J Endocrinol Reprod 18 (2014) 29 : 29-36 **Original Research**

Effect of exogenous gonadal steroids on reproductive functions of the Indian pygmy field mouse *Mus terricolor*

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Summary

Different thresholds of gonadal steroids exert stimulatory or inhibitory effects on GnRH and gonadotropin release. During the reproductively active phase (RAP), the concentration of endogenous gonadal steroids remains high while, during reproductively inactive phase (RIP), it remains low. During RIP the HPG axis is sensitive to gonadal steroids but the circulatory levels of testosterone and estradiol remain low. During this phase one can observe conveniently the effects of gonadal steroids on the HPG axis in male or female rodents. Therefore, the aim of the present study was to find the effects of testosterone and estradiol on the reproductive functions of male and female Indian pygmy field mouse, *Mus terricolor*, during RIP. The male mice were injected aquaviron (commercially available testosterone, 1mg/100g body weight) while the female mice received estradiol-benzoate (25µg/100g body weight) for 15 consecutive days during the RIP. After completion of the treatment, a significant increase in the weights of gonads and accessory sex organs was noted in both the sexes. The biochemical constituents of accessory sex organs such as epididymal sialic acid, seminal vesicular fructose and uterine protein content reflected significant elevation accompanied by increased levels of plasma testosterone, estradiol and progesterone. Histologically, the gonads and accessory sex organs exhibited increased cellular activity. However, the gonadal cholesterol was significantly decreased in both the sexes. Over all, administration of gonadal steroids to both male and female mice accelerated the gonadal recrudescence but did not inhibit the reproductive functions when administered during the RIP. Therefore, it can be inferred from the present study that during the RIP the HPG axis is sensitive to gonadal steroids and, hence, exogenous gonadal steroids induce gonadal activity.

Key words: Gonadal steroids, *Mus terricolor*, reproduction, tropical rodent, seasonal breeder

Introduction

The production, release, and actions of GnRH, FSH and LH are influenced by feedback actions of the gonadal steroid hormones (Tilbrook et al., 2000; Marshall et al., 2001). Different thresholds of gonadal steroids exert both stimulatory and inhibitory effects on GnRH and gonadotropin release. During the reproductively active phase (RAP), the concentration of endogenous gonadal steroids remains high while, during reproductively inactive phase (RIP), the endogenous gonadal steroids remain low.

Testosterone is a steroid hormone, primarily produced by Leydig cells in the testicles of mammals, involved in the regulation of several male reproductive processes. Its levels differ between active and inactive phases of the reproductive cycle in the seasonal breeders (Sanford et al., 1974; Gomes and Joyce, 1975). It has role in promoting and maintaining spermatogenesis as well as secondary sexual characteristics. In particular, this steroid hormone is regarded as the prime mediator of life history trade-offs between parental care and mating effort, or between reproduction and survival (Wingfield et al., 2001; Hau, 2007). During breeding season, high plasma testosterone concentration facilitates mating effort in males (Ketterson et al., 1992). Correlative and experimental evidence also indicates that higher testosterone concentration can lead to increased mating success (Reed et al., 2006). Similarly, estradiol is produced primarily by the ovary and maintains libido and sexual behavior in the female having negative and positive feedback effects on gonadotropin secretion.

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Mus terricolor is a seasonally breeding rodent (Arora, 2013). Till date there is no report that deals with the effects of exogenous gonadal steroids, testosterone and estradiol, on the reproductive functions and breeding cycle of either sex of *M. terricolor*. The present study was, therefore, designed to observe the effects of exogenously administered gonadal steroids (testosterone and estradiol) on the reproductive functions of this wild rodent during its reproductively inactive phase (RIP) i.e., in the month of June. During inactive phase of the reproductive cycle the axis is sensitive but the circulatory gonadal steroids (testosterone and estradiol) remain at minimum levels. This is the suitable phase when one can delineate the effect of gonadal steroids on the HPG axis in male and female rodents. We divided the study into two parts:

Part I: Effects of exogenous testosterone on the reproductive functions of male *M. terricolor*.

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

Materials and methods

Maintenance of animals

All the experiments were conducted in accordance with the institutional ethical practice, within the framework of Institutional Animal Ethics Committee under CPCSEA. Experiments were performed during the reproductively inactive phase (RIP) of the animal. The mice were collected from the fields in the vicinity of Varanasi (Lat. 25º, 18' N; Long. 83º, 1'E), India, following the methods as described (Bardhan and Sharma, 2000; Singh et al., 2009; Basu et al., 2012; Basu and Singaravel, 2013).

After two weeks of acclimation to laboratory conditions, healthy young adult male mice and non-pregnant female mice of average weight 11.0 ± 1.0 g were randomly selected. They were kept in commercial polypropylene cages during experiments and maintained in a well-ventilated room exposed to ambient conditions (27 ± 2ºC, with gentle ventilation). Mice were fed with commercial food pellets along with wheat, paddy/rice and water *ad libitum*.

Experimental groups

Part I: Twelve young adult male mice were randomly assigned to two groups of six each. Mice in the first group served as control while those in the second group were given intramuscular testosterone injection (1mg/100 g body weight, Ahmad and Haldar, 2010) for 15 days. Commercial testosterone (Aquaviron) in ethanol base was diluted in normal saline (0.9%) up to desired concentration and administered during morning hours (10:00-11:00 h). Control animals received injections of 0.9% normal saline intramuscularly for the same duration.

Part II: Twelve young adult female mice were divided into two groups of six each. Mice in the first group served as control while those in the second group were given intramuscular estradiol-benzoate injection. Estradiol benzoate was dissolved in ethanol and diluted in normal saline (0.9%) up to the desired concentration and injected through intramuscular route during morning hours (10:00-11:00 h) at a dose of 25µg/100g body weight (Singh, 1992) for 15 consecutive days. Control animals received 0.9% normal saline intramuscularly for the same duration.

