

Effects of L-Thyroxine on the Reproductive Functions of the Indian Pygmy Field Mouse *Mus terricolor*

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Abstract

Besides being potent regulators of energy balance, thyroid hormones are necessary for the maintenance of seasonal reproductive changes in many species of mammals and birds. The thyroid gland is known to be crucial for the seasonal transition in seasonal breeders from breeding to non-breeding. Many workers have reported that thyroid ablation prevents the animal from entering the non-breeding state in both males and females of different species of animals and, in such a situation, the breeding state is sustained indefinitely. No report exists to date about the involvement of the thyroid gland in the reproduction of the pygmy field mouse *Mus terricolor*. L-thyroxine was administered subcutaneously to both male and female mice for 15 consecutive days during the reproductively active phase. L-thyroxine treatment led to a significant reduction in the weights of gonads and accessory sex organs along with biochemical constituents like epididymal sialic acid, seminal vesicular fructose and uterine protein. Regressive changes in the histology of the gonads and accessory sex organs were observed which can be attributed to the reduced levels of plasma testosterone in males and plasma estradiol and progesterone in females of the L-thyroxine-treated mice. However, there was a significant increase in the levels of plasma T3, T4, and gonadal cholesterol in both sexes following L-thyroxine treatment. Both hypo- and hyperthyroidism can influence gonadal activity. The observations in the present study suggest that hyperthyroidism leads to the transition of reproductively active gonads to inactivity substantiating the hypothesis that high levels of thyroxine can accelerate the reproductive inactivity in this rodent.

Keywords: *Mus terricolor*; Seasonal reproduction, Thyroid, Reproduction, Tropical rodent

1. Introduction

The annual reproductive cycle of seasonal breeders is regulated in part by environmental factors (photoperiod, temperature, humidity, rainfall, and food availability) and endogenous changes in the brain-pituitary-target endocrine axis that are independent of environmental influences¹. Metabolically active thyroid gland is known to play a pivotal role in the control of reproduction in seasonal breeders. The importance of thyroid hormones

in seasonal reproduction was first demonstrated in starlings by Woitkewitsch in the year 1940² wherein it was shown that thyroidectomy prevented the seasonal decline in the testicular activity and sustained the breeding status indefinitely. Since, then many studies have proved that thyroid hormones are necessary for the maintenance of seasonal reproductive changes in many species of mammals such as golden hamster^{3,4}, Indian palm squirrel *Funambulus pennanti*^{5,6}, sheep⁷, edible dormouse⁸ and several species of birds^{9,10}.

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The thyroid gland appears to be crucial for the seasonal transition in seasonal breeders from the breeding to the non-breeding phase. Many workers have reported that thyroid ablation prevents the animal from entering the non-breeding state in both males and females of different species of animals such as rat^{11,12}, Indian palm squirrel⁶, Syrian hamster¹³, and mouse¹⁴.

Besides being a potent factor in the regulation of seasonal reproduction, the metabolic hormone, thyroxine (T₄), has been implicated in the physiological regulation of energy balance as well as in maintaining normal reproductive function in mammals^{15,16}. Thyroxine and tri-iodothyronine are involved in tissue differentiation and growth, and the regulation of numerous bodily functions, mainly by affecting metabolic rate. Thyroid hormones act on many different target tissues, stimulating oxygen utilization and heat production in every cell of the body. The overall effects are to increase the basal metabolic rate, make more glucose available to cells, stimulate protein synthesis, increase lipid metabolism, and stimulate cardiac and neural functions^{4,17}.

The appropriate timing of various processes, necessary for the survival and reproductive fitness and success of seasonal breeders, is achieved by the interaction of various endogenous factors (including the thyroid gland) which consequently drives seasonal changes in the reproductive activity of the animal. The Indian pygmy field mouse *Mus terricolor* is a seasonal breeder¹⁸ but no report about the involvement of the thyroid gland in its reproduction exists. Therefore the present study was undertaken to find the response of the reproductive functions of this rodent to the thyroid hormone action.

The study has aimed to explore the effects of exogenous L-thyroxine administration on the reproductive functions of male and female *M. terricolor* during the reproductively active phase. The study was divided into two parts:

Part I: Effects of exogenous L-thyroxine on the reproductive functions of male *M. terricolor*.

Part II: Effects of exogenous L-thyroxine on the reproductive functions of female *M. terricolor*.

2. Materials and Methods

2.1 Animals and Their Maintenance

The experiments were performed according to the Institutional practices and within the framework of the revised Animal (Specific Procedure) Act of 2007 of

the Government of India on animal welfare. The study was conducted during the reproductively active phase of the animal. The male and female animals were collected from the crop fields in the vicinity of Varanasi (Lat. 25°, 18' N; Long. 83°, 1'E), India, following the methods as described^{19,20}.

The animals were brought to the laboratory and acclimatized for 2 weeks. Healthy young adult male mice and non-pregnant female mice of average weight 11±1.0 g were randomly selected. For the duration of the experiments, the animals were kept in commercial polypropylene cages and maintained in a room at 27±2.0°C, with gentle ventilation. The animals were fed commercial food along with wheat and rice and had free access to water.

2.2 Experimental Groups

Part I: Randomly selected twelve young adult male animals were assigned to two different groups of six each. The first group of mice was considered as a control and was treated with normal (0.9%) ethanolic saline. The animals in the second group received L-thyroxine.

Part II: Similarly, the female mice were divided into two groups with six animals each. The normal (0.9%) ethanolic saline-treated group was the control while the mice in the second group were treated with L-thyroxine.

L-thyroxine (Sigma-Aldrich Chemicals, USA) was dissolved in 0.01 N NaOH solution and diluted in normal saline up to the desired concentration. The injections were given through a subcutaneous route during the afternoons for 15 consecutive days in the reproductively active phase of the animal. The dose of thyroxine was 35 µg/100 g body Wt²¹.

2.3 Sample Collection

After a gap of 24 h from the last injection, the animals were weighed and sacrificed under total anesthesia. Heparinized tubes were used to collect the trunk blood. The heparinized blood was kept at -80°C till the ELISA. The levels of plasma estradiol and progesterone (Biotron Diagnostics Inc., Hemet, California, USA), T₃ and T₄ (Elabscience Biotechnology Co., Ltd., Houston, Texas, USA), and testosterone (DIAMETRA, Italy; Lot no. DKO002), were measured in the blood.

In males testis, epididymis, and seminal vesicles, and in females ovary and uterus were dissected on ice. The tissues were cleaned properly of blood and extra tissue.

