การแช่แข็งน้ำเชื้อและการแยกเพศอสุจิในฝูงสุกรปลอดโรคพ่อ-แม่พันธุ์

นายชาญยุทธ ตรีทิพย์สกุล

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BOAR SEMEN FREEZING AND SPERM SEXING IN A SPECIFIC DISEASE-FREE NUCLEUS HERD

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ชาญยุทธ ตรีทิพย์สกุล : การแช่แข็งน้ำเชื้อและการแยกเพศอสุจิในฝูงสุกรปลอดโรคพ่อ-แม่พันธุ์
(BOAR SEMEN FREEZING AND SPERM SEXING IN A SPECIFIC DISEASE-FREE NUCLEUS HERD)
อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ศ.น.สพ.ดร.มงคล เตชะกำพุ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.น.สพ.ดร.เผด็จ ธรรมรักษ์, อ.น.สพ.ดร.เสรี กุญแจนาค, 84 หน้า.

การศึกษาปัจจัยที่มีผลกระทบต่อการผลิตน้ำเชื้อในสุกรในเขตพื้นที่ร้อนชื้น จากน้ำเชื้อจำนวน 19,966 ครั้ง ของพ่อสุกรที่มีสถานะปลอด PRRS จำนวน 517 ตัว แบ่งเป็น สายพันธุ์แท้ (ดูร็อค (D) จำนวน 164 ตัว และเปียแทรง (P) จำนวน 31 ตัว) และสายพันธุ์ผสม (แลนด์เรซxยอร์คเชียร์ (LY) จำนวน 268 ตัวและเปียแทรงxดูร็อค (PD) จำนวน 54 ตัว) ผลการตรวจคุณภาพน้ำเชื้อจาก ปริมาตร (มล.) ความเข้มข้น (x10^6 sperm/มล.) และจำนวนอสุจิทั้งหมดต่อการหลั่งต่อครั้ง (x10^9sperm/มล.) ตามรายเดือนและตามฤดูกาล(ฤดูร้อน,มีนาคม-มิถุนายน:ฤดูฝน,กรกฎาคม-ตุลาคม:ฤดูหนาว,พฤศจิกายน-กุมภาพันธ์) เมื่อเปรียบเทียบตามช่วงฤดูกาลพบว่าจำนวนอสุจิทั้งหมดต่อการหลั่งต่อครั้งในฤดูหนาวนั้นมีค่าสูงกว่าเดือนสิงหาคมถึงตุลาคมในฤดูฝน (P<0.05) ในขณะที่ค่าความเข้มข้นในช่วงต้นฤดูหนาว (พฤศจิกายนและธันวาคม) มีค่าต่ำกว่าช่วงฤดูร้อนและช่วงฤดูฝนในเดือนกรกฎาคม (P<0.05) และผลกระทบจากพันธุ์พบว่าค่าจำนวนอสุจิทั้งหมดต่อการหลั่งต่อครั้งของพันธุ์ผสม LY มีค่าสูงกว่าสายพันธุ์แท้ D และ P แสดงว่าพันธุ์และฤดูกาลมีผลกระทบกับพ่อสุกรพันธุ์แท้มากกว่าในพันธุ์ผสมและ PD ซึ่งข้อมูลความสมบูรณ์พันธุ์ (จำนวนลูกสุกรเกิดทั้งหมด (TB) และลูกสุกรมีชีวิต(BA)) ที่ใช้น้ำเชื้อจากสายพันธุ์ดูร็อคระหว่างเดือนมีนาคม-มิถุนายนและตุลาคมถึงธันวาคมมีแนวโน้มเพิ่มขึ้น และพบว่า TB และ BA มีค่าต่ำสุดในเดือนตุลาคม (P< 0.05) สรุปได้ว่าฤดูกาลและพันธุ์มีผลกระทบต่อการผลิตน้ำเชื้อของพ่อพันธุ์สุกรปลอดโรค PRRS ที่เลี้ยงในโรงเรือนระบบปิด EVAP

การศึกษาความสามารถของพ่อสุกรในการแช่แข็งน้ำเชื้อและผลประสิทธิภาพการผลิตในการผสมเทียม

การทดลองที่1 จุดประสงค์ของการศึกษา คือ การใช้เครื่อง CASA ตรวจประเมินรูปแบบการเคลื่อนที่ของน้ำเชื้อสุกรแช่แข็งภายหลังทำละลายทันทีและในอีก 60 นาทีถัดมาจาก ตัวอย่างน้ำเชื้อจำนวน 41 ตัวอย่าง จำแนกเป็นแลนด์เรซ 14 ตัวอย่าง ยอร์คเชียร์ 12 ตัวอย่าง และดูร็อค 15 ตัวอย่าง โดยประเมินผลการเคลื่อนที่ ความสมบูรณ์ของเยื่อหุ้มอสุจิและรูปแบบการเคลื่อนที่ของอสุจิ โดยทำการตรวจวัดทันทีและภายหลังการเก็บรักษาที่ 38°C จึงทำการตรวจอีกครั้งใน 60 นาทีถัดมา รูปแบบของการเคลื่อนที่จำแนกเป็น วิถีการเคลื่อนที่แบบโค้ง (VCL, ไมโครเมตรต่อวินาที), วิถีเคลื่อนที่แบบตรง (VSL, ไมโครเมตรต่อวินาที),วิถีการเคลื่อนที่แบบเฉลี่ย(VAP, ไมโครเมตรต่อวินาที),linear coefficient (LIN, ร้อยละ), amplitude of lateral head displacement (ALH, ไมโครเมตร), ร้อยละการเคลื่อนที่ผลการศึกษาพบว่าค่าร้อยละการเคลื่อนที่และวิถีการเคลื่อนที่แบบตรงแตกต่างอย่างมีนัยสำคัญทางสถิติในระหว่างสายพันธุ์ (P<0.05) และรูปแบบของวิถีการเคลื่อนที่ยังลดลงอย่างมีนัยสำคัญเมื่อเปรียบเทียบระหว่างภายหลังการทำละลายทันทีกับการเก็บรักษาต่อที่ 60 นาทีถัดมา (P<0.05) อย่างไรก็ตามไม่พบความแตกต่างทั้งในร้อยละการเคลื่อนที่และ LIN เมื่อผ่านระยะเวลาการเก็บรักษาที่ 60 นาที

การทดลองที่2 จุดประสงค์ คือ การศึกษาความสามารถในการแช่แข็งน้ำเชื้อ รวมถึงผลของการใช้ 50%น้ำเลี้ยงตัวอสุจิเป็นตัวทำละลายและผลการผสมเทียมด้วยน้ำเชื้อแช่แข็งในฟาร์มสุกร โดยทำการแช่แข็งน้ำเชื้อจากพ่อสุกร 115 ตัว แบ่งเป็น สายพันธุ์แท้ (เบิร์กเชียร์ 4 ตัวและดูร็อค 29 ตัว) และสายพันธุ์ผสม (แลนด์เรซxยอร์คเชียร์ (LY) จำนวน 53 ตัวและเปียแทรงxดูร็อค (PD) จำนวน 29 ตัว) โดยจัดแบ่งกลุ่มตามอายุของพ่อสุกรและตามความสามารถในการแช่แข็งภายหลังการทำละลายเปรียบเทียบด้วย 50% HSP ในการทดสอบความสมบูรณ์พันธุ์ ได้ทำการผสมเทียมแม่สุกรหย่านมจำนวน 86 ตัว แบ่งเป็นกลุ่มควบคุมที่ผสมเทียมด้วยน้ำเชื้อสดจำนวน 43 ตัวและกลุ่มทดลองที่ชักนำให้เป็นสัดด้วยฮ์อรโมน PG600® จำนวน 43 ตัว และผสมเทียมด้วยน้ำเชื้อแช่แข็ง (HFT) ผลการศึกษาพบว่าค่าการเคลื่อนที่ของอสุจิหลังการทำละลายในกลุ่มพ่อสุกรพันธุ์ผสมที่มีอายุมากกว่า 2 ปี (LY และ PD) มีการสูงกว่ากลุ่มพันธุ์แท้ (P<0.05) และพบพ่อสุกรที่มีค่าความสามารถในการแช่แข็งที่ดีจำนวน 10 ตัวจาก 53 ตัว ส่วนในการละลายน้ำเชื้อด้วย 50%HSP จะให้ผลลดลงเมื่อเปรียบเทียบกับการทำละลายด้วย BTS และไม่พบการแตกต่างอย่างมีนัยสำคัญของ FR, TB และ BA (P>0.05)ระหว่างกลุ่มผสมเทียมที่ใช้น้ำเชื้อสดและน้ำเชื้อแช่แข็ง

การศึกษาการแยกเพศอสุจิด้วยวิธีปั่นเหวี่ยงด้วยสารละลาย percoll gradient และผลการผสมเทียม โดยทำการศึกษาในน้ำเชื้อ 15 ตัวอย่างจากพ่อสุกร 5 ตัว (3ครั้ง/ตัว) และทำการปั่นเหวี่ยงน้ำเชื้อผ่านสารละลาย percoll gradient จำนวน 8 ชั้น ที่มีความเข้มข้นต่างกันที่ 90, 80, 75, 60, 55,45, 30 และ 20% จากล่างขึ้นบนตามลำดับ จากนั้นเปรียบเทียบความแตกต่างร้อยละประชากร X และ Y จากน้ำเชื้อส่วนบน (45,55%) และส่วนล่าง (80,90%) ภายหลังการปั่นเหวี่ยงเทียบกับน้ำเชื้อสดในตอนเริ่มต้น ด้วยวิธี quantitative PCR ในการทดสอบประสิทธิภาพผสมเทียมทำการศึกษาด้วยการผสมเทียมแม่สุกรหย่านม จำนวน 78 ตัว โดยวิธี IUI โดยใช้น้ำเชื้อแยกเพศอสุจิส่วนล่าง (กลุ่มทดลอง, N=39) ภายหลังการปั่นเหวี่ยงของพ่อสุกร จำนวน 2 ตัว เปรียบเทียบกับการใช้น้ำเชื้อสด (กลุ่มควบคุม, N=39) ผลการศึกษาพบว่าการปั่นเหวี่ยงน้ำเชื้อผ่านสารละลาย percoll gradientสามารถเพิ่มกลุ่มประชากรอสุจิ X ได้ทั้งในน้ำเชื้อส่วนบนและส่วนล่างภายหลังการปั่นเหวี่ยง (P<0.05) ถึงแม้ว่าผลสัดส่วนของลูกเพศเมียต่อเพศผู้ของจำนวนลูกทั้งหมดต่อครอกในกลุ่มทดลองจะมีค่าสูงกว่ากลุ่มควบคุม แต่อย่างไรก็ตามการศึกษาในครั้งนี้ไม่พบความสัมพันธ์กันอย่างมีนัยสำคัญระหว่างสัดส่วนเพศของจำนวนลูกทั้งหมดต่อครอกเมื่อเปรียบเทียบการผสมเทียมระหว่างน้ำเชื้อในกลุ่มควบคุมและกลุ่มทดลอง (P>0.05)

