CHAPTER II

VARIATIONS IN ASCORBIC ACID AND CHOLESTEROL CONTENTS OF LIVER, BLOOD AND GONADS AS RELATED TO REPRODUCTIVE CYCLES

Ascorbic acid (AA) - vitamin C - is known to play varied significant roles in the general body metabolism and more particularly in steroidogenesis. As regards the first point, AA is known to fecilitate several metabolic processes involving transfer of electrons (Chinoy, 1969, 70 a & b, 71, 72 a & b) by virtue of its capacity to form reversible oxidation-reduction complexes. Further it is well known that AA, its monoascorbate and dehydroascorbate, are also intimately involved in the steroidogenesis (Szent Gyorgii, 1957; Bacq and Alexander, 1961; Chinoy and Seethalaxmi, 1978; Chinoy and Rao, 1979). Based on their work on toad testis, Biswas (1969) and Biswas and Deb (1970) have shown that dehydroascorbate activates the enzyme Δ^5-3β-hydroxysteroid dehydrogenase (3β-HSDH), one of the key enzymes for several steps in the inter-conversion of steroids. Chinoy and Seethalaxmi (1978) have reported an inter-relationship between the tissue levels of AA and testosterone. Kotak (1979) has discussed changes in AA levels in liver, blood and gonads of pigeons during breeding and non-breeding seasons. Beneficial influences of AA under hot climatic conditions (Pardue *et al.*, 1983) and on semen production in cocks and egg

production in hens maintained in hot conditions has already been reported (Coates, 1971).

With regard to biosynthesis of AA, animal kingdom reveals interesting facets. Most invertebrates, including insects, as well as fishes, lack the ability to synthesize AA. This is thought to be due to their negligible requirements (Chatterjee, 1973). Capacity to synthesize AA begins with microsomal fractions of amphibian kidney, it resides in reptilian kidney and is transferred to mammalian liver (Chatterjee, 1973). Among mammals guinea - pigs, primates and Indian bat lack the ability to synthesize AA due to the absence of one of the enzymes responsible for the conversion of L-gulonate to L-ascorbate. In birds, a phylogenic trend in respect of sites of synthesis of AA has been shown to be present by Ray Chaudhuri and Chatterjee (1969), and Chatterjee (1973). In the pigeons and chicken, birds of the primitive group, kidney is the site of AA synthesis, whereas among recent order Passeriformes it is liver. Further, it has been shown that in some Passeriformes like house crow C. spelendens and common myna A. tristris both kidney and liver are capable of synthesizing AA; with liver taking major part (Roy and Guha, 1958). Red-vented bulbul Pycnonotus cafer is totally unable to synthesize AA and has to depend on dietary sources (Chatterjee et al.,1968).

It is a well established fact that all steroid hormones (androgens, oestrogens, progesterone and adrenocortical hormones) can be synthesized from cholesterol. Cholesterol is also involved in several other metabolic processes. At the first step in synthesis of steroids, cholesterol is converted to pregnenolone. Smith et al., (1985) have reported that in each of the tissue that produces steroid hormone, it is the rate-limiting step, which is controlled by various pituitary "tropic" hormones, e.g. the LH for gonads and ACTH for the adrenal cortex. Pregnenolone is further converted to either progesterone or 17α-hydroxy pregnenolone both of which in turn are converted through several alternative routes to Δ^4-androstenedione. Microsomal enzyme system referred as aromatase complex converts testosterone to oestrogens. During the synthesis of steroid hormones from cholesterol, a ring of the steroid is oxidized by the 3β-HSDH. Bartke (1971) has reported in mouse testis that the esterified form of the cholesterol serves as the precursor for androgenic steroids, whereas Smith et al. (1985) have reported that cholesterol is stored in the leydig cells as cholesterol ester, and some cholesterol enters in the leydig cells from the plasma cholesterol pool.