The weight of the tissues was recorded using an electronic balance (Denver Instruments, Gottingen, Germany). Testis, epididymis, and seminal vesicles of the left side in the male mice and ovary and uterine horn of the same side in the female mice were fixed in Bouin's fluid for histological studies. The tissues of the right side were used for biochemical estimations of gonadal cholesterol, epididymal sialic acid, seminal vesicular fructose, and uterine protein.

2.4 Histology

The fixed tissues were processed for routine histological procedures. Some 6- μ m sections were deparaffinized and stained using Ehrlich's hematoxylin and eosin. The stained slides were observed in a microscope (Leica MPV-3, Germany) and documented.

2.5 Biochemical Estimations

The estimation of gonadal cholesterol was done using the manufacturer's protocol (BioLab Diagnostics, India). The concentrations of epididymal sialic acid and seminal vesicular fructose were determined following the methods of Aminoff²², and Linder and Mann²³, respectively. Bradford method²⁴ was adopted for measuring uterine protein.

2.6 Hormonal Analysis

2.6.1 ELISA for Testosterone

Following the manufacturer's instruction, 25 μ L of the sample, control, and standard were added to each well of the ELISA plate. One hundred microlitres of the enzyme conjugate solution and 100 μ L of the testosterone antiserum were added afterward. The incubation of the plate was done for one hour at room temperature with mild shaking. The wells were aspirated and washed thrice with double distilled water. The plate was further incubated at room temperature for 30 minutes after adding 100 μ L of the TMB chromogenic solution to each well. Finally, 100 μ L of stop solution (0.2 M H₂SO₄) was added. The absorbance was recorded at 450 nm using a microplate ELISA reader (BioTek).

2.6.2 ELISA for Estradiol and Progesterone

As per the manufacturer's protocol, 25 μ L of control, standard, and sample were added to each well in the ELISA plate. Further, 100 μ L of enzyme conjugate solution

was added. The plate was incubated for two hours with mild shaking at room temperature. Next, the wells were aspirated and washed three times with a wash solution. One hundred microlitres of the TMB chromogenic solution (substrate) was added and incubated at room temperature for 30 minutes in dark. One hundred microliters of stop solution were added finally. The absorbance was recorded at 450 nm.

2.6.3 ELISA for T3 and T4

The plasma levels of T3 and T4 in the respective groups were determined according to the manufacturer's instructions. ELISA kit for the assay of T3 and T4 was purchased from Elabscience Biotechnology Co. Ltd (Catalog No. E-EL-H1871 and E-EL-M1364, respectively). As per the manufacturer's instruction, 100 μ L of standard, control, and sample were added to each well of the ELISA plate. The incubation was done for 90 minutes at room temperature followed by the addition of 100 μ L of the Biotinylated Detection Ab. The ELISA plate was incubated with mild shaking at room temperature for one hour. The wells were aspirated and washed thrice. Then, 100 μ L of the HRP conjugate was added to each well and the plate was incubated further at room temperature for 30 min. The wells were again aspirated and washed five times. After that 90 μ L of substrate reagent was added to the plate and incubated for 15 minutes at room temperature. Finally, 50 μ L of stop solution was added and the absorbance was recorded at 450 nm.

2.7 Statistical Analysis

Statistical analysis of the data was performed with Student's 't'-test. The differences were considered significant when $p \leq 0.05$.

3. Results

3.1 Body Weight

No significant difference was observed in the body weight between saline-treated controls and L-thyroxine-treated male mice (Figure 1.1). Similarly, no significant change was observed in the body weight of the L-thyroxine-treated female mice as compared with the controls (Figure 1.14).

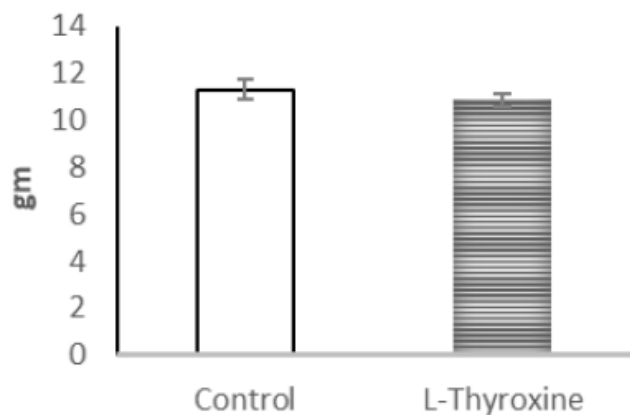


Figure 1.1 Bar graphs showing non-significant change in body weight of male *M.terricolor* following L-thyroxine injections.

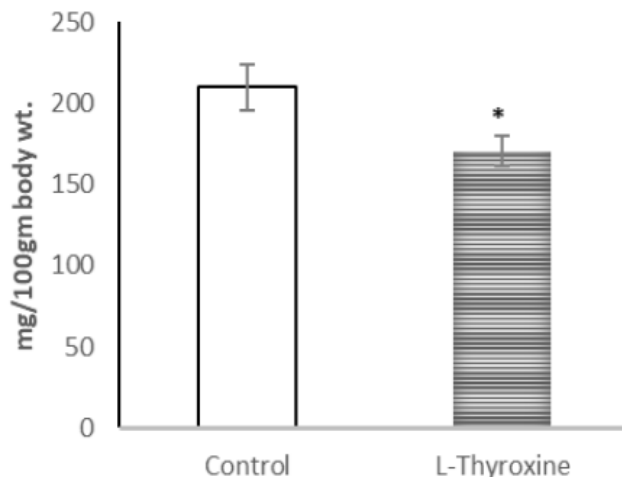


Figure 1.4 Bar graphs showing the significant reduction in relative seminal weight following L-thyroxine injection.

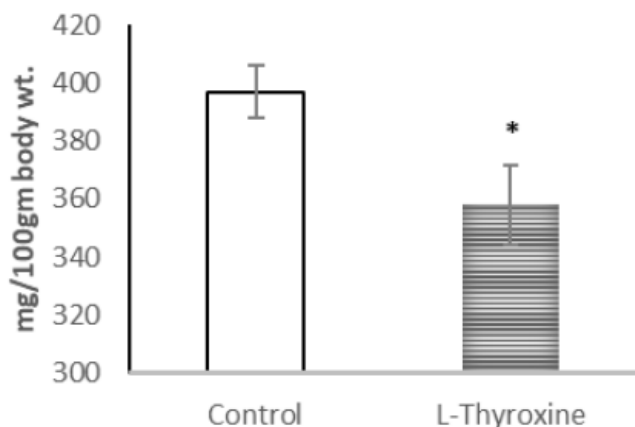


Figure 1.2 Bar graphs showing the significant reduction in relative testicular weight following L-thyroxine injections.

