UNIVERSITI PUTRA MALAYSIA

GONADAL STRUCTURE AND ANDROGEN ACTIVITY OF THE MALE LESSER MOUSE DEER (TRAGULUS JAVANICUS OSBECK)

SRIYANTO

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DOCTOR OF PHILOSOPHY
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2011

GONADAL STRUCTURE AND ANDROGEN ACTIVITY OF THE MALE LESSER MOUSE DEER (*TRAGULUS JAVANICUS* OSBECK)

By

SRIYANTO

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Doctor of Philosophy

August 2011

Specially dedicated to:

My beloved late father and my mother,

TRISNOWIDOYO
Mrs. JATUN TRISNOWIDOYO

My Siblings,

SANDIYEM AND SAIMA
WALUYO RAHARJO AND PURWANINGSIH
SRIYANA AND QOMARUL
SRI WAHYONO AND NUR FITRI

My beloved wife and Children,

SHARMY PRASTITI
DIFFA FADHIL SRIYANTO
AKHTAR ALFARISI SRIYANTO
FATHIA AZZAHRA SRIYANTO

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor Philosophy

GONADAL STRUCTURE AND ANDROGEN ACTIVITY OF THE MALE LESSER MOUSE DEER (*TRAGULUS JAVANICUS* OSBECK)

By

SRIYANTO

August 2011

Chairperson: Professor Mohd. Zamri Saad, DVM, PhD

Faculty: Veterinary Medicine

The lesser mouse deer (*Tragulus javanicus*), belongs to the suborder Ruminantia and as the smallest ungulate species in the world is a common native to the tropical rain forest of Southeast Asia. The population of lesser mouse deer is threatened by illegal hunting and habitat destruction for many years. Thus, conservation and management of this animal is becoming important. One of the keys for successful conservation and breeding of wild animals is the management and knowledge of the reproductive system. Currently, limited information is available on the reproductive physiology of the lesser mouse deer. Furthermore, the reproductive physiology with regards to the reproductive activity of the lesser mouse deer is mainly unknown, creating gaps in understanding the reproduction of this animal.

As there is limited information on their reproductive especially of the male animal, this study was conducted with the objective to determine the gross morphological and histological features on the male reproductive organs, the testosterone levels and profiles, the distribution and appearance of steroidogenic enzymes and the gonadal

regulatory hormones in the testis to provide better understanding on the male reproductive activity of lesser mouse deer.

This study describes the general structures of the male reproductive system of lesser mouse deer, which include gross and histological morphology, analysis of testosterone concentration levels and the profile, based on the testosterone levels in fecal samples. The samples were collected from 3 adult male lesser mouse deer for a period of 6 months covering both dry and wet months. They were subjected to high performance liquid chromatography (HPLC) techniques to determine the testosterone levels. Using immunohistochemistry staining, distribution and localization of gonadal regulatory hormones, sex steroid hormones and converting steroidogenic enzymes in the testis were determined.

Generally, the male reproductive organ consisted of two oval-shaped testes, 14 mm in length and 10 mm in diameter, which were located within the scrotum. This was followed by the ducts that consisted of the epididymis and vas deferens of 117 mm long, and the accessory organs consisted of ampullae, seminal vesicles, prostate and bulbo-urethral glands, and finally the penis. The penis of lesser mouse deer was fibroelastic type with sigmoid flexure, resembling that of the boar and other ruminants. The glans penis was twisted at the end, resembling that of the boar but was twisted one and a half turns clockwise whereas the twisted end of the glans penis of boar is counter-clockwise.

