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Spikes of The Traditional Female Hormones (Estrogen and Progesterone) during Breeding Seasons in Adult Male African Fruit Bat (*Epomops franqueti)*

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ABSTRACT

The spikes in some traditional female hormones (estrogen and progesterone) during breeding seasons in adult male African fruit bat (Epomops franqueti) were investigated. A total of 60 adult male bats, five per month, weighing between 96.45 to 98.87g were used. The bats were captured using mist nets with dimensions measuring 50 feet × 35 feet × 45 feet. After anaesthetizing each bat, the testes were harvested and weighed, and prepared for gross and histological studies. Blood samples were collected from each bat into plain sample bottles for assays of estrogen and progesterone. Hormonal analysis was performed using the ELISA technique. The results of the study showed higher testicular investment in the wet season than dry season. Germinal epithelium was significantly (P<0.05) thicker with higher number of sperm cells in the bats during the rainy season as compared to those during the dry period of the year. Overall, estrogen and progesterone peaked during the wet season, while the lowest values were recorded during the dry periods of the year. The spikes in estrogen and progesterone, traditionally considered female hormones during the breeding season corresponded to the pattern of peak in testosterone in the African fruit bat. Conclusively, estrogen and progesterone may be vital as testosterone in breeding seasonality of Epomops franqueti.

Keywords: *African fruit bat, Epomops franqueti, estrogen, germinal epithelium, hormone, progesterone,*

1.0 INTRODUCTION

Bats undergo seasonal breeding which is regulated by sex hormones. The major sex hormones secreted by stimulation of the gonadotropins are: estrogen and testosterone (Fochi *et al*., 2013). Testosterone is the primary male hormone while estrogen is regarded as the primary female hormone (Hamilton *et al*., 2017, Clark *et*

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al., 2018). This male primary hormone has been studied during the reproductive activities in certain male bat, *Rhinolophus capensis* (Bernard, 1986a). The outcome of a study showed a patterned-based hormonal profile relating to surge in the male primary hormones (Bernard, 1986b). Also, recently, it was reported that at specific period of the year, during the wet season, reproductive activities in the bat (*Epomops franqueti)* (Tomes, 1860), is mostly high when serum testosterone reached its peak production (Ekeolu *et al*., 2022) so that the surge and reduction in the hormone also influence the size, and cellular structures of the bat's testis (Bueno *et al*., 2023) but in *Artibeus jamaicensis* and *Artibeus lituratus*, continuous release of the hormone barely has a morphological relationship with their reproductive status (Duarte and Talamoni, 2009). However, testosterone being the main hormone that induces the male secondary sexual characteristics has been reported not to be sole determinant of the adult male reproductive activities (Hess, 2003). Estrogen and progesterone have been said to play major roles in male secondary sexual development and reproductive activity (Hess, 2003, Oettel and Mukhopadhyay, 2004).

The source of estrogen in the male reproductive tract may not be directly from certain glands but when testosterone is produced, it is conducted to the epididymis through epididymal fluid in the lumen. It is then transformed to dihydrotestosterone by the 5α-reductase enzyme that are mostly located in the cells of the epithelium, and then to estrogen by aromatase. The activation and deactivation of testosterone receptor and estrogen receptor α in the epithelial cells control their morpho-physiology, prevents apoptosis, regulating their protein expression and secretion, so that sperm maturation and storage is ensured (Beguelini *et al*., 2015). Research also has shown estrogen to be a vital hormone for prostate homeostasis that regulates the glands morpho-functional activities through the receptors ERα and ERβ (Prins and Korach, 2008). The effect of estrange on certain species of fruit bats, *Dyacopterus spadecius*, in Malaysia and *Pteropus capistrastus* in New Guinea, has resulted in lactation and high level of estrogen helped to inhibit the production of milk by these male bats with evidence of seasonal cyclicity in mammary gland development as seen in the female (Francis *et al*., 1994). Estrogen in the male reproductive tract of mammalian species plays an important role in male fertility (Cooke *et al*., 1991). Beguelini *et al* (2014) also observed that estrogen in the male reproductive tracts of *Myotis nigricans* was a factor that regulated the reproductive activities in the microbat, synchronizing with the pattern of surge and fall in the testosterone production. The common vampire bat, *Desmodus rotundus* also displayed seasonal breeding pattern which the secretion of estrogen by the reproductive tract regulated (Silva *et al*., 2020).