จากผลการศึกษาทั้งหมด พบว่าพันธุ์และฤดูกาลมีผลต่อการผลิตน้ำเชื้อของพ่อสุกรปลอดโรค PRRS ที่ถูกเลี้ยงในระบบ EVAP และในการเก็บรักษาพันธุกรรมของพ่อสุกร ด้วยวิธีการแช่แข็งน้ำเชื้อนั้นควรทำการประเมินความสามารถของการแช่แข็งน้ำเชื้อของพ่อสุกรก่อนการเก็บรักษาเพื่อให้ได้ประสิทธิภาพการผสมเทียมที่ดี ส่วนในการศึกษาวิธีการแยกเพศอสุจิในน้ำเชื้อพบว่าในการใช้น้ำเชื้อที่ผ่านการปั่นเหวี่ยงด้วยสารละลาย percoll gradient สามารถเพิ่มร้อยละของจำนวนประชากร X ได้ในน้ำเชื้อส่วนล่าง และให้ผลการผสมเทียมที่มีสัดส่วนลูกเพศเมียสูงขึ้นเมื่อเทียบกับการใช้น้ำเชื้อสดตั้งต้น ดังนั้นการประยุกต์นำเทคโนโลยีด้านระบบสืบพันธุ์ไปใช้ในระบบอุตสาหกรรมการผลิตสุกร ควรคำนึงถึงการใช้เทคโนโลยีที่ถูกดัดแปลงให้นำไปใช้ได้ง่ายและมีต้นทุนต่ำ แต่ยังคงให้ประสิทธิภาพที่ดีในการผลิตสุกรในรูปแบบอุตสาหกรรม

ภาควิชา สูติศาสตร์ เธนุเวชวิทยา และวิทยาการสืบพันธุ์	ลายมือชื่อนิสิต .....
สาขาวิชา วิทยาการสืบพันธุ์สัตว์	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....
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	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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CHANYUTH TRETIPSKUL : BOAR SEMEN FREEZING AND SPERM SEXING IN A SPECIFIC DISEASE-FREE NUCLEUS HERD. ADVISOR : PROF. MONGKOL TECHAKUMPHU, Ph.D, Doctoratde 3e cycle, CO-ADVISOR : ASSOC. PROF. PADET TUMMARUK, D.V.M., Ph.D, SERI KOONJAENAK, D.V.M., Ph.D, 84 pp.

The factors influencing of boar semen production were evaluated a total of 19,966 ejaculates from 517 PRRS free boars (2 purebred; 164D, 31P) and (2 crossbred; 268LY, 54PD). Semen parameters; volume (ml), sperm concentration ($x10^6$ sperm/ml) and total number of sperm per ejaculate ($x10^9$ sperm/ejaculate) were evaluated in relation to seasons as summer (Mar-Jun), rainy (Jul-Oct) and winter (Nov-Feb) and breed differences. Seasoning effected, the total number of sperm per ejaculate in winter was higher than a period in rainy season (Aug-Oct) (P<0.05) while the concentration in early winter (Nov and Dec) was lower than summer and a month in rainy (Jul) (P<0.05). Affect from breed, the total sperm production of LY was higher than D and P (P=0.03). Fertility data using D semen (TB and BA) tended to increase during Mar-Jun and Oct-Dec but the lowest TB and BA in Oct (P< 0.05). It can be concluded that the sperm production was influenced both of breeds and seasons what was found in PRRS free boars kept in EVAP in Thailand.

Boar semen freezability and in vivo fertility

EXP1 was to evaluate the sperm motility of FT boar semen including motility, PMI and motility pattern by using CASA after thawing (T_0) and at 60 min (T_{60}) after incubation at 38°C. Forty-one ejaculates from 14L, 12Y and 15D boars were freeze-thawed and evaluated. Motion parameters including VCL(μm/s), VSL(μm/s), VAP(μm/s), LIN(%), ALH (μm) and total motility (MS-CASA;%) were measured. The results revealed that total motility and VSL of T_0 differed (P<0.05) among breeds. Some motion characteristics of FT boar semen i.e., VSL, VAP, VCL and ALH significantly decreased an hour after post-thawing (P<0.05). However, there was no significant difference in MS-CASA, and LIN between T_0 and T_{60} groups.

EXP2 was to study the freezability and influence of 50%HSP as thawing medium and fertility data in sows after IUI with HFT semen in field. Semen from one hundred-fifteen boars including purebred (4B, 26D) and crossbred (53LY, 29PD) were classified as 3 groups by age and freeze-thawed with and without presenting 50% HSP then evaluated by CASA for classification as good or poor freezability. For artificial insemination, eighty-six sows were inseminated as 43 control sows (fresh semen) and 43 treatment sows (HFT semen). All sows in treatment group were induced estrus by PG600®. The percentage of subjective motility of crossbred was significantly different higher than purebred (P<0.05). In addition, post thawed motility in boar, age >2 yr, in LY and PD was higher significant different (P<0.05) while D was lower significant different (P<0.05). For thawing, of ten were classified as good freezability from fifty-three boars showed significant difference in motility, MS-CASA, VAP, VSL, LIN and viability (P<0.05) between BTS and 50%HSP groups. However, the beneficial effected of 50%HSP did not present in our study. For fertility data, there were no significant difference between control and treatment groups in FR, TB and BA (P>0.05). In summary, boar individuality with good freezability should be tested for achievement fertility data in field.

Boar semen sexing using percoll gradient and *in vivo* testing after insemination

The objectives aimed to study the possibility to use discontinuous gradient centrifugation to sex boar spermatozoa and test by *in vivo* fertilization. To evaluate the efficiency of percoll-gradient centrifugation for sperm sexing, semen samples from fifteen ejaculates of five boars were studied (three replications). Eight layers of percoll-gradient concentration was used to separate semen as 90, 80, 75, 60, 55, 45, 30 and 20%, respectively. Fresh semen was placed on top and then centrifuged. Before (fresh semen) and after centrifugation (upper part; concentration 45% and 55% and lower part; concentration 80% and 90%) were collected into each aliquot; one, unprocessed initially fresh semen/ the other two aliquots, upper and lower part, respectively. All samples were extracted DNA then processed by modified quantitative PCR to calculate the percentage of X- and Y- bearing spermatozoa with standard curve. Two sets of primers were designed on specific AMELX and Y-chromosome (SRY) genes with SYBR green. The percentage of difference in each X- and Y- spermatozoa population was compared to initial fresh semen. *In vivo* testing, two boars with presenting the normal fertility were included in this study. The control group (unsex semen) was routinely processed while the treatment group (sexed semen) was processed by discontinuous percoll gradient centrifugation. The seventy-eight wean sows were included in this experiment. Seventy-eight sows were inseminated by IUI as control (N=39) with unsex semen and treatment groups (N=39) with the lower part of semen identified as X dominance. The 21-day CR, FR, TB and sex ratio of total born (female:male) were collected to compare as P<0.05. The result of the difference of percentage of X- and Y- bearing spermatozoa population showed significant difference (P<0.05) in lower part to which was compared each individual fresh semen. Unfortunately, the result did not relate to the significant difference all of the parameters (P>0.05) for the fertility data, however, the sex ratio of total born (female:male) in treatment group seems higher than control. In conclusion, the significant difference in percentage of X- spermatozoa populations by percoll gradient centrifugation can be enhanced by discontinuous percoll gradient centrifugation. Nevertheless, the effect of sex ratio from in vivo fertilization did not relate closely to the insemination with the shifting of the difference of X- or Y- spermatozoa populations.

The results of all studies indicated that breeds and seasons could effect on semen production of boar kept in EVAP. In addition, the classification of boar as freezabiltiy to preserve their genetics as frozen semen form should be done to be acceptable fertility data. Percoll-gradient centrifugation can slightly shift the percentage of sex of sperm population, however, it did not significantly relate to sex ratio of TB. Finally, the application of reproductive technology should be simplified and adapted with easy and economical concepts but high efficiency for commercial pig production in field.

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LIST OF ABBREVIATIONS

AI	artificial insemination
ALH	amplitude of lateral head displacement
B	Berkshire
BA	number of piglets born alive per litter
BCF	beat cross frequency
BTS	Beltsville Thawing Solution
°C	Celsius
CASA	computer-assisted sperm analysis
cm	centimeter
CR	conception rate
D	Duroc
EVAP	evaporative cooling system
FR	farrowing rate
FT	frozen-thawed
GLM	general linear model
HFT	heterospermic frozen-thawed
HSP	heterologous seminal plasma
IC	intracervical insemination
IUI	intrauterine insemination
kg	kilogram
L	Landrace
LY	Landrace x Yorkshire
LEY	lactose-egg yolk
LIN	linearity
LN_2	liquid nitrogen
LSM	least square mean
min	minute
ml	milliliter
mm	millimeter
P	Pietrain
PCR	polymerase chain reaction
PD	Pietrain x Duroc
PRRS	porcine reproductive and respiratory syndrome

sec	seconds
SD	standard deviation
SEM	standard error of mean
sHOST	short hypoosmotic swelling test
STR	straightness
TB	number of total piglets born per litter
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight line velocity
Y	Yorkshire
μg	microgram
μl	microliter

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

1.1.1 General background

Thailand is one of the leaders of pig production in Asia with 900,000 sows and 13 million pig produced per year. The pork consumption per capita is currently 13.7 kg per year per person (The trade council of Denmark, 2009). The genetic and general management are in the same standard as many developed countries, for example, breeds, housing, waste management by biogas system including biosecurity. About 80% of pigs are produced from intensive farming systems and 56% of these are from farms with over 1,000 sows. From 1980s, large commercial farms were rapidly increased either integrated company owned (8.5%) or private independent (47.5%) farms (Cameron, 2000). Until around 1990, swine production systems were usually housed on a single site, because of labor savings and convenience. Health and performance concerns have since caused many swine operations to house the various production phases at different sites to further minimize contact between pigs of different ages. This is either a two-site or a three-site system. A two-site system has breeding and gestation/farrowing at one site and nursery to finisher pigs at a separate site, while a three site also places the nursery at a separate site. In the last few years, some producers have constructed “wean to finish” barns where pigs go immediately after weaning, and stay until market. This combines the nursery and grow-finish phases of production. These barns provide substantially more space per pig than is needed initially, but provide the advantage of only moving pigs once during their lifetime. This reduces stress on the animals and saves labor since buildings are not cleaned until the hogs are marketed. Nowadays, a contrating farms have become to big scale to produce the hogs by cooperation to the company where support feeds, breeds and management by the multi-sites which may have one or more breeder units at Site 1, providing weaners for up to eight weaner rooms or buildings (seven weeks production) at Site 2, and finishers for up to16 grow-out buildings (14 weeks production) at Site 3. Each batch of weaner and finisher room will hold one week’s production and where separate buildings.