Sample collection

After 24 h of the last injection, mice were weighed and anesthetized. Blood was collected by cardiac puncture in heparinized tubes. Plasma was separated and stored at -80ºC till the ELISA, for measurement of the levels of testosterone (DIAMETRA, Lot no. DKO002), estradiol and progesterone (Biotron Diagnostics Inc., Hemet, California, USA), was carried out.

Testis, epididymis and seminal vesicles in males and ovary and uterus in females were dissected out on ice, blotted free of blood, cleaned from extra tissues, and weighed in an electronic balance (Denver Instruments, Gottingen, Germany). Testis, epididymis and seminal vesicles of left side in male mice, and ovary and uterine horn of left side in female mice were fixed in Bouin's fluid for histology while those on right side were kept for biochemical estimations of testicular and ovarian cholesterol, epididymal sialic acid, seminal vesicular fructose and the uterine protein.

Histology

After fixation in Bouin's fluid testis, epididymis, seminal vesicles, ovary and uterus were processed for routine histological analysis. Six µm thick sections were deparaffinized, stained with Ehrlich's hematoxylin and eosin. The stained sections were observed in a research microscope (Leica MPV-3, Germany) and documented.

Biochemical estimations

Concentration of cholesterol in testis and ovary was estimated using kits and as per manufacturer's protocol (BioLab Diagnostics, India). The concentrations of sialic acid in the epididymis and fructose in the seminal vesicles were determined according to the methods of Aminoff (1961) and Linder and Mann (1960), respectively. The protein content of the uterus was quantified adopting Bradford (1976) method.

Hormone analysis

ELISA for Testosterone

ELISA kit for testosterone assay was purchased from Dia Metra (Lot No: DKO002). According to the manufacturer's instruction, 25 µl of standard, control and sample were added to each well of the ELISA plate followed by addition of 100 µl of the enzyme conjugate solution and 100 µl of the testosterone antiserum. The ELISA plate was incubated with mild shaking at room temperature for one hour. Wells were aspirated and washed thrice with double distilled water. Then, 100 µl of the TMB chromogenic solution was added to each well and the plate was incubated at room temperature for 30 minutes. Finally, 100 µl of stop solution (0.2 M H_2SO_4) was added and the absorbance was recorded at 450 nm using an ELISA microplate reader (BioTek).

ELISA for estradiol and progesterone

The plasma levels of estradiol and progesterone were estimated using respective ELISA kits (Biotron Diagnostics Inc. Hemet, California, USA) according to the manufacturer's instructions. 25 µl of standard, control and sample were added to the wells of ELISA plate followed by addition of 100 µl of enzyme conjugate solution. The wells were then incubated with mild shaking at room temperature for two hours. The wells were aspirated and washed three times with wash solution. Then, 100 µl of the TMB chromogenic solution (substrate) was added to each well and the plate was incubated at room temperature for 30 minutes in dark. Finally, 100 µl of stop solution was added and the absorbance was recorded at 450 nm.

Statistical analysis

Statistical analysis of the data was performed adopting Students' '*t*'-test. The differences were considered significant when $P < 0.05$.

Results

Body weight

No significant difference was observed in the body weight of saline-treated control and testosterone-treated male mice (Fig. 1.1). Similarly, no significant difference in body weight was observed between saline-treated control and estradiol benzoate-treated female mice (Fig. 1.1).

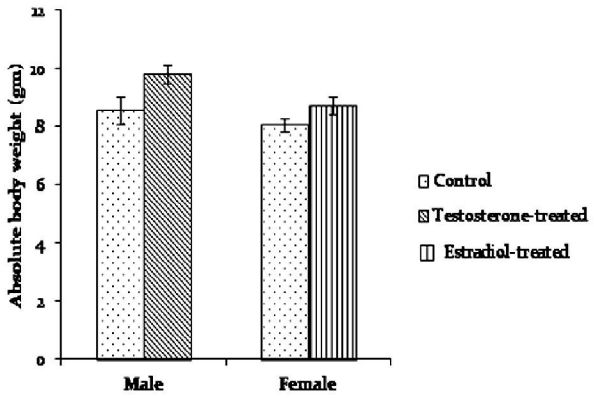


Fig.1.1

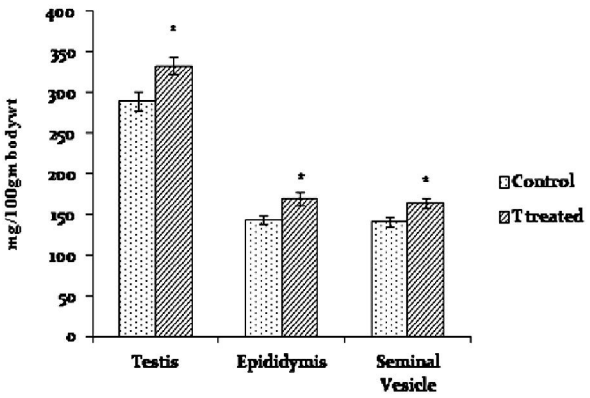


Fig.1.2

Fig. 1.1: Effect of exogenous testosterone and estradiol on the body weight of male and female *M. terricolor,* respectively. No significant change was found after the treatment. Values represent mean ± SEM of six animals.

Fig. 1.2: Effect of exogenous testosterone on the relative weight of testis, epididymis and seminal vesicle of *M. terricolor*. Note the significant increase in the testicular, epididymal and seminal vesicular weight in the treated mice. Values represent mean ± SEM of six animals. * Significance of difference, $P < 0.05$ with respect to control.

