



UNIVERSITI PUTRA MALAYSIA

***OESTRUS CYCLE, FEMALE REPRODUCTIVE SYSTEM MORPHOLOGY
AND TEMPERAMENT AND SPERM ATTRIBUTE OF RUSA DEER
(*Rusa timorensis* BLAINVILLE) IN CAPTIVITY***

MOHAMMED BAKARI MAHRE

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MOHAMMED BAKARI MAHRE

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

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DEDICATION

This thesis is dedicated to ALLAH; the Lord of the world; ‘the omniscience, the omnipotence’.



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

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October 2014

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Faculty: Veterinary Medicine

The reproductive biology of the Rusa deer (*Rusa timorensis*) farmed in Malaysia is not known. Therefore, this study was conducted to determine certain aspects of the reproductive biology of *R. timorensis* raised in the tropics with the objectives to investigate the temperament, describe the morphology of the female reproductive system, establish the oestrous cycle, develop a reference data for evaluation of reproductive status of *R. timorensis* and determine the oestrus response and pregnancy rate following oestrus synchronization with cloprostenol and natural mating as well as to determine the fertility potentials of the male *R. timorensis* through semen collection and evaluation. Seventeen adult *R. timorensis* hinds were selected for evaluation of temperament. To evaluate the temperament, well established methods from previous studies on cattle and red deer were adopted using flight time, crush test score and plasma cortisol assay. Description of the morphology of the female reproductive system were performed on two adult hinds after slaughter. Different reproductive structures were measured using a pair of calipers and a long string. Histology and Scanning Electron Microscopy were done according to the standard methods. To establish the oestrous cycle, five healthy adult hinds were selected for peripheral blood sampling to gauge the plasma progesterone levels by radioimmunoassay. Vaginal smears were also taken to characterize the oestrus stages of *R. timorensis* using vaginal cytology. Semen were collected from two and five fertile stags during the first and second breeding season respectively for semen evaluation. Semen samples were collected from the stags using an electro-ejaculator. The ejaculate was processed and samples prepared for light and scanning electron microscopy (SEM) according to standard methods. To determine the oestrus response and pregnancy rate in *R. timorensis* following oestrus synchronization with a prostaglandin analogue, oestrus was synchronized in eight cycling hinds with 500 µg cloprostenol given intramuscularly at 10 days apart. Eight other *R. timorensis* hinds were not-synchronized and served as control. Blood samples, collected every three to four days for 29 days from the first cloprostenol injection, were analyzed for plasma progesterone concentrations. Oestrus was recorded based on observation of oestrus signs during the period of blood sampling. The hinds were bred 24h after the second cloprostenol injection and all hinds were subjected to transrectal ultrasonography to assess pregnancy status. The results of this study have demonstrated that flight time, crush score and plasma cortisol concentration could be used for selecting *R. timorensis*

based on temperament for the breeding herd. The morphology of the female reproductive system of *R. timorensis* is similar to that observed in domestic ruminants except that the uterus did not have an interconual ligament and unlike domestic ruminants, the left ovary is slightly larger than the right ovary which indicates that it is physiologically more active. Unlike the cows and goats, the cervix of *R. timorensis* is characterized by six cervical rings projecting into the cervical canal. This feature should be taken into account when designing an Artificial insemination catheter for transcervical passage of semen during Artificial insemination in this species. Two cell types were observed in the epithelium of the reproductive tract: ciliated cells and non-ciliated secretory cells. Based on the cyclic basal plasma progesterone levels and the observations of changes in the cellular pattern of the vaginal epithelium, the mean length of oestrous cycle of *R. timorensis* deer farmed in Malaysia was estimated to be 19.2 ± 1.3 days with a range of 18 to 21 days. On the basis of changes in plasma progesterone concentrations, cloprostenol (prostaglandin analogue) induced oestrus in only five of the eight treated hinds. The other three treated hinds showed progesterone values (0.8 ng/mL) which appeared to be too low to indicate presence of a corpus luteum for the drug to act on. Five treated hinds and four control hinds displayed standing oestrus. Conception occurred in four treated and four control hinds. In the present study, oestrus synchronization has been successfully achieved using prostaglandin analogue. However, future studies need to refine synchronization regimes to increase oestrus synchrony and pregnancy rate. No significant difference ($P > 0.05$) was found between sperm attributes in comparison between different stags and different months of the fertile seasons. Semen volume as well as pH, sperm concentration, general motility, progressive motility, and viability were 2.2 ± 0.29 ml, 7.2 ± 0.17 , $886.3 \pm 39.7 \times 10^6$ spermatozoa/ml, 78.7 ± 2.01 %, 80.8 ± 1.85 % and 83.2 ± 0.85 % respectively. Morphological analysis showed low percentage (13.9 ± 2.88 %) of abnormal spermatozoa. In conclusion, the study provided baseline data on the reproductive biology of male and female *R. timorensis*, which will facilitate future researches that would lead to propagation of this vulnerable species of animal. The results obtained from this study can be used as a general reference by deer farmers in Peninsular Malaysia and local zoos to evaluate the reproductive status of *R. timorensis*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia dalam memenuhi keperluan bagi Ijazah Doktor Falsafah

