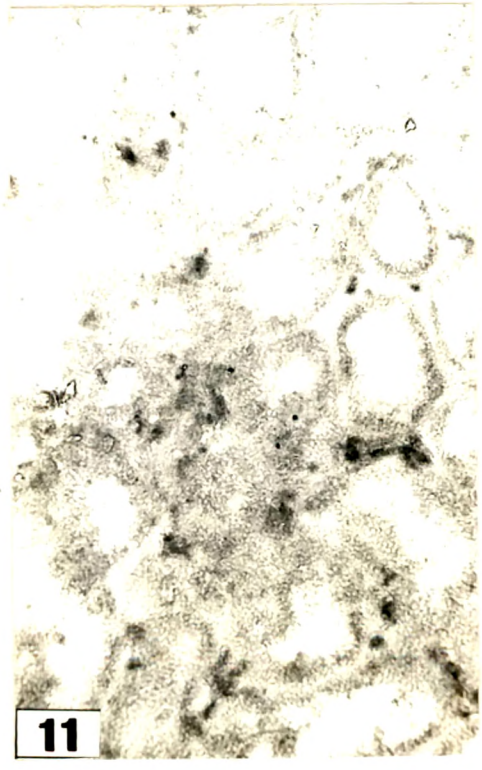

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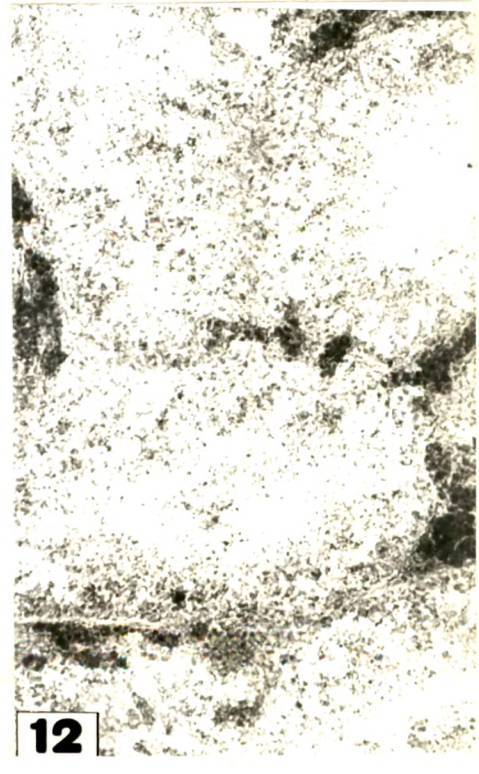

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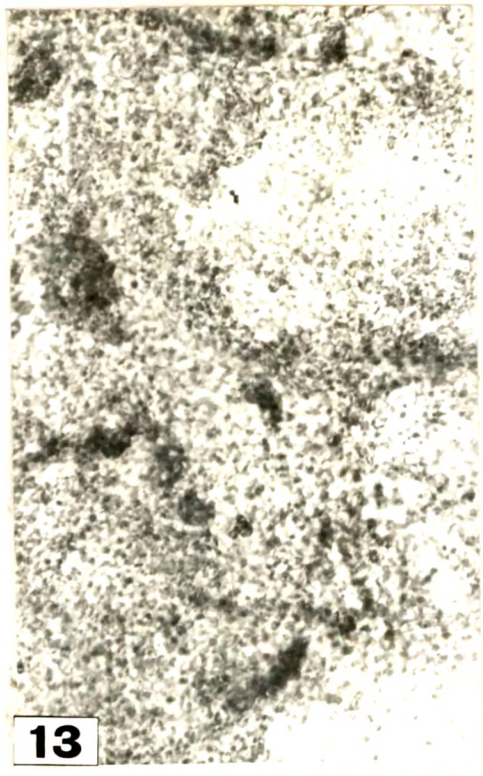
EXPLA NATION TO FIGURES

Fig. 13. 17β-HSDH (control) during breeding phase. X 200.

Fig. 14. 17β-HSDH (T) during non-breeding (June) phase. Dense lipids filled in the seminiferous tubules noticeable. X 200.

Fig. 15. 3β-HSDH (control) during breeding phase. X 100.



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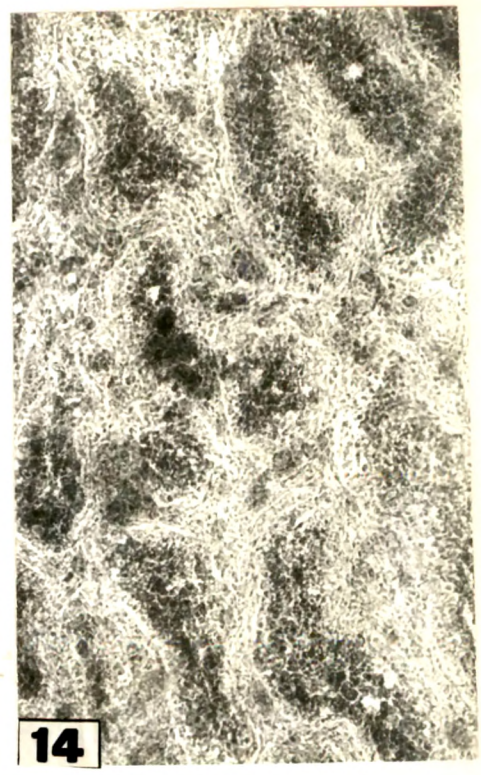

