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Histological, Histochemical and Immunohistochemical Characterization of the Testis of Double-Spurred Francolin (*Francolinus Bicalcaratus)* in A Reproductive Cycle

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ABSTRACT

The Double-spurred Francolin (*Francolinus bicalcaratus*) is a feral bird and remains undomesticated with very little known about its reproductive capability, hence, this study was carried out to know about the structural integrity of its testis. Birds (n=5), were obtained during the dry and rainy seasons from their natural habitat in Kano, stabilized and acclimatized for two weeks in the Experimental Animal House of the Veterinary Anatomy Department, University of Ibadan. The Birds were weighed, sedated and sacrificed by decapitation. The testis was removed and fixed in neutral buffered formalin and then processed histochemically for Reticulin fibres using Gordon and Sweet's stain and Collagen fibres using Verhoff-Van Gieson stain. Paraffin-embedded sections of each fixed testes were cut and stained with Haematoxylin and Eosin, then immunostained for structural proteins with monoclonal antibodies against Vimentin, Desmin, Pancytokeratin and Alpha smooth muscle Actin. Results indicate that the mean morphometric values for testis and epididymis observed were highest at late rainy season and lowest at early dry season for all indexes except for the testicular capsule thickness that was lowest at late rainy season and highest at late dry season. The testicular capsule and peritubular layer immunostained for alpha smooth muscle actin, desmin, pancytokeratin and vimentin and were thickest at late rainy season. The capsule was histochemically positive for collagen fibres mostly restricted to the tunica Albuginea. We also, observed that the peritubular wall was positive for reticulin, thickest at late dry season. We therefore, conclude that capsular and peritubular tissues of the testis are similar to that of Muscuvy duck, quail and domestic fowl. The testicular capsule is rich in collagen and would be actively involved in keeping the testis from total collapse when it regresses at late dry season.

Keywords: Histology, Histochemistry, immunohistochemistry, Testis, Morphometrics *Francolinus bicalcaratus*

INTRODUCTION

The Double-spurred Francolin occurs in dry grasslands, open savanna, palm groves and cultivated areas of West Africa from Senegal east to northern Cameroon and southern Chad[1].

It is a resident breeder in tropical West Africa, but there is a small and declining isolated population in Morocco[2].

Urbanization, new farm settlement, hunting as well as the use of agricultural pesticides in their habitat[3] suggest a possibility of extinction. A threat to extinction thus exist for this prized bird[4].

Works have been carried out on the male reproductive organs of the domestic fowl[5,6]. Aire and Ozegbe[7] in their work on morphometry, histology, ultrastructure and immunohistochemistry of testicular capsule and peritubular tissues of Japanese quail, domestic fowl, turkey and duck, measured the thickness of the testicular capsule in these species as well as immunolocalized some cytoskeletal proteins.

Furthermore, works had been done to suggest a seasonal variation in morphology and physiology of the reproductive tract in the male avian[8]. The organization of the myoid cell layers of the seminiferous tubules also differs among species[9]. Smooth muscle-like peritubular cells have been reported in the domestic fowl[10] and quail[11].

Carvalho et al.[12] reported that the testicular capsule did not form septum that penetrate the interstitium in greater rhea whereas, in rooster, duck and quail septum project from the capsule into the interstitium as reported by[13,14,15].

The double-spurred Francolin has remained elusive to bird lovers and scientist, thereby leading to lack of knowledge on structure and physiology of its' reproductive organ, hence this study was carried out to characterize the structure of the testis of the male bird in a reproductive cycle.

MATERIALS AND METHODS

Twenty adult male double-spurred Francolin, weighing 350-500gm, were obtained in four batches, of 5 birds each and stabilized. The Seasons were given as: Early Rainy Season (ERS): April to June, Late Rainy Season (LRS): July to September, Early Dry Season (EDS): October to December, Late Dry Season (LDS): January to March. Then acclimatized/stabilized under natural day light/dark for two weeks at the Experimental Animal House, Department of Veterinary Anatomy, Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria, before the on-set of sample collection. Ethical Approval for the project was obtained from University of Ibadan Animal care and use research ethics approval-ACUREC with certificate number UI-ACUREC/18/0114.

Five of the stabilized birds each season were used to study the change in the structure of the tissues of the testis.