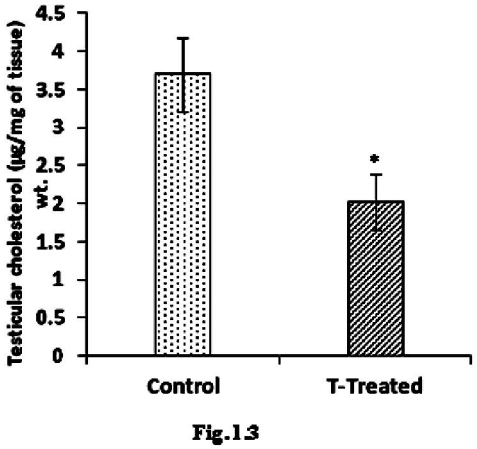


Fig.1.3

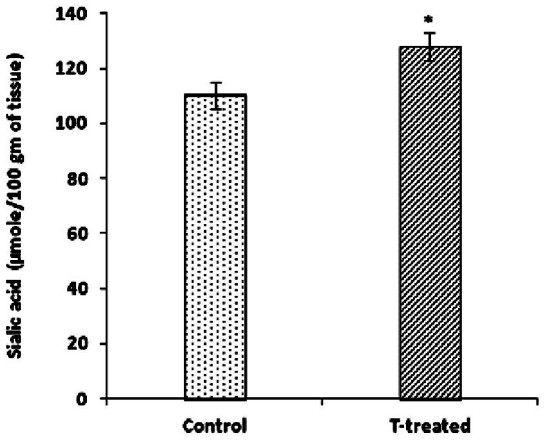


Fig.1.4

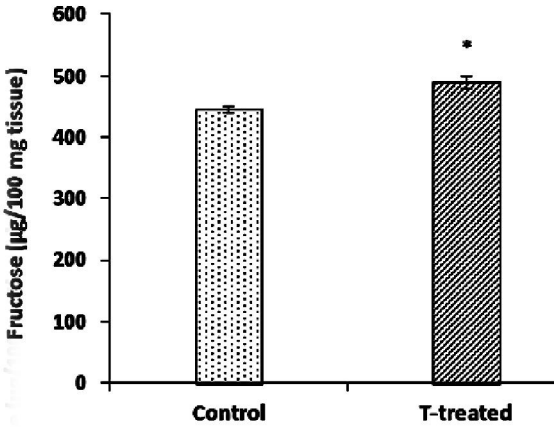


Fig.1.5

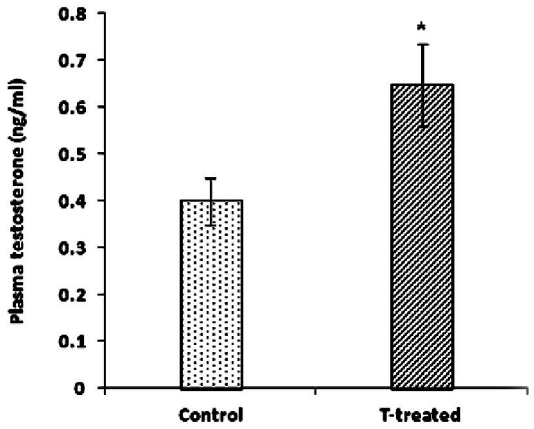


Fig.1.6

Fig. 1.3: Effect of exogenous testosterone on the testicular cholesterol of *M. terricolor*. Note the significant reduction in the concentration of testicular cholesterol after treatment. Values represent mean ± SEM of six animals.

* Significance of difference, $P < 0.05$ with respect to control.

Fig. 1.4: Effect of exogenous testosterone on the epididymal sialic acid of *M. terricolor*. Note the significant increase in the concentration of epididymal sialic acid after the treatment. Values represent mean ± SEM of six animals.

* Significance of difference, $P < 0.05$ with respect to control.

Fig. 1.5: Effect of testosterone injections on the seminal vesicular fructose of *M.terricolor*. Note the significant increase in the concentration of seminal vesicular fructose after the treatment. Values represent mean ± SEM of six animals.

* Significance of difference, $P < 0.05$ with respect to control.

Fig. 1.6: Effect of exogenous testosterone on the level of plasma testosterone of *M. terricolor*. Note the significant increase in the level of plasma testosterone after the treatment. Values represent mean ± SEM of six animals.

* Significance of difference, $P < 0.05$ with respect to control.

