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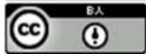

THE ROLE OF SEX HORMONES AND ROS VALUES IN THE CONTROL OF ANDROLOGY IN MALE DOUBLE-SPURRED FRANCOLIN (*FRANCOLINUS BICALCARATUS*)

[1]Olakunle Olutoye Osinubi, [1]Oluwasanmi Olayinka Aina, [1]Peter Chuka Ozegbe

[1]Department of Veterinary Anatomy, University of Ibadan, Ibadan, Nigeria

Corresponding author: Dr. Oluwasanmi Olayinka Aina. Email: ainasanmi@gmail.com

ORCID ID: https://orcid.org/0000-0001-8030-4431

ABSTRACT

Background: This study explored the relationship between andrology, seasonal sex hormone fluctuations, and ROS values in adult male Francolinus bicalcaratus while establishing baseline parameters. **Methods:** Double-spurred Francolins (n=5 per season) were randomly selected in both dry and rainy seasons from their natural habitat. They were carefully stabilized with dewormer, antibiotics, and multivitamins, then acclimatized for two weeks at the Experimental Animal House, University of Ibadan, Nigeria. The birds were weighed, sedated, and 3 to 5 ml of blood was collected from the jugular vein in lithium heparinized bottles for serum sex hormone analysis. Testes were excised, weighed, washed with a 1.15% KCL solution, and processed for complete andrology and oxidative stress assays. **Results:** Sperm count, activity, and morphological characteristics peaked during late rainy seasons, coinciding with increased mating and hatchability. This correlated with high serum testosterone and low ROS titres in the testes. Conversely, early dry seasons witnessed declines in serum testosterone, sperm parameters, and hatchability due to increased ROS titres from food scarcity. Late dry seasons saw further declines in sex hormone levels and elevated ROS titres, leading to the absence of sperm cells. **Conclusion:** This study highlights how sex hormone and ROS titres influence sperm cell viability in double-spurred Francolins, with significant seasonal variations. These factors mainly support spermatogenesis and fertility during the breeding season, characterized by food abundance and cover. Dry season sex hormone values serve as baselines, while wet season sperm count and ROS levels represent baseline data for this bird species.

Keywords: *Francolinusbicalcaratus,* seasonality, oxidative stress, andrology, sex hormones

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INTRODUCTION

Francolinusbicalcaratus is a resident breeder in tropical West Africa. Its territory extends from Senegal to Cameroon. It is a seasonal breeder and is hard to find during the wet season, when they breed. They are feral birds and attempts at domestication have not been successful, hence little is known about their physiology and fertility in their natural environment. There are reports of extermination programmes using traps and poisons in farming communities around natural habitats of double-spurred fowl lately, which has adversely affected their natural physiology and breeding preferences, hence the need for this study.

Seasons expose male and female birds to demand which impact their circulating blood parameters. Male birds fight intruders, exhibit aggressive behaviour and defend territory against competitors and predators while females incubate eggs but remain vigilant of intruders and predators (Kamiński*et al.*, 2014).

Majority of bird population in the world are tropical, behavioural changes are observed which varies from one season to another and little is known about the reproductive biology of these birds (Patankar*et al.,* 2021). Annual cycles in plasma levels of

luteinizing hormone (LH), testosterone and dihydrotestosterone (DHT), as well as seasonal changes in the ultrastructure of Leydig cells, have been studied in free-living adult and juvenile male Great Tits from south-west Sweden (Röhss, and Silverin, 1983).

