CHANGES IN THE TESTICULAR ACTIVITY IN MALES OF A PTEREPODIDAE BAT *ROUSETTUS LESCHENAULTI*

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BY

VANLALREMPUIA

DEPARTMENT OF ZOOLOGY.

SUPERVISOR: Dr. VIKAS KUMAR ROY

Submitted in partial fulfilment of the requirements for the degree of

Master of Philosophy in Zoology of

Mizoram University, Aizawl.

Abstract

Bats are unique mammals with respect to certain features, such as echolocation, flying and their habit and habitats. Despite certain peculiarities, bats do exhibits some unique phenomena in the reproductive physiology. The process of delayed ovulation, fertilization, implantation and development makes them an idea animal to study the uniqueness of reproduction. In male bats, the extended sperm survival in the epididymis and the asynchrony in the spermatogenesis, and accessory sex gland function, and along with mating are unique features of male reproduction. As the Chiroptera (bats) inhabited both in the tropical and temperate zones they show a great diversity in their reproductive pattern. The temperate bats are considered to be seasonal breeders with their characteristic precisely correlate with low temperature and hibernation. However the intensity of rainfall along with the food availability in summer also has a great influence on this pattern. While bats from tropical regions do not hibernate and their reproduction is greatly influenced by their habitat in which they lived, so they are directly influenced by temperature changes which takes place seasonally, rainfall, latitude and their food resources.The male reproductive system plays a vital function in storage and production of spermatozoa and their transportation in viable state to the genital tract of the female.

Reproductive patterns of *Rousettus leschenaulti*

Gametogenesis in bats is prolonged and exhibits unique patterns of hormonal changes. Studies on *Rousettus leschenaulti* bats suggest the adult testes of *Rousettus leschenaulti* shows great variations in their weight during different months of annual reproductive cycle. A better understanding of how does seasonal reproductive activity change in the testis of this species, would contribute to the knowledge generation for bat reproduction in the male. The literature searched on the testicular activity of *Rousettus leschenaulti* showed only a few studies, which was focused on the. It has been suggested that male fruit bat *Rousettus leschenaulti* show two peaks in their testicular activity corresponding to the two pregnancy cycles of the female. First peak occurs during February–March and second occurs during October-

November, however, suggested that December to June testicular activity is regressed. These results showed some discrepancy. The detailed aspects of spermatogenesis in the male bat *Rousettus leschenaulti* have not been investigated. The previous study have shown the cytomorphology of different types of germ cells, frequency of appearance of these stages, pattern of spermatogonial stem cell renewal and per cent degeneration of various germ cells in *Rousettus leschenaulti*. However, studies of proliferation markers with respect with testicular activity have not been investigated. This is one of the reasons based for selection of present work for detailed investigation.

Although much is known about the reproductive phenomena and associated endocrine characteristics of various bat species, the mechanism regulating the testicular activity remains to be elucidated in the *Rousettus leschenaulti*. As the role of androgen and estrogen are important for spermatogenesis, no study has been conducted on the modulation of their receptor during the reproductive and non-reproductive period in this species. Therefore, we aimed to investigate the seasonal changes in the steroid hormone along with its receptor and important makers in the testis of *Rousettus leschenaulti.*In the present study, we investigated the changes take place in the testis of adult males of an Indian fruit bat *Rousettus leschenaulti* during their reproductive and non-reproductive phases. The present study also measured the different circulating steroid hormones, androstenedione, testosterone and estrogen along the receptors of androgen and estrogen. The important markers of steroidogenesis such as LHR, and 3βHSD were also investigated. The histological study showed a marked change in the architecture of testis. The seminiferous tubules in the month of September showed few spermatogonia and Sertoli cells with without any advance germ cells. The active spermatogenesis was observed in the month of October and November with advance stage of germ cells such as spermatogonia, primary spermatocytes, round spermatid, elongated spermatid and sperm. However, in month of November, testis also showed some evidence of degeneration, furthermore, these degenerative changes become more pronounced in the months of December and January, followed by the improvement in the February-March. The testes collected in the months of May showed sign of regression, like presence of

vacuole and devoid of some germ cells. Thus, these results further strengthen the presence of two peaks of spermatogenesis with a transient suppression. The two peaks of testicular activities with a transient suppression have also been reported in the other bat species, *Scotophilus heathi* and *Plecotus auritus*. The transient suppression of spermatogenesis during December-January coincides with decreased environmental temperature. The gonadosomatic index, the ratio of gonad to body weight, is an important measure for gonadal activity, and it has been shown that males of *Rousettus leschenaulti* do not reach sexual maturity until they attain a body weight of 73 g and it was suggested that this is minimum body weight, which could show the sign of spermatogenesis, however in our present study we observed that spermatogenesis have been shown in the testis of males weighing below 73 g which is believed to be influence by the difference in environmental factors based on the geographical locations. The change in the body weight did not show marked variation during the calendar months. The testes showed two peaks, one in October-November and second in the February-March, and the overall weight of testis was higher during second peak. It may also be suggested that out of two peaks, the second peak could be more efficient in terms of sperm production, as the testis weight was higher during second peak. To best of our knowledge, no study has been conducted in any bat species in relation to sperm count and physiological difference between two peaks, therefore, further study would be required to strengthen the this hypothesis.

The two peaks of spermatogenesis further coincide with increase circulating testosterone. The circulating testosterone also showed two peaks with a decline in December-January, which suggests that testosterone might regulate the testicular activity in *Rousettus leschenaulti* and suppressed spermatogenesis could be due to low testosterone during winter i.e. December-January. Our results also showed the expression of AR was elevated in November, and after a decline in January, again the expression of AR increased in February-March, these finding further supports the androgen mediated regulation of spermatogenesis during two peaks and a transient suppression could be due to low testosterone levels. The serum testosterone concentration was high during the breeding period, during February-March and October-November, whereas the rapid decrease takes place in the months of May,

June and August in *Rousettus leschenaulti;* however, other male hormone like androstenedione and steroidogenic makers has not been investigated in this species. The androstenedione levels showed highest levels in October and there was decline in December-January and followed by an increase in the level in February. These results also suggest that during the two peak of spermatogenesis, the testicular steroidogenesis has also been elevated. The increase androstenedione could also be converting to testosterone by 17βHSD for spermatogenesis. The western blot analysis of LHR also showed an elevation in October, February and March, which may be responsible for increase testosterone during both peaks, although a surprisingly increase in December has been noticed despite low level of testosterone and androstenedione. However, our present study have not measured the circulating LH levels so we cannot certain that it was due to low LH level although it is believed that the unexpected high-rise expression of LHR despite low testosterone and androstenedione could be due to negative feedback. The immunohistochemical study of 3βHSD showed intense staining in the Leydig cells in the month of November, February and March. These results also suggest an elevated steroidogenesis during the two peaks of spermatogenesis.

The circulating estrogens in the present study also showed a marked change during the calendar months, with a coincidence of its increased levels during the two peaks of spermatogenesis. The very striking observation was observed that in the month of December, the levels of circulating estrogens were very low. To further find out the possible action of estrogens, we have measured the expression of two estrogens receptor, ERα and β in the testis by western blot analysis. Our results showed that two receptor changes during the testicular activity and the expression also coincide with two peaks of spermatogenesis, with increase expression of both receptors during the second peaks. On the very interesting note, the expression of ERα was very low in the December, which further coincides with low circulating estrogens. These finding suggests that transient suppression of spermatogenesis in the December could be due to low estrogen and its signaling, however, during two peaks of spermatogenesis both androgen and estrogens could be important regulators in the testis of *Rousettus leschenaulti*. It has been shown that in the testis expression of

ERα and β are variable and its expression depends on the species, however, estrogen regulates the testicular activity. It has also been documented that ERβ has a ubiquitous expression in male reproductive organs, whereas ERα has greater specificity, and has been shown to express ERα in every species. Since the proliferation is an important event in the spermatogenesis, therefore, we also investigated the expression of proliferating marker, PCNA in the testis of *Rousettusleschenaulti* immunohistochemistry. The immunolocalization of PCNA revealed that testis undergo proliferation during the two peaks of spermatogenesis and during December-January the testicular proliferation slow down as it was evident by the PCNA immunostaining. Furthermore, a transient suppression was coincides with low proliferation and less expression of AR and ERα. Thus it may be hypothesized that the two peaks of spermatogenesis in *Rousettus Leschenaulti* might be regulated by the androgen and estrogens, and suppressed activity of testis could be due to impair signaling of estrogens by ERα. Estrogen signaling has been shown to regulate the testicular function like proliferation. The overall a halt in spermatogenesis can further be hypothesized that during the December-January, the environmental temperature is low and the mature female in the roost are in stages of pregnancy therefore during this time additional mating may not be required. As it was reported earlier that during December also some non-parous female mate, thus it may be suggested some spermatozoa may also be stored in the cauda epididymis for additional fertilization despite low spermatogenesis in the testis. Thus, changes in the quality and quantity of spermatozoa in the epididymis also important which could be correlated with the testicular activity. Therefore, further study would be required to decipher the exact mechanisms of two peaks of spermatogenesis with a transient halt in the winter by considering the hypothalamus, pituitary and testicular axis along with quality and quantity of spermatozoa in the epididymis.

CERTIFICATE

This is to certify that ***"Changes in the testicular activity in males of a Pterepodidae bat, Rousettus leschenaulti''***is written by **Vanlalrempuia**bearing **M.Phil Regn. No.: MZU/M.Phil./592 of 12.06.2020**and has written this thesis under my supervision.

He has fulfilled all the required norms laid down within the **Master of Philosophy** regulations of Mizoram University. This dissertation is truly the result of his owned investigation. Neither the dissertation as a whole nor any part of it was submitted by any other University for any research degree.

(DrVIKAS KUMAR ROY)

Assistant Professor and Supervisor

Department of Zoology

Mizoram University

DECLARATION

I,**Vanlalrempuia**, bearing **M.Phil Regn. No.: MZU/M.Phil./592 of 12.06.2020**, hereby declare that the subject matter of this dissertation ***"Changes in the testicular activity in males of a Pterepodidae bat, Rousettus leschenaulti''*** is the record of work done by me, that the contents of this dissertation did not form the basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the dissertation has not been submitted by me to any other University or Institute.

This is being submitted to Mizoram University for the **Degree of Master of Philosophy in Zoology.**

VANLALREMPUIA

(Research Scholar)

Dr HT LALREMSANGA **Dr VIKAS KUMAR ROY**

(Head of Department) **(Supervisor)**

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Dated: Aizawl (VANLALREMPUIA)

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1. INTRODUCTION

Bats are unique mammals with respect to certain features, such as echolocation, flying and their habit and habitats. Despite certain peculiarities, bats do exhibits some unique phenomena in the reproductive physiology. The process of delayed ovulation, fertilization, implantation and development makes them an idea animal to study the uniqueness of reproduction. In male bats, the extended sperm survival in the epididymis and the asynchrony in the spermatogenesis, and accessory sex gland function, and along with mating are unique features of male reproduction **(Roy and Krishna, 2010)**.

Bats are the only mammals having the ability to flyand they have extensive reproductive strategies along with roosting and feeding habits**(Prior, 1999)**. From the intermediate mesoderm of the hindgut, the excretory organs and gonads are embryologicaly developed and the gonadal ridge formed as a precursor for gonad development **(Tilmann and Capel, 1990)**. The gonads came off as a thick ridge-like structure which became thicker and thicker as it differentiates into primordial germ cells of gonads **(Noden and De Lahunta, 1985)**. The mammalian male reproductive tract usually include paired testes and associated epididymis, vasa differentia, urethra, penis and accessory sex complex, where the accessory sex gland complex consists of prostrate, ampullary, seminal vesicles, urethra and cowper's glands **(Krutzsch, 1979)**. Male accessory organs have great adaptability in form along with the distribution of species, these glands provide difference substances like fructose, sialic acid, citric acid, proteins and zinc to the ejaculate **(Rajalakshmi and Prasad, 1970; Mokkapati and Dominic, 1976; Sapkal, 1986)**. Large availability of food resources coincide with the periods of reproduction is believed to be the motive forces other than the annual course of reproduction in mammals **(Bronson, 1985)**, which is quite correct in the temperate regions where plenty accessibility of food vary considerably due to distinct climatic seasonality. In the tropical regions where there is fairly distribution of food sources along the year, animals reproduce several times in a year which lead to unclear synchronization of reproductive cycles in the population. While in the temperate zone there is a usual synchronization with pronounced seasonality of reproduction **(Bronson and Heideman, 1994)**. As the Chiroptera (bats) inhabited both in the tropical and temperate zones they show a

great diversity in their reproductive pattern (**Jerrett, 1979**). The temperate bats are considered to be seasonal breeders with their characteristic precisely correlate with low temperature and hibernation. However the intensity of rainfall along with the food availability in summer also has a great influence on this pattern (**Krutzsch, 1979; Grindal et al., 1992; Lewis, 1993**). While bats from tropical regions do not hibernate and their reproduction is greatly influenced by their habitat in which they lived, so they are directly influenced by temperature changes which takes place seasonally, rainfall, latitude and their food resources **(Fleming et al., 1972; Taddei, 1976)**.The male reproductive system plays a vital function in storage and production of spermatozoa and their transportation in viable state to the genital tract of the female **(Krutzsch, 1979)**

In *Rousettus leschenaulti* the testis of sexually active male located in the temporary scrotal pouches while in the immature and non-sexually active males the testis position in the abdominal region. This position of testis is used to distinguish between the sexually active males and non-sexually active males. Female's experiences two pregnancies in quick succession along a year, first in the month of November and December, deliver in March-April. The second pregnancy takes place immediately after parturition with deliveries occurring in July. Meanwhile the testis of the male is also showing two peak periods of spermatogenetic activity, one in October–November and another during February–March, in between these peaks a slight regression takes place in January. Males are sexually active from October to April followed by the inactive period from May to September and spermatogenesis ceases completely after April in the next breeding season (**Nerkar and Gadegone, 2017**). While in case of grey headed fruit bat *Pteropus poliocephalus* prior to their mating, adult males show seasonal changes in the size of testis where maximal testis size shown during the autumn season **(Nelson, 1963)** and following the breeding season there was a regressive changes in the testis which include decrease in Leydig cell nuclear diameter and in the diameter of the seminiferous tubules. The adrenal also showing greater weight in the breeding season in comparison with the non-breeding season, on the other hand testicular testosterone is much higher than adrenal

testosterone with less contribution of adrenal gland in the breeding season increase in the peripheral testosterone **(McGuckin and Blackshaw, 1987, 1991)**.