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Estrogen can also be derived from pregnenolone (Aron *et al.*, 2004). Progesterone is an important sex hormone that is synthesized from pregnenolone. It is considered a primary hormone of the female animal that is periodically produced during the reproductive cycle, regulating the normal morpho-physiological activities of the female reproductive biology (Lydon *et al*., 1995). Against the backdrop that progesterone is also known to be a female hormone, the importance in male animal has always been reduced (Frye *et al*., 2021). Therefore, serum concentrations of progesterone in male animals, even though relatively lower than what is obtained in females, is said to be significant (Fochi *et al*., 2013). The Ley dig's cells of the testes help in the synthesis of progesterone through the activities of 3β hydroxysteroid dehydrogenase. In addition, hormone and expression of its receptors in the male reproductive tracts has been reported (Fochi *et al*., 2013). For example, its presence has been established in the male accessory gland. The expression of its receptors increases with estrogen even though the interaction of progesterone and prostate gland is poorly comprehended. It is however observed to play a physiological role in the gland Fochi *et al* (2013). Therefore, progesterone not being restricted to the female gender has been documented to also play significant role in spermiogenesis, sperm cell acrosomal reaction and testosterone biosynthesis (Oettel and Mukhopadhyay, 2004). The morphophysiological effect of estrogen on the germinal epithelium helps spermatogenesis and spermiation processes (D'Souza *et al*., 2005).

Although, the pattern of distribution of alpha and beta estrogen receptors in the testis of the large fruit eating bat, *Artibeus lituratus, a tropical fruit eating bat has been said to be seasonal, and cell-and stage-specific manner (*Oliveira *et al*., 2009) and there are several reports on the morphophysiological role of estrogen on the reproductive biology of mammalian male gender (Danzo, 1986, Nitta *et al*., 1993, Hess *et al*., 1995). The literatures available on estrogen as an important hormone in the regulation of the reproductive activities in the male microchiropteran and the temperate bats are few, and scarce in African fruit bats while reports on progesterone in the male megachiropteran is almost lacking for both the bats of the temperate and the tropical regions, hence there is the need to investigate the seasonal estrogen and progesterone profile in the African fruit bat (*Epomops franqueti).*

2.0 MATERIALS AND METHOD

2.1 Experimental Animals: A total of 60 bats were trapped and used for this study. The bats were caught every month of the year at Faculty of Arts, University of Ibadan, using mist net, the method of Ekeolu and Ozegbe (2012). The mesh dimension of the mist net was 1´X 1´. The mist net was suspended to form a wall

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50ft high and bed of 35ft wide, and 45ft wide. The wall and bed were made between two trees respectively. The walls made by the mist net between the two trees were relatively stretched out compare to the sagging bed. The trapped bats in the mist net were immediately removed while wearing a tough leather glove. They were put into a specially designed metal cage to avoid any form of injury to the animal, and then transported from the field to the laboratory for acclimatization. The animals were fed Almond fruits and drank potable water. The bodyweight of the bats was taken using Microvar® weighing balance (Microvar, U.K). They were anaesthetized using ketamine HCl (Biotechnica Pharma Global (BPG), China) at 25mg/kg body weight intramuscularly on the medial side of the thigh muscle.

2.2 Histology: The live weight of each bat was measured using electric weighing balance (Microvar, U.K). The bats were anaesthetized with ketamine HCl at 25mg/kg body weight, through intramuscular administration on the medial aspect of the thigh. The testes were harvested and weighed. The testes were then fixed in Bouin's fluid, and prepared for paraffin sections of 5mm. The sections were stained with hematoxylin and eosin (H & E) on histology slides, covered with cover slips using DPX. The stained tissues were viewed and histomorphometry of the germinal epithelium was carried out under light microscope (TSview software). Also, the vesicular glands were dissected, weighed, fixed with 4% neutral buffered formalin and prepared for paraffin section, stained with H&E then observed under light microscope. Gonadosomatic index (GSI) was calculated using the formula below:

$$GSI = \frac{Weight\ of\ testis\ (g)}{Weight\ of\ animal} \times 100$$

2.3 Statistical analysis: Data were expressed as mean ± standard error of the mean (mean ± SEM). Data were then subjected to t-tests, followed by Duncan's post-hoc test, to compare the means. Values of $P < 0.05$ were considered significant (Jones, 1994). Graph pad 4.0 for windows (San Diego, California, USA) software was used for the analysis.

