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ERRATUM

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 is a feral bird and remains undomesticated with very little known about its reproductive capability. This study aimed to know 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 testis 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 indicated that the mean histomorphometric values for testis and epididymis observed were highest in the early rainy season and late rainy season but lowest in the early dry season for all indexes except for the testicular capsule thickness which was lowest at the 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 during the late rainy season. The capsule was positive for collagen fibres mostly restricted to the *tunica albuginea.* The testicular capsule is rich in collagen and would be actively involved in keeping the testis from total collapse when it regresses in the late dry season.

Keywords: histology, histochemistry, immunohistochemistry, testis, morphometrics *Francolinus bicalcaratus*

INTRODUCTION

The Double-spurred Francolin occurs in dry grasslands, open savannas, 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. An extinction threat thus exists 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 have 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 a septum that penetrates the interstitium in greater rhea whereas, in rooster, duck and quail septum project from the capsule into the interstitium as reported by [13-15].

The double-spurred Francolin has remained elusive to bird lovers and scientists, thereby leading to a lack of knowledge on the 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 350-500 g, were obtained in four batches, of 5 birds each and stabilized. The Seasons were given as Early Dry Season (EDS): October to December, Late Dry Season (LDS): January to March; Early Rainy Season (ERS): April to June, Late Rainy Season (LRS): July to September. They were 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 onset of sample collection. Ethical Approval for the project was obtained from the 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. Fixed testicular tissues were passed through increasing alcohol concentration of 70 % for 1 hour, 90 % thrice, 1 hour each and 100 % twice, 1 hour 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 a warm water bath and later dried in an oven before staining with Hematoxylin 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).

Immunohistochemistry: Paraffin-embedded sections of testes were immunostained for the structural proteins with monoclonal antibodies against vimentin, desmin, pancytokeratin and alpha smooth muscle actin during the late dry season and late rainy season. Sections 5 µm thick were cut and mounted on slides pre-coated 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, photographed and analyzed using image J® software on a light microscope.

Verhoeff-Van Gieson Stain: Cut testis section is 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 the dish for 20 minutes at room temperature, and rinse quickly in tap water to remove most of the Verhoff stain. Add 1% Ferric chloride for up to 5 minutes (the 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, and rinse quickly with tap water to remove excess stain. Do not leave in water and immediately transfer to a 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 coverslip. The slides were viewed, photographed and analyzed using image J® software on a light microscope.

Gordon and Sweet's staining protocol for Reticulin: Cut testis section 5 µm 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. All numerical data obtained were expressed as means ± the standard error of means. The data were subjected to Analysis of Variance (ANOVA) and DMRT with P value set at 0.05.

RESULTS

Histomorphometrics of the testis, epididymis and testicular capsule

The mean ± SEM values of seminiferous parameters significantly increased at LRS as shown in table 1, but the mean ± SEM values of epididymal parameters significantly increased at ERS as indicated in table 2, while the testicular capsule thickness significantly increased at LDS as shown in table 3.

The reticulin fibers form mesh work that binds the tubular basement membranes to the interstitium. It was found to be sparse and loose at LDS but abundant and more organized at the ERS and LRS. In the interstitium, the Leydig cells were observed to be made up of spherical to ovoid-shaped nuclei with very little cytoplasm. The nuclei were prominent at LRS with dense cytoplasm but became shattered with pale cytoplasm at LDS. The interstitium was penetrated by a more extracellular matrix of fibroblast-like cells at LDS compared to LRS as indicated in Figure 1.

Collagen was demonstrated in the outer testicular capsule wall at varying amounts throughout the seasons using Verhoff-Van Gieson Stain. The LDS recorded the thickest collagen fibres. The collagen fibres were intensely localized at the outer serous layer and the tunica Albuginea as shown in Figure 2.

Alpha smooth muscle actin, desmin, pancytokeratin and vimentin antibodies

Alpha smooth muscle actin was more reactive at LRS because of myoid cells in the wall of seminiferous tubules and testicular capsule.

The LRS was more reactive for desmin due to myoid cells in the wall of the seminiferous tubules, testicular capsule and blood vessels. Desmin defines a few blood vessels in the interstitium along with a few macrophages at LDS.

The LRS testis was more reactive for pancytokeratin because of the presence of these fibers in cells around the peritubular layer, the testicular capsule and blood vessels. The intensity decreases with the arrival of LDS when the testis regresses.

The LRS showed more reaction due to the presence of vimentin in the cells of the peritubular layer, blood vessels and membrane boundaries at the base of germinal epithelium. The membrane boundaries are more defined in the LDS testis where the remains of Sertoli cell boundaries in the regressing testis were revealed. In the testicular capsule, vimentin is found around blood vessels and the intensity increases at LRS as shown in Figure 3.