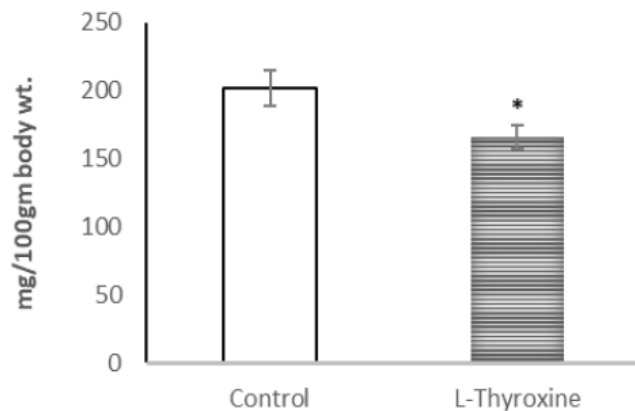


Figure 1.3 Bar graphs showing the significant reduction in relative epididymal weight following L-thyroxine injections.

3.2 Weight of Gonads and Accessory Reproductive Organs

Significant reduction in the relative weights of testis, epididymis, and seminal vesicles of the L-thyroxine-treated male mice as compared with the controls was found (Figure 1.2, 1.3, and 1.4, respectively). Likewise, L-thyroxine treatment in female mice resulted in a significant reduction in the relative weights of the ovary and uterus as compared with the controls (Figure 1.15 and 1.16, respectively).

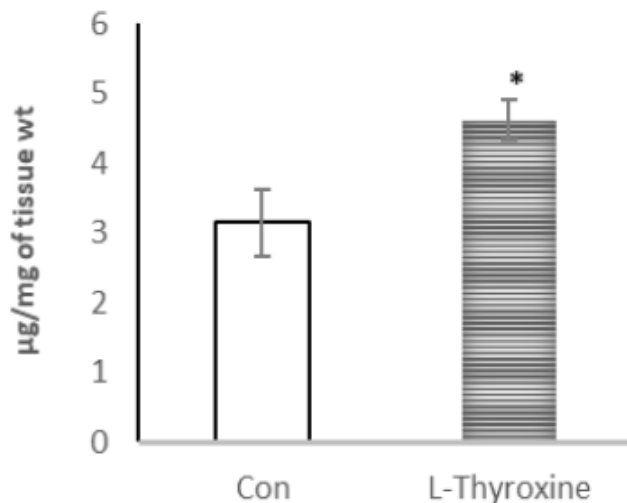


Figure 1.5 Bar graphs showing the significant elevation in the concentration of cholesterol following L-thyroxine injections.

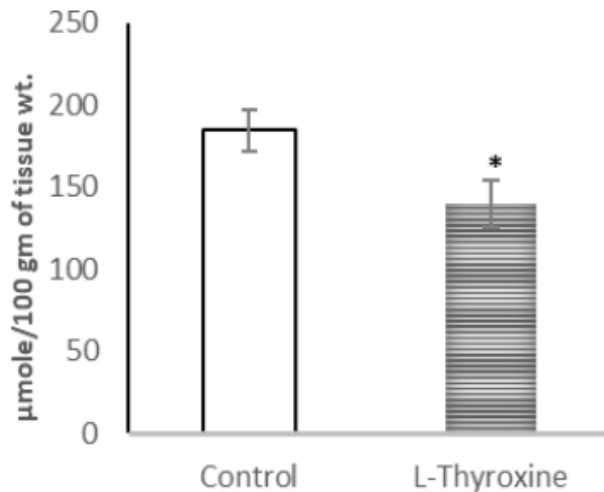


Figure 1.6 Bar graphs showing the significant reduction in the concentration of sialic acid following L-thyroxine injections.

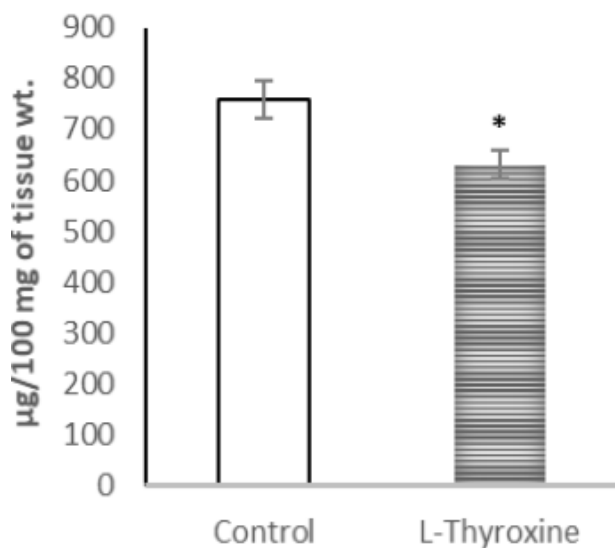


Figure 1.7 Bar graphs showing the significant reduction in the concentration of seminal vesicular fructose following L-thyroxine injections.

3.3 Biochemical Estimations

L-thyroxine treatment caused a significant increase in the concentrations of testicular (Figure 1.5) and ovarian (Figure 1.17) cholesterol as compared with their respective controls. By contrast, significant reductions were noticed in the concentrations of sialic acid in the epididymis (Figure 1.6), fructose in the seminal vesicles (Figure 1.7), and protein in the uterus (Figure 1.18) of L-thyroxine-treated mice as compared with controls.

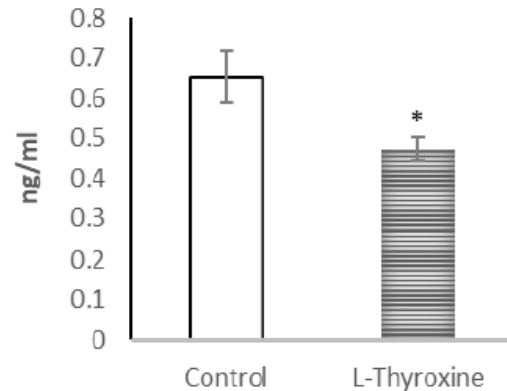


Figure 1.8 Bar graphs showing the significant reduction in the level of plasma testosterone following L-thyroxine injections.

