CHAPTER 3

THE ASCORBIC ACID AND CHOLESTEROL CONTENTS IN THE BREEDING AND NON-BREEDING MALE AND FEMALE AND HELPER FEMALE OF JUNGLE BABBLER.

INTRODUCTION ~

Ascorbic acid is known to play varied significant roles in the general body metabolism due to its ability to participate in the metabolic reactions as a oxidizing as well as reducing agent. It gets oxidized to dehydorascorbic acid and thereby prevents many enzymes and co-factors from being rapidly reduced during oxido-reduction reactions (Mayes, 2000).

Ascorbic acid for long has also been related with fertility/reproduction *via* its three principal functions namely its promotion of collagen synthesis, its role in hormone production and its ability to protect cells from free radicals (Chinoy and Rao, 1979; Luck *et al.*, 1995; Biswas *et al.*, 1996). The reactions in the steroid biosynthesis pathway which are accompanied by formation of oxygen radicals have been correlated with the levels of antioxidant *i.e.* ascorbic acid, and plasma progesterone levels indicate that the antioxidative mechanisms are activated to handle the process of

steroidogenesis in bovine corpora lutea (Rapaport *et al.,* 1998). Luck *et al.,* 1995 has reviewed the involvement of ascorbic acid with fertility in both testis and ovaries. During gonadal cycle, both the gonads exhibit phases of tissue regeneration and regression as well as peptide and steroid secretion the activities that are assumed to be ascorbate dependent. Further, ascorbic acid may also prevent gametes from getting damaged by free radicals during their production and fertilization. Ascorbic acid has a synergistic action with testosterone for the potentiation of its anabolic action for increasing the testicular germ cell maturation and enhancing the activity of a number of androgen dependent enzymes (Chinoy *et al.,* 1978). In Guinea pigs, which are unable to synthesize ascorbic acid and has to depend on dietary ascorbic acid as vitamin, the deficiency of the same has been reported to influence their reproductive activities (Chinoy *et al.,* 1986), this effect is primarily at the testis level, and the androgen deprivation and the anti-fertility effects are probably secondary effects indicating that ascorbic acid is essential for maintaining the physiological integrity of androgen target reproductive organs. Biswas *et al.,* (1996) have also related the effects of ascorbic acid on testicular steroid dehydrogenase activity and testosterone concentration and observed that a significant positive stimulation of enzyme activity is under the influence of ascorbic acid thereby increasing the testosterone concentration.

Since, ascorbic acid is essential for the formation of intercellular materials and structural elements as well as for metabolic activities,

most of the animals have developed the abilities to synthesize it. Most of the birds and mammals are known to synthesize ascorbic acid in their tissues (Chinoy, 1972). Biosynthesis of ascorbic acid in non – passerine birds occurs in the kidneys, whereas in higher passerines the kidneys as well as the liver possess this capacity (Raychaudhari and Chaterjee, 1969). The present investigation reports the concentration of ascorbic acid in liver, intestine, kidney and gonads of breeding and the non – breeding males and females and helper females of Jungle Babbler (*Turdoides striatus*).

Cholesterol is an important sterol, which is widely distributed and is a constituent of all animal cells. It has various important roles which include functions as a special transport agent for unsaturated fatty acids in the blood plasma, as a precursor of bile acids in liver, and as a precursor of various steroid hormones. Of the many tissues, liver is the major site of cholesterol synthesis followed by intestine, carcass and skin in chicken (Yeh and Leveille, 1973). In the birds, liver plays a major role in the synthesis of the major amount of cholesterol and its esterified derivatives and its release into the blood for the facilitation of the gonadal development (Lofts and Murton, 1973). Although, most attention has been given to liver, other tissues capable of cholesterol synthesis are intestine or the GI tract and skin (Dietschy and Weigh, 1974; Murray *et al.,* 2000). In addition to synthesis cholesterol is also absorbed as well as excreted in the GI tract. It is excreted by the GI tract as a catabolic steroid product *viz* bile acids or

as cholesterol itself (Murray *et al.,* 2000). In the GI tract it can be re-esterified and supplied back to liver for further circulation with other absorbed lipids in the form of protomicrons via portal blood (Bensadoun and Rothfeld, 1972).