Not only the pig production system or disease but also genetics can effect on the breeding performance. The reproductive performance of purebred sows in Thailand is not as good as the performance reported in Europe (Muirhead and Alexander, 1997) especially total number of piglets born per litter (10.77 vs 9.8; Europe vs Thailand) (Kunavongkrit and Heard, 2000; Huang et al, 2002). This reason caused the import great grand parents (GGP) from European countries in order to improve the pig wean per sow per year (Kunavongkrit and Heard, 2000). The breed of Landrace (L) and Yorkshire (Y) line was dam line to produce a female which is mated with a Duroc boar as the terminal line. In addition, the external factors affect on breeding performance in Thailand can express into three criterias; i) climate; three seasons which are many variation during day and night time. The low litter size in the rainy (July-September) might be the result of summer (March-May) by early embryonic death (Tantasuparak et al. 1997); ii) diseases; by virus, bacteria, parasite can cause or induce abortions and SMEDI syndromes (stillborn, mummified, embryonic death and fertility) (Kunavongkrit and Heard, 2000); and iii) Nutrition and management; the major issues are the quality of feed and mycotoxin which was produced by mold.

To dissiminate the genetic and disease control inside herd, Artificial insemination (AI), has successfully been implemented to the swine industry worldwide for many decades including Thailand. The process consisted of semen collection, dilution for preservation and insemination into female pigs (Polge, 1956; Salamon and Maxwell, 1995).

The major advantages of AI include disease control and genetic improvement (Bathgate et al., 2008[a]). It has been the most important management tool leading to improved herd productivity in farm animals (Leman and Rodeffer, 1976). The principle benefit of this technique is accelerated markedly rate of genetic improvement by inseminating multiple females with the semen from a genetically superior and disease-free male. It has been found that some diseases such as Porcine Reproductive and Respiratory Syndrome (PRRS), Aujesky's disease (AD) or Brucellosis can transmitted via semen. Then, the semen should be collected from disease-free boar for AI.

Moreover, AI has been helping to prevent the spreading of venereal diseases by natural mating. AI in swine was initiated in Russia in the early 1900s (Bathgate et al., 2008[b]). Sows were inseminated with 100-150 ml of fresh, extended semen on the second day of estrus. An average conception rate of 70% has been reported. Despite the importance of these achievements, it was not until after World War II that fresh boar semen was used successfully (Polge, 1956). According to Gauthier (2004),

approximately 90% of the commercial swine herds in the province of Québec, Canada use AI with fresh semen; between 1983 and 2003, back fat thickness decreased 7 mm and the interval from birth to market (at 100 kg) decreased by 26 days. In Thailand, recently a high number of AI were performed over 90% of pig farms in the country (Tummaruk et al., 2001; Tummaruk et al, 2004). Use of liquid boar semen for AI, preserved for up to 3–5 days at 16–20ºC, constitutes the core of the AI routinely performed by the pig breeding industry, covering more than 99% of AIs in pigs done around the world (Wagner and Thibier, 2000).

1.1.2 Importance and rationale

Porcine reproductive and respiratory syndrome virus (PRRSV) infection has been well known as very important disease of swine due to this can cause severe economic losses and lead to trade barriers. In female, PRRSV causes reproductive failure including reduced conception rates, elevations in the percentage of regular, as well as irregular returns to estrus and sporadic early abortions, increased repeat breeding, increased stillbirths, abortions, early farrowings, and increased number of pigs born weak or dead (Yaeger et al., 1993). In male, transient lethargy, depression, inappetence, and mild pyrexia, loss of libido and decreased semen quality not only but decreased in motility and the percentage of spermatozoa with normal acrosome but also increased in morphoabnomalies and cytoplasmic droplets have been reported with PRRSV (Prieto et al., 1996; Shin et al., 1997). Moreover, PRRSV can be transmitted by artificial insemination via semen, it is the one of route for infection that can be effected on both of the reproductive performances (Guérin and Pozzi, 2005).

1.1.2.1 Semen freezing

Cryopreserved semen was used in 1975 with mainly for five purposes: gene bank for genetic valuable animals and/or sire lines or dam lines, semen bank for emergency supply, export of genetic materials, domestic use in certain remote areas and for experimental or research proposes (Hofmo and Grevle, 2000). However, the limitation of FT is considering about semen quality after thawing due to boar sperm is susceptible to the temperature fluctuation during freezing and thawing process (Holt, 2000). According to genetic improvement by importing new dam or sire line, the international exporting live animals are strictly regulated especially gilts or boars have been quarantined for long time before entering to the new herds. To solve the problem is the transportation with the FT semen. Since there was no report that piglet born from

using frozen-thawed semen exported from Thailand to aboard, it may be a chance for implement this technique to the commercial breeding farm.

1.1.2.2 Semen sexing

A sex predetermination in livestock production is in great demand (Johnson, 2000). This can be done before fertilization by semen sexing or after fertilization by embryo sexing (Seidel, 2007). Controlling the sex of offspring prior to conception permits the livestock industry to produce the desirable sex to take the advantage to sex limited, economically flexible management for the producer and permits faster genetic process, higher productivity, improves animals welfare by decreasing obstetric difficulties in cattle, avoiding castration in pigs, and producing less environmental impact due to the elimination of unwanted sex before they grow up to adulthood (Rath and Johnson, 2008). Pig production would benefit from sex pre-selection if it was economically available by allowing for the production of male and female crossbred lines (Johnson et al., 2005). The ideal application by pig producers is the use of sex-sorting technology in nucleus herds, particularly for producing female lines. If pig producers are marketing gilts, the male pigs produced are ‘‘by-products’ (Vazquez et al., 2009). Flow cytometery is one of the acceptable sperm sexing technique which was refered to the most accuracy but it is very expensive and time consuming (Suh et al., 2005). If we look for general usage of sperm sexing at farm level, we have to try the easy, cheap and convenient method to extend the lifespan and sort boar spermatozoa for shifting to the desirable sex.

Cryopreservation combined with a sex selection, male or female dominance will be interested for preserving desired genetics. Freezing and processing protocols in combination with sex-sorted sperm, however, are not optimal for commercial application in swine, as in other mammalian species (Johnson et al., 2005; Bailey et al., 2008)

1.2 Literature review

1.2.1 Use of fresh and frozen boar semen

Using conventional AI, pigs are often inseminated several times (i.e. two or three times) during oestrus. A large volume of semen (80–100 mL) containing high numbers of spermatozoa (usually 2–3 x 10^9 as total sperm) is deposited into the posterior region of the cervix by a disposable catheter, emulating the situation that occurs in this species during natural mating. However, the limit of fresh semen is the long term preservation, it can be solved by using cryopreserved semen. This is a particular limitation when using cryopreserved semen, where the post-thawed viability of sperm is lower than for liquid semen, and the inseminate dose must be increased to (5000–6000)×10^6 sperm (Johnson et al., 2000). At present, while the use of frozen semen for AI is considered a basic component for cattle breeding worldwide (Curry, 2000), meanwhile the situation in the pig industry is diametrically different. Efficient application of frozen-thawed boar semen in commercial AI program is now feasible due to improvements in cryopreservation protocols and the development of new insemination procedures. Even though more sperm are required per dose of frozen semen than fresh, cryopreservation greatly facilitates the distribution of agriculturally desirable genes, because the semen can easily be transported over long distances or held until female is in estrus, and a stock of frozen semen enables the continued use of a sire that is dead or ill (Bailey et al., 2008). Moreover, the development of new procedure for AI, such as intrauterine or deep intrauterine insemination, has decreased the number of frozen-thawed spermatozoa required for acceptable fertility. Bolarín et al, (2006) revealed that the interval deep intrauterine insemination to ovulation provided a major explanation for fertility differences between farms when frozen-thawed spermatozoa was used.

Recently, cryopreservation of boar semen has been established in Thailand and shown the number of total piglets born per litter (TB) after intra-uterine insemination (IUI) using FT boar semen. It was relatively high and comparable to those obtained from AI using fresh semen (Buranaumnuay et al., 2008; Buranaamnuay et al., 2010) by demonstration about 9.0 piglets born alive per litter (BA) after IUI using FT boar semen. However, this study was conducted as an experiment and relatively low number of sows were included (Buranaumnuay et al., 2010). IUI with FT boar semen is however an interesting technique that should be applied to a high genetic resource and specific diseases free herd [e.g., Aujesky's disease, Porcine reproductive and respiratory syndrome (PRRS)].

1.2.2 Semen production

Leman and Rodeffer (1976) reported that puberty of boar occurred between 5 and 8 months, and the number of sperm and the volume of ejaculate increase until the boar reaches 18 months. The ejaculates at this time was between 20 and 80 × 10^9 sperms in 200–400 ml of semen. Diehl et al. (1979) reported that the number of sperm and the volume of ejaculate were (30–60)×10^9 sperm and 150–200 ml, respectively. The sperm production of the boars is affected by several factors such as breed, season, nutrition and housing (Kunavongkrit et al., 2005). Moreover, the differences among individual boars were also differences among breeds not only between purebred and crossbred also between lines (Sonderman and Luebbe, 2008). To produce the good fertility rate, semen for AI must be examined to ensure the success. The subjective parameters as motility, concentration, volume etc. are used for semen evaluation but the quality depend on individual assessment. The evaluation of sperm motion parameters using computer-assisted sperm analysis (CASA) reflects the correlation between sperm motility and fertility results and provides a way of objectively classifying a given population of spermatozoa (Holt et al., 2007). CASA allows an objective assessment of the sperm cell including motion, velocity and morphology (Verstegen et al., 2002). Moreover, CASA will help more investigation of semen quality in boar station.

1.2.2.1 Effect on semen production: Breed and season

There were significant among breed different in sperm production (Kozdrowski and Dubiel, 2004) and generally the larger breed, such as Yorkshire tended to produce a greater volume of semen per ejaculate and greater numbers of sperm cells over a period. Park and Yi (2002) showed that Yorkshire boars produced higher semen volume compared to Duroc boars among seasons. However, sperm concentration did not differ significantly between Duroc and Yorkshire boars among seasons. Sperm motility of frozen-thawed sperm in Yorkshire boars was higher than in Duroc boars in spring and summer. Normal acrosome of frozen-thawed sperm in Yorkshire boars was higher than in Duroc boars in winter (Park and Yi et al., 2002). However, both quantitative and qualitative differences in these relationships among boars are present and a given semen quality estimate that is a good predictor of *in vivo* or *in vitro* fertilization for one boar, may not be applicable for others.