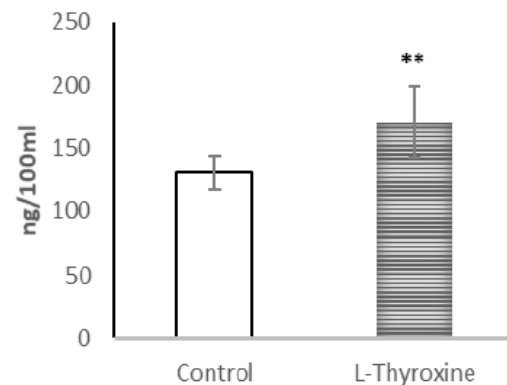


Figure 1.9 Bar graphs showing the significant elevation in the concentration of plasma T3 level of male *M. terricolor* following L-thyroxine injections.

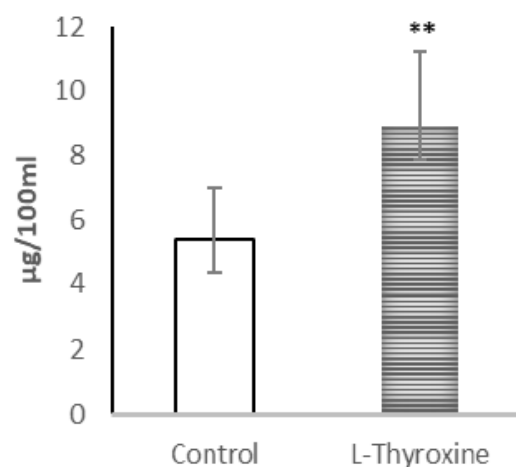


Figure 1.10 Bar graphs showing the significant elevation in the concentration of plasma T4 level of male *M. terricolor* following L-thyroxine injections.

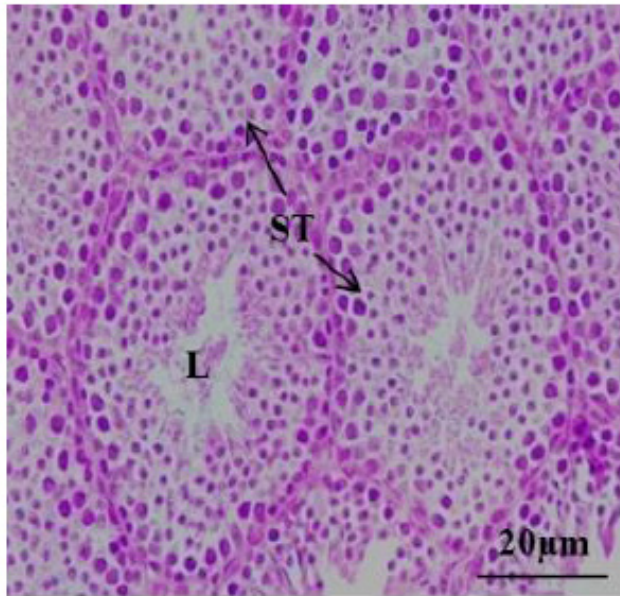


Figure 1.11A. T.S. of the testis of *M.terricolor* following saline injections. Note the active seminiferous tubules showing full spermatogenic activity. EL: Epithelial lining, L: Lumen, ST: Seminiferous Tubules.

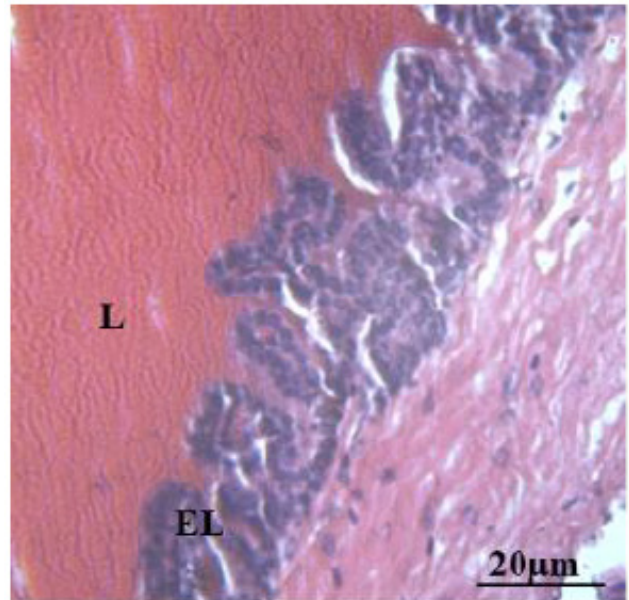


Figure 1.12A. T.S. of the seminal vesicle of *M.terricolor* following saline injections. Note the wide lumen filled with secretions. EL: Epithelial lining, L: Lumen.

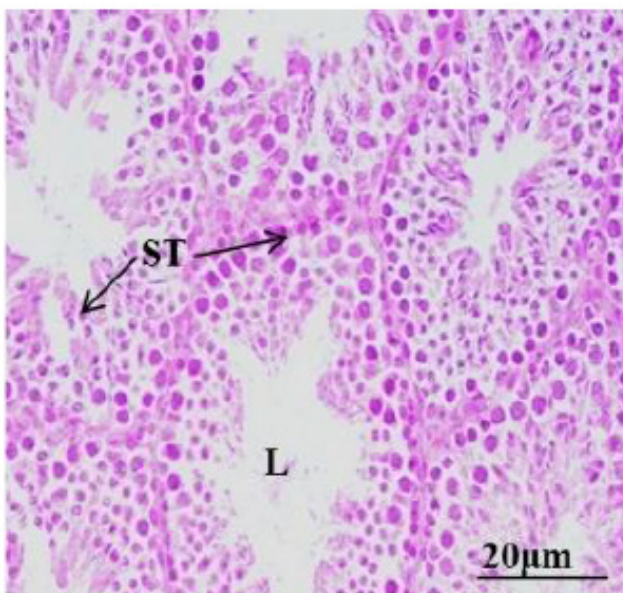


Figure 1.11B. T.S. of the testis of *M.terricolor* following L-thyroxine injections. Note the seminiferous tubules showing suppression of spermatogenic activity and lumina devoid of spermatozoa. EL: Epithelial lining, L: Lumen, ST: Seminiferous Tubules.