Renal tissue to a certain extent seems to have a possible influence in cholesterol metabolism. Since cholesterol is a chief precursor of steroidogenesis, the delta-4 pathway of steroid metabolism is observed in kidneys (Baillie *et al.*, 1966). The co- factor involved in steroid synthesis, the ascorbic acid is synthesized in the avian kidney which possibly suggests the role of renal tissue in steroid metabolism. One of the important enzyme of this enzyme complex, the 3â- HSDH has been shown to be under the influence of Ascorbic acid in the toad testis (Biswas and Deb, 1970) and the 17 â- HSDH another important enzyme catalyzing the conversion of dehydroepiandrosterone to androsteindione has been reported to increase its activity after incubation with ascorbic acid in rat testis (Biswas *et al.,* 1996) thereby stimulating the synthesis of testosterone.

In this chapter ascorbic acid and cholesterol contents in liver, intestine, kidney and gonads of breeding and non-breeding Jungle Babblers with that of helper females is evaluated.

MATERIALS AND METHODS ~

Birds were procured from a local animal supplier and they were sacrificed as early as possible to avoid effect of caging. Tissues *viz.* liver, intestine, kidney and gonads (testis and ovary) were dissected out, blotted free of blood and tissue fluids, weighed and kept in refrigerator for short periods till processed further.

Ascorbic acid

To estimate ascorbic acid the method of Roe (1954) was employed. Parts of the tissues were homogenized in prechilled mortars with 5 ml cold 6% TCA. TCA is known to reduce pH, stabilize the ascorbic acid and prevent its catalytic oxidation. Norit an activated animal charcoal acting both as oxidizing agent as well as a clarifying reagent for removal of pigments was added. The solution was mixed well and allowed to stand for 15 minutes and filtered through Whatman filter paper No. 42. 2 ml aliquot was taken in the test tube and 2 drops of thiourea (which acts as a reducing agent) and 0.5 ml of 2, 4 DNPH (which acts as a mild oxidizing agent) were added to all the tubes. The contents of the tubes were mixed thoroughly and left for incubation in a boiling water bath for 15 minutes. At the end of the incubation, tubes were transferred to ice bath and 2.5 ml of 85 % H_2SO_4 was added and allowed to stand for 30 minutes. The color developed was read at 540 nm in the photocolorimeter. Ascorbic acid

in the tissue is expressed as gm of Ascorbic acid/ 100 gm of tissue weight.

Cholesterol

Other parts of all the tissues were used for the estimation of total cholesterol contents. Cholesterol was extracted in 3:1 chloroform - methanol mixture. The fresh tissues were crushed in test tube with a glass rod to which 2 ml of chloroform: methanol mixture was added and left overnight and the supernatant was collected next day. For the estimation of cholesterol, 2 ml of the extract was taken in separate tubes and dried totally by keeping the tubes in air oven. The cholesterol contents of the tissues were estimated by employing the method described by Crawford, (1958). After drying the tubes (containing the extract), 3 ml of $FeCl_3$ was added to each test tube and boiled for 5 minutes. After cooling 2 ml of concentrated H_2SO_4 was added and mixed thoroughly. The brown color developed was measured after 30 minutes on the colorimeter at 540 nm. Cholesterol is expressed as mg of Cholesterol/ 100 mg of tissue weight.