The study of testosterone profile revealed fluctuating testosterone levels between 0.01 and 17.90 ppm, but the levels were significantly increased ($p<0.05$) in the wet

months. Peak testosterone levels were observed at approximately 10-day intervals, particularly during the wet months of October, November and December, which correlated well with the 16-day estrus cycle of the females. These results were supported by investigation on the immunolocalization of steroidogenic enzymes and gonadal hormones in the testis of the lesser mouse deer during dry and wet months. Testicular steroidogenesis of the lesser mouse deer occurs at different sites within a testis. The 3β-HSD enzyme appeared in Leydig cells, Sertoli cells and spermatogonia while the cytochrome P450scc enzyme appeared only in the Leydig cells. The inhibin alpha hormone, on the other hand, was observed stronger in Sertoli cells. This suggests that in the wet months, Leydig cells are more active in producing steroidogenic enzymes and gonadal hormone, thus, correlated well with earlier observations that the breeding season for the lesser mouse deer was during the wet months. On the other hand, Sertoli cells were active in producing the enzyme in both dry and wet months.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

GONADAL STRUCTURE AND ANDROGEN ACTIVITY OF THE MALE LESSER MOUSE DEER (*TRAGULUS JAVANICUS* OSBECK)
(STRUKTUR GONADAL DAN AKTIVITI HORMON ANDROGEN PELANDUK (TRAGULUS JAVANICUS OSBECK)JANTAN)

Oleh

SRIYANTO

Ogos 2011

Pengerusi: Profesor Mohd. Zamri Saad, DVM, PhD

Fakulti : Perubatan Veterinar

Pelanduk atau kancil (*Tragulus javanicus*), tergolong dalam suborder Ruminantia dan spesis haiwan berkuku terkecil di dunia yang terdapat dalam hutan hujan tropika di Asia Tenggara. Terkini, populasi kancil telah turun mendadak akibat ancaman pemburuan liar dan pemusnahan habitat. Dengan demikian, pemuliharaan dan pengurusan haiwan ini menjadi penting. Salah satu kunci kejayaan pemuliharaan dan captif kehidupan liar termasuk pengurusan dan pengetahuan tentang sistem pembiakan. Memandangkan yang maklumat amat terhad terhadap fisiologi reproduksi berkaitan dengan aktiviti pembiakan kancil, terdapat jurang yang luas yang perlu difahami. Oleh itu, pengetahuan tentang organ pembiakan jantan, kadar testosteron, pengedaran dan penampilan enzim steroidogenik dan hormon gonad seperti inhibin dan aktivin pada testis akan memberikan kefahaman yang lebih baik terhadap kegiatan pembiakan jantan haiwan ini.

Oleh kerana maklumat berkaitan organ pembiakan pelanduk jantan adalah terhad maka kajian ini telah dijalankan bertujuan untuk menentukan ciri morfologi dan histologi pada organ pembiakan jantan, paras testosteron dan profil, pengedaran dan rupa enzim steroidogenic dan hormon gonad pengawalseliaan dalam testis untuk memberi kefahaman yang lebih baik terhadap aktiviti pembiakan kancil jantan.

Kajian ini menggambarkan struktur sistem pembiakan pelanduk jantan termasuk morpologi dan histologi, analisis tahap kepekatan testosteron dan profil berdasarkan mengukur paras testosteron yang diekstrak daripada sampel najis. Sampel ini diambil daripada 3 ekor pelanduk jantan dewasa untuk tempoh 6 bulan yang meliputi kedua-dua bulan kemarau dan tengkujuh. Sampel dianalisis dengan tehnik kromatografi cecair prestasi tinggi (HPLC) untuk menentukan paras testosteron. Menggunakan teknikal Immunohistokimia untuk melihat pengagihan dan pentaburan hormon gonad pengawalseliaan, hormon steroid seks dan enzim steroidogenic dalam testis.

Secara umumnya organ pembiakan haiwan pelanduk jantan terdiri daripada dua testis yang berbentuk bujur, 14 mm panjang dan 10 mm, yang terletak di dalam skrotum, saluran epididimis dan vas deferens berukuran 117 mm panjang, sementara organ aksesori terdiri daripada ampullae, vesikel seminalis, prostat dan kelenjar bulbo-urethra, dan akhirnya zakar. Zakar kancil yang kurang jenis fibroelastic dengan lenturan sigmoid, menyerupai babi dan ruminan lain. Penghujung glans penis kancil adalah bengkok, menyerupai babi jantan. Namun begitu, penghujung glans penis kancil adalah mengikut arah jam dengan satu setengah pusingan sedangkan penghujung glans penis babi adalah berlawanan arah jam.