As early as 1908, Chamy has reported on cyclic appearance and discharge of cholesterol and lipids in

interstitial leydig cells as well as seminiferous tubules. The latter phenomenon has been suggested as the basis for the seasonal fluctuations in the sensitivity of the leydig cells as well as germinal epithelial elements to the gonadotropins (Lofts, 1961; van Oordt and Lofts, 1963). Further, a direct relationship between the number of gonadotrophes and the variation in sensitivity to gonadotropins has also been clarified. Thus a close relationship has been shown to exist between the secretary activity of the pituitary gonadotrophes and lipid cycles of the interstitial cells (van Oordt, 1961).

In the light of above facts it was thought desirable to study seasonal variations in levels of AA and cholesterol (total - TC - and esterified - EC - components) in liver, blood/blood plasma and gonads of Bank myna and Brahminy myna as related to reproductive cycles.

MATERIAL AND METHODS

Bank myna Acridotheres ginginianus and Brahminy myna Sturnus pagodarum were used for the present investigation. The comparative study of male and female birds of both species was undertaken. Birds were collected from a local animal supplier and they were then sacrificed as early as possible to avoid effect of caging.

Blood was collected from jugular vein before sacrificing the birds. Part of the blood was used directly for estimation of AA whereas other part was centrifuged to collect plasma for estimation of plasma cholesterol levels. Liver and gonads were dissected out, blotted free of blood and tissue fluids, weighed and kept in refrigerator for short periods till processed further.

To estimate AA content of whole blood, 1 ml of blood was added to 6% chilled Trichloroacetic acid (TCA). Part of liver and gonads were homogenized in prechilled mortars with cold 6% TCA. TCA is known to reduce pH, stabilize the vitamin and prevent its catalytic oxidation. AA in the homogenate was then oxidized to dehydroascorbate by shaking with activated charcoal for fifteen minutes with frequent stirring. It was then filtered through Whatman filter paper No. 42. 4 ml aliquots were used for estimation of AA employing dinitrophenylhydrazine method of Roe (1954).

Other part of the liver, gonad, and the blood plasma were used for the estimation of total and esterified cholesterol contents. Cholesterol was extracted in 3 : 1 (V:V) ether : alcohol mixture. The fresh tissue was crushed in test tube, with a glass rod, to which 2 ml of ether : alcohol mixture was added. These tubes were stored in the refrigerator till processed further. During further

processing, test tubes were kept in the waterbath maintained at 65°±2°C for 10 minutes and then centrifuged for 5 minutes at 3000 rpm. The supernatant was collected in a separate graduated test tube. The process was repeated two times more, adding every time similar volumes of fresh ether : alcohol mixture. Approximately 6 ml of supernatant was collected in this way. Finally the volume of supernatant was adjusted to 10 ml by adding adequate amount of ether : alcohol mixture. Aliquots of 2 ml were used for the estimation of total as well as esterified cholesterol employing method of Crowford (1958).

For the estimation of cholesterol, the extract was dried totally keeping the tubes in an air oven at 55°±2°C. 3 ml of $FeCl_3$ were added to the dry residue and the test tube was kept in the boiling waterbath for 5 minutes. To this, after cooling the tube, 2 ml of conc. H_2SO_4 was added and mixed thoroughly avoiding bubbling. The brown colour developed was measured after 30 minutes in a spectrophotometer at 540 nm. For estimation of esterified cholesterol, 2 ml of whole cholesterol sample was evaporated to approximately 1 ml by keeping the tubes in air oven at 65°C. To each of the tube, 1 ml of 1% digitonin solution was added and mixed thoroughly by shaking. The tubes were then allowed to stand for about 15 minutes and then centrifuged for 10 minutes at 3000 rpm.

The supernatant was completely removed by decantation. Thereafter, the residue was resuspended in 4 ml of acetone and then recentrifuged for 10 minutes at 3000 rpm. The precipitates of digitonide cholesterol was estimated by the above mentioned method. The values thus obtained give free cholesterol content. The amount of esterified cholesterol was obtained by substracting the amount of free cholesterol from that of the total cholesterol.

