การคัดเลือกตัวอสุจิเพื่อลดผลกระทบจากความผิดปกติของตัวอสุจิในเสือลายเมฆ

นางสาววัลยา ทิพย์กันทา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาการสืบพันธุ์สัตว์ ภาควิชาสูติศาสตร์-เธนุเวชวิทยาและวิทยาการสืบพันธุ์
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558

OVERCOMING SPERM ABNORMALITIES BY SPERM SELECTION IN CLOUDED LEOPARDS (*Neofelis nebulosa*)

Miss Wanlaya Tipkantha

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Theriogenology
Department of Obstetrics Gynaecology and Reproduction
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2015

Thesis Title	OVERCOMING SPERM ABNORMALITIES BY SPERM SELECTION IN CLOUDED LEOPARDS (*Neofelis nebulosa*)
By	Miss Wanlaya Tipkantha
Field of Study	Theriogenology
Thesis Advisor	Associate Professor Dr. Kaywalee Chatdarong, D.V.M., M.Sc., Ph.D.
Thesis Co-Advisor	Dr. Pierre Comizzoli, D.V.M., Ph.D.

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Veterinary Science
(Professor Dr. Roongroje Thanawongnuwech, D.V.M., M.Sc., Ph.D.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Dr. Padet Tummaruk, D.V.M., M.Sc., Ph.D.)

.....Thesis Advisor
(Associate Professor Dr. Kaywalee Chatdarong, D.V.M., M.Sc., Ph.D.)

.....Thesis Co-Advisor
(Dr. Pierre Comizzoli, D.V.M., Ph.D.)

.....Examiner
(Assistant Professor Dr. Theerawat Tharasanit, D.V.M., Ph.D.)

.....Examiner
(Dr. Theerawat Swangchan-uthai, D.V.M., Ph.D.)

.....External Examiner
(Dr. Boripat Siriaroonrat, D.V.M., M.Sc., Ph.D.)

.....External Examiner
(Associate Professor Dr. Nikorn Thongtip, D.V.M., Ph.D.)

วัลยา ทิพย์กันทา : การคัดเลือกตัวอสุจิเพื่อลดผลกระทบจากความผิดปกติของตัวอสุจิในเสือลายเมฆ (OVERCOMING SPERM ABNORMALITIES BY SPERM SELECTION IN CLOUDED LEOPARDS (*Neofelis nebulosa*)) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. สพ.ญ. ดร. เกวลี ฉัตรดรงค์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. ปิแอร์ คอมิซโซลี, 101 หน้า.

เสือลายเมฆเป็นหนึ่งในสัตว์ป่าตระกูลเสือใกล้สูญพันธุ์ของเอเชียตะวันออกเฉียงใต้ ประชากรในธรรมชาติลดลงอย่างต่อเนื่องจากการบุกรุกถิ่นที่อยู่อาศัยและการล่า การเพาะขยายพันธุ์ในสภาพการเพาะเลี้ยงมีความสำเร็จค่อนข้างจำกัดเนื่องจากพฤติกรรมความดุร้ายของตัวผู้ การผลิตน้ำเชื้อที่มีตัวอสุจิโครงสร้างผิดปกติสูงและความไม่แน่นอนของการตกไข่ในตัวเมีย การพัฒนาเทคนิคช่วยการสืบพันธุ์จึงมีความจำเป็นเพื่อนำมาช่วยในการเพาะขยายพันธุ์เสือลายเมฆ การศึกษานี้มีวัตถุประสงค์เพื่อ 1) ศึกษาคุณลักษณะของน้ำเชื้อของเสือลายเมฆที่อยู่ในสภาพการเพาะเลี้ยงของประเทศไทย 2) ประเมินคุณสมบัติของตัวอสุจิที่ผ่านการคัดเลือกด้วยวิธีปั่นแยกเดี่ยว 3) ศึกษาสาเหตุของการเกิดตัวอสุจิหางขดและการลดผลกระทบด้วยวิธีการคลายหาง 4) ประเมินการตอบสนองของระบบสืบพันธุ์เพศเมียภายหลังการให้ฮอร์โมนเพศจากภายนอก และ 5) พัฒนาเทคนิคการผสมเทียมด้วยวิธีการส่องกล้องและปล่อยน้ำเชื้อบริเวณท่อนำไข่ โดยทำการศึกษาในกลุ่มประชากรเสือลายเมฆที่อยู่ในสภาพเพาะเลี้ยง (เพศผู้ 11ตัว และเพศเมีย 6 ตัว) ในระหว่างปี 2556 – 2558 การศึกษาในเพศผู้ ทำการประเมินคุณภาพน้ำเชื้อ (22 ตัวอย่าง) ที่ได้จากการรีดเก็บด้วยการกระตุ้นด้วยกระแสไฟฟ้า ผลการตรวจประเมินน้ำเชื้อพบว่าตัวอสุจิของเสือลายเมฆมีลักษณะความผิดปกติทางโครงสร้างจำนวนมาก (63.9 ± 2.0%) และพบลักษณะหางขดมากที่สุด (13.5 ± 0.5 %) ทั้งนี้ ในกลุ่มเสือตัวผู้ที่มีการจับคู่แล้วพบค่าความสมบูรณ์ของอะโครโซมมากกว่าและตัวอสุจิที่มีความผิดปกติส่วนคอน้อยกว่ากลุ่มอยู่เดี่ยวอย่างมีนัยสำคัญ ($P < 0.05$) จึงได้ทำการศึกษาเปรียบเทียบการปั่นล้างและปั่นแยกเดี่ยวในสารคอลลอยด์เพื่อแยกตัวอสุจิที่มีคุณภาพดีก่อนการแช่แข็ง (12 ตัวอย่าง) ผลการศึกษาพบว่าค่าเฉลี่ยของการเคลื่อนไหว ความสมบูรณ์ของอะโครโซมและตัวอสุจิหางปกติ ในกลุ่มปั่นแยกเดี่ยวภายหลังการแช่เย็นและแช่แข็งมากกว่ากลุ่มควบคุม ($P < 0.05$) นอกจากนี้การทดสอบความสามารถในการปฏิสนธิของตัวอสุจิภายหลังการอุ่นละลายด้วยวิธีการปฏิสนธิภายนอกร่างกายกับโอโซไซต์ของแมวบ้าน พบว่าตัวอสุจิในกลุ่มที่ผ่านการคัดเลือกด้วยวิธีปั่นแยกเดี่ยวมีอัตราการปฏิสนธิมากกว่ากลุ่มควบคุม ($P < 0.05$) การศึกษาเพื่อแยกสาเหตุของการเกิดลักษณะตัวอสุจิหางขดเป็นจำนวนมากในน้ำเชื้อเสือลายเมฆ (11 ตัวอย่าง) ด้วยการตรวจประเมินความสมบูรณ์ของผนังเซลล์ด้วยสารละลายความเข้มข้นร่วมกับการใช้สาร Triton-X 100 (TX) ความเข้มข้นร้อยละ 20 ในการช่วยคลายหางขดเพื่อประเมินว่าลักษณะตัวอสุจิหางขดที่พบเป็นลักษณะความผิดปกติที่เกิดก่อนหรือหลังกระบวนการหลั่งน้ำเชื้อ โดยแบ่งกลุ่มทดลองออกเป็น 2 กลุ่ม ได้แก่ กลุ่มที่พบตัวอสุจิหางขดมากกว่าร้อยละ 10 และกลุ่มที่ผ่านการปั่นแยกเดี่ยวมีตัวอสุจิหางขดไม่เกินร้อยละ 6 ผลการศึกษาพบว่าอัตราการคลายหางขดก่อนและหลังการเติมสารความเข้มข้นต่ำร่วมกับสาร TX ไม่แตกต่างกันในแต่ละกลุ่ม ดังนั้น ลักษณะการเกิดอสุจิหางขดส่วนใหญ่ที่พบอาจเกิดก่อนกระบวนการหลั่งน้ำเชื้อ แต่อย่างไรก็ตามในกลุ่มที่ทำการปั่นแยกเดี่ยวมีจำนวนตัวอสุจิที่สามารถคลายหางได้มากกว่ากลุ่มควบคุม การศึกษาในเสือเพศเมีย ศึกษาการตอบสนองของระบบสืบพันธุ์ภายหลังได้รับฮอร์โมนเพศจากภายนอกจากการเปลี่ยนแปลงของระดับเมตาโบไลต์ของฮอร์โมนเอสโตรเจนและโปรเจสเตอโรนในอุจจาระ แบ่งเป็น 2 กลุ่ม ดังนี้ กลุ่มที่ 1 (จำนวน 2ตัว) ให้ฮอร์โมน eCG ขนาด 300 IU และ pLH 1500 IU ห่างกัน 82 ชั่วโมง และกลุ่มที่ 2 (จำนวน 3 ตัว) ให้ฮอร์โมน eCG ขนาด 200 IU และ pLH 1000 IU ห่างกัน 82 ชั่วโมง ภายหลังการกระตุ้นด้วยฮอร์โมนพบว่าเสือทั้ง 5 ตัว แสดงอาการเป็นสัดและการเปลี่ยนแปลงของระดับฮอร์โมน ภายหลังการตรวจประเมินการตอบสนองของระบบสืบพันธุ์ต่อด้วยการส่องกล้อง พบว่าเสือในกลุ่มที่ 1 มีฟอลลิเคิลขนาดใหญ่หลายใบ จึงได้ทำการเก็บโอโอไซต์ด้วยวิธีการเจาะดูดผ่านช่องท้องภายหลังการให้ฮอร์โมน pLH 29 ชม. และนำไปปฏิสนธิภายนอกร่างกายกับน้ำเชื้อแช่แข็ง พบการแบ่งตัวเป็นตัวอ่อนระยะ 2 เซลล์ 1ใบ (11%) ในกลุ่มที่ 2 เสือทั้ง 2 ตัวมีการตกไข่จึงได้ทำการย้ายฝากตัวอ่อนเข้าในแม่รับ 43 ชม.หลังผสมและทำการทดลองผสมเทียมด้วยน้ำเชื้อแช่แข็งที่ผ่านการคัดเลือกผ่านทางปีกมดลูกภายหลังการให้ฮอร์โมน pLH 44 ชม. แต่ในสัตว์ทั้งสองตัวไม่พบรายงานการตั้งท้อง การศึกษาวิธีการผสมพันธุ์ด้วยเทคนิคการส่องกล้องและปล่อยน้ำเชื้อบริเวณท่อนำไข่ โดยทำการศึกษาในตัวเมีย 4 ตัว ที่ได้รับ eCG 200 IU และ pLH 1000 IU ห่างกัน 82 ชม การตรวจระบบสืบพันธุ์หลังการให้ pLH 44 ชม พบตัวเมียที่ไม่เคยมีลูกเกิดการตกไข่จากรังไข่ทั้ง 2 ข้าง จึงได้ทำการผสมเทียมเข้าบริเวณท่อน้ำไข่ ด้วยน้ำเชื้อแช่เย็นจากตัวผู้ 2 ตัว มีจำนวนอสุจิเคลื่อนไหว 8 ล้าน และ 2.7 ล้านตัว ภายหลังการผสมเทียมพบการเพิ่มขึ้นของฮอร์โมนโปรเจสเตอโรนสูงสุดที่ 25 วัน หลังการให้ pLH และคงอยู่ที่ระดับ 128.4 µg/g นาน 65 วันก่อนลดลงตามลำดับ ภายหลังการผสมเทียม 90 วัน พบการคลอดลูกเสือสุขภาพดี จำนวน 2 ตัว จากการศึกษานี้จะเห็นได้ว่า การคัดเลือกตัวอสุจิคุณภาพดีร่วมกับการประยุกต์ใช้เทคนิคช่วยการผสมพันธุ์ที่เหมาะสมช่วยเพิ่มโอกาสความสำเร็จในการเพาะขยายพันธุ์เสือลายเมฆเพื่อคงความหลากหลายทางพันธุกรรมในกลุ่มประชากรได้ต่อไป

ภาควิชา	สูติศาสตร์-เธนุเวชวิทยาและวิทยาการสืบพันธุ์	ลายมือชื่อนิสิต	.....
สาขาวิชา	วิทยาการสืบพันธุ์สัตว์	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
ปีการศึกษา	2558	ลายมือชื่อ อ.ที่ปรึกษาร่วม	.....

5575402931 : MAJOR THERIOGENOLOGY
KEYWORDS: CLOUDED LEOPARD / TERATOSPERMIA / SPERM SELECTION / SINGLE LAYER SENTIFUCATION / HETEROLOGOUS IN VITRO FERTILIZATION / ARTIFICIAL INSEMINATION

WANLAYA TIPKANTHA: OVERCOMING SPERM ABNORMALITIES BY SPERM SELECTION IN CLOUDED LEOPARDS (*Neofelis nebulosa*). ADVISOR: ASSOC. PROF. DR. KAYWALEE CHATDARONG, D.V.M., M.Sc., Ph.D., CO-ADVISOR: DR. PIERRE COMIZZOLI, D.V.M., Ph.D., 101 pp.

Clouded leopard (CL) is an endangered felid species of Southeast Asia. Wild populations are declining due to habitat loss and hunting in range countries. CL breeding success in captivity is limited due to male aggression, high incidence of abnormal sperm and uncertain ovarian activity of females. Therefore, the development of optimal assisted reproductive technologies for this species is recommended by CL specialist group. This study aimed to 1) investigate sperm characteristic of captive clouded leopards in Thailand; 2) determine the functional properties of sperm after selection with the single layer centrifugation (SLC) method; 3) identify the origin of coiled tail defects and mitigate them using a demembranation approach; 4) assess the reproductive organs and ovarian activity after hormonal treatment; and 5) develop laparoscopic oviductal artificial insemination technique for CL. Captive CL (n=17, 11 males and 6 females) have been studied during 2013 - 2015. In male study, twenty-two ejaculates were collected by electroejaculation and evaluated. The high proportion of morphologically abnormal sperm (63.9 ± 2.0%) was observed with tightly coiled tail as a major defect (13.5 ± 0.5 %). CL males that are paired with females showed significantly higher percentage of sperm with intact acrosome, and lower number of sperm with bent midpiece/cytoplasmisc droplet than the males housed singly ($P < 0.05$). Two sperm preparation methods including 1) simple washing; or 2) SLC, was applied prior to cryopreservation (n=12). Sperm motility, % sperm with intact acrosome and % sperm with normal tail in chilled and frozen-thawed semen samples were increased after SLC treatment. The heterologous IVF in the SLC-processed group showed significantly higher fertilization success than the simple washing group ($P < 0.05$). To investigate the potential origin of coiled tail, 11 ejaculates were evaluated and equally allocated to simple washing (control group; resulting in about 10% coiled tails after treatment) and SLC (treated sample; resulting in about 6 % coiled tails after treatment). Aliquots of semen were subjected to hypo-osmotic swelling (HOS) test and demembranation test using 20% Triton X-100 (TX). After TX treatment, most of the coiled tail in raw ejaculates could not be uncoiled or opened by TX indicating that the cause of coiled sperm tails may be caused by testicular origin. SLC demonstrated its ability to decrease the primary sperm defects of raw semen. SLC-selected spermatozoa were prone to be mitigated after demembranation. In females study, fecal estradiol (E2) and progesterone (P4) metabolites of adult female were quantified by enzyme immunoassay (EIA). Females were given exogenous gonadotropins; Group 1 (n=2) 300 IU eCG/1500 IU of pLH (82 h interval); and Group 2 (n=3) 200 IU eCG/1000 IU pLH (82 h interval). Ovarian assessment was performed at 29 h, 44 h and 96 h post pLH administration using laparoscopy. Estrus signs have been observed in all females. Oocytes were collected from two females of group 1 by OPU technique and were subjected to homologous IVF with post-thawed SLC-selected spermatozoa. A two-cell stage embryo was transferred to recipient (n=1) with no conception. For AI, the ovulated female (n=1) were inseminate with frozen-thawed selected sperm at 44 h after pLH administration. However, no pregnancy occured after 90 days post insemination. The optimized stimulating protocol for AI (eCG 200 i.u. and pLH 1000 i.u. with 82 hr interval) was applied in four CL females. Ovarian assessment was performed at 44 hr after pLH administration. One nulliparous female ovulated from both ovaries. Semen of two adult males with total motile sperm of 8×10^6 (M1) and 2.7×10^6 (M2) were used for AI by laparoscopic oviductal technique. Increasing of fecal progesterone concentration after AI presented the peak at day 25 post pLH injection and maintained at 128.4 µg/g dry feces during 65 day post AI indicating that the female had conceived. Delivery of two healthy CL cubs occurred on day 90 of gestation. In conclusion, sperm selection for good quality spermatozoa with optimal assisted techniques can be promising tools to assist in conservation breeding and sustaining genetic variation of this endangered species in the future.

Department:	Obstetrics Gynaecology and Reproduction	Student's Signature	.....
Field of Study:	Theriogenology	Advisor's Signature	.....
Academic Year:	2015	Co-Advisor's Signature	.....

ACKNOWLEDGEMENTS

The authors' PhD program and this study was supported by grants from the Royal Golden Jubilee PhD program of Thailand Research Fund (PHD/0100/2555), the 90th Anniversary of Chulalongkorn University Fund, Thailand Research Fund and Research Unit of Obstetrics and Reproduction in Animals. This study was taken place at the Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, the Thailand Clouded Leopard Consortium Project, Khao Kheow Open Zoo and Bureau of Conservation and Research, Zoological Park Organization of Thailand. I would like to express my deeply grateful acknowledgement to Assoc.Prof. Dr.Kaywalee Chatdarong, for supervising the project planning and publication writing skill from the beginning through the end of my PhD research and her support, encouragement and giving me a chance to learn many kinds of techniques of Assisted Reproductive Technologies (ARTs) which could be useful for conducting the advance research in the future. I also thank Dr.Pierre Comizzoli for co-supervising in USA both personal and scientific teaching, his support and main technical advice on my research. I would like to thanks, Dr. Sumate Kamolnorranath and Dr. Boripat Siriaroonrat, for inspiring me to the wildlife conservation works and giving me a great chance to learn and work with various wonderful jobs. This PhD study could not be done without the greatly support from the Thailand Clouded Leopard Consortium Project team. Many thanks for Mr.Bill Wood, Ms.Napapat Sumrantin and clouded leopard keepers team (Yok, P'Dong, Plern) who assisted animal management and kindly helping on most of animal preparation procedure throughout my study. I also thank Dr.Umapron Maikeaw and Khao Kheow Open Zoo team, Dr Kwanruen Duangsa-ard and Chiangmai Zoo team, Dr. Saowaphang Sananu and Dusit Zoo team, for helping on animal anesthesia, sample collection, AI procedure in this study and for their kindness and friendship. Many thanks Dr. Paweena Thuwanut, Dr. Chommanart Thongkittidilok and Dr. Saritvich Panyaboriban for her kindly suggestion and helping on laboratory techniques. Thank you Dr.Nae Tanpradit for his kindly help and all assisting throughout my experimental studies, technical supports and advices. I would like to thank Ms.Junpen Suwimonteerabutr for her suggestion, helping me setting up all of my laboratory works and chemical/equipment preparation. Thanks to Dr.Nikorn Thongtip and Dr. Ampika Thongphakdee for advise me about scientific ideas and shared the knowledge of reproductive physiology. I would like to thank Asst. Prof. Dr. Theerawat Tharasanit for helping me to learn and set up the embryo production laboratory. I also would like to thank Ms. Saifon Yapila and Mr. Chainarong Punkong for her and his assistance in hormonal analysis. Thanks to Dr.Worawidh Wajjawulku, Dr.Sirinart Chaichanathong for helping on molecular genetic analysis. I also would like to special thanks to Assoc. Prof. Dr. Padet Tummaruk and Dr. Em-on Olanratmanee for helping me with statistical analysis. I also would like to thanks Dr. Nucharin Songsasen and Dr. Budhan Pukazhenthi for giving me the excellent opportunity to learn the research, conservation knowledge, laboratory practice and always taking care of me during my research time in USA. I am so grateful to other OGR staffs and postgraduate students for our friendship and encouragement and helping me in document preparation. Finally, I would like to dedicate this work to my family for their support, understanding, encouragement and always being right beside me. They are the most important part in my life.

CONTENTS

Page

Page

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AI	artificial insemination
ARTS/ART	assisted reproductive technologies
CH	corpus hemorrhagecum
CL	corpus luteum
Cm	centimeter
DTT	dithiothreitol
E_2	estradiol or 17β- estradiol
eCG	equine chorionic gonadotropin
EE	electro-ejaculation
ET	embryo transfer
g	gram
h	hour
hCG	human chorionic gonadotropin
HIC	head in coiled tail
HOS	hypo osmotic swelling
IM	intramuscular
IVF	in vitro fertilization
IVM	in vitro maturation
IVC	in vitro culture
IU	international unit
Kg	kilogram
LH	luteinizing hormone
LOAI	laparoscopic oviductal artificial insemination
m	month
mOsm	milliosmole
mg	milligram
min	minute
mL	milliliter

mM	millimolar
mm	millimeter
ng	nanogram
OPU	ovum pick-up
pg	pictogram
P4	progesterone
SAS	statistical analysis system
SD	standard deviation
SEM	standard error
SLC	single layer centrifugation
TX	triton-X
v/v	volume/volume
y	year
µL	microliter
µg	microgram

CHAPTER I

Introduction

1.1 Importance and Rationale

The clouded leopards (*Neofelis nebulosa*) are medium-sized wild felid living in the tropical forest of Southeast Asia (Kitchener et al., 2006). To date, the wild populations are declining due to the over exploitation, habitat destruction, range defragmentation, and illegal hunting (Grassman L et al., 2015). They are now classified as a vulnerable species by the IUCN red list of threated species that indicating the risk to extinction in near future. In wild population, they are considered as an arboreal species due to spending considerable amount of time on the tree for resting and hunting in the primary evergreen tropical forest where virtual observation is difficult. Accordingly, little is known of their biology and behavior. Most current knowledge of their social behavior and reproductive biology are acquired from zoo-based population. It has been stated that this species is one of most challenges and difficult to breed naturally in captivity. A high incidence of male aggression and lethal attack on females have frequently occurred when unfamiliar adults of different sex are introduced for pairing even in estrous period (Yamada and Durrant, 1989; Howard et al., 1997). Pairing at pre-pubertal age (< 1 y) is likely to success in several breeding pairs but it need long-term observation. As the population decline is critical, assisted reproductive technologies (e.g. gamete cryopreservation, artificial insemination) along with natural breeding augmentation are required as an equal level to recover its population. Moreover, these techniques enable movement of genetic materials rather than living animals between geographically different populations and can also be used to breed sexually incompatible individuals (Wildt and Roth, 1997).

Moreover, two decades, the fundamental studies on reproduction of clouded leopard have conducted to gain better understanding of their biology and physiology serving for develop the optimal assisted reproductive techniques in this species.

According to previous studies, semen and sperm characteristic of adult males demonstrated a high proportion of abnormal morphological sperm or the teratospermic characteristics is commonly found in captive population (Wildt et al., 1986b; Pukazhenthi et al., 2006a). These studies stated that the possible cause of high incidence of sperm abnormality may be the lack of genetic diversity of the originally small founder population (Pukazhenthi et al., 2001). The malformed sperm is well recognized that do not participate in fertilization according to the reduced ability to capacitate, undergo the acrosome reaction, bind and penetrate the zona pellucida and condense within ooplasm (Pukazhenthi et al., 2001; Neubauer et al., 2004). Moreover, the defect sperm are susceptible to cold-induced acrosome damage that impaired the sperm quality after freezing-thawing cycle (Pukazhenthi et al., 2006b). Thus, selection of morphologically normal sperm may enhance post-thawed survival and fertilizing ability. Recently, sperm selection technique using single-layer centrifugation (SLC) through colloid has improved quality of ejaculated spermatozoa (Morrell et al., 2009a; Morrell et al., 2009b; Morrell et al., 2010; Lindahl et al., 2012; Alvarez-Rodriguez et al., 2016) and epididymal spermatozoa (Chatdarong et al., 2010). However, the SLC technique has never been applied in endangered felid species, especially those of teratospemic donors such as the clouded leopards (*Neofelis nebulosa*). Therefore, it is interesting to investigate whether the SLC could improve quality of male gametes from valuable donors who are affected by teratospermia.

Thailand is one of the range countries of habitats for the clouded leopards. To conserve this valuable species, an international consortium between Thailand and the united states (USA) consisting of five parties; 1) the Zoological Park Organization of Thailand (ZPO), 2) Smithsonian's National Zoological Park (NZP), 3) Nashville Zoo, 4) Point Defiance Zoo & Aquarium (PDZA), and 5) the Clouded Leopard Species Survival Plan® has established a clouded leopard breeding and research program at Khao Kheow Open Zoo (KKOZ) in Chonburi province of Thailand in 2002. Over the decade, 70% live cubs have been produced from calm breeding pairs (20% of population). There are about 32% adult individuals have lived unpaired without production (Marti and Breitbeil, 2013). Therefore, natural breeding in captivity has not been sufficient to sustain a viable population. To help these unpaired animals having a possibility to

produce offspring will need assisted reproductive technologies. Artificial insemination (AI) represents a powerful tool to propagate and manage genetically variation in various domestic species. To date, laparoscopic intraoviductal (LO) AI was superior in that ten folds fewer numbers of motile sperm were required to achieve a satisfactory pregnancy outcomes comparing to intrauterine technique (Tsutsui et al., 2001). Moreover, LOAI has already proven to produce non-domestic cats included the ocelot (*Leopardus pardalis*) and three viable kittens in the Pallas' cat (*Otocolobus manul*) (Swanson, 2012). The procedure likely benefit to the teratospermic donor as clouded leopards because they produce low number of structurally normal spermatozoa. In addition, intact acrosome spermatozoa tend to dramatically decline after post-thawed. Therefore, to select small population of good quality spermatozoa in term of motility, normal morphology, intact acrosomal and DNA would be benefit for applying with this novel technique.