Kajian analisis profil testosterone mendedahkan tahap testosteron di antara 0.01 dan 17.90 ppm, tetapi tahap testosteron pada bulan hujan ternyata ($p < 0.05$) lebih tinggi. Puncak tertinggi testosteron adalah pada setiap 10-hari kitaran, terutama pada bulan hujan iaitu Oktober, November dan Disember. Ini adalah selaras dengan kitaran estrus 16 hari pada haiwan betina. Keputusan ini disokong oleh pemeriksaan terhadap kedudukan enzim steroidogenik dan hormon gonad testis kancil semasa bulan kemarau dan hujan. Steroidogenesis kancil terdapat di lokasi yang berbeza dalam testis. Enzim 3β-HSD terdapat dalam sel Leydig, sel Sertoli dan spermatogenia, sedangkan enzim cythochrome P450scc hanya terdapat dalam sel Leydig. Hormon inhibin lebih banyak dalam sel Sertoli. Hal ini menunjukkan bahawa pada bulan hujan, sel Leydig lebih aktif dalam menghasilkan enzim steroidogenik dan hormon gonad. Ini sehingga berhubungan baik dengan cerapan sebelumnya bahawa musim pembiakan untuk kancil adalah selama bulan hujan.

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In this thesis, I have tried to explain about the structure and activity of the reproductive system of male lesser mouse deer (*Tragulus javanicus*) using gross observations, light microscopy, immunohistochemistry and high performance liquid chromatography (HPLC) including utilization of non-invasive monitoring methods such as fecal steroid hormone analyses to assess testosterone levels. This fundamental data would be important for revealing the possible assisted reproductive studies. Some of the results have been published in *Pertanika Journal Tropical Agricultural Science*. Other parts of the results have been presented in the 3rd

International Meeting on Asian Zoo/Wildlife Medicine and Conservation (AZWMC) in Bogor (2008) and 22nd Veterinary Association Malaysia Congress and 4th International Meeting on Asian Zoo/Wildlife Medicine and Conservation in Kuala Lumpur (2010), FAO/IAEA International Symposium on Sustainable Improvement of Animal Production and Health (2009), First Malaysian Association of Veterinary Pathology (MAVP) Conference in Sarawak (2009) and 2nd Malaysian Association of Veterinary Pathology (MAVP) Conference in Sabah (2010).

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May Allah Subhana wa ta'ala bless you all. Thank You.

I certify that a Thesis Examination Committee has met on August 22, 2011 to conduct the final examination of Sriyanto on his thesis entitled "**Gonadal Structure and Androgen Activity of the Male Lesser Mouse Deer (*Tragulus javanicus* Osbeck)**" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Mohamed Ali bin Rajion, PhD
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Date: 28 October 2011

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that is has not been previously and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or other institutions.

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S R I Y A N T O

Date : 22 August 2011

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	percentage
β	Beta
^{o}C	degree celcius
μg	Microgram
μl	Microliter
μm	Micrometer
μM	Micromolar
AB	alcian blue
BSA	bovine serum albumin
dH_2O	distilled water
ELISA	enzyme linked immunosorbent assay
EDTA	ethylene-diamine-tetraacetic acid (disodium salt)
g	Gram
H_2O	Water
H & E	hematoxylin and Eosin
HPLC	high performance liquid chromatography
IgG	immunoglobulin G
kg	kilogram
L	Liter
M	Molar
m	Meter
mg	Milligram
min	Minute

mL	Milliliter
mM	milliMolar
Na_2HPO_4	di-sodium hydrogen phosphate
NaCl	natrium chloride or sodium chloride
NaH_2PO_4	sodium di-hydrogen phosphate
ng	Nanogram
nm	Nanometer
OD	optical density
PBS	phosphate buffer saline
PAS	periodic acid staining
pH	puissance hydrogen (hydrogen-ion concentration)
psi	Pounds per square inch
rpm	rotation per minute
TBE	tris-boric EDTA
TBS	tris-buffer saline
UV	ultra-violet

CHAPTER 1

INTRODUCTION

The lesser mouse deer (*Tragulus javanicus*), the smallest ungulate species in the world (Nowak, 1991), is a native of tropical rain forests of Southeast Asia (Medway, 1969). This animal is interesting from several view points: the animal is regarded as the smallest ruminant and has been proposed to be a model in biomedical and ruminant research (Kudo *et al.,* 1997). On the other hand, the lesser mouse deer shows larger proportion of carcasses and therefore is suitable as an alternative meat source (Ismail *et al.*, 1992). In addition, this animal is an interesting exhibit in the zoo and other conservation areas for the purpose of education and tourism.