2.4 Blood sample collection and hormonal Assay: Blood sample was collected in universal bottle and allowed to coagulate at 25ºC, room temperature for one hour. It was then centrifuged for 15minutes at a speed of 5,000 r/m revolutions and the serum stored in Eppendorf tube at a temperature of 4ºC. The serum samples in Eppendorf tube were transported to the laboratory in icepacks for hormonal assay. The serum hormonal profile for estrogen (E) and progesterone (P), were

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assayed for each of the five animals for each month of the year using specific Kits and well labeled ELISA microplate immunoassay and microplate reader for the two hormones (estrogen and progesterone). Commercial ELISA kits were used to quantify the reproductive serum hormones abiding strictly to the manufacturers guide and procedures (Ekeolu *et al*., 2022). The principle and procedure for assaying the sex hormones was similar for the two reproductive hormones using estrogen coagulate for estrogen and progesterone coagulate for progesterone.

To assay estrogen, twenty-five (25µl) microliters of each of the calibrators, control and serum samples of each of the five male bats for each month were aliquoted into the microliter wells in duplicate to minimize error. Hundred (100 µl) microliters of the conjugate were added into the wells, slowly shaking for 30seconds and then incubated for one hour (1hr) at a temperature of 37°C. The incubated mixture in the microplate wells was transferred into another jar using transferring pipette and the wells blot-dried with absorbent paper. The microplate wells were then washed with wash solution several times, following the kit manufacturer's guide. For colour reaction, hundred (100 µl) microliters of (Tetra methyl benzidine substrate) TMB-substrate were added into the wells. It was left in a dark cupboard at room temperature of 37°C to incubate. The reaction was terminated by adding hundred and fifty (150 µl) microliters of stopping reagent into the wells. The degree of the reactivity was quantified with a spectrophotometer (Robonik 11-2000, England) by plotting on Y and X axes for the two hormones. The serum concentration of the hormones in each sample was estimated by locating the point of intersection of the average absorbance of the sample duplicates on the vertical axis of the graph to its complementary concentration on the horizontal axis of the graph. The test validity criteria for each of the assay were met in accordance with the test kit manufacturer's instructions.

2.5 Ethical Approval: The protocol for this research was approved by the Research and Ethics Committee of the Faculty of Veterinary Medicine, University of Ibadan, Nigeria (UI-ACUREC/19/0001).

3.0 RESULTS AND DISCUSSION

The average body weight of the fruit bat used for this investigation when measured was 97.66 ± 0.72g. The average body length was 8.35 ± 0.31cm. There was no significant difference between the body weight obtained in the wet and dry seasons ($P< 0.05$). There was significant difference in the average weights obtained for the

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testis during the wet and dry seasons at P< 0.05. The mean weight of the testis was 0.161±0.02

The mean GSI for the wet and dry seasons were 0.18% and 0.16% respectively. The maximum value for GSI obtained during seasons of the year was 0.18% with a mean testicular weight of 0.176g while for dry season, the GSI value was 0.16 with annual mean testicular weight of 0.160g. The germinal epithelium at 187.50±12.82µm during the wet season was thicker than the germinal epithelium at 58.60± 15.13µm with a wider luminal diameter than that of the wet season. There were numerous spermatocytes in the germinal epithelium of the seminiferous tubules during the wet season. The luminal surface of the germinal epithelium had weak and dead sperm cells during the dry seasons. During the wet season, round spermatids migrate along the cytoplasm of the Sertoli cells in the ad luminal area of the seminiferous tubule. There were copious number of spermatozoa as well as secretions at some regions, within the lumen of the epididymis, and the cytoplasm of the principal epithelial cells were obviously foamy in appearance than in the epididymis during the dry season (Figure 1: A, B, C & D, Table 1 & 2).