Table 1: Variations in the Ascorbic acid content of male and female Jungle Babblers

	MALE		FEMALE		
	Breeding	**Non-breeding**	**Breeding**	**Non-breeding**	**Helpers**
LIVER	6.20 ± 0.601	4.85 ± 0.429	4.4 ± 1.01	4.97 ± 0.732	4.2 ± 0.37
INTESTINE	3.11 ± 0.310	4.07 ± 0.550	4.92 ± 0.63	5.725 ± 0.732	4.31 ± 0.58
KIDNEY	4.4 ± 0.726 *	2.68 ± 0.524	2.52 ± 0.24	2.36 ± 0.686	2.81 ± 0.45
GONADS	3.46 ± 0.537	4.93 ± 0.679	1.85 ± 0.41	2.55 ± 0.369	2.4 ± 0.25

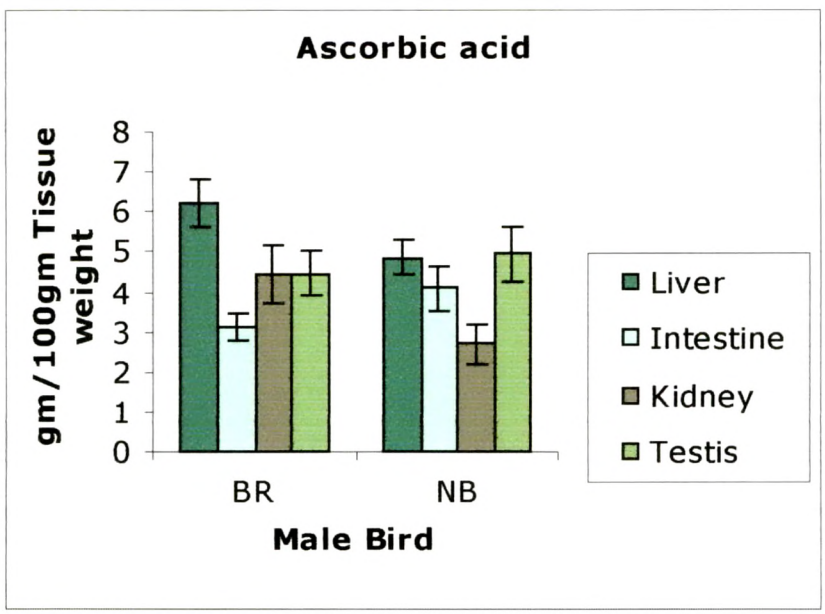
* $P < 0.05$

Table: 2 Variations in the Cholesterol content of male and female Jungle Babblers

	MALE		FEMALE		
	BREEDING	**NON-BREEDING**	**BREEDING**	**NON-BREEDING**	**HELPERS**
LIVER	0.10 ± 0.014	0.14 ± 0.024	0.14 ± 0.011	0.095 ± 0.02	0.12 ± 0.014
INTESTINE	0.10 ± 0.015	0.10 ± 0.018	0.12 ± 0.013	0.12 ± 0.027	0.13 ± 0.010
KIDNEY	0.14 ± 0.01	0.12 ± 0.007	0.12 ± 0.014	0.056 ± 0.02**	0.11 ±0.006**
GONADS	0.029 ± 0.001	0.041 ± 0.001	0.02 ± 0.013	0.040 ± 0.001	0.011 ±0.007*

* P <0.05 ** P < 0.005

Figure 1: **Ascorbic acid content of gonadal and extra-gonadal tissues of breeding and non-breeding male and female Jungle Babblers along with helper females.**