Testosterone is a primary sex hormone and the main factor for spermatogenesis and sexual behaviour, so the seasonal secretion of testosterone is vital and could limit the male reproductive capability during some periods in a year (**Todini et al., 2007**). It is also considered being the most dynamic gonadal factor for the regulation of FSH secretion **(Araki et al., 2000)**; testosterone and FSH are needed to access full reproductive ability. For sperm maturation, transducer signals from FSH and testosterone are required in the somatic sertoli cells of testis **(Walker and Cheng, 2005)**. The large-eared free-tailed bats (*Otomops martiensseni*) have spermatogenic activity or an increase in plasma testosterone that is coincide with the changes in the ultrastructure of leydig cells; this is also shown by mammals **(Kayanja and Mutere, 1978)**. In the male vespertilionid Bat (*Scotophilus heathi*) two peak periods of serum testosterone concentration were seen, the first peak occurs during recrudescence that coincides closely to the period of active spermatogenesis and the second peak comes closely with the period of mating or active spermatogenesis **(Singh and Krishna, 1996, Roy and Krishna, 1996)**. While low plasma testosterone during the winter coincides with the period of testicular inactivity, the testosterone concentration comes to its lowest level during the month of March **(Singh and Krishna, 1996)**. However in the species of Fruit bats, such as *Artibeus lituratus* spermatogenesis continues throughout the year in different regions and as the female get into the estrus stage, the sperm readily formed in the males. In South eastern Brazil, there has a report that their reproductive period coincides with the rainy season based on their studies on the seasonal testis activity which is followed by regression in the testis before the next cycle of testicular recrudescence. But other analysis has also shown that males of these species are considered to be fertile the whole year as they shown continuous spermatogenesis (**Tamsitt and Valdivieso, 1963;Oliveira et al.,2009;Duarte and Talamoni, 2010**).

In endemic species of insectivorous vespertilionid bat (*Myotis nigricans*), observation from Panama have shown that the female exhibit a unique reproductive cycle in which the females have three parturition peaks **(Wilson and Laval, 1974)**.

The gestation period takes approximately 60 days with the first parturition peak takes place in February. This first peak is followed by the postpartum oestrus leading to the second peak that is April to May and August. The last peak is followed by a decline in the reproductive activity which last till late December **(Wilson and Laval, 1974)**. Some findings also revealed that the male *Myotis nigricans* has spermatogenic cycle which is similar to the cycle in female where spermatogenesis is slow or stopped in the month of September, October and November with no sperm storage. From the specimen observed in Mexico it is found that the species resemble close to the temperate region bats in their reproductive strategy, and they have shown great geographically variable with respect to their reproductive cycle and the males are known to be the main factor that controls the seasonality in this species **(Wilson and Findley, 1971)**.

2. REVIEW OF LITERATURE

Bats belonging to order Chiroptera constitute the second largest order of mammals next to rodents. The numbers of species are greatly abundant in the tropical region and to some extend their population may exceed that of rodents. They show great variation with reference to their habitat, foods availability and mating systems (**Altringham, 1996; McCracken and Wilkinson, 2000**). In consideration to their global distribution and the variation exhibit by the members of the order Chiroptera less attention has been given to the reproductive pattern of male. The structural details of the reproductive system are generally not studied and little is known on the function and physiological control of reproduction in male **(Krutzsch, 2000)**. Bats reproductive cycle somehow depends on both the environment as well as the physiological factors **(Racey and Entwistle, 2000)**. Further they are grouped into two suborders: Megachiroptera (megabats) and Microchiroptera (microbats). There are approximately 175 species of megabats and 790 species of microbats. Megachiroptera belong to one family, the Pteropodidae. Microchiroptera are a diverse group and classified into 17 families. These bats are most universally distributed mammals apart from man and rodents. Most of the bat species are seasonal breeders. This makes them ideal animal models for the study of the problems related to reproduction **(Rasweiler, 1990; Abhilasha and Krishna, 1996; Srivastava and Krishna, 2006)**.Seasonally reproductive species in the tropical-zone generally exhibit increased plasma testosterone concentration during the breeding season, and there is corresponding synchronous stimulus of both spermatogenesis and sex accessory gland functions. A close correlation of increased circulating testosterone concentration with peak spermatogenesis has been widely reported in most tropical bat **(Singh and Krishna, 2000)**, suggesting a direct role of testosterone in spermatogenesis. Not only the testosterone rather estrogen may also play important role in the spermatogenesis of mammalian testis including bats **(Oliveira et al., 2009)**.

Several authors have reviewed the progress of research on Chiroptera reproduction from time to time, which have broadened the frontiers of knowledge and still work the male reproduction in bats being continued **(Wimsatt, 1979; Carter, 1970; Krutzsch, 1979; Gustafson, 1987; Crichton and Krutzsch, 2000; Kawamoto,**

2003; Krishna and Bhatanagar, 2010; Silva et al., 2020; Farias et al, 2020; Reeves and Hoffman, 2020).

Bats display astonishing ecological and evolutionary diversity and serve as important models for studies of a variety of topics. The remarkable longevity of spermatozoa in the genital tract (sperm storage), asynchrony of the period of copulation and that of ovulation (delayed ovulation), morphological and chemical specialization of Graafian follicle, delayed implantation and delayed development are among the unique features associated with the reproduction in female bats **(Racey, 1982; Bernard, 1989; Krishna, 1999)**. Among males the most striking features are prolonged retention of sperm in the epididymis and reproductive asynchrony of the periods of spermatogenesis and copulation **(Wimsatt, 1969; Gustafson, 1979, 1987; Racey, 1982)**.

Reproductive patterns of *Rousettus leschenaulti*

Gametogenesis in bats is prolonged and exhibits unique patterns of hormonal changes. Studies on *Rousettus leschenaulti* bats suggest the adult testes of *Rousettus leschenaulti* shows great variations in their weight during different months of annual reproductive cycle **(Rudey and Dhamani, 2017; Masram, 2015)**. A better understanding of how does seasonal reproductive activity change in the testis of this species, would contribute to the knowledge generation for bat reproduction in the male. The literature searched on the testicular activity of *Rousettus leschenaulti*showed only a few studies, which was focused on the testis **(Rudeyand Dhamani, 2017; Masram, 2015; Saidapur and Patil, 1992)**. It has been suggested that male fruit bat *Rousettus leschenaulti* show two peaks in their testicular activity corresponding to the two pregnancy cycles of the female. First peak occurs during February–March and second occurs during October-November **(Masram 2015)**, however, **Rudey and Dhamani (2017)** suggested that December to June testicular activity is regressed. These results showed some discrepancy. The detailed aspects of spermatogenesis in the male bat *Rousettus leschenaulti*have not been investigated. The previous study by **Saidapur and Patil (1992)** have shown the cytomorphology of different types of germ cells, frequency of appearance of these stages, pattern of

spermatogonial stem cell renewal and per cent degeneration of various germ cells in *Rousettus leschenaulti*. However, studies of proliferation markers with respect with testicular activity have not been investigated. This is one of the reasons based for selection of present work for detailed investigation.

Although much is known about the reproductive phenomena and associated endocrine characteristics of various bat species, the mechanism regulating the testicular activity remains to be elucidated in the *Rousettus leschenaulti*. As the role of androgen and estrogen are important for spermatogenesis, no study has been conducted on the modulation of their receptor during the reproductive and non-reproductive period in this species. Therefore, we aimed to investigate the seasonal changes in the steroid hormone along with its receptor and important makers in the testis of *Rousettus leschenaulti.*

3. OBJECTIVES

OBJECTIVES

1. To evaluate serum hormones (Testosterone, androstenedione and estradiol) during different reproductive phases.
2. To study the spermatogenic cycle during different reproductive phases.
3. To study the activity of germ cell proliferation in the testis during different reproductive phases.

4. MATERIALS AND METHODS

4.1. Sample collection:

All the experiments were conducted in accordance with the principles and procedures approved by Mizoram University Institutional Animal Ethical Committee (MZUIAEC), Mizoram University, Aizawl. The experimental procedures were performed to minimize the pain in the animals. The specimens of *Rousettus leschenaulti* were netted randomly from their cave in Khamrang, a Village in Thingdawl Block in Kolasib District of Mizoram (23.9331° N, 92.6543° E). Animals were trapped alive in a cage and then transported to the laboratory with constant supply of food and water. The specimen was anesthetized by Ketamine injection and weight on balance, it was then sacrificed by decapitation, blood (1-2 ml) was collected, and the serum was separated out from blood sample within 1 hour and stored at –20° C until it was used for hormonal analysis. Testis was dissected out from the scrotal sacs, and kept in freeze or fix (Bouin's fluid for 24 hours). This was later used for further analysis i.e. histopathological evaluation, immunocytochemistry and western blotting.

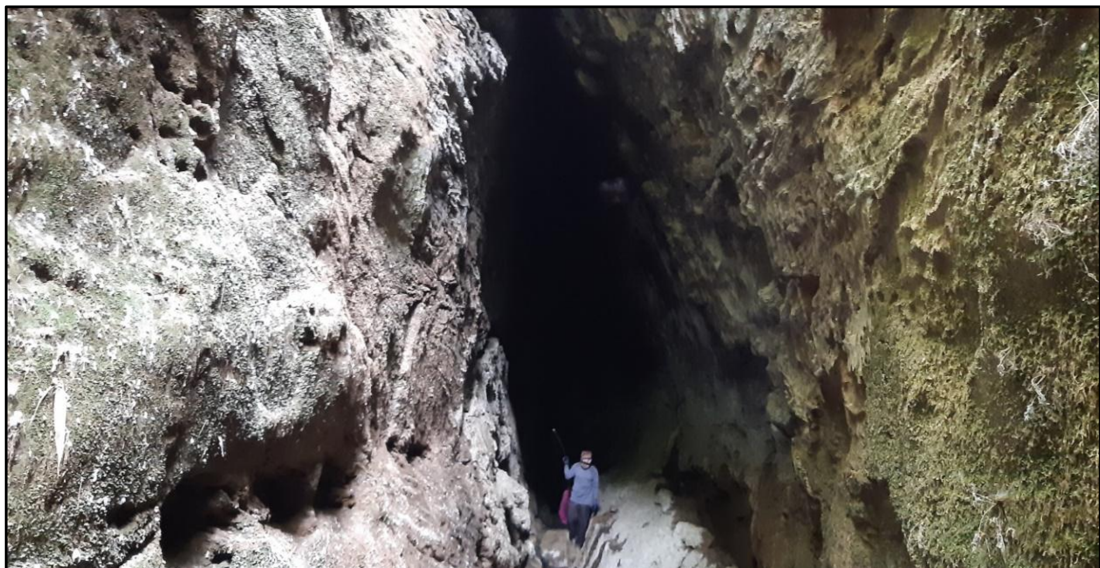


Fig. 1: Site of sample collection (Bat cave, Khamrang).

4.2. Histology:

One testis of each bats were immersed in Bouin's fixative, the next day the tissues were transferred to 70% ethyl alcohol. The transferring should be done before 24 hours to avoid brittleness. For wax block preparation the selected tissue was transferred to 90% and 100% ethyl alcohol involving two changes for 1 hour each. The tissue was placed in xylene for 5 to 10 minutes and transferred to hot air oven in a mixture of melted wax and xylene (1:1) with temperature between 50ºC to 60ºC for 40 minutes, it was then infiltrated with molten waxes (Wax 1, Wax 2 and Wax 3) for 40 minutes each. The temperature of the oven must not exceed 60ºC to prevent over cooking of the tissue. Finally the tissue sample was fixed in paraffin wax block. Paraffin section of 6µm thick were obtained using microtome and fixed in slide. The tissues were deparaffinised in xylene involving two changes for 10 minutes and the slides were transferred to different grades of ethyl alcohol (100%, 90%, 70%) which also involves two changes for 10 minutes each. The slides were then immersed in distilled water and the stained with hematoxylene and eosin and were further dehydrated with series of ethyl alcohol in order of increasing concentration (70%, 90% and 100%). The slides were mounted after proper dehydration and cleared in xylene which was further mounted with DPX and photographed using camera-mounted binocular microscope (Model E200, Nikon, Tokyo, Japan).

4.3. Testis morphometry:

Measurement of seminiferous tubules diameter

Five testes from each month were selected and ten tubular sections which are round or nearly round were chosen and the diameter of each tubule was measured using ocular micrometer of 40X magnification and findings were noted as per method described earlier **(Guneli et al., 2008)**.