Popwell and Flowers (2001) showed the comparisons were made among boars, farrowing rates, numbers of pigs born alive, and monospermic penetration rates were significantly different, but progressive motility, normal head and tail morphology,

and acrosome morphology were not. However, when comparisons were made among ejaculates within individual boars, there were significant effects of semen quality on both *in vivo* and *in vitro* fertility. Although the semen analysis was done routinely for predicting the fertility before fertilization, Gadea (2005) suggested the development of new sperm tests such as the binding and penetration of zona pellucida were more powerful to explain the fertility parameter of the variability in sperm fertilizing protential among fertile boars. From the reviewed of Gil et al. (2008) showed that the spermatozoa from Yorkshire boars can penetrated at significantly higher rates than those from Landrace and Duroc breeds, but the resulting fertilization was also more polyspermic for *in vitro* fertilization (IVF).

1.2.2.2 Semen evaluation technique: CASA

It has been demonstrated that the assessment of sperm motility using light microscopy resulted in a 30-60% (Amann, 1989; Chan et al., 1990; Cancel et al., 2000) variability caused by the evaluator's skills and cannot see the motility characteristic pattern. In recent years, the motility characteristics of spermatozoa have been evaluated by a CASA (Mortimer et al., 1995; Holt et al., 1997). CASA allows the objective determination of the concentration of spermatozoa, a variety of motility parameters, and the assessment of morphological aberrations (Thurston et al., 1999). There are some reports that have revealed the relationship of the sperm fertility with motility patterns or have tested whether sperm fertility is predictable motility characteristics (Mortimer et al., 1995; Holt et al., 1997). Although, Suzuki and Nagai (2003) showed that there was no distinct pattern between fertility *in vitro* and motility parameters, there were many investigators suggested the prospective research studies (*in vivo* or *in vitro*) were anticipated using CASA as a tool for identification of factors influencing sperm function. The advantages of CASA system were accurately profiles sperm for a variety of motion characteristics (i.e. forward progressive motility (FPM), linearity, velocity, beat cross frequency), and it is plausible that certain motion parameters might influence (increase or decrease) the fertilization process (Didion, 2008). The disadvantages of CASA are related to the cost of the equipment, the extreme need of validation, quality control, and standardization of the measures realized (Verstegen et al., 2002).

1.2.3 Cryopreservation of boar semen

Cryopreservation is one of technique to preserve and extend shelf life of boar semen. Polge (1956) stated that boar spermatozoa had, consistently, shown a higher sensitivity to cold shock and freezing compared with bull semen. Freezing boar semen started in 1975 (Hammerstedt et al, 1990; Großfeld et al., 2008) and the semen was frozen in Maxi straws using a programmable freezer. The frozen semen technology could be helped to support of genetic to export to customers and for establishing semen for gene bank.

1.2.3.1 Cryo-injuries

Boar spermatozoa are exposed to many processes during freezing and thawing procedures. One of these is lipid peroxidation, which causes damage to the sperm membranes and impairs metabolic energy (Großfeld et al., 2008). Cryopreservation can induce cold shock, a direct result of sudden cooling that is a cause of disruption and/or increased permeability of the plasma membrane (Buhr et al., 1994; Fernandez-Santos et al., 2006). In general, a final glycerol concentration of 2–3% in the freezing media, cooling rates of -30 to -50 °C/min, and thawing rates of 1200–1800 °C/min resulted in the best sperm survival (Großfeld et al., 2008). Moreover, the formation of lethal intracellular ice crystal and reduce acrosome and plasma membrane damage can be affected to the survival of spermatozoa (Hu et al., 2009). There were many studies to improve the post-thaw qualities by addition of antioxidants or chelating agent (e.g. catalase, vitamin E, glutathione, butylated hydroxytoluene or superoxide dismutase) to the still standard egg-yolk based cooling and freezing media for boar semen, effectively prevented this damage. The most popular diluents for boar spermatozoa freezing were glucose and egg yolk (Polge et al., 1970); Tris, fructose, EDTA, citric acid, glucose and egg yolk (Visser and Salamon, 1974); Tris, glycine, citric acid, glucose and egg yolk (Obando et al., 1984). Currently, disaccharide trehalose had been found to be able to resist dehydration or freezing in a number of plants and animals (Westh and Ramlv, 1991). Trehalose could form hydrogen bonds with the polar head groups of phospholipids to prevent fusion events of juxtaposed membranes (Anchordoguy et al., 1987). Thus, trehalose had been widely used as a cryoprotectant for spermatozoa (Dalimata and Graham, 1997; Graham and Morcé, 2005).

Although, egg yolks are commonly used in diluents in order to improve the freezability of semen. Maldjian et al., (2005) reported the role of lipids is the eventual exchanges of lipid components between spermatozoa and the yolk-based diluents during cryopreservation by adding docosaheaenoic acid (DHA) in medium.

1.2.3.2 Boar semen freezability

The survival of frozen–thawed sperm is affected by many factors, extensively reviewed by Salamon and Maxwell (1995). These factors include the basic types and concentrations of ingredients used in the semen extenders, the concentration of glycerol or other cryoprotectants, packaging, freezing and thawing rate, as well as the quality of semen used for freezing. Park and Yi (2002) studied in the relationship between serum testosterone and freezability during seasons. This report revealed that when serum testosterone concentrations were higher in Duroc and Yorkshire boars among seasons, semen volume, sperm concentration and frozen-thawed sperm viability were higher. Not only in swine but also in equine, Janett et al. (2003) demonstrated that individual and seasonal differences occurred in semen quality of Franches–Montagnes stallions. When collection of semen in autumn (September, October, November) demonstrated good quality, especially regarding sperm morphology, and were more suitable for cryopreservation because of better motility in frozen-thawed semen collected during autumn than in winter.

Andrabi et al. (2008) indicated that using duck egg yolk extender had showed the higher post-thawed motility and percentage of normal acrosome than Guinea fowl egg yolk, Indian indigenous hen (Desi) egg yolk and commercial hen egg in buffalo bull spermatozoa. El-Alamy and Foote (2001) proved that the compounded semen extenders can be affected on the freezability of ram semen. Furthermore, Roca et al. (2006) found that boar was the most important factor explaining the variability among ejaculates in sperm survival in cryopreservation.

The use of several small straws (either mini- or medium-straws) to preserve one insemination dose is, however, impractical under field condition. Eriksson et al. (2002) tested their protocol and FlatPack container maintains high sperm viability post thaw were satisfied for exporting frozen boar semen in field.

Although the breed boar effect is one of criterias for cryopreservation, there are large variations in freezability of the semen between breeds and between individual boars within the same breeds (Buranaamnuay et al., 2009). Supplementation of antioxidants such as L-cysteine, glutathione and water soluble-vitamin E can improve

the semen quality in different of breed boars (Kaeoket et al., 2008). The addition of 3% fish oil to daily boar ration significantly increased the content of docosahexaenoic acid (DHA) in spermatozoa; however, DHA-enriched semen did not show improved freezability (Maldjian et al., 2005). Not only types of cryopreservation extenders but cryopreservation conditions affected the sperm freezability. According to the recent study revealed that the use of 3% glycerol and thawed at 1800 °C/min showed the best post-thaw quality results (Herna´ndez et al., 2007).

1.2.4 Semen sexing

There were several methods for sex pre-selection, for example, flow cytometry, swim-up technique (Yan et al., 2006) and discontinuous percoll-gradient centrifugation (Kobayashi et al., 2004). The best technology is based on the well-known difference in X- and Y- sperm in the amount of DNA presenting.

1.2.4.1 Concept of sperm sexing

Separation of X- and Y-bearing spermatozoa may be accomplished on the basis of physical differences such as detected by flow cytometry, or by detection of chemical differences in membrane characteristics. The techniques must satisfy three criteria (Jafar and Flint, 1996). First, The procedure must result in a significant shift in the proportion of the Y- and X-bearing sperm population. Second, it must not interfere with in vivo or in vitro fertilization. Third, it must result in progeny (embryos or live neonates) that reflect the previously determined proportions of the X- and -bearing spermatozoa. The first criterion may be satisfied by analysis of the populations by reliable method such as flowcytometry, Fluorescence in situ hybridization (FISH) and quantitative polymerase chain reaction (qPCR); the second and third criteria require artificial insemination or *in vitro* fertilization and embryo transfer.

1.2.4.2 Flowcytometry

Although, flow cytometry is being applied in livestock, zoo animals and in humans with a success rate of 90-95% in shifting the sex ratio of offspring (Johnson et al., 2005; Johnson, 2008) and the efficiency of the sorting procedure has increased with development of improved nozzle designs and adaptation to high speed cell sorting, the usefulness remain limited in pig industry (Vazquez et al., 2009). Flow cytometry is the technique which can be sexed sperm for many mammalian species by sorting at about 90% accuracy. Since the number of sexed sperm produced per unit time is limited

(Seidel, 2003; Seidel, 2007), the flow cytometry has not been applied in practice in pig due to the high sperm numbers needed for optimum fertility in the female (Suh et al., 2005). This fact has made it economically impractical for the pig artificial insemination industry to sex boar sperm for commercial pig production.

1.2.4.3 Colloidal centrifugation

Centrifugation fractionation of semen is commonly done to improve sperm quality by allowing sperm separation based on their isopycnic points (Edmond et al., 2012). Using different concentrations of colloid with silica particles to make discontinuous density gradients, semen will be separated by specific gravity into layers. Furthermore, colloidal silica does not effect on osmotic stress when it was added into culture medium to formulate the layer with high specific gravity to separate dense cells. Single layer centrifugation through a colloidal with species-specific formulation (Androcoll-E™) can enhance a sub-population of high motile stallion spermatozoa with normal morphology (Morrell et al., 2009). However, one of well known discontinuous density-gradient media containing silane-coated silica particles colloid is Percoll® which have been used various assisted reproductive techniques with high recovery of high quality sperm (Centola et al., 1998). This technique might shift the sub-population spermatozoa via the different amount of DNA content between X- and Y-bearing spermatozoa. According to Kobayashi et al. (2004) showed the using of Percoll® separation in bull semen could sort the X- and Y-bearing spermatozoa which the bottom part was significantly different in X-bearing spermatozoa. Moreover, Suzuki and Nagai (2003) revealed that Percoll-separated spermatozoa had increased the higher percentage of motile and progressively motile spermatozoa than those that were not separated.

Regarding to the genetic and good management system, it is then interesting to study the feasibility to set up the semen freezing and sexing for swine industry in Thailand.