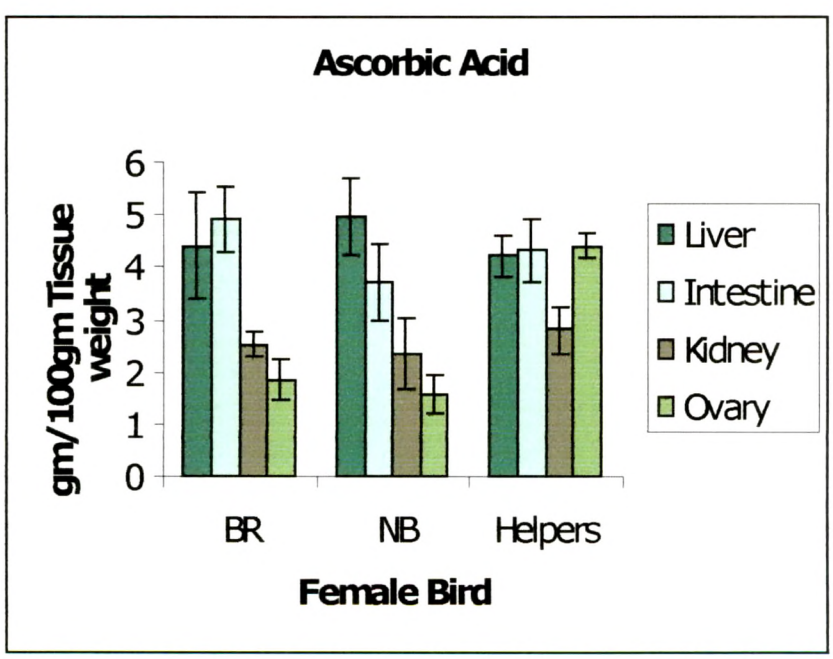
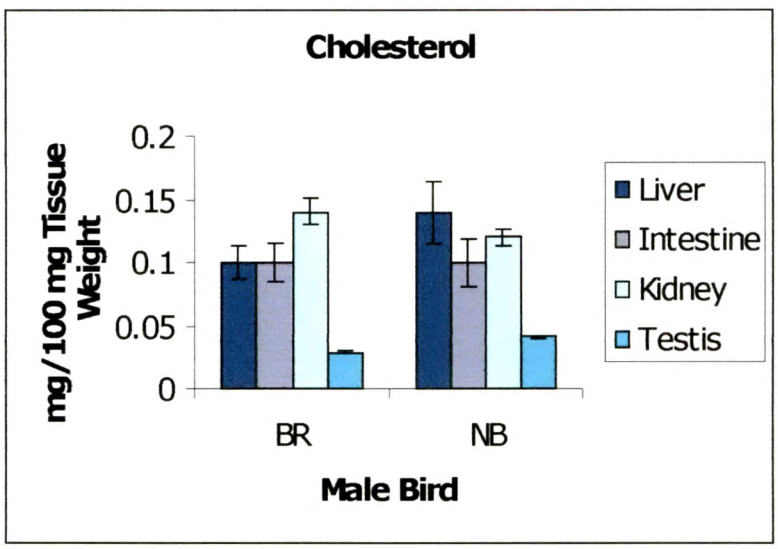


Figure 2: Cholesterol content of gonadal and extra-gonadal tissues of breeding and non-breeding male and female Jungle Babblers along with helper females.