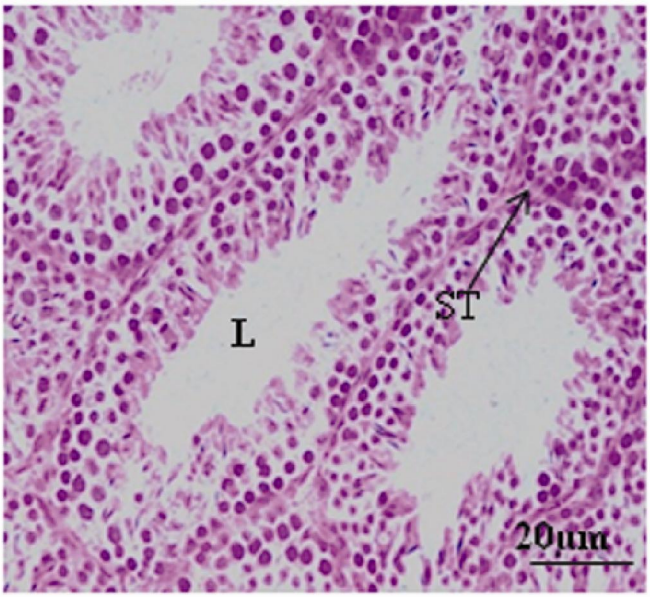


Fig. 1.7A

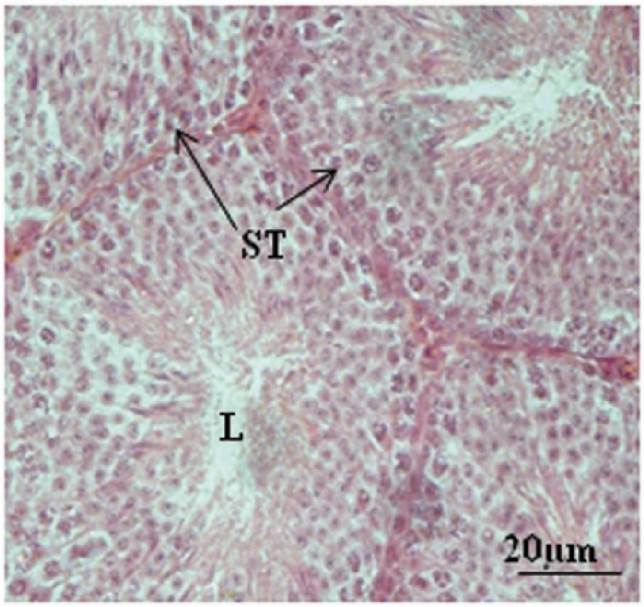


Fig. 1.7B

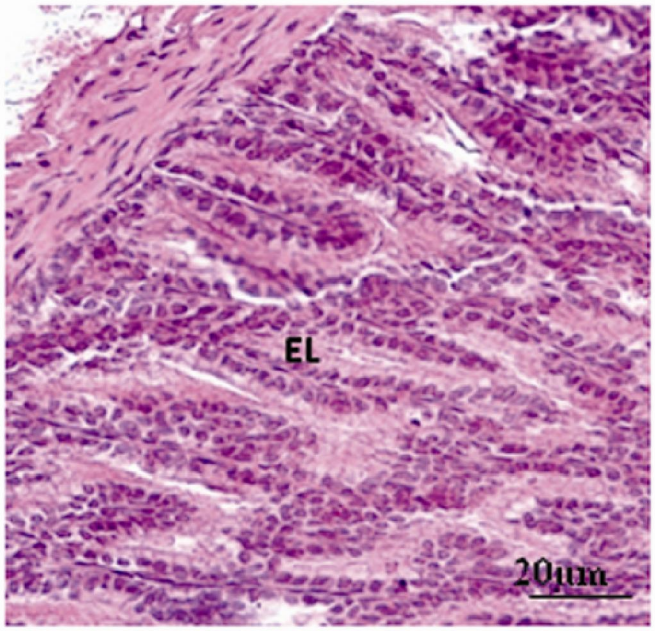


Fig. 1.8A

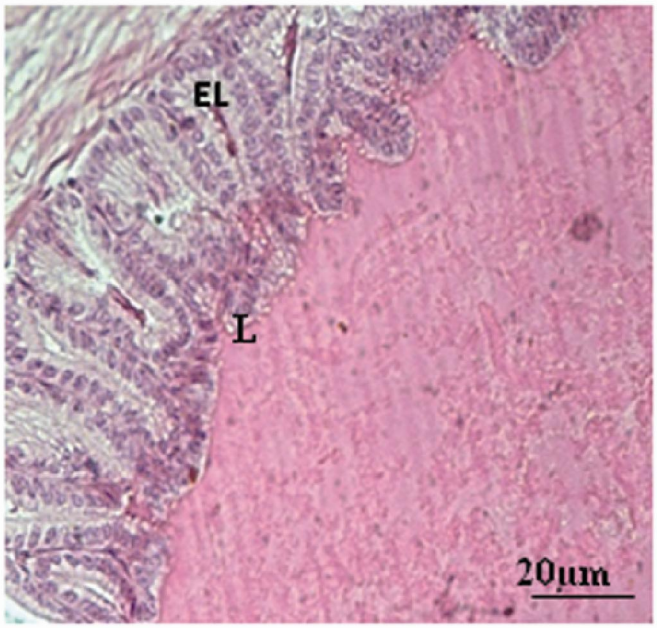


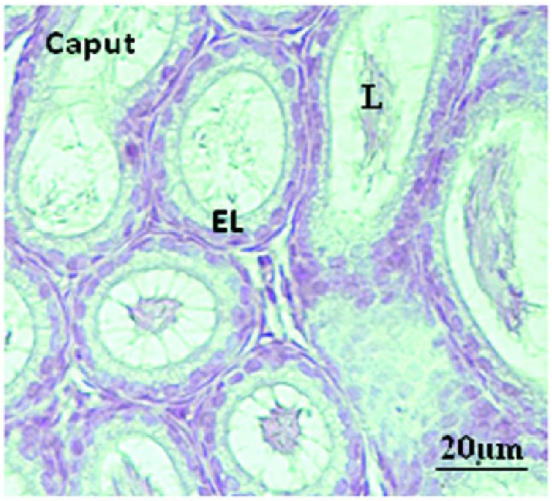
Fig. 1.8B

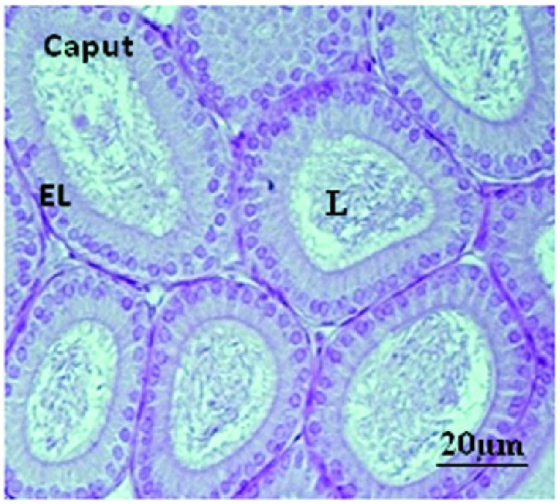
Fig. 1.7A: T.S. of the testis of *M. terricolor* following saline injections. Note the regressed seminiferous tubules, devoid of spermatozoa. EL: Epithelial lining, L: Lumen, ST: Seminiferous Tubules.