Measurement of Germinal epithelial height (GEH) of the seminiferous tubules

Ten tubular sections which were round or nearly round were measured from each testis. The GEH was obtained in the same tubule utilized for determining the tubular diameter **(Franca et al., 2003).**

4.4. Immunohistochemistry:

The presence and distribution ofPCNA, 3 βHSD in the testis during the active and quiescence periods were studied by immunohistochemistry (n= 5 for each period). The Bouin's fixed testis tissues which were kept in 70 % ethanol were further dehydrated in a series of ethanol solutions in increasing order of concentrations (90% and 100%) twice for one hour each. The tissues were then immersed in xylene and subsequently infiltrated with molten paraffin. Using Leica rotary microtome (model RM2125 RTS), the tissues were sectioned at 6 μm thick and then embedded in a sterile glass slides. The slides were then transferred to xylene, two changes for 10 minutes each for deparaffinization and rehydrated in different grades of ethanol in a decreasing order of concentration (100%, 90% and 70%) so as not to excessively distort the tissue, which was then put in distilled water and PBS for 10 minutes each. The slides were then blocked by incubating in a blocking buffer [goat serum 1:100 diluted in PBS (Lot # A0515, Santa Cruz Biotechnology, Inc., CA, USA)] for 30 minutes at room temperature, which were further incubated overnight at 4°C in a humidified chamber using primary antibody against Proliferating cell nuclear antigen (PCNA) (1:200, Rabbit polyclonal IgG, Cat# sc7907, Santa Cruz Biotechnology Inc. Dallas, USA). Unbound antibody was washed off using PBS and then incubated in Horse-radish Peroxidase (HRP) conjugated goat-anti-rabbit secondary IgG antibody (1:500, Merck Specialties Pvt. Ltd, Mumbai, India) for 3 hours. Unbound antibodies were washed off by PBS and incubated for 10 minutes at room temperature in a solution containing 0.6 mg/ ml solution of 3, 3-diaminobenzidine tetra hydrochloride dihydrate (DAB) in Tris–HCl (pH 7.6) and 0.01% H_2O_2 till brown colour develops. Dehydration was done using different grades of alcohol in increasing order of concentrations and then cleared in xylene. It was then mounted with DPX and the

slides were examined and photographed using camera-mounted binocular microscope (Model E200, Nikon, Tokyo, Japan).

4.5. Serum hormone analysis:

Blood sample prepared and stored in -20ºC was used for hormone analysis, total serum concentrations of , testosterone ,androstenedione, estrogen were measured using a human ELISA kit.

Determination of serum testosterone:

Quantification of total serum concentration of testosterone was done using human ELISA kit (Testosterone Cat# DKO002, DiaMetra, Italy) as directed by the manufacturer's instructions. In brief, 25 µL of samples and calibrators was added to the wells of ELISA plate. Then, 100 µL of the enzyme conjugate was added to these wells. ELISA plate was incubated for 1hour at 37°C. Then each well was aspirated and washed with 300 µL wash solution three times followed by addition of 100 µL Tetramethylbenzidine (TMB) substrate to each well and incubated for 15 minutes at room temperature in the dark. Finally, 100 µL of stop solution was added and absorbance was taken at 450 nm using microplate ELISA reader.

Androstenedione:

Androstenedione was measured using human ELISA kit (Androstenedione Cat# DKO008, DiaMetra, Italy) as the given manufacturer's instructions. This is a competitive immunoenzymatic colorimetric for determination of Androstenedione concentration in serum samples. The androstenedione (antigen) present in the sample competes with the conjugated with horseradish peroxidase (HRP) for binding to limited number of antibodies coated on the microplate. 25 µL of samples and calibrators was added to the wells of ELISA plate, then 200 µL of the enzyme conjugate was added to each wells. The plate was incubated at 37°C for 1hour, which was then washed three times with 300 µL wash solution. 100 µL Tetramethylbenzidine (TMB) substrate was added to each well and incubated in the dark for 15 minutes at room temperature. Then, 100 µL of stop solution was added and absorbance was taken at 450 nm using ELISA reader.

Estradiol:

Serum estradiol was measured using human ELISA kit (Estradiol Cat# DKO003, DiaMetra, Italy) in accordance with the manufacturer's instructions. In brief, 25 μL of samples and calibrators was added to the wells of ELISA plate followed by the addition of 200 μL of the enzyme conjugate to these wells. Incubation for 2 hours was done in 37°C, after which the content in the well was removed and washing of the wells was done three times with 300 μL of diluted wash solution. 100 μL of Tetramethylbenzidine (TMB) substrate was added to each well and incubated in the dark for 30 minutes at room temperature, finally stop solution of 100 μL was added and the absorbance was read at 450 nm against the blank within 5 minutes.

4.6.Western blotting:

For the preparation of tissue total protein extract , testis from each month were homogenized in a chilled suspension buffer containing 150 mM Sodium chloride (NaCl), 50 mMTris-Hydrochloric acid (HCl), pH 8.0, 0.1 % SDS,1 g/mL Aprotinin, 1 mM (PMSF) and 1 mM (EDTA). The homogenates were centrifuged at 4 °Cat 25,000 g for 15 min and supernatants were collected. The protein concentration from each month was estimated by Bradford method (Marion M. Bradford, 1976). The samples were then denatured in SDS-sample buffer (62·5 mMTris, 2% SDS, 10 % glycerol) by boiling for 10 minutes. 50 μg/well of each month were loaded in 10 % SDS-PAGE along with protein marker and the gel was electrophoresed at 100 V for 3 hours. The resolved proteins were then transferred onto a polyvinylidineflouride membrane (Millipore India Pvt. Ltd., Bangalore, India), using wet transfer at 4° C overnight. The blots were then blocked with 5 % non-fat dry milk with PBS (10 mM, 7.5 pH) and 0.1 % Tween 20 and incubated for 30 min at room temperature, after which it was then incubated with primary antibodies; Androgen receptor (AR) (Rabbit polyclonal IgG, 1:1000 diluted with Blocking buffer, #E-AB-31380, Elabscience, Wuhan, China), Estrogen receptor α (ER) (Rabbit polyclonal IgG,1:1000 diluted with Blocking buffer, #E-AB-31380, Elabscience, Wuhan, China), Estrogen receptor β (ER) (Rabbit polyclonal IgG, 1:1000 diluted with

Blocking buffer, #CWK-F12, DSHB, Department of Biology, Iowa, USA), β-tubulin (1:1000 diluted with Blocking buffer, Rabbit polyclonal IgG #E-AB-20033, Elabscience, Wuhan, China) for overnight at 4 ◦C.The blot were washed with PBS-Tween 20 for 2 changes, and incubated with horse-radish peroxidase conjugated secondary antibody (1:4000) for 3 hours at room temperature. After washing with PBS-Tween, the blots were finally detected by chemiluminescence (ECL) (cat no-1705060, BioRad, Hercules, CA, USA) and developed with x-ray film. The protein band was then analysed with Image J software (1.38x, NIH, Bethesda, MD, USA). The density of each band was normalized to the density of the β-Tubulin band that was used as an internal control.

4.7. Statistical analysis:

All data were expressed as mean±SEM (Standard error of mean) and were analysed by one-way analysis of variances (ANOVA) followed by Tukey's test to compare the data from different month. The data was considered significant if $p < 0.05$.

5. RESULTS

5.1. Changes in the body weight and testis weight during different calendar months

The seasonal changes in the body weight and testis of *R. leschenaulti* have been shown in the **Fig. 2**. The body weight of male bat did not show marked variation throughout the calendar months. A significant increase in the body weight was noticed from September to October (One-way ANOVA followed by post hoc, Tukey's test $F_{7, 32} = 5.28$, $p<0.05$). From October to November, a significant decline followed by an increase in body weight was observed. From December onwards body weight did not show pronounced change **(Fig. 2A)**.

Testis weight showed a significant higher weight in February-March compared to the other months (One-way ANOVA followed by post hoc, Tukey's test $F_{7, 32} = 36.12$, $p<0.05$). A significant increase in the testis weight was also observed in the October from September **(Fig. 2B)**

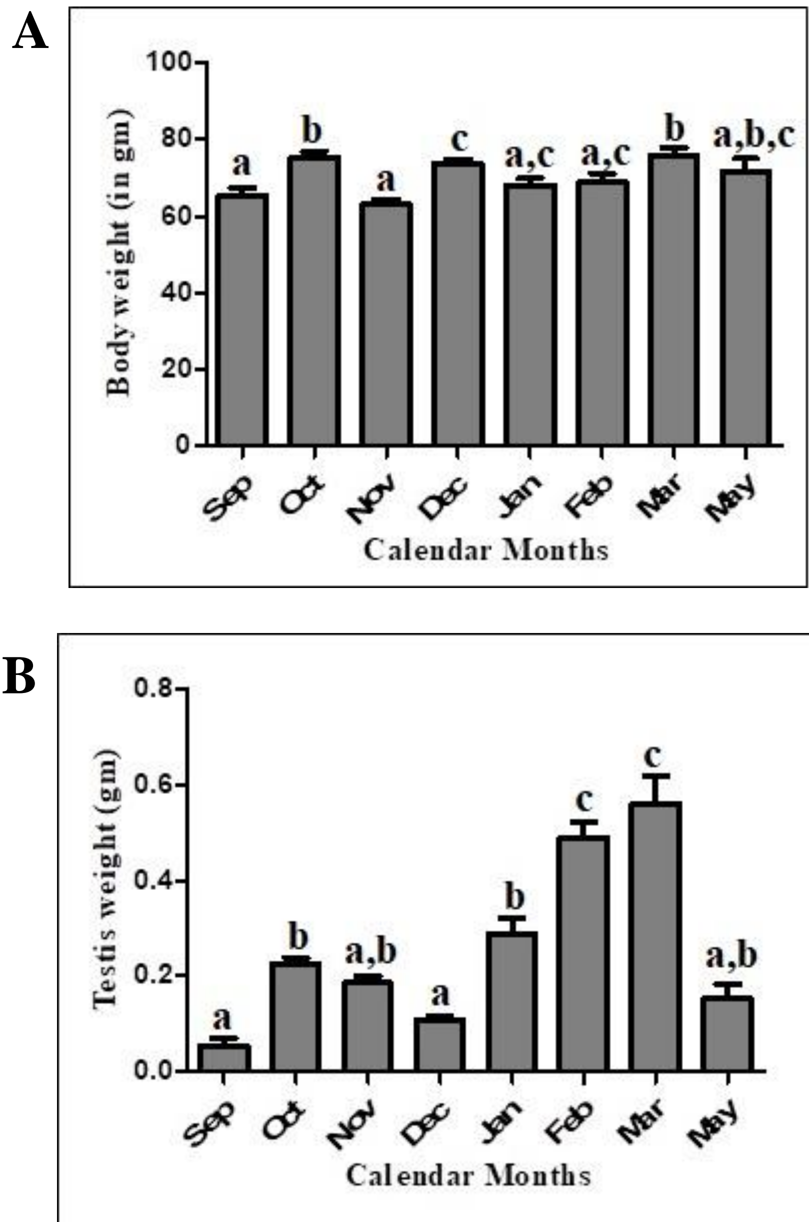


Fig. 2: Changes in the (A)body weightand (B)testis weightduring different calendar months.Data were represented as mean±SEM. (*: *P*<0.05). Different alphabet letter above the bar graph indicates statistically significant differences between groups.

5.2 . Changes in the testicular histoarchitecture during different calendar months

The histological changes during the different calendar months exhibited conspicuous changes in the germ cell types and histoarchitecture (**Fig. 3**). The seminiferous tubules of testis collected in September were having few spermatogonia and Sertoli cells with any advance germ cells of spermatogenic stage. The interstitium showed the presence of Leydig cells in the September. The seminiferous tubules of October showed active spermatogenesis which were evidenced by the presence of advance stage of germ cells such as spermatogonia, primary spermatocytes, round spermatid, elongated spermatid and sperm. Leydig cells of October also showed a clear cytoplasm. The seminiferous tubules of November testis also showed an evidence of spermatogenesis, however, some degenerative changes were also noticed such as presence of vacuoles and presence of few sperm compared to October. These transient degenerative changes further elevated in the months of December and January, where more vacuolization were noticed. Furthermore, the round, elongated spermatids and sperms were a very few in the December-January. The histological study further revealed that an improvement from February to March. The seminiferous tubules of February and March showed an evidence of active spermatogenesis, which was reflected by the presence of primary spermatocytes, round, elongated spermatid and sperm. The testis collected in the months of May showed sign of regression, the seminiferous tubules were having vacuole and devoid of some germ cells, such as elongated spermatids and sperm.

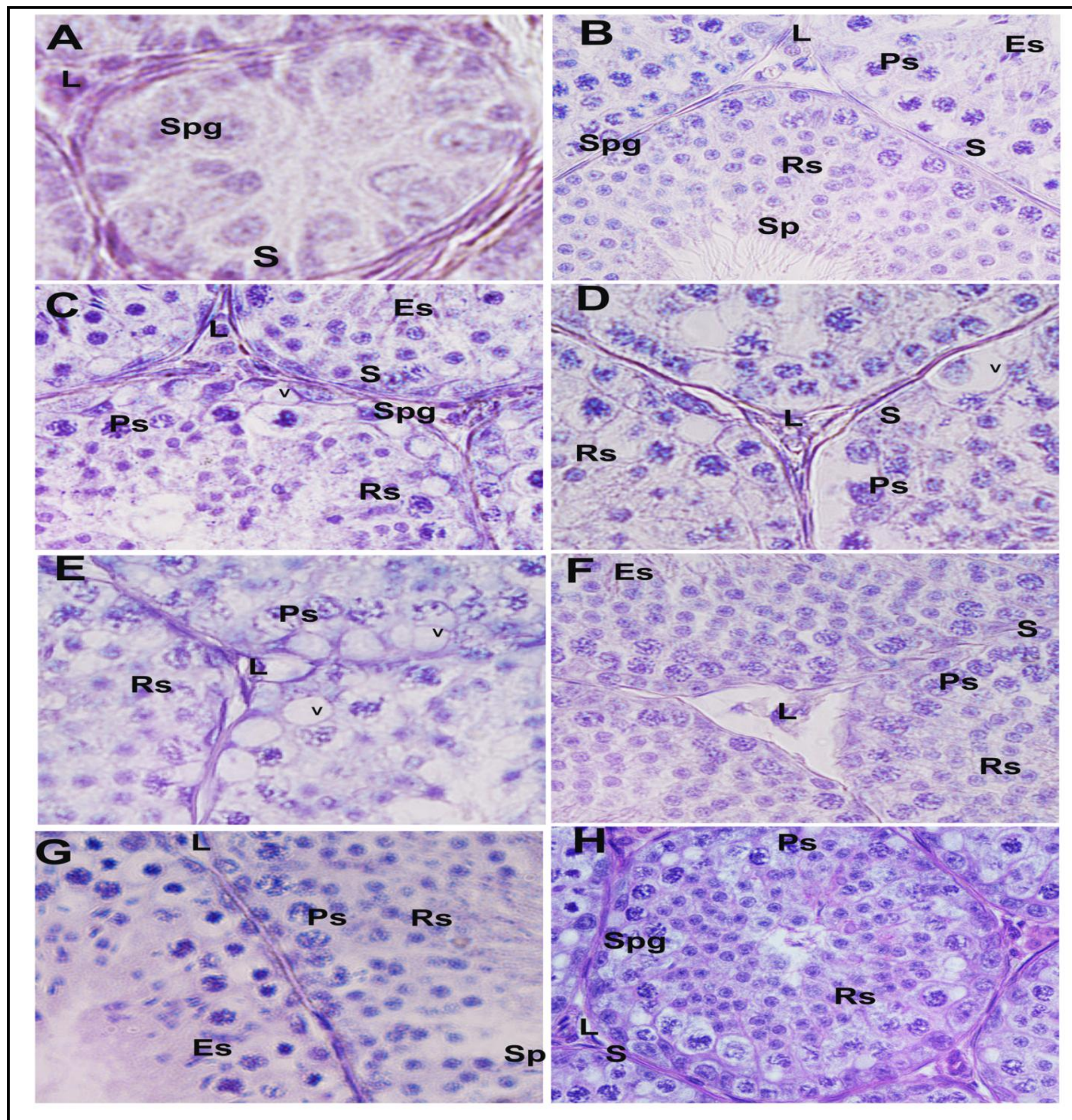


Fig. 3: Histoarchitecture of testis during different calendar months.