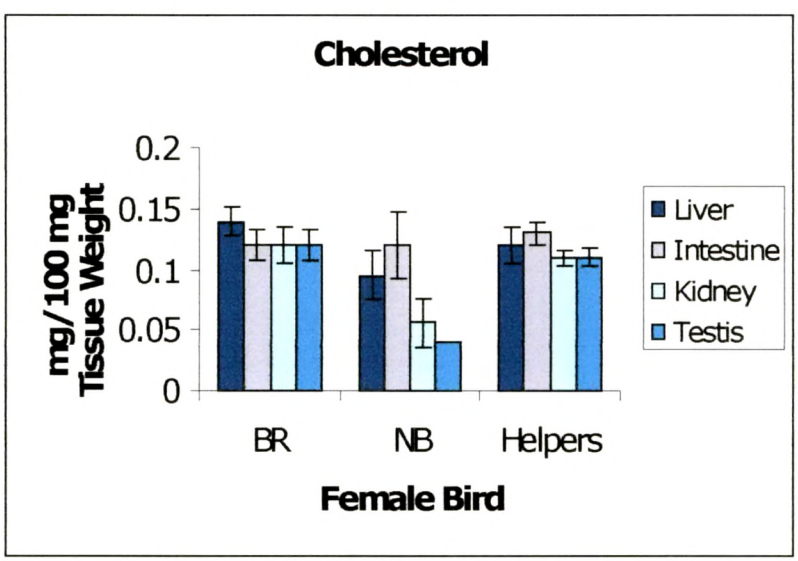
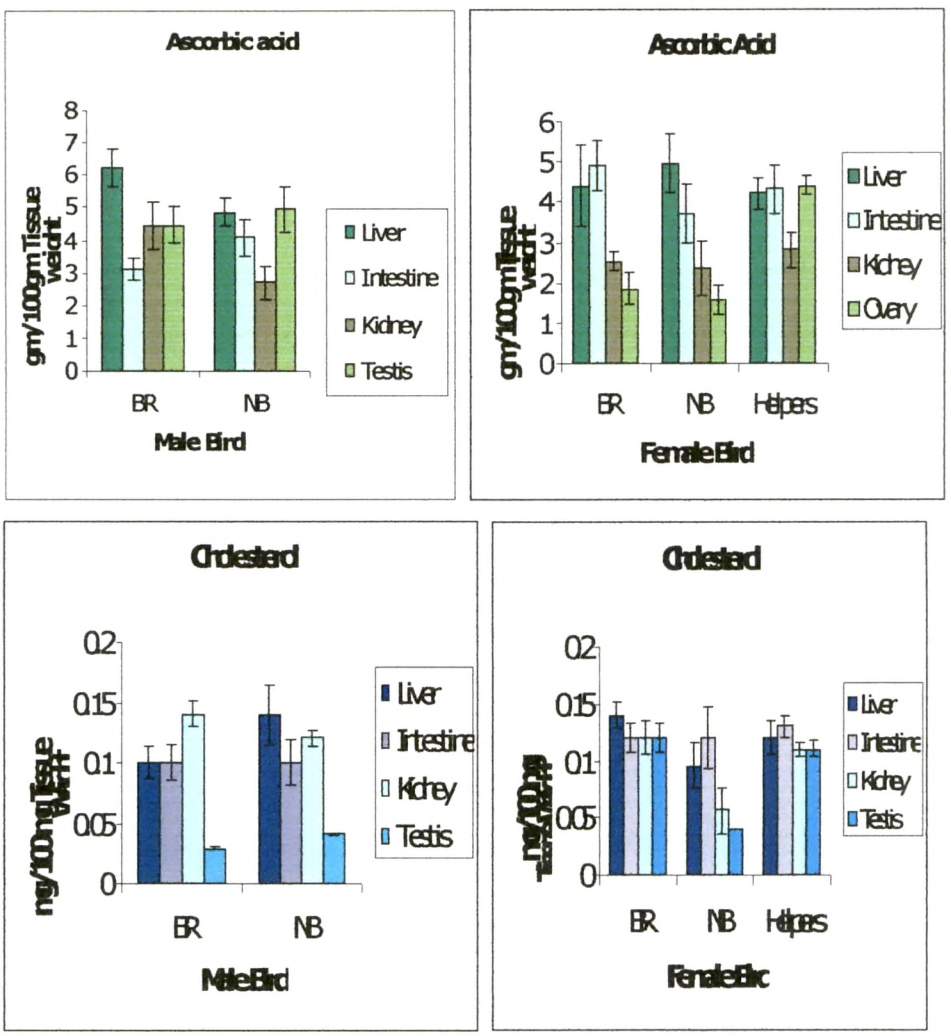


Figure 3: Ascorbic acid and Cholesterol content of gonadal and extra-gonadal tissues of breeding and non-breeding male and female Jungle Babblers along with helper females



RESULTS ~

The tissues studied were liver, intestine, kidney, and gonads of both breeding and non-breeding male and female Jungle Babblers *(Turdoides striatus)* as well as helper females. Variations as related to the reproductive state in Ascorbic acid and cholesterol contents of various tissues are given in table 1 and 2; Fig 1, 2 and 3 respectively.