Table 1: Seminiferous tubular morphometrics of adult male double-spurred Francolin at four seasons

	EDS	LDS	ERS	LRS
LD (µm)	50.64 ± 1.91^{b}	54.79 ± 2.06^{b}	43.96 ± 1.82^{c}	69.49 ± 2.42^{a}
EH (µm)	7.316 ± 1.75^{c}	14.57 ± 1.09^{b}	11.12 ± 0.40^{b}	57.64 ± 1.22^{a}
TD (µm)	59.65 ± 2.03^{c}	69.36 ± 2.52^{b}	55.07 ± 1.89^{c}	127.1 ± 2.933^{a}

EDS- Early Dry Season; LDS- Late Dry Season; ERS- Early Rainy Season; LRS- Late Rainy Season; LD- luminal diameter; EH- epithelial height; TD- tubular diameter. Data with different superscripts on the same row are statistically significant ($p<0.001$).

Table 2: Epididymal morphometrics of adult male double-spurred Francolin at four seasons

EPIDI	EDS	LDS	ERS	LRS
LD (µm)	28.37 ± 1.22^{b}	28.51 ± 0.91^{b}	48.79 ± 2.39^{a}	44.33 ± 3.37^{a}
EH (µm)	6.95 ± 0.95^{c}	11.00 ± 0.89^{b}	17.62 ± 1.49^{a}	15.53 ± 2.00^{a}
TD (µm)	35.32 ± 1.17^{b}	39.59 ± 0.07^{b}	67.41 ± 2.83^{a}	59.86 ± 4.93^{a}

EDS- Early Dry Season; LDS- Late Dry Season; ERS- Early Rainy Season; LRS- Late Rainy Season; LD- luminal diameter; EH- epithelial height; TD- tubular diameter. Data with different superscripts on the same row are statistically significant ($p<0.001$).

Table 3: Testicular capsule thickness of adult male double-spurred Francolin at four seasons

	EDS	LDS	ERS	LRS	P value
Mean (µm)	78.71 ± 6.02^{a}	82.55 ± 3.73^{a}	51.44 ± 1.06^{b}	16.91 ± 1.31^{c}	***

EDS- Early Dry Season; LDS- Late Dry Season; ERS- Early Rainy Season; LRS- Late Rainy Season; LD- luminal diameter; EH- epithelial height; TD- tubular diameter. Data with different superscripts on the same row are statistically significant ($p<0.001$).

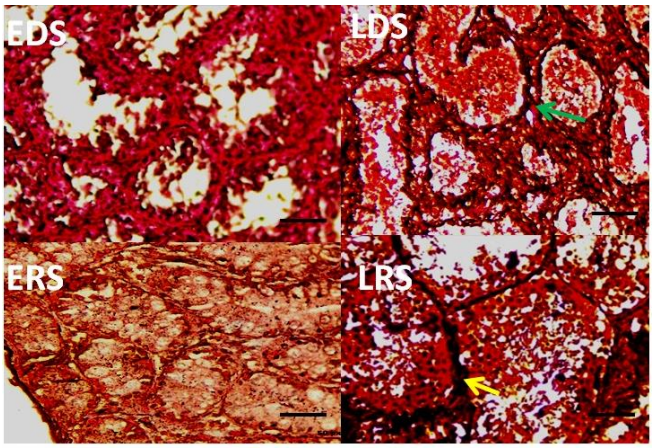

Figure 1: Cross section of the testis of DSF to show reticulin fibres: Green arrow- sparse & loose reticulin; Yellow arrow-organized & abundant reticulin. Gordin & Sweet's stain. EDS- Early Dry Season; LDS- Late Dry Season; ERS- Early Rainy Season; LRS- Late Rainy Season. Scale bar = 25µm