1.2 Literature reviews

1.2.1 Taxonomy and biology of the clouded leopard

The clouded leopards (*Neofelis nebulosa*) are medium-sized wild felid living in the tropical forest of Asia. According to the taxonomy, they belong to the order Carnivora, family Felidae and genus Neofelis. Historically, only one species was recognized and four subspecies were classified according to morphological and geographic variation (Ellerman et al., 1966; Kitchener, 1998; Kitchener et al., 2006). Currently, there are two species of clouded leopard existing in the world classified by the molecular distinction (Wilting et al., 2007; Wilting et al., 2011) include 1) mainland Asia clouded leopard (*Neofelis nebulosa*) and 2) Sunda clouded leopard (*Neofelis diardi*). The mainland Asia species has three subspecies distribute along the Southern to Eastern part of Asia whereas the Sunda or Sundaland species is endemic to Borneo and Sumatra islands Peninsula (Table 1 and Figure 1).

Table 1 Current species, subspecies and the geographical distribution of the clouded leopard. Reprinted and modified from Kitchener et al. (2006) with permission from Elsevier.

Species	Geographical distribution
Neofelis nebulosa	
Subspecies	
Neofelis nebulosa nebulosa	Southeast Asia and China
Neofelis nebulosa macrosceloides	India, Nepal, Sikkim, Bhutan
Neofelis nebulosa brachyuran	Taiwan
Neofelis diardi	Sumatra, Borneo

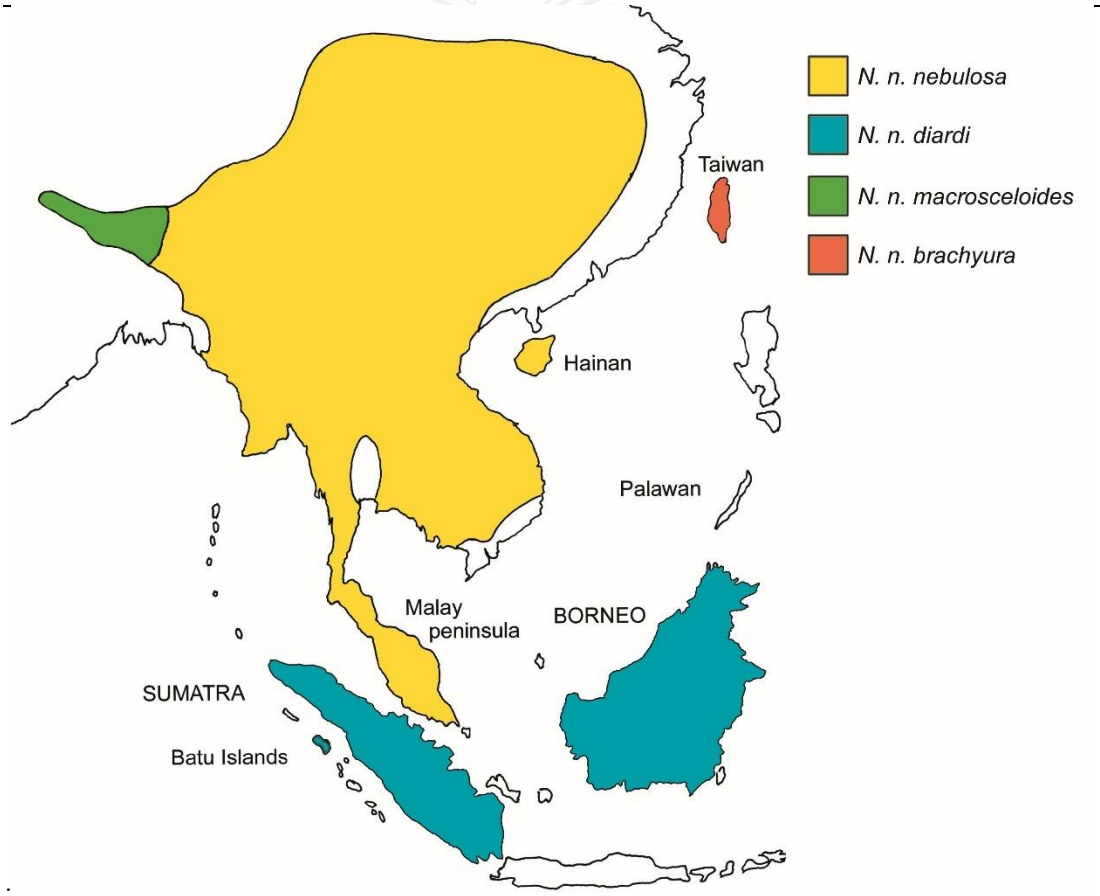


Figure 1 Geographic range of clouded leopard species and subspecies. Modified from Kitchener et al. (2006) with permission from Elsevier.

The mainland clouded leopards are mainly living in the tropical forest of Southeast Asia (Kitchener et al., 2006). They have a beautiful coat skin look like the cloud patterns. Generally, they have long body structure (1.3 – 2 meters), short legs and very long tails (about the body size). The body weight of adult individuals ranges from 11-20 kilograms and males tend to be larger than females (Figure 2). In ecology system, clouded leopards play important role to control the variety of prey species from small to medium-sized vertebrates including terrestrial and arboreal species that make the ecosystem balance. To date, few studies have performed in wild population. A few study on clouded leopard biology and ecology was mainly gained from Sunda clouded leopard revealed that they are solitary. Male has a home range overlapping to several females. Both species are threaded by human activities and invasions to their range habitats resulting in declining of the wild population.

Figure 2 Physical morphologies of adult female (left) and male (right) clouded leopards (*Neofelis nebulosa*) taken at Khao Kheow Open Zoo in 2016. This figure was provided by Bill Wood.

1.2.2 Reproductive physiology of the clouded leopard

Over two decades, the clouded leopards in the North American population have performed the systematic studies gaining valuable information for better understanding on clouded leopard reproductive physiology. The reproductive performance and circannual hormonal profiles in both males and females (Wildt et al., 1986a; Wildt et al., 1986b). In the males, semen quality, serum cortisol, luteinizing hormone (LH) and testosterone concentrations were influenced by natural fluctuations in photoperiod with the increasing of number of motile spermatozoa per ejaculate and peaked in June and July of the subtropical climate in the North America (Wildt et al., 1986b). A high proportion of structurally abnormal sperm with the predominant deformity being a primary defect (tightly coiled tail) was presented in studied population (Wildt et al., 1986b; Pukazhenthi et al., 2006a). In contrast, the retrospective study in female reproductive performance revealed the peak of female parturition in March and April, indicating the estrus period occurred from late of December through February (Wildt et al., 1986a). This physiological asymmetry in peak reproductive performance between males and females likely contributes to the poor offspring production of captive clouded leopard in the North America.

The study of non-invasive fecal steroid monitoring in females clouded leopards provided useful information on the reproductive cycle (Brown et al., 1995). The fecal estradiol or 17β- estradiol (E_2) profiles demonstrated the duration of estrus cycle of 24 ± 2 days with estrus lasting 6 ± 1 days; the gestation length was 89 ± 2 days; non-pregnant luteal phase was 47 ± 2 days. Moreover, the females clouded leopard in North America experienced a seasonal anestrus during the late summer and early fall (Brown et al., 1995). In addition, the monitoring long-term of fecal progesterone (P_4) concentration throughout the year revealed that they could ovulate spontaneously in the absence of mating. The fecal steroid hormone profiles serve as a useful adjunct to develop assisted reproductive technologies, especially the hormonal induction of ovulation for AI. Consequently, it was applied to develop a timing laparoscopic

intrauterine AI that was the first success of producing two clouded leopard cubs (Howard et al., 1996).

1.2.2. Sperm maturation in the felids

The development and maturation of mammalian spermatozoa occur in epididymal regions. Ultrastructure of epididymal ducts are different according to different regions. In feline, epididymal ducts are divided into six regions (1 to 6) according to the morphometric and histological characteristics (Axner, 2006). Briefly, regions 1 to 4 are localized within the caput where increase in sperm concentration is marked; region 5 is in the corpus, and region 6 is in the cauda of the epididymis. Each segment is lined with different type of epithelial cell that function on sperm development and maturation by producing certain characteristics of epididymal fluid. The epididymal fluid plays important roles on producing specific luminal environmental which is generally low in pH, bicarbonate, Na^+ and Ca^{2+} concentrations, but high in K^+ concentration and osmolality. This environment is able to maintain sperm viability for several weeks. Motility of spermatozoa occurs when sperm environment is changed during ejaculation. Seminal fluid in the ejaculate contributes to elevation of pH and bicarbonate, Na^+, and Cl^- that likely promote motility (Axner, 2006). Moreover, the rapid change of sperm environment causes K^+ decrease and change of osmotic milieu. Most spermatozoa liberate their distal droplets after diluted in hypo-osmotic fluid (Cooper and Yeung, 2003). However, the previous study revealed that diluting cat epididymal sperm with hypo-osmotic fluid (288 mOsm PBS) reduced the percentage of distal droplets but induced sperm tail bending than the formal saline fixative (Axner et al., 2004). Investigations of composition of epididymal fluid in various species are beneficial for developing extenders suitable for sperm preservation in individual species (Sanchez-Partida et al., 1997; Luvoni, 2006). Most recently, the occurrence of a high incidence of sperm tail defects from ejaculated samples and epididymal samples in a male domestic cat resembling the known 'Dag-like' defect (Villaverde et al., 2013). The ‘Dag-like’ defect is a heavily coiled sperm tails containing several axonemal units enclosed in the same common cell membrane. This

phenomenon has been previously described in other domestic species which may be involved a testicular origin.

1.2.3 Sperm characteristic of wild felids and clouded leopard

The Felidae is comprised of 37 species. Most species are currently threatened or endangered by extinction. The previous studies of semen traits of felid species revealed that about 70 % of all species are commonly found teratospermia (Pukazhenthi et al., 2001; Pukazhenthi et al., 2006b) with over 60 % morphologically abnormal sperm. This phenomenon is common in human males and well considered that the deformed sperm do not fertilize oocytes even the normal shape spermatozoa from teratospermic donors have also reduced function capacity (Pukazhenthi et al., 2006b). The study of ejaculate characteristics of the clouded leopards indicated that sperm concentration and motility were comparative similar to other wild felid species (Wildt et al., 1986b; Howard, 1993) but there was high incidence of pleiomorphic spermatozoa with around 15 % normal sperm and >40% with deranged acrosome (Wildt et al., 1986b; Pukazhenthi et al., 2006a).

The ultrastructure of clouded leopard spermatozoa have been reported by (Wildt et al., 1986b). The investigation of sperm under the light and transmission electron microscopy (TEM) revealed that the morphological traits of clouded leopard spermatozoa was commonly share the characteristics similar to other species. The general characteristics were 1) spermatozoa had an electron dense nucleus, 2) the acrosome was moderately dense and covered approximately 75% of the nucleus, 3) the neck region contains proximal centriole and basal plate, 4) the midpiece region consists of the axial filament surrounded by nine outer coarse fibers and mitochondrial sheath and 5) the flagellum region display the axial filament and fibrous sheaths within the cell membrane.

1.2.4. Sperm assessment

Evaluation of semen is aimed to characterize semen traits or to diagnose male infertility. Generally, male fertility can be assessed in two categories 1) descriptive

evaluation or conventional semen profile, and 2) sperm functional assay. The descriptive evaluation provides general semen qualities such as proportion of motile and progressively motile sperm, concentration of sperm, and morphologically normal sperm. To date, computer assisted sperm analysis (CASA) has been used for some routine diagnostic applications such as sperm concentration and movement pattern (WHO, 2010). It has high precision and provides quantitative data on the kinematic parameters of spermatozoa in terms of forward progression and hyper-activated motility, a characteristic of capacitated cells. Moreover, CASA can estimate concentration and movement characteristic significantly related to fertilization rates *in vitro* and *in vivo*, as well as to time to conception (Liu et al., 1991; Barratt et al., 1993; Irvine et al., 1994; Donnelly et al., 1998; Garrett et al., 2003; Shibahara et al., 2004). However, fertility prediction of a semen samples cannot be done by CASA alone. It need to be integrated with other functional tests. Moreover, it have to carefully validated the system because the diversity of sperm responses to changes in the microenvironment was reported in previous researches (Amann and Waberski, 2014)

The descriptive sperm count and morphology indicate fertilization and pregnancy outcome. Nevertheless, this evaluation is not able to detect the functional deficiencies responsible for the unexplained infertility (Aitken et al., 1984). Recently, the functional assays have been developed to predict the fertilizing capacity of spermatozoa in various species such as human. Moreover, some parameters as the excessive generation of reactive oxygen species (ROS) and DNA integrity can provide the information both on the ability of fertility capacity and the ability of spermatozoa to support normal embryonic development. The assessment of sperm function included *in vitro* sperm-oocyte interaction, sperm-zona pellucida (ZP) binding assay or zona-free penetration assay. These techniques have shown some promise to evaluate fertility in various species e.g. human sperm (Aitken, 2006) domestic bovids (Brackett et al., 1982), and deer (Comizzoli et al., 2001). The study in the clouded leopards demonstrated that the percentage of morphologically normal sperm was significantly increased and the recovered sperm were capable to bind and penetrate the zona-pellucida of oocytes *in vitro* (Howard and Wildt, 1990). In endangered species,

heterologous *in vitro* fertilization (IVF) offers an alternative method to test sperm function (Roth et al., 1998) due to the poor availability of oocytes in wild species. Accordingly, oocytes of various domestic species such as hamster, domestic cat and bovine were used to assess the sperm fertilizing ability and to analyze the first cell cycle. For example, The hamster zona-free oocyte has been studied for sperm penetration of Indian leopard (Jayaprakash et al., 2001), domestic cat oocytes replaced oocytes of leopard cats (Andrews et al., 1992), tiger (Donoghue et al., 1992), cheetah (Roth et al., 1995), fishing cat (Thiangtum et al., 2006), Pallas's cat (Swanson et al., 2006), ocelot (Stoops et al., 2007), Iberian lynx (Ganan et al., 2009), black-footed cat (Herrick et al., 2010) and sand cat (Herrick et al., 2010) for testing binding and penetrating capability. In addition, this assay has been applied to assess the in vitro fertility cryopreserved spermatozoa of sika deer and red deer epididymal spermatozoa using heterologous IVF with zona-free bovine oocytes (Comizzoli et al., 2001).

1.2.5. Sperm cryopreservation

Cryopreservation and storage of biological materials i.e. germ cells (spermatozoa, oocytes), somatic cells can be facilitated management and conservation of endangered species (Wildt and Roth, 1997). Frozen-thawed spermatozoa have been achieved widely used to produce offspring via artificial insemination and in vitro production of embryo in domestic animals. Nevertheless, it has been noted that cryopreservation affect to the quality of in vitro fertilization (IVF) and in vitro development (IVD) of embryos (Comizzoli et al., 2001). Freezing and thawing are major causes of damage to sperm in various aspects such as unequal distribution of cryoprotectant, osmotic stress during dehydration and rehydration, phase transitions in the plasma membrane, and oxidative damage. Cryopreservation has been reported to cause changes in sperm morphology (mitochondria, acrosome, and tail), motility, mitochondrial activities, and viability (O'Connell et al., 2002). In the clouded leopards, the sperm cryopreservation study has focused on effects of permeating (glycerol) and non-permeating (raffinose) cryoprotectants (Pukazhenthi et al., 2006a). The study of Pukazhenthi et al. (2006a) showed that removal of glycerol from the semen extender

resulted in damaged acrosome whereas removal of raffinose had no effect on acrosome integrity. Moreover, raffinose seemed to mitigate the osmotic effect of glycerol. Unlike the clouded leopards, the benefits of raffinose have been noted in other teratospermic species.

1.2.6. Sperm selection using single-layer centrifugation through a colloid

The techniques of sperm selection have been developed and applied to assist reproduction. To date, two major methods are used as standard preparation techniques to recover and select good quality of sperm. There are density centrifugation and swim-up procedure (Paasch et al., 2007). Sperm selection technique using SLC through a colloid has shown its capability to select morphologically normal sperm from fresh human semen (Prakash et al., 1998), improve proportion of sperm with normal midpiece and tail of stallion (Morrell et al., 2009a; Morrell et al., 2009b) and improve quality of epididymal cat sperm (Chatdarong et al., 2010). Concurrently, SLC using Androcoll-E^{TM} significantly improved all the sperm parameters of stallion semen including sperm head morphometry, DNA integrity, motility, membrane integrity, viability, and mitochondrial membrane potential (Macias Garcia et al., 2009). Using SLC, the incidence of certain abnormalities, e.g. proximal cytoplasmic droplets and mid-piece defects of the stallion semen were significantly reduced (Morrell et al., 2009b). Moreover, the frozen-thawed stallion semen processed by SLC has also increased percentage of progressively motile sperm (Cerny et al., 2012).

1.2.7. Reproductive cycle and ovarian control in felids

To date, assisted reproductive techniques such as AI and *in vitro* fertilization/embryo transfer (IVF/ET) are powerful tools for managing endangered species (Pelican et al., 2006). However, the success of such techniques is still low to be applied in most species (<20%). The major constraints are the variable responses and sensitivities to gonadotropin treatment, especially in species that exhibit spontaneous ovulation (Brown, 2011). In Felidae, ovulatory patterns exhibit a range from restrict induced to combination of induced and spontaneous (Brown, 2006).

Therefore, to develop an appropriate dosage of exogenous gonadotropin to stimulate ovarian activity in these species is extremely difficult. Although the studies of ovarian control using exogenous gonadotropin have been performed for decades, appropriate dosage has not yet been identified (Graham et al., 2006; Pelican et al., 2006b). More recently, the use of synthetic progestins and GnRH analogs to temporarily down-regulate the ovaries prior to gonadotropin stimulation showed promising results in felids (Pelican et al., 2006a; Pelican et al., 2008). The use of progestin implantation (Levonorgestrel or Norplant®) prior to gonadotropin stimulation produced more than doubled the embryo yield in the domestic cat after IVF (Pelican et al., 2005) and resulted in consistent suppression of follicular and predictable ovulations for AI (Pelican et al., 2005) whereas GnRH agonist synchronized follicular phase but did not induce complete ovarian down regulation (Pelican et al., 2008). Moreover, progestin exposure prior to gonadotropin stimulation produced more follicles and improved embryo development after *in vitro* fertilization in the domestic cats (Pelican et al., 2010). Alternatively, progestin (altrenogest) in oral preparation could rapidly suppress at initiation of follicular activity and the return to normal follicular activity after progestin withdrawal was observed in the domestic cats (Stewart et al., 2010).

Use of exogenous gonadotropin regimen for AI has commonly use eCG followed by hCG 80-84 h to stimulate ovarian before AI. This protocol has successfully produced living offspring of domestic cat (Tsutsui, 2006) and eight species of wild felids including tigrina; *Leopardus tigrinus* (Swanson and Brown, 2004), snow leopard; *Uncia uncia* (Roth et al., 1997), cheetah; *Acinonyx jubatus* (Howard et al., 1997), tiger; *Pathera tigris* (Donoghue et al., 1992), and clouded leopard; *Neofelis nebulosa* (Howard et al., 1996). However, the pregnancy rate has been variable with only three species (domestic cat, leopard cat, and cheetah) having a consistently respectable incidence at >45% of pregnancy success (Pelican et al., 2006b). In clouded leopards, the dosage of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) were reported as 100 IU and 75 IU, respectively (Howard et al., 1996; Howard et al., 1997) as an optimal eCG/hCG dosage. Nevertheless, only 60% of the original gonadotropin-treated population was observed ovulation. Moreover, the multiple endocrine

responses following gonadotropin stimulation were documented including ovarian hyperstimulation and luteal insufficiency (Brown et al., 1995). More recently, the modified gonadotropin regimen using 100 IU eCG with 1000 IU pLH produced consistent ovulatory responses and superior synchronization in domestic cat compare to the standard eCG/hCG regimen. In addition, using this regimen in domestic cat recipient prior to laparoscopic embryo transfer of non-frozen or frozen thawed IVF embryo has produced viable kittens from hereditary disease models (Swanson, 2012).

1.2.8. Artificial insemination (AI) in the domestic cats and wild felids

Artificial insemination is an important technique to propagate populations of the domestic animals with highly producing trait and the endangered species with valuable genetic. Moreover, AI may be necessary when natural mating is not successful, particularly for animals that are separately housed in distance location (Tsutsui, 2006). Generally, in most animals mating, semen is deposited intravaginally then transported to the fertilization site at the ampulla. In domestic cats, the success of AI is influenced by; 1) sperm deposition site, 2) the number of sperm to be inseminated, 3) types of sperm storage prior to insemination (fresh ejaculate, chilled, or cryopreserved sperm), and 4) ovarian induction protocol (Tsutsui, 2006). To date, AI is divided into 3 methods according to the site of insemination including; 1) intravaginal insemination, 2) intrauterine insemination and 3) intratubal insemination. These methods have been reported with varying success rates. Fresh semen deposited intravaginal showed the higher conception rate when inseminate with higher number of sperm (50% with 10-50 x 10^6 sperm and 78% with 80 x 10^6 sperm, respectively). Moreover, deeper sperm deposition sites such as intrauterine and intratubal insemination, required lower number of sperm with acceptable conception rate. The conception rate was 50 and 80 % following deposition of 6.2 x 10^6 and 8 x 10^6 sperm at intrauterine site whereas deposited 4 x 10^6 spermatozoa at intratubal site obtained 43 % conception rate (Tsutsui, 2006). While frozen-thawed sperm are used, only intrauterine insemination is recommended (Chatdarong et al., 2007). However, the primary limitation with intrauterine AI requires high number of motile sperm to ensure sperm penetration

through the uterotubal junction into the oviducts to achieve fertilization. More recently, artificial insemination at oviductal site has successfully produced viable offspring in domestic cat using laparotomy (Tsutsui, 2006) and laparoscopy (Swanson, 2012).

In clouded leopard, the application of AI technology to produce offspring in the clouded leopards was conducted by Howard et al. (1996). The systematic studies involving hormone stimulation of ovarian activity, sperm processing, timing of insemination, and site of sperm deposition were subsequently performed finding. According to species differences in sensitivity of ovaries to exogenous gonadotropin, optimum dosage of hormonal regimen for inducing follicular development and ovulation is essential. The results demonstrated a highly sensitivity to exogenous gonadotropins as eCG and hCG. The excessive follicular development was observed to the production of 30 follicles using 200 IU eCG, whereas the lower doses (50 and 100 IU eCG) appeared to minimize ovarian hyperstimulation and showed more natural ovulatory response (Howard et al., 1997). The evaluation of ovarian activity indicated that ovulation occurred at 38-39 h after hCG treatment. In addition, the previous study using laparoscopic examination indicated that this species is spontaneous ovulation. In this study, five females were inseminated using a laparoscopic intrauterine AI procedure and the semen was deposited into the proximal aspect of uterine horn with various sperm concentrations (range 13-89 x 10^6/ml). From this effort, only female clouded leopard treated with 100 IU eCG + 75 IU hCG and inseminated with 89 x 10^6 sperm at 45 h post- hCG, successfully produced two live cubs after AI 89 day (Howard et al., 1996).

1.3 Thesis Objectives

1. To investigate semen characteristic of the clouded leopards in captivity in Thailand
2. To evaluate the use of SLC to select morphological normal sperm of the clouded leopards
3. To assess the functional ability of clouded leopard cryopreserved spermatozoa

4. To assess the reproductive organs of females clouded leopard after hormonal treatments
5. To develop intraoviductal insemination technique in the clouded leopards

1.4 Hypothesis

1. Semen of captive clouded leopard in Thailand are teratospemic
2. Morphologically normal sperm are improved by single-layer centrifugation
3. Selected sperm are capable of penetrating domestic cat oocytes
4. Reproductive organs of females clouded leopard response to hormonal treatments
5. Intraoviductal insemination using selected sperm results in pregnancy

1.5 Keywords

Clouded leopard, teratospermia, sperm selection, single-layer centrifugation, heterologous in vitro fertilization, artificial insemination

1.6 Research merits

1. The application of assisted reproductive technology
2. The appropriate protocol for ovarian induction
3. The knowledge on reproductive biology and physiology

CHAPTER II

Influence of living status (single vs. paired) and centrifugation with colloids on the sperm morphology and functionality in the clouded leopard (*Neofelis nebulosa*)

2.1 Abstract

The objectives of the present study were to evaluate sperm characteristic of captive clouded leopards in Thailand and examine the structural and functional properties of sperm after selection with the single layer centrifugation (SLC) method. Twenty-two ejaculates from 11 captive clouded leopards (four housed in pair and seven housed singly) were collected and assessed for semen traits during 2013 – 2015. Twelve fresh ejaculates were chosen from seven males and each was divided to two sperm preparation methods; 1) simple washing, or 2) SLC. Cryopreservation was performed after semen preparation. Sperm qualities after selections including motility, progressive motility, sperm motility index, viability, acrosome integrity, DNA integrity and morphology were evaluated in fresh, chilled and frozen-thawed samples. In addition, functionality of sperm after cryopreservation was tested by heterologous *in vitro* fertilization using the domestic cat oocytes. The ejaculates contained sperm motility of 52.5-91.3% (76.8 ± 2.0%, mean ± SE). High proportion of morphologically abnormal sperm (63.9 ± 2.0%) was observed with tightly coiled tail as a major defect (13.5 ± 0.5 %). Regarding the different living status, the pairing males had significantly higher percentage of sperm with intact acrosome (47.9 ± 3.4 and 38.4 ± 2.8%) and lower number of sperm with bent midpiece and droplet (7.1 ± 0.6 and 10.2 ± 0.5%) than the males living singly. The sperm motility index, intact acrosome and sperm with normal tail in the fresh and chilled semen samples were improved by the SLC. In the post-thawed semen, the SLC selected higher numbers of viable sperm (2.2 ± 34.1 and 1.8 ± 27.9%) and sperm with intact acrosome (44.5 and 66.1 ± 1.8 2.3 ±%) than the simple washing. Also, the tightly coiled tail sperm was lower in the SLC-processed than the simple washed samples (6.2 ±1.0 and 11.2 0.6 ±%). The heterologous IVF in the

SLC-processed group had significantly higher fertilization rate (29.3 ± 1.6%) than the simple washing group (15.8 ± 0.9 %). In conclusions, ejaculates of the clouded leopards living in Thailand demonstrated teratospermic characteristic similar to the previous reports from other continents. SLC is a promising tool to select the morphologically normal sperm of the teratospermic donors. The successes of assisted reproductive technology could be enhanced by the improved quality of post-thaw sperm in this species.