Ascorbic acid (Table 1 and Fig 1)

Liver: The ascorbic acid content of liver in breeding males is non-significantly higher than in the non – breeding males which were 6.20 ± 0.60 (gm/100gm tissue wt) and 4.85 ± 0.42 gm respectively. In the breeding females hepatic ascorbic acid content was 4.4 ± 1.01 gm while in the non – breeding females it was 4.97 ± 0.073 gm. Helper female showed nearly equal ascorbic acid content to that of the breeding females 4.2 ± 0.37 (gm/100gm tissue wt).

In intestine a reverse pattern is noted. In males the ascorbic acid content was non-significantly higher in the non – breeding males than in the breeding males, at 4.07 ± 0.55 (gm/100gm tissue wt) and 3.11 ± 0.310 gm respectively. In breeding females, the intestinal ascorbic acid was 4.92 ± 0.63 gm whereas it decreases non – significantly in the non – breeding females to 5.72 ± 0.73 gm; while in case of helper females the levels were nearly same to that of the breeding females at 4.31 ± 0.58 (gm/100gm tissue wt).

Kidney of breeding males had 4.4 ± 0.22 (gm/100gm tissue wt) ascorbic acid whereas that in the non-breeding males was significantly low at 2.68 ± 0.52 gm. The female Jungle Babblers in three different breeding states had almost constant levels of renal ascorbic acid with breeding females having 2.52 ± 0.24 gm, the non-breeding females 2.36 ± 0.68 gm and in the helper females' 2.81 ± 0.45 gm/100gm tissue wt.

Testis of Jungle Babblers had non-significantly higher ascorbic acid in the breeding males than in the breeding males at 4.93 ± 0.67 (gm/100gm tissue wt) and 3.46 ± 0.53 gm respectively. Ovaries also showed the same pattern wherein ascorbic acid content were non-significantly low in the breeding females than in the non – breeding females at 1.85 ± 0.41 gm and 2.55 ± 0.36 gm respectively; while in the helper females it was nearly equal to non-breeding females at 2.4 ± 0.25 gm/100gm tissue wt.

Cholesterol (Table 2 and Fig 2)

The cholesterol content in the liver of non – breeding and the breeding males Jungle Babblers were 0.14 ± 0.024 and 0.10 ± 0.014 mg/100mg tissue wt respectively whereas in breeding females, it was 0.14 ± 0.011 mg and in helper females it was 0.12 ± 0.010 mg respectively which decreases in the non – breeding females to 0.095 ± 0.02 mg/100mg tissue wt.

No significant difference was seen in the intestinal cholesterol content of breeding and non-breeding males and females as well as helper females. The intestine content fluctuated marginally around 0.1 mg/ 100 mg of tissue weight in all the categories of Jungle Babblers.

The kidney of breeding and non-breeding male and breeding and helper female Jungle Babblers also did not show any significant variations in cholesterol content and had about 0.1 mg /100 mg of tissue. In the non-breeding females, the renal cholesterol content were significantly low at 0.056 $\pm$ 0.02 mg.

The testes of non-breeding males had significantly higher cholesterol content at 0.041 $\pm$ 0.001 (mg/100mg tissue wt) as compared to the non-breeding testes at 0.029 $\pm$ 0.001 mg respectively. Ovaries showed nearly same levels in the breeding and helper females at 0.012 $\pm$ 0.013 mg and 0.011 $\pm$ 0.007 mg respectively which were significantly higher than the non – breeding ovaries at 0.040 $\pm$ 0.001 (mg/100mg tissue wt).

DISCUSSION ~

Ascorbic acid has long been associated with fertility. The mechanism of action of Ascorbic acid in reproductive tissues has been done by several workers. On one hand, ascorbic acid has been reported to influence gonadal steroidogenesis in Toad testis (Biswas, 1969; Biswas and Deb, 1970) and rat testes (Biswas *et al.,* 1996; Chinoy *et al.,* 1978). Ascorbic acid is also essential for maintaining the physiological integrity of the androgen target reproductive organs (Chinoy *et al.,* 1986). On the basis of the involvement of Ascorbic acid in promotion of collagen synthesis, its role in hormone production and its ability to protect cells from free radicals, Luck *et al.,* (1995) have emphasized the role of Ascorbic acid in male and female fertility.