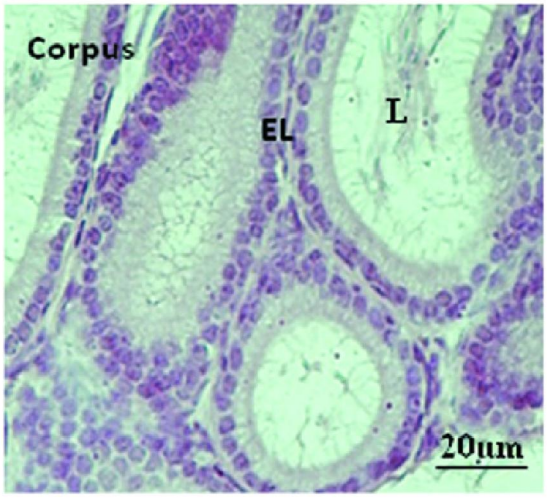
Fig. 1.7B: T. S. of the testis of *M. terricolor* following 15 days of testosterone injections. Note the active seminiferous tubules showing full spermatogenic activity. EL: Epithelial lining, L: Lumen, ST: Seminiferous Tubules.

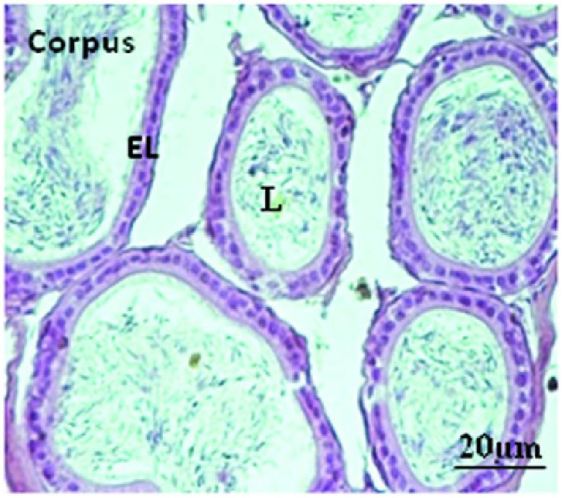
Fig. 1.8A: T.S. of the seminal vesicle of *M. terricolor* following saline injections. Note the regressed condition as indicated by excessive inward ramification of epithelial lining and scanty secretion in the reduced lumen. EL: Epithelial lining, L: Lumen.

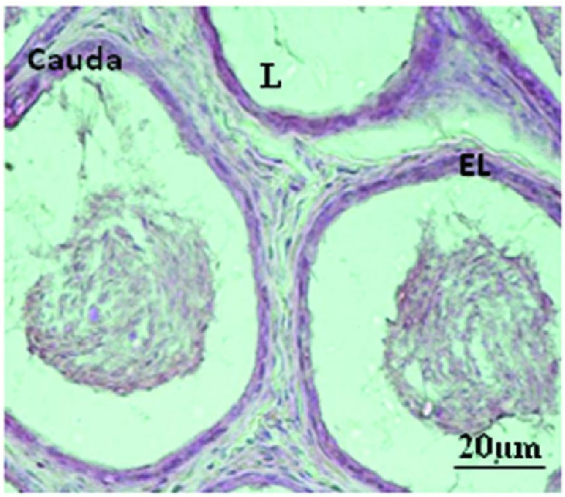
Fig. 1.8B: T.S. of the seminal vesicle of *M. terricolor* following 15 days of testosterone injections. Note the active condition of the organ as indicated by reduction in inward ramification of epithelial lining and the lumen filled with large amount of secretions. EL: Epithelial lining, L: Lumen.











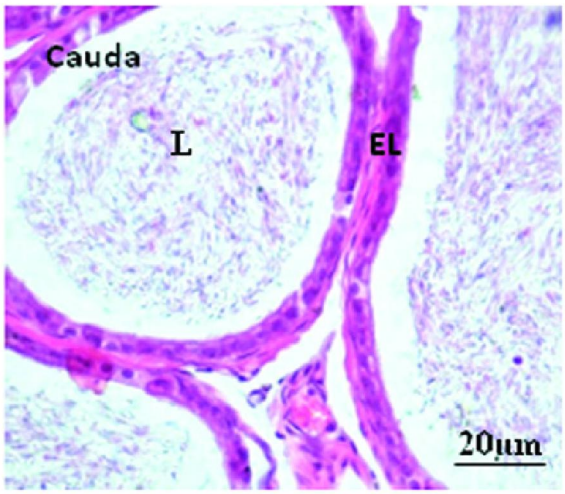


Fig. 1.9A

Fig. 1.9B

Fig. 1.9A: T.S. of the epididymis (caput, corpus and cauda) of *M. terricolor* following saline injections. Note the lumina of epididymal tubules devoid of spermatozoa, containing debris of spermatozoa. Also note the increase in fibromuscular stroma. EL: Epithelial lining, L: Lumen.

Fig. 1.9B: T.S. of the epididymis (caput, corpus and cauda) of *M. terricolor* following 15 days of testosterone injections. Note the lumen containing large number of spermatozoa. Also note the decrease in fibromuscular stroma . EL: Epithelial lining, L: Lumen.