The reproductive activities in the African fruit bat (*Epomops franqueti)* (Tomes, 1860), were high during the wet seasons. The histomorphometry and histology of the seminiferous tubules and epididymis validates the reports from the previous seasonal study on this species of African fruit bat as there was high investment on the testicular parenchyma during the wet season with a GIS of 0.18% but 0.16% in dry season. These values fall in range of the previous study (Ekeolu *et al*., 2020, Ekeolu *et al*., 2022). The foamy appearance of the cytoplasm of the epididymal epithelium in the African fruit bat during the wet season indicated active secretory activities which aid in sperm cells maturation and capacitation. Flickinger (1983) reported similar secretory function of the epididymal epithelium in mice, which aid the maturation of the sperm cells (Toshimori *et al*., 1990).

The numerous elongated sperm cells along the cytoplasm of the Sertoli cells indicated the influence of estrogen on the more active support mechanism of Sertoli-germ cell proliferation when there was pronounced reproductive activity as previously seen in rat (Macheroni *et al*., 2020), and in bat, *Artibeus lituratus* (Oliveira *et al*., 2012) noticeable during the wet season. Therefore, the favorable micro environments in the testis and epididymis that elicited spermatogenesis, spermiation, and spermatozoa storage and concentration were probably under the control of estrogen since it exhibited seasonal changes which correlate with the seasonal changes in testosterone, FSH, and LH profiles in the African fruit bat (*Epomops franqueti*) (Ekeolu *et al*., 2022). The increase thickness of the germinal epithelium during the wet season suggested proliferation and differentiation of the

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germ cells, with more spermatozoa in the lumen of the seminiferous tubules than it was observed during the dry seasons.

Figure 1 (A and B), during the wet season shows the numerous spermatocytes (**SE**), forming a thick germinal epithelium (**GE**), and a narrow lumen (**L**) of the seminiferous tubules. Inset shows elongated spermatids (**ES**) along the cytoplasm of the Sertoli cell (**SC**). Also, note the foamy appearance of the epididymal epithelium (**Ep**), with vacuole (black arrow) in the cytoplasm of the principal cells (**PC**) and the clumped spermatozoa (**Sp**) in the epididymal lumen. Inset shows secretion (**Sr**) within the epididymal lumen and the connective tissue (**CT**). In C and D, during the dry season, note the thin germinal epithelium (**GE**), and the wide lumen (**L**) of the seminiferous tubules with weak and dying spermatozoa (**Sp**) at the luminal surface were observed. The lumen of **L** of the epididymis is empty while the epithelium of the epididymis appeared less foamy. Note the connective tissue (**CT**) X 400 (H&E).

The mean weights of the vesicular gland during the wet and dry seasons were 0.60±0.07 g and 0.57±0.04 g. The acinar lumen of the vesicular glands was filled with secretory granules during the wet season and the epithelial cells appeared simple low cuboidal with connective tissue traversing the parenchyma of the gland. During the dry season the lumen of the vesicular gland appeared rather empty and the epitheliums were folded up each other. The simple cuboidal epithelium appeared stratified with two to three layers (Figure 2: A & B).

In Figure 2(A), during the wet season, the copious secretions (**Sr**) within the lumen (**L**) of the gland, the epithelium (**Ep**) with low cuboidal cell, and round nucleus (**Nu**) were observed. The connective tissue (**CT**) transverse the parenchyma and form septate (St). Also, the foamy appearance of the epithelium was noted. In Figure 2(B), during the dry season, the folds of epithelial tissue (**Ep**) were observed upon each other, giving a pseudo-stratified layer of the epithelium. The epithelial cell was cuboidal with round nucleus (**Nu**). Also note the apparently empty glandular lumen (**L**), and the connective tissue (**CT**).

The morpho physiology displayed by the vesicular gland was seasonal. The secretory cells were active during the wet season, with cuboidal epithelium while the entire gland is surrounded by connective tissue similar to the reports of Sohn *et al*. (2021). Our finding is also similar to the report of Sarangi *et al*. (2021) where the vesicular gland in the bull was more active in winter than in summer. During the dry season vesicular gland epithelial cells appeared somewhat stratified and inactive with sparse or no secretory granules in the lumen of the acini. This is similar to the seasonal variation observed in the vesicular gland of *Eidolon helvum* (Abiaezute *et al*., 2021)