2.2 Introduction

Most of the non-domestic felids, particularly that are endangered, present a high incidence of teratospermia, defined as ejaculate traits of ≥ 60% structurally abnormal sperm (Wildt, 1994; Pukazhenthi et al., 2001). The etiology of teratospermia is unknown but it was observed in the populations with an extreme genetically monomorphism, i.e. cheetahs (*Acinonyx jubatus*) (Wildt et al., 1983; Wildt et al., 1987; Crosier et al., 2007), diminished genetic variation due to geographically isolated population, i.e. Asiatic lions (*Panthera leo persica*) (Wildt et al.,1987) and Florida panthers (*Puma concolor coryi*) (Barone et al., 1994), and low gene diversity in small captive population, i.e. clouded leopards (*Neofelis nebulosa*) (Wildt et al., 1986b; Pukazhenthi et al., 2001). The previous study revealed that teratospermic donors possessed poor reproductive performance in term of low fertility rates both *in vivo* and *in vitro* (Pukazhenthi et al., 2006b). It has been hypothesized that the pleiomorphic sperm cause retarded fertilizing function including delayed capacitation, compromised acrosomal reaction (Long et al., 1996), disrupted protein tyrosine phosphorylation (Pukazhenthi et al., 1996; Pukazhenthi et al., 1998), impaired sperm metabolic function (e.g glycolysis) (Terrell et al., 2010), and reduced ability to bind, penetrate zona pellucida and decondense within ooplasm (Howard et al., 1993; Neubauer et al., 2004). In addition, the defect sperm have been found in association with reactive oxygen species (ROS) overproduction in the seminal plasma that further damages the overall population of sperm cells (Aziz et al., 2004). Although discarding of the seminal plasma could lessen the detrimental effects of ROS, the pleiomorphic sperm remained in the re-suspended pellet continuously generate ROS in semen(de Lamirande and Gagnon,

1995).

To date, there are two standard semen preparation techniques to recover and select good quality sperm; density centrifugation and swim-up procedure (Paasch et al., 2007). With minimal damages to sperm cells, the density centrifugation using single layer centrifugation (SLC) through a colloid has shown its capability to select morphologically normal sperm from fresh human semen (Prakash et al., 1998), reduce proportion of certain abnormalities sperm (i.e. proximal cytoplasmic droplets and midpiece defects) and improve sperm motility, viability and chromatin integrity of stallion sperm (Cerny et al.; Morrell et al., 2009a; Morrell et al., 2009b) improve percentages of motile sperm, sperm with intact membrane and DNA of cat epididymal sperm (Chatdarong et al., 2010), and decrease the number of damage acrosome in brown bear sperm (Alvarez-Rodriguez et al., 2016). However, the SLC technique has never been applied in endangered felid species, especially those of teratospemic donors such as the clouded leopards (*Neofelis nebulosa*). The ejaculate characteristic of the clouded leopards demonstrated a high incidence of pleiomorphic sperm with >40% deranged acrosome (Wildt et al., 1986b; Pukazhenthi et al., 2006a). The defect sperm are susceptible to cold-induced acrosome damage that impaired the sperm quality after freezing-thawing cycle (Pukazhenthi et al., 2006a). Therefore, this study aimed to assess the semen and sperm characteristics of the captive clouded leopards in Thailand. In addition, we investigated whether the SLC could improve quality of male gametes from valuable donors who are affected by teratospermia.

2.3 Materials and Methods

All chemicals used in this study were purchased from Singma-Aldrich (St.Louis, MO, USA), unless otherwise indicated. Androcoll-F was provided by Prof. Morrell (Clinical Science, Swedish University of Agricultural Science, Uppsala, Sweden).

2.3.1 Experimental design

Ejaculates of 11 adult male clouded leopards (n=22) were evaluated for semen traits and sperm characteristics. Sperm quality parameters included sperm motility (0-

100%), progressive motility (0 - +5), sperm motility index (SMI), membrane integrity, acrosome integrity, DNA integrity and morphology. Ejaculates contained ≥ 60% motile sperm and sperm concentration ≥ 100 x 10^6 sperm/mL were submitted to sperm selections using SLC or simple washing prior to cryopreservation (n = 12). Aliquots of fresh, chilled and frozen-thawed sample were assessed. Sperm parameters were compared between SLC and simple wash group after frozen-thawed. Moreover, *in vitro* heterologous fertilization was performed to evaluate sperm functional ability. Percentage of pro-nuclear formation was compared between groups.

2.3.2 Animals

Adult male clouded leopards (n=11; aged 3-11 years) (animal numbers 1 to 11) (Table 1), member of the Clouded Leopard Consortium Project living at Khao Kheow Open Zoo (Chonburi, Thailand) were included. Semen collection was performed over two years (November 2012 - March 2015) during breeding seasons (November – March). Of 11 animals, four were in the pairing process whereas the others lived single. The pairing males were kept next to the females and allowed to stay together only during the estrus periods. All single males were individually kept nearby the female enclosure with occasionally see and smell but rear encounter. During the experiment, all males were kept in the large enclosure and provided food and enrichment according to the husbandry protocol recommended for the clouded leopard. The study was approved by the animal care and use committee of the zoological park organization under the Royal Patronage of H.M. the King and the Ethical Committee for Animal use at Chulalongkorn University (#13310079).

Table 2 Age and living status of clouded leopards at first semen collection. Semen containing >60% motile sperm and concentration >100x10^6 spermatozoa/mL are subjected to single-layer centrifugation (SLC study).

ID	Age at first semen collection		Living status	Number of ejaculate	SLC study
	Year	Month			
1	9	7	Singleton	A	-
2	3	5	Singleton	A, B, C	A, B
3	3	8	Singleton	A, B, C	A, B
4	6	3	Singleton	A, B, C	B, C
5	3	7	Singleton	A, B	B
6	6	9	Singleton	A	-
7	4	0	Singleton	A	-
8	11	0	Paring	A	A
9	2	8	Paring	A, B	A, B
10	7	10	Paring	A, B, C, D	A, B
11	11	1	Paring	A	-

2.3.3 Semen collection and evaluation

Semen of each male was collected 1 to 4 times constituted 22 ejaculates in total (Table 1). Animals were anesthetized using the combination of ketamine hydrochloride (5-10 mg/kg; Alfasan International B.V., Holland) and xylazine hydrochloride (0.5-1 mg/kg; Troy Laboratories Pty Limited, Australia), and maintained surgical plane stage with isoflurane (0.5-5%; Piramal Critical Care Inc., PA, USA) inhalation during the procedure. After the procedure completed, all animals were given yohimbine hydrochloride (0.125 mg/kg; Bomac Pty Limited, Austratia) as an antidote and closely monitored until complete recovery. The reproductive organs (testis, penis, prepuce and prostate glands) were palpated and the testicular volume was measured. Semen was collected using electroejaculation method followed the

standard protocol for felids (Howard, 1993). In brief, a lubricated rectal probe (1.6 cm diameter with three longitudinal electrodes) was placed on the prostate glands. Ejaculate stimulation was performed using electroejaculator (P.T. Electronics, Boring, Oregon) at electricity of 2-5 voltages for three series. Ejaculate samples were collected into small plastic cups (6 OZ).

Fresh ejaculate was immediately evaluated for appearance, volume and pH. The semen evaluation was followed the protocol described for carnivore species (Howard, 1993) and the previous studies in the clouded leopards (Wildt et al., 1986b; Pukazhenthi et al., 2006a). Sperm motility (0-100 %) and progressive motility status (0-5) were determined on fields at 200x magnification under phase contrast microscope. Sperm motility index (SMI) was calculated (Howard and Wildt, 1990). Sperm concentration and total number of spermatozoa per ejaculate was measured using hemocytometer chamber. Fresh ejaculate contained more than 60 % sperm motility and 100 x 10^6 spermatozoa per mL was selected for SLC study.

An aliquot of fixed raw ejaculate (5µL) in 100 µL of 0.3 % glutaraldehyde in PBS was evaluated for sperm morphology (500 spermatozoa) under 1000x magnification of phase contrast microscope (Pukazhenthi et al., 2006a). The sperm was classified as normal otherwise having one of the following abnormalities: 1) head abnormalities including microcephalic, macrocephalic and bicephalic; 2) acrosome abnormalities including missing or damaged acrosomal membranes; 3) midpiece abnormalities including abnormal or missing midpiece; a bent midpiece with and without retained cytoplasmic droplet; 4) tail abnormalities including tightly coiled tail, bent tail with and without retained cytoplasmic droplet and 5) other abnormalities including spermatid and detached head.

Aliquots of fresh, chilled and frozen-thawed sample were assessed for sperm membrane integrity using eosin-nigrosin staining (in field) (Bjorndahl et al., 2003) or dual staining technique using SYBR-14 and propidium iodide (PI) (Dead/Alive kit, Molecular Probe Inc., Eugen, OR, USA) (in laboratory). For eosin-nigrosin staining, at least 200 sperm were assessed using bright-field microscopy at a 1000x magnification under oil

immersion. Unstained sperm and sperm with a slight pink or red appearance restricted to the neck region were classified as live while those showed pink to red coloration were assessed as dead. For SYBR-14/PI staining, two hundred spermatozoa were evaluated and classified into three categories: live sperm (green, stained with SYBR-14), dead sperm (red, stained with EthD-1), and moribund sperm (both green and red) under the epifluorescence microscope (at 1000x magnification).

For acrosome integrity evaluation, an 5 µL aliquot of fresh, chilled and frozen-thawed sperm sample was smeared on the glass slide and membrane permeabilized with 95% ethanol for 30 sec then was stained using the double fluorescent labeling techniques (FITC-PNA and PI staining) (Axner et al., 2004). Acrosome integrity of 200 spermatozoa was assessed under the epifluorescence microscope and classified into three categories: intact acrosome (stained with bright green from FITC-PNA at the acrosome cap), damaged acrosome (stained with green and red), and missing acrosome (stained with red from PI).

Sperm DNA integrity was evaluated using acridine orange (AO) staining (Thuwanut et al., 2011). An 10 µL aliquot of fresh, chilled and frozen-thawed sample was gently smeared on a glass slide and air-dried then fixed in a freshly prepared mixture of methanol-glacial acetic acid (Carnoy's solution; 3:1 v:v) for 24 h. After fixation, the slide was air-dried and stained with AO staining solution (1% AO diluted in distilled water) for 5 min, rinsed in a stream of distilled water and air-dried. Two hundred spermatozoa were assessed using an epifluorescence microscope. The heads of sperm cells with normal DNA integrity (double- stranded) emitted green fluorescence, whereas those with denatured or single-stranded DNA showed orange, yellow, or red fluorescence.

2.3.4 Sperm separation, cryopreservation and thawing

The semen sample with over 60 % sperm motility and 100 x 10^6 spermatozoa/mL was equally divided into two aliquots for single-layer centrifugation (SLC) and simple washing. For simple washing, an aliquot was subjected to centrifugation at 300 x g for 8 min. The supernatant was discarded and the sperm

pellet was re-suspended with Tris egg yolk extender I (EYT-I) containing 3.025 Tris, 1.4% (w/v) citric acid, 0.8% (w/v) glucose, 3% (v/v) glycerol, 20% (v/v) egg yolk, 0.06% sodium benzylpenicillin and 0.1% (w/v) streptomycin sulfate in distilled water [30] with the half volume of the final volume that constitute to the sperm concentration of 10 – 30 x 10^6 sperm/mL. The extended sample was cooled from room temperature to 4 ºC. After 1 h incubation, Tris egg yolk extender II (EYT-II) containing EYT-I plus 1% Equex STM paste (Nova Chemical Sales, Inc., Scituate, MA, USA) and 7% (v/v) glycerol was added as equal volume of EYT-I. After adding the EYT-II, semen was loaded into two 0.25-mL pre-cooled straws. For cryopreservation, loaded straws were placed at 4 cm over liquid nitrogen vapor using a two-step cryomethod. At thawing, each straw was held rapidly in air for 10 sec and then immersed in 37°C water bath for 30 sec. The straws were emptied into an empty prewarmed tube containing 250-µL thawing medium (3.025 g Tris, 1.4% (w/v) citric acid, 0.8% (w/v) glucose, 20% (v/v) sodium benzylpenicillin and 0.1% (w/v) streptomycin sulphate in 100 mL distilled water).

Sperm selection through SLC was performed (Chatdarong et al., 2010). Briefly, an aliquot (1 mL) of semen sample diluted with Tris buffered solution was slowly released into a 15-mL centrifuge tube to top of 2-mL colloid layer (Androcoll-F; SLU, Sweden). The gradient was centrifuged at 300 x g at 25 ºC for 20 min. After centrifugation, the upper layer was slowly removed. The bottom layer was slowly discarded until around 0.5 mL left. The sperm pellet was transferred to a clean tube, resuspended with the extenders and processed for cryopreservation according to the above protocol described for simple washing method.

2.3.5 Heterologous *in vitro* fertilization

Cryopreserved sperm were thawed and examined for their *in vitro* fertilizing ability using matured domastic cat oocytes. The protocol used for oocyte maturation and *in vitro* fertilization (Pukazhenthi et al., 2000; Sananmuang et al., 2011) were applied. In brief, groups of 20-30 cumulus-oocytes complex (COC) were cultured in 500 µL of *in vitro* maturation (IVM) medium ($NaHCO_3$-M199 supplemented with 1.0 mM sodium pyruvate, 2.0 mM l-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin,

4 mg/mL BSA) at 38.5 °C in a humidifed atmosphere of 5% CO_2 for 24 h. After maturation, oocytes were washed and transferred to 50 µL drop of IVF medium (Tyrode's balanced salt solution containing 1% (v/v) mininum essential media (MEM) nonessentail amino acids (NEAA), 6 mg/mL bovine serum albumin (BSA), 100 IU/mL penicillin, 30 µg/mL heparin, 1mM L-glutamine, 0.36 mM sodium pyruvate, and 0.11 mM calcium lactate). Groups of 8-10 oocytes were co-incubated with frozen-thawed sperm (5 x 10^5 motile sperm/mL) in IVF medium at 38.5 °C and 5% CO_2 in air. After 18 h of co-incubation, excessive sperm was removed by gentle pipetting and presumptive zygotes were washed and fixed in 4% (w/v) paraformaldehyde then stained with fluorescent DNA labeling (4',6-diamidino-2-phenylindole; DAPI). The nuclear status of oocytes were examined using an epifluorescent microscope (x1000 magnification). Oocyte maturation status was classified as germinal vesicle (GV), metaphase I (MI), metaphase II (MII) and fertilization status (confirmation of pronuclei or blastomere).

2.3.6 Statistical analysis

Parameters of semen quality were presented as means ± SE. Data analyses were performed using a statistical software package (Statistical Analysis System software, version 9.0, SAS Institute, Cary, NC, USA). Sperm parameters including motility, motility index, viability, acrosome integrity, DNA integrity, sperm morphology and number of oocyte with pronuclear formation between samples from males living singly and in pairing process, and between samples undergone simple washing and SLC, were compared using student t-test and Wilcoxon. Animals were included in the statistical model as a random effect. Differences between experimental groups were considered statistically significant when $P \leq 0.05$.

2.4 Results

The testicular volume and circumference of the 11 males measured while performing the semen collections were averaged 20.8 ± 0.7 cm^3 (range 17.2 – 27.4) and 14.1 ± 0.3 cm (range 12.1 – 18.5), respectively. The general background of each male, number of ejaculates obtained and ejaculates that were selected to the SLC study were demonstrated (Table 2). The semen traits demonstrated individual variation

of volume, pH, osmolality among males while they were not significant different between the single and paring males. Interestingly, the higher number of sperm with intact acrosome was higher in the semen samples of the paring males than the single males ($P < 0.05$) (Table 3). Moreover, although the other sperm parameters showed a trend of higher means in the paring male group, there was no significant difference between the male groups.

Table 3 Semen traits (Mean ± SE) of clouded leopards live single and in pairing process during November 2012 to March 2015.

Parameter	Single (7 males, 14 ejaculates)	Pairing (4 males, 8 ejaculates)	Overall (11 males, 22 ejaculates)	
			Mean	Range
Volume (µL)	422.6 ± 73.9	394.4 ± 73.3	412.4 ± 53.1	101 – 1,059
pH	7.7 ± 0.1	7.6 ± 0.1	7.6 ± 0.1	7 – 8.5
Osmolality (mOsm)	356.8 ± 11.9	370.0 ± 15.0	361.5 ± 9.2	308 - 418
Motility (%)	75.5 ± 3.1	79.2 ± 1.4	76.8 ± 2.0	52.5 – 91.3
Progressive motility (0-5)	3.4 ± 0.1	3.4 ± 0.2	3.4 ± 0.1	2 - 4
Sperm motility index	72.0 ± 2.4	73.3 ± 2.2	72.5 ± 1.7	56.3 – 85.6
Sperm concentration ($x10^6$/mL)	168.6 ± 37.8	196.6 ± 75.2	178.8 ± 35.5	8.3 - 672
Total sperm ($x10^6$)	57.2 ± 12.0	63.6 ± 24.4	59.5 ± 11.4	2.9 - 213
Viability (%)	81.7 ± 2.3	83.8 ± 1.6	82.5 ± 1.5	66.5 – 94.5
DNA intact (%)	97.9 ± 0.4	98.7 ± 0.2	98.2 ± 0.3	94.5 – 99.5
Acrosome intact (%)	38.4 ± 2.8^a	47.9 ± 3.4^b	41.9 ± 2.3	20.5 – 56
Normal morphology (%)	33.3 ± 2.2	40.9 ± 3.5	36.1 ± 9.2	14.0 – 57.5

Within row, different superscripts (a and b) differ ($P \leq 0.05$).

The clouded leopard males in this study presented a high incidence of structurally abnormal sperm reflecting teratospermia characteristic. The tightly coiled tail and acrosome abnormality were the most common sperm defects (13.5 ± 0.5 % and 10.8 ± 0.6 %, respectively) (Table 4). Other abnormalities including macrocephalic, microcephalic, bicephalic, bent midpiece, bent tail with and without retained cytoplasmic droplet, proximal and distal droplet were also observed. When the sperm morphology of the two groups was compared, semen from the paring males contained

a lower number of sperm with bent midpiece containing cytoplasmic droplet than those of the single living males ($P < 0.05$) whereas the other defects were not statistically significant.

Table 4 Detailed sperm morphology (Mean ± SE) of clouded leopards live single and in pairing process during November 2012 to March 2015.

	Single (7 males, 14	Pairing (4 males, 8	Overall (11 males, 22 ejaculates)	
Morphology (%)	ejaculates)	ejaculates)	Mean	Range
Abnormal sperm	2.2 ± 66.7	59.0±3.5	2.0 ± 63.9	86.0 – 42.5
Macrocephalic	5.9 ± 0.8	4.7 ± 0.7	0.6 ± 5.5	10.6- 1.5
Microcephalic	2.3 ± 0.4	3.0 ± 0.5	0.3 ± 2.6	5.5 – 0.5
Bicephalic	1.0 ± 0.2	0.5 ± 0.1	0.1 ± 0.4	2.0 – 0.0
Abnormal acrosome	10.9 ± 0.8	10.6 ± 1.1	0.6 ± 10.8	17.0 – 6.6
Tightly coiled tail	13.7 ± 0.7	13.1 ± 0.9	0.5 ± 13.5	18.0 – 8.8
Bent midpiece with droplet	10.2 ± 0.5[a]	7.1 ± 0.6[b]	0.5 ± 9.1	13.5 – 5.0
Bent midpiece without droplet	4.6 ± 0.7	4.3 ± 1.2	0.6 ± 4.5	12.3 – 1.5
Bent tail with droplet	7.9 ± 1.1	5.9 ± 1.2	0.9 ± 6.5	18.0 – 0.0
Bent tail without droplet	3.4 ± 0.5	3.1 ± 1.0	0.5 ± 2.8	9.0 – 0.0
Proximal droplet	4.3 ± 0.9	4.1 ± 0.6	0.6 ± 4.3	11.0 – 1.0
Distal droplet	2.2 ± 0.6	1.5 ± 0.4	0.4 ± 1.8	8.0 – 0.0
Detached head/tail	3.1 ± 0.6	4.0 ± 0.9	0.5 ± 2.2	9.3 – 0.0

Within row, different superscripts (a and b) differ ($P \leq 0.05$).

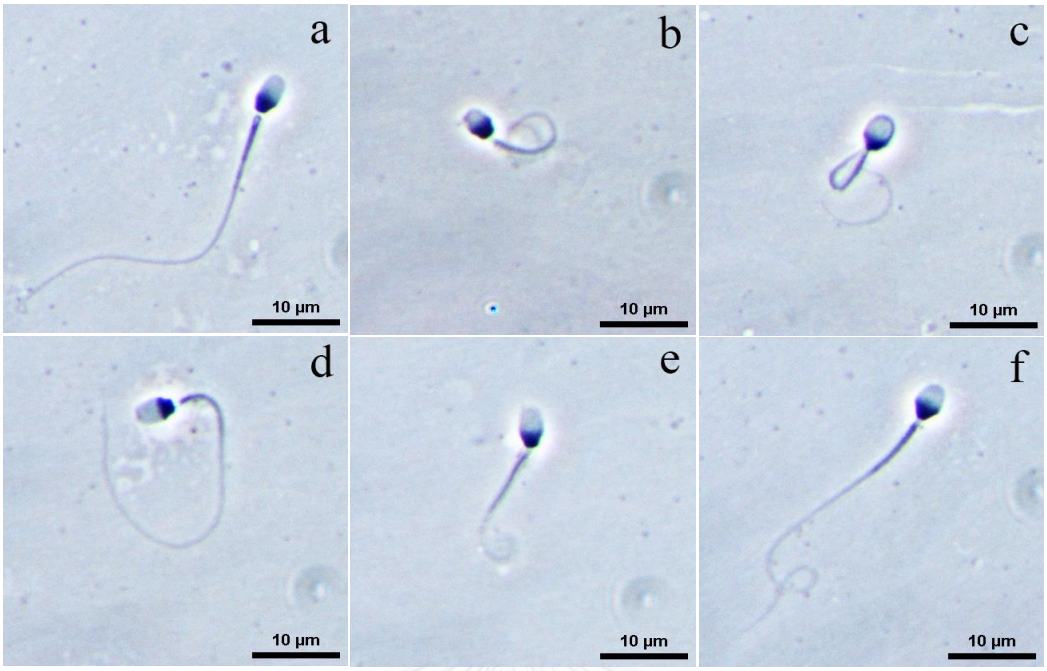


Figure 3 Photomicrograph of clouded leopard sperm evaluated under the phase contrast microscopy (1000x): (a) normal spermatozoa (b) abnormal acrosome and tightly coiled tail (c) macrocephalic and bent neck with cytoplasmic droplet (d) bent neck without cytoplasmic droplet (e) bent tail with cytoplasmic droplet (f) bent tail without cytoplasmic droplet

Of 22 ejaculates, 12 ejaculates from 7 males were selected for the sperm selection study. The sperm recovery rate after SLC processed was 10.5 ± 8.8 %. The sperm parameters of the fresh semen after SLC-processed showed a better percentage of sperm motility index, intact acrosome and normal tail ($P < 0.05$) (Table 5). After chilling, sperm parameters related with motility including progressive motility and SMI, viability and normal morphology were compromised ($P < 0.05$) whereas after cryopreserved, all of the above parameters including DNA and acrosome integrity were affected ($P < 0.05$). After chilling, the number of motile sperm with intact acrosome and normal tail of SLC group were higher than the other ($P < 0.05$). Cryopreserved in the SLC-processed group presented a better number of normal sperm with intact acrosome after thawing ($P < 0.05$). The number of abnormal sperm tail reduced after SLC processed ($P < 0.05$). The tightly coiled tail sperm in the SLC-processed semen was lower than the initial fresh sample ($P < 0.05$) and the simple washed semen both

after chilled and post-thawed ($P < 0.05$) (Tables 5 and 6). The others sperm defects were not significantly different.

Testing of fertilizing ability of the frozen-thawed clouded leopard sperm with the domestic cat oocytes showed the fertilizing feasibility. The frozen-thawed sperm obtained from the SLC-processed semen performed higher fertilization rate than those from simple washing (simple-washed, 15.8 ± 0.9 %; SLC-processed, 29.3 ± 1.6 %, mean ± SE, $P < 0.05$).

Table 5 Comparisons of clouded leopard sperm parameters between semen undergone simple washing and single-layer centrifugation (SLC) in fresh, chilled and frozen-thawed samples (Mean ± SE, n=12).