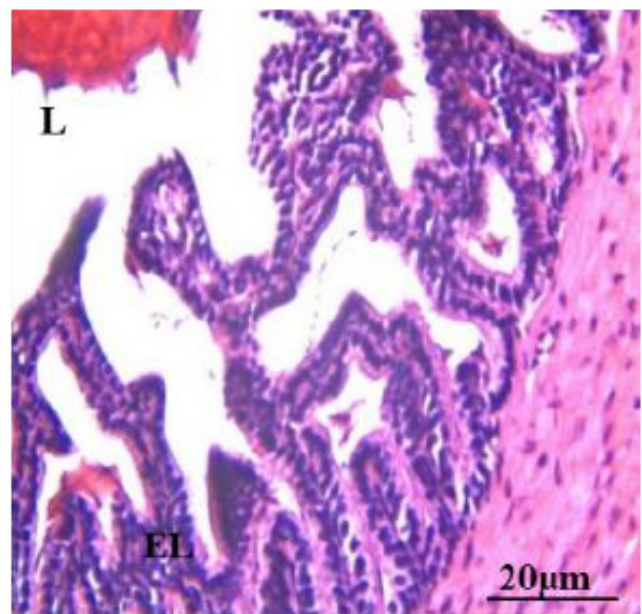


Figure 1.12B. T.S. of the seminal vesicle of *M.terricolor* following L-thyroxine injections. Note the excessive inward ramification of epithelial lining and scanty secretion in the reduced lumen. EL: Epithelial lining, L: Lumen.

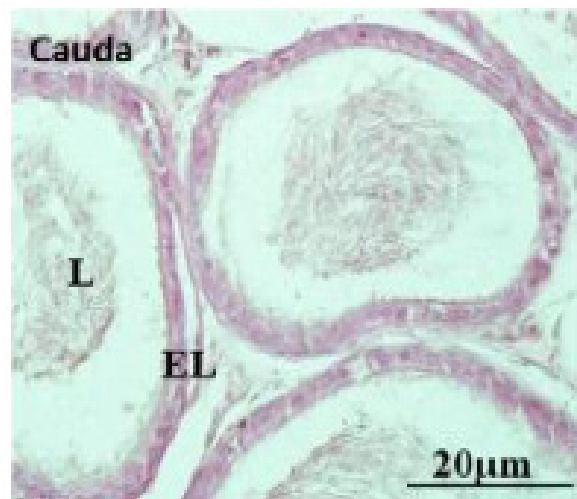
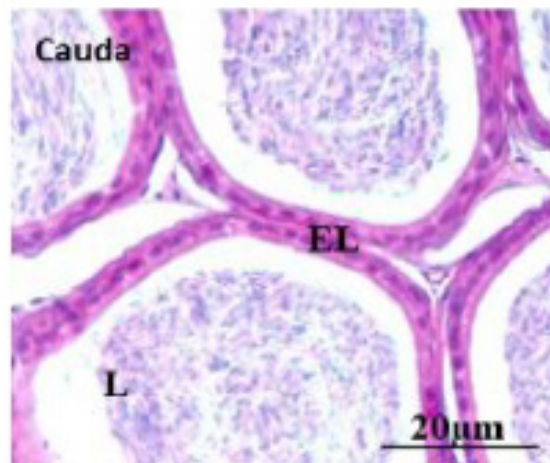
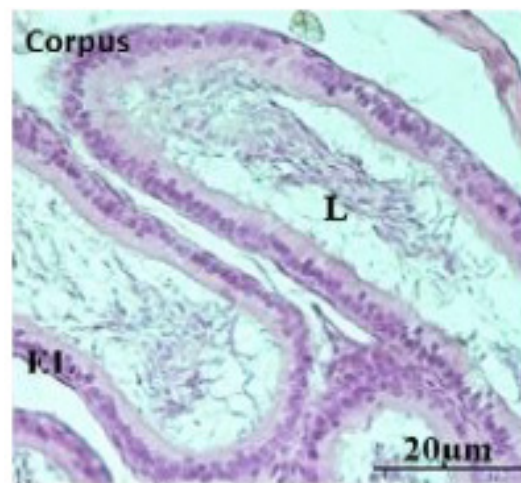
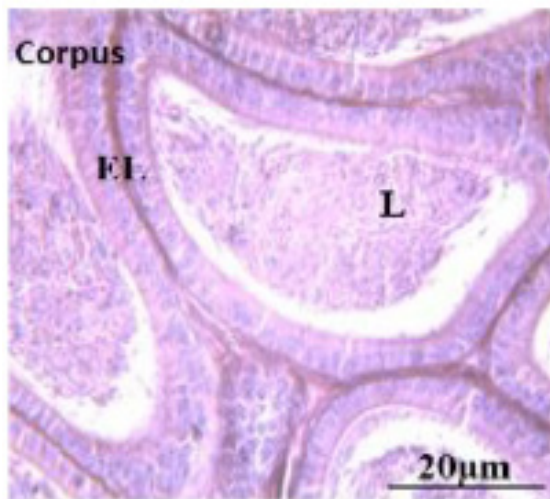
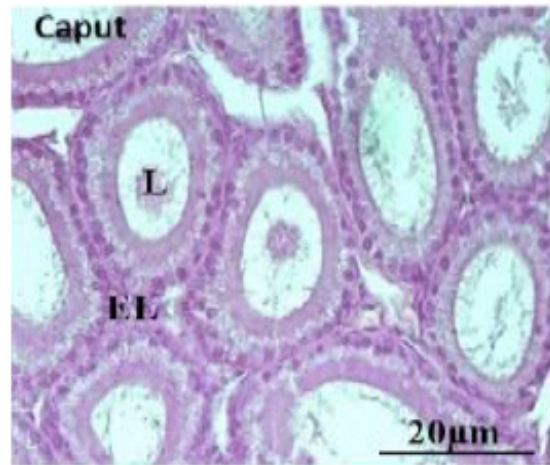
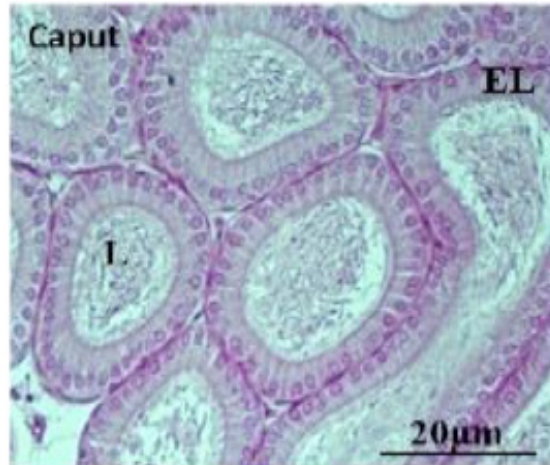


Figure 1.13A. T.S. of the epididymis (Caput, Corpus and Cauda part) of *M. terricolor* following saline injections. Note the lumina contain large number of spermatozoa. EL: Epithelial lining, L: Lumen.