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The vesicular gland is a major component of the accessory sex gland that produced the seminal fluid required by spermatozoa for survival that eventually will swim through the female genital tract for fertilization of the ovum. Our study showed that the vesicular gland was seasonally active. This followed the pattern in the straw-colored fruit bat vesicular gland (Abiaezute *et al*., 2021). Although it has been shown that estrogen regulate secretory activity of the prostate gland (Albernaz *et al*., 2021), literature on the influence of estrogen on vesicular gland is rare. Although the testosterone influences the growth of the vesicular gland, the spike in the progesterone during the wet season, synchronizing with the profile of the testosterone (Ekeolu *et al*., 2022), suggesting that progesterone may also seasonally modulate the secretory function of the vesicular gland epithelium in *Epomops franqueti* since it influences the biosynthesis of testosterone. Although the production of progesterone in the prostate gland has been mentioned (Fochi *et al*., 2013), however, we are not sure if progesterone has a direct influence on the secretory function of the vesicular gland, that is literatures on the presence of progesterone receptors in the vesicular gland of chiropteran is almost non-existence.

There was a decline in the serum concentration of estrogen level from January to April. The least value was obtained in the month of March. There was a sharp increase between the months of April and June with a slight fall in July followed by a slight increase in August. Then, there was a sharp fall to October. The fall was almost maintained through the months of November to December (Figure 3). The progesterone serum concentration maintained a low level from the month of January during the late dry to the month of May. Between May and July, the level of the serum concentration of progesterone rose. And there was a sharp increase between July and October. Then, there was sharp increase with the peak in September. The decline in the serum concentration of progesterone was maintained from the month of October to December, from early dry season to late dry season (Figure 4).

There was interplay between both the traditional male and female hormones on the male reproductive tracts which helps in the functionality of the structures. With inadequacy or without either of this group of hormones, the reproductive activity in the male induced by interactions of these hormones with the reproductive tract is impaired (Sohn *et al*., 2023). Therefore, the spikes in serum estrogen and progesterone levels during the peak of wet season synchronize with the period bat testicular weight and cellular investment was higher. The period of least production of serum estrogen and progesterone also aligned with low testicular weight and parenchyma investment of the testis during the seasons. This increased reproductive activity indicated the influence of the surge in serum estrogen level in *Epomops*

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franqueti suggesting its essentiality in spermatogenesis and may also serve as pheromones to induce female receptivity during the peak of reproductive activity similar to the observation in *Eptesicus fuscus* (deCatanzaro *et al*., 2014). Also increased serum progesterone level in *Epomops franqueti* is an indication of increased spermiogenesis, and stimulation of testosterone production.

4.0 CONCLUSION

The study investigated the spikes in some traditional female hormones (estrogen and progesterone) during breeding seasons in adult male African fruit bat (*Epomops franqueti)*. A total of 60 bats were trapped and used for this study. The bats were caught every month of the year at Faculty of Arts, University of Ibadan, and using mist net. The live weight of each bat was measured using electric weighing balance. Data were expressed as mean ± standard error of the mean. It suggests that the increased serum level of estrogen and progesterone which are traditionally the female hormones have seasonal influence on the reproductive activity in the African fruit bat (*Epomops franqueti).* Further work using immunohistochemistry to investigate the role of estrogen and progesterone in the seasonal breeding of the African fruit bat (*Epomops franqueti)* is proposed.

Table 1: The weights of African fruit bat, the testis and gonadosomatic index

Seasons	WOA (g)	WOT (g)	GSI (%)	WOV(g)
Wet	98.87 ± 0.32	0.176 ± 0.001^{a}	0.18	0.60 ± 0.07^{a}
Dry	96.45± 0.39	0.160 ± 0.001^{b}	0.16	0.57 ± 0.04^{a}
AS	97.66± 0.72	0.168 ± 0.003	0.17	0.59±0.04

**Means with different superscripts within columns are significantly different (P < 0.05).*
WOA: Weight of Animal, WOT: Weight of Testis (combined),
GSI: Gonadosomatic Index, AS: Annual Season.