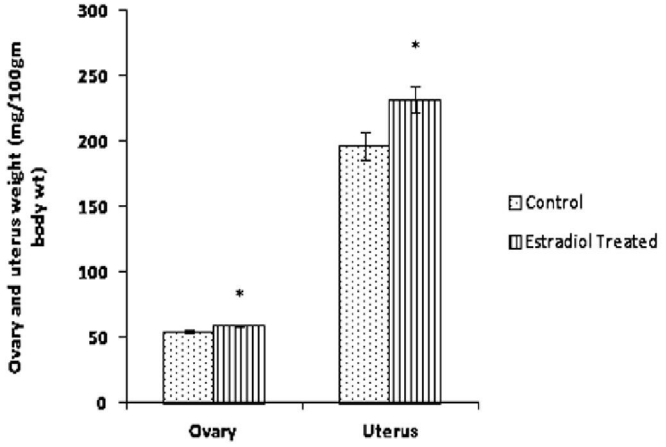


Fig. 1.10

Fig. 1.10: Effect of exogenous estradiol injections on relative ovary and uterus weight of *M. terricolor*. Note the significant increase in ovarian and uterine weight after treatment. Values represent mean ± SEM of six animals. * Significance of difference, $P < 0.05$ with respect to control.

Weights of gonads and accessory reproductive organs

Significant increase was noticed in the relative weights of gonads in both sexes of the gonadal steroid-treated groups (Fig. 1.2 and 1.10). Weights of the epididymis (Fig. 1.2) and seminal vesicles (Fig. 1.2) in the males and uterus (Fig. 1.10) in the females also reflected an increasing trend in the gonadal steroid-treated groups as compared with the control.

Biochemical estimations

Significant decrease was noticed in the concentrations of testicular (Fig. 1.3) and ovarian (Fig. 1.11) cholesterol in testosterone-treated and estradiol benzoate- treated groups as compared with their respective control groups. However, the concentrations of sialic acid in the epididymis (Fig. 1.4) and fructose in the seminal vesicles (Fig. 1.5) increased significantly in testosterone-treated male mice as compared with control. Likewise, a significant increase was noticed in the concentration of uterine protein in estradiol benzoate-treated female mice as compared with the control (Fig. 1.11).

Hormone analysis

Significant increase was noticed in the levels of plasma testosterone (Fig. 1.6) in male, and plasma estradiol

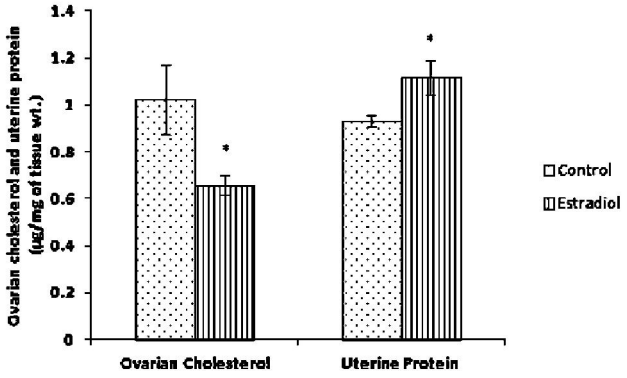


Fig. 1.11

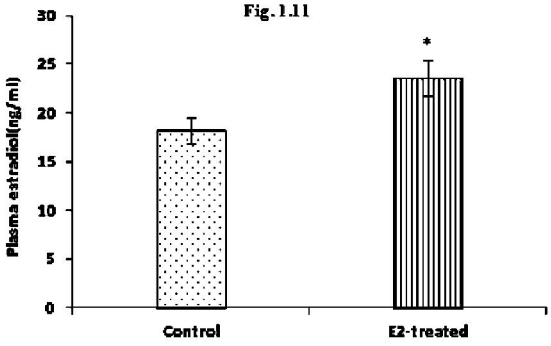


Fig. 1.12

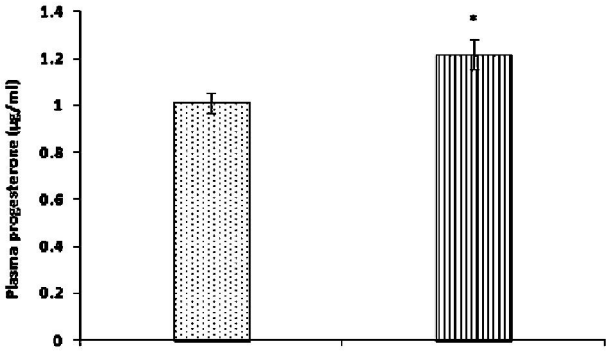


Fig. 1.13

Fig. 1.11: Effect of estradiol injections on the ovarian cholesterol and uterine protein of *M. terricolor*. Note the significant reduction in the concentration of ovarian cholesterol and a significant elevation in the concentration of uterine protein in treated group. Values represent mean ± SEM of six animals. * Significance of difference, $P < 0.05$ with respect to control.

Fig. 1.12: Effect of estradiol injections on the level of plasma estradiol of *M. terricolor*. Note the significant increase in the level of plasma estradiol in the treated group. Values represent mean ± SEM of six animals. * Significance of difference, $P < 0.05$ with respect to control.

Fig. 1.13: Effect of estradiol injections on the level of plasma progesterone of *M. terricolor*. Note the significant increase in the level of plasma progesterone after the treatment. Values represent mean ± SEM of six animals. * Significance of difference, $P < 0.05$ with respect to control.