1.3 Objectives of the study

1. To investigate the sperm production of disease freeboar in relation to breed, seasonal influences and fertility data
2. To study the boar semen cryopreservation and semen sexing

CHAPTER II

FACTORS INFLUENCING BOAR SEMEN PRODUCTION

(this part was presented in Thai J. Vet. Med)

2.1 Abstract

This study was carried out to investigate the factors of season and boar breed (purebred and crossbred) on sperm production in a boar stud which has been free from porcine reproductive and respiratory syndrome (PRRS) more than ten years and kept in evaporative cooling system, in relation to season and breed influence. Semen production data from 19,966 ejaculates of 517 boars (164 Duroc, 31 Pietrain, 268 Landrace x Yorkshire: LY, 54 Pietrain x Duroc: PD) were collected. Semen parameters; volume (ml), sperm concentration ($x10^6$ sperm/ml) and total number of sperm per ejaculate ($x10^9$ sperm/ejaculate) were evaluated. The semen production was shown by month and group in relation to season as summer (Mar-Jun), rainy season (Jul-Oct) and winter (Nov-Feb). On average, the semen volume, concentration, and total sperm per ejaculate were 249.7±97.5 ml, 335.7±95.9$x10^6$ sperm/ml and 78.9±28.4$x10^9$ sperm/ejaculate, respectively. Effect of season, the total number of sperm per ejaculate in winter was higher than a period in rainy season (Aug-Oct) ($P< 0.05$) while the concentration in early winter (Nov and Dec) was lower than summer and a month in rainy (Jul) ($P< 0.05$). Effect of breed, the total sperm production of LY crossbred boar (88.2±27.2 x 10^9 sperm/ejaculate) was higher than purebred Duroc boar (60.2±21.9 x 10^9 sperm/ejaculate, $P = 0.01$) and purebred Pietrain boar (76.5±21.8$x10^9$ sperm/ejaculate, $P = 0.03$). The seasonal variation effect was most pronounced in purebred Duroc and Pietrain boars rather than LY and PD crossbred boars. The fertility data using Duroc semen (total number of piglets born and born alive) tended to increase during Mar-Jun and Oct-Dec. The lowest total number of piglets born and born alive were shown in Oct ($P< 0.05$). It can be concluded that seasons and breeds influencing the sperm production was found in boars kept in EVAP with free PRRS in Thailand.

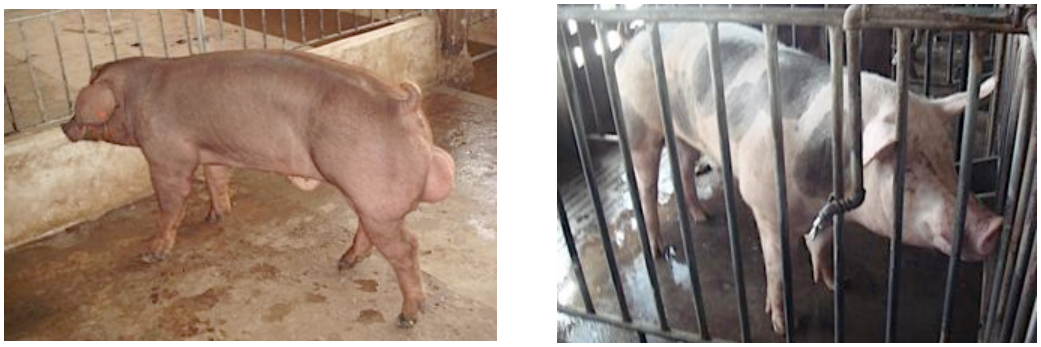
2.2 Introduction

Sperm production of boars is affected by several factors such as breed, season, nutrition and housing (Ciereszko et al., 2000; Kunavongkrit et al., 2005; Huang et al., 2010). It was found that the semen production of purebred and crossbred boar including breed lines was different (Sonderman and Luebbe, 2008). Thailand is situated in tropical zone where the temperature is normally above 30^{0}C for several months of the year, Reduction in pig production and reproductive performance are significantly observed during hot periods. As a result, evaporative cooling system is normally used for housing in Thailand to ensure good semen production. This system helps to reduce the seasonal variation of the sperm production in Duroc boars (Suriyasomboon et al., 2004). Moreover, the semen must be produced from disease-free boar to prevent disease transmission via semen. It has been known that porcine reproductive and respiratory syndrome virus (PRRSV) infection is a very important disease of swine due to its transmission from infected boar to several sows which subsequently affects reproductive performances (Guérin and Pozzi, 2005). The establishment of isolated free PRRS boar station is required for semen distribution to sow herds, which has been successfully done in Thailand. The objectives of the present study were to investigate the sperm production of boar stud which has been free from PRRS in Thailand in relation to breed, seasonal influences and fertility data in a few sow herds.

2.3 Materials and methods

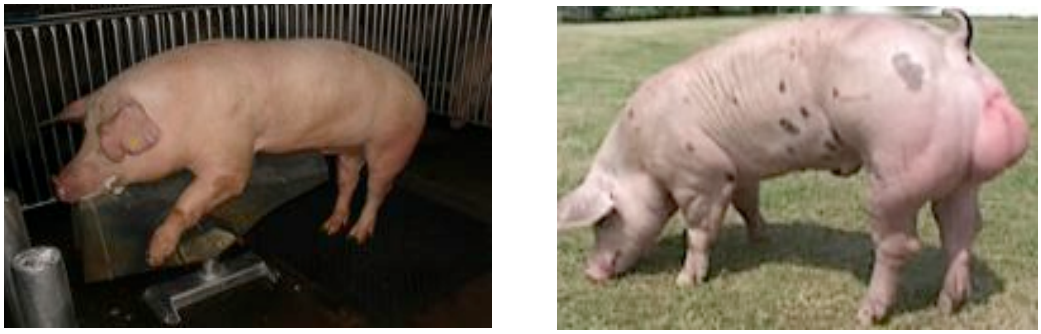
2.3.1 Data of semen production

This study was based on semen production collected from a central isolated boar station in the eastern part of Thailand during the period of January 2006 until December 2009. This station provided semen for AI to three breeder farms (n= 10,000 sows) all year round. All breed of boars in this study was depicted in Fig1. A total of 19,966 ejaculates were collected from 517 boars with two purebreds (Duroc, n= 164; Pietrain, n= 31) and two crossbred boars (Landrace x Yorkshire: LY, n= 268; Pietrain x Duroc: PD, n= 54). All boars were trained and identified as proven sires from their fertility data of artificial insemination in PRRS free herds. The seronegative of PRRS was tested with the LAB (ISO/IEC17025:2005) and had been operated for at least 10 years before the start of the study.



(a) Duroc (b) Pietrain

Purebred



(c) Landrace x Yorkshire (d) Pietrain x Duroc

Crossbred

Figure1 Breed of boars in this study
(Purebred: (a) and (b); Crossbred: (c) and (d))

2.3.2 Semen collection and evaluation

The ejaculates were collected routinely using the gloved-hand method and evaluated macroscopically and microscopically as subjective motility assessment. The gel free semen parameters including volume (ml) measured by weight, sperm concentration (x 10^6 sperm/ml) measured by Spermacue® (Minitube, Germany) and total number of sperm per ejaculates (x 10^9 sperm/ejaculate) calculated by multiplying ejaculating volume and sperm concentration were evaluated. To relate to seasonal effect, the data of daily temperature and humidity were recorded during the studying period.

2.3.3 General herd management and serology monitoring

Each boar was kept in individual pens (9 m^2/boar) and was fed on 2.2-3.0 kg/day of commercial feed containing 14-18% crude protein. The boars had access to water *ad libitum* via nipple. The young replacement boars produced from a GGP farm classified as free PRRS status were penned in the quarantine area at least 2 weeks before being trained. The clinical disease and serial blood monitoring were observed during quarantine period. After absolutely free PRRS evidence, all replaced boars were trained and the semen was collected at least twice for evaluation for volume, concentration and motility. To maintain PRRS negative status, all boars were routinely tested for seronegative (S/P ratio < 0.2) by ELISA (HerdChek-PRRS®; IDEXX, Laboratories Inc., Westbrook, MA, USA) and PCR from Animal Health and Technical Service Office.

2.3.4 Season, temperature and humidity

Semen production was calculated in relation to season as summer (Mar-Jun), rainy (Jul-Oct) and winter (Nov-Feb). Temperature and humidity were recorded once a day. The Max-Min thermometer which was hung at the center of the housing about 170 cm above the floor was used to record the temperature inside EVAP. Temperature was record every day about 7-8 am, after each recording the device was set to measure a new figure for the next day. The percentage of humidity in EVAP was recorded as the average humidity by using a digital device (TEMP1000®; Italy) which had 2 hygro-sensors located about 20 meters far from each other along the front and back of the housing. The outside temperature and humidity were recorded from The Eastern Part of Thai Meterological Department, Thailand. The two temperature variables were defined as the number of hot days per month (maximum temperature > 25°C) and the number of optimum days per month (maximum temperature $\leq$ 25°C) (Auvigne et al., 2010).

2.3.5 Fertility data collection

To find the relationship between sperm production and fertility, data were collected from three breeder farms located around this boar stud not more than 50 km. All of them were EVAP system housings. Semen usage, 62,404 of fertility data of Duroc boar semen from the boar stud during January 2006 until December 2009, were included in this study. This fertility data comprised total piglet born and born alive.

2.3.6 Statistical analysis

The sperm production data were analyzed using general linear mixed model procedure (MIXED) of SAS version 9.0 (SAS®, NC, USA). The models included boar breeds, year and month in which the semen was collected as fixed effect and included boar identity tested within breed as random effect. Least-squared means were obtained and compared among breeds using Tukey-Kramer test. The total number of piglets born were analyzed using general linear model procedure (GLM) to compare among the month of farrowing data from each farm during 2006-2009. $P < 0.05$ was regarded as a significant difference.

2.4 Results

On the average, semen production resulted as descriptive data in each breed is presented in Table 1. Across the breeds, the average semen volume, concentration, and total sperm per ejaculate were 249.7±97.5 ml, *335*.7±95.9x10^6 sperm/ml *and 78.9*±28.4x 10^9 sperm/ejaculate, respectively. Duroc boar presented the lowest mean value of volume, 170.0±67.8 ml, but possessed the highest concentration. For the overall sperm output (78.9±28.4 sperm/ejaculate), LY had the highest mean value of total sperm per ejaculate (88.2±27.2 x 10^9 sperm/ejaculate).