**KITARAN ESTRUS, PERANGAI DAN MORFOLOGI SISTEM
PEMBIAKAN BETINA SERTA CIRI SPERMA RUSA (*RUSA TIMORENSIS*
BLAINVILLE) DALAM KURUNGAN**

Oleh

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Biologi pembiakan rusa (*Rusa timorensis*) yang ditenak di Malaysia tidak diketahui. Oleh itu, kajian ini dilakukan untuk menentukan aspek biologi pembiakan *R. timorensis* yang ditenak di tropika dengan objektif untuk mengenalpasti perangai, menghuraikan morfologi sistem pembiakan betina, menentukan kitaran oestrus, mewujudkan data rujukan untuk menilai status reproduksi *R. timorensis* dan menentukan tindakbalas estrus serta kadar kebuntingan selepas pensinkronian estrus dengan kloprosternol dan pengawanan semulajadi serta menentukan potensi kesuburan *R. timorensis* jantan melalui pengumpulan dan penilaian semen. Tujuh belas ekor *R. timorensis* betina dipilih untuk penilaian perangai. Untuk menilai perangai, kaedah yang telah terbukti dari kajian terdahulu pada lembu dan rusa merah digunakan untuk masa lari, skor ujian pasung dan asai kortisol plasma. Penghuraian mengenai morfologi sistem reproduksi betina dibuat keatas dua rusa betina dewasa selepas disembelih. Pelbagai struktur pembiakan diukur menggunakan tali panjang dan angkup. Histologi dan mikroskopi elektron penskanan dilakukan mengikut kaedah piawai. Untuk menentukan kitaran estrus, lima ekor rusa betina dewasa yang sihat dipilih untuk pensampelan darah bagi mengukur paras porsesteron plasma menggunakan kaedah radioimmunoasai. Saput vagina juga diambil untuk menggambarkan ciri peringkat estrus *R. timorensis* menggunakan kaedah sitologi vagina. Semen dikumpul daripada dua dan lima ekor rusa pejantan subur masing-masing semasa musim pembiakan pertama dan kedua untuk penilaian semen. Sampel semen dikumpul daripada pejantan menggunakan kaedah elektroejakulator. Semen diproses untuk penyediaan kaedah mikroskopi electron penskanan dan cahaya mengikut kaedah piawai. Untuk menentukan tindakbalas estrus dan kadar kebuntingan *R. timorensis* selepas pensinkronian estrus menggunakan analog prostaglandin, estrus tersinkroni bagi lapan ekor rusa betina dengan 500 µg kloprosternol yang diberikan secara suntikan intraotot selang 10 hari. Lapan ekor lagi *R. timorensis* betina lain tidak disinkroni dan bertindak sebagai kawalan. Sampel darah diambil setiap 3-4 hari untuk selama 29 hari bermula dari suntikan pertama kloprosternol dan dianalisis untuk kepekatan progesteron plasma. Estrus direkod berdasarkan pemerhatian petanda estrus semasa pensampelan darah. Rusa betina dibiak 24 jam selepas suntikan kedua kloprosternol dan kesemua rusa dilakukan ultrasonografi transrektum untuk menilai status kebuntingan. Keputusan kajian ini menunjukkan masa lari, skor ujian pasung dan kepekatan kortisol plasma boleh digunakan untuk memilih gerompok pembiakan