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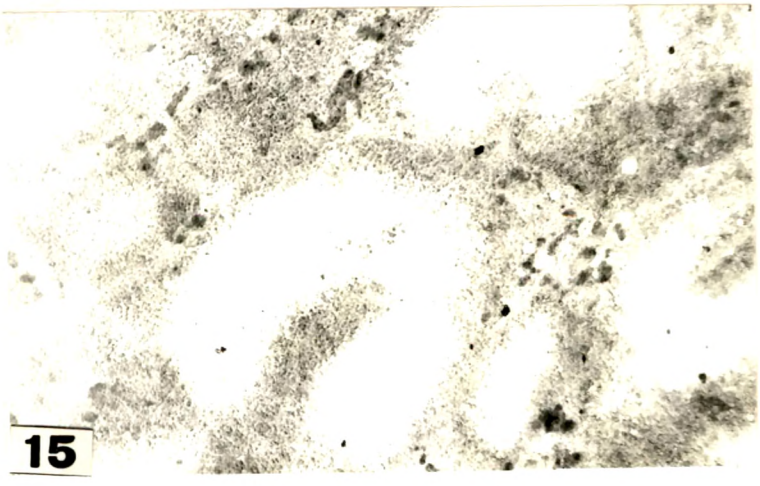

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and early August, condition remained same, intensity being stronger when pregnenolone was employed as substrate; as compared to that with DHEA (Fig. 11). From late August onwards, once again the enzyme activity was observed to show a gradual increase. Peak enzyme activity was observed in the months of September and October. Leydig cells reacted positively, particularly when DHEA was the substrate. The enzyme activity became relatively mild in November; a moderate increase in December was descernible.

17β-hydroxysteroid dehydrogenase:

This enzyme was later demonstrated during the probable breeding (March-April) and non-breeding (June-July) phases. High activity was seen in the Leydig cells and moderate in the lining of the seminiferous tubules during the breeding phase using estradiol-17β and testosterone as substrates (Figs. 10 and 12 respectively). The activity however, was not as intense as 3β-HSDH (DHEA) during the same months (Figs. 5 and 6). 17β-HSDH (T) activity was greater than 17β-HSDH (E). The enzyme intensity was very feeble during the non-breeding phase (Fig. 14).

DISCUSSION

Lofts and Murton (1973) have reported that the cyclic waxing and waning of cellular lipids, which though in itself

is insufficient to implicate unequivocally a steroid synthesizing role, is a useful index of the functional activity of the gonadal tissue. Sudden depletion of lipids and cholesterol that preceeds the peak of breeding activity in many reptilian species is indicative of an increased utilization of precursor material for androgen production (Lofts, 1968).

Presence of $\triangle^5$-3β-HSDH enzyme activity in the interstitial tissue has been demonstrated by histochemical techniques (Arvy, 1962; Woods and Domm, 1966), attributing its role in catalyzing the conversion of $\triangle^5$-3β-hydroxysteroids to $\triangle^4$-3-ketosteroids (Samuels et al., 1951); a step important in the biosynthesis of progesterone and other androgenic steroids such as androstenedione. Deane and Rubin (1965) rightly pointed out that the presence of this enzyme in the Leydig cells provides a strong evidence of their capacity for steroid biosynthesis. Many workers have reported the presence of $\triangle^5$-3β-hydroxysteroid dehydrogenase activity in the Leydig cells and seminiferous tubule lining of avian testes viz. Gallus gallus (Botte, 1963; Arvy, 1962); Gallus domesticus, (Chieffi, 1964; Narbiatz and Kolodny, 1964; Tingari, 1973); Eider (Gorman, 1974); white breasted water hen (Bhujle and Nadkarni, 1974, 1976), and chicken-pheasant hybrids (Purohit et al., 1977). Garnier et al. (1973) have reported observations on testicular cycles

of the pekin duck employing ultrastructural (Leydig and Sertoli cells), histochemical (Δ^5-3β-HSDH) and biochemical (GLC of androgens in plasma and testes) parameters.