Oxidative stress occurs in cells and tissues as a result of chemical metabolism of organic constituents. These chemical processes (oxidation and peroxidation) produce radicals referred to as reactive oxygen species (ROS), which are removed by the body´s anti-oxidant system as rapidly as possible (Puppel*et al.,*2015) (Pryor *et al.,* 2006, Szabo *et al.,* 2007). It has been discovered in Seychelles warblers (*Acrocephalussechellensis*), that a seasonal variation in oxidative stress in terms of generation of ROS was attributed to low availability of food (Van de Crommenacker*et al.,* 2011). Sperm cells are highly vulnerable to free radicals, and sperm quality and male fertility are critically affected by oxidative stress. It has been demonstrated that brightly coloured males are better protected against oxidative stress damage (Helfenstein *et al*., 2010). Recently, sexual ornaments, particularly carotenoid-based colourful traits, have been proposed to depend on a male's capacity to resist oxidative stress and thus to signal sperm quality. A study on great tits *Parus major*, in which adults are sexually dichromatic in carotenoid-based breast plumage, gave the first evidence that ornaments and sperm quality may be linked through oxidative stress. When experimentally subjected to oxidative stress resulting from increased workload, less colourful males suffered a greater reduction in sperm motility and swimming ability, and increased levels of sperm lipid peroxidation compared to more colourful males. Moreover, the level of sperm lipid peroxidation was negatively correlated with sperm quality. Finally, carotenoid supplementation increased sperm quality of less colourful males, suggesting that pale males are deficient in carotenoid antioxidants (Helfenstein *et al*., 2010). There is a dearth of research data on the reproductive biology of *Francolinusbicalcaratus* and alterations in the hormonal status and ROS values through the seasons.

MATERIALS AND METHODS

Hormonal assay: Samples of blood were obtained from the left jugular vein, using 18 guage needle and syringe and put in lithium heparinized bottles (about 3 ml). The samples for serology/hormonal assay were centrifuged at 3500 rpm for 5 minutes using centrifuge (Gallenkamp, England) and the supernatant collected into ependorf tubes (MicropointDiagnostica, China). The tubes were stored at -20º C prior to subsequent hormonal assay (Follicle stimulating hormone (FSH), luitenizing hormone (LH) and testosterone) using commercially available kits (Dialab, Germany).

Oxidative stress assay: The Birds were weighed, sedated and sacrificed by decapitation. The testes were freshly harvested from five birds each season, rinsed in 1.15% potassium chloride in 50mM Tris-HCL buffer at pH 7.4, weighed and quickly refrigerated. Thawed slices of these organs were then mixed in 10% phosphate buffered saline (1:100) and homogenized on ice. These were then decanted into test tubes and centrifuge at 10000rpm for 15 minutes at 4º C. The supernatants were decanted and used to run assay for six oxidative stress markers each season. Catalase activity, superoxide dismutase, reduced glutathione, glutathione-s transferase and lipid peroxidation assays were done using standard methods. Catalase activity was done using hydrogen peroxide as substrate, according to the method of Clairborne, (1995). Superoxide dismutase assayed by

the method described by Misra and Fridovich, (1972). Reduced glutathione was assayed by the method of Beutler et al. (1963), Glutathione-S transferase was assayed by the method of Habig et al. (1974). Lipid peroxidation was quantified as Malondialdehyde (MDA) according to the method described by Farombi et al. (2000) and expressed as μM MDA/g tissue. Milligramme protein determination (MG protein) was done using Bradford protein assay method.

Andrology: The caudal end of the vas deferens from the excised testis were gently lifted, cut and placed in a normal saline solution. Fine needle aspirate were taken from the epididymis and dropped on clean glass slides and 0.9 % normal saline was added to view sperm motility, then drops of Eosin and Negrosin stain were added to the slides to study sperm morphology and characteristic for five birds each season, by standard method. as described by Zemjanis, (1970). Ethical Approval for this study was obtained from the University of Ibadan: UI-ACUREC/18/0114