(A) September; (B) October; (C) November; (D) December; (E) January; (F) February; (G) March; (H) May. Note that the testis underwent a profound spermatogenesis in October, February, and March. The testes were regressed in September and May. In December and January, they show a sign of degenerative process.

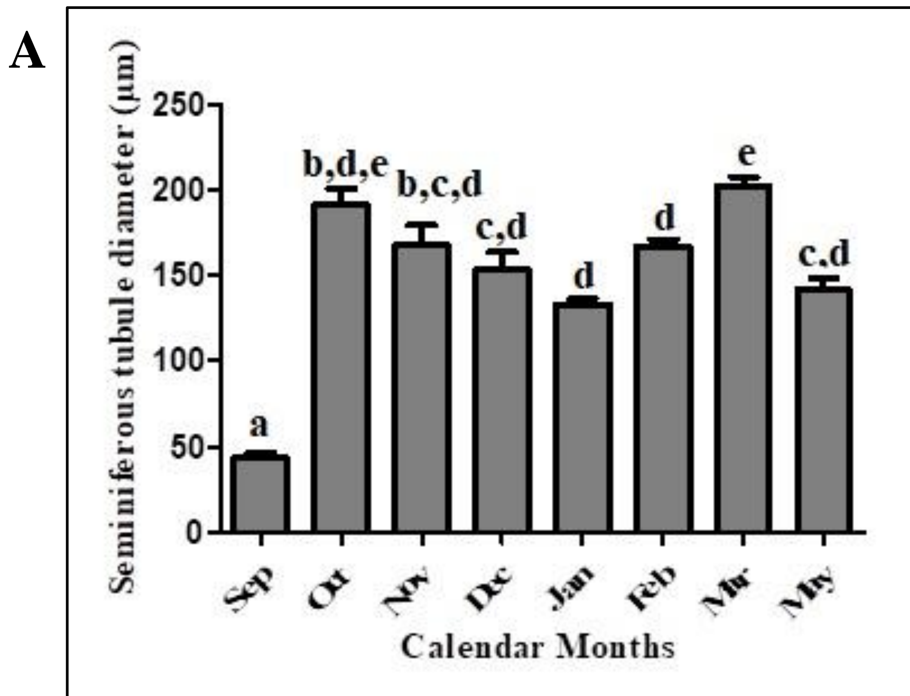
Spg- Spermatogonia, S- Spermatid, L- Leydig cell, Ps- Primary spermatocyte, Rs- Round spermatid, Es- Elongated spermatid, Sp- Sperm, V- vacuole.

5.3 . Changes in the testicular histomorphometric parameters during different calendar months

To evaluate the changes in the testicular activity during different reproductive phases, histomorphometrical study of testis was performed. The two parameters, seminiferous tubule diameter and germinal epithelium height of testis showed noticeable variations(**Fig. 4).** The seminiferous tubule showed a minimum diameter in the month of September compared to the other months (One-way ANOVA followed by post hoc, Tukey's test $F_{7, 32} = 43.66$, $p<0.0001$). Followed by increased in the October-November, the diameter of tubules showed a significant decline in January compared to October and March **(Fig. 4A)**.

The germinal epithelium height of testis also showed a similar trend with the diameter with minor change(**Fig. 4B**). The germinal epithelium height also exhibited a significant decrease in the September compared to the other months (One-way ANOVA followed by post hoc, Tukey's test $F_{7, 24} = 22.70$, $p<0.0001$).

A



B

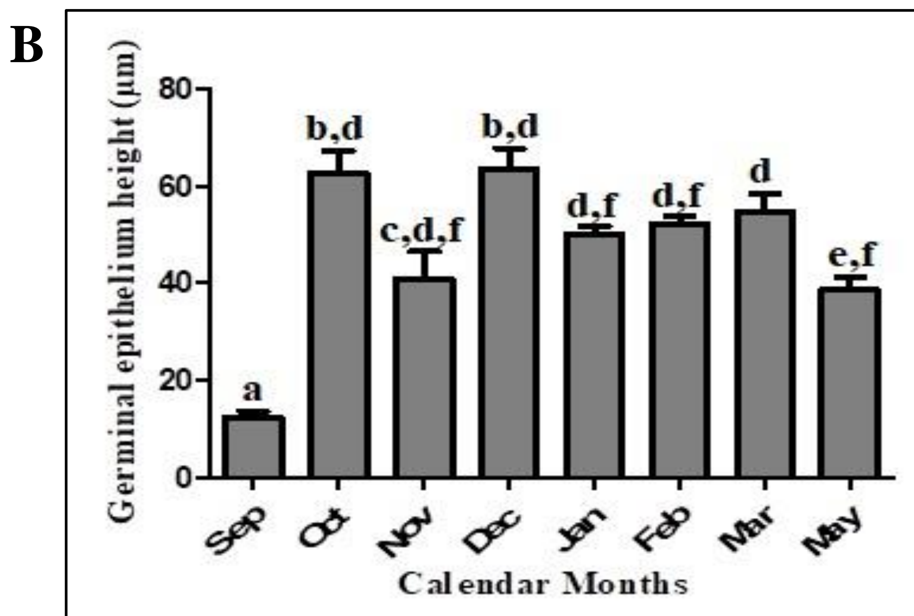


Fig. 4: Histomorphometric study of testis.

(A)Seminiferous tubule diameter. (B) Germinal epithelium height.

Data were represented as mean±SEM. Different alphabet letter above the bar graph indicates statistically significant differences between groups (a,b,c,d, :*p<0.0001*).

5.4. Changes in the circulating Androstenedione levels during different calendar months

The circulating steroid hormone levels of androstenedione, testosterone and estradiol exhibited a marked variation during the different calendar months from September to May **(Fig.5)**. The circulating androstenedione levels showed a peak in the month of October (One-way ANOVA followed by post hoc, Tukey's test $F_{7, 24} = 46.50$, $p<0.0001$).

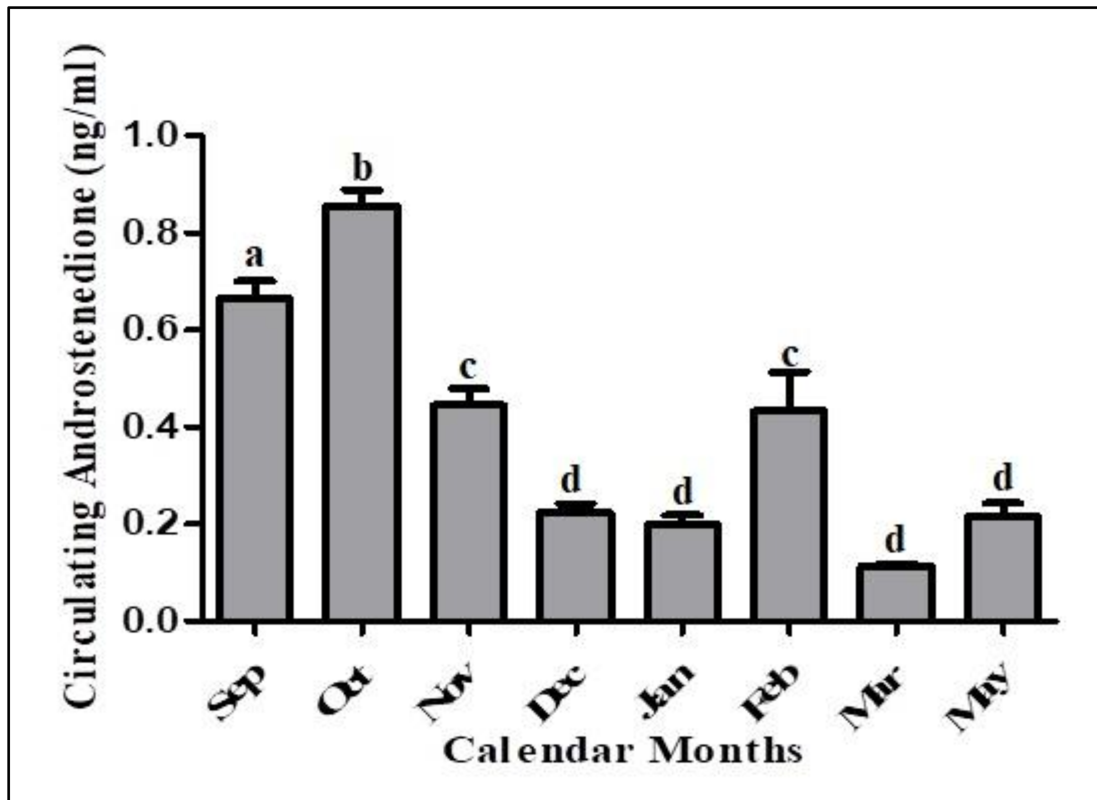


Fig. 5: Changes in the circulating Androstenedione levels during different calendar months. The data are represented as the mean ± SEM. Different alphabet letter above the bar graph represents statistically significant differences between groups (a,b,c,d : $p<0.0001$).

5.5. Changes in the circulating Testosterone levels during different calendar months

The levels of male hormone, testosterone also showed variation during the different months(**Fig. 6**). The testosterone levels showed a significant elevation in months of October and November from September (One-way ANOVA followed by post hoc, Tukey's test $F_{7, 24}= 5.68$, $p<0.0001$). Form September, the levels of testosterone decreased significantly in the months of December and January followed by an elevation in the February and March compared to December and January.

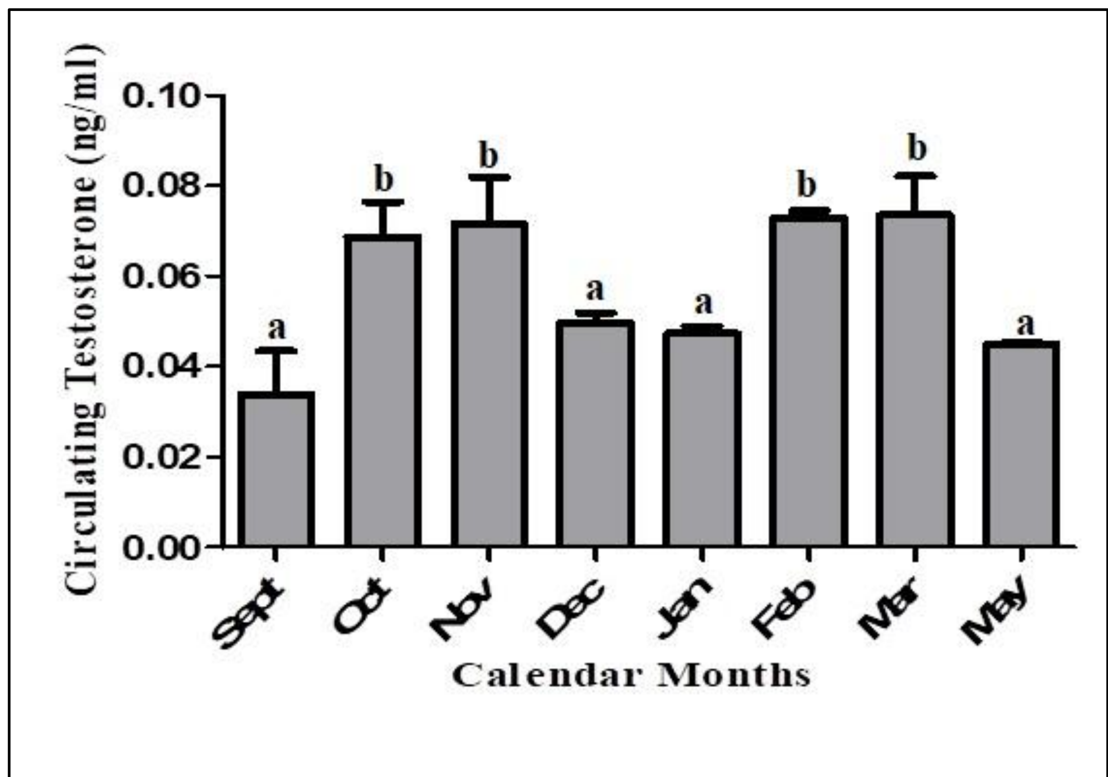


Fig. 6:Changes in the circulating Testosterone levels during different calendar months. The data are represented as the mean ± SEM. Different alphabet letter above the bar graph represents statistically significant differences between groups (a,b : $p<0.0001$).