(%) Parameter	Fresh		Chilled		Post-thawed	
	Simple washing	SLC	Simple washing	SLC	Simple washing	SLC
Motility	1.7^{A} ± 77.1	1.5^{A} ± 78.3	1.1^{B} ± 61.7	1.1^{B} ± 64.6	1.8^{C} ± 27.1	1.9^{C} ± 32.1
Progressive motility	0.1^{A} ± 3.5	0.1^{A} ± 3.8	0.1^{A} ± 3.2	0.1^{B} ± 3.5	0.1^{B} ± 1.5	0.1^{C} ± 1.7
Sperm motility index (SMI)	1.5^{Aa} ± 73.3	0.9^{Ab} ± 77.5	1.1^{Ba} ± 62.9	1.2^{Bb} ± 66.9	1.7^{C} ± 27.9	1.6^{C} ± 32.3
Viability	1.8^{A} ± 78.8	1.8^{A} ± 81.9	1.4^{B} ± 64.7	1.9^{B} ± 67.1	1.8^{Ca} ± 27.9	2.2^{Cb} ± 34.1
Intact acrosome	2.8^{Aa} ± 41.2	2.2^{Ab} ± 48.9	1.5^{A} ± 34.8^{a}	2.3^{A} ± 44.5^{b}	2.2^{Ba} ± 24.3	2.1^{Bb} ± 31.2
Intact DNA	0.4^{A} ± 98.1	0.3^{A} ± 98.5	0.5^{AB} ± 97.7	0.3^{AB} ± 98.0	1.7^{B} ± 96.3	1.6^{B} ± 96.9
Normal morphology	2.3^{A} ± 45.8	2.9^{A} ± 51.5	2.9^{B} ± 35.2	3.2^{B} ± 40.1	2.1^{Ca} ± 24.6	2.1^{Cb} ± 31.6
Normal head	3.6 ± 65.7	4.4 ± 68.1	3.9 ± 54.8	2.9 ± 61.6	2.7 ± 33.0	3.5 ± 40.4
Normal tail	5.9 ± 48.0^{a}	2.8 ± 71.5^{b}	5.1 ± 39.9^{a}	1.8 ± 66.1^{b}	2.7 ± 24.3^{a}	2.7 ± 34.2^{b}

Within the same treatment, (Simplewashing and SLC), values with different letter (A-C) differ ($P < 0.05$). Between treatments, values with different superscript (a and b) differ ($P < 0.05$).

Table 6 Detailed sperm morphology of fresh semen processed through simple washing and single-layer centrifugation (SLC) (mean ± SEM) (n=12 ejaculates)

Morphology (%)	Simple washing	SLC
Macrocephalic	5.0 ± 0.8	4.0 ± 0.7
Microcephalic	2.0 ± 0.2	2.3 ± 0.6
Bicephalic	0.4 ± 0.0	0.8
Abnormal acrosome	9.6 ± 0.9	11.4 ± 1.7
Tightly coiled tail	11.2 ± 0.6[a]	6.2 ± 1.0[b]
Bent midpiece with droplet	7.4 ± 0.7	6.3 ± 0.8
Bent midpiece without droplet	3.7 ± 1.0	6.8 ± 1.7
Bent tail with droplet	4.6 ± 0.4	3.1 ± 0.7
Bent tail without droplet	2.1 ± 0.6	2.8 ± 0.3
Proximal droplet	3.0 ± 0.9	2.8 ± 0.3
Distal droplet	1.6 ± 0.5	1.4 ± 0.1
Detached head/tail	4.0 ± 0.6	4.8 ± 0.4
Abnormal sperm	54.2 ± 2.3	48.5 ± 3.0

Within row, with different superscripts (a-b) differ ($P < 0.05$).

2.5 Discussion

This study demonstrated the first application of a colloid centrifugation to select sperm from a teratospermic species. This was also the first systematic semen evaluation in a proper genetic management population established by the Clouded Leopard Consortium Project in Thailand after the preliminary study in the wild-caught founders of this population since 2000. Using the same sperm evaluation method, the semen collected in this study contained a high proportion of morphologically abnormal sperm similar to the former study. The sperm preparation technique using SLC apparently could improve the sperm parameters, presenting the better post-thawed sperm quality in the felid species possessed teratospermia. The frozen-thawed selected sperm also demonstrated its functional ability to form a pronuclear in

matured domestic cat oocytes *in vitro* with a higher percentage than the conventionally prepared spermatozoa.

The anticipated teratospermia in the clouded leopard ejaculates in this study was in accordance with the report in the North American zoos, demonstrating the consistently high proportion of pleomorphic spermatozoa (> 80%) with a severe cryosensitivity (Pukazhenthi et al., 2006a) throughout the year (Wildt et al., 1986b). Those studies assumed the cause of high incidence of abnormal sperm to be related to a low genetic diversity of the originally small number of founders in this species. However, the preliminary study in the wild-caught founders of the males in this study stated that an improper nutritional management and stress were the factors compromising the ejaculate quality (Brown et al., 1995). For our current study results presented a poor sperm quality in overall ejaculate traits although this study was examined in the variance genetic individuals and they were raised in the husbandry managed according to the consortium guideline for this species. The better sperm quality in this study compared to the previous study might be the positive effect during the high breeding activity period while the semen collections were performed. Therefore, the high incidence of pleiomorphic sperm without influenced by the seasonality as was shown in the former study in clouded leopard (Wildt et al., 1986b) and cheetah (Crosier et al., 2007).

Our findings on overall semen traits showed the tendency that the pairing males demonstrated a better quality than those housed singly. We assumed that the better sperm quality involved with the spermatogenesis. The extrinsic factors such as visualization, smelling and/or touching with an estrous female stimulate the hypothalamic-pituitary-genital (HPG) axis that further increase producing the androgenic steroid hormones (testosterone and androgen) which are necessary for spermatogenesis (Johannisson et al., 2009). The frequent chances to interact with the females during estrous period may affect the male reproductive physiology that result in producing better quality of sperm in paired males than those single livings. However, there was a few pairing males available for this present study due to the rest of the

males were paired for natural breeding purpose. Therefore, the long-term study of the sexual hormone influencing sperm characteristic in the paired male, paring male, and single males would gain beneficial information for the male clouded leopard reproductive physiology in captivity.

The application of SLC to select sperm subpopulation of better quality in this study demonstrated the improving of the number of motile sperm, normal sperm tail and intact acrosome in fresh ejaculates. These results were similarly to the previous studies that indicated the ability of SLC to select good quality spermatozoa both prior to cool storage or cryopreservation (Morrell et al., 2009a; Morrell et al., 2009b; Morrell et al., 2010; Martinez-Alborcia et al., 2012). Although, all parameters were tended to decline after cooling process (chilling and freezing), the higher sperm quality in the SLC-processed than the control samples was observed. In this experiment, the percentage of viable sperm and normal morphological sperm showing higher than control group after post-thaw. This supported that the elimination of poor quality sperm subpopulation before improves the freezability of sperm as similar to the previous finding in boar sperm (Martinez-Alborcia et al., 2012) , donkey ejaculates (Ortiz et al., 2015). The ability to improve the abnormal sperm tail by SLC was also shown in this current study. The declining of moreover 50% of coiled and bent tail with droplet from the initial samples were observed. To select sperm with normal morphology and better quality particularly sperm motility using SLC would be benefit post-thawed quality as well as artificial insemination. The defect sperm has been found in association with reactive oxygen species (ROS) overproduction that could further damages the overall population of sperm cell. Besides morphology, an acrosome integrity of SLC-processed showed admitted percentage after thawing. This was consistent with the recent report in brown bear (*Ursus arctos*) stated that acrosome damage was lower in samples processed through the colloid Androcoll-Bear (Alvarez-Rodriguez et al., 2016). Thus, to apply the SLC for selecting in teratospermic donors is the other promising technique to improve quality of sperm as the swim-up had shown its capacity for recovering motile and structurally normal spermatozoa in teratospermic cat (Howard et al., 1991).

Previous studies indicated that clouded leopard spermatozoa are susceptible to cold-induced damage (Pukazhenthi et al., 2006a). The severity of poor quality frozen-thawed spermatozoa was also observed in the present study. Sperm qualities after chilling and frozen-thawed protocols tend to reduce in all measured parameters in both groups. This result confirmed a highly cryo-sensitivity of spermatozoa in this species, as observed in other species of Felidae. However, the functional test using a heterologous IVF after thawing demonstrated that selected sperm had a better fertilization success than that of control group. This finding correlated with the improved intact acrosome percentage after thawing in SLC-processed group. Although, it is clearly shown that SLC could improve the quality of clouded leopard sperm, there were some limitations in applying this method. In this study, a challenge in sperm selection using SLC was the limitation of total volume of raw ejaculate and variation of the initial sperm concentration. Thus, a lower yield of sperm will be recovered by this method. Moreover, the small amount of sample may be negatively affected by centrifugation as stated in rat sperm study (Varisli et al., 2009).

CHAPTER III

Mitigation of sperm tail abnormalities using demembranation approach in the Clouded Leopard (*Neofelis nebulosa*)

3.1 Abstract

Captive clouded leopards (*Neofelis nebulosa*) produced high proportion of morphologically abnormal spermatozoa (mainly tail defects) that can limit sperm movement and conception. The aim of the study was to better identify the origin of those defects and mitigate them using a demembranation approach. Fresh ejaculates (1-2 ejaculations/male; n=11) were evaluated and equally allocated to simple washing (control group; resulting in about 10% coiled tails after treatment). Semen processed through colloid centrifugation to reduce the number of sperm with tail defects was defined as treated sample (resulting in about 6 % coiled tails after treatment). Aliquots of semen (control and treated samples) were incubated in HOS (60 mOsm fructose solution) containing 5 mM dithiothreitol (DTT) (a reducing agent) to prevent oxidation of sperm tail at 37 °C for 45 min. Thereafter, 5 µL of 20% Triton X-100 (TX) (a detergent) was added to the HOS/DTT-treated samples (15µL) and incubated at room temperature for 5 min prior to evaluate the proportion of sperm with tightly coiled-tail. The results demonstrated that the semen contained 78.9 ± 3.0 % swollen sperm (bent and coiled). After HOS/DTT incubation, the control samples and sperm selected samples presented 73.4 ± 3.1 % and 73.9 ± 2.5 % swollen sperm indicating membrane intact, respectively. Moreover, the proportion of sperm with tightly coiled-tail was not different between groups. After TX treatment, most of the coiled tail in the raw ejaculates could not be uncoiled or opened by TX indicating that the cause of coiled sperm tails may be from testicular origin. Moreover, the proportion of sperm with tightly coiled-tail was lower in the sperm selected group than control group (18.8 ± 3.8% and 26.5 ± 3.4; P = 0.1) whereas the proportion of sperm that were opened up by TX was higher in the sperm selected group (53.6 ± 10.4 and 21.1 ± 7.9%; P = 0.06). The results indicated that TX was able to uncoil half of the tightly coiled sperm in the

semen undergone preparation. In conclusion, the coiled sperm in the clouded leopard semen were likely not a defect of sperm volume regulation during post-ejaculate (osmotic swelling) but pre-ejaculate origin. Semen preparation process demonstrated its ability to lessen the primary sperm defects of raw semen and selected spermatozoa that were prone to be mitigated after demembranation.

3.2 Introduction

Clouded leopard (*Neofelis nebulosa*) is an endangered felid species that are difficult to breed in captivity due to male aggression and lethal attack on female (Yamada and Durrant, 1989). The previous studies on semen traits of adult captive males revealed the high incidence of pleiomorphic spermatozoa with a major of coiled tail defect (Wildt et al., 1986b; Pukazhenthi et al., 2006a). The coiled tails are the defects of either midpiece or tail of sperm that are possibly caused by testicular or epididymal origins. The different types of coiled sperm tail included: 1) the proximal coiled tail localized near the head, 2) the distal coiled tail, and 3) the tail coiled around the head or head-in-coiled (HIC) (Yeung et al., 2009). The cause of HIC was associated with genetic background whereas other coiled sperm tails may involve with epididymal factors (Yeung et al., 2009). The phenomenon of a high proportion of coiled or bent sperm tail in the ejaculated semen has been reported in many domestic species, i.e. bulls (Blom, 1966), stallions (Hellander et al., 1991), goats (Molnar et al., 2001), dogs (Rota et al., 2008) and cats (Villaverde et al., 2013). This phenomenon has been termed as a 'Dag defect' or 'Dag-like defect' that is caused by testicular origin (Rota et al., 2008; Villaverde et al., 2013) that can lead to subfertility (Blom, 1966; Wenkoff, 1978). However, bending or coiling of the sperm tail can occur as a cell swelling phenomenon responding to osmotic change in a hypo-osmotic solution (HOS) or in abnormal epididymal secretion (Cooper and Yeung, 2003; Yeung et al., 2006). The hypothesis was that the coiled sperm tail that caused by post-ejaculated osmotic swelling when sperm are exposed to the seminal plasma can be reversed by a detergent (Yeung et al., 1999). Unless, the coiled form originated from pre-ejaculation; 1) testicular origin or 2) early stage of epididymis before mixed with seminal plasma, characterized by plasma membrane being oxidized and created sulfide bond (S-S) between the outer dense

fibers and fibrous sheath of sperm tail membrane (Yeung et al., 2009). These kinds of tails are fixed in the coiled form that could not be reversed by TX.

The modified HOS test by adding a reducing agent (dithiothreitol; DTT) and then demembranized using a detergent (Triton-X 100; TX) has been applied to distinguish whether sperm coiling of infertile patients was caused by spermatogenic factors or osmotic swelling (Yeung et al., 2009). The sperm with coiled-tail that reversed by demembranation was accounted for post-ejaculation cause whereas the sperm that could not be uncoiled were the defect sperm of pre-ejaculation origin. In the clouded leopards, there were no studies to investigate the possible causes of high incidence of coiled-tail sperm This present study aimed to 1) identify the origin of coiled tail defects whether it is from pre- or post- ejaculation and 2) mitigate coiled tail sperm defects using a demembranation approach for more understanding on the possible causes of a high producing of coiled sperm and possible way to overcome the effect from sperm abnormalities in this species.

3.3 Materials and Methods

All chemicals and reagents were purchased from Sigma-Aldrich Chemical (St.Louise, MO, USA). Unless, otherwise indicated.

3.3.1 Animals and semen collection

Semen of seven adult clouded leopards (aged 3-11 years old) kept at Khao Kheow Open Zoo (Chonburi, Thailand) were collected (n=9; 1-2 ejaculates per male) using an electro-ejaculator (P.T. Electronics, Boring, Oregon) under surgical anesthesia. Three series of electrical stimuli episode (2-5 voltage; 80 stimuli) were performed following the semen collection standard protocol for carnivores (Howard, 1993).

3.3.2 Semen quality evaluation

Fresh semen sample from each individual was initially evaluated for a conventional semen and sperm analysis including volume measured by micropipette, pH by pH paper, osmolality by osmometer, percentage of subjective sperm motility under a phase contrast microscope (200x magnification), sperm concentration by haemocytometer, percentage of sperm viability by eosin-nigrosin staining under a light

microscope (1000X magnification), percentage of acrosome integrity by double fluorescence labeling (FITC-PNA/PI) under a fluorescence microscope (1000X magnification) (Axner et al., 2004) and percentage of normal sperm morphology by fixed sperm in 0.3% glutaraldehyde in phosphate buffered saline under phase contrast microscope (1000X magnification) (Pukazhenthi et al., 2006a).

3.3.3 Sperm preparation

After basic seminal trait evaluation, the semen sample of each individual was equally divided and processed by simple washing and single layer centrifugation (SLC) through colloids (Felicoll®, Swedish University of Agricultural Science, Uppsala, Sweden, kingly provided by Prof. Jane Morrell. For simple washing, the semen aliquot was centrifuged at 300X g for 8 min. The supernatant was discarded and the sperm pellet was re-suspended with Tris buffered solution. For SLC method, the sperm selection was performed following the protocol for the domestic cats (Chatdarong et al., 2010). The sperm morphology under the phase-contrast microscope (400X magnification) was performed in both groups.

3.3.4 Hypo-osmotic solution (HOS) test

The protocol of hypo-osmotic solution (HOS) test used in this study was modified from Comercio et al. (2013). Aliquots (5 µl) of each sample (raw ejaculate, simple washed and SLC-selected) were treated with 50 µl of hypo-osmotic (HOS) solution (60 mOsm/kg of fructose solution) and incubated at 37 °C for 45 min. Two hundred spermatozoa from each sample were evaluated for percentage of sperm swollen (presented as bent and coiled tail) under the phase contrast microscope (400X). After HOS evaluation, the samples (simple washed and SLC-selected) were added with 5 mM dithiotheiton (DTT), a reducing agent to prevent oxidation of sperm tail that was assumed to occur during HOS incubation.

3.3.5 Demembranation test of coiled sperm

Aliquot (15 µl) of raw and treated samples (simple washed and SLC-processed) was added with 20% Triton X-100 (TX) (5 µl). All treated samples were incubated at room temperature for 5 min and evaluated under the phase contrast microscope

(400X). A total of 200 sperm cells were examined per sample. The tail morphology was categorized into three types including: 1) straight tail, 2) bent tail and 3) tightly coiled tail. The percentage of sperm with tightly coiled tails before and after TX treatment was compared among groups.

3.3.6 Statistical analysis

Percentages of sperm with coiled tail were compared between raw semen and HOS treated samples prior to and after TX treatment using student's t-test. Animal was assigned as random effect. Values were presented as mean ± SEM. Differences between experimental groups were considered statistically significant when P-value less than 0.1.

3.3.7 Experimental design

Ejaculated semen of seven clouded leopards (n=9 ejaculates) were collected by eletroejaculation. Fresh semen sample from each individual was initially evaluated for a conventional semen and sperm analysis. Single layer centrifugation (SLC) through colloids (Felicoll®) was used to prepare sperm before undergo the experiments. Sperm membrane function of raw ejaculated, simple washed and SLC-processed samples was evaluated using hypo-osmotic solution (HOS) test. Demembration was performed by adding TX to raw ejaculated, simple washed and SLC-processed samples Percentages of tail morphology were recorded as straight, bent and coiled tail. Percentages of swollen sperm tail presenting a tightly coiling appearance were compared among groups before and after TX treatment. The percentage of sperm opened up was calculated from the percentage of tightly coiled tail subtracted by those after TX treatment. Differences between experimental groups were considered statistically significant when $P < 0.1$.

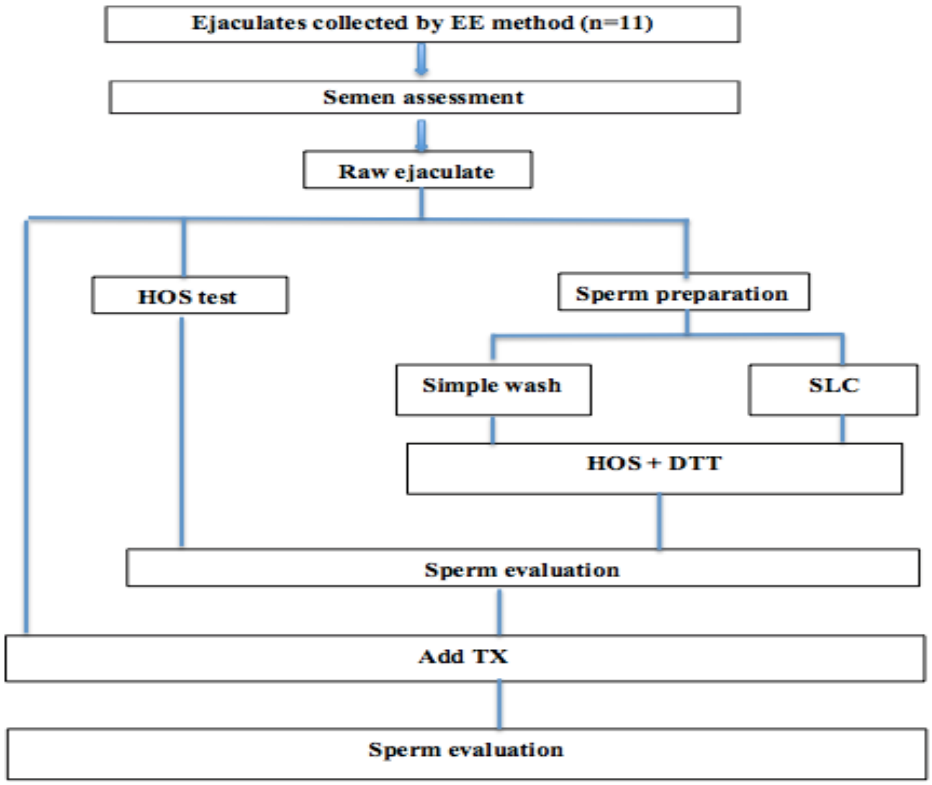


Figure 4 Diagram of experimental design

3.4 Results

3.4.1 Ejaculate traits

Traits of ejaculates (n=9; clear opaque to milky appearance are presented in Table 1. Samples contained a high proportion of abnormal morphologically sperm with low intact acrosome (Table 7). Abnormalities were related to head segment defects (34.5% ± 2.9 %; macrocephalic, microcephalic, abnormal acrosome and detached head) and tail defects (65.5 % ± 3.4 %; bent midpiece and tail with or without droplet, proximal and distal droplet, tightly coiled-tail). Tightly coiled tails were the major defect (13.4% ± 1.0%) across samples whereas head in coiled (HIC) was not observed in all samples. In prepared sperm by simple wash and SLC contained a percentage of tightly coiled-tail 11.7 ± 1.9% and 5.9 ± 0.9%, respectively.

Table 7 Fresh ejaculate traits (Mean ± SEM) of captive clouded leopards (n=9).

Parameter	Mean ± SEM	Range
Volume (µL)	411.6 ± 81.3	175 – 1,059
pH	7.4 ± 0.1	7 – 7.5
Osmolality (mOsm)	354.8 ± 10.1	321.5 – 422.5
Motility (%)	75.2 ± 3.0	52.5 – 91.3
Progressive motility (0-5)	3.3 ± 0.2	2 – 4
Sperm motility index	71.3 ± 2.5	56.3 – 85.6
Sperm concentration ($x10^6$/mL)	154.6 ± 35.7	26.7 – 465
Total sperm ($x10^6$)	59.2 ± 13.4	7.4 – 152.9
Viability (%)	80.8 ± 2.2	66.5 – 93
DNA intact (%)	97.8 ± 0.5	94.5 – 99.5
Acrosome intact (%)	41.9 ± 2.2	26.5 – 59
Normal morphology (%)	34.5 ± 2.9	21 – 57.5

3.4.2 Sperm membrane functional test

Clouded leopard responded to the HOS test with 62.0 – 92.5 % (78.9 ± 3.0 %) of swollen sperm cells. The HOS-treated sample from two sperm prepared methods (simple wash and SLC) presented the lower percentage of swollen sperm compared to the raw samples (73.4 ± 3.1% and 73.9 ± 2.5%).

3.4.3 Effects of demembranation on coiling of sperm tail

After treatment with TX, the uncoiled sperm in raw ejaculated group did not differ from the initial samples (Table 8). In HOS-treated samples, the percentage of tightly coiled-tail sperm in the simple washed group (>10% coiled sperm) tended to decrease after TX treatment and the percentage of ability to open plasma membrane of TX did not differ from the raw semen. In contrast, SLC-selected group (<6% coiled tail sperm) demonstrated the higher proportion of uncoiled sperm and the percentage of opened plasma membrane than the control group after TX treatment (P = 0.06).

Table 8 Effects of demembranation by 20% Triton-X 100 (TX) on sperm coiling in raw semen and hypo-osmotic swollen sperm prepared by simple washing-processed and SLC-processed samples (Mean ± SEM, n=9)

Treatment	Before TX	After TX	Opened by TX
Raw semen	13.9 ± 0.8	11.9 ± 0.6	12.8 ± 3.8
Simple washing, HOS/DTT (~10% coiled sperm)	34.9 ± 3.2	26.5 ± 3.4[a]	21.1 ± 7.9[A]
Single Layer Centrifugation, HOS/DTT (~6% coiled sperm)	41.7 ± 4.4	18.8 ± 3.8[b]	51.6 ± 10.4[B]

Within column value with different (a-b) differ (P = 0.1) and different captital letter differ (P = 0.06).

3.5 Discussion

This study demonstrated that most of the tightly-coiled tail defects in the clouded leopards could not be reversed by demembranation using the detergent. However, when semen processed through colloids, it presented a lower proportion of tightly coiled tail than the conventional semen prepared by simple washing. The demembranation test in SLC-treated group demonstrated that about 50 % of initial coiled sperm could be uncoiled by TX. These results implied that semen preparation was a useful tool to remove the coiled sperm that were defined as primary defects. The sperm with coiled tail defect remained in the SLC-processed samples could be mitigated, indicating secondary sperm defects. In addition, this was the first demonstration of sperm plasma membrane ability in the clouded leopard to maintain the volume equilibrium of sperm cells in hypo-osmotic environment.

Generally, the HOS test was applied to evaluate the functional integrity of sperm plasma membrane (Ramu and Jeyendran, 2013) to predict and diagnose male infertility (Van der Ven et al., 1986; Jeyendran et al., 1992) and to determine sperm

viability prior to sperm selection for ICSI (Verheyen et al., 1997). It has been stated that the percentage of swollen sperm by HOS test was correlated to the vital staining test using eosin (Schrader et al., 1986). In our finding, we also found the correlation on both methods (HOS and supravital stain using eosin-nigrosin) in all sample groups (data not shown). Thus, it would be useful to apply the HOS test as an alternative sperm evaluation method for predicting the fertilizing potential of spermatozoa. Moreover, this method is field-friendly due to its simple protocol and does not require advanced equipment for evaluation. In this study, we applied two sperm selection methods (simple washing and SLC) that are the standard sperm preparation methods for removing seminal plasma of felid semen (Morrell et al., 2009b). The result of membrane functional of processed sperm tended to decline from the initial samples. We assumed that the physical stress by centrifugation and osmotic stress by extender solution might affect the sperm membrane integrity as it was shown in the rat sperm (Varisli et al., 2009).

The demembranation test using TX in our experiment demonstrated a low efficacy to uncoil sperm compared with the previous study in infertile patients (Yeung et al., 2009). The variation of percentages of sperm tail defects responding to TX was observed among males indicating the individual factor resulted in high standard deviations. About 30% of uncoiled sperm in the raw samples were assumed to have a strength bonding that could not be lysed by a non-ionic surfactant as TX. In addition, only 21 % of coiled tail sperm in the HOS-treated sample could be opened by TX. This figure was similar to the previous reported in head-in-coiled (HIC) sperm defect type in human (Yeung et al., 2009).

The evidences supported that the cause of coiling tail sperm of animal in this study might be the pre-ejaculation origin possibly in the testis or upper section of epididymis where the oxidation of sperm membrane occurred (Yeung et al., 2009). The cross section of coiled sperm defect caused by testicular origin has been characterized transactional electron microscopy (TEM) in humans (Yeung et al., 2009) and domestic cats (Villaverde et al., 2013). Thus, further investigation on electron microscopic appearance of the tightly coiled-tail sperm in clouded leopard sperm is suggested.