RESULTS

Andrology

Two seasons, late dry season and early rainy season witnessed zero sperm count and sperm activity while late rainy season and early dry season witnessed sperm count and activity. The mean sperm motility (P < 0.05) value increased from EDS ([a]) value of 42±3.74 to LRS ([aa]) value of 80±3.16. The mean live/dead cells (P < 0.05) values increased from EDS ([b]) value of 71±3.32 to LRS ([bb]) value of 96.8±0.73. The mean sperm count (P < 0.05) value increased from EDS ([c]) value of 92.8±3.68 to LRS ([cc]) value of 135±5.06. The mean tailless head (P > 0.05) value increased from EDS ([d]) value of 156.2±11.55 to LRS ([dd]) value of 159.2±59.58. The mean headless tail (P > 0.05) value increased from EDS ([e]) value of 38±5.15 to LRS ([ee]) value of 177±39.06. The mean rudimentary tail (P < 0.05) value increased from EDS ([f]) value of 0.4±0.24 to LRS ([ff]) value of 11±4.47. The mean bent tail (P > 0.05) value increased from EDS ([g]) value of 2587.2±477.9 to LRS ([gg]) value of 3235.6±459.7. The mean bent tail (P > 0.05) value increased from EDS ([g]) value of 2587.2±477.9 to LRS ([gg]) value of 3235.6±459.7. The mean curved tail (P < 0.05) value increased from EDS ([h]) value of 5.6±0.5099 to LRS ([hh]) value of 8.4±0.6. The mean curved midpiece (P > 0.05) value increased from EDS ([i]) value of 1957.8±207.8 to LRS ([ii]) value of 3033.8±503.3. The mean bent midpiece (P < 0.05) value increased from EDS ([j]) value of 1533.4±157.1[j] to LRS ([jj]) value of 3433.8±499.2. The mean looped tail (P < 0.05) value increased from EDS ([k]) value of 0.4±0.25 to LRS ([kk]) value of 11±4.47. The mean total count (P > 0.05) value increased from EDS ([l]) value of 369.8±18.4 to LRS ([ll]) value of 403±2.00. However, the mean abnormal sperm count (P < 0.05) value increased from EDS ([m]) value of 38.2±3.918 to LRS ([mm]) value of 55.6±3.918.

Serology: The mean luiteinizing hormone (P > 0.05) value increased from EDS ([*]) value of 10.4±0.93 to LDS ([**]) value of 12.2±0.86 and increased from EDS ([$]) value of 10.4±0.93 to LRS ([$$]) value of 12.2±0.86. The mean follicle stimulating hormone (P > 0.05) value increased from EDS ([+]) value of 7.8±1.16 to LDS ([++]) value of 9.2±0.86, it also increased from EDS ([>]) value of 7.8±1.16 to ERS ([>>]) value of 9±0.55 and increased from EDS ([<]) value of 7.8±1.16 to LRS ([<<]) value of 9.2±0.86. The mean values of testosterone (P < 0.05) rose from 2.48±0.39 at EDS ([~]) to 3.66±0.21 at LRS ([~~]), it also rose from EDS (-) value of 2.48±0.39 to ERS (—) value of3.64±0.13 and increased (P > 0.05)

from EDS (!) value of 2.48±0.39 to LDS (!!) value of 3.32±0.

Oxidative stress assay: The mean value of SOD testis ($P < 0.05$) decreased from 0.380982±0.10 at LDS (y) to 0.082204±0.05 at ERS (y) and increased ($P > 0.05$) from 0.082204±0.05 at ERS (z) to 0.292424±0.03 at LRS (zz). The mean value of CAT testis ($P < 0.05$) decreased from 0.169728±0.06 at EDS (%) to 0.022791±0.01 at ERS (%%) and increased from 0.022791±0.01 at ERS (&) to 0.12595±0.03 at LRS (&&).However, the mean value for glutathione S transferase (GST) testis ($P < 0.05$) decreased from 0.02004±0.002 at EDS (*) to 0.000756±0.0002 at LDS (**), it also decreased from 0.02004±0.002 at EDS (#) to 0.000311±0.003 at ERS (##) and decreased from 0.02004±0.002 at EDS (@) to 0.000271±0.0001 at LRS (@@). The mean value ($p< 0.05$) of lipid peroxidation testis decreased from 0.091705±0.01 at EDS (<) to 0.00803±0.001 at LDS (<<), it also decreased from 0.091705±0.01at EDS (~) to 0.018998±0.003 at ERS (~~) and decreased from 0.091705±0.01 at EDS (µ) to 0.010961±0.003 at LRS (µµ). The mean value ($p < 0.05$) of reduced glutathione testis decreased from 4.883621±0.14 at EDS ($) to 3.969828±0.04 at LDS ($$), it also decreased from 3.969828±0.04 at LDS (+) to at ERS (++) and increased from 3.969828±0.04 at LDS (!) to at LRS (!!), but increased from 1.87069±0.04 at ERS (>) to 5.198276±0.42 at LRS (>>).