RESULTS

The tissues studied were liver, blood/blood plasma and gonads of male and female birds of both species. Seasonal variations as related to the phases of reproductive cycles in AA and cholesterol (Total - TC and esterified - EC) are given in Table 2.1 and Fig. 2a for male birds and Table 2.2 and Fig. 2b for female birds.

Ascorbic acid :

The testicular levels showed more or less similar trend of variations in both the species; in that there was a noticeable decrease from PR to BR and gradual but steady increase from BR to PS. However, the testicular AA level exhibited further increase from PS to NB in case of Bank myna but no such increase was evident in the other species. The ovarian AA levels in both the species of mynas revealed

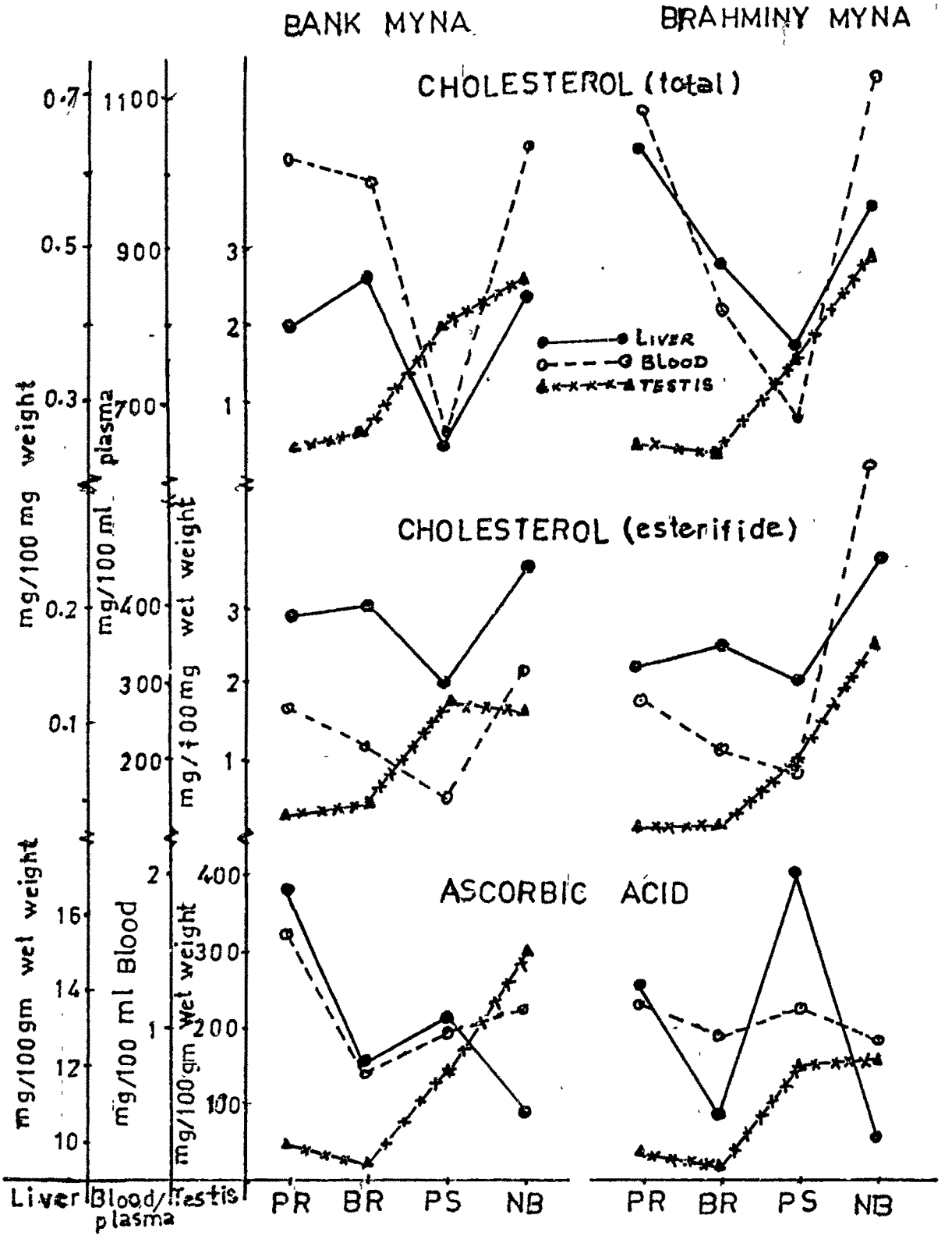
Seasonal variations in the levels of Ascorbic acid and cholesterol in two species of myna as related to female reproductive cycle