In conclusions, the sperm defects in the ejaculates of clouded leopards in this study demonstrated a majority of fixed form of coiled tail that could not be reversed by demembranation using TX, implying the defect of pre-ejaculation origin.

CHAPTER IV

Hormonal profile of female clouded leopards during hormone induction

4.1 Abstract

Endangered clouded leopards are difficult to breed in captivity because of mate incompatibility. To date, information on gonadal steroidogenic activity of female clouded leopards provide from the North American population. Captive breeding program have been established to produce animals with value genetically, but as yet none are self-sustaining. The objectives of this study were to: 1) determine the ovarian steriodogenic function; and 2) examine the effect of exogenous gonadotropins for ovulation induction. Fecal samples were collected 1–7 days/week for 12 months from five adult females housed at Khao Kheow Open Zoo in Thailand. Fecal estradiol (E_2) and progesterone (P_4) metabolites were quantified by enzyme immunoassay (EIA). Of five individuals, two females (F1, F2) were given 300 IU eCG/ 1500 IU pLH (82 h interval) and three females (F3 – F5) were administrated with 200 IU eCG/1000 IU pLH (82 h interval). Ovarian assessment was performed at 29 h, 44 h and 96 h post pLH administration. Of five females, 4/5 animals were observed estrus signs. Based on fecal estrogen and progestagen metabolite concentrations, the overall estrous cycle length was (Mean ± SD) 41.5 ± 18.7 days. Birth records over the past 10 years indicated young are born throughout the year. The ovarian assessment by laparoscope presented that two females (40%, 2/5) have ovulated whereas others demonstrated multiple pre-ovulatory follicles. Oocytes were collected by OPU technique and were subjected to homologous IVF with post-thawed SLC-selected spermatozoa. A two-cell stage embryo was transferred to recipient (F3) with no conception. For AI, an ovulated female (F5), frozen-thawed semen were inseminated at 44 h after pLH administration with no pregnancy occur after 90 days post insemination. In conclusion, the exogenous regimen using eCG/pLH presented a promising ovarian response that will be useful for breeding management and assisted reproductive techniques like artificial insemination (AI), *in vitro* fertilization (IVF) or embryo transfer (ET).

4.2 Introduction

Endangered clouded leopards, medium-sized wild felid that challenge to breed in captivity. Previous studies on female reproductive physiology revealed that they are regularly presented a spontaneous ovulation. To develop an appropriate of exogenous gonadotropin regimen to stimulate ovarian activity in this species was extremely difficult. According to the exhibition of spontaneous ovulation was commonly found in this species (Brown et al., 1995; Howard et al., 1997; Pelican et al., 2006a). A common exogenous gonadotropin regimen use eCG followed by hCG 80-84 h to stimulate ovarian activity has successfully produced living offspring of domestic cat (Tsutsui, 2006) and eight species of wild felids (Donoghue et al., 1992; Howard et al., 1997; Roth et al., 1997; Swanson and Brown, 2004) including clouded leopard; *Neofelis nebulosa* (Howard et al., 1996; Howard et al., 1997). Nevertheless, the pregnancy rate was low in most endangered wild felid species. In addition, previous study of given exogenous gonadotropin treatments in clouded leopard demonstrated multiple endocrine responses including ovarian hyper-stimulation and luteal insufficiency (Brown et al., 1995). Currently, the modified gonadotropin regimen using eCG/pLH presented more consistent ovulatory responses compare to the standard eCG/hCG regimen and promising produce viable kitten by the laparoscopic embryo transfer and AI in domestic cat (Swanson, 2012).

The aims of this study were to 1) determine the ovarian activity of captive female clouded leopard in natural light exposure 2) assess the effect of hormone induction to the ovarian activity using the laparoscopy observation and the change of hormone concentration in fecal metabolite by the enzyme immunoassay (EIA). The hypothesis of this study was presumed that reproductive organs of female clouded leopard response to the exogenous gonadotropin treatment. The data from this study could be applied to further study in ARTs application.

4.3 Materials and Methods

4.3.1 Animals

Female clouded leopards (n=5) age between 2 to 10 years (Table 9) kept in the same condition as the males in Thailand Clouded Leopard Consortium Project at Khao Kheow Open Zoo (KKOZ), Chonburi province and Chiangmai Zoo (CMZ), Chiangmai province. All females were exposed to natural fluctuations in photoperiod and were housed singly in the steel cage but they can visual or smell neighboring males except two females in Chiangmai Zoo were kept together. Fecal samples were collected daily for 12 months for analysis of hormone profile including estrogen and progesterone metabolites.

Table 9 Inventory of female clouded leopards used in this study

ID	House name	Location	Transponder #	Birth date	Age (Y)
F1	Mesa	KKOZ	122762460A	26-Apr-04	10
F2	Thapthim	KKOZ	123211735A	12-Sep-04	10
F3	Grading	KKOZ	900012000507371	6-Sep-10	4
F4	Gradum	KKOZ	900012000500107	6-Sep-10	4
F5	F1	CMZ	900006000007352	24 Nov 12	2

4.3.2 Fecal hormone extraction

Fecal samples of all females were collected in the enclosure during 08.00 – 09.00 AM daily, sealed in plastic bags and stored at -20 ºC before extraction. Fecal samples were extracted to quantify estrogen and progesterone concentration using assays validated for domestic cat (Brown et al., 1994). In brief, fecal sample were dried in the conventional oven at 60°C for 4-5 days and kept frozen at -20 °C until extraction. Dried and powered fecal sample (0.2 ± 0.01g) was boiled in 90% ethanol in distilled water, vortexed for 1 min and boiled in water bath (96 ºC) for 20 min. The boiled sample will be centrifuged 2,500 g for 20 min then the supernatant was recovered. The second

extraction was performed by re-suspended the pellet with 5 mL of 90% ethanol then boiled and centrifuged at 3,500 g for 15 min and reconstituted in 1 mL methanol and briefly vortexed. The extracted sample was diluted 1:10 in steroid dilution buffer (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, 0.15 M NaCl, pH 7.0) and stored in polypropylene tubes at -20 ºC until enzyme immunoassay (EIA) analysis.

4.3.3 Fecal hormone analysis

Estrogen and progesterone metabolites were quantified by enzyme immunoassays (EIA) using antibodies (estradiol - 17 β (E_2), polyclonal, R-0008); progesterone monoclonal, CL-425) and horseradish peroxidase-conjugated tracers provided by C.J. Munro (University of California, Davis, CA, USA). EIA assay was performed in 96-well, flat-bottomed microtiter plates (Nunc Maxisorp, Fisher Scientific Inc., Pittsburgh, PA, USA) coated overnight (16-24 h) with antiserum (50 µl; estrogen, 1:12500; progesterone, 1:8000 dilutions) in coating buffer (0.05 M $NaHCO_3$, pH 9.6). Plates were washed (0.05% Tween 20 in 0.15 M NaCl solution) to remove unabsorbed antibody for five times using microplate washer. An aliquot of 50 µl of standards estradiol - 17 β (E_2) [sigma #E8875], progesterone [sigma #P0132], 1.50 – 400 pg/well diluted in EIA assay buffer (NaH_2PO_4, Na_2HPO_4, NaCl, BSA, pH 7.0) was added to well in duplicate and maintained at room temperature for 2 hours. Thereafter, plates were washed 5 times and added 100 µl of substrate buffer (H_2O_2, citric acid buffer, pH 0.4). After incubation for 50 – 60 min, the optical density (OD) was read using a microplate reader (TECAN Sunris microplate reader, Salzburg, Austria) at 405 nm when 0 pg standard wells reached an OD of 0.9-1. Serial dilutions of pooled fecal extracts produced a displacement curve that was parallel to the standard curve (R^2 = 0.99). Recovery of known amounts of estradiol - 17 β (E_2) and progesterone standards added to a pooled of fecal extracts demonstrated mass recovery. Assay sensitivity were 0.039 ng/mL for the estrone glucuronide EIA and 0.015 ng/mL for the progesterone EIA. All interassay CVs were < 10% base on binding of high (30%) and low (70%) control samples. The inter-assay variation was < 10%. Data are presented as micrograms per gram dry feces.

4.3.4 Exogenous gonadotropin regimens

4.3.4.1 Gonadotropin stimulation

Females were observed for the estrus signs prior to inject the exogenous gonadotropin regiments. Hormones were administered in non-estrus females. Two protocols were trial as followed 1) 200 IU eCG (Folligon, Intervet International B.V., Kirkland, canada) followed by 1000 IU pLH (Sioux Biochemical, Iowa, USA) (n=3) and 2) 300 IU eCG/1500 pLH (n=2) with 82 h interval. The ovarian and reproductive tract assessment were performed using a 10-mm diameter straight forward telescope (Karl storz endoscope, Tuttlingen, Germany) at 1) 29 h (n=2), 44 h (n=2) and 96 h (n=1) post pLH administration. The hormonal program presented in Figure 5.

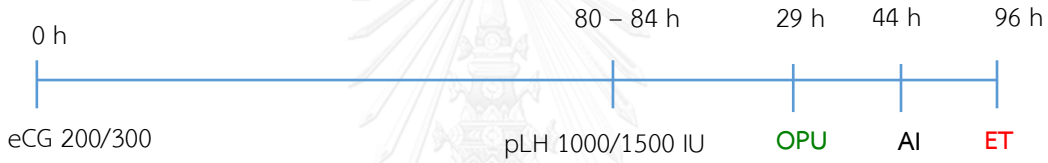


Figure 5 Exogenous gonadotropin regimens program and time of reproductive tract and ovarian assessment post pLH injection.

4.3.4.2 Assessment of ovarian activity

The reproductive tract and ovaries was inspected using standard laparoscopic approaches (Howard et al., 1996). Briefly, animals were placed in a head-down, supine position. A 10 mm diameter laparoscope was inserted at the abdominal midline. All aspects of each ovary were examined. A Veress needle was inserted transabdominal to measure the size of ovarian structures. The follicles were classified into 2 types; pre-ovulatory follicles (flatted or slightly raised, clear area measuring 2-6 mm diameter) and postovulatory corpus luteum (CL: opaque, reddish-yellowish structures raise above ovarian surface). Female with multiple pre-ovulatory follicles was conduct the ovum pick up (OPU) to retrieve oocytes using laparoscopic visualization. Female with at least one CL was classified as postovulatory then was subjected to AI.

4.3.5 Sperm preparation

Approximately 60 min before AI or IVF procedure, semen straws were thawed rapidly in air for 10 sec then in 37 °C water bath for 30 sec. Thawed semen was added in an empty prewarmed tube containing 250-µL thawing medium (3.025 g Tris, 1.4% (w/v) citric acid, 0.8% (w/v) glucose, 20% (v/v) sodium benzylpenicillin and 0.1% (w/v) streptomycin sulphate in 100 mL distilled water). Thawed sample was washed twice by centrifugation. The total sperm concentration was adjusted to 50 – 100 x 10^6 sperm/mL for AI and 5 x 10^5 sperm/mL for IVF.

4.3.6 Laparotomy intrauterine AI

All animals were anesthetized proposing to reproductive assesment. In female who presented an ovulation was inseminate using a cryopreserved sperm from SLC selected was used for inseminate by laparotomy intrauterine. In brief, an incision was made along the medium line and the uterine horns were exposed. A modified blunt 18G needle was inserted into the central region of the uterine lumen, and semen was inseminated into the tip of the uterine horn using an injector consisting of a 20G indwelling needle connected to a 1 mL syringe. The insemination was done in both uterine horns.

4.3.7 Homologous *in vitro* fertilization and embryo transfer

Collected oocytes by OPU were assessment and good quality oocytes were cultured in 500 µL of *in vitro* maturation (IVM) medium ($NaHCO_3$-M199 supplemented with 1.0 mM sodium pyruvate, 2.0 mM l-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, 4 mg/mL BSA) at 38.5 °C in a humidifed atmosphere of 5% CO_2 for 24 h. (Sanamung et al., 2010; 2011). After maturation, oocytes were washed and transferred to 50 µL drop of IVF medium (Tyrode's balanced salt solution containing 1% (v/v) mininum essential media (MEM) nonessentail amino acids (NEAA), 6 mg/mL bovine serum albumin (BSA), 100 IU/mL penicillin, 30 µg/mL heparin, 1mM L-glutamine, 0.36 mM sodium pyruvate, and 0.11 mM calcium lactate). The matured oocytes were co-incubated with frozen-thawed sperm (5 x 10^5 motile sperm/mL) in IVF medium at 38.5 °C and 5% CO_2 in air. After 24 h of co-incubation, excessive sperm was removed by

gentle pipetting. A part of Presumptive zygotes were washed and trasferred to 50 µL of synthetic oviductal fluid (SOF) containg 4 mg/mL BSA, 100 µg/mL streptomycin and 100 IU/mL peniciliin (IVC-1 medium). The zygotes were cultured and reassessed at 36-40 h post insemination (hpi). The 2 – 4 cells zygote was transferred to the recipient at 43 hpi. The unfertilized oocytes were fixed in 4% (w/v) paraformaldehyde then stained with fluorescent DNA labeling (4',6-diamidino-2-phenylindole; DAPI). The nuclear status of oocytes were examined using an epifluorescent microscope (1000x magnification).

4.3.8 Statistical analysis

Values are presented as the mean ± standard error of the mean (SEM). Baseline estrogen and progesterone metabolite concentrations were determined by an iterative process in which high values that exceeded the mean + 1.5 times the standard deviation (SD) were excluded and the mean recalculated until no values exceeded the mean + 1.5 SD. The estrous cycle length was calculated as the number of days from the first increase in fecal progesterone concentrations until the next increase. The data of number of follicles and corpora lutea, are presented as means ± SD. The pregnancy female was presents as percentage. The results were analyzed using descriptive analysis.

4.4 Results

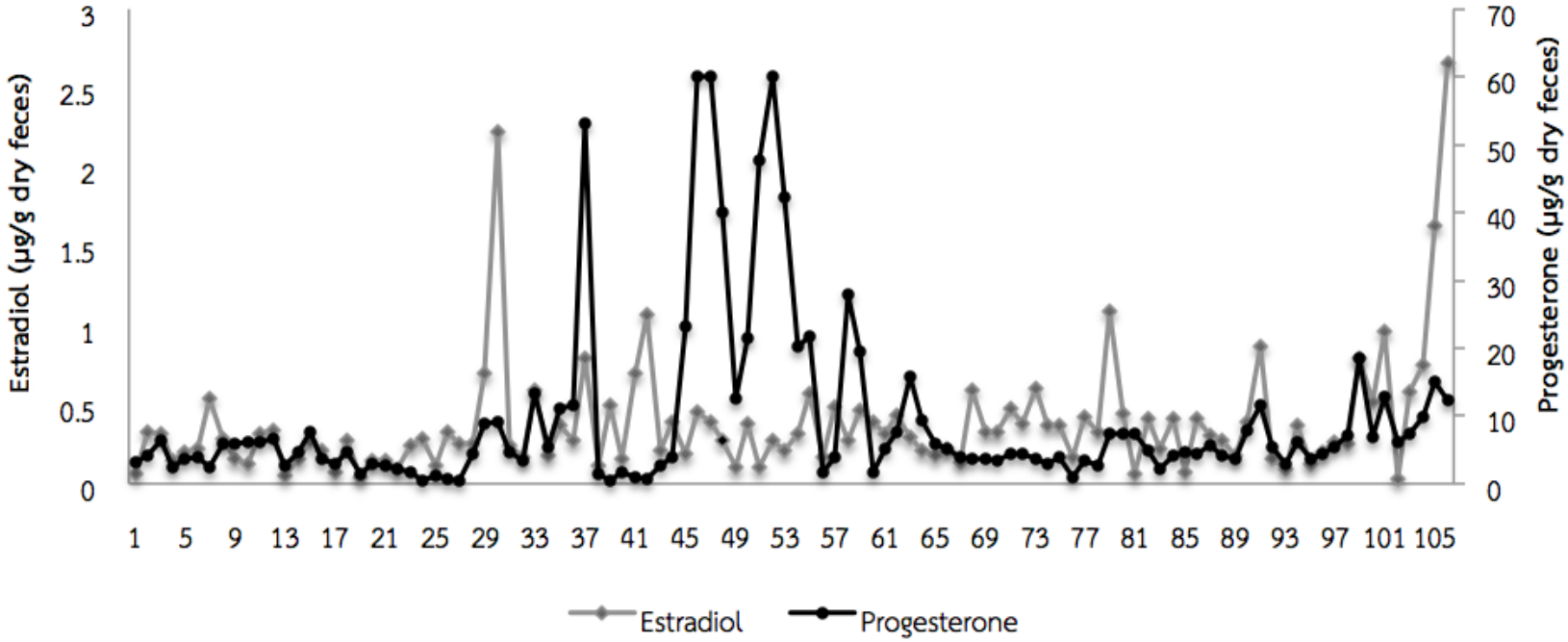
4.4.1 The effect of exogenous gonadotropin to ovarian activity

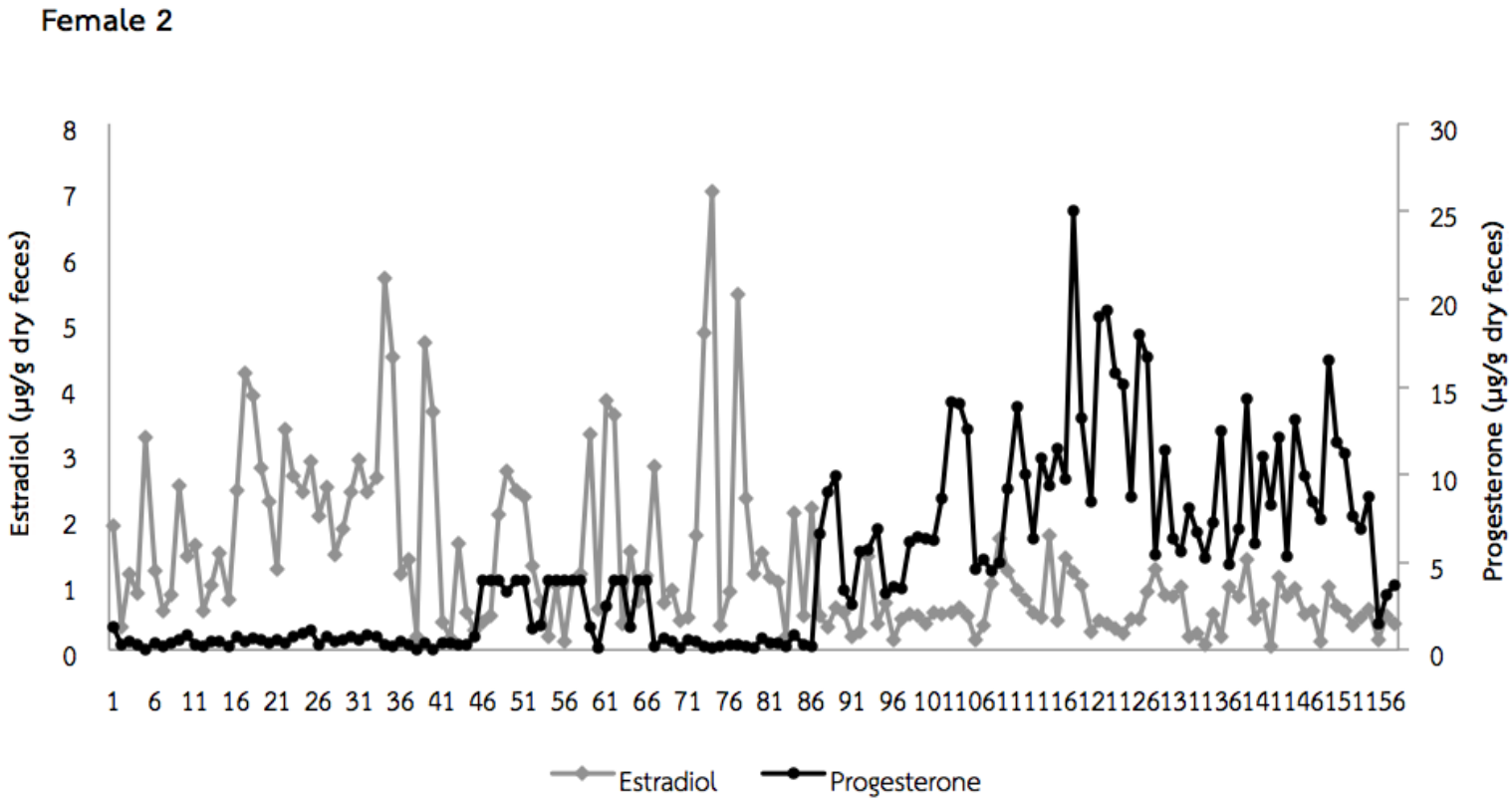
The observation of estrus behavior signs was observed in both group of hormonal regimens with varied signs from weak to strong estrus signs (rolling, restlessness and lordosis). However, there was one female (F4) not showing the estrus sign. The progesterone concentration of all females before exogenous hormone were at baseline (Mean ± SD) of E2 was 0.49 ± 0.15 and P4 6.03 ± 0.5 µg/g dry feces. The increase of progesterone was seen on Day 2 after pLH administration in F1 and F3 (Figure 6). However, the increase of fecal estradiol metabolite has seen in females who received 300 IU eCG/1500 pLH. The hormonal concentration was correlated to ovarian response that examine by visual laparoscope. The ovarian sizes and structures were measured (Table 10).

Table 10 Ovarian structure measured through the laparoscopy in clouded leopards treated with 200 IU eCG followed by 1,000 IU pLH at 82 h interval.

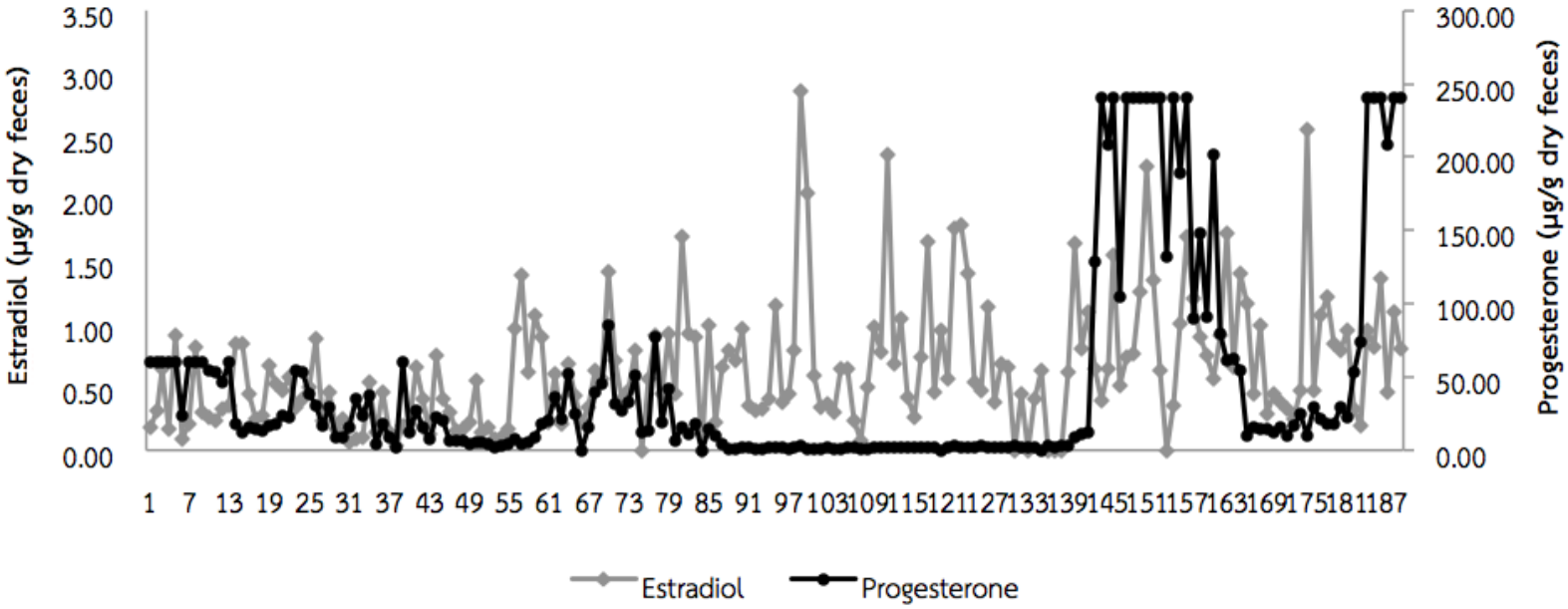
Female	Ovary size (cm)		Follicle (mm, number)		Corpus hemorrhagicum	
	Right	Left	Right	Left	Right	Left
F1	1.0×0.5×0.4	1.0×0.5×0.4	4 (1)	4 (1)	CH (2)	CH (2)
F2	1.2×0.7×0.5	1.2×0.7×0.5	6(1), 4(2), 2 (2)	6 (1), 4(1)	-	-
F3	1.2×0.5×0.4	1.2×0.5×0.4	4(3)	6(1)	-	CL (1)
F4	1.2×0.5×0.4	1.2×0.5×0.4	-	-	CL (2)	CL (1)
F5	1.2x0.6x0.4	1.2x0.6x0.4	-	-	CL (1)	CH (2)

Female 1


Estradiol (µg/g dry feces)
Progesterone (µg/g dry feces)
3
2.5
2
1.5
1
0.5
0
70
60
50
40
30
20
10
0
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69 73 77 81 85 89 93 97 101 105
Estradiol
Progesterone


Female 2
Estradiol (µg/g dry feces)
Progesterone (µg/g dry feces)
0
1
2
3
4
5
6
7
8
0
5
10
15
20
25
30
1 6 11 16 21 26 31 36 41 46 51 56 61 66 71 76 81 86 91 96 101 106 111 116 121 126 131 136 141 146 151 156
Estradiol
Progesterone