Table 1- Mean sperm count and morphology at breeding and post breeding season

	Motility (%)	Live|Dead (%)	Count(Millions/Ml)	Tailesshead	Headlesstail	Rudimentarytail
EDS	42±3.74a	71±3.32b	92.8±3.68c	156.2±11.55d	38±5.15e	0.4±0.24f
LRS	80±3.16aa	96.8±0.73bb	135±5.06cc	159.2±59.58dd	177±39.06ee	11±4.47ff

	BENTTAIL	CURVEDTAIL	CURVEDMIDPIECE
EDS	2587.2±477.9g	5.6±0.5099h	1957.8±207.8i
LRS	3235.6±459.7gg	8.4±0.6hh	3033.8±503.3ii

	BECENTMIDPIE	LOOPEDTAIL		TOTAL
EDS	1533.4±157.1j	0.4±0.25k		369.8±18.4l
LRS	3433.8±499.2jj	11±4.47kk	403±2.00ll	

	ABNORMAL COUNT
EDS	38.2±3.918m
LRS	55.6±3.918mm

**Different superscripts indicate significant differences in the mean values within groups (p value of 0.05)

Table 2- Mean serological values at four seasons

	LH (iU/L)	FSH (iU/L)	TESTOSTERONE (ng/dL)
EDS	10.4±0.93*$	7.8±1.16+><	2.48±0.39!–~
LDS	12.2±0.86**	9.2±0.86++	3.32±0.21!!
ERS	12.4±1.21	9±0.55>>	3.64±0.13—
LRS	12.2±0.86$$	9.2±0.86<<	3.66±0.21~~