Trimmed testicular tissues were passed through increasing alcohol concentration of 70 % for 1 hour, 90 % thrice, 1 hour each and 100 % twice, 1hour each for proper dehydration. This was followed by clearing of the dehydrated tissue in xylene twice, 2 hours each and then embedded in paraffin wax at 60º C. The tissue blocks produced were then sectioned at 5µm using a rotary microtome (Leica USA). These sections were then mounted on clean glass slides, floated on warm water bath and later dried in an oven before staining with Haematoxylin and Eosin (H & E). The slides of the testis and epididymis were examined under the light microscope at x100 and x400 magnification and the following measurements were taken: tubular diameter, luminal diameter, epithelial height and testicular capsule thickness, using Motic MC 2000 image capture module (Motic China Group).

Paraffin-embedded sections of testes were immunostained for the structural proteins with monoclonal antibodies against vimentin, desmin, pancytokeratin and alpha

smooth muscle actin at late dry season and late rainy season. Sections 5 µm thick were cut and mounted on slides precoated with polylysine, deparaffinized and rehydrated. Immunostaining of slides for alpha smooth muscle actin, pancytokeratin, desmin and vimentin was performed as recommended by Dako Cytomation (Denmark), the supplier of the LSAB+ Kit (HRP) used in this study. The slides were viewed and photographed and analyzed using image J software on a light microscope

Verhoff-Van Gieson Stain: Cut testis section is covered with coverslip and placed in Baker's solution in a Columbia staining dish (Thomas scientific #8542-CL2) for 10 minutes at room temperature. Wash with 3 exchanges of tap or deionized water. Add Verhoff's staining solution to dish for 20 minutes at room temperature, rinse quickly in tap water to remove most of the Verhoff stain. Add 1% Ferric chloride for up to 5 minutes (section should still be dark). Rinse quickly in tap water to remove excess 1% Ferric chloride. Immediately add Van-Gieson"s stain for 2 minutes, rinse quickly with tap water to remove excess stain. Do not leave in water and immediately transfer to ceramic rack (Thomas scientific#8542—E40). Dehydrate in ascending alcohol solutions (50%, 70%, 80%, 95% X2, 100% X2) in Columbia staining dishes, clear with xylene (3-4X), mount cover slip. The slides were viewed and photographed and analyzed using image J software on a light microscope

Result: nuclei- blue to black, muscle myofibril- tan to brown, connective tissue fibrils- blue-black and collagen- red-purple

Gordon and Sweet's staining protocol for Reticulin: Cut testis section 5 micron is deparaffinized with xylene, then taken through alcohol to water. It is oxidized in acidified potassium permanganate for 3 minutes, rinsed in distilled water and decolorized with 2% oxalic acid for 1 minute, rinsed in distilled water and mordant in 4% iron alum for 10 minutes, rinsed in distilled water and impregnated in ammonical silver solution for 11 seconds. This is followed by rinsing in distilled water and reduced 10% aqueous formalin for 2 minutes. Counter stained with neutral red for 2 minutes, dehydrated, cleared and mounted. The slides were viewed, photographed and analyzed using image J software on a light microscope. Result: reticulin fibres stain black, nuclei stain red. All numerical data obtained were expressed as means ± the standard error of means.The data were subjected to Analysis of Variance (ANOVA)

RESULTS

Microstereolgy of the Testes, Epididymis and Testicular Capsule: The mean value of seminiferous tubule's luminal diameter LD ($P < 0.05$) decreased from 69.49±2.42 at LRS to 54.79±2.06 at EDS, the mean value of seminiferous tubule's epithelia height EH ($P < 0.05$) decreased from 57.64±1.22 at LRS to 7.316±1.75 at EDS and the mean value of seminiferous tubule's tubular diameter TD ($P < 0.05$) decreased from 127.1±2.933 at LRS to 59.65±2.03 at EDS

Epididymal Microstereology: The mean value of the epididymal luminal diameter (LD) ($P < 0.05$) decreased from 44.33±3.37 at LRS to 28.37±1.22 at EDS, the mean value of epithelia height EH ($P < 0.05$) decreased from 15.53±2.00 at LRS to 6.95±0.95 at EDS and the mean value of epididymal tubular diameter TD ($P < 0.05$) increased from 35.32±1.17 at EDS to 59.86±4.93 at LRS

Measurement of the Testicular Capsule: The mean value of the testicular capsule ($P < 0.05$) decreased from 82.55±3.73 at LDS to 16.91±1.31 at LRS

Reticulin- The reticulin fibres form mesh-work that bind the tubular basement membranes to the interstitium. It was found to be thick and loose at LDS but thin and more organized at the LRS. In the interstitium, the Leydigs cells were observed to be made up of spherical to ovoid-shaped nucleus with very little cytoplasm. The nuclei were prominent at

LRS with dense cytoplasm but become shattered with pale cytoplasm at LDS. The interstitium was penetrated by more extracellular matrix of fibroblast-like cells at LDS compared to LRS.