Tissues	Ascorbic acid			Total Cholesterol			Esterified Cholesterol		
	Liver*	Blood**	Ovary*	Liver@	Blood plasma@@	Ovary@	Liver@	Blood plasma@@	Ovary@
Seasons				Bank myna					
PR	11.84 ±1.35	1.05 ±0.13	48.72 ±4.6	0.408 ±0.04	947.72 ±158.81	1.05 ±0.13	0.219 ±0.03	369.46 ±64.74	0.278 ±0.03
BR	9.64 ±1.04	0.81 ±0.09	32.38 ±6.63	0.585 ±0.05	840.58 ±44.08	2.46 ±0.39	0.17 ±0.02	467.44 ±53.61	0.591 ±0.1
PS	14.09 ±1.21	1.05 ±0.17	80.68 ±10.95	0.319 ±0.07	334.12 ±64.27	1.76 ±0.2	0.18 ±0.01	116.63 ±11.67	0.484 ±0.09
NB	13.11 ±0.32	1.06 ±0.17	176.89 ±37.92	0.552 ±0.04	1026.18 ±88.46	2.74 ±0.26	0.219 ±0.02	366.3 ±30.05	1.21 ±0.13
				Brahminy myna					
PR	13.14 ±1.89	1.17 ±0.13	90.76 ±14.76	0.515 ±0.03	963.02 ±56.03	0.91 ±0.1	0.211 ±0.01	238.12 ±32.13	0.418 ±0.08
BR	10.73 ±1.96	0.83 ±0.14	28.01 ±4.7	0.442 ±0.05	874.15 ±81.7	1.17 ±0.16	0.178 ±0.02	216.00 ±38.27	0.447 ±0.03
PS	12.69 ±1.07	1.69 ±0.28	33.04 ±4.34	0.402 ±0.02	592.01 ±95.11	0.96 ±0.08	0.171 ±0.02	303.35 ±27.13	0.473 ±0.03
NB	8.02 ±0.9	0.69 ±0.07	66.91 ±8.58	0.805 ±0.13	989.07 ±89.41	2.04 ±0.27	0.248 ±0.1	363.33 ±44.96	1.234 ±0.25

Values expressed as mean±S.E.