5.6. Changes in the circulating Estradiol levels during different calendar months

The circulating Estradiol concentration during the different months were also estimatedand showed some variation in the concentration(**Fig. 7**). The circulating estrogen levels were found to be significantly lower (One-way ANOVA followed by post hoc, Tukey’s test $F_{7, 24}=10.69$, $p<0.0001$) in December compared to all other months, except October.

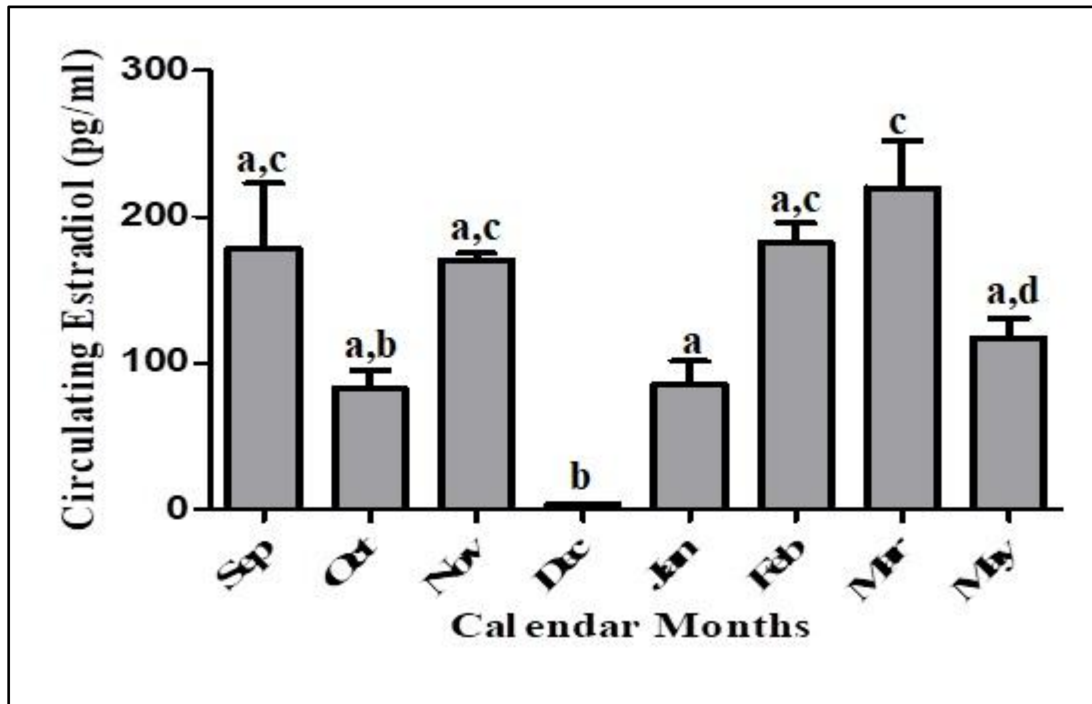


Fig. 7:Changes in the circulating Estradiol levels during different calendar months. The data are represented as the mean ± SEM. Different alphabet letter above the bar graph represents statistically significant differences between groups (a,b,c,d : $p<0.0001$).

5.7. Immunolocalization of steroidogenic marker, 3βHSD in the testis

To evaluate the Leydig cell activity in relation to steroidogenesis, immunostaining of 3βHSD was performed **(Fig. 8)**. The staining of 3βHSD exhibited it abundance in the Leydig cells and showed a distinct variation. A mild immunostaining of 3βHSD was observed in the Leydig cells of testis collected in September, December, January and May. Leydig cells in the months of October, November, February and March showed intense immunostaining.

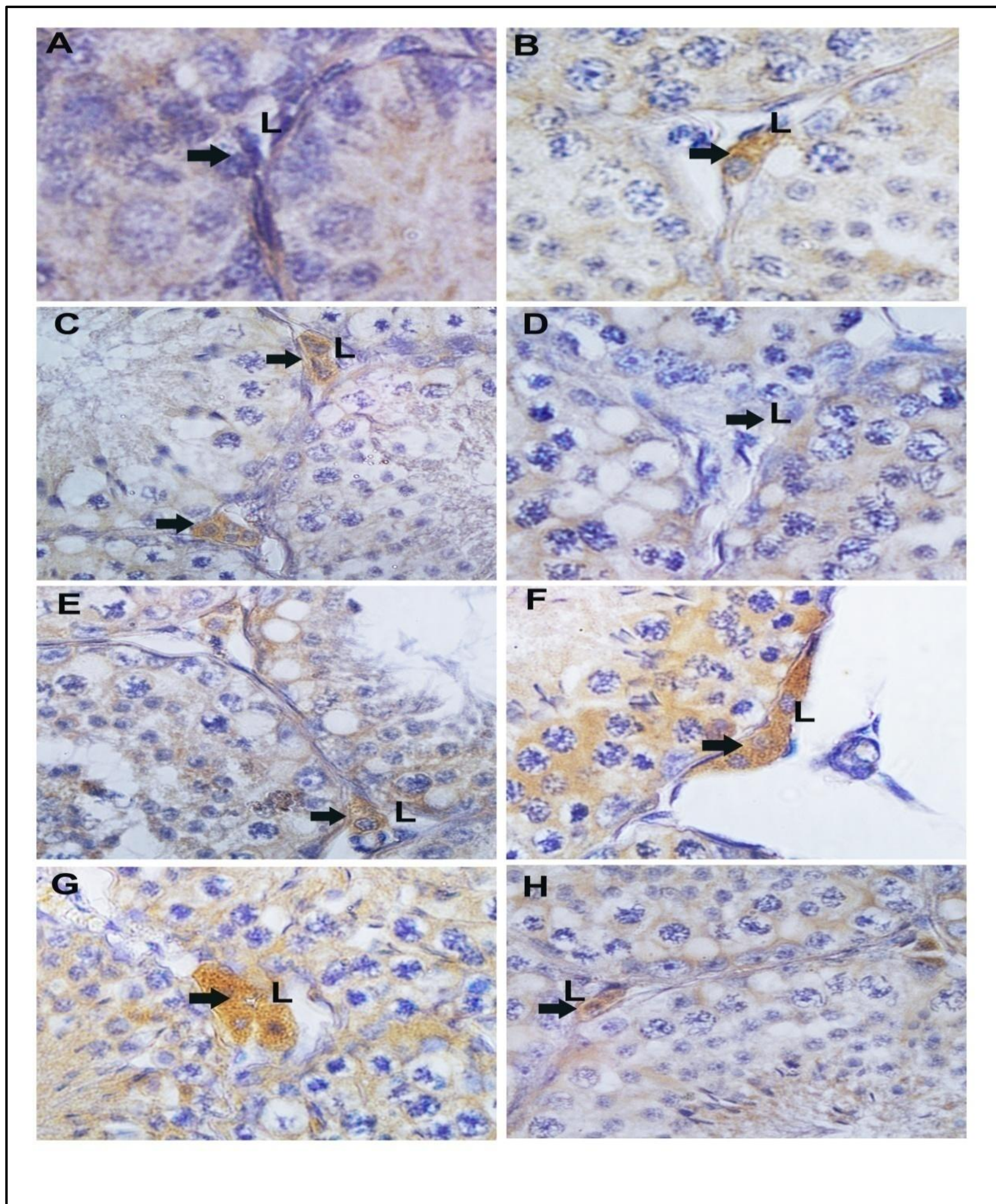
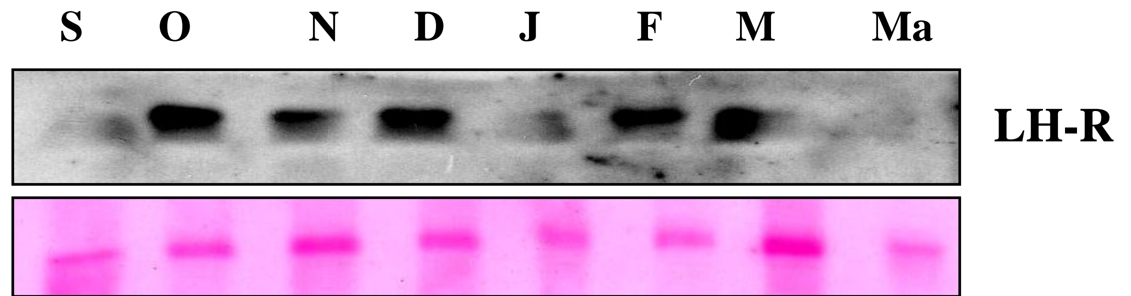


Fig. 8: **Immunolocalization of steroidogenic marker, 3βHSD in the testis.**

(A) September; (B) October; (C) November; (D) December; (E) January; (F) February; (G) March; (H) May. L- Leydig cell.

5.8. Expression of LH-R in the testis of *R. leschenaulti* during different calendar months

The western blot of LH-R followed by densitometric analysis showed distinct changes in the expression during different reproductive phases**(Fig. 9)**.Expression of LH-R increased significantly in October-November compared to September (One-way ANOVA followed by post hoc, Tukey's test $F7, 24=46.54$, $p<0.0001$). Furthermore, LH-R showed an increase in December followed by a decline in January. The expression of LH-R again showed an elevation in February followed by decline in March and May.



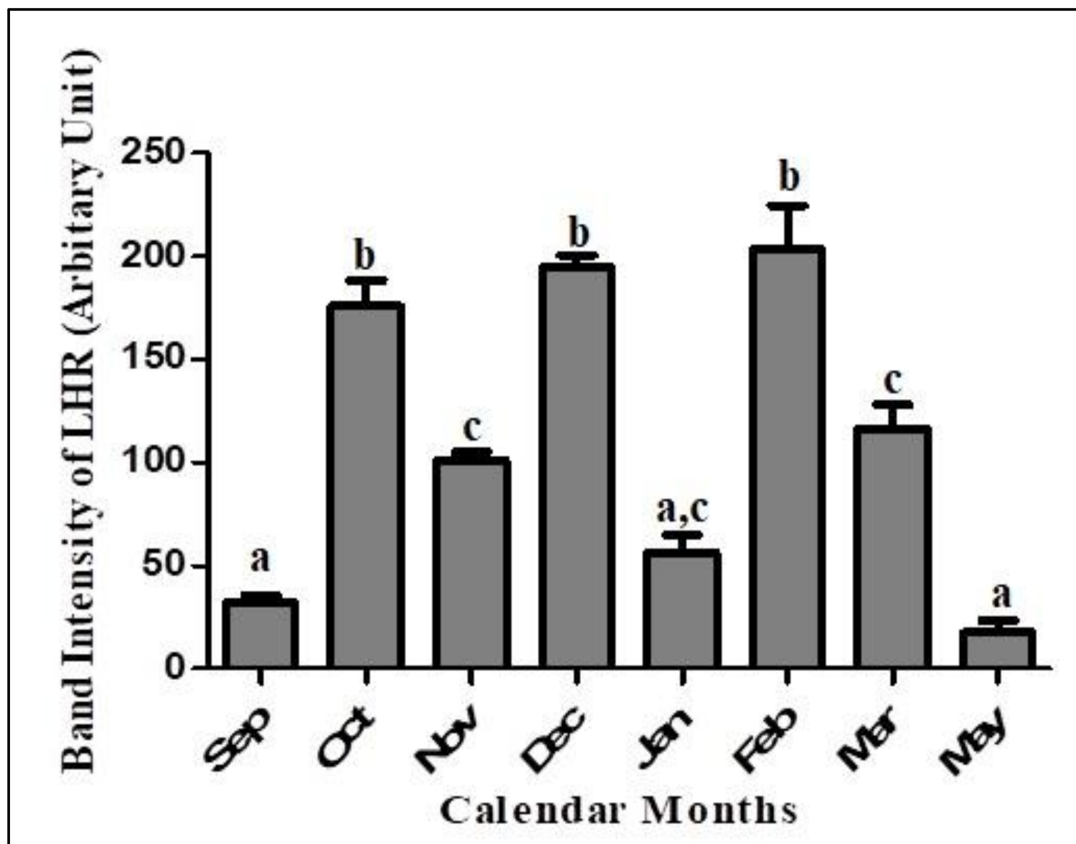
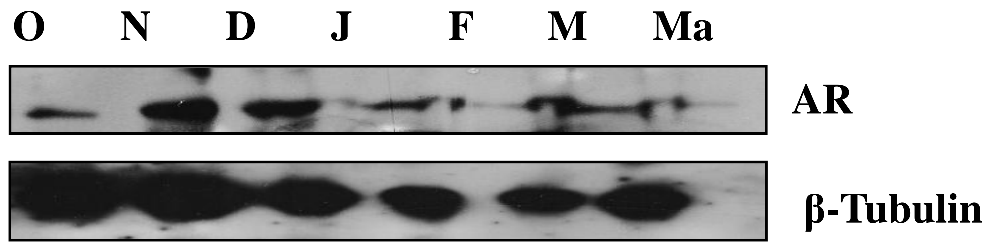


Fig. 9: **Immunoblotting of LH-R followed by densitometric analyses. The data are represented as the mean ± SEM. Different alphabet letter above the bar graph represents statistically significant differences between groups (a,b,c:*P*<0.05).**

S- September; O- October; N- November; D- December; J- January; F- February; M- March; Ma- May.

5.9. Expression of AR in the testis of R. leschenaulti during different calendar months

To find out the role of androgen in the testicular activity, expression of AR was measured by western blot analysis(**Fig. 10**). The expression of AR showed an increase in the November-December compared to the October, followed by a decline in January. The expression of AR further elevated in the March followed by a decline in the May. The expression of AR was found to be significantly in March compared to the other months (One-way ANOVA followed by post hoc, Tukey's test $F_{6, 21}=44.21$, $p<0.0001$). The expression of AR was found to be low in the October, January and May.