Currently, the population of the lesser mouse deer is threatened by illegal hunting and habitat destruction. According to the 2007 International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species, there are 6,306 species of animals that have become threatened all over the world, with 1,528 animal species are reported as critically threatened, including 162 species of mammals. *Tragulus javanicus* is listed in the Red List by IUCN as data deficient and this partly reflects the lack of clarity over how many species of this animal there are in the natural habitat and also the lack of information on *Tragulus javanicus*. Dedicated field investigations of status are urgently warranted, and the status of Red List species should be reviewed regularly in light of current uncertainty and concerns (IUCN-World Conservation Union).

Conservation and management of wildlife animal species is becoming important. One key success in the conservation and breeding strategy of wildlife animals includes the knowledge and manipulation of the reproductive system. There is only limited information available on the reproductive system of the lesser mouse deer. In order to understand the science of reproduction, it is necessary to include anatomy, histology, physiology, endocrinology, embryology, pathology, cytology, microbiology and nutrition. However, the reproductive anatomy and physiology with regard to the reproductive activity of the lesser mouse deer are not yet known. Therefore there is still wide gap to the understanding of the reproduction of this animal, especially of the male animals.

In males, the pattern of reproductive activity can be studied by measuring the testosterone levels, the androgenic hormone that is essential to maintain sexual function, germ cell development and accessory sex organs of males (Payne and Youngblood, 1995). Among seasonal breeding animals, testosterone levels have been shown to a peak at mating time; this is not known in the male lesser mouse deer. Besides androgen, oestrogen is another important hormone that regulates part of the male endocrine system. Previous studies have indicated that various processes of spermatogenesis are impaired following inappropriate exposure to oestrogen, highlighting the importance of a normal balance between androgen and oestrogen for the male fertility (Donnel, 2003).

Synthesis of steroid hormones such as androgen and oestrogen requires steroidogenic enzyme activities. The enzymes such as cholesterol side chain cleavage cytochrome (P450scc), and 3-ß-hydroxysteroid dehydrogenase (3ßHSD) are required to convert

cholesterol into testosterone, while cytochrome P450 aromatase (P450arom) is responsible to convert oestrogen from testosterone (Bhasin *et al.*, 2003; Donnel 2003). Therefore, knowledge on the distribution and presence of steroidogenic enzymes in the testes will provide better understanding of the synthesis and proportion of steroid hormones in a species. In addition to the steroid hormones, others hormones are also secreted by the testes and play an important role in the reproduction function. These hormones include the gonadal regulatory hormones such as inhibin and activin, which function in regulating secretion of FSH from the pituitary gland (Vale *et al.*, 1986; Vale *et al.,* 1988).

The activity of spermatogenesis begins at puberty. During spermatogenesis, morphological changes occur in the Sertoli and spermatogenic cells i.e. the spermatogonia, spermatocytes and spermatids. The end product of spermatogenesis is the elongated spermatids, which are released during spermiasis to enter the epididymis and undergo maturation. The processes of spermatogenesis are controlled by the pituitary and gonadal hormones (Kurohmaru *et al.,* 2000). .

As there is limited information on the reproductive physiological data of the male lesser mouse deer, *Tragulus javanicus*, this study was conducted with the following objectives:

1. To describe the gross morphology and histological features of the male reproductive organ of lesser mouse deer (This includes the morphological

changes of the seminiferous tubules and spermatogenic cells during spermatogenesis at light microscopic levels).

2. To evaluate variation the testosterone concentrations of the male lesser mouse deer during in the research period both in dry and wet season.

3. To immunolocalize the site of gonadal regulatory hormones (activin, inhibin), and sex steroid hormones (progesterone, androgen and oestrogen) converting steroidogenic enzymes in the testes by specific immunohistochemistry.

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