Table 2: Histomorphometry of the seminiferous tubule of AFB

Seasons	STD (µm)	SLD (µm)	GH (µm)
Wet	287.70± 7.41	89.83 ± 9.32^{a}	187.50 ± 12.82^{a}
Dry	178.80 ± 6.12	126.10 ± 10.36^{b}	58.60 ± 15.13^{b}
AS	233.25 ± 6.73	107.92±9.84	166.7 ±18.53

**Mean with different superscripts within columns are significantly different (P < 0.001).*
STD: Seminiferous tubular diameter, SLD: Seminiferous tubule luminal diameter, GH: Germinal epithelial height

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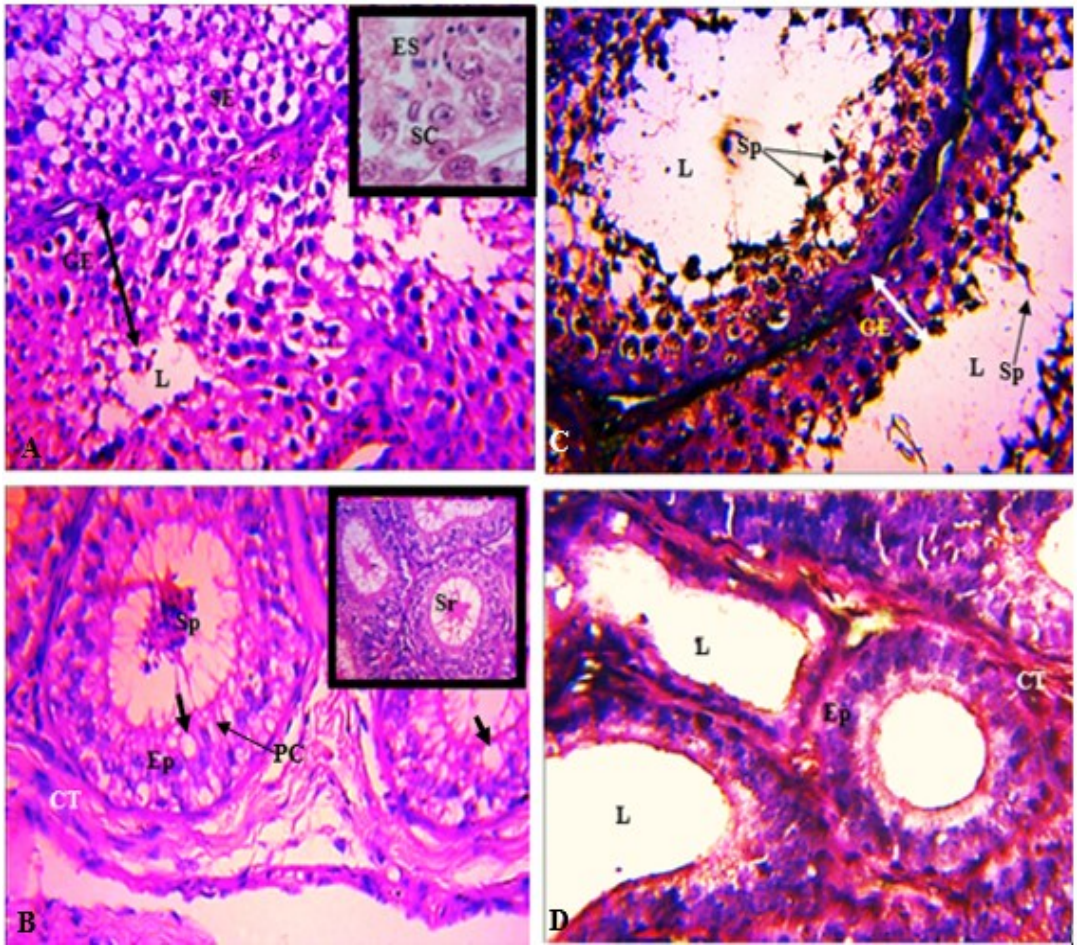

Figure 1: The photo micrograph of the testis and epididymis of Epomops franqueti.