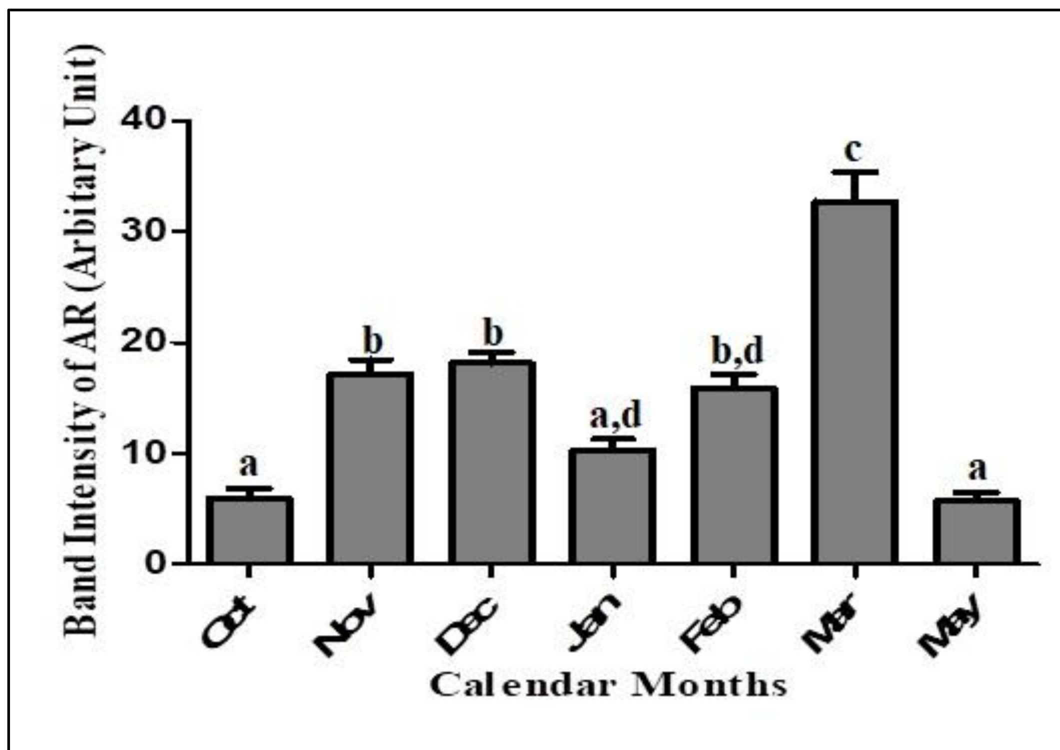


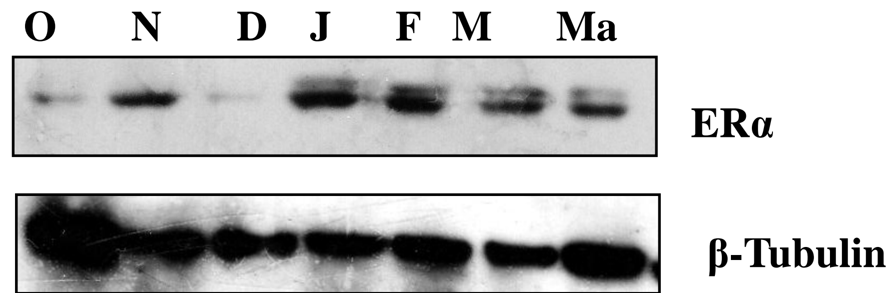
Fig. 10: **Expression of AR in the testis. The data are represented as the mean ± SEM. Different alphabet letter above the bar graph represents statistically significant differences between groups (a,b,c,d:*$p<0.0001$*).**

S- September, O- October, N- November, D- December, J- January, F- Febuary, M- March, Ma- May.

5.10. Expression of ERα and β in the testis of *R. leschenaulti* during different reproductive phases

To explore the possible role of estrogen on the testicular activity, expression of ERα and β were analysed by western blot methods. The expression of ERα showed a significant elevated peak in the month of February compared to the other months (One-way ANOVA followed by post hoc, Tukey's test $F_{6,\ 21}=125.1$, $p<0.0001$). The expression of ERα was found to be significantly low in the October and December(**Fig. 11**).

On the other hand, expression of ER β was significantly low in the October compared to the months (One-way ANOVA followed by post hoc, Tukey's test $F_{6,\ 21}=158.7$, $p<0.0001$). From October onwards to November and December the expression of ER β showed an increasing trend with no change between November-December. Furthermore, the expression of ER β from January to May showed no pronounced change, however, a peak was observed in the month of February **(Fig. 12)**.



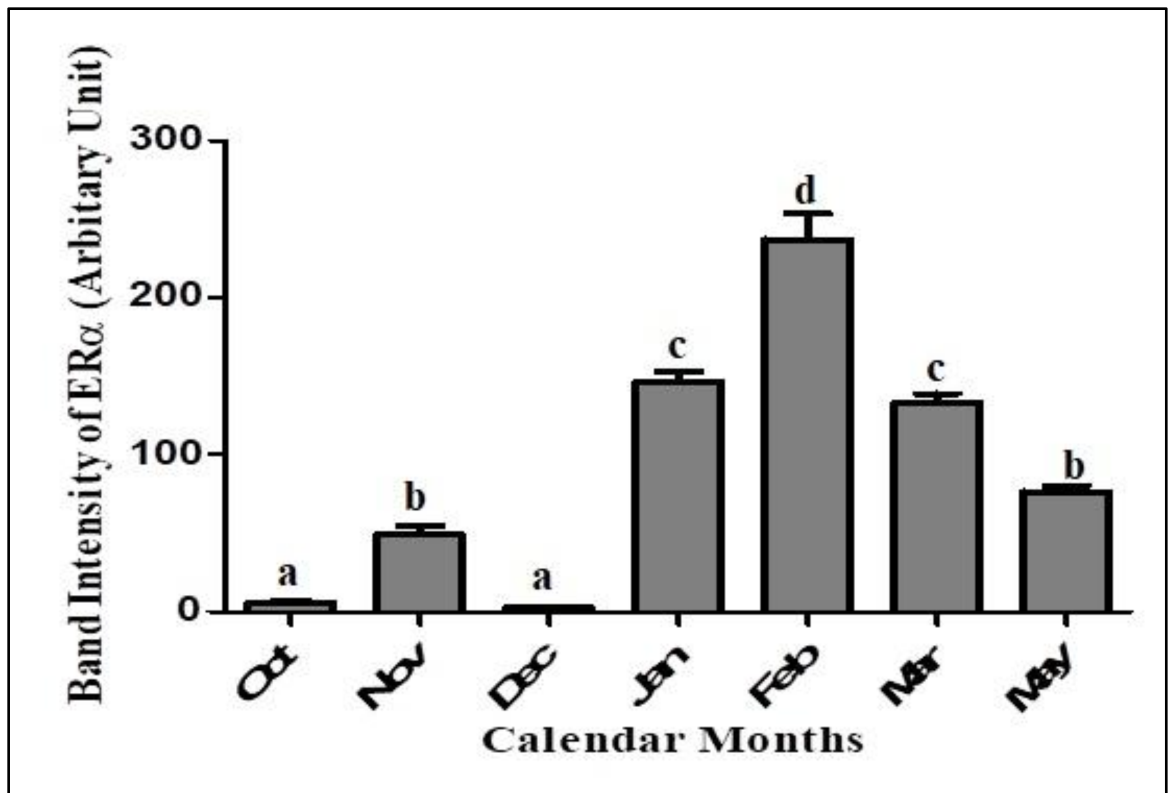
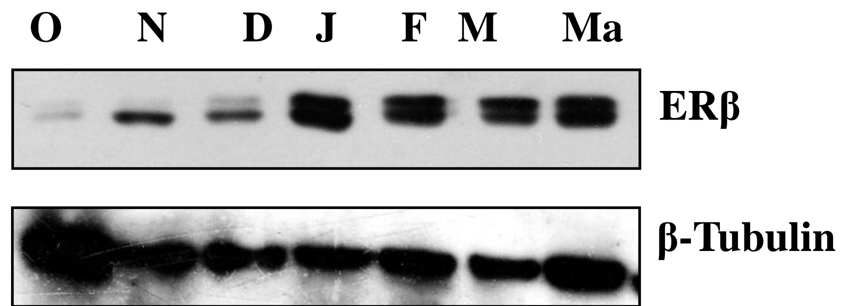


Fig. 11: **Expression of ERα in the testis. Different alphabet letter above the bar graph represents statistically significant differences between groups (a,b,c:*p<0.0001*). O- October, N- November, D- December, J- January, F- February, M- March, Ma- May.**



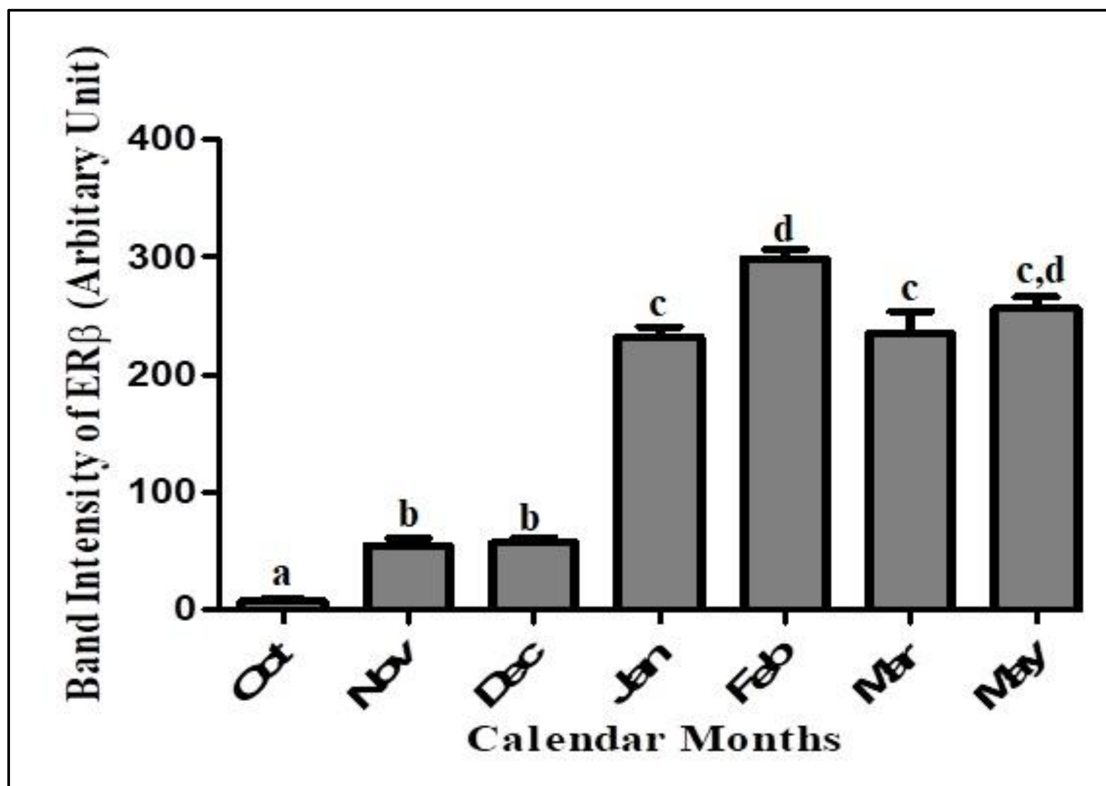


Fig. 12: **Expression of ERβ in the testis. Different alphabet letter above the bar graph represents statistically significant differences between groups (a,b,c,d:*p<0.0001*). O- October, N- November, D- December, J- January, F- February, M- March, Ma- May.**

5.11. Immunolocalization of germ cell proliferation marker, PCNA in the testis

To evaluate the testicular germ cell proliferation in the testis of male bat, *R. leschenaulti*, the immunolocalization of PCNA was performed. The immunostaining of PCNA showed a distinct appearance in the seminiferous tubules. The immunostaining PCNA showed its abundance in the spermatogonia and primary spermatocytes. The seminiferous tubules of September showed mild staining in the germ cells, spermatogonia in particular. In October, an increase in the PCNA was observed in the spermatogonia and primary spermatocytes. The PCNA showed a similar staining pattern in the November. A very faint and a few PCNA immunostaining were seen in the seminiferous tubules of December-May (**Fig. 12**).