To elucidate the same in birds, ascorbic acid and cholesterol levels were quantified in liver, intestine, kidney and gonads of both the breeding and the non-breeding male and female Jungle Babblers along with the helper females that show the ovarian development but with moderate oviducal development.

In Jungle Babbler the variations in ascorbic acid levels of breeding and non-breeding birds are not significant in both the sexes as well as the helper birds. The significant differences are noted only in renal ascorbic acid of male birds though the significance level is low. Ascorbic acid is known to participate in tissue metabolism via the formation of its free radical (Chinoy *et al.,* 1978) and tissue distribution and synthesis of ascorbic acid are testosterone dependent

in rat (Chinoy and Rao, 1979). As noted in chapter 2, compared to other species of birds, male Jungle Babblers, members of the social flock, show lower testosterone levels. Probably this is reflected by lower metabolic fluctuations between breeding and non-breeding states, further reflecting lower formation of free radicals compared to other birds showing distinct breeding and non-breeding activities and hence lower variations in hepatic ascorbic acid levels. In rats, liver is the site of ascorbic acid synthesis whereas in birds liver and /or kidney is/are the ascorbic acid synthesizing sites (Raychaudhari and Chaterjee, 1969). From the variations in the ascorbic acid levels of kidney it seems that in Jungle Babbler kidney seems to be the main site of Ascorbic acid synthesis, hence, the expected influence of testosterone is exhibited by the kidney. In female rats, concentration, metabolic turnover and synthesis of ascorbic acid in liver and adrenal are dependent of estrogen. No such dependence seems to be occurring in liver, intestine and kidney of Jungle Babbler females which are having a helper to perform many of the reproduction related activities except egg laying. The difference in ascorbic acid level of the two being noted only in the ovaries with helper female ovary having ascorbic acid equal to that of the non-breeding female. The evaluation of estrogen levels in these birds can help in coming to a more definite conclusion.

In female birds, the contribution from dietary sources seems to be higher during non – breeding state. Ascorbic acid is very loosely

held in the tissue of intestine and that the small intestine is capable of accumulating double the normal amount of ascorbic acid even when animal's body stores are depleted (Oelrichs and Kratzing, 1980). Though liver is the prime Ascorbic acid synthesizing site, Ascorbic acid deficiency provokes several metabolic disorders in the liver, one of which is impaired cholesterol transformation (Jenkins, 1980). In humans, a significant positive correlation between serum HDL cholesterol and Ascorbic acid is reported in man and woman (Itoh *et al.*, 1990). A contradictory relationship of ascorbic acid and cholesterol has been established. Among non-human, in Baboon, the stress of captivity causes decrease in the serum ascorbic acid and an increase in the serum cholesterol (Kotze, 1975) while in cockerels addition of ascorbic acid to 5% fatty acid diet increased heart and liver cholesterol (Klopfenstein and Clegg, 1980). Dietary intake of ascorbic acid stimulates the synthesis of cholesterol from acetate and mevalonate in Baboon liver and increases the turnover rate of cholesterol body pool (Kotze, 1975). Jenkins (1980), showed that pregnant guinea pigs receiving low doses of L- ascorbic acid develop a condition namely hypercholesterolemia. The group receiving higher dose showed an increased biliary secretion of bile acids.

No significant differences in the cholesterol content of liver, kidney and intestine of breeding and non-breeding Jungle Babblers as well as helpers are noted, except a significantly lower level of cholesterol in the kidney of non-breeding females. In Jungle Babbler

kidney, higher 17 â-Hydroxysteriod dehydrogenase (HSDH) is observed in the non-breeding birds (Sapna, unpublished data) indicating probable involvement of kidney in steroid metabolism. The non-significant accumulation of cholesterol in testis is noted in non – breeding gonads. These are probably the reflections of social nature of Jungle Babbler as 6-8 individuals of the flock show the similar foraging activities and mainly feed on carbohydrate and protein rich diet. Hence no significant differences in the cholesterol in the breeding, non-breeding and helper birds.