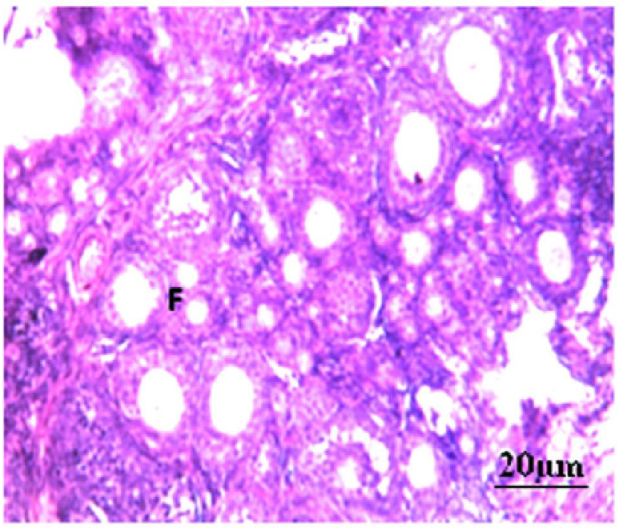


Fig. 1.14A

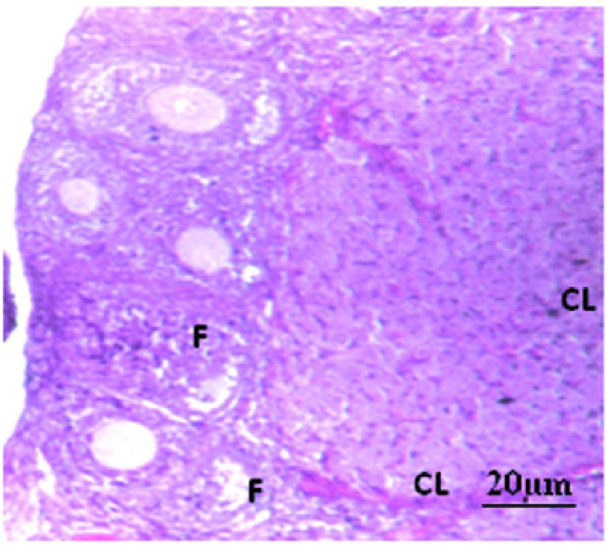


Fig. 1.14B

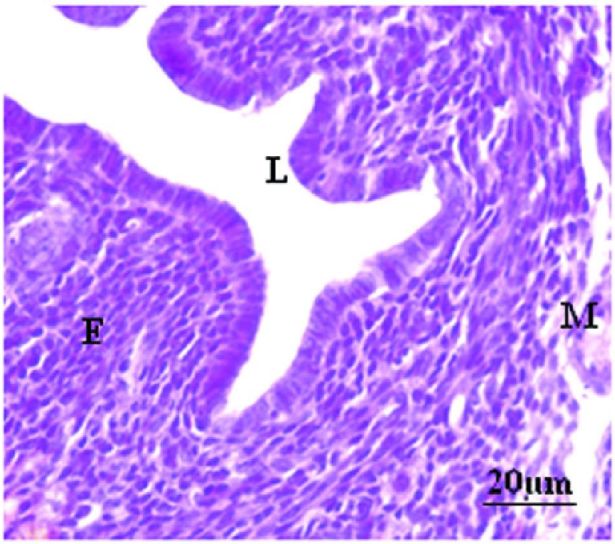


Fig. 1.15A

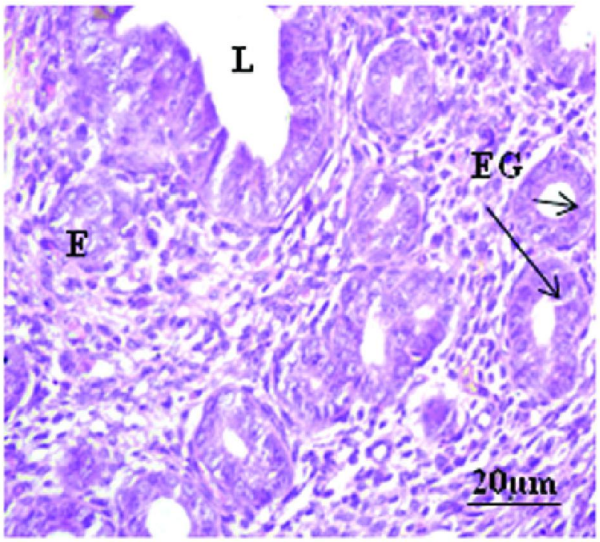


Fig. 1.15B

Fig. 1.14A: T.S. of the ovary of *M. terricolor* following saline injections. Note the presence of only primordial and primary type of follicles. F: Follicle.

Fig. 1.14B: T.S. of the ovary of *M. terricolor* after administration of estradiol. Note the presence of well developed corpora lutea and follicles at different stages of development. CL: Corpus luteum, F: Follicle.

Fig. 1.15A: T.S. of the uterus of *M. terricolor* following saline injections. Note the regressed endometrium and non-proliferated endometrial glands. E: Endometrium, EG: Endometrial glands, L: Lumen, M: Myometrium.

Fig. 1.15B: T.S. of the uterus of *M. terricolor* showing histological changes following administration of estradiol. Note the well developed endometrium and the proliferated endometrial glands. E: Endometrium, EG: Endometrial glands, L: Lumen.

(Fig. 1.12) and progesterone (Fig. 1.13) in the female mice when compared with their respective control groups.

Histological observations

The spermatogenic activity was suppressed in the testis of control animals. Lumina of the majority of the seminiferous tubules were devoid of spermatozoa, containing sperm debris and only few stages of germ cells (Fig. 1.7A). However, testosterone injection caused stimulation of spermatogenic activity as evidenced by successive stages of transformation of spermatogonia into spermatozoa in a majority of the seminiferous tubules. The lumina of the tubules were packed with spermatozoa (Fig. 1.7B).