Table 1 Means, standard deviation (SD) and range of sperm production in Duroc (D), Pietrain (P), Landrace x Yorkshire (LY) and Pietrain x Duroc (PD) boars kept in EVAP

Variables	Breed	N	Mean±SD	Range
Volume (ml)	D	5,763	170.0±67.8	50-497
	P	749	249.5±72.2	65-486
	LY	11,729	293.8±86.0	50-500
	PD	1,725	250.7±76.1	58-500
	All	19,966	249.7±97.5	50-500
Concentration	D	5,253	381.6±88.9	75-600
(x 10^6sperm/ml)	P	659	318.3±91.2	97-600
	LY	11,232	310.0±89.6	80-600
	PD	1,283	381.1±93.3	105-600
	All	18,427	335.7±95.9	75-600
Total sperm per ejaculate	D	5,229	60.2±21.9	12-255
(x 10^9 sperm)	P	654	76.5±21.8	19-230
	LY	11,058	88.2±27.2	17-284
	PD	1,279	76.4±26.5	15-203
	All	18,220	78.9±28.4	12-284

Temperature (°C) and humidity (%RH) between outside and inside EVAP system are shown in Fig 2. EVAP system enables the outside temperature to be lowered about 4-6°C while the humidity was increased due to its spraying and cooling system. The numbers of days with a maximum temperature of >25°C were classified as hot days; and the days with a maximum temperature of $\leq$ 25°C as optimum days (Fig 3). It was revealed that the hot days were more than twenty days a month in Mar-Jun (summer) and Jul-Oct (rainy) and all the days in April and September were hot days.

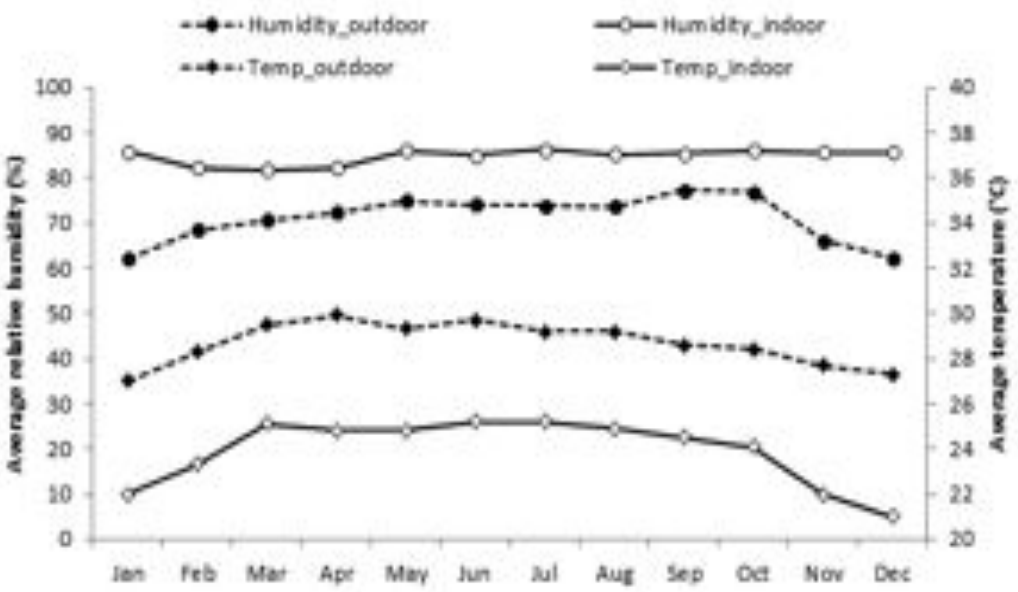


Figure 2 Average temperature and humidity outside and inside the boar stud equipped with evaporative cooling system (Summer, Mar-Jun; Rainy, Jul-Oct; and Winter, Nov-Feb)

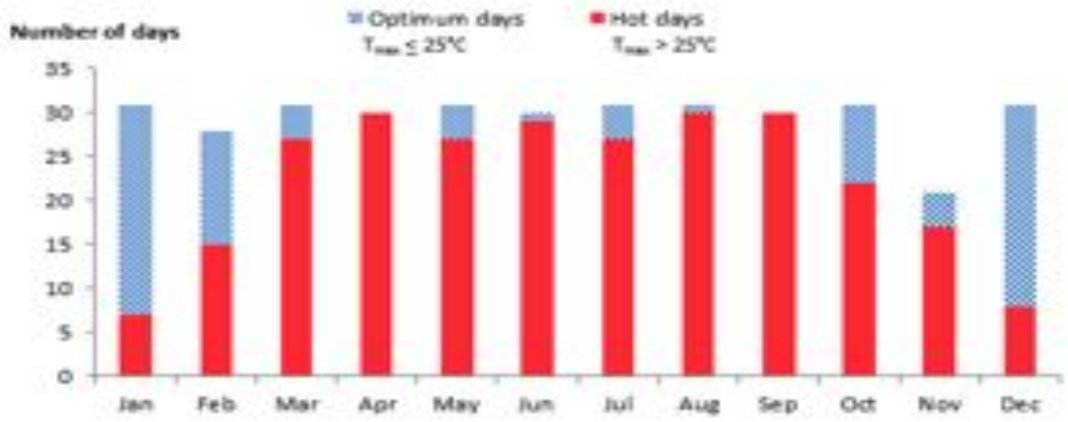


Figure 3 Number of hot days (maximum temperature >25°C) with the month of inside temperature of the boar stud equipped with evaporative cooling system (Summer, Mar-Jun; Rainy, Jul-Oct; and Winter, Nov-Feb)

Monthly sperm production is presented in Fig 4 ((a) volume, (b) concentration and (c) total sperm per ejaculate). The volume was obviously significantly different in early winter (Nov and Dec) ($P<0.05$). On the contrary, the obviously significant difference of concentration was found during middle rainy to early winter (Aug to Dec) ($P<0.05$). Accordingly, the total number per ejaculate during Nov to Dec had no difference ($P> 0.05$) but during Aug to Oct had a significant difference ($P< 0.05$).

The sperm production in each breed is presented in Fig 5 ((a) volume, (b) concentration and (c) total sperm per ejaculate) in each average parameter production. Across the months, LY had the *highest volume and total* sperm production ($P<0.05$) throughout the year, while Pietrain possessed the lowest concentration and total sperm production ($P<0.05$). Duroc produced the lowest volume but produced the highest concentration all year round ($P<0.05$). The total sperm production of LY crossbred boar was higher than Duroc ($P = 0.01$) and Pietrain ($P = 0.03$).

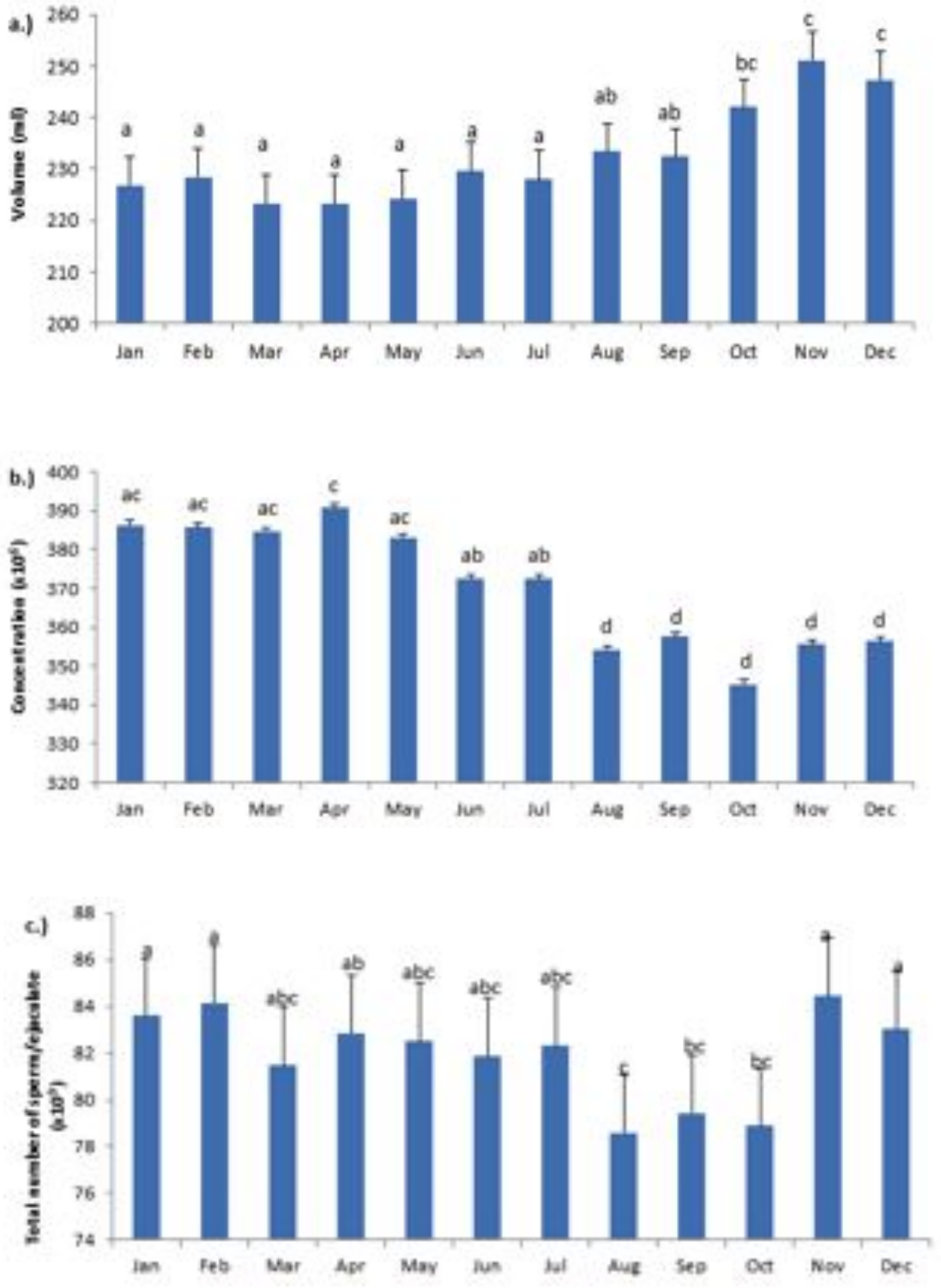


Figure 4 Semen production (a. volume, b. concentration and c. total number of sperm) compared by months with ^{a,b,c} as different superscripts indicating significant difference (P<0.05) (Summer, Mar-Jun; Rainy, Jul-Oct; and Winter, Nov-Feb)