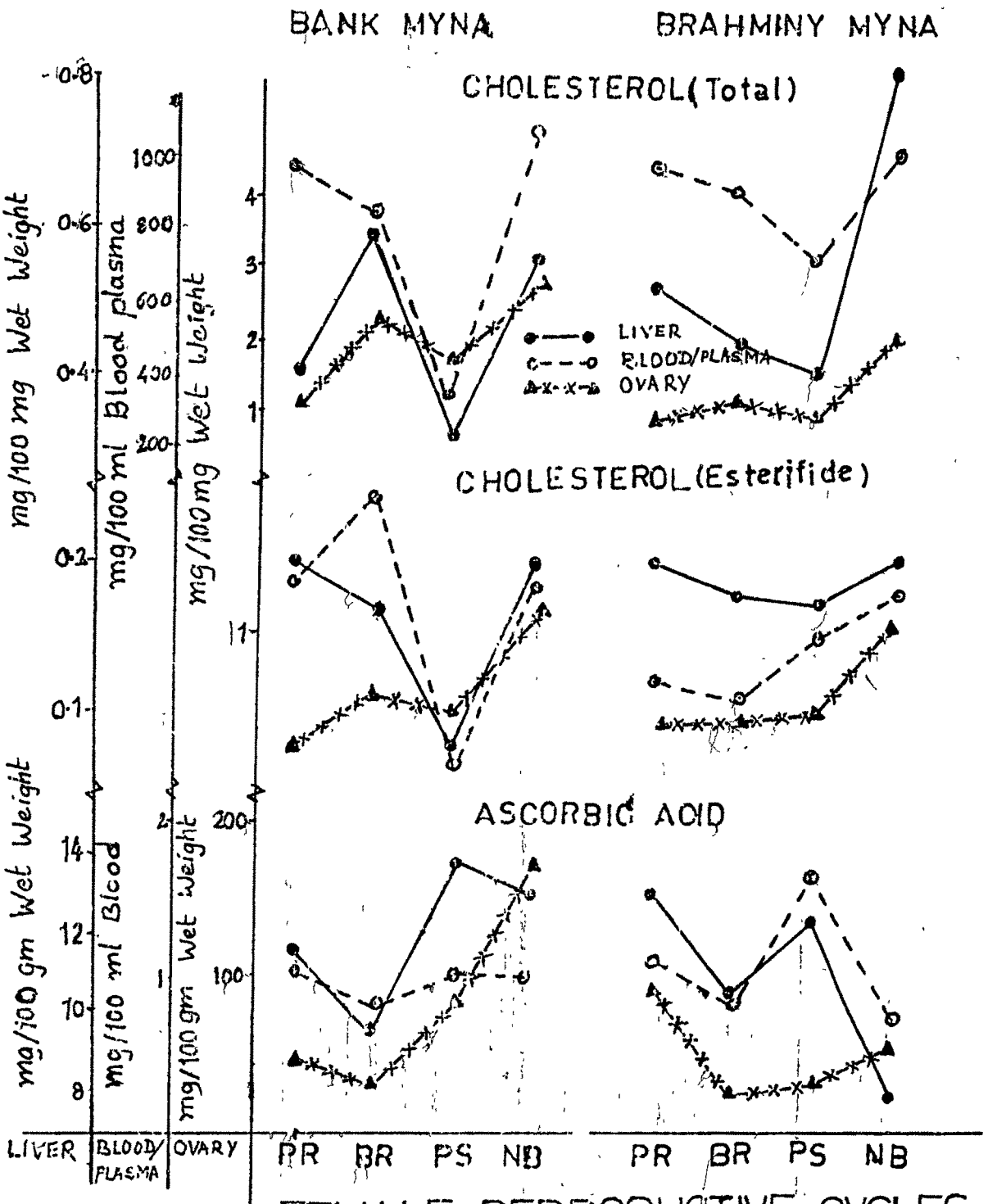
* - mg/100 gm fresh tissue weight ** - mg/100 ml blood

@ - mg/100 mg fresh tissue weight @@ - mg/100.ml blood plasma.



MALE REPRODUCTIVE CYCLES

Fig. 2a



FEMALE REPRODUCTIVE CYCLES

Fig. 2b

parallel trends of variation. There was reduction from PR to BR and a gradual increase through the rest of the phases, however, that of Bank myna was more intense and overshot the levels recorded at previous phases. In case of Brahminy myna though there was an increase in ovarian AA content it did not reach even the PR level during NB.