**Different superscripts indicate significant difference in the mean values within groups (p value of 0.05)

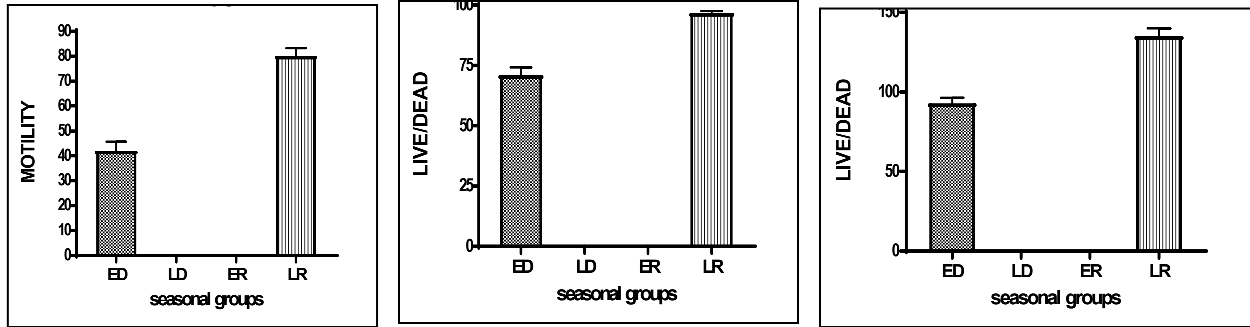


Figure 1- graph showing the activity of sperm cells in a reproductive cycle

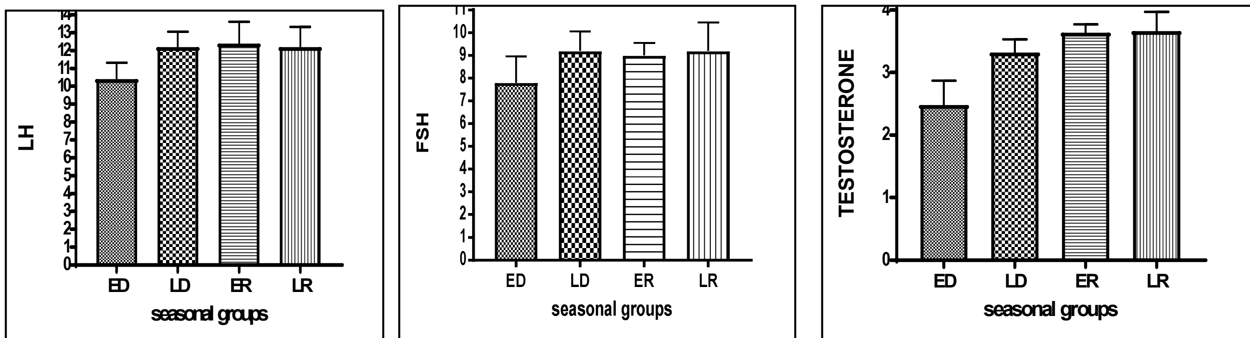
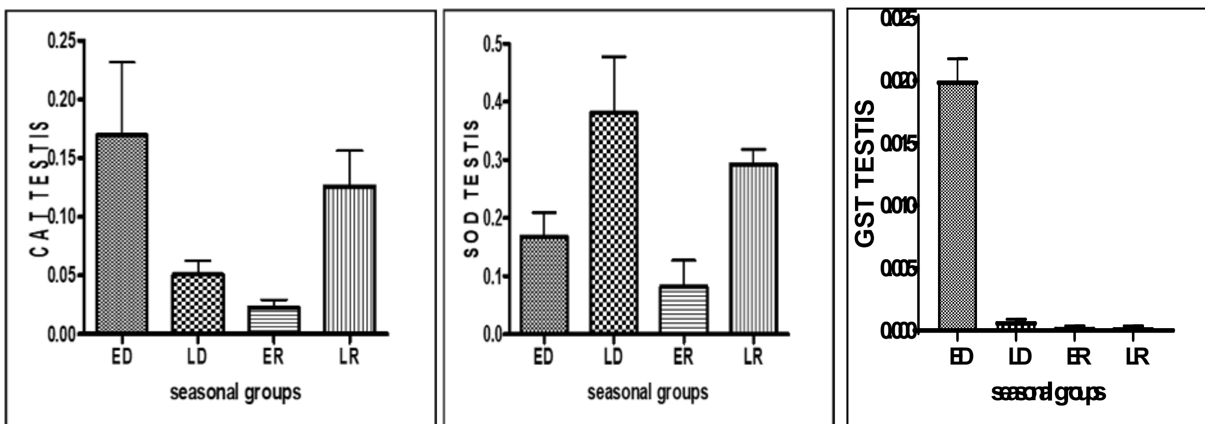


Figure 2- graph showing serum distribution of sex hormones at four seasons

Table 3- Mean values of ROS assay for testes at four seasons

Testis	S0D	CAT	GST	GSH	LPO
EDS	0.168132 ± 0.04	$0.169728 \pm 0.06^{\%}$	$0.02004 \pm 0.002^{*\#@}$	$4.883621 \pm 0.14^{\$}$	$0.091705 \pm 0.01^{<\sim\mu}$
LDS	0.380982 ± 0.10^{y}	0.050713 ± 0.01	$0.000756 \pm 0.0002^{**}$	$3.969828 \pm 0.04^{\$\$+!}$	$0.00803 \pm 0.001^{<<}$
ERS	0.082204 ± 0.05^{yyz}	$0.022791 \pm 0.01^{\%\%\&}$	$0.000311 \pm 0.003^{\#\#}$	$1.87069 \pm 0.04^{++>}$	$0.018998 \pm 0.003^{\sim\sim}$
LRS	0.292424 ± 0.03^{zz}	$0.12595 \pm 0.03^{\&\&}$	$0.000271 \pm 0.0001^{@@}$	$5.198276 \pm 0.42^{!!>>}$	$0.010961 \pm 0.003^{\mu\mu}$