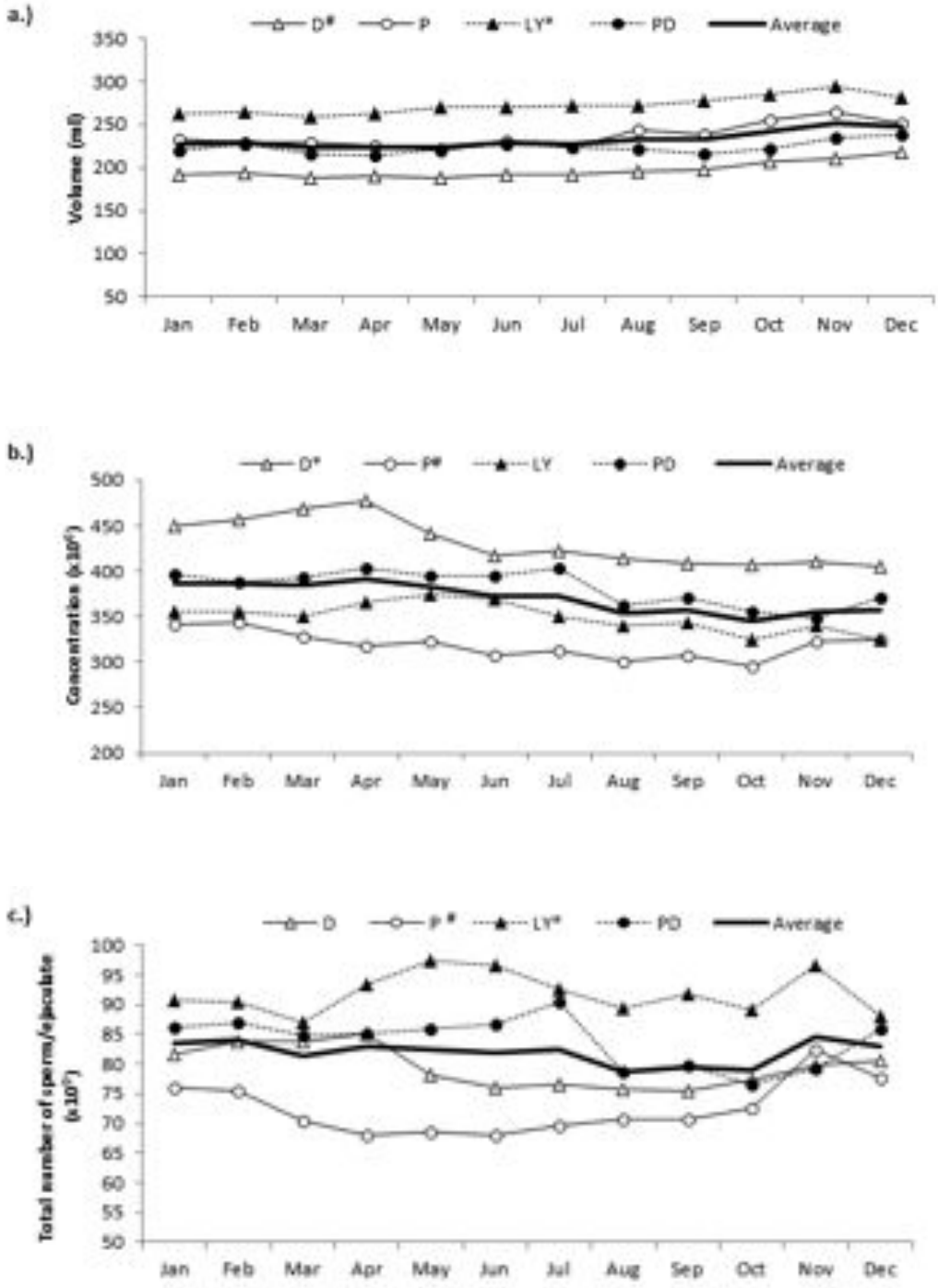


Figure 5 Semen production (a. volume, b. concentration and c. total number of sperm) in Duroc (D), Pietrain (P), Landrace x Yorkshire (LY) and Pietrain x Duroc (PD) boars by months with $^{*, \#}$ as different superscripts in each legend indicating significant difference (P<0.05) as the highest and the lowest in each parameter among breed, respectively

The fertility data, 2006-2009, was recorded from sow herds by using Duroc semen, as shown in Fig 6. The total number of both piglets born and born alive tended to increase during summer (Mar-Jun) and Oct-Dec. Meanwhile, in June to August, the total number of piglets born and born alive tended to decrease. The lowest total born and born alive piglets was shown in October (P<0.05).

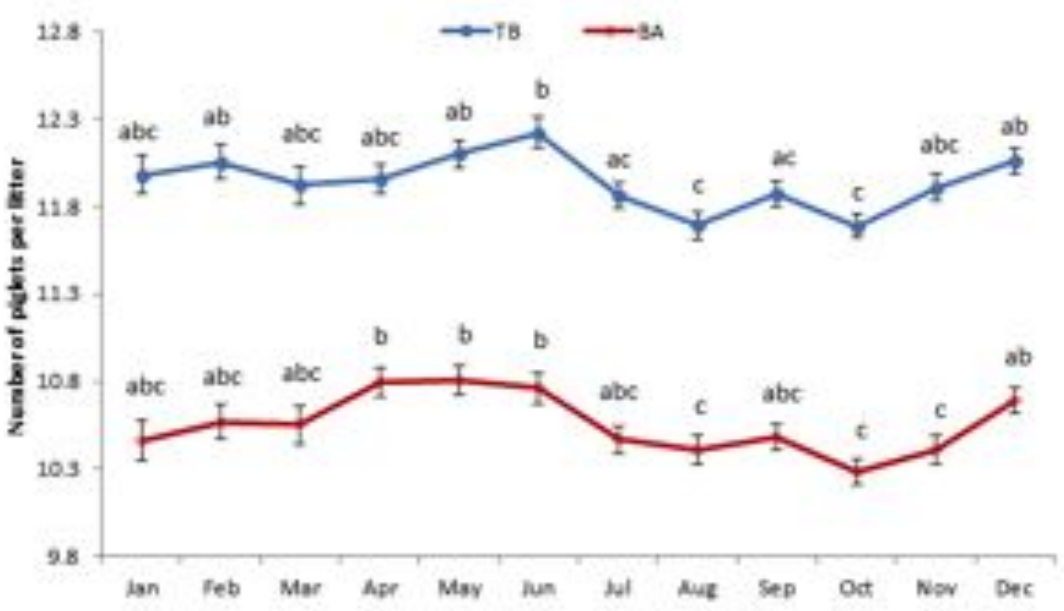


Figure 6 Least-squared means and standard error of total piglets born by months
[a,b,c] as different superscripts in each line indicating significant difference (P<0.05) (Summer, Mar-Jun; Rainy, Jul-Oct; and Winter, Nov-Feb)

2.5 Discussion

In this chapter, the result showed that the sperm production differed among the breeds. According to Park and Yi, (2002) Yorkshire boars produced higher semen volume compared to Duroc boars among seasons. This result resembles Wolf and Smital (2009) who revealed that the reduction in volume could be seen significantly different in Duroc when compared among breeds. Generally, Duroc had the lowest semen volume and highest sperm concentration, whereas the sire line of Yorkshire had the highest semen volume and the lowest sperm concentration. Trudeau and Sanford (1990) suggested that the volume of semen can be altered in adult Landrace boar by season which that decreased in semen quality preceding summer so that increasing in ambient temperature and the presence or absence of female pigs alters seasonal patterns.

Purebred boars were more sensitive to fluctuation of temperature than crossbred in the volume and total number of sperm per ejaculation, which tended to be lower in purebred during the summer, agreeing with Sonderman and Luebbe (2008). In Thailand, high humidity during rainy (Jun-Oct) might contribute to the seasonal influence on the sperm output. Meanwhile, sperm output varied with season, including high values in autumn and winter and low ones in spring and summer in the Czech Republic (Smital et al., 2004; Smital, 2005; Smital, 2010). Even though there was a report that boars exposed to increasing or decreasing photoperiod between seasons could affect sperm concentration due to testicular function (Sancho et al, 2004), in this study there was slightly decrease concentration in winter. It is assumed that Thailand, which is located in the tropical zone, has no effect of photoperiod on boars kept in EVAP system. However, semen production decreasing can be caused by management such as vaccination program or the interval-time resting of boar semen collection.

Suriyasomboon et al. (2006) revealed that the pattern of seasonal effect on reproductive performance in tropical area differed from that found in northern Europe and USA due to the differences in seasons. Normally, there are three seasons in tropical area (summer, rainy and winter) while there are four seasons in northern Europe and USA (winter season: Dec to Feb, spring: Mar to May, summer: Jun to Aug and autumn: Sept to Nov). Since swine gestation period spends approximately four months, farrowing at that time of season come from the insemination using semen during the previous season as sequence (summer, rainy and winter). From this study, these corresponded to insemination occurred in middle to late winter (Jan, Feb), the increase of total piglets born was found during summer. On the other hand, the insemination occurring during summer to early rainy season shows the lower production all period of rainy. The good or bad fertility data in tropical area could be caused by semen quality, however, one must consider that poor fertility data might be affected by the factors from female such as ovarian function, stress resulting to abortion or management. Finally, we concluded that season and breed can effect on boar sperm production eventhough boars were kept in EVAP. Fluctuation tempertature is also important to control for good semen production.

CHAPTER III

BOAR SEMEN FREEZABILITY AND *IN VIVO* FERTILITY

3.1 Experiment 1 A discrimination of motility pattern of frozen-thawed boar semen using CASA (this part was presented in Thai J. Vet. Med)

3.1.1 Abstract

Computer-assisted sperm analysis (CASA) was initiated to reduce subjective bias on the motility assessment and to discriminate a series of motility patterns of boar semen. The objectives of this present study were to evaluate the sperm motility pattern of frozen-thawed (FT) boar semen at 0 and 60 min after thawing using CASA. Forty one ejaculates from Landrace (n=14), Yorkshire (n=12) and Duroc (n=15) boars were cryopreserved and included in the experiment. The semen was thawed at 37°C for 30 sec. The post-thawed sperm qualities including subjective motility, plasma membrane integrity and motility pattern were determined immediately after thawing (T_0) and at 60 min (T_{60}) after incubation at 38°C. All motility parameters were recorded. Motion parameters including curvilinear velocity (VCL, μm/s), linear velocity (VSL, μm/s), mean velocity (VAP, μm/s), linear coefficient (LIN, %), amplitude of lateral head displacement (ALH, μm) and total motility (MS-CASA, %) were measured. The results revealed that total motility and VSL assessed by CASA after thawing (T_0) differed ($P<0.05$) among breeds. Some motion characteristics of FT boar semen i.e., VSL, VAP, VCL and ALH significantly decreased an hour after post-thawing ($P<0.05$). However, there was no significant difference in MS-CASA, and LIN between T_0 and T_{60} groups.