There were obvious sex-specific differences within the species as far as hepatic and plasma AA contents were concerned. The trend of variation in the AA level of liver and plasma : in case of male Bank myna and female Brahminy myna and those in case of female Bank myna and male Brahminy myna were comparable. The noticeable point of difference being that in the former pattern PR levels were maximum and in the latter case they were much lower. An additional point worth noting in respect of variation from PS to NB was that the change was maximally apparent in the case of hepatic tissue of male Brahminy myna and hepatic as well as plasma levels in female Brahminy myna, whereas such was not the case in the both the sexes of Bank myna.

Cholesterol (TC and EC) :

The overall pattern of variation in the case of male gonads exhibited a rising trend throughout BR to PS to NB whereas in the case of female gonads the rising trend was more apparent from PS to NB.

A gradual decrease was noted in hepatic as well plasma TC level in case of female Brahminy myna from PR to BR to PS whereas the trends in case of male Brahminy myna during the same phases were precipetous. As against this, in case of male and female Bank myna, the trends in hepatic and plasma TC variations were comparable (Fig. 2 a and b). The overall variation in EC levels in both species and the sexes showed a decrease from BR to PS, except those of plasma EC levels in female Brahminy myna. An increase from PS to NB was common to both sexes of both species. Exceptional difference worthy of notice was concerned with rise in plasma EC levels during PR to BR in the case of female Bank myna only.

DISCUSSION

It is a well recognized fact that in steroidogenic tissues, depletion of AA is known to occur during active steroidogenases (Szent Gyorgii, 1957; Breitenbach, 1962; Bratcher and Kent, 1971; Chinoy and Seethalaxmi, 1977; Muddeshwar *et al.*, 1984). Sex-dependent differences in AA content have been reported in six out of eight tissues studied in rats (Stubbs and Mackernan, 1967). Sex dependent differences in the AA synthesizing enzyme activity of the liver has been reported to be more androgen dependent (Stubbs *et al.*, 1967; Chinoy and Rao, 1979). In pigeon

higher gonadal, hepatic and renal AA content have been reported during non-breeding season, when Δ^5-3β-HSDH activity in the gonads was at its minimum level (Kotak, 1979). Dieter (1969), Majumdar and Chatterjee (1974) and Chinoy et al. (1979) have demonstrated tissue localization and synthesis of AA to be under the control of testosterone in cockreals and rats.

On the basis of histochemical observations on the tissue AA level in pigeon, Chinoy (1972 a) reported following descending order —
Brain>liver>ovary>pancreatic acini>kidney>adrenals>testis.
However, on the basis of quantitative assessment, Kotak (1979) found that the tissue AA levels exhibited a pattern in descending order as gonads>liver>kidney. Thus, it seems that there is enough evidence available from the literature on a positive relationship between the tissue AA content and steroidogenesis. By now it is well known that the liver plays a major role in synthesizing and releasing into the blood major amount of cholesterol and its esterified derivatives for the facilitation of gonadal development in the birds in general (Lofts and Murton, 1973) and that of female birds in particular (McIndoe,1971).

In the light of the above discussion, it was thought desirable to deal with the variations in the parameters studied here in a comparative way, first in respect of

the female reproductive phases and then in the case of the male birds of the two species. It would also be of interest to refer to species-specific and then sex-specific differences wherever they occur. Further, from the perusal of the data presented here, it became apparent that instead of the information emanating from separate consideration of either total or esterified components of cholesterol moieties that from paying attention to the ratios between these two components provided better indications about the trends in variations, hence, further discussion would follow this line of thinking in deriving the necessary inferences.

Female birds :

In both the species of birds the AA levels were higher during PR in case of all the three tissues and that there was a clearcut decrease in all the tissues during the transition from PR to BR. This trend clearly indicated a positive involvement of AA in steroidogenic activity as corroborated by variation in EC : TC levels as evinsced from the consideration of the ratio between latter two components. From this it could be inferred that comparatively lower proportions of esterified cholesterol component is more favourable for enhanced gonadal steroidogenesis in the Bank myna. However, this seems to be not so obvious in the case of Brahminy myna.