Taking into consideration the above mentioned facts on lipids and hydroxysteroid dehydrogenase, accumulation of neutral and total fat and moderately low intensity of Δ^5-3β-HSDH in the Leydig cells in January, February, and very low in late May, June, July and to a certain extent in November, it could be surmised that:

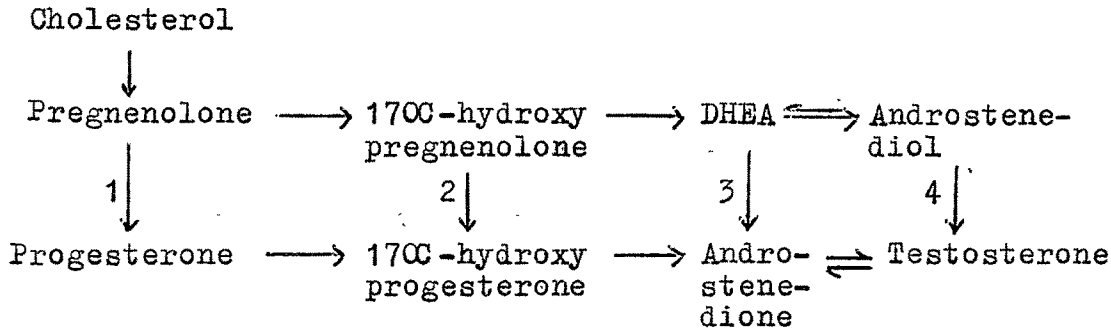
1. the lipids, particularly the cholesterol positive lipids, were getting accumulated probably due to reduced utilization for androgen synthesis and

2. weak activity of one of the cardinal enzymes in steroid hormone biogenesis i.e. Δ^5-3β-HSDH suggested a minimum steroid hormone production. The earlier work (Chapter I) has revealed that testicular cholesterol concentration in feral pigeons was very high in the months of February, June and July together with high levels of total lipids, a fact corroborating the above stated histochemical findings. Testicular steatogenesis observed in the month of June deserves a special mention. In this month, moderate amounts of sudanophilic lipids were characteristically localized in the seminiferous tubules whereas no lipids were encountered in the interstitium, the phenomenon being a typical

picture of regressive testis. Generally speaking, the tubules remain in this lipoidal state for some time and then with the onset of recrudescence, the sudanophilic material rapidly disappears (Lofts and Murton, 1973). In Columbid species however, a massive post-nuptial tubular steatogenesis does not occur, and the quantity of Sertoli lipid is very much less (Lofts et al., 1967). It was perhaps due to this reason that testes with dense or heavily loaded lipid tubules were never observed throughout the year. All these facts suggest that the months of January, February, late May, June, July and to a certain extend November, were the non-breeding months.

Two annual breeding peaks, on the basis of distribution patterns of lipids and intensity of steroid dehydrogenase activity, were distinctly observed in the months of March, April, early May and late August, September, and October, whereas an insignificant simulation to breeding activity could be observed in the months of November and December. During full breeding phases, lipids in the Leydig cells were relatively very low and were observable as very small patches (Fig. 1) and the enzyme activity was intense (Figs. 6, 8 & 12). Quantitative data have shown depleting levels of gonadal lipids and cholesterol during these months (Chapter I). Cholesterol positive lipids were in all probability, getting utilized from the interstitial

tissue as was evident from the high activity of HSDH, thereby indicating that androgen production was proceeding at an enhanced rate. High activity of Δ^5-3β-HSDH both with DHEA and pregnenolone obviously pointed at an accelerated turnover through steps 1 and 3 of the following metabolic flowsheet leading to the production of androgens.



All reactions from 1 to 4 are catalyzed by Δ^5-3β-HSDH. (Baillie et al., 1966)

Employing DHEA as the substrate, androstenedione could be formed, which in turn could be converted to testosterone and vice versa. Since progesterone is an important intermediate metabolite in the biosynthesis of steroid sex hormones including androgens, strong activities of the enzyme with pregnenolone in breeding phases were not surprising.

Lofts and Marshall (1959) have reported on the presence of progesterone (chromatographically) in case of birds with regressed gonads containing heavily lipoidal

tubules with a lipid free intersitium. However, Lofts and Murton pointed out later in 1973 that more rigorous criteria for specific identification of the steroid are required to affirm conclusively such observations. In this context it could be mentioned that, during the current investigation, it was noted that a relatively strong enzyme activity of Δ^5-3β-HSDH with pregnenolone as the substrate, as compared to that with DHEA, during non-breeding phases was possibly needed to produce chiefly progesterone that may be required for other purposes.

With respect to androgen metabolism, the interconversions between testosterone & androstenedione and dehydroepiandrosterone (DHEA) & androstenediol are catalyzed by 17β-HSDH (Baillie et al., 1966). High activity of this enzyme during breeding phase could mean that the turnover rate of testosterone and other androgens was possibly high. Low enzyme intensities observed during June and July probably indicated decreased androgen outpute by the testes. Since the activity of 3β-HSDH (DHEA) was distinctly more than 17β-HSDH (T) during the breeding phase, it is possible that the pathways involving the former enzyme may be preferred for the production of testosterone than those catalyzed by 17β-HSDH. Less 17β-HSDH activity (as compared to 3β-HSDH) has also been reported in the testis of crow-phesant (Bhujle and Nadkarni, 1976).

In short, from the above discussion, it is evident that male pigeons exhibit two annual breeding peaks, one in summer (March to May) and the other in late wet-summer to early winter (late August to October). June-July was apparently the typical regressive stage.