Female 3


Estradiol (µg/g dry feces)
Progesterone (µg/g dry feces)
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
1 7 13 19 25 31 37 43 49 55 61 67 73 79 85 91 97 103 109 115 121 127 133 139 145 151 157 163 169 175 181 187
Estradiol
Progesterone

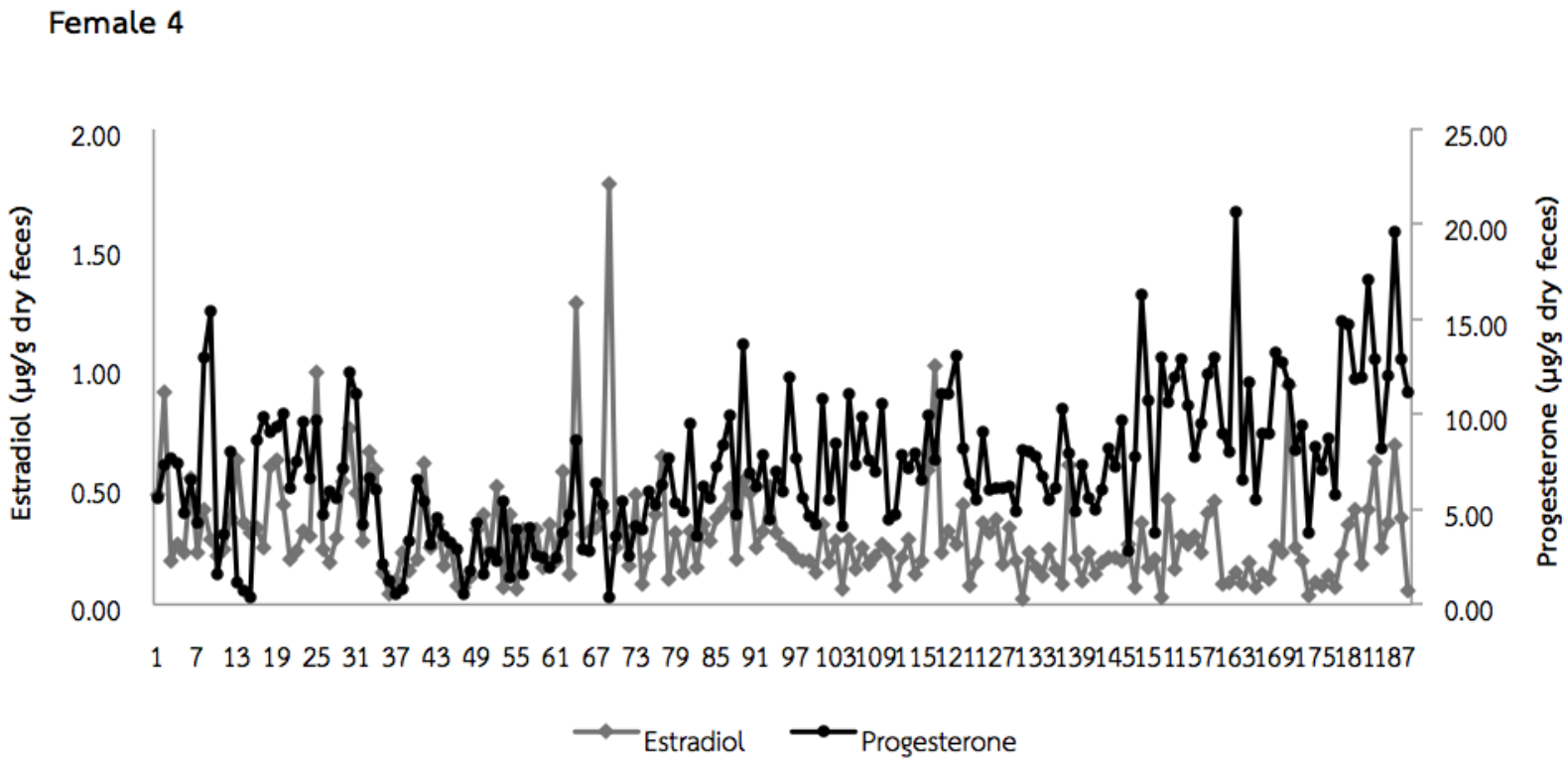


Figure 6 Longitudinal profiles of fecal estrogen and progesterone metabolites during estrous cycle and given exogenous hormone from four female clouded leopards from January 2013 through December 2013.

4.4.2 Laparotomy intrauterine AI

A female (F5) presented the corpora hemorrhagica (CH) indicating the ovulation, the laparotomy intrauterine AI was performed in this female. Frozen-thawed semen from SLC-treated of previous study in chapter 2 was used to inseminate with 40 x 10^6 and 30 x 10^6 total motile spermatozoa. The only pregnancy observation by keepers has been done according to the limitation of enclosure available for keeping animals therefore fecal samples could not be collected as usual. The parturition was not report after 90 days after insemination indicate that the animal was not conceived.

4.4.3 Homologous IVF and embryo transfer

Of 2 females (F1, F2), received 300 IU eCG/ 1500 IU pLH subjected to stimulate follicular formation demonstrated multiple pre-ovulatory follicles (Table 9). In addition, F4 who received 200 IU eCG/ 1000 IU pLH that initially proposed to AI presented only pre-ovulatory follicles and was not present the corpora hemorrhagica (CH). Therefore, the oocytes collection by OPU was performed in this female. All retrieval oocytes (n=9) from 3 females consisted of matured and immatured oocytes (Table 11) were incubated in IVM medium for 5 h prior to perform IVF. After 24 h incubation, only one oocyte has cleaved (11.1 %; 1/9). Embryo (2 cell stage) was transferred to recipient (F3) at 43 h post IVF. Fecal progesterone metabolize samples were determined for pregnancy monitoring. The progesterone concentration increased in first 2-week period after pLH administration and gradually decreased after ET. Moreover, the parturition was not occurred after 90 days post ET indicated that zygote was not implanted.

Table 11 In vitro fertilization of matured oocytes retrieved from two females (F1, F2)

Details	F1	F2
Sperm preparation from sire no.	M1	M2
Post thaw motility	20	80
Sperm concentration (for IVF)	5×10^5	5×10^5
Sperm volume per drop (µl)	5	7
Number of oocyte undergo IVF	5	6
# Clevage @ 24 hpi	0	1 (2 cells)

4.5 Discussion

This study demonstrated the preliminary results of exogenous gonadotropin to ovarian activity as a first time challenge in naïve individuals. The variation of sensitivity to gonadotropin hormone was found. The regimen of 300 IU eCC plus 1500 IU pLH likely to optimal dose for stimulate follicle formation in clouded leopard. Meanwhile, the treatment for AI was also shown a promising ovarian response. However, the sample size of this study was limited because they are endangered species and most of captive individual purposed to natural breeding program.

Female clouded leopards have been shown to be that difficult to stimulate ovarian induction because they are frequently presented spontaneous ovulation without copulation. Previous study revealed that a high progesterone concentration interfered the given gonadotropin with unclear explanation of mechanism in this species. Generally, the persistent CL on ovaries maintained the progesterone concentration level for pregnancy or anestrus of normal estrus period. The determination of a metabolized hormone concentration could predict the ovarian activity real-time from serum or urine sample. However, most species in Felidae tend to excrete the metabolized form of sex hormone through the fecal. This was advantage

to study in wildlife species due to it is non-invasive technique and is not generate stress to animals. However, the results is retrospection that could not use to predict the ovarian status prior to given exogenous hormone. In this present study, we combined the behavior sign to initially select non-estrous cycle for the experiment. However, in some female exhibited strong estrous signs but after visually ovarian assessment presented only small size of pre-ovulatory and the estrogen was also at the baseline level. These results revealed uncorrelated of animal behavior, hormonal concentration and ovarian activity in some animals.

To date, hormonal regimens of ovarian induction has developed and proven for domestic species. The common regimen using eCG/hCG was frequently presented significant formation of ancillary follicles (Swanson, 2012). Therefore, the current study used eCG/pLH regimens because it has been stated that this alternative hormonal treatment would improve the pregnancy rate without significant formation of ancillary follicles. Moreover, pLH has a short half-life metabolism than hCG and the combination of eCG/pLH produced consistent ovulatory responses and superior synchronization in domestic cat compare to the standard eCG/hCG regimen (Magarey et al. 2005). The regular spontaneous ovulation of clouded leopards resulted in unconsistency of ovarian response to given exogenous gonadotropins. The difficult to predict ovarian activity and a poor response to given gonadotropin treatment has been reported (Pelican et al., 2006b). To date, ovarian suppression prior to ovarian induction using gonadotropin presented a more predictable response in various studies of felids (Pelican et al., 2008; Pelican et al., 2010;Stewart et al., 2012) including clouded leopard (Pelican et al., 2006a). The modification of the protocol in further studies would be interested. However, there were not studies on prolong effect of ovarian suppression in this species therefore it would be better to apply a proven protocol that demonstrated less side effects on reproductive function.

CHAPTER V

The successful laparoscopic oviductal artificial insemination in the clouded leopard (*Neofelis nebulosa*) in Thailand.

5.1 Abstract

Challenges of breeding clouded leopards (*Neofelis nebulosa*) in captivity have been addressed due to a high incidence of mate incompatibility, teratospermic characteristic of the males, and uncertain ovulation pattern in the females. Pairing at pre-puberty aged promised a natural breeding achievement but it needed long-term observation. To date, assisted reproductive techniques are admitted as potential tools to manipulate the unable naturally breeding individuals and to manage suitable genetic variation. This study was performed in unsuccessful paired females (n=4; aged 4.5 – 5 years) kept at Khao Kheow Open Zoo, Thailand. Fecal hormone of all females (estrogen and progesterone metabolites) was extracted and determined by enzyme immunoassay (EIA) for assessing ovarian activity. Females were injected hormone equine chorionic gonadotropin (eCG) 200 IU. and porcine luteinizing hormone (pLH) 1000 IU) with 82 h interval. Ovarian assessment was performed by laparoscope at 44 h after pLH administration. One nulliparous female aged 4.5 years (F1) presented the ovulation (1/4; 25%) on both site of ovaries (n= 4). Semen of two adult males was collected by electroejaculation and prepared as chilled semen (at 4 °C) for insemination. Total motile sperm of 8 x 10^6 (M1) and 2.7 x 10^6 (M2) were used for artificial insemination (AI) in F1 by laparoscopic intraoviductal technique. Increasing of fecal progesterone concentration after AI presented the peak at day 25 post pLH injection and maintained at 128.4 µg/g dry feces during 65 days post AI indicated that the female had conceived. The delivery of two healthy cubs from F1 occurred on a 90-day of gestation. Microsatellite markers analysis of cubs was assessed and subsequently confirmed that M1 is father of both cubs.

5.2 Introduction

The clouded leopard (*Neofelis nebulosa*), a medium-sized (10-20 kg) felid living in the tropical rain forest of Southeast Asia, is regarded as vulnerable due to direct exploitation, range fragmentation and reduction in habitat quality (Grassman L et al., 2015). Being an arboreal species(Kitchener et al., 2006), observation of sexual behavior and mating *in situ* of the clouded leopards is restricted, contributing to insufficient information to facilitate captive environment for natural breeding. Other major challenges to conduct successful breeding management are: 1) mating incompatible behavior; a male often lethally attacks the smaller female (Brown, 2011), 2) teratospermic characteristic of the males (Wildt et al., 1986b), and 3) uncertain ovulation pattern in the females; 40% of the population exhibited spontaneous ovulation in the absence of mating (Brown et al., 1995). Also, assisted reproductive technologies such as sperm cryopreservation, artificial insemination (AI) and *in vitro* fertilization (IVF) in felids have been developed with limited successes. The ovarian hyper-stimulation and variable individual sensitivity to exogenous gonadotropin treatment were reported in many studies (Howard et al., 1996; Howard et al., 1997; Pelican et al., 2006a; Pelican et al., 2006b; Pelican et al., 2008) . The only AI success using fresh semen in the clouded leopards was reported since 1996 (Howard et al., 1996).

In 2002, the coalition of international partners, Thailand and the United State has agreed to create a viable and self-sustaining population of the clouded leopards at Khao Kheow Open Zoo in Chonburi province of Thailand. Over the decade, 70% live cubs have been produced from calm breeding pairs (8 of 40) (20%). The rest of the animals have lived unpaired without production (13 of 40, 32.5%), 11 (27.5%) were pre-pubertal and 8 (20%) were aging animals (Marti and Breitbeil, 2013). To help the unpaired clouded leopards having the possibility to propagate offspring, AI represents a powerful tool to manage these genetically valuable endangered species. Although transcervical and laparoscopic intrauterine AI in the domestic cats were claimed as non-invasive approaches (Chatdarong et al., 2007), laparoscopic intraoviductal AI was superior in that ten folds fewer numbers of motile sperm were required to achieve a

satisfactory pregnancy outcomes (Tsutsui et al., 2001). The procedure likely benefited the teratospermic characteristic of the clouded leopards that usually presented high rates of post-thawed sperm acrosomal disruption (Pukazhenthi et al., 2006a). Recently, a laparoscopic intraoviductal AI has been conducted in nondomestic felids, resulting in one viable kitten in the ocelot (*Leopardus pardalis*) and three viable kittens in the Pallas' cat (*Otocolobus manul*) (Swanson, 2012).

Except the appropriate AI technique, ovarian stimulation regimen is an important factor influencing the pregnancy outcomes. The standard follicullogenesis and ovulation stimulation regimen consisting of equine chorionic gonadotropin (eCG) followed by human chorionic gonadotropin (hCG) demonstrated undesired results including unovulated, residual follicles (Howard et al., 1996; Howard et al., 1997) and highly variable pregnancy success after AI (Donoghue et al., 1992). Their long half-lives (5 days for eCG and 4 days for hCG) caused secondary follicular growth and ovulations leading to compromised embryo migration and implantation. Alternatively, eCG treatment followed by porcine luteinizing hormone (pLH) has demonstrated improvement of pregnancy results by inducing ovulation without significant formation of ancillary follicles in the domestic cats (Conforti et al., 2013). This promising result is worth propagated to the female clouded leopards, the bigger-sized cats than the domestic cats. The knowledge would be presented as an assisted reproduction model for other wild cat species both *in situ* and *ex situ*.

5.3 Materials and Methods

5.3.1 Animals

Four female (age ranged 4.5 – 5.0 years) (F1, F2, F3 and F4) and two male clouded leopards (M1 and M2) aged 5 and 10 years, respectively, housed at Khao Kheow Open Zoo (KKOZ), Chonburi province, Thailand (latitude 13°″44′21 N and longitude ″0′59°100 E), were included. Two females (F1and F2) were kept together with no chance to confront with the males whereas the other two females (F3 and F4) were paired with adult males for natural breeding purpose. They were provided food, water and environmental enrichment based on the husbandry guideline for the clouded

leopard (Marti and Breitbeil, 2013) and exposed to natural photoperiod throughout the years. Estrus cycle stage was monitored for follicular and luteal phase according to behavioral observation and fecal steroid hormone analysis. F1 and F2 females received second exogenous gonadotropin for estrus induction at 12 m earlier whereas the others were naïve. All procedures were approved by the Ethical Committee for Animal Use at the Zoological Organization Park under the Royal Patronage of H.M. the King and the Ethical Committee for Animal use at Chulalongkorn University (#13310079).

5.3.2 Gonadotropin treatment

In the absence of behavioral signs of estrus (non-estrus) and fecal progesterone levels being at baseline (non-luteal), the female was administered 200 IU eCG intramuscullar. (Folligon, Intervet International B.V., Kirkland, Canada) followed by 1000 IU pLH intramuscular. (Sioux Biochemical, Iowa, USA) 82 h later. The progesterone baselines of F1, F2, F3 and F4 prior to eCG administration were 2.9 ± 2.3, 9.3 ± 3.5, 6.6 ± 2.4, 5.4 ± 2.2 µg/g dry feces, respectively. The eCG was administered on Julian Day 65th and 66th in F1 and F2, and F3 and F4, respectively.

5.3.3 Fecal hormone extraction and analysis

For assessment of ovarian activity, fecal samples were collected from the floor in the enclosure during 08.00-09.00 a.m. every 1-7 days interval for 10-15 m. The duration was covered before and after given the exogenous hormones. All samples were dried using a conventional oven at 60°C for 4-5 days and kept frozen at -20 °C until extraction. Steroid hormone extraction was modified from the previously developed and validated assay for felids (Brown et al., 1994). In brief, 0.2 g (± 0.01) of well-mixed, dried and powdered fecal samples was added in 5 mL a glass tube containing 90% ethanol in distilled water, vortexed for 1 min and boiled in water bath (96 °C) for 20 min. The pre-boiled volume was maintained by adding 90% ethanol during boiling. The boiled sample was centrifuged at 2,500 g for 20 min and supernatant was obtained. The second extraction was performed by re-suspended the pellet with 5 mL of 90% ethanol then boiled and centrifuged at 3,500 g for 15 min. Two supernatants were combined and boiled in the warm water bath (96 °C) until

dried then reconstituted in1 mL methanol. Reconstituted sample was dried-down and reconstituted in 1 mL dilution buffer (NaH_2PO_4, Na_2HPO_4, NaCl, pH 7.0), vortexed for 1 min, transferred to microcentrifuge tube (1.5 mL) and stored at -20 until further analysis. Extraction efficiencies were 97.7% (coefficient of variance [CV] <10%) for estrogen and 87 % (CV<10%) for progesterone based on the recovery of the respective standard added to dried feces before extraction.

Estrogen and progestagen metabolites were quantified by enzyme immunoassays (EIA) using antibodies (estradiol - 17 ß (E_2), polyclonal, R-0008); progesterone monoclonal, CL-425) and horseradish peroxidase-conjugated tracers provided by C.J. Munro (University of California, Davis, CA, USA). EIA assay was performed in 96-well, flat-bottomed microtiter plates (Nunc Maxisorp, Fisher Scientific Inc., Pittsburgh, PA, USA) coated overnight (16-24 h) with antiserum (50 µl; estrogen, 1:12500; progesterone, 1:8000 dilutions) in coating buffer (0.05 M $NaHCO_3$, pH 9.6). Plates were washed (0.05% Tween 20 in 0.15 M NaCl solution) to remove unabsorbed antibody for five times using microplate washer. An aliquot of 50 µl of standards estradiol - 17 ß (E_2) [sigma #E8875], progesterone [sigma #P0132], 1.50 – 400 pg/well diluted in EIA assay buffer (NaH_2PO_4,Na_2HPO_4, NaCl, BSA, pH 7.0) was added to well in duplicate and maintained at room temperature for 2 h. Thereafter, plates were washed 5 times and added 100 µl of substrate buffer (H_2O_2, citric acid buffer, pH 0.4). After incubation for 50 – 60 min, the optical density (OD) was read using a microplate reader (TECAN Sunris microplate reader, Salzburg, Austria) at 405 nm when 0 pg standard wells reached an OD of 0.9-1. Serial dilutions of pooled fecal extracts produced a displacement curve that was parallel to the standard curve (R^2 = 0.99). Recovery of known amounts of estradiol - 17 ß (E_2) and progesterone standards added to a pooled of fecal extracts demonstrated mass recovery. Assay sensitivity were 0.039 ng/mL for the estrone glucuronide EIA and 0.015 ng/mL for the progesterone EIA. All interassay CVs were < 10% base on binding of high (30%) and low (70%) control samples. The inter-assay variations were < 10%. Data are presented as micrograms per gram dry feces.

5.3.4 Animal observation and ovarian assessment

Behavioral estrus characterized by rubbing and rolling, restlessness and calling was detected in F1 and F2 on Day 3 after the eCG administration. None of the estrus signs in the other females were observed. The rise of progesterone was seen on Day 12 after pLH administration in F1 whereas the fecal samples were not consecutively obtained in the other females due to the dramatically decrease in food intake after anesthesia for abdominal laparoscopy. However, the increase of fecal estradiol metabolite above the baseline was not observed throughout the study in all females although large follicles were presented. During laparoscopy, the ovarian sizes and structures were measured (Table 12). The corpora hemorrhagica (CH) were seen only in F1 (Fig. 7), thus, the oviductal laparoscopic AI was performed in this female. The other females contained a large follicle of >5 mm. The rise of progesterone metabolite indicating successful ovulation was demonstrated with the peaks (F1=500 µg/g feces and F2=62 µg/g feces) observed 25 days after pLH injection. The progesterone rise was maintained until 47 (F1) and 45 days (F2) after pLH to and then started to decline to the baseline on Julian day 132nd and 126th, respectively (Fig. 8).

Table 12 Ovarian structure measured through the laparoscopy in clouded leopards treated with 200 IU eCG followed by 1,000 IU pLH at 82 h interval

Female	Ovary size (cm)		Follicle (mm, number)		Corpus hemorrhagicum	
	Right	Left	Right	Left	Right	Left
F1	1.2×0.6×0.4	1.2×0.6×0.4	4 (1)	-	CH (2)	CH (2)
F2	1.2×0.6×0.5	1.2×0.7×0.5	4(1), 2 (1)	6 (1)	-	-
F3	1.2×0.5×0.4	1.2×0.6×0.4	4(1), 6 (1)	4(3), 2 (3)	-	-
F4	1.2×0.5×0.4	1.2×0.7×0.5	4(2), 2 (1)	6(1), 2 (1)	-	-

5.3.5 Semen collection and processing

Semen collection was performed in two males (aged 10 and 11 y) using electroejaculation on the same day of AI. Ejaculate samples were evaluated for its qualities (Table 13). Motility and progressive motility was assessed subjectively under phase-contrast microscope. Sperm count was performed in a hemocytometer chamber. Sperm viability, acrosome and DNA integrity was evaluated using eosin-nigrosin staining (Bjorndahl et al., 2003), the double fluorescent labeling techniques (FITC-PNA and PI staining) (Axner et al., 2004), and acridine orange (AO) staining (Thuwanut et al., 2011), respectively. The semen was subjected to centrifugation (300 x g, 20 min) and supernatant was discarded. Sperm pellet was re-suspended with tris egg yolk extender (contains 3.025 Tris, 1.4% (w/v) citric acid, 0.8% (w/v) glucose, 3% (v/v) glycerol, 20% (v/v) egg yolk, 0.06% sodium benzylpenicillin and 0.1% (w/v) streptomycin sulphate in distilled water). The extended sample was cooled from room temperature to 4 ºC. Total motile sperm of 8 x 10^6 and 2.7 x 10^6 was used for AI.

5.3.6 Oviductal laparoscopic AI procedure:

AI was conducted 44 h after the pLH administration in one out of four females (F1) that presented CH. Anesthesia was induced using the combination of ketamine hydrochloride (5-10 mg/kg; Alfasan Internatinal B.V., Woerden, Holland) and xylazine

hydrochloride (0.5-1 mg/kg; Troy Laboratories Pty Limited, Smithfield, Australia). Surgical plane stage was maintained using isoflurane (0.5 - 3%) inhalation during the procedure. Prior to AI, the chilled semen (10 µL) was prepared for insemination. The female was placed in ventral recumbency with her hind end elevated. A 10-mm diameter straight forward telescope (Karl Storz endoscope, Tuttlingen, Germany) was used to assess the ovaries and reproductive tract prior to AI. Ovarian follicles and CH were counted and measured (Table 12). Laparoscopic oviductal AI was performed as previously described (Swanson, 2012). In brief, the grasping forceps with a tapered serrated tip (MDS Incorporated, Brandon, FL, USA) were used to grip the craniomedial edge of ovarian bursa and allowed visualization of the abdominal oviductal ostium (Figure 7). A polypropylene i.v. catheter (16 g, 3.2-inch length; Terumo Medical Corporation, Elkton, MD, USA) was placed through the ventral abdominal wall dorsal to the ovary and inserted a blunted spinal needle (20 g, 4-inch length; Terumo Medical Corporation, Elkton, MD, USA) that attached to 1 mL syringe containing 10 µl extended semen. The AI needle was passed through the oviductal ostium and the semen was deposited about 2 cm. deeply into the oviduct. After AI, the female was kept in the same cage with the female cagemate (F2). The pregnancy monitoring was closely observed by keepers and close circuit television. Fecal hormone was also monitored. The delivery of two healthy cubs from F1 occurred on Day 90 after AI.