Verhoff-Van Gieson (VVG) Stain: Collagen was demonstrated in the outer testicular capsule wall at varying amount throughout the seasons using Verhoff-Van Gieson Stain. The LDS recorded the thickest collagen fibres and this is most likely serving to prevent the testis from collapsing as it regresses at LDS.
The collagen fibres were intensely localized at outer serous layer and the tunica Albuginea.

Alpha smooth muscle actin, desmin, pancytokeratin and vimentin, antibodies: Alpha Smooth muscle actin: Alpha Smooth muscle actin were more reactive at LRS because of availability of abundant myofibroblast in the wall of seminiferous tubules and testicular capsule.

Desmin: The LRS was more reactive due to desmin investment into the wall of the seminiferous tubules, testicular capsule and blood vessels at this period. In the LDS, desmin was able to define a few blood vessels in the interstitium along with few macrophages, indicating the clean up of dead cells in the regressing testis.The incorporation of desmin into the testis suggest a role in the movement of lumen content.

Pancytokeratin:The LRS testis was more reactive because of the presence of fibroblast-like cells around the peritubular layer, the testicular capsule and blood vessels. The intensity decreases with the arrival of LDS when the testis regresses.

Vimentin:TheLRS showed more reation due to the presence of vimentin in the cells of the pertubular layer, peritubular blood vessels and membrane boundaries in the germinal epithelium. The membrane boundaries are more defined in the LDS testis where the remains of sertoli cell bundaries in the regressing testis were revealed. In the testicular capsule, vimentin is found around blood vessels and the intensity increase at LRS.

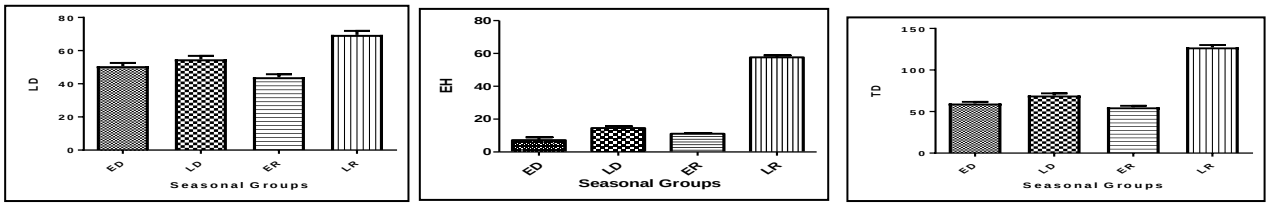

Figure 1 Graph of mean microsterology values of the testis, A-luminal diameter, B-epithelial height, C- tubular diameter

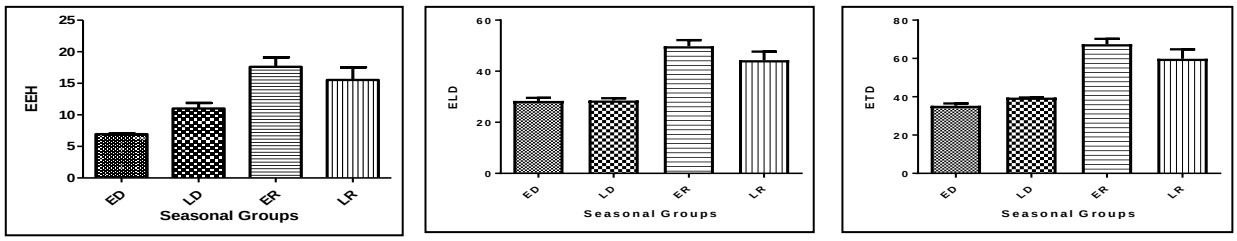

Figure 2 Graph showing epididymal microsterology values, A- epithelial height, B-luminal diameter, C- tubular diameter