Histological features of the caput, corpus and cauda epididymides of the control mice showed regressed condition as evidenced by flattening of the epithelial lining, presence of sperm debris in the lumina and increase in fibromuscular stroma (Fig. 1.9A). By contrast, testosterone injections resulted in appearance of normal histology of these three regions of the epididymis (Fig. 1.9B).

The seminal vesicles of control mice appeared regressed as indicated by excessive inward ramification of the epithelial lining and scanty secretion in the reduced lumen (Fig. 1.8A). Administration of testosterone, however, caused almost normal histological features as evidenced by enhanced amount of secretion in the wide lumen with reduced inward ramification of the epithelial lining (Figs. 1.8B).

The ovary of the vehicle-treated mice showed only primordial and primary follicles while corpora lutea and antral follicles were absent (Fig. 1.14A). By contrast, estradiol treatment caused appearance of many Graafian follicles, antral follicles and corpora lutea in the ovary along with other developing follicles (Fig. 1.14B).

The transverse sections of uterus of the vehicle-treated animals showed underdeveloped endometrium without proliferation of the uterine glands. Further, the uterine lumen also appeared wide (Fig. 1.15A). On the other hand, the uterus of the estradiol-treated females showed well developed endometrium with proliferated endometrial glands and reduced lumen (Fig. 1.15B).

Discussion

The results of the present study justify the role of gonadal steroids in the reproductive cycle of this tropical seasonally breeding rodent. Our results are in agreement with the previous reports that have suggested a stimulatory action of gonadal steroids during the RIP of animals like *Funambulus pennanti*, a seasonally breeding rodent (Singh, 1992; Vidhu, 1992) as well as in a seasonally breeding rodent *Psittacula cyanocephala* (Maitra and Ghosh, 1981).

A stimulatory effect on the reproductive functions of *M. terricolor* was observed following administration of gonadal steroids (testosterone in male mice and estradiol-benzoate in female mice). Significant increase in the weights of testis and accessory sex organs of the testosterone-treated *M. terricolor* was in consistence with the findings reported in mice following administration of testosterone Oenanthate (Bansal and Davies, 1986) and in *Funambulus pennanti* following testosterone propionate injection (Vidhu, 1992). Further, a significant elevation was noted in the level of plasma testosterone while a significant reduction in testicular cholesterol was noted. Cholesterol could be a marker of steroidogenesis as it is one of the major precursors required for steroidogenesis. There was a significant increase in the biochemical constituents of the accessory sex organs, i.e., sialic acid in the epididymis and fructose in the seminal vesicles, following testosterone injection. Similar findings were reported earlier by Rudolf and Starnes (1954) in the rat and Hupp et al. (1961) in the boar. The increase in biochemical constituents and weights of the accessory sex organs might be due to their androgen dependence that can be correlated to the increased plasma testosterone in the androgen-treated group. The histological observations of these organs further reflected the stimulatory effect of testosterone. The elevated testosterone level might have stimulated the process of spermatogenesis which is evidenced by presence of a abundant spermatozoa in the lumina of the seminiferous tubules in the testis and in the epididymis. All these changes can be attributed to increase in the level of plasma testosterone in the androgen-treated mice. Earlier, Maitra

and Ghosh (1981), while working with an avian species of tropics, *Psittacula cyanocephala*, reported that testosterone propionate administered during sexually resting phase resulted in spermatokinesis and differentiation of the Leydig cells.

A similar trend of stimulatory effects of estradiol benzoate treatment was observed in female mice. Administration of estradiol benzoate resulted in increase in ovarian and uterine weights as reported earlier by Kim et al. (1984) in the hamster and Singh (1992) in the Indian palm squirrel *Funambulus pennanti*. Significant elevations as noted in uterine protein (uterine protein is supposed to be required for preparation, impregnation and proliferation of uterus) and plasma estradiol in estradiol-treated groups in this study have also been reported in the Indian palm squirrel *Funambulus pennanti* (Singh, 1992). An inverse relation between plasma estradiol and cholesterol as observed in female mice is similar to that noticed in males regarding the testosterone and cholesterol. Histological observations of ovary and uterus also indicated the stimulatory effects of estradiol-benzoate treatment. Besides primordial, primary and secondary follicles, the presence of Graafian and antral follicles along with corpora lutea was also observed in the transverse section of the ovary of estradiol-treated mice. Uterine sections of estradiol-treated mice exhibited marked proliferative changes as compared to the regressed organ of control mice. The estradiol injections might have stimulated the development of primary and secondary follicles up to the level of Graafian follicles, antral follicles, ovulation and luteinization. A significant increase was also noticed in the level of plasma progesterone in the estradiol-treated mice when compared with the control mice indicating the stimulatory effect on steroidogenesis.

Over all, it was found that gonadal steroids in both male and female mice could not stimulate the reproductive functions to highly significant level when administered during the RIP. A stimulatory effect was noted in most of the parameters considered which were, however, not up to the level as in reproductively active phase (RAP) of the animal recorded by us. The anti- and pro-gonadotropic effects depend upon the sexual states of the animal. We opted to treat *M. terricolor* with gonadal steroids (testosterone in male mice and estradiol benzoate in female mice) during its sexually inactive phase. During RIP the plasma levels of gonadal steroids remain low, so when the gonadal steroids were administered exogenously it might be possible that it did not reach to the required threshold level to give negative feedback to the HPG axis and might have induced a slow gonadotropin release. Therefore, it can be inferred from the present study that gonadal steroids play a pivotal role in maintaining the reproductive functions and the annual reproductive cycle of this tiny rodent.

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