Figure 1.13B. T.S. of the epididymis (Caput, Corpus and Cauda part) of *M. terricolor* following L-thyroxine injections. Note the lumina devoid of spermatozoa, containing debris of spermatozoa. EL: Epithelial lining, L: Lumen.

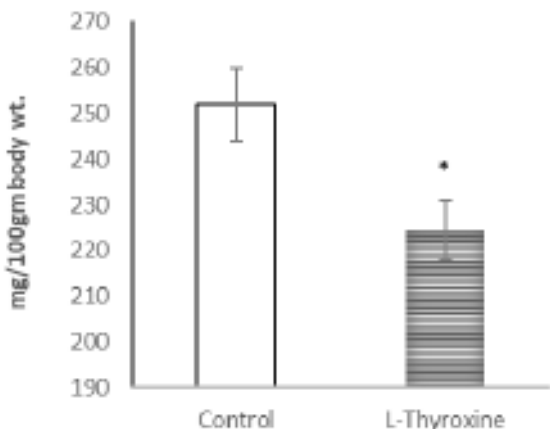


Figure 1.16. Bar graphs showing significant reduction in relative weight of uterus of female *M.terricolor* following L-thyroxine injections.

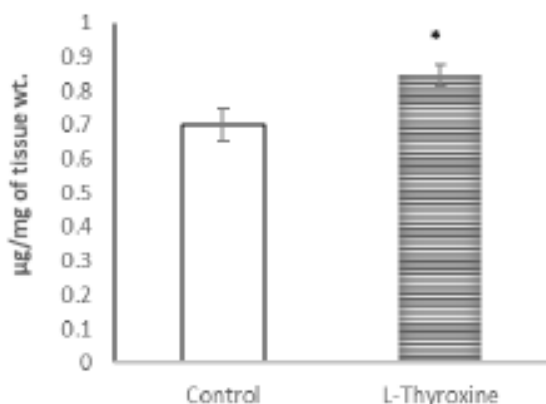


Figure 1.17. Bar graphs showing the significant elevation in ovarian cholesterol of *M.terricolor*. following L-thyroxine injections.

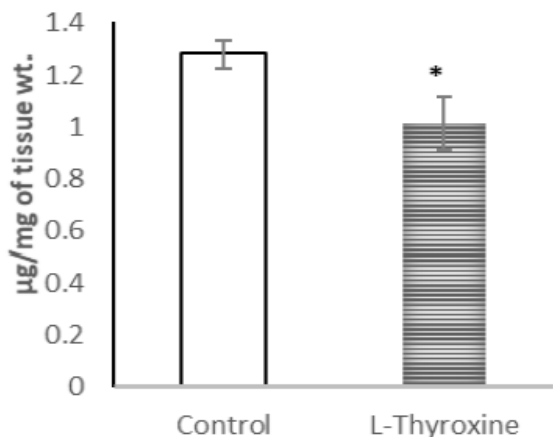


Figure 1.18. Bar graphs showing significant reduction in the concentration of uterine protein of *M.terricolour* following L-thyroxine injections.

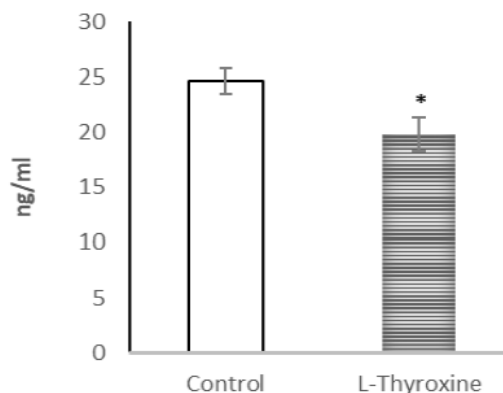


Figure 1.19. Bar graphs showing significant reduction in the level of plasma estradiol of *M.terricolor* following L-thyroxine injections.

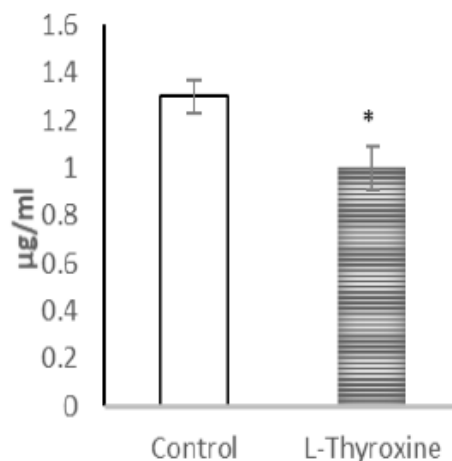


Figure 1.20. Bar graphs showing significant reduction in the level of plasma progesterone of *M.terricolor* following L-thyroxine injections.

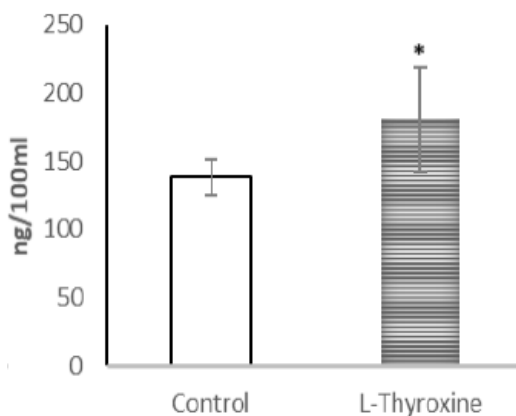


Figure 1.21. Bar graphs showing the significant elevation in the concentration of plasma T3 level of female *M.terricolor* following L-thyroxine injections.

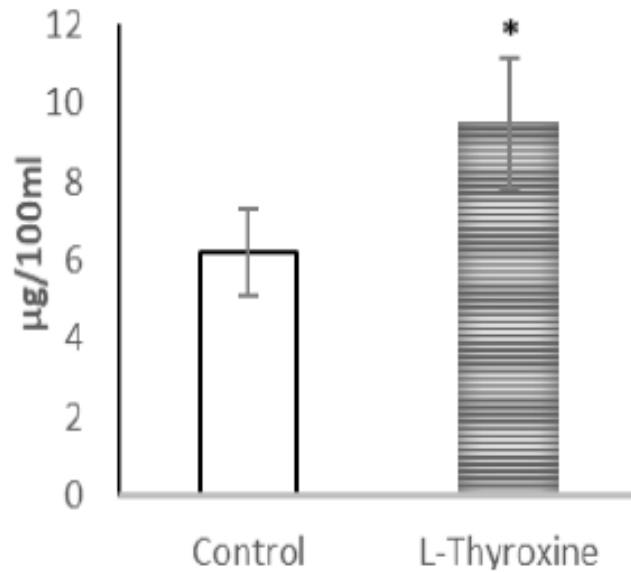


Figure 1.22. Bar graphs showing the significant elevation in the concentration of plasma T4 level of female *M.terrlicolor* following L-thyroxine injections.