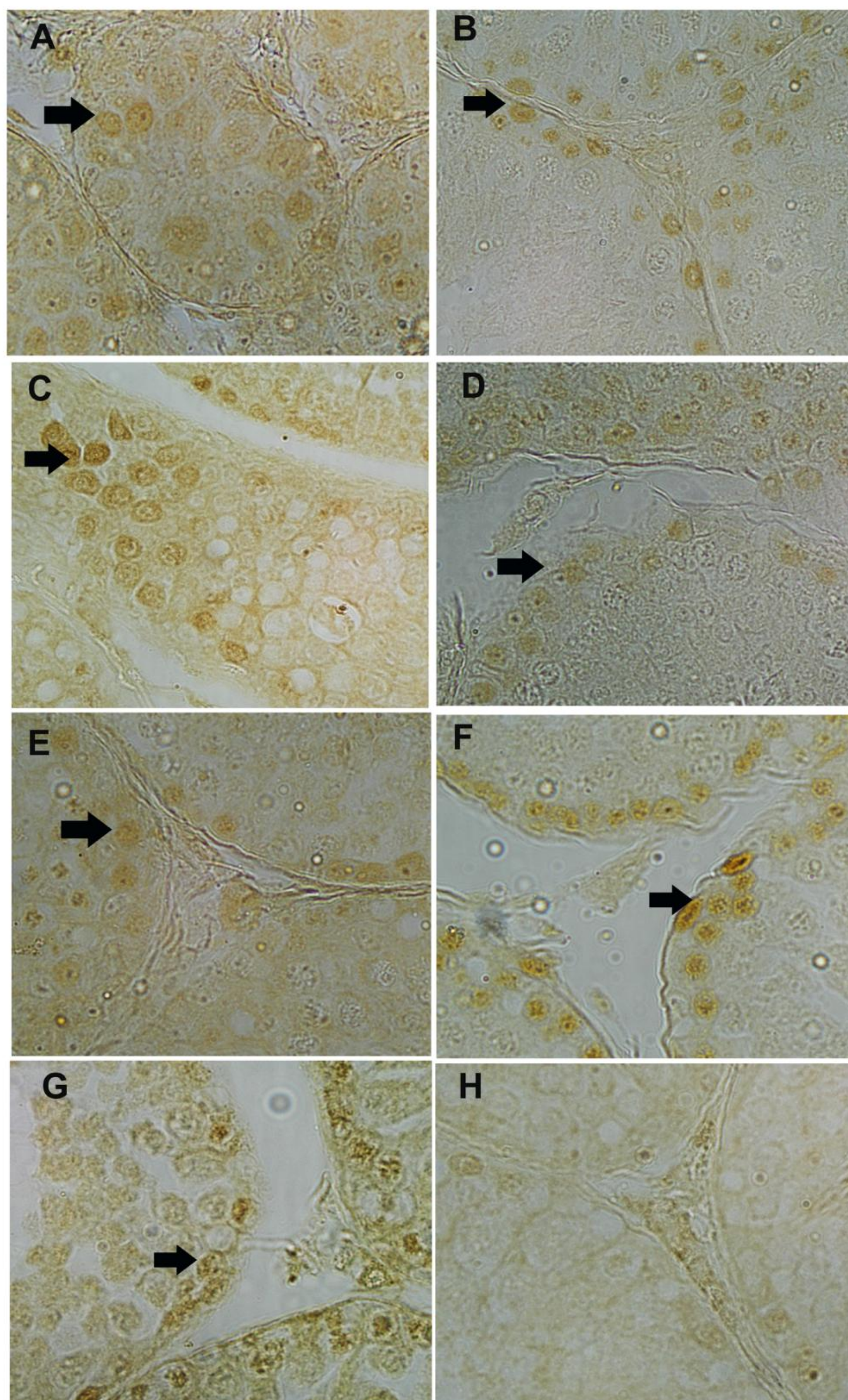


Fig. 12: Immunolocalization of PCNA in the testis.

(A) September. (B) October. (C) November. (D) December. (E) January. (F) February. (G) March. (H) May.

6. DISCUSSION

In the present study, we investigated the changes take place in the testis of adult males of an Indian fruit bat *Rousettus leschenaulti* during their reproductive and non-reproductive phases. The present study also measured the different circulating steroid hormones, androstenedione, testosterone and estrogen along the receptors of androgen and estrogen. The important markers of steroidogenesis such as LHR, and 3βHSD were also investigated. The histological study showed a marked change in the architecture of testis. The seminiferous tubules in the month of September showed few spermatogonia and Sertoli cells with without any advance germ cells. The active spermatogenesis was observed in the month of October and November with advance stage of germ cells such as spermatogonia, primary spermatocytes, round spermatid, elongated spermatid and sperm. However, in month of November, testis also showed some evidence of degeneration, furthermore, these degenerative changes become more pronounced in the months of December and January, followed by the improvement in the February-March. The testes collected in the months of May showed sign of regression, like presence of vacuole and devoid of some germ cells. Thus, these results further strengthen the presence of two peaks of spermatogenesis with a transient suppression **(Gopalakrishna, 1977)**. The two peaks of testicular activities with a transient suppression have also been reported in the other bat species, *Scotophilus heathi* and *Plecotus auritus***(Roy and Krishna, 2010; Entwistle, 1998)**. The transient suppression of spermatogenesis during December-January coincides with decreased environmental temperature. It has been shown that testis of sexually active male located in the temporary scrotal pouches while in the immature and non-sexually active males the testis position in the abdominal region **(Nerkar and Gadegone, 2017)**. Both physiological and environmental factors play a vital role in the reproductive pattern of bats **(Racey and Entwistle, 2000)**. The suppressed testicular activity in winter has also been in the insectivorous bat, *Scotophilus heathi***(Roy and Krishna, 2010)**. It is very clear that change in the seasonal temperature play important role in testicular activity and it has been suggested that temperature may be a proximate influence on bat **(Jolly and Blackshaw, 1988)**. It has been shown that bat from tropical zone also show the reproductive patterns of temperate region **(Wilson and Findley, 1970)**. The peaks of

spermatogenesis have also been reported in *Eptesicusfurinalis* and *Myotis nigricans*(**Krutzsch, 1979;Beguelini et al., 2014**).

The gonadosomatic index, the ratio of gonad to body weight, is an important measure for gonadal activity, and it has been shown that males of *Rousettus leschenaulti* do not reach sexual maturity until they attain a body weight of 73 g and it was suggested that this is minimum body weight, which could show the sign of spermatogenesis **(Gopalakrishna and Choudhari, 1977)**, however in our present study we observed that spermatogenesis have been shown in the testis of males weighing below 73 g which is believed to be influence by the difference in environmental factors based on the geographical locations. The change in the body weight did not show marked variation during the calendar months. The testes showed two peaks, one in October-November and second in the February-March, and the overall weight of testis was higher during second peak. It may also be suggested that out of two peaks, the second peak could be more efficient in terms of sperm production, as the testis weight was higher during second peak. To best of our knowledge, no study has been conducted in any bat species in relation to sperm count and physiological difference between two peaks, therefore, further study would be required to strengthen the this hypothesis.

It is well known that male hormone; testosterone stimulates and maintains the spermatogenesis in mammals including bats **(Berensztein et al., 2006; Oliveira et al., 2009; Gustafsonand Shemesh, 1976)**. The two peaks of spermatogenesis further coincide with increase circulating testosterone. The circulating testosterone also showed two peaks with a decline in December-January, which suggests that testosterone might regulate the testicular activity in *Rousettus leschenaulti* and suppressed spermatogenesis could be due to low testosterone during winter i.e. December-January. Androgens require a receptor, called androgen receptor to elicit the biological function in the testis **(Wang et al ., 2009; Davey et al ., 2016)**. Our results also showed the expression of AR was elevated in November, and after a decline in January, again the expression of AR increased in February-March, these finding further supports the androgen mediated regulation of spermatogenesis during two peaks and a transient suppression could be due to low testosterone levels. The

serum testosterone concentration was high during the breeding period, during February-March and October-November, whereas the rapid decrease takes place in the months of May, June and August in in *Rousettus leschenaulti* (**Masram, 2015**); however, other male hormone like androstenedione and steroidogenic makers has not been investigated in this species. The androstenedione levels showed highest levels in October and there was decline in December-January and followed by an increase in the level in February. These results also suggest that during the two peak of spermatogenesis, the testicular steroidogenesis has also been elevated. The increase androstenedione could also be converting to testosterone by 17βHSD for spermatogenesis. The testicular testosterone is synthesized manly by the Leydig cells and it is under control of LH (**Zirkin and Papadopoulos, 2018; Abiaezute et al., 2020**). The western blot analysis of LHR also showed an elevation in October, February and March, which may be responsible for increase testosterone during both peaks, although a surprisingly increase in December has been noticed despite low level of testosterone and androstenedione. However, our present study have not measured the circulating LH levels so we cannot certain that it was due to low LH level although it is believed that the unexpected high-rise expression of LHR despite low testosterone and androstenedione could be due to negative feedback. The immunohistochemical study of 3βHSD showed intense staining in the Leydig cells in the month of November, February and March. It has been shown that 3βHSD is an important enzyme in steroid biosynthetic pathway in the testis (**Keeney et al., 1992; Okuyama et al., 2014**). These results also suggest an elevated steroidogenesis during the two peaks of spermatogenesis.

The spermatogenesis in mammals, including bats, is not only regulated by testosterone rather estrogen has also been shown to regulate the testicular activity and spermatogenesis (**Bouma and Nagler, 2001; D'Souza et al., 2005; Oliveira et al., 2009**). The circulating estrogens in the present study also showed a marked change during the calendar months, with a coincidence of its increased levels during the two peaks of spermatogenesis. The very striking observation was observed that in the month of December, the levels of circulating estrogens were very low. To further find out the possible action of estrogens, we have measured the expression of two

estrogens receptor, ERα and β in the testis by western blot analysis. Our results showed that two receptor changes during the testicular activity and the expression also coincide with two peaks of spermatogenesis, with increase expression of both receptors during the second peaks. On the very interesting note, the expression of ERα was very low in the December, which further coincides with low circulating estrogens. These finding suggests that transient suppression of spermatogenesis in the December could be due to low estrogen and its signalling, however, during two peaks of spermatogenesis both androgen and estrogens could be important regulators in the testis of *R. leschenaulti*. It has been shown that in the testis expression of ERα and β are variable and its expression depends on the species, however, estrogen regulates the testicular activity **(Carreauand Hess, 2010)**. It has also been documented that ERβ has a ubiquitous expression in male reproductive organs, whereas ERα has greater specificity, and has been shown to express ERα in every species **(Hess et al., 2001; Hess 2002).** Since the proliferation is an important event in the spermatogenesis, therefore, we also investigated the expression of proliferating marker, PCNA in the testis of *R. Leschenaulti* by immunohistochemistry. The immunolocalization of PCNA revealed that testis undergo proliferation during the two peaks of spermatogenesis and during December-January the testicular proliferation slow down as it was evident by the PCNA immunostaining. Furthermore, a transient suppression was coincides with low proliferation and less expression of AR and ERα. Thus it may be hypothesised that the two peaks of spermatogenesis in *R. Leschenaulti*might be regulated by the androgen and estrogens, and suppressed activity of testis could be due to impair signalling of estrogens by ERα. Estrogen signalling has been shown to regulate the testicular function like proliferation (**Chimento et al., 2020; Akingbemi, 2005**). The overall a halt in spermatogenesis can further be hypothesized that during the December-January, the environmental temperature is low and the mature female in the roost are in stages of pregnancy therefore during this time additional mating may not be required. As it was reported by **Gopalakrishna and Choudhari (1975)** that during December also some non-parous female mate, thus it may be suggested some spermatozoa may also be stored in the cauda epididymis for additional fertilization despite low spermatogenesis in the testis. Thus, changes in the quality and quantity

of spermatozoa in the epididymis also important which could be correlated with the testicular activity. Therefore, further study would be required to decipher the exact mechanisms of two peaks of spermatogenesis with a transient halt in the winter by considering the hypothalamus, pituitary and testicular axis along with quality and quantity of spermatozoa in the epididymis.

7. SUMMARY

- The present study for the first time showed seasonal changes in three circulating steroid hormones, androstenedione, testosterone and estradiol in *Rousettus leschenaulti.*

- The histological analysis of testis also showed two peaks of spermatogenesis with a transient suppression during December-January, as reported earlier.

- The first peak during October-November and second peak during February-March showed intense and more PCNA positive germ cells in the seminiferous tubules compared to the December-January, which suggests that testicular proliferation also suppressed during December-January.

- The transient suppression of spermatogenesis during December-January coincides with decreased levels of circulating androstenedione and testosterone along with reduced expression of androgen receptors.

- The elevated expression of LH-R also coincides with the increased testosterone and androstenedione, which suggests that two peaks of spermatogenesis may be regulated by LH-R mediated androgen biosynthesis.

- An increase expression of LH-R in December despite low testosterone and androstenedione remains unclear. The possible explanation of increase expression of LH-R in December, which is a period of testicular suppression, could be due to low circulating LH levels. Although present study has not measured the circulating LH levels, so further study is required to explain the increase LH-R in December. This is also a limitation of present study.

- The histological analysis also showed an initiation of mild testicular regression in November and the suppression of spermatogenesis in December-January could be an outcome of testicular regression stared in November.

- The unusual very low levels of circulating estrogen and decreased ERα could also be responsible for suppressed testicular proliferation and spermatogenesis, as estrogen has been shown to play important role in testicular proliferation.

- Furthermore, the possible driven force for a transient suppression of testicular activity in December-January may be related to two important factors, first decreased environmental temperature during December-January, second, the mature female in the roost are in stages of pregnancy and this time additional mating may not be required. Although it cannot be ruled out that some spermatozoa may also be stored in the cauda epididymis, if some females may requires the sperm for fertilization. Thus, changes in the quality and quantity of spermatozoa in the epididymis also important.