R. timorensis berdasarkan perangai. Morfologi sistem pembiakan betina *R. timorensis* adalah serupa dengan ruminan belajinak kecuali uterus tidak mempunyai ligamen interkornu dan tidak seperti ruminan belajinak, ovari kiri didapati lebih besar berbanding ovari kanan yang menunjukkannya lebih aktif secara fisiologi. Tidak seperti lembu dan kambing, servik *R. timorensis* mempunyai ciri enam gegelang servik mengunjur ke salur servik. Ciri ini perlu diambil kira apabila merekabentuk kateter permanian beradas untuk laluan transervik semen semasa permanian beradas spesies ini. Dua jenis sel diperhatikan pada epitelium trakus pembiakan: sel perembes bersilia dan tanpa silia. Berdasarkan paras progesteron plasma basal siklik dan pemerhatian perubahan pada corak sel epitelium vagina, purata kitaran estrus rusa *R. timorensis* yang ditenak di Malaysia dianggarkan selama 19.2 ± 1.3 hari dengan julat 18-21 hari. Berdasarkan perubahan kepekatan progesteron plasma, kloprosternol (analog prostaglandin) estrus teraruh pada hanya lima daripada lapan ekor rusa betina yang dirawat. Tiga ekor rusa lain yang dirawat menunjukkan nilai progesteron (0.8 ng/mL) yang kelihatannya amat rendah bagi menunjukkan kewujudan korpus luteum untuk membolehkan drug beraksi keatasnya. Lima ekor rusa yang dirawat dan empat ekor rusa kawalan menunjukkan estrus berdiri. Konsepsi berlaku pada empat ekor rusa yang dirawat dan empat ekor rusa kawalan. Tiada sebarang perbezaan bererti ($P > 0.05$) dalam tindakbalas estrus dan kadar kebuntingan di antara rusa kawalan dan yang dirawat. Rusa betina *R. timorensis* menunjukkan darjah ketinggian relatif bagi pensinkronian estrus secara semulajadi dan oleh itu pensinkronian buatan estrus menggunakan analog prostaglandin adalah tidak efektif. Tiada perbezaan bererti ($P > 0.05$) diperhatikan pada ciri sperma dibandingkan di antara rusa pejantan dan perbezaan bulan semasa musim subur. Isipadu semen, pH, kepekatan sperma, kemotilan am, kemotilan progresif dan daya maju masing-masing adalah $2.2 \pm 0.29 \text{ ml}$, 7.2 ± 0.17 , $886.3 \pm 39.7 \times 10^6$ spermatozoa/ml, $78.7 \pm 2.01\%$, $80.8 \pm 1.85\%$ dan $83.2 \pm 0.85\%$. Analisis morfologi menunjukkan peratus spermatozoa yang abnormal adalah rendah ($13.9 \pm 2.88\%$). Sebagai kesimpulan, kajian ini memberikan data asas biologi pembiakan *R. timorensis* jantan dan betina, yang berupaya membantu kajian di masa hadapan ke arah pembiakan haiwan spesies rentan ini.

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

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LIST OF ABBREVIATIONS

ART	assisted reproductive technology
CIDR	controlled internal drug release
D	Days
ECG	equine chorionic gonadotropin
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
H & E	haematoxylin and eosin
H	Hour
Kg	Kilogram
LH	luteinizing hormone
Mg	Milligram
mL	Milliliter
Mm	Millimeter
Mv	Microvilli
Ng	Nanogram
P ₄	progesterone
PGF _{2α}	prostaglandin F _{2α}
PMSG	pregnant mare serum gonadotropin
QC	quality control
RIA	radioimmunoassay
SEM	standard error of mean
SPSS	statistical package for social sciences
TPU	Taman Pertanian Universiti
UPM	Universiti Putra Malaysia

CHAPTER 1

GENERAL INTRODUCTION

The livestock industry in Malaysia is a valuable sector of the nation's economic development which provides employment and source of animal protein (meat, milk) to the population. Cattle, buffaloes, goats, sheep, pigs and poultry (chickens) are the most popular livestock in Malaysia. However, in recent years, the deer has also become important in Malaysia as an alternative source of meat, fur and velvet.

The delayed domestication of *Rusa timorensis* may be associated with their poor temperament and to date there is no published information on the temperament of the farmed *R. timorensis*. Understanding of *R. timorensis* temperament and selection programme for its evaluation in a breeding herd is important for the successful farming of *R. timorensis*.

Most of the farmed deer in Malaysia are of different species imported from different countries such as Australia, New Zealand, Mauritius, Indonesia and New Caledonia. The reproductive biology of *R. timorensis* farmed in Malaysia is unknown. This study was conducted to determine certain aspects of reproductive biology of *R. timorensis* farmed in Malaysia. The *R. timorensis* is listed as a vulnerable species by the International Union for Conservation of Nature and Natural Resources (IUCN, 2008). The lack of information on the reproductive biology of *R. timorensis* has cautioned investigation on artificial breeding of this 'vulnerable' species.

Successful assisted reproductive technology (ART) requires an in-depth knowledge in the basic aspects of reproductive biology. Very few mammalian species have been studied for the details of their reproductive biology and many of those studied were livestock and laboratory animals (Lasley *et al.*, 1994 and Wildt *et al.*, 1997) and a few studies about wild species (Comizzoli *et al.* 2000).

Deer species have different seasonal reproductive patterns; breeding is not seasonal in the axis deer (*Axis axis*), rusa deer (*Rusa timorensis*) and sambar deer (*Cervus unicolor*) whereas the red deer (*Cervus elaphus*) and sika deer (*Cervus nippon*) are seasonal breeders (Jabbour *et al.*, 1997). Treatment with melatonin is used as a method to control the circannual cycle of reproductive activity in the red deer (Asher *et al.*, 1994) but not for all seasonal species. The oestrous cycle length and gestation periods also vary between the different deer species.