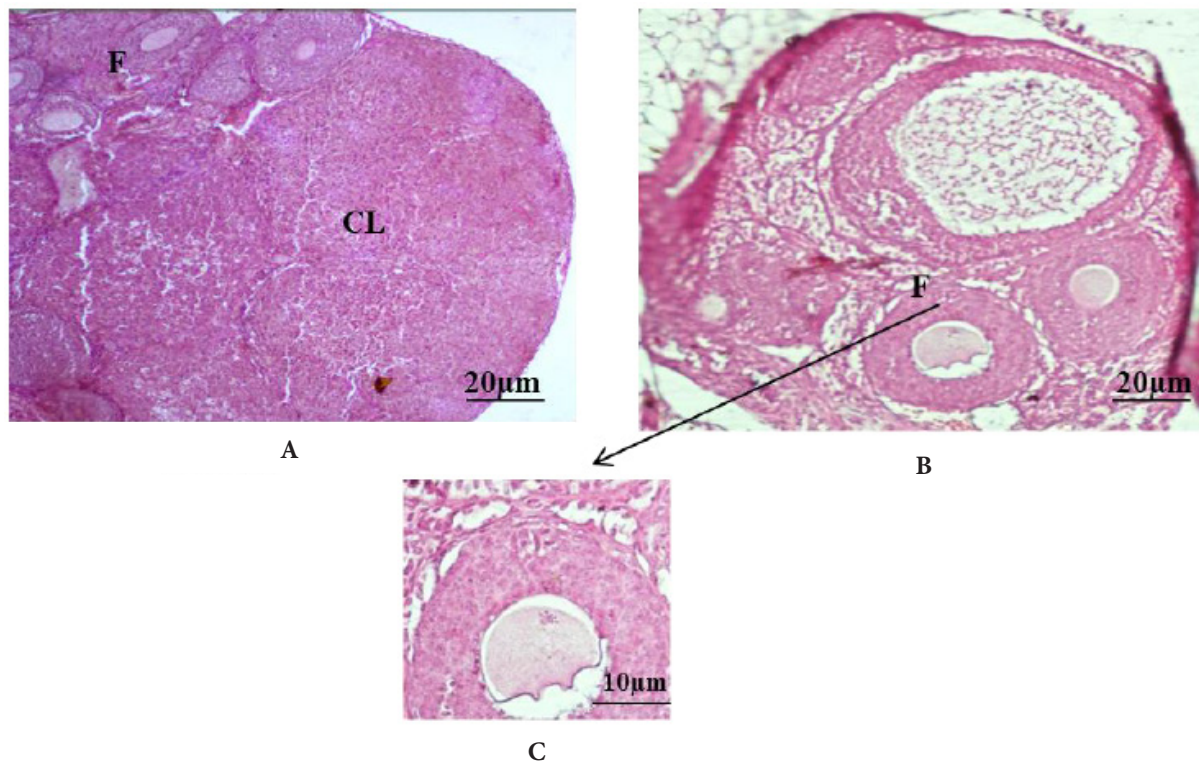


Figure 1.23. (A) T.S. of the ovary of *M.terrlicolor* following saline injections. Note the presence of antral follicles and well developed corpus luteum. CL: Corpus Luteum, F: Follicle. (B). T.S. of the ovary of *M.terrlicolor* showing regressive histological changes following administration of L-thyroxine. Note the absence of corpora lutea and presence of abnormal follicle. F: Follicle. (C). Section of the ovary of *M.terrlicolor* showing an abnormal follicle.

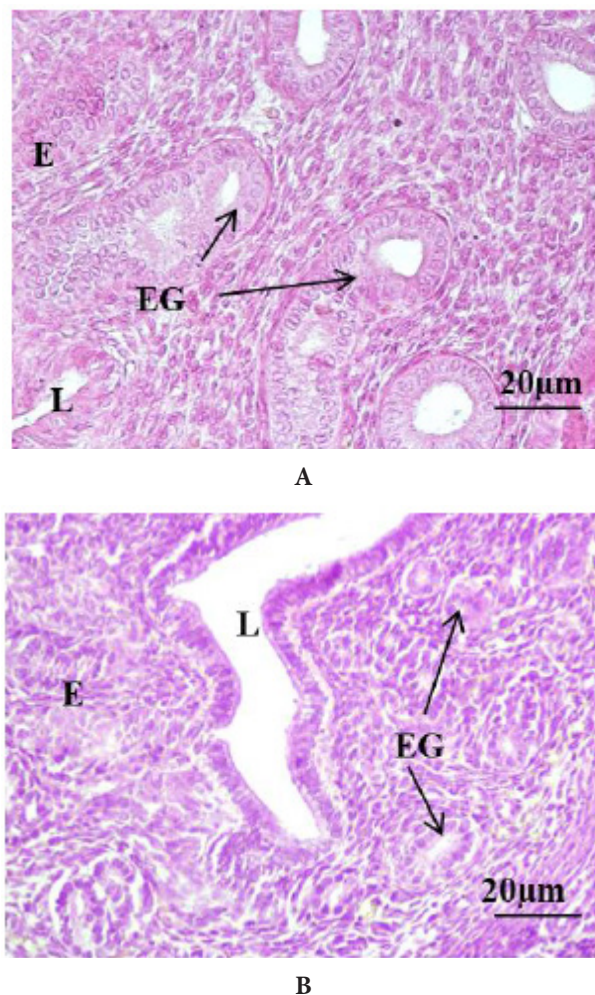


Figure 1.24. (A) T.S. of the uterus of *M.terricolor* following saline injections. Note the developed endometrium with several proliferated endometrial glands. EG: Endometrial glands, L: Lumen. (B). Transverse section of the uterus of *M.terricolor* showing regressive histological changes following administration of L-thyroxine. Note the non-proliferated endometrial glands in the regressed endometrium and wide lumen. EG: Endometrial glands, L: Lumen.