APPENDICES

PARTICULARS OF THE CANDIDATE

NAME OF CANDIDATE: VANLALREMPUIA

DEGREE:MASTER OF PHILOSOPHY

DEPARTMENT:ZOOLOGY

TITLE OF DISSERTATION: Changes in the testicular activity in males of a Pterepodidae bat, *Rousettus leschenaulti*

DATE OF ADMISSION:08.08.2019

COMMENCEMENT OF SECOND SEM/DISSERTATION:01.02.2020

APPROVAL OF RESEARCH PROPOSAL

1. **DRC:**02.06.2020
2. **BOS:**02.06.2020
3. **SCHOOL BOARD:**12.06.2020

MZU REGISTRATION NO.:1396/2014

M.Phil. REGISTRATION NO. & DATE:MZU/M.Phil./592 of 12.06.2020

DATE OF SUBMISSION:

Head

Department of zoology

REFERENCES

1. Abiaezute, C.N., Oguejiofor, C.F., Nwaogu, I.C., Obidike, I.R. and Igwebuike, U.M. (2020). Seasonal changes in sperm parameters, testicular histology and circulating levels of reproductive hormones in the male African straw-colored fruit bat (*Eidolon helvum*). *Asian Pacific Journal of Reproduction, 9(4), p.197*.
2. Akingbemi, B.T. (2005). Estrogen regulation of testicular function. *Reproductive Biology and Endocrinology, 3(1), p.51.*
3. Altringham, J.D., McOwat, T. and Hammond, L. (1996). Bats: Biology and Behaviour: Oxford University Press. *New York*, *262*.
4. Beguelini, M.R., Góes, R.M., Taboga, S.R., Morielle-Versute, E. (2014). Two Periods of Total Testicular Regression Are Peculiar Events of the Annual Reproductive Cycle of the Black Myotis Bat, *Myotis nigricans* (Chiroptera: Vespertilionidae). *ReprodFertil Develop 26: 834–846.*
5. Berensztein, E.B., Baquedano, M.S., Gonzalez, C.R., Saraco, N.I., Rodriguez, J., Ponzio, R., Rivarola, M.A., Belgorosky, A. (2006). Expression of aromatase, estrogen receptor alpha and beta, androgen receptor, and cytochrome P-450scc in the human early prepubertal testis. *Pediatr. Res. (6) 740–744.*
6. Bouma, J. and Nagler, J.J. (2001). Estrogen receptor-alpha protein localization in the testis of the rainbow trout (Oncorhynchusmykiss) during different stages of the reproductive cycle. *Biol. Reprod. (65), 60–65.*
7. Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.(72) 248–254.*
8. Bronson, F.H. (1985). Mammalian reproduction: an ecological perspective. *Biology of reproduction*, *32(1), pp.1-26.*
9. Bronson, F.H. (1994). Seasonal regulation of reproduction in mammals. *The physiology of reproduction*.
10. Carreau, S. and Hess, R.A. (2010). Oestrogens and spermatogenesis. *Philosophical Transactions of the Royal Society B: Biological Sciences, 365(1546), pp.1517-1535.*

11. Chimento, A., De Luca, A., Nocito, M.C., Avena, P., La Padula, D., Zavaglia, L. and Pezzi, V. (2020). Role of GPER-Mediated Signaling in Testicular Functions and Tumorigenesis. *Cells, 9(9), p.2115.*
12. D'Souza, R., Gill-Sharma, M.K., Pathak, S., Kedia, N., Kumar, R. and Balasinor, N. (2005). Effect of high intratesticular estrogen on the seminiferous epithelium in adult male rats. Mol. *Cell Endocrinol. 241, 41–48.*
13. Davey, R.A. and Grossmann, M. (2016). Androgen receptor structure, function and biology: from bench to bedside. *The Clinical Biochemist Reviews, 37(1), p.3.*
14. Duarte, A.P.G. and Talamoni, S.A. (2010). Reproduction of the large fruit-eating bat Artibeus lituratus (Chiroptera: *Phyllostomidae*) in a Brazilian Atlantic forest area. *Mammalian Biology, 75(4), pp.320-325.*
15. Fleming, T.H., Hooper, E.T. and Wilson, D.E. (1972). Three Central American bat communitis: structure, reproductive cycles, and movement patterns. *Ecology, 53(4), pp.555-569.*
16. Gopalakrishna, A. (1977). Breeding habits and associated phenomena in some Indian bats. Part I *Rousettus leschenaulti* (Desmarest) meguchirotera.
17. Grindal, S.D., Collard, T.S., Brigham, R.M. and Barclay, R.M. (1992). The influence of precipitation on reproduction by Myotis bats in British Columbia. *American Midland Naturalist, pp.339-344.*
18. Gustafson, A.W. and Shemesh, M. (1976). Changes in plasma testosterone levels during the annual reproductive cycle of the hibernating bat, *Myotis lucifugus*with a survey of plasma testosterone levels in adult male vertebrates. *Biology of Reproduction, 15(1), pp.9-24.*
19. Hess, R.A. (2003). Estrogen in the adult male reproductive tract: a review. *Reproductive Biology and Endocrinology*, *1(1), p.52.*
20. Jerrett, D.P. (1979). Female reproductive patterns in non-hibernating bats. *Reproduction*.
21. Kayanja, F.I.B. and Mutere, F.A. (1978). The fine structure of the testis of the insectivorous bat, Otomops martiensseni. *In Proc. Fourth Znt. Bat Res. Conf (Vol. 245).*

22. Keeney, D.S. and Mason, J.I. (1992). Regulation of expression of 3β-hydroxysteroid dehydrogenase is mediated by cAMP in rat Leydig cells and H540 rat Leydig tumor cells. *The Journal of Steroid Biochemistry and Molecular Biology, 43(8), pp.915-922.*
23. Krutzsch, P.H. (1979). Male reproductive patterns in non-hibernating bats. *Reproduction, 56(1), pp.333-344*.
24. Krutzsch, P.H. (2000). Anatomy, physiology and cyclicity of the male reproductive tract. In *Reproductive biology of bats, pp. 91-155.*
25. Lewis, S.E. (1993). Effect of climatic variation on reproduction by pallid bats *(Antrozouspallidus). Canadian Journal of Zoology, 71(7), pp.1429-1433.*
26. McCracken, G.F. and Wilkinson, G.S. (2000). Bat mating systems. In *Reproductive biology of bats, pp. 321-362.*
27. McGuckin, M.A. and Blackshaw, A.W. (1987). Seasonal changes in spermatogenesis (including germ cell degeneration) and plasma testosterone concentration in the grey-headed fruit bat, Pteropus poliocephalus. *Australian Journal of Biological Sciences*.
28. McGuckin, M.A. and Blackshaw, A.W. (1991). Seasonal changes in testicular size, plasma testosterone concentration and body weight in captive flying foxes (*Pteropus poliocephalus* and *P. scapulatus*). *Reproduction, 92(2), pp.339-346.*
29. Mokkapati, S. and Dominic, C.J. (1976). Sites of production of fructose and citric acid in the accessory reproductive glands of three species of male chiropterans. *Biology of reproduction, 14(5), pp.627-629*.
30. Nelson, J.E. (1963). The biology of the flying fox (genus Pteropus) in south-eastern Queensland.
31. Nerkar, A.A., Gujar, M.P. and Gadegone, M.M. (2017). Ultrastructure of the Secretory Epithelial Cells of the Bulbo-Urethral Glands (Cowper's gland) in the Indian Fruit Bat, Rousettus leschenaulti (Desmarest) During the Reproductive Cycle. *International Journal of Morphology.*

32. Noden, D.M. and De Lahunta, A. (1985). Derivatives of the intermediate mesoderm: reproductive organs. In *The Embryology of Domestic Animals, pp. 322-342*.
33. Okuyama, M.W., Shimozuru, M., Yanagawa, Y. and Tsubota, T. (2014). Changes in the immunolocalization of steroidogenic enzymes and the androgen receptor in raccoon (Procyonlotor) testes in association with the seasons and spermatogenesis. *Journal of Reproduction and Development, pp.2013-122.*
34. Oliveira, R.L., Oliveira, A.G., Mahecha, G.A., Nogueira, J.C. and Oliveira, C.A. (2009). Distribution of estrogen receptors (ERα and ERβ) and androgen receptor in the testis of big fruit-eating bat *Artibeus lituratus* is cell-and stage-specific and increases during gonadal regression. *General and comparative endocrinology, 161(2), pp.283-292.*
35. Racey, P. A., and Entwistle, A. C. (2000). Life-history and reproductive strategies of bats. In *'Reproductive Biology of Bats', pp. 363–414.*
36. Rajalakshmi, M. and Prasad, M.R.N. (1970). Sites of formation of fructose, citric acid and sialic acid in the accessory glands of the giant fruit bat, Pteropus giganteusgiganteus (Brünnich). *Journal of Endocrinology, 46(4), pp.413-NP*.
37. Sapkal, V.M. (1986). Biochemical studies on the male accessory structures of *Rousettus leschenaulti*. *Myotis, 24, pp.79-86.*
38. Singh, K. and Krishna, A. (1996). Seasonal changes in circulating serum concentration and in vitro testicular secretion of testosterone and androstenedione in the male vespertilionid bat, *Scotophilus heathi*. *Journal of Experimental Zoology, 276(1).*
39. Taddei, V.A. (1976). The reproduction of some Phyllostomidae (Chiroptera) from the northwestern region of the state of São Paulo. *Boletim de Zoologia da Universidade de São Paulo, 1, pp.313-330.*
40. Tamsitt, J.R. and Valdivieso, D. (1963). Reproductive cycle of the big fruit-eating bat, Artibeus lituratus Olfers. *Nature, 198(4875), pp.104-104.*

41. Tilmann, C. and Capel, B. (1999). Mesonephric cell migration induces testis cord formation and Sertoli cell differentiation in the mammalian gonad. *Development, 126(13), pp.2883-2890.*
42. Todini, L., Malfatti, A., Terzano, G.M., Borghese, A., Pizzillo, M. and Debenedetti, A. (2007). Seasonality of plasma testosterone in males of four Mediterranean goat breeds and in three different climatic conditions. *Theriogenology, 67(3), pp.627-631.*
43. Walker, W.H. and Cheng, J. (2005). FSH and testosterone signaling in Sertoli cells. *Reproduction, 130(1), pp.15-28.*
44. Wilson DE and Findley J.S. (1970). Reproductive cycle of a Neotropical insectivorous bat, *Myotis nigricans*. *Nature 225(5238).*
45. Wilson, D.E. and Findley, J.S. (1971). Spermatogenesis in some Neotropical species of Myotis. *Journal of Mammalogy, 52(2), pp.420-426.*
46. Wilson, D.E. and LaVal, R.K. (1974). Myotis nigricans. *Mammalian species, (39), pp.1-3.*
47. Zirkin, B.R. and Papadopoulos, V. (2018). Leydig cells: formation, function, and regulation. *Biology of reproduction, 99(1), pp.101-111.*

BRIEF BIO-DATA OF CANDIDATE

Name: Vanlalrempuia

Father's name: Vanlalnghaka

Date of birth: 2nd May 1996

Marital Status: Single

Nationality: Indian

Religion: Christianity

Contact: +919612608514

Email ID: victorlalrempuia@gmail.com

Educational qualification

Name of Exam	Year	Board	Subject	Percentage	Division
HSLC	**2012**	**MBSE**	**General**	**76.2 %**	**Distinction**
HSSLC	**2014**	**MBSE**	**Science**	**62.2 %**	**First**
B.Sc	**2017**	**MZU**	**Zoology**	**72.3 %**	**First**
M.Sc	**2019**	**MZU**	**Zoology**	**63.75 %**	**First**
M.Phil (Course Work)	**2020**	**MZU**	**Zoology**	**75 %**	**First**

Permanent Address: H.No. C-55, Venghlun, Darlawn, Aizawl, Mizoram -796111.

Present Address: SZ-B-6B, Zemabawk North, Aizawl, Mizoram-796017.

VANLALREMPUIA

CERTIFICATE ON PLAGIARISM CHECK

Name of Research Scholar/Student	Vanlalrempuia	
M.Phil. Registration Number	MZU/M.Phil./592 of 12.06.2020	
Title of M.Phil. dissertation	Changes in the testicular activity in males of a Pterepodidae bat, *Rousettus leschenaulti*	
Name & Institutional Address of the Supervisor	Dr. Vikas Kumar Roy Department of Zoology Mizoram University Aizawl-796004, India	
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- Presented paper entitled **"Effect of Metforminon uterine histopathological changes in D-galactose treated mice"** in the three days International Webinar organized by the Department of Biotechnology, Pachhunga University College (PUC), and Mizoram University (MZU) from 24th-26th June 2020.

- Presented paper entitled, **"Seasonal Changes in the Testicular Histology and Histomorphometry of Indian Fruit Bat *Rousettus leschenaultia*"** in the 2nd Annual Convention of North East (India) Academy of Science and Technology (NEAST) & International Seminar on Recent Advances in Science and Technology (IRSRAST) during 16th–18th November 2020 (Virtual) organized by NEAST, Mizoram University, Aizawl-796004, Mizoram (India) .

INTERNATIONAL WEBINAR ON EMERGING TRENDS IN BIOTECHNOLOGY

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This is to certify that Ms./Mr./Dr./Prof. VANLALREMPUIA has presented a paper entitled ***"EFFECT OF METFORMIN ON UTERINE HISTOPATHOLOGICAL CHANGES IN D-GALACTOSE TREATED MICE"*** in the three days International Webinar organized by the Department of Biotechnology, Pachhunga University College (PUC), and Mizoram University (MZU) from 24th-26th June 2020.

Dr. Tawnenga	**Prof. S.K.Mehta**	**Dr. Prashant K. Singh**	**Dr. Mukesh K. Yadav**	**Dr. Liansangmawii Chhakchhuak**
Principal PUC	*Organising Secretary*	*Co-Ordinator*	*Co-Ordinator*	*Head, Dept. of Biotechnology, PUC*

2nd Annual Convention of North East (India) Academy of Science and Technology (NEAST)
&
International Seminar on Recent Advances in Science and Technology (ISRAST)
(16th -18th November 2020)
(Virtual)

11215150020

Certificate

This is to certify that

Mr./Ms./Dr./Prof. Vanlalrempuia
Dept. of Zoology, Mizoram Univ., Aizawl, Mizoram

has attended and presented an Oral Presentation entitled, "Seasonal Changes in the Testicular Histology and Histomorphopetry of Indian Fruit Bat Rousettus leschenaultia" in the 2nd Annual Convention of North East (India) Academy of Science and Technology (NEAST) & International Seminar on Recent Advances in Science and Technology (IRSRAST) during 16th-18th November 2020 (Virtual) organized by NEAST, Mizoram University, Aizawl-796004, Mizoram (India).

Prof. Diwakar Tiwari	**Prof. R. Lalthantluanga**
General Secretary, NEAST	President, NEAST

North East (India) Academy of Science & Technology (NEAST), Mizoram University, Aizawl-796004 (India)
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