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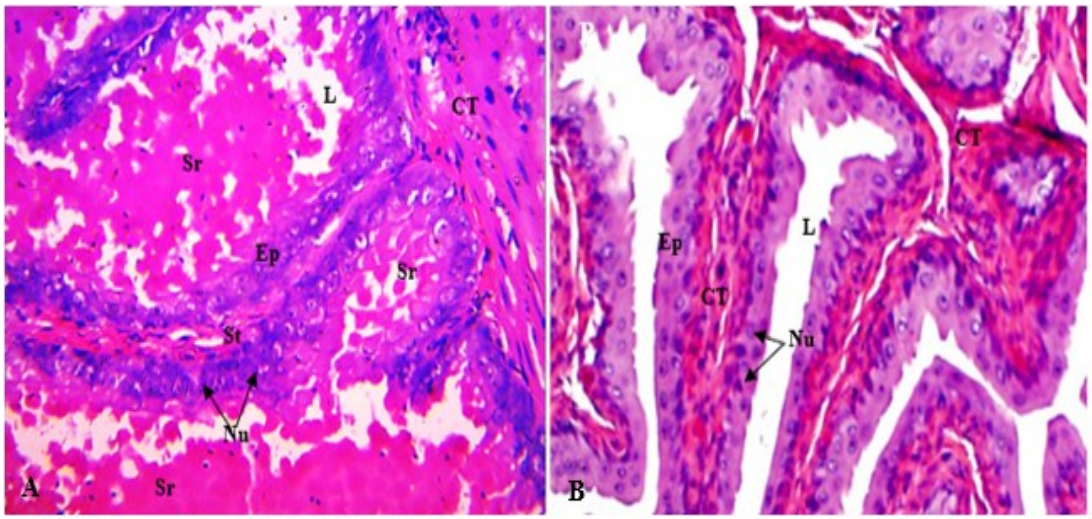

Figure 2: The photo micrograph of the vesicular gland of epididymis of Epomops franqueti.

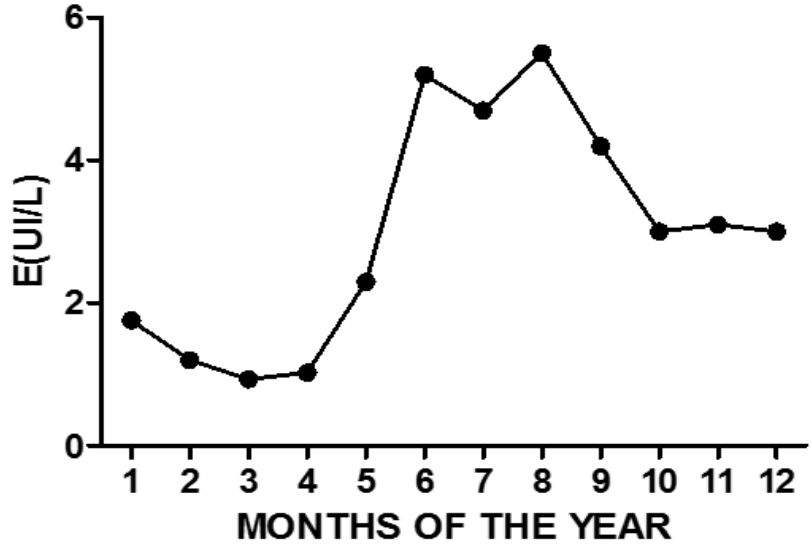

Figure 3: The serum estrogen concentration profile through the wet and dry seasons of the year in adult male Epomops franqueti

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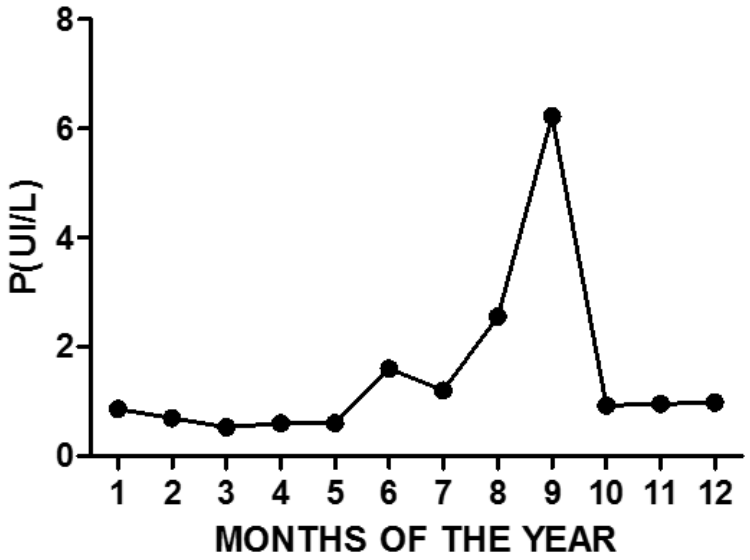

Figure 4: The serum progesterone concentration profile through the wet and dry seasons of the year in adult male Epomops franqueti

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