3.4 Hormonal Analysis

The levels of plasma T3 (Figures 1.9 and 1.21) and T4 (Figures 1.10 and 1.22) increased to significant levels in both sexes of mice following L-thyroxine injection. On the other hand, L-thyroxine treatment led to a significant decrease in the levels of plasma testosterone (Figure 1.8) in male and plasma estradiol (Figure 1.19) and progesterone

(Figure 1.20) in female mice when compared with their respective controls.

3.5 Histological Observations

Most of the seminiferous tubules in the testis of control mice showed full spermatogenic activity with successive stages of transformation of spermatogonia into spermatozoa (Figure 1.11(A)). On the other hand L-thyroxine treatment induced mild regressive changes in the seminiferous tubules as evidenced by thinning of tunica propria, and loss of a few stages of spermatocytes. The Lumina of the majority of tubules were also devoid of spermatozoa (Figure 1.11(B)).

The caput, corpus, and cauda epididymides of the control mice showed normal histological features (Figure 1.13(A)). These three regions of the epididymis of the L-thyroxine-treated mice exhibited almost normal histology except that the lumina of the tubules contained a few broken heads (Figure 1.13(B)).

The seminal vesicles of the control mice showed normal histological features with a wide lumen containing a large amount of secretion (Figure 1.12(A)). By contrast, the seminal vesicles of L-thyroxine-treated mice showed marked degenerative changes, as indicated by the excessive inward ramification of the epithelial lining and scanty secretion in the reduced lumen (Figure 1.12(B)).

The ovary of the vehicle-treated control mice showed corpora lutea along with numerous small and medium-sized antral follicles (Figure 1.23(A)). However, the ovarian sections of the mice treated with L-thyroxine showed inhibition of ovulation as reflected by the absence of corpora lutea and the presence of only primordial and primary follicles. The presence of some abnormal follicles was also noted in the sections of the ovary (Figure 1.23(B)).

The uterus of the control mice exhibited well-developed endometrium, a narrow lumen, and several proliferative endometrial glands (Figure 1.24(A)). L-thyroxine treatment caused marked regressive changes in the uterus as evidenced by reduced endometrium, regressed endometrial glands, and the appearance of the wide lumen (Figure 1.24(B)).

4. Discussion

Both hyperthyroidism and hypothyroidism are known to be detrimental to reproduction. A marked difference between species and even between strains within the same

species in the response to the altered status of thyroid action exists²⁵. So far there is no evidence of a normal reproductive cycle in mammals when there is a chronic or acute alteration in thyroid function. In this regard, the thyroid gland appears to have a specific influence on the regulation of the reproductive function of *Mus terricolor*.

In the present study, the L-thyroxine treatment caused a significant decrease in the weight of gonads and accessory sex organs. It also led to a significant decrease in biochemical constituents like sialic acid in the epididymis and fructose in the seminal vesicles in males and uterine protein in females, while the gonadal cholesterol was significantly increased in both sexes. Histological observations in males revealed inhibited spermatogenesis in the testis, reduced spermatozoa in the lumen of the epididymis, and marked degenerative changes in the seminal vesicles. In females, histology of the ovary and uterus reflected anoestrus-like condition as evidenced by the presence of only developing follicles and the absence of antral follicles and corpora lutea and non-proliferative endometrium, respectively. Our results are consistent with the findings reported in European starlings² and ewes²⁶ where pharmacological doses of thyroxine have been reported to increase the circulating thyroid hormone thereby leading to premature termination of breeding activity in intact animals.

Many plausible mechanisms have been proposed according to which exogenous thyroxine may affect reproduction. The most likely mechanism is the involvement of thyroxine through the neuroendocrine axis rather than its direct actions on the gonads. The possibility is that the elevated thyroid hormones act directly on the GnRH neurosecretory system, decreasing GnRH and thus suppressing gonadotropin release. The authors in²⁷ demonstrated that the thyroid gland is required for the endogenously generated switch in function of the GnRH neurosecretory system which leads to the end of the breeding season of the ewe. Further, there are two viewpoints regarding the suppression of gonadotropins in the presence of thyroid hormones. Some reports suggest it to be due to steroid-dependent suppression i.e., negative feedback from gonadal steroids^{27,28} while Shi and Barrel²⁹ associated it with a steroid-independent mechanism.

Moreover, thyroid hormones are assumed to act directly on the brain. The authors in³⁰ demonstrated that small doses of T_4 inhibited the secretion of LH

when applied centrally in thyroidectomized sheep. Later, Anderson *et al.*, with the help of micro-implants containing T_4 , confirmed the premammillary and ventromedial preoptic area as the target sites for thyroid hormone action⁷.

Several studies on thyroid hormone metabolism report that the expression of the enzymes type 2 deiodinase (DIO2) and type 3 deiodinase (DIO3) is critical for seasonal changes in mammals. The DIO2 is reported to convert T_4 to a more active T_3 and DIO3 converts T_4 to inactive reverse triiodothyronine (rT_3) as well as to inactive diiodothyronine (T_2)³¹. The ratio of these two enzymes is supposed to regulate the availability of T_3 in the hypothalamus³². The action of these two deiodinases has been repeatedly reported by different workers in different species such as Djungarian hamster^{33,34}, Syrian hamster³⁵, and European hamster³⁶. Incidences of oxidizing stress situations have been reported regarding l-thyroxine-induced hyperthyroidism^{37,38}. Incidences of hyperthyroid condition due to elevation in prooxidant to antioxidants ratio lead to faster aggregation of oxidatively damaged molecules and thus oxidative stress and damage of certain organs, specifically the liver. However, we have not performed the toxicity or oxidative damage tests to develop the discussion further along these lines.

In the present experiment, a premature termination of breeding activity was evidenced by administering exogenous L-thyroxine (35 $\mu\text{g}/100\text{g}$ Body wt.). The thyroid hormone is known for its reproductive effects. Both hypo- and hyperthyroidism can influence gonadal activity. Our observations suggest that hyperthyroidism leads to reproductively active gonads to inactivity suggesting that the high level of thyroid hormone can accelerate the reproductive inactivity in this rodent. It might be possible that the action of two enzymes, DIO2 and DIO3 is responsible for the seasonal changes in this tiny rodent which will be checked in the future.

5. Acknowledgments

The authors thank University Grants Commission, New Delhi, for financial support to Ms. Sweta Arora in the form of a Research Fellowship (RFSMS). The gift of instruments from the Alexander von Humboldt Foundation, Bonn, Germany, to Prof. C. Haldar is gratefully acknowledged.

6. References

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