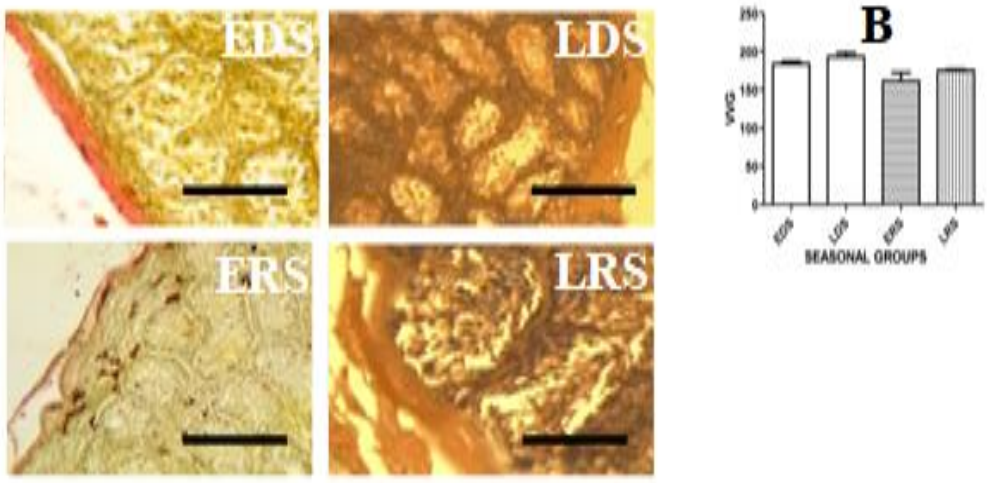

Figure 2: Cross section of the testis of DSF to show collagen fibers using Verhoff-van Gieson stain (VVG), all seasons were positive for collagen as indicated by intensity graph B. Scale bar = 25 µm.

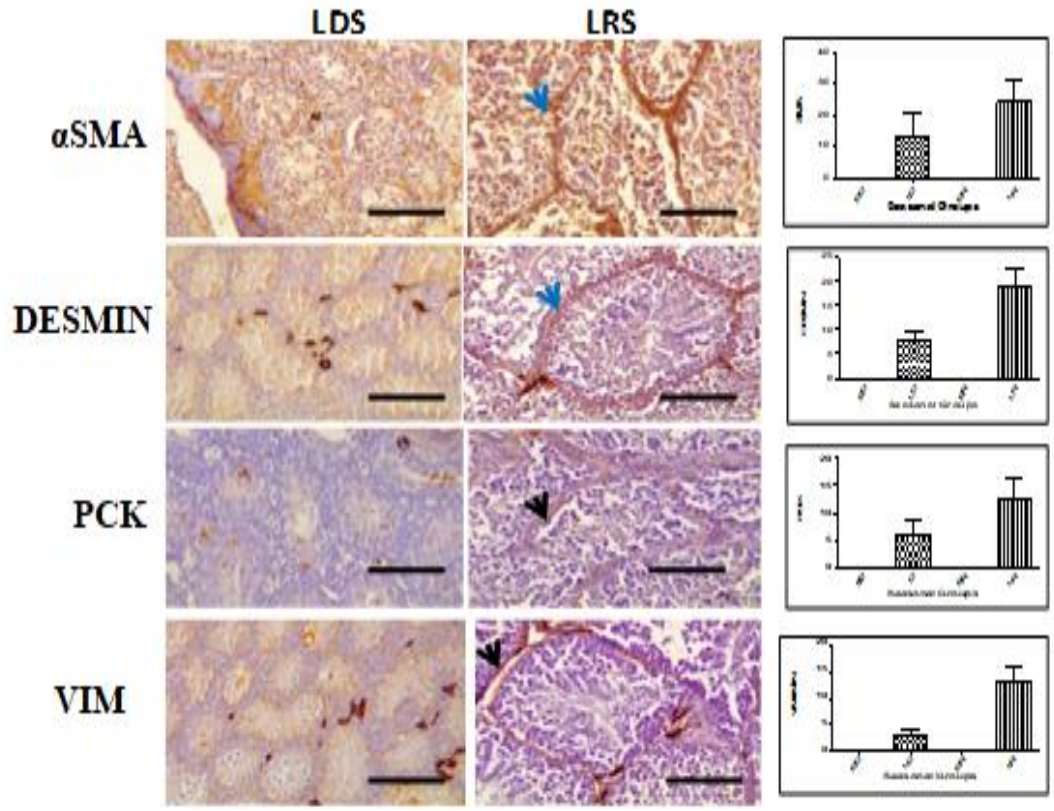

Figure 3: Immunohistochemistry to show cytoskeletal proteins: blue arrows- myoid +ve cells, black arrows- intermediate filaments. Scale bar = 25 µm

DISCUSSION

Morphometric values obtained in the testes and epididymis of double-spurred Francolin in a reproductive cycle indicated an 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] and 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 cells that are separated by a meshwork of reticulin fibres. The walls are separated by a population of irregularly oval Leydig's cells packed in an 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, a thin serous outer layer, a thick middle tunica albuginea and a loose inner layer. The myoid cell layers of the tubule were found to be made up of desmin and alpha smooth muscle actin beyond the reticulin fiber 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 wall of adult male double-spurred Francolin but the work of Aire and Ozegbe[7] could only immunostain 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 was however lower than that recorded for quail, turkey and duck[7].