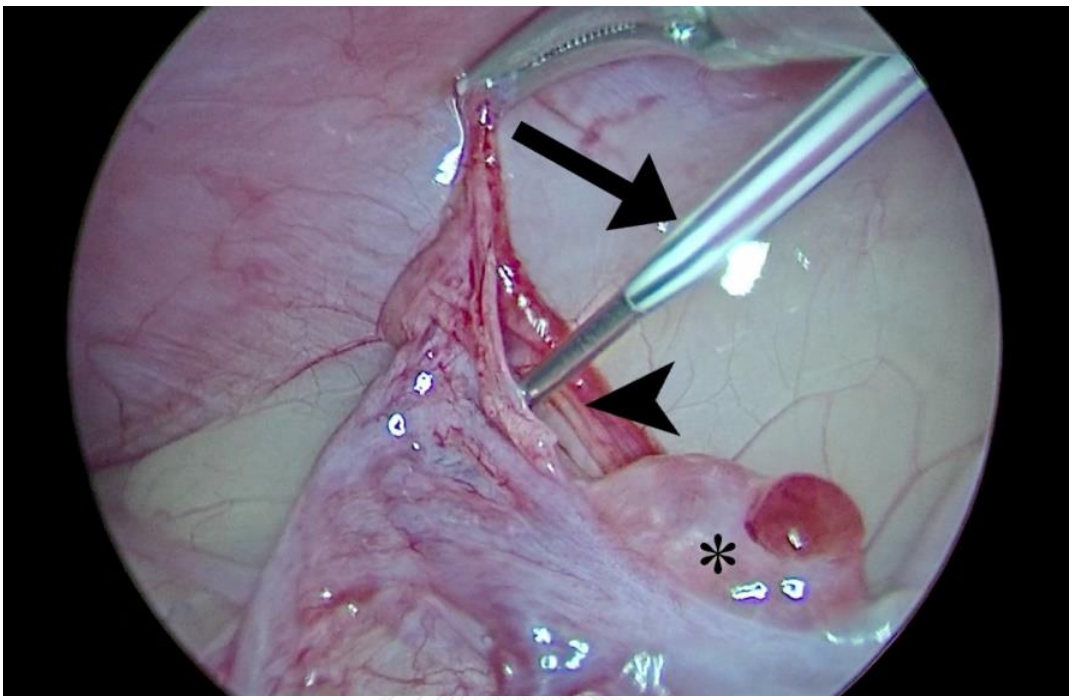


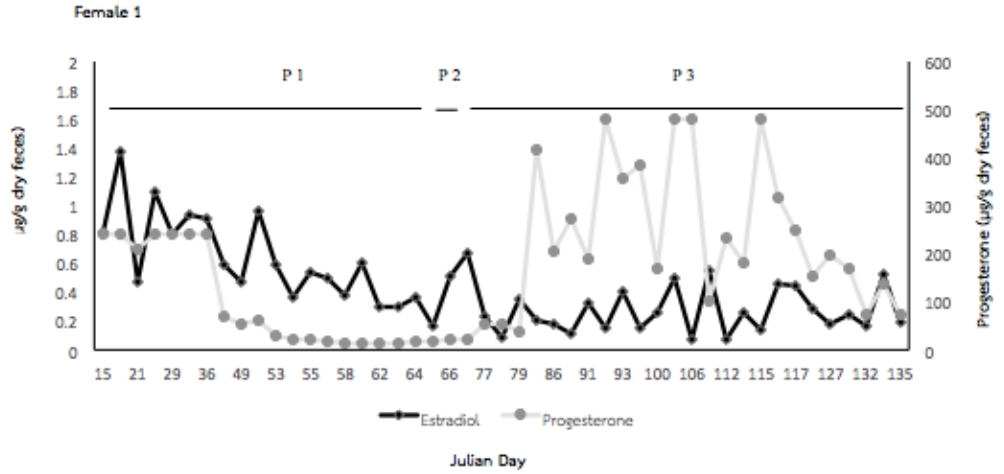
Figure 7 Laparoscopic view of oviductal AI using the modified stainless steel needle (black arrow) passed through a polypropylene i.v. catheter into the oviductal ostium (black arrow head) nearby the ovary (black asterisk) for sperm deposition.

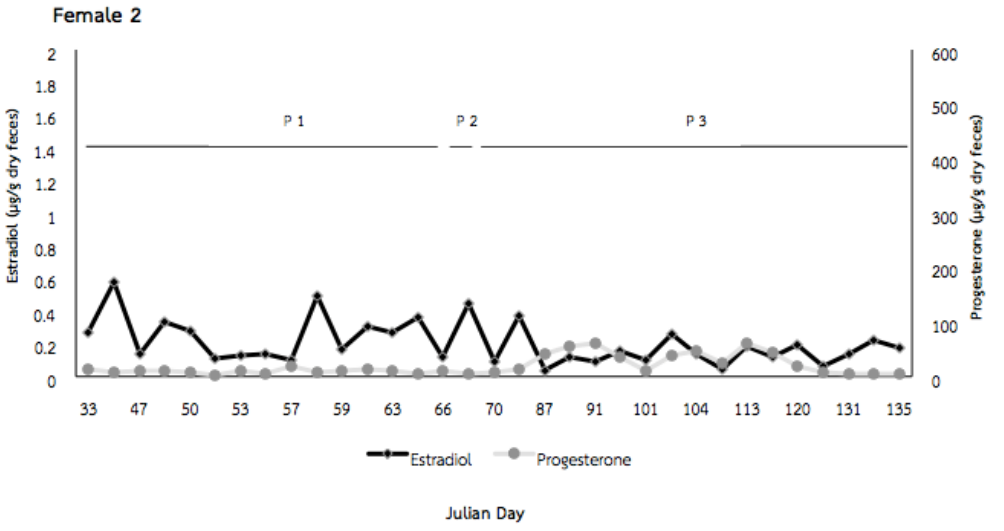
Table 13 Sperm quality of chilled semen used for AI in the clouded leopards (M1 and M2).

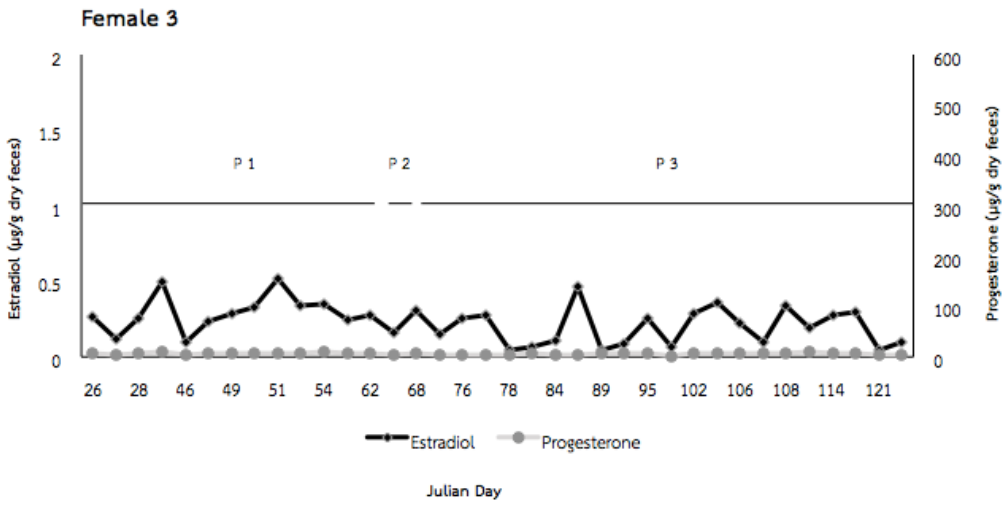
Parameter	M1 Mei	M2 Sakda
Fresh semen		
Ejaculate volume (µL)	650	360
Sperm concentration (x10^6/mL)	17.6	8.25
Total sperm count (x10^6)	11.4	3.9
Motility (%)	70	80
Progressive motility	+3	+3
Viability (%)	76	81
Intact membrane (%) (HOS test)	78	66
Intact acrosome (%)	56.3	52
Intact DNA (%)	99	99
Head normal (%)	47.5	31.8
Tail normal (%)	51.5	34
Chilled semen		
Inseminated volume (µL)	10	10
Motility (%)	70	70
Progressive motility	+3	+3
Total sperm inseminated (x10^6)	11.4	3.9
Total motile sperm number (x10^6)	8	2.7

Table 14 The average of fecal hormone metabolite values (progesterone and estradiol) in female clouded leopards that subsequently treated with equine chorionic gonadotropin (eCG) and porcine luteinizing hormone (pLH) prior to intratubal laparoscopic artificial insemination (Mean ± SD).

Hormone (µg/g dry feces)	Female	Period		
		Period 1: 45 days before estrous induction	Period 2: Estrous induction to artificial insemination	Period 3: 65 days after artificial insemination
Progesterone	Female 1 (Gra Ding)	2.95 ± 2.27	22.72 ± 0.92	238.06 ± 147.92
	Female 2 (Gra Dum)	9.25 ± 3.54	5.63 ± 1.13	30.82 ± 20.14
	Female 3 (Sen Lek)	6.61 ± 2.38	5.71 ± 0.49	5.79 ± 1.91
	Female 4 (Sen Yai)	5.40 ± 2.21	1.85 ± 0.56	2.40 ± 0.92
Estradiol	Female 1 (Gra Ding)	0.62 ± 0.31	0.59 ± 0.11	0.26 ± 0.15
	Female 2 (Gra Dum)	0.25 ± 0.15	0.27 ± 0.25	0.15 ± 0.08
	Female 3 (Sen Lek)	0.29 ± 0.12	0.23 ± 0.11	0.19 ± 0.11
	Female 4 (Sen Yai)	0.07 ± 0.04	0.11 ± 0.06	0.12 ± 0.05


Female 1
P 1
P 2
P 3
µg/g dry feces)
Progesterone (µg/g dry feces)
15 21 29 36 49 53 55 58 62 64 66 77 79 86 91 93 100 106 112 115 117 127 132 135
Estradiol
Progesterone
Julian Day


Female 2
P 1
P 2
P 3
Estradiol (µg/g dry feces)
Progesterone (µg/g dry feces)
33 47 50 53 57 59 63 66 70 87 91 101 104 113 120 131 135
Estradiol
Progesterone
Julian Day


Female 3
P 1
P 2
P 3
Estradiol (µg/g dry feces)
Progesterone (µg/g dry feces)
26 28 46 49 51 54 62 68 76 78 84 89 95 102 106 108 114 121
Estradiol
Progesterone
Julian Day

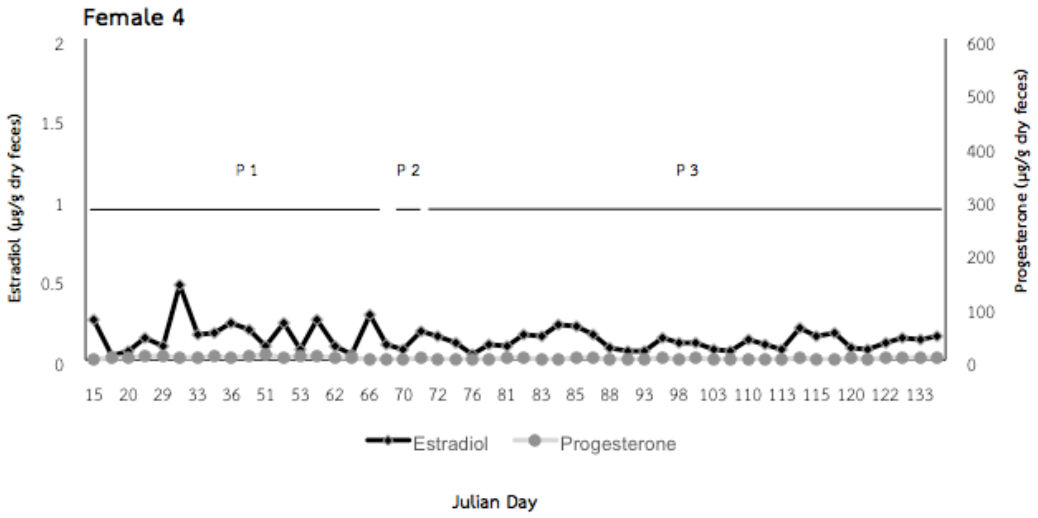


Figure 8 Representative fecal estradiol and progesterone metabolite patterns in female clouded leopards (n = 4; female 1 to female 4) that subsequently treated with equine chorionic gonadotropin (eCG) and porcine luteinizing hormone (pLH) prior to laparoscopic oviductal artificial insemination. P1 = 45 days before estrous induction; P 2 = estrous induction to artificial insemination and P 3= 65 days after artificial insemination

5.3.7 Parentage testing

Three blood samples from parents (2 males; M1 and M2 and female; F1) were collected purposing to examine the parentage of cubs. Genomic DNA was extracted) by modified phenol-chloroform methodNelson and Krawetz, For .(1992cubs, hair samples were collected and the genomic DNA was extracted by modified dilution protocol using Phire Animal Tissue Direct PCR Kit (Thermo Fisher Scientific Inc.,Waltham, MA, USA). Microsatellite analysis was performed using11 microsatellite markers that were previously studied in domestic cat (*Felis catus*) and Sumatran tiger (*Panthera tigris sumatrae*) including; FCA 045, FCA 082, FCA 144, FCA 261, FCA 310, 6HDZ 003, 6HDZ 057, 6HDZ 064, 6HDZ 089, 6HDZ817 and 6HDZ 859 (Menotti-Raymond et al., 1999; Williamson et al., 2002). The samples were analyzed by two-step PCR using Fusion® Hot start Taq DNA polymerase II (Fisher Scientific Inc.,Waltham, MA, USA). Genotyping was performed using a CEQ system (CEQTM8000 Genetic Analysis System; Beckman Coulter, Inc.) and GenomeLab DNA Size Standard Kit—400 internal

standard sizes— were used to assess PCR product size. Of 11 microsatellite markers, two markers (FCA 045 and 6HDZ 817) revealed thatboth cubs are cubs of M1.

5.4 Discussion

This present report demonstrated the first successful of the laparoscopic oviductal artificial insemination in the endangered clouded leopard. This was only second time of AI success in this species since Howard et al. has reported in 1996. The advantages of this technique were 1) less invasive procedure 2) reduce the number of the motile sperm number require for insemination 3) high conception rate when perform the AI after ovulation. In this approach, we found only 25% of females (1/4) revealed well responsive to the hormone stimulation regiment whereas mainly females likely to less response to the exogenous gonadotropin hormone. We assumed that the variation of ovarian response might be because the initial different stages of ovarian activity prior to given exogenous hormones.

It has been noted that artificial insemination (AI) is a powerful tool to propagate and manage the genetically valuable endangered species. In Felidae, the AI technique was mostly developed in domestic cats as a model species. Recently, the cross-species applicability of AI technique to non-domestic cats has progressively applied with a promising outcome. Laparoscopic technique could minimally invasive and traumatic to the animal comparing to the other surgical technique (i.e. laparotomy). In our procedure, most animals showed normal wound healing after surgery procedure. However, the decrease of appetite was observed for 2-3 days without other additional problems. According to a high incidence of abnormal morphologically sperm is commonly found in male clouded leopard (Wildt et al., 1986b; Pukazhenthi et al., 2006a). This characteristic can be limited sperm movement and fertilization thus small amount of good quality sperm could be retrieved for further application. The laparoscopic oviductal (LO) AI technique has been shown a high pregnancy rate by using low sperm number (Swanson, 2012). In our current approach, we could collect small amount of total motile spermatozoa of each male that available for AI (8×10^6

and 3 x 10^6). In addition, an exogenous gonadotropin treated female (F1) presented four corpus hemorrhagicum (CH) indicated the ovulation. Therefore, we decided to inseminate on both sides of oviduct to enhance a chance of successful that consequently the female has conceived. This achievement was supported that fewer motile spermatozoa are required for LOAI and can be accounted for the first success of applicable technique in a medium-sized felid as clouded leopard.

Apart from laparoscopic technique, the ovarian response to the synchronization protocol is one of key of successful. The exogenous gonadotropin hormone play๗ a role to initiate folliculogenesis, induce follicular maturation and ovulation. In our research, we used eCG/pLH regimens because our hypothesis presumed that this alternative regiment would improve the pregnancy results by inducing ovulation without significant formation of ancillary follicles (Conforti et al., 2013) and adverse effects to the ovarian activity. Moreover, the single injections of these two gonadotropins could reduce time to approach the animals that would be compromised the stress comparing to a multiple injection regiment. According to ovarian assessment by laparoscope, we found the variation of ovarian responsive. Only one female demonstrated ovulation after 44 h pLH injection whereas other females presented some pre-ovulatory follicles on the ovaries (3-8 follicles). Although, all females had a dominant follicle (6 mm sized) but from the hormone profile results seemed that only female (F2) ovulated after procedure. We assumed that the initial different stages of ovarian activity on the injected date influenced to a given exogenous hormone. In regularly spontaneous ovulator as clouded leopard, it difficult to predict ovarian activity and a poor response to given gonadotropin treatment has been stated(Pelican et al., 2006b). Alternatively, ovarian suppression prior to gonadotropin stimulation presented a more predictable response in various studies of felid (Pelican et al., 2008; Pelican et al., 2010; Stewart et al., 2010) including clouded leopard (Pelican et al., 2006a). In clouded leopard, the recent researches on GnRH agonist, leuprolide acetate (Lupron®) showed promising to inactivate ovarian activity but altered ovarian sensitivity resulting in unovulation were observed (Pelican et al., 2006a). Meanwhile, progestin administration by implantation (Levonorgestrel) or orally (Altrenogest) in

domestic cat demonstrated successful improvement of ovarian response. The short term suppression ovarian activity using progestin implants resulting in a more consistent response to gonadotropin stimulation included spontaneous ovulation (Pelican et al., 2008), enhanced IVF embryo production (Pelican et al., 2010) and improved ovulation induction for AI (Pelican et al., 2006b). In addition, priming with oral progestin effectively down regulated folliculogenesis and increased ovarian sensitivity to exogenous gonadotropins (Stewart et al., 2010). These promising effects of progestin on ovarian control in domestic cat would be benefit for further study to other species that prone to spontaneous ovulation i.e. clouded leopards or fishing cats.

CHAPTER VI

General discussion and conclusion

6.1 Overcoming sperm abnormalities

Semen of clouded leopard contained high proportion of abnormal morphological spermatozoa with abnormal acrosome and tightly coiled tail as major defects that affect to its functionality. For sperm cell, acrosome and tail segment play important roles on the fertilization. To improve acrosome integrity and sperm motility, sperm selection using single layer centrifugation (SLC) through colloid has currently demonstrated as a proven technique applying for ejaculate or epididymal semen of domestic species with more application in non-domestic species. These current studies demonstrated a promising technique to overcome the effect of sperm abnormalities by selecting good quality spermatozoa prior to apply further assisted reproductive techniques. It was well recognized that acrosome of clouded leopard severe sensitive to an ultra-low temperature during cryopreservation resulting in extremely decline of intact acrosome in frozen-thawed semen. The SLC could select normal spermatozoa with intact acrosome significantly higher than the conventional simple washing sperm preparation. Although, after freezing the dramatically decreasing of acrosome intact has also seen similar to the other method but the functional test on its ability to fertilize *in vitro* showed that selected spermatozoa has a better on fertilization rate. The tightly coiled tail spermatozoa obtained from male clouded leopards of this study are likely to the defect of pre-ejaculate origin that difficult to uncoil by recent demembranation technique. However, to mitigate such a sperm tail defect, the modified SLC sperm selection and demembranation technique could lessen the primary sperm defects of raw semen and could be selected spermatozoa that were prone to be mitigated after demembranation.

The female ovarian function study using the exogenous gonadotropins (eCG/pLH) revealed the sensitivity variation in naïve individuals. The regimen eCG and

pLH demonstrated the optimal dose for stimulate follicle formation and showed a promising ovarian response. The available animals for the study was limited and variation response of ovaries found in the recent experiment. Therefore, we could not perform the oviductal insemination using selected spermatozoa for our first challenge of the laparoscopic oviductal artificial insemination (LOAI). However, the first successful of LOAI using non-selected spermatozoa also promised that this technique has the potential for propagating the unpaired and unproduced naturally individuals of this species.

6.2 Suggestion for further investigations

Most of our study we emphasized on male gamete biology and its function. The main objective of this study was to improve male gamete quality for further used *in vivo* fertilization with living female that unpaired individuals. In sperm preparation part has demonstrated some progressive on improving sperm cell qualities for further fertilization. However, we did not get the information about male physiology. The gap of this study is lack of the information on the correlation of gamete producing quality, animal behavior and reproductive performance that could be measuring from fecal testosterone metabolite. Therefore, the study on male hormonal pattern is needed in further investigation. Apart from male, study in female clouded leopards is also challenging. Although, our trial treatment program presented a promising pregnancy results but it was not consistent and difficult to predict the ovarian activity. Ovarian suppression prior to ovulation induction protocol have been applied with a promising results on stimulate ovarian activity and more predictable of ovulation result in high pregnancy rate and viable kitten. To apply the protocol in clouded leopard would be interested. However, the effect of administration suppressed ovarian hormone need to be considered whether it could be affected to reproductive organ function or not. It would be better to study in model species prior to apply to this endangered species.

6.3 Conclusion

This current study has demonstrated that sperm characteristic of captive clouded leopards in Thailand present a high proportion of abnormality morphology.

This was correlated to the first hypothesis that semen of captive clouded leopards in Thailand contained a high proportion of abnormally morphological sperm or teratospermia. Although, in this population has genetically variation and most of former donors are wild caught. This finding in present study is also similar to previous reports of the clouded leopard sperm characteristic in the North American population and other felids species keeping in captivity.

According to the high proportion of malformed sperm in this studied individuals, we presumed that those abnormal spermatozoa could be decreased by sperm selection as presented in several domestic species who were affected by poor sperm quality. However, this is the first demonstration to apply the gradient selected method through the colloid in a known teratospermic species. The current results revealed that the morphologically normal sperm has been increased by SLC. Two main sperm parameters involving to fertilization as acrosome integrity and normal sperm tail showed a significantly increased percentage after SLC processed. Therefore, the results of this part were correlated to the second hypothesis indicting that SLC could be improved the number of normal spermatozoa.

The selected spermatozoa were used for further investigation on their ability to fertilize with the maturated oocytes. In this study, we have performed both homologous and heterologous IVF. In homologous IVF, we could be retrieved small amount of good quality oocytes for IVF therefore the percentage of cleavage was low. In the meantime, the heterologous IVF revealed that the post-thawed selected spermatozoa presented a better number of fertilization than the non-selected group. This was correlated to the third hypothesis that indicated the selected sperm are capable of penetrating domestic cat oocytes.

In the fourth experiment was switched to the female part. In the present study, we applied the novel hormone regimen in naïve animals using the combination of eCG/pLH. We presumed that these exogenous gonadotropins would able to stimulate the ovarian function of adult female clouded leopards. The results showed the variation of ovarian responses. We assumed that the variation of responsive would

result from the different of initial ovarian stage during hormone treatment. Moreover, the regular spontaneous ovulation of clouded leopards also resulted in inconsistency of ovarian response to the given exogenous gonadotropins. However, the adverse effects such as the formation of ancillary follicles or ovarian cyst were not found. Therefore, this regimen is promising regimen for ovarian induction in further apply of assisted reproductive techniques.

The artificial insemination technique using laparoscopic intrauterine has been succeeded in clouded leopard. However, the former technique needed the large number of motile sperm for insemination. In this study, we applied the insemination via intraoviductal to reduce the number of motile sperm used for inseminated dose and had a hypothesis that insemination using the selected spermatozoa resulted in pregnancy., According to the limitation of available females for insemination in this current experiment, only two females were used for insemination. The selected spermatozoa were used for intrauterine insemination whereas non-selected group was used for intraoviductal insemination. The result of pregnancy in female animal that undergone oviductal insemination demonstrated that this technique is the promising tool for applying to propagate clouded leopard that could not breed naturally.

In summary, the application of sperm selection using SLC through colloid revealed that normal spermatozoa with fertilizing ability were retrieved. Although, small amount of sperm was recovered after process but selected spermatozoa promised to enhance the successful for further applications. The studies have gained the better understanding of reproductive biology and physiology in term of semen characteristic and reproductive cycle of female clouded leopards in range country. The application of proven selection sperm technique as SLC will be shown the possibly to select good sperm quality for further application such as AI or IVF. Moreover, the development of ovarian control protocol using novel modified hormonal treatment regimens has gained more information of ovarian response to exogenous gonadotropins. In addition, laparoscopic oviductal AI with frozen-thawed selected

spermatozoa may be an optimal choice using assisted reproductive technique in this species.

REFERENCES

Aitken RJ 2006. Sperm function tests and fertility. Int J Androl. 29(1): 69-75; discussion 105-108.

Aitken RJ, Best FS, Warner P and Templeton A 1984. A prospective study of the relationship between semen quality and fertility in cases of unexplained infertility. J Androl. 5(4): 297-303.

Alvarez-Rodriguez M, Alvarez M, Anel-Lopez L, Lopez-Uruena E, Manrique P, Borragan S, Morrell JM, de Paz P and Anel L 2016. Effect of colloid (Androcoll-Bear, Percoll, and PureSperm) selection on the freezability of brown bear (Ursus arctos) sperm. Theriogenology. 85(6): 1097-1105.

Amann RP and Waberski D 2014. Computer-assisted sperm analysis (CASA): capabilities and potential developments. Theriogenology. 81(1): 5-17 e11-13.

Andrews JC, Howard JG, Bavister BD and Wildt DE 1992. Sperm capacitation in the domestic cat (Felis catus) and leopard cat (Felis bengalensis) as studied with a salt-stored zona pellucida penetration assay. Mol Reprod Dev. 31(3): 200-207.

Axner E 2006. Sperm maturation in the domestic cat. Theriogenology. 66(1): 14-24.

Axner E, Hermansson U and Linde-Forsberg C 2004. The effect of Equex STM paste and sperm morphology on post-thaw survival of cat epididymal spermatozoa. Anim Reprod Sci. 84(1-2): 179-191.

Aziz N, Saleh RA, Sharma RK, Lewis-Jones I, Esfandiari N, Thomas AJ, Jr. and Agarwal A 2004. Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. Fertil Steril. 81(2): 349-354.

Barratt CL, Tomlinson MJ and Cooke ID 1993. Prognostic significance of computerized motility analysis for in vivo fertility. Fertil Steril. 60(3): 520-525.

Bjorndahl L, Soderlund I and Kvist U 2003. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. Hum Reprod. 18(4): 813-816.

Blom E 1966. A new sterilizing and hereditary defect (the "Dag defect") located in the bull sperm tail. Nature. 209(5024): 739-740.

Brackett BG, Cofone MA, Boice ML and Bousquet D 1982. Use of zona-free hamster ova to assess sperm fertilizing ability of bull and stallion. Gamete Research. 5(3): 217-227.

Brown JL 2006. Comparative endocrinology of domestic and nondomestic felids. Theriogenology. 66(1): 25-36.

Brown JL 2011. Female reproductive cycles of wild female felids. Anim Reprod Sci. 124(3-4): 155-162.

Brown JL, Wasser SK, Wildt DE and Graham LH 1994. Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. Biol Reprod. 51(4): 776-786.

Brown JL, Wildt DE, Graham LH, Byers AP, Collins L, Barrett S and Howard JG 1995. Natural versus chorionic gonadotropin-induced ovarian responses in the clouded leopard (Neofelis nebulosa) assessed by fecal steroid analysis. Biol Reprod. 53(1): 93-102.