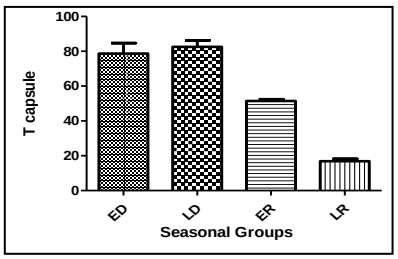

Figure 3 Mean testicular capsule thickness in a reproductive cycle

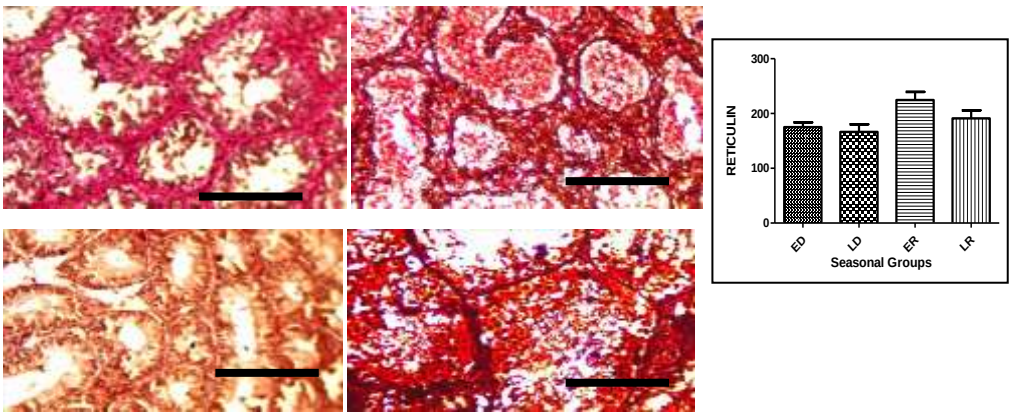

Figure 4 Photomicrograph of the testis of adult male double-spurred Francolin, A-Early dry season, B-Late dry season, C-Early rainy season, D-Late rainy season, E-graph of seasonal quantification of the reticulin. Gordon and sweet's stain (Reticulin) X400.

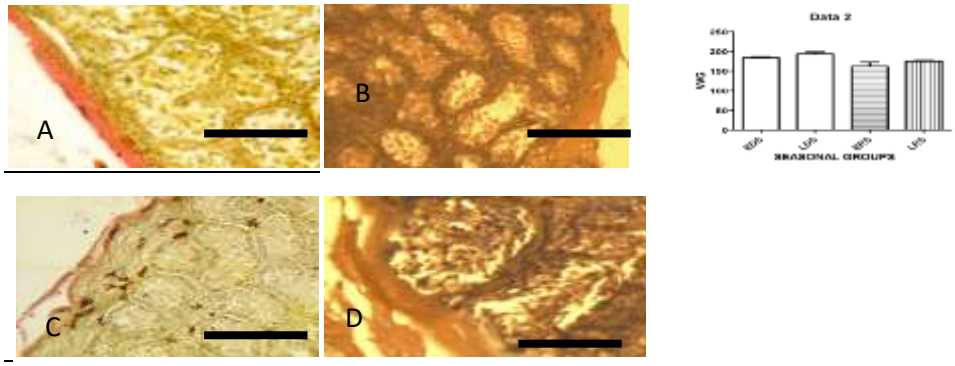

Figure 5 Photomicrograph of the testis of double–spurred Francolin demonstrating the distribution of collagen. A- early dry season, B-late dry season, C-early rainy season, D-late rainy season VVG X400