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 Muscovy 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 in the late dry season.

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Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES

1. Little R. Terrestrial gamebirds and snipes of Africa. 1st, Jacana media, Johannesburg, South Africa 2016; 301.
2. BirdLife International."*Pternistis bicalcaratus"*. The IUCN Red List of Threatened Species. IUCN. 2016: e.T22678803A92788993. doi:10.2305/IUCN.UK.2016-3.RLTS.T22678803A92788993.en. Retrieved 14th of January, 2018.
3. Adekola O.E., Odewumi O.S. and Agbelusi E.A. Avian pests of cereal crops in rainforest and savanna agro-ecological zones, Ondo State, Nigeria. Journal of Research in Forestry, Wildlife and Environment 2019; 11, 1.
4. Fayenuwo JO, Olakojo SA, Akande M, Amusa NA, Olujimi OA. Comparative evaluation of vertebrate pest damage on some newly developed quality Protein maize (QPM) varieties in south-western Nigeria. Africa Journal of Agricultural Research 2007; 2(11), 592-585.
5. Lake PE. The male reproductive tract of the fowl. Journal of Anatomy 1957;1, 16-29
6. King AS. Aparelho urogenital das aves. In: Getty, R., Sisson and Grossman. *Anatomia dos animais domésticos*.5ª ed. Rio de Janeiro, Interamericana 1986; 2, 1805-13.
7. Aire TA, Ozegbe PC. The testicular capsule and peritubular tissue of birds: morphometry, histology, ultrastructure and immunohistochemistry. Journal of Anatomy 2007;201(6), 731-740.
8. Morton MI, Peterson IE, Burns DM, Allan N. Seasonal and age related changes in plasma testosterone levels in mountain white-crowned sparrows. The Condor 1990;92,1, 166-173
9. Bustos-Obregon E. Ultrastructure and function of the Lamina propria of mammalian seminiferous tubules. Andrologia 1976;8, 179-185.
10. Rothwell B, Tingari MD. The ultrastructure of the Boundary tissue of the seminiferous tubule

in the testis of the domestic fowl (*Gallus domesticus*). Journal of Anatomy 1973;114, 321–328.
11. Van Nassauw I, Harrison F, Callebaut M. Smooth muscle Cells in the peritubular tissue of the quail testis. European Journal of Morphometrics 1993;31, 60–64.
12. Carvalho SFM, Freneau BN, Frerneau GE. Aspects of the Macroscopic Testicular and Epididymal Morphology in the Greater Rhea, *Rhea Americana* (Linneaus– 1758) Birds. Anatomia, Histologia, Embryologia 2014;44,4, 255-261. doi: 10.1111/ahe.12133
13. King AS. Birds' urogenital system. The male genital organs. In: Sisson and Grossman. The Anatomy of Domestic Animals. (R. Getty, ed.). Filadelfia: Saunders, 1975;2, 1927–1935.
14. Lake PE. The male reproduction. in Physiology and Biochemistry of the domestic fowl. Bell, DJ and Freeman, BM London, New York: academic press 1971; 3, 1411-1447.
15. Artoni SM, Orsi AM, Lamano-Carvalho TI, Vicentini CA, Stefanini MA. Seasonal morphology of the domestic quail (*Coturnix coturnix japonica*) testis. Anatomy Histology and Embryology, 1999;28, 217-20.
16. Gerzilov V, Bochukov A, Penchev G, Petrov P. Testicular development in the Muscovy duck (*Cairinamos chata*). Bulgarian Journal of Veterinary Medicine 2016;19, 8–18
17. Lin M, Jones R, Blackshaw A. The cycle of the seminiferous epithelium in the japanese quail (*coturnix coturnix japonica*) and estimation of its duration. Reproduction 1990;88, 481–490.
18. Sherkawy FA, Abdelnaby AA, Osman GS, Elsogheer GA, Salaheldeen EM. Immunohistochemical expression of beta–catenin in urinary bladder urothelial carcinoma. SVU-IJMS, 2020;4(1) 26-29.
19. Dharani P, Kumary SU, Venkatesan S, Joseph C, Geetha R. Morphometry and histology of the testicular capsule and peritubular tissue of testis of guinea fowl (Numida meleagris). Indian Journal of Veterinary Anatomy 2017;29, 67–9.
20. Kannan TA, Venkatesan S, Geetha R. Histochemical studies on the testis of the Japanese quail. Indian Veterinary Journal 2008;85, 1129–30.