The vaginal epithelium is influenced by hormonal changes during the oestrous cycle, allowing cyclic monitoring of the various reproductive stages in different species of animals (Miroud and Noakes, 1990; Ola *et al.*, 2006). The vaginal cytology of *R. timorensis* in Malaysia has not been reported before. Therefore, the present study also aimed to characterize the oestrus stages of *R. timorensis* using vaginal cytology.

The progesterone (P₄) concentration measured during the various oestrous cycle stages and pregnancy provided important information about reproductive status of the deer (Yamauchi and Matsuura, 2009). Plasma P₄ concentrations were determined to monitor the luteal function, oestrous cycle and seasonality of reproduction in Fallow

deer (Asher, 1985, Asher *et al.*, 1986, 1988). Plasma P₄ concentrations have also been used to predict ovulation and detect oestrus in some deer species such as the red deer (Adam *et al.*, 1985, Kelly *et al.*, 1982, Asher *et al.*, 1997, Asher *et al.*, 2000, Garcia *et al.*, 2002, Garcia *et al.*, 2003 and Asher *et al.*, 2011), Chita deer (Chapple *et al.*, 1993), white-tail deer (Plotka *et al.*, 1980) and Pere Davids deer (Curlewis *et al.*, 1988). No published information is yet available on the relationship between progesterone profiles and the oestrous cycle of *R. timorensis* raised in captivity in the tropics. The duration and progesterone profiles of the oestrous cycle of *R. timorensis* farmed in Malaysia have not been documented.

Few studies exist on the anatomy and histology of the female reproductive system of *R. timorensis*. To our knowledge, the first and only description of the female reproductive system of *R. timorensis* was reported by Hamilton *et al.* (1960) which briefly stated that the animal has a cotyledonary placentation with three large uterine caruncles. Information with respect to other parts of the female reproductive system of *R. timorensis* is not yet available. Therefore, this study describes the morphology of the female reproductive system of *R. timorensis* farmed in the tropics, encompassing the gross anatomical and histological aspects.

Examination of the structure and function of mammalian spermatozoa was conducted with the aid of light and electron microscopy (Hafez and Kanagawa, 1973). Microscopic description of normal and abnormal sperm cells of some deer species have been reported by Wislocki (1949), Bierschwal *et al.* (1970), Dott and Utsi (1971), Andersen (1973), Gosch *et al.*, (1989), Wahid *et al.*, (2000) and Amare (2009). To date, there is no published information on the microscopic description of normal and abnormal sperm of *R. timorensis*.

The ability of prostaglandin F_{2α} to prematurely regress the corpus luteum has been documented in the red deer (Fisher *et al.* 1994; Asher *et al.* 1995), wapiti (Glover, 1985), fallow deer (Jabour *et al.* 1993; Asher *et al.* 1989), Pere David's deer (Curlewis *et al.* 1988), white tail deer (Haigh, 1984; Magyar *et al.* 1988) and reindeer (Ropstad *et al.* 1996). However, no information is available on the efficacy of cloprostenol in inducing oestrus in *R. timorensis*.

To date, there is no published information on the reproductive status of *R. timorensis* farmed in Malaysia and to our knowledge, the reproductive biology of *R. timorensis* has not been well documented. The scarcity of information on the reproductive biology of *R. timorensis* warrants investigation on artificial breeding of this vulnerable species.

1.1 Hypotheses of the study

Ho1 = The oestrous cycle of *R. timorensis* is similar to that of other deer species.

Ho2 = Treatment with 500 µg cloprostenol given 10 days apart is an effective method of oestrus synchronization in *R. timorensis*.

Ho3 = The morphology of the female reproductive system of *R. timorensis* is similar to domestic ruminants.

Ho4 = Flight time test and crush test score are effective for evaluating the temperament of *R. timorensis*.

Ho5 = Semen characteristics of *R. timorensis* are similar to those of other deer species.

1.2 General Objective: The general objective of the study was to determine certain aspects of reproductive biology of *R. timorensis* farmed in Malaysia.

1.3 Specific Objectives of the study

The specific objectives of this study were:

1. To investigate the temperament of *R. timorensis* and determine its relationship to stress.
2. To establish the oestrous cycle of *R. timorensis* raised in captivity in the tropics.
3. To describe the morphology of the female *R. timorensis* reproductive system.
4. To determine the fertility potential of the male *R. timorensis* through semen collection and evaluation.
5. To determine the oestrus response and pregnancy rate in *R. timorensis* following oestrous synchronization with cloprostenol and natural mating respectively.
6. To develop a reference data for evaluation of the reproductive status of female *R. timorensis*.

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