Ascorbic acid influences steroid synthesis by stimulating the 17â HSDH activity in the testis of the rat by increasing the rate of oxidation of reduced NADP and thereby stimulating the activity of 17 â-HSDH to synthesize testosterone (Biswas *et al.,* 1996) and $Ä^5$-3 â-HSDH activities in toad testis (Biswas, 1969; Biswas and Deb, 1970). The increased requirement of ascorbic acid in the breeding male Jungle Babblers probably comes from the liver and kidney. Comparatively lower ascorbic acid and cholesterol levels in general could be due to its flocking or social nature. In Bank Myna (*Acridotheres ginginianus*) and Brahminy Myna (*Sturnus pagodarum*) which have distinct breeding and non-breeding seasons as well as move around in pairs, distinct differences in ascorbic acid and cholesterol levels during breeding and non-breeding seasons have been reported from our laboratory (Padate, 1990). These birds also show higher testosterone levels (Sapna, 2002) when compared to Jungle Babblers (Chapter 2).

Almost equal ascorbic acid levels in liver, intestine and kidney of breeding and helper females indicate its equal role in general metabolism whereas accumulation of the same in the ovary of the helper female to that of the non-breeding females indicates subdued steroidogenesis in helper females as is in the non-breeding females. Accumulation of ascorbic acid in the non-breeding gonads has been reported by several workers (Ambadkar and Kotak, 1976; Ambadkar and Padate, 1993; 1995). Both the gonads, the testis and the ovary exhibit the cycles of tissue remodeling and of peptide and steroid secretion that can be assumed to be ascorbate dependent. The reactions of steroid hormone biosynthesis are accompanied by the formation of oxygen radicals (Rapaport *et al.,* 1998) and ascorbic acid may also prevent gametes from damage by free radicals during gametogenesis and fertilization (Luck *et al.*, 1995). They suggested that the supply of ascorbic acid to the ovary might be limiting factor in the ability of the pre-ovulatory follicle to grow in response to gonadotropin stimulation. The dietary deficiency of ascorbic acid affects the androgen dependent factors in the reproductive tissues-testis, epididymis and vas deferens and the accessory sex glands and caused an "androgen deprived effect" in these target organs of male rat (Chinoy *et al.,* 1986). This in turn alters their internal composition and causes changes in their metal ion profile and the morphology, motility and density of spermatozoa in epididymis and vas- deferens. This demonstrates that the ascorbic acid is essential for maintaining

the physiological integrity of the androgen target reproductive organs (Chinoy *et al.,* 1986). Ascorbic acid in testis acts as a co-factor wherein it influences testicular steroid dehydrogenase by stimulating the enzyme activity resulting in altered testosterone levels (Biswas *et al.,* 1996). According to Pintauro and Bergan (1982), ascorbic acid increases the conversion of pregnenolone to delta 4 steroids and decreases its conversion to delta 5 steroids. They support a general inhibitory effect of high ascorbic acid on the steroid hydroxylation and a possible regulatory role of ascorbic acid on the conversion of pregnenolone to delta-4 and delta-5 steroids. In birds also the association of the ascorbate with chicken steroidogenesis has been suggested (Byrd *et al.,* 1993). The levels of ascorbic acid present in the testis of the breeding and the non – breeding Jungle Babbler males suggest the utilization of ascorbic acid as a co-factor in steroidogenesis in the breeding male and its accumulation in the non- breeding males in the absence of steroidogenesis.

Jungle Babbler having a longer breeding season as well as sharing its parental responsibilities with other individuals of the flock, comparatively lower testosterone levels have been found (Chapter 2) than those species which show distinct breeding and non-breeding seasons. This is probably reflected by lower fluctuations in the ascorbic acid and cholesterol levels in all the tissues studied as well as gonads.