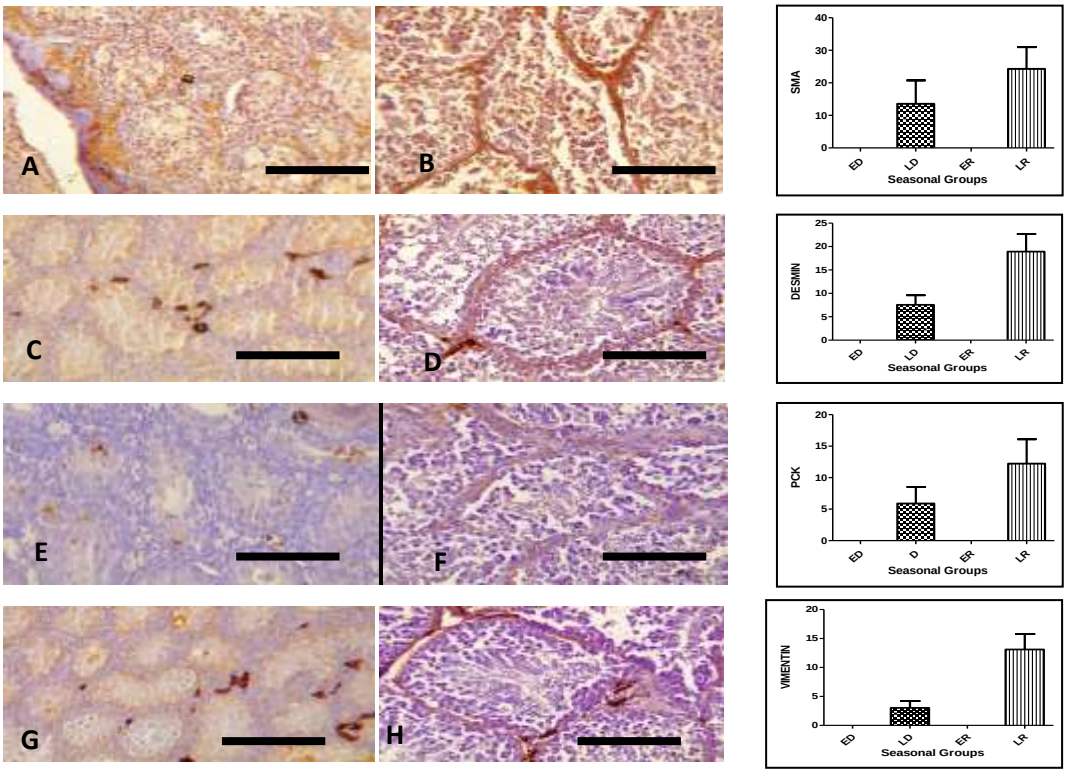

Figure 6 Photomicrgraph of the immunohistochemistry of cytoskeletal proteins of the testis of the adult male double-spurred Francolin, A-non-active season SMA, B- active season SMA, C- non-active season desmin, D- active season desmin, E- non-active season PCK, F- active season PCK, G- non-active season vimentin, H- active season vimentin. X400

DISCUSSION

Morphometric values obtained in the testes and epididymes of double-spurred Francolin in a reproductive cycle indicated increase in values towards the late rainy season except for testicular capsule thickness that decreased towards the late rainy season. This is similar to values obtained for Muscovy duck[16], quails[17].

The work was able to show that the seminiferous tubular wall in the male double-spurred Francolin is made up of a basal lamina tightly surrounded by two layers of myoid-like cells that are separated by a meshwork of reticulin fibres. The walls are separated by a population of irregularly oval Leydig's cell packed in extracellular matrix, with blood vessels, lymphatic vessels and nerve fibres. The work also showed that the testicular capsule in the male double-spurred Francolin is composed of three layers, thin serous outer layer, thick middle tunica albuginea and the loose inner layer.

The myoid-like cell layers of the tubule were found to be made up of vimentin and pancytokeratin near the base of the germinal epithelium and desmin and alpha smooth muscle actin beyond the reticulin fibre close to the interstitium. This arrangement is similar to that found in the domestic fowl[10].

The work observed the presence of alpha smooth muscle actin and desmin in high concentration during the late rainy season in the testicular capsule. This is similar to the work of Aire and Ozegbe[7] on quail, domestic fowl and duck. The work immunostained for vimentin and pancytokeratin uniformly in the testicular wall but Aire and Ozegbe[7] found only vimentin in quail testis. Desmin, actin, vimentin and pancytokeratin were immunostained in the peritubular myoid-like cells of adult male double-spurred Francolin but the work of Aire and Ozegbe[7] could only immunostained for actin and desmin in quail, domestic fowl, duck and turkey and faintly for vimentin and not cytokeratin in turkey

The presence of collagen fibres in the testicular capsule is limited to the tunica serosa and albuginea and does not penetrate the interstitium. This arrangement is similar to that found in dove [18], fowl[19], in quail[20] and in duck[16].

Measurement of the testicular capsule thickness showed that the capsules were thickest during the late dry season and thinnest during the late rainy season. This is different from what was observed in Guinea fowl by[19] that found no difference in thickness between seasons. The capsular thickness were however, lower than that recorded by[7] for quail, turkey and duck

CONCLUSION

We therefore, conclude that the testicular morphometrics, histology and peritubular tissue immunohistochemistry in the adult male double-spurred Francolin are similar to that of Muscuvy duck, quail and domestic fowl. The testicular capsule is rich in collagen and would be actively involved in keeping the testis from total collapse when it regresses at late dry season.

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CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

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