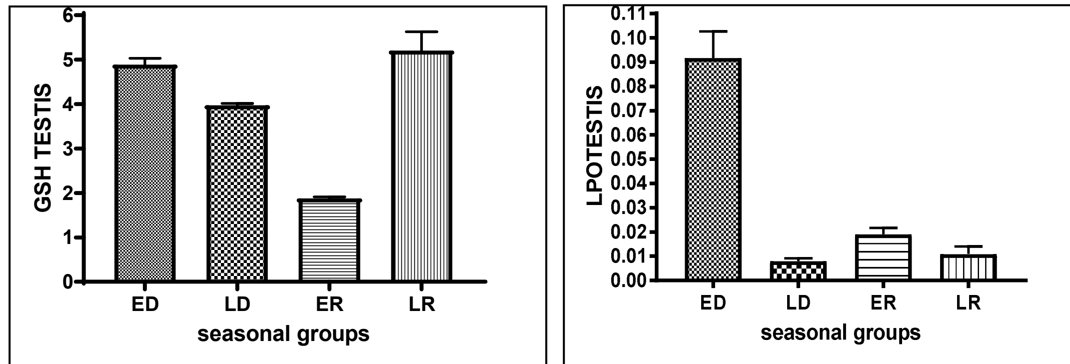


Figure 3- graph of mean ROS values

**Different superscripts indicate significant differences in the mean values within groups (p value of 0.05)

DISCUSSION

The serum/plasma Luteinizing Hormone, Follicle stimulating hormone and testosterone increased towards the breeding season with the testosterone having the highest surge to support spermatogenesis. Testosterone level in the serum/plasma rose by 1.2X towards the breeding season and declined subsequently in double-spurred Francolin. Similar surges have been seen in song birds (Deviche et al., 2014), (Smith *et al.,* 1997) and Magang ganders (Shi *et al.,* 2007). The importance of the surge is to maintain spermatogenesis as evidenced by values of sperm count and characteristic recorded and this was also seen in the work on Penguins (Williams, 1992), Mallards (Donham, 1979), Ostriches (Degan*et al.,* 1994), Geese (Dittami, 1981), Greypartridges(Fraissinet,1987) and Japanese common pheasants (Sakai and Ishii, 1986) that showed seasonal changes in the serum testosterone and Luteinizing Hormone levels during reproductive cycle. This work was able to show that testosterone levels changed in parallel with spermatogenic activity in the testis. In the seasonally breeding birds, testicular activity is cyclic and in some species like the male double-spurred Francolin, there is evidence that these cyclic changes may, in part at least be endogenous according to serological values recorded. This is also evident in most wild birds as observed by Marshall and Serventy, 1958. Serum level of sex hormones revealed a non-active season decline in testosterone, follicle stimulating hormone and the luiteinizing hormone leading to almost complete breakdown of germinal epithelium. A row of spermatogonia and sertoli cells in the seminiferous tubules was observed in male double spurred Francolin at this period. Testosterone and follicle stimulating hormone level returns to season's high at breeding to sustain spermatogenesis. Kauatcho et al. (2015) got similar result with work on varying age groups of Japanese quail. Sperm count and activity were observed at late rainy season and early dry season. The breeding season count was higher and mating occurred at this season. This is similar to the work of Birkhead et al. (1993) that associated increased testicular activity at breeding season with increased semen production.

The decrease in values of SOD and CAT in the testis towards the breeding season could serve to protect the testis against oxidative damage as sperm cells are being produced..The dry season's values of GST and LPO represent a period of cellular breakdown and scarcity of food

The GSH values for the testes were found to be highest at the breeding season (LRS) which is also the period of high protein

buildup while the LPO values of the testes went down at the same season signifying the buildup of steroids in sex hormone. It has been discovered in Seychelles warblers (*Acrocephalussechellensis*), that a seasonal variation in oxidative stress in terms of generation of ROS was attributed to low availability of food (Van de Crommenacker*et al.,* 2011).

CONCLUSION

We, therefore conclude that sperm cell viability in double-spurred Francolin is regulated by sex hormones and ROS titres. These factors are seasonally controlled and could only support spermatogenesis and fertility at the breeding season which corresponds to period of abundance of food and cover. The dry season value of sex hormones could be assumed to be the baseline sex hormone values while the wet season values of sperm count and ROS will represent the baseline data for the bird.

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