Cerny KL, Hughes S, Campos JR, Coleman RJ, Troedsson MHT and Squires EL Fertility of Mares Inseminated With Frozen-Thawed Semen Processed by Single Layer Centrifugation Through a Colloid. Journal of Equine Veterinary Science. 32(5): 289-291.

Cerny KL, Hughes S, Campos JR, Coleman RJ, Troedsson MHT and Squires EL 2012. Fertility of Mares Inseminated With Frozen-Thawed Semen Processed by Single Layer Centrifugation Through a Colloid. Journal of Equine Veterinary Science. 32(5): 289-291.

Chatdarong K, Axner E, Manee-In S, Thuwanut P and Linde-Forsberg C 2007. Pregnancy in the domestic cat after vaginal or transcervical insemination with fresh and frozen semen. Theriogenology. 68(9): 1326-1333.

Chatdarong K, Thuwanut P and Morrell JM 2010. Single-layer centrifugation through colloid selects improved quality of epididymal cat sperm. Theriogenology. 73(9): 1284-1292.

Comercio EA, Monachesi NE, Loza ME, Gambarotta M and Wanke MM 2013. Hypo-osmotic test in cat spermatozoa. Andrologia. 45(5): 310-314.

Comizzoli P, Mauget R and Mermillod P 2001. Assessment of in vitro fertility of deer spermatozoa by heterologous IVF with zona-free bovine oocytes. Theriogenology. 56(2): 261-274.

Conforti VA, Bateman HL, Schook MW, Newsom J, Lyons LA, Grahn RA, Deddens JA and Swanson WF 2013. Laparoscopic oviductal artificial insemination improves pregnancy success in exogenous gonadotropin-treated domestic cats as a model for endangered felids. Biol Reprod. 89(1): 4.

Cooper TG and Yeung CH 2003. Acquisition of volume regulatory response of sperm upon maturation in the epididymis and the role of the cytoplasmic droplet. Microsc Res Tech. 61(1): 28-38.

Crosier AE, Marker L, Howard J, Pukazhenthi BS, Henghali JN and Wildt DE 2007. Ejaculate traits in the Namibian cheetah (Acinonyx jubatus): influence of age, season and captivity. Reprod Fertil Dev. 19(2): 370-382.

de Lamirande E and Gagnon C 1995. Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects. Hum Reprod. 10 Suppl 1: 15-21.

Donnelly ET, Lewis SE, McNally JA and Thompson W 1998. In vitro fertilization and pregnancy rates: the influence of sperm motility and morphology on IVF outcome. Fertil Steril. 70(2): 305-314.

Donoghue AM, Johnston LA, Seal US, Armstrong DL, Simmons LG, Gross T, Tilson RL and Wildt DE 1992. Ability of thawed tiger (Panthera tigris) spermatozoa to fertilize conspecific eggs and bind and penetrate domestic cat eggs in vitro. J Reprod Fertil. 96(2): 555-564.

Ellerman JR, Morrison-Scott TCS and . BM 1966. Checklist of Palaearctic and Indian mammals 1758 to 1946 / by J.R. Ellerman and T.C.S. Morrison-Scott. Vol. N/A. BM(NH), London :.

Ganan N, Gonzalez R, Garde JJ, Martinez F, Vargas A, Gomendio M and Roldan ER 2009. Assessment of semen quality, sperm cryopreservation and heterologous

IVF in the critically endangered Iberian lynx (Lynx pardinus). Reprod Fertil Dev. 21(7): 848-859.

Garrett C, Liu DY, Clarke GN, Rushford DD and Baker HW 2003. Automated semen analysis: 'zona pellucida preferred' sperm morphometry and straight-line velocity are related to pregnancy rate in subfertile couples. Hum Reprod. 18(8): 1643-1649.

Graham LH, Byers AP, Armstrong DL, Loskutoff NM, Swanson WF, Wildt DE and Brown JL 2006. Natural and gonadotropin-induced ovarian activity in tigers (Panthera tigris) assessed by fecal steroid analyses. Gen Comp Endocrinol. 147(3): 362-370.

Grassman L, Lynam A, Mohamad S, Duckworth JW, Bora J, Wilcox D, Ghimirey Y and Reza A 2015. *Neofelis nebulosa*. The IUCN Red List of Threatened Species 2015: e.T14519A50656369, http://dx.doi.org/10.2305/IUCN.UK.2015-4.RLTS.T14519A50656369.en.

Hellander JC, Samper JC and Crabo BG 1991. Fertility of a stallion with low sperm motility and a high incidence of an unusual sperm tail defect. Vet Rec. 128(19): 449-451.

Herrick JR, Campbell M, Levens G, Moore T, Benson K, D'Agostino J, West G, Okeson DM, Coke R, Portacio SC, Leiske K, Kreider C, Polumbo PJ and Swanson WF 2010. In vitro fertilization and sperm cryopreservation in the black-footed cat (Felis nigripes) and sand cat (Felis margarita). Biol Reprod. 82(3): 552-562.

Howard J 1993. Semen collection and analysis in carnivores. In: Zoo & Wild Animal Medicine Current 3 ed. ME. Fowler (ed). Philadelphia: W.B. Saunders. 390-399.

Howard J, Bush M and Wildt DE 1991. Teratospermia in domestic cats compromises penetration of zona-free hamster ova and cat zonae pellucidae. J Androl. 12(1): 36-45.

Howard J, Byers AP, Brown JL, Barrett SJ, Evans MZ, Schwartz RJ and Wildt DE 1996. Successful ovulation induction and laparoscopic intrauterine artificial insemination in the clouded leopard (Neofelis nebulosa). Zoo Biology. 15(1): 55-69.

Howard J and Wildt DE 1990. Ejaculate-hormonal traits in the leopard cat (Felis bengalensis) and sperm function as measured by in vitro penetration of zona-free hamster ova and zona-intact domestic cat oocytes. Mol Reprod Dev. 26(2): 163-174.

Howard JG, Donoghue AM, Johnston LA and Wildt DE 1993. Zona pellucida filtration of structurally abnormal spermatozoa and reduced fertilization in teratospermic cats. Biol Reprod. 49(1): 131-139.

Howard JG, Roth TL, Byers AP, Swanson WF and Wildt DE 1997. Sensitivity to exogenous gonadotropins for ovulation induction and laparoscopic artificial insemination in the cheetah and clouded leopard. Biol Reprod. 56(4): 1059-1068.

Irvine DS, Macleod IC, Templeton AA, Masterton A and Taylor A 1994. A prospective clinical study of the relationship between the computer-assisted assessment of human semen quality and the achievement of pregnancy in vivo. Hum Reprod. 9(12): 2324-2334.

Jayaprakash D, Patil SB, Kumar MN, Majumdar KC and Shivaji S 2001. Semen characteristics of the captive Indian leopard, Panthera pardus. J Androl. 22(1): 25-33.

Jeyendran RS, Van der Ven HH and Zaneveld LJ 1992. The hypoosmotic swelling test: an update. Arch Androl. 29(2): 105-116.

Johannisson A, Morrell JM, Thoren J, Jonsson M, Dalin AM and Rodriguez-Martinez H 2009. Colloidal centrifugation with Androcoll-E prolongs stallion sperm motility, viability and chromatin integrity. Anim Reprod Sci. 116(1-2): 119-128.

Kitchener A 1998. The Natural History of the Wild Cats. In: Cornell University Press.

Kitchener AC, Beaumont MA and Richardson D 2006. Geographical variation in the clouded leopard, Neofelis nebulosa, reveals two species. Curr Biol. 16(23): 2377-2383.

Lindahl J, Dalin AM, Stuhtmann G and Morrell JM 2012. Stallion spermatozoa selected by single layer centrifugation are capable of fertilization after storage for up to 96 h at 6 degrees C prior to artificial insemination. Acta Vet Scand. 54: 40.

Liu DY, Clarke GN and Baker HW 1991. Relationship between sperm motility assessed with the Hamilton-Thorn motility analyzer and fertilization rates in vitro. J Androl. 12(4): 231-239.

Long JA, Wildt DE, Wolfe BA, Critser JK, DeRossi RV and Howard J 1996. Sperm capacitation and the acrosome reaction are compromised in teratospermic domestic cats. Biol Reprod. 54(3): 638-646.

Luvoni GC 2006. Gamete cryopreservation in the domestic cat. Theriogenology. 66(1): 101-111.

Macias Garcia B, Morrell JM, Ortega-Ferrusola C, Gonzalez-Fernandez L, Tapia JA, Rodriguez-Martinez H and Pena FJ 2009. Centrifugation on a single layer of colloid selects improved quality spermatozoa from frozen-thawed stallion semen. Anim Reprod Sci. 114(1-3): 193-202.

Marti K and Breitbeil B 2013. Thailand Clouded Leopard (*Neofelis nebulosa*) Consortium Population Viability Analysis Report. AZA Species Survival Plan, Lincoln Park Zoo, Chicago, IL.

Martinez-Alborcia MJ, Morrell JM, Parrilla I, Barranco I, Vazquez JM, Martinez EA and Roca J 2012. Improvement of boar sperm cryosurvival by using single-layer colloid centrifugation prior freezing. Theriogenology. 78(5): 1117-1125.

Molnar A, Sarlos P, Fancsi G, Ratky J, Nagy S and Kovacs A 2001. A sperm tail defect associated with infertility in a goat--case report. Acta Vet Hung. 49(3): 341-348.

Morrell JM, Dalin AM and Rodriguez-Martinez H 2009a. Comparison of density gradient and single layer centrifugation of stallion spermatozoa: yield, motility and survival. Equine Vet J. 41(1): 53-58.

Morrell JM, Johannisson A, Dalin AM and Rodriguez-Martinez H 2009b. Morphology and chromatin integrity of stallion spermatozoa prepared by density gradient and single layer centrifugation through silica colloids. Reprod Domest Anim. 44(3): 512-517.

Morrell JM, Rodriguez-Martinez H and Johannisson A 2010. Single layer centrifugation of stallion spermatozoa improves sperm quality compared with sperm washing. Reprod Biomed Online. 21(3): 429-436.

Nelson JE and Krawetz SA 1992. Purification of cloned and genomic DNA by guanidine thiocyanate/isobutyl alcohol fractionation. Anal Biochem. 207(1): 197-201.

Neubauer K, Jewgenow K, Blottner S, Wildt DE and Pukazhenthi BS 2004. Quantity rather than quality in teratospermic males: a histomorphometric and flow cytometric evaluation of spermatogenesis in the domestic cat (Felis catus). Biol Reprod. 71(5): 1517-1524.

O'Connell M, McClure N and Lewis SE 2002. The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Hum Reprod. 17(3): 704-709.

Ortiz I, Dorado J, Acha D, Galvez MJ, Urbano M and Hidalgo M 2015. Colloid single-layer centrifugation improves post-thaw donkey (Equus asinus) sperm quality and is related to ejaculate freezability. Reprod Fertil Dev. 27(2): 332-340.

Paasch U, Grunewald S and Glander HJ 2007. Sperm selection in assisted reproductive techniques. Soc Reprod Fertil Suppl. 65: 515-525.

Pelican KM, Brown JL, Wildt DE, Ottinger MA and Howard JG 2005. Short term suppression of follicular recruitment and spontaneous ovulation in the cat using levonorgestrel versus a GnRH antagonist. Gen Comp Endocrinol. 144(2): 110-121.

Pelican KM, Spindler RE, Pukazhenthi BS, Wildt DE, Ottinger MA and Howard J 2010. Progestin exposure before gonadotropin stimulation improves embryo development after in vitro fertilization in the domestic cat. Biol Reprod. 83(4): 558-567.

Pelican KM, Wildt DE and Howard JG 2006a. GnRH agonist Lupron (leuprolide acetate) pre-treatments prevent ovulation in response to gonadotropin stimulation in the clouded leopard (Neofelis nebulosa). Theriogenology. 66(6-7): 1768-1777.

Pelican KM, Wildt DE, Ottinger MA and Howard J 2008. Priming with progestin, but not GnRH antagonist, induces a consistent endocrine response to exogenous gonadotropins in induced and spontaneously ovulating cats. Domest Anim Endocrinol. 34(2): 160-175.

Pelican KM, Wildt DE, Pukazhenthi B and Howard J 2006b. Ovarian control for assisted reproduction in the domestic cat and wild felids. Theriogenology. 66(1): 37-48.

Prakash P, Leykin L, Chen Z, Toth T, Sayegh R, Schiff I and Isaacson K 1998. Preparation by differential gradient centrifugation is better than swim-up in selecting sperm with normal morphology (strict criteria). Fertil Steril. 69(4): 722-726.

Pukazhenthi B, Laroe D, Crosier A, Bush LM, Spindler R, Pelican KM, Bush M, Howard JG and Wildt DE 2006a. Challenges in cryopreservation of clouded leopard (Neofelis nebulosa) spermatozoa. Theriogenology. 66(6-7): 1790-1796.

Pukazhenthi B, Noiles E, Pelican K, Donoghue A, Wildt D and Howard J 2000. Osmotic effects on feline spermatozoa from normospermic versus teratospermic donors. Cryobiology. 40(2): 139-150.

Pukazhenthi BS, Long JA, Wildt DE, Ottinger MA, Armstrong DL and Howard J 1998. Regulation of sperm function by protein tyrosine phosphorylation in diverse wild felid species. J Androl. 19(6): 675-685.

Pukazhenthi BS, Neubauer K, Jewgenow K, Howard J and Wildt DE 2006b. The impact and potential etiology of teratospermia in the domestic cat and its wild relatives. Theriogenology. 66(1): 112-121.

Pukazhenthi BS, Wildt DE and Howard JG 2001. The phenomenon and significance of teratospermia in felids. J Reprod Fertil Suppl. 57: 423-433.

Pukazhenthi BS, Wildt DE, Ottinger MA and Howard J 1996. Compromised sperm protein phosphorylation after capacitation, swim-up, and zona pellucida exposure in teratospermic domestic cats. J Androl. 17(4): 409-419.

Ramu S and Jeyendran RS 2013. The hypo-osmotic swelling test for evaluation of sperm membrane integrity. Methods Mol Biol. 927: 21-25.

Rota A, Manuali E, Caire S and Appino S 2008. Severe tail defects in the spermatozoa ejaculated by an English bulldog. J Vet Med Sci. 70(1): 123-125.

Roth TL, Armstrong DL, Barrie MT and Wildt DE 1997. Seasonal effects on ovarian responsiveness to exogenous gonadotrophins and successful artificial insemination in the snow leopard (Uncia uncia). Reprod Fertil Dev. 9(3): 285-295.

Roth TL, Weiss RB, Buff JL, Bush LM, Wildt DE and Bush M 1998. Heterologous in vitro fertilization and sperm capacitation in an endangered African antelope, the scimitar-horned oryx (Oryx dammah). Biol Reprod. 58(2): 475-482.

Sananmuang T, Tharasanit T, Nguyen C, Phutikanit N and Techakumphu M 2011. Culture medium and embryo density influence on developmental competence and gene expression of cat embryos. Theriogenology. 75(9): 1708-1719.

Sanchez-Partida LG, Setchell BP and Maxwell WM 1997. Epididymal compounds and antioxidants in diluents for the frozen storage of ram spermatozoa. Reprod Fertil Dev. 9(7): 689-696.

Schrader SM, Platek SF, Zaneveld LJ, Perez-Pelaez M and Jeyendran RS 1986. Sperm viability: a comparison of analytical methods. Andrologia. 18(5): 530-538.

Shibahara H, Obara H, Ayustawati, Hirano Y, Suzuki T, Ohno A, Takamizawa S and Suzuki M 2004. Prediction of pregnancy by intrauterine insemination using CASA estimates and strict criteria in patients with male factor infertility. Int J Androl. 27(2): 63-68.

Stewart RA, Pelican KM, Brown JL, Wildt DE, Ottinger MA and Howard JG 2010. Oral progestin induces rapid, reversible suppression of ovarian activity in the cat. Gen Comp Endocrinol. 166(2): 409-416.

Stoops MA, Bond JB, Bateman HL, Campbell MK, Levens GP, Bowsher TR, Ferrell ST and Swanson WF 2007. Comparison of different sperm cryopreservation procedures on post-thaw quality and heterologous in vitro fertilisation success in the ocelot (Leopardus pardalis). Reprod Fertil Dev. 19(5): 685-694.

Swanson W and Brown J 2004. International training programs in reproductive sciences for conservation of Latin American felids. Animal reproduction science. 82: 21-34.

Swanson WF 2012. Laparoscopic oviductal embryo transfer and artificial insemination in felids--challenges, strategies and successes. Reprod Domest Anim. 47 Suppl 6: 136-140.

Swanson WF, Maggs DJ, Clarke HE, Newell AE, Bond JB, Bateman HL and Kennedy-Stoskopf S 2006. Assessment of viral presence in semen and reproductive

function of frozen-thawed spermatozoa from Pallas' cats (Otocolobus manul) infected with feline herpesvirus. J Zoo Wildl Med. 37(3): 336-346.

Terrell KA, Wildt DE, Anthony NM, Bavister BD, Leibo SP, Penfold LM, Marker LL and Crosier AE 2010. Evidence for compromised metabolic function and limited glucose uptake in spermatozoa from the teratospermic domestic cat (Felis catus) and cheetah (Acinonyx jubatus). Biol Reprod. 83(5): 833-841.

Thiangtum K, Swanson WF, Howard J, Tunwattana W, Tongthainan D, Wichasilpa W, Patumrattanathan P and Pinyopoommintr T 2006. Assessment of basic seminal characteristics, sperm cryopreservation and heterologous in vitro fertilisation in the fishing cat (Prionailurus viverrinus). Reprod Fertil Dev. 18(3): 373-382.

Thuwanut P, Chatdarong K, Bergqvist AS, Soderquist L, Thiangtum K, Tongthainan D and Axner E 2011. The effects of antioxidants on semen traits and in vitro fertilizing ability of sperm from the flat-headed cat (Prionailurus planiceps). Theriogenology. 76(1): 115-125.

Tsutsui T 2006. Artificial insemination in domestic cats (Felis catus). Theriogenology. 66(1): 122-125.

Tsutsui T, Tanaka A and Hori T 2001. Intratubal insemination with fresh semen in cats. J Reprod Fertil Suppl. 57: 347-351.

Van der Ven HH, Jeyendran RS, Al-Hasani S, Perez-Pelaez M, Diedrich K and Zaneveld LJ 1986. Correlation between human sperm swelling in hypoosmotic medium (hypoosmotic swelling test) and in vitro fertilization. J Androl. 7(3): 190-196.

Varisli O, Uguz C, Agca C and Agca Y 2009. Various physical stress factors on rat sperm motility, integrity of acrosome, and plasma membrane. J Androl. 30(1): 75-86.

Verheyen G, Joris H, Crits K, Nagy Z, Tournaye H and Van Steirteghem A 1997. Comparison of different hypo-osmotic swelling solutions to select viable immotile spermatozoa for potential use in intracytoplasmic sperm injection. Hum Reprod Update. 3(3): 195-203.

Villaverde AI, Fioratti EG, Ramos RS, Neves RC, Cardoso GS, Landim-Alvarenga FC and Lopes MD 2013. High incidence of 'Dag-like' sperm defect in the domestic cat. J Feline Med Surg. 15(4): 317-322.

Wenkoff MS 1978. A sperm mid-piece defect of epididymal origin in two Hereford bulls. Theriogenology. 10(4): 275-281.

Wildt DE 1994. Endangered Species Spermatozoa: Diversity, Research, and Conservation. In: Function of Somatic Cells in the Testis. Andrzej Bartke (ed). New York, NY: Springer New York. 1-24.

Wildt DE, Bush M, Howard JG, O'Brien SJ, Meltzer D, Van Dyk A, Ebedes H and Brand DJ 1983. Unique seminal quality in the South African cheetah and a comparative evaluation in the domestic cat. Biol Reprod. 29(4): 1019-1025.

Wildt DE, Howard JG, Chakraborty PK and Bush M 1986a. Reproductive physiology of the clouded leopard: II. A circannual analysis of adrenal-pituitary-testicular relationships during electroejaculation or after an adrenocorticotropin hormone challenge. Biol Reprod. 34(5): 949-959.

Wildt DE, Howard JG, Hall LL and Bush M 1986b. Reproductive physiology of the clouded leopard: I. Electroejaculates contain high proportions of pleiomorphic spermatozoa throughout the year. Biol Reprod. 34(5): 937-947.

Wildt DE, O'Brien SJ, Howard JG, Caro TM, Roelke ME, Brown JL and Bush M 1987. Similarity in ejaculate-endocrine characteristics in captive versus free-ranging cheetahs of two subspecies. Biol Reprod. 36(2): 351-360.

Wildt DE and Roth TL 1997. Assisted reproduction for managing and conserving threatened felids. International Zoo Yearbook. 35(1): 164-172.

Wilting A, Buckley-Beason VA, Feldhaar H, Gadau J, O'Brien S J and Linsenmair KE 2007. Clouded leopard phylogeny revisited: support for species recognition and population division between Borneo and Sumatra. Front Zool. 4: 15.

Wilting A, Christiansen P, Kitchener AC, Kemp YJ, Ambu L and Fickel J 2011. Geographical variation in and evolutionary history of the Sunda clouded leopard (Neofelis diardi) (Mammalia: Carnivora: Felidae) with the description of a new subspecies from Borneo. Mol Phylogenet Evol. 58(2): 317-328.

Yamada JK and Durrant BS 1989. Reproductive parameters of clouded leopards (Neofelis nebulosa). Zoo Biology. 8(3): 223-231.

Yeung CH, Barfield JP and Cooper TG 2006. Physiological volume regulation by spermatozoa. Mol Cell Endocrinol. 250(1-2): 98-105.

Yeung CH, Sonnenberg-Riethmacher E and Cooper TG 1999. Infertile spermatozoa of c-ros tyrosine kinase receptor knockout mice show flagellar angulation and maturational defects in cell volume regulatory mechanisms. Biol Reprod. 61(4): 1062-1069.

Yeung CH, Tuttelmann F, Bergmann M, Nordhoff V, Vorona E and Cooper TG 2009. Coiled sperm from infertile patients: characteristics, associated factors and biological implication. Hum Reprod. 24(6): 1288-1295.

APPENDIX

Publications

1. Influence of living status (single vs. Paired) and centrifugation with colloids on the sperm morphology and functionality in the clouded leopard (*Neofelis nebulosa*)
Journal: Theriogenology, accepted
2. Mitigation of sperm tail abnormalities using demembranation approach in the clouded leopard (*Neofelis nebulosa*)
Journal: Reproduction in Domestic Animals (supplement), accepted
3. Beneficial effect of extracellular Adenosine 5'-Triphosphate (ATPe) treatment on the Indochinese leopard (*Panthera pardus delacouri*) sperm quality after cryopreservation
Journal: Reproduction in Domestic Animals (supplement), submitted
4. Dynamic changes in mitochondrial DNA, distribution and activity within cat oocytes during folloculogenesis
Journal: Reproduction in Domestic Animals (supplement), submitted
5. The successful laparoscopic oviductal artificial insemination in the clouded leopard (*Neofelis nebulosa*) in Thailand
Manuscript

Proceeding

1. Semen characteristics and sperm cryopreservation of captive clouded leopards (*Neofelis nebulosa*) in Thailand. RGJ Seminar Series XCIX (99) "Innovative Reproductive Technology for Wildlife". Khao Kheo Open Zoo, Chonburi, Thailand, November 20, 2013
2. Sperm selection using single layer centrifugation improves normal morphology of captive clouded leopard (*Neofelis nebulosa*) spermatozoa. RGJ-Ph.D. Congress XVI, Jomtien Palm Beach Hotel, Chonburi, Thailand, June 11-13, 2015.
3. Sperm selection improves normal morphology of captive clouded leopard (*Neofelis nebulosa*) spermatozoa. Chulalongkorn University Veterinary Conference 2015, Siam Paragon, Bangkok, Thailand, April 20-22, 2015.

4. Colloid centrifugation selects clouded leopard (*Neofelis nebulosa*) sperm with intact acrosome and normal tail. Chulalongkorn University Veterinary Conference 2016, Chulalongkorn University, Bangkok, Thailand, April 20-22, 2016.
5. Hypo-osmotic test in cat epididymal sperm. Chulalongkorn University Veterinary Conference 2016, Chulalongkorn University, Bangkok, Thailand, April 20-22, 2016.

Awards

Best poster presentation award, RGJ-Ph.D. Congress XVI, Jomtien Palm Beach Hotel, Chonburi, Thailand, June 11-13, 2015.

VITA

Miss Wanlaya Tipkantha was born on May 31th 1980 in Chiangmai province. She graduated with Degree of Doctor of Veterinary Medicine (DVM) from Faculty of Veterinary Medicine, Chiangmai University in 2004. After graduated, she enrolled and worked at Bureau of Conservation and Research, Zoological Park Organization. In 2011 she got a Master Degree of Science from Agricultural Biotechnology, Kasetsart University. In 2013, she received a scholarship from the Royal Golden Jubilee PhD program of Thailand Research Fund to conduct a PhD program of Theriogenology at the Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. Her research is focusing on overcoming sperm abnormalities by sperm selection in clouded leopard. For her PhD research studies, she has published two papers as first author are 1) Influence of living status (single vs. Paired) and centrifugation with colloids on the sperm morphology and functionality in the clouded leopard (Neofelis nebulosa) in Theriogenology journal and 2) Mitigation of sperm tail abnormalities using demembranation approach in the Clouded leopard (Neofelis nebulosa) in Reproduction in Domestic Animals (supplement) journal. She has also submitted two manuscripts as a co-author included 1) Beneficial effect of extracellular Adenosine 5'-Triphosphate (ATPe) treatment on the Indochinese leopard (Panthera pardus delacouri) sperm quality after cryopreservation and 2) Dynamic changes in mitochondrial DNA, distribution and activity within cat oocytes during folloculogenesis in Reproduction in Domestic Animals (supplement) journal. In addition, she has prepared the manuscript in the topic the successful laparoscopic oviductal artificial insemination in the clouded leopard (Neofelis nebulosa) for submission.