A further interesting fact about Bank myna, that came to light, pertains to the role of intestine as far as the cholesterol component is concerned. There was a distinct lowering of EC moiety (from 0.53 to 0.29) in the liver even in the face of ovarian increase of EC along with TC component. As reported elsewhere in this thesis (Chapter I) there was a noticeable increase in the gonadal lipid content along with marked rise in the gonadal weight only in Bank myna. Under these conditions it was observed (Chapter III) that the intestinal uptake as well as re-esterification of cholesterol was enhanced, in all probability a menifestation of influence of increasing levels of female sex hormones. Here it would be worthwhile citing the findings of Kotak (1979). in respect of increased enzyme activities concerning steroids in the intestinal mucosa of pigeon during the breeding season. Therefore, the present author is tempted to suggest that, at least in female Bank myna, the oestrogens apparently enhance the role of intestine in supplying esterified cholesterol through chylomicrons, particularly in the face of reduced feed from the liver under these circumstances. However, it should be noted here that this was not so in the case of female Brahminy myna.

During the transition from BR to PS a decrease was seen in TC and EC levels in three tissues studied and also as mentioned elsewhere in the thesis (Chapter I), decrease

in gonadal total lipids and gonadal weight was noted. During this period accumulation of AA was also very apparent. This probably indicated a subdued level of steroidogenesis in the ovaries, since loss in gonadal weight was not so abrupt in female birds as compared to those of male birds.

During transition from PS to NB hepatic TC as well as EC rose to much higher levels with decrease in gonadal weights. These changes are logically indicative of ovarian regression and losses due to egg laying. During this transition however, the hepatic and plasma AA exhibited a decrease whereas the gonad showed a significant increase in the level of the same, thereby suggesting arrest of steroidogenic function.

In the light of the above discussion, it would be logical to infer that the female gonads of both species regressed comparatively slowly during the transition from BR to PS to NB as opposed to what occurred in the case of male birds. This finds support in the findings reported elsewhere (Chapter I) as far as the graded loss of ovarian weight and supportive lowering of gonosomatic indices are concerned. This amounts to saying that the subdued ovarian steroidogenic activity during the transition from BR to PS is, in all probability, for supporting the post-breeding activities (brooding and nurture of young ones). Thereafter,

the ovarian regression was clear, when accumulation of AA, maximal decrease in gonadal weight and GSI were obvious.

Whatever have been said for Bank myna was also applicable to the female Brahminy mynas but the variations were comparatively much less intense.

On an overall basis, it could be seen from the variation in the ratios of EC : TC that 30 to 40% of esterified component in the hepatic and plasma apparently favour accumulation of cholesterol positive lipids in the gonads of both species of mynas. Why this should be so is not easy to explain from the data at hand. Further work is necessary to understand this situation properly.

Male birds :

On a comparative basis, it could be suggested that in case of male birds steroidogenesis appears to occur mostly during the transition from PR to BR. Thereafter, the regressive changes, as indicated by the levels of cholesterol (TC and EC), AA, gonadal weight, GSI and cholesterol positive gonadal lipids (Chapter I), continued to decline through BR to PS to NB. Moreover, in case of male birds of both species, whatever has been said about subdued steroidogenesis during post-breeding period in case of female birds, did not seem to hold true. Hence, the

testicular regression appeared to be sharp and final, that is to say that the spermatogenic arrest is more or less sudden. This may be the reason for lesser interest exhibited by male birds in respect of post-breeding behaviour in both the species of myna. Another interesting point, apparent from the data at hand, seemed to be related to a comparatively sharp recrudescence of male gonads as opposed to those of the females (NB to PR variations). This also explains an early expression of male sexual behavioural patterns (occupation of territory and seeking of mates) in case of both the species. This demands further investigation in respect of the pituitary functions and titres of gonadotropins as well as gonadal hormones in these two species to substantiate the inferences drawn here.