3.1.2 Introduction

The assessment of semen quality is important for the success of artificial insemination in pigs. It can be routinely assessed by subjective visual examination under a phase contrast microscope e.g. individual progressive motility, concentration and morphology etc., or by evaluation of farrowing performances which remains time-consuming of high cost. Microscopic techniques have limitations including subjectivity, variability, the small number of sperm analyzed and poor correlation with fertilizing potential (Rijsselaere et al., 2005). It has been demonstrated that the assessment of

sperm motility using light microscopy resulted in a 30-60% (Amann, 1989; Chan et al., 1990; Cancel et al., 2000) variability caused by the evaluator's skills. Computer-assisted sperm analysis (CASA) provides objective and detailed information on various motility characteristics and morphometric dimensions that cannot be identified by conventional light microscopic semen analysis (Rijsselaere et al., 2005). The use of sperm motion parameters via computer-assisted sperm analysis (CASA) reflects the correlation between sperm motility and fertility results and provides a way of objectively classifying a given population of spermatozoa with respect to their motility (Holt et al., 2007). CASA allows the objective assessment of the sperm cell characteristics including motion, velocity and morphology (Verstegen et al., 2002). Time lapse and multiple exposure photography were the forerunners of sperm motility analysis by CASA. These techniques involved recording the movement of live spermatozoa on microscope slides by opening the shutter for a fixed period so that the cell movements produced continuous tracks on the exposed film. The motion parameters typically derived using automated CASA systems provide information about the velocity, linearity and lateral displacement of sperm heads as they progress along their trajectories (Didion, 2008; Rijsselaere et al., 2005). Our previous study found that the breed of boar and individual boars within the same breed significantly influenced most of the post-thaw sperm parameters (Buranaamnuay et al., 2009). However, the use of CASA to evaluate the FT boar semen was performed. Moreover, a thermal resistance test (38°C for 60 min) for post-thawed semen qualities was not evaluated. This technique showed itself to be a potential method for evaluating the survival capacity of spermatozoa *in vitro* (Koonjaenak et al., 2007). The objectives of the present study were to evaluate the motility pattern of frozen-thawed boar semen at 0 and 60 min after thawing in three breeds of boars using CASA.

3.1.3 Materials and methods

3.1.3.1 Semen collection, freezing and thawing

Each sperm- rich fraction of forty one ejaculates from 15 purebred boars (5 Landrace; 14 ejaculates, 5 Yorkshire; 12 ejaculates and 5 Duroc; 15 ejaculates) aged between one and three years old were included in the experiment. The boars are routinely used in two commercial swine herds in Nakorn Pathom province, Thailand. The ejaculates were collected once a week using the gloved-hand technique and were kept in an insulated thermos flask during transport to the laboratory within 40 min after

collection. Fresh semen with a minimum of 70% sperm motility was used for freezing with some modifications following Westendorf et al. (1975) and Gadea et al. (2004). The semen was diluted with isothermal Beltsville thawing solution (BTS; Minitüb, Abfüll-und Labortechnik GmbH & Co. KG, Germany) extender at a ratio of 1:1 (v/v). The diluted semen was kept at 15°C for 2 h and centrifuged at 800 xg for 10 min. The supernatant was discarded and the pellet resuspended (about 1 to 2:1) with lactose-egg yolk (LEY) extender (80 ml of 11% lactose solution and 20 ml egg yolk). After further cooling to 5°C over a 90-min period, two parts of the semen were mixed with one part of extender III (LEY extender and 9% glycerol with 1.5% Equex-STM®). The final concentration of sperm frozen was approximately $1x10^9$ spermatozoa/ml with 3% glycerol. The straws were sealed with PV powder at the open end of the straws before being placed in liquid nitrogen (LN2) vapour at 4 cm above the level of LN2 for 10 min and then plunged into LN_2. The frozen boar semen was stored in LN_2 (-196°C) and thawed using the protocol in a 37°C water-bath for 30 sec and the post-thaw sperm quality evaluated immediately to consider the total motility (Buranaamnuay et al., 2009).

3.1.3.2 Hypoosmotic swelling test (Hos-test)

The plasma membrane integrity (PMI) of the spermatozoa was assessed using the short hypo-osmotic swelling test (Hos-test) described by Perez-Llano et al. (2001), with some modifications (Koonjaenak et al., 2007). Briefly, aliquots of each semen sample (100 μl) were incubated at 38°C for 10 min, with 1,000 μl of either hypo-osmotic (75 mOsm/kg) or iso-osmotic (300 mOsm/kg) solution. The solutions were prepared with fructose and Na-citrate in distilled water and final osmolarity was measured by freezing point depression. Following the 10-min incubation, 200 μl of the semen-hypo-osmotic solution was fixed in 1000 μl of hypo-osmotic solution plus 5% formaldehyde (Merck Co. Ltd., Boeco, Germany) for later evaluation. Sperm coiling was assessed by placing 20 μl of well- mixed sample on a warm slide, which was covered with a cover slip before being observed under a light microscope (x 1,000) and 200 spermatozoa per slide were counted. To determine the percentage of sperm with intact membranes, the proportion of coiled tail sperm from the control sample (300 mOsm/kg) was subtracted from the results of the hypo-osmotic condition.

3.1.3.3 Computer-assisted sperm analysis

The subjective motility of fresh and frozen-thawed semen was evaluated using a light microscope at 400x magnification (Dott and Foster, 1979). The motility of diluted frozen-thawed semen was assessed using the CASA system (Halminton Thorne Biosciences IVOS, Version 12 TOX IVOS, Beverly, USA). A 80 μl aliquot of the thawed semen was re-extended with 920 μl of pre-warmed BTS (37°C) to obtain a final concentration of $40x10^6$ spermatozoa/ml. A 5 μl aliquot was placed in a pre-warmed container (37°C). The cell motion analyzer provided a stage warmer to allow for sample distribution upon the sample chamber and to pre- warm samples. A waiting period of 1 min preceded each measurement (Smith and England, 2001; Iguer- Ouada and Verstegen, 2001). After this primary assessment (T_0), the thawed semen was placed in an incubator at 38°C for 60 min (T_{60}) (Thermal resistance test) before being assessed again by CASA (Koonjaenak et al., 2007). To select cells from debris, the camera recognized the position of the sperm heads in successive frames. Spermatozoa heads were marked with a different color to enable the observer and the analyzer to differentiate between the different motility parameters. In addition, all the motility parameters were recorded for a single sperm cell.

3.1.3.4 Determination of the motility pattern of FT boar semen

Each semen sample was measured twice, 3 fields were evaluated per sample and 100 cells per field were evaluated. Motion parameters consisted of (1) Curvilinear velocity (VCL, μm/s), the instantaneously recorded sequential progression along the whole trajectory of the spermatozoon per unit of time,(2) linear velocity (VSL, μm/s), the straight trajectory of the spermatozoa per unit of time (= straight line distance from the beginning to the end of the track divided by time taken), (3) mean velocity (V AP , μm/s), the mean trajectory of the spermatozoa per unit of time, (4) Linear coefficient (LIN, %), the ratio of the straight displacement in the sum of elementary displacements during the time of the measurement which is defined as (VSL/VCL) x 100, (5) Amplitude of lateral head displacement (ALH, μm), which is the mean width of sperm head oscillation (Aleporou-Mairinou et al., 2001) and (6) total motility (MS-CASA), expressed as the percentage of spermatozoa with VCL >15 μm/s.

3.1.3.5 Statistical analysis

The statistical analyses were performed using SAS (SAS version 9.0, Cary, NC, USA). Descriptive statistics were used to describe semen quality after thawing. Pearson's correlation was used to evaluate the correlation among sperm parameters. The differences of post-thawed sperm qualities between 0 and 60 min were analyzed using a paired *t*-test. The difference between breeds was evaluated using the General linear model procedure (GLM). The least-square means were obtained and were compared using Student's t test. $P < 0.05$ was regarded as a significant difference.

3.1.4 Results

On average, the subjective motility of FT boar semen was 28.2% (range 5% to 45%), while PMI was 18.5% (range 3% to 45%). The subjective motility was 29.0±2.4% in Duroc (n=15), 30.3±2.4% in Landrace (n=14) and 25.3±2.4 in Yorkshire (n=12) (P=0.05). PMI was 19.4±2.9% in Duroc, 18.1±2.9% in Landrace and 17.9±2.9% in Yorkshire (P=0.05). The LIN, VSL, VAP, VCL and ALH at 0 and 60 min after thawing are presented in Table 2. Sperm parameters determined by CASA differed between T_0 and T_{60} was presented in Table 3. A thermal resistance test revealed that there were no significant differences in all parameters among the three breeds.

The post-thawed motion parameters varied among individuals and among breeds (Table 2). On average, the VSL in Landrace was significantly higher than Duroc (30.3 vs 40.0 μm/sec (P<0.05). VAP, VCL and the ALH significantly decreased in all breeds after incubation (Table 3). There was no significant difference for the subjective motility and PMI among breeds. The progressive motility and VSL of Landrace was higher than Duroc and Yorkshire (Table 2). No significant difference in motility (%), LIN (%), VSL, VAP, VCL and ALH at T60 (P>0.05) was found among the breeds.

Table 2 Least square means (LSM) ± standard error of the mean (SEM) of sperm parameter post-thaw (PT) in semen collected from boar. Samples examined for sperm motility were examined immediately by CASA (n = number of ejaculation)

Parameter	After thawing(T_0)			After thermal resistance test (T_{60})		
	Duroc (n=15)	Landrace (n=14)	Yorkshire (n=12)	Duroc (n=15)	Landrace (n=14)	Yorkshire (n=12)
MS-CASA (%)	4.7±2.9[a]	15.4±3.0[b]	20.9±3.2[b]	7.7±3.0[a]	10.3±3.1[a]	10.1±3.4[a]
LIN (%)	39.1±4.9[a]	44.4±5.0[a]	49.8±5.5[a]	48.7±5.6[a]	44.2±5.8[a]	52.8±6.3[a]
VSL (μm/sec)	30.3±2.9[a]	40.0±3.0[b]	35.9±3.2[a,b]	27.7±3.1[a]	32.2±3.3[a]	29.1±3.5[a]
VAP (μm/sec)	59.1±5.9[a]	61.0±6.1[a]	59.0±6.6[a]	38.3±3.6[a]	41.3±3.7[a]	36.5±4.0[a]
VCL (μm/sec)	115.3±13.3[a]	114.1±13.7[a]	109.9±14.8[a]	75.2±7.1[a]	82.8±7.4[a]	63.3±8.0[a]
ALH (μm)	5.9±0.8[a]	8.1±0.8[a]	7.2±0.9[a]	3.8±0.9[a]	4.4±0.9[a]	4.7±1.0[a]

[a,b] Means with different superscripts in the row are significantly between breed ($P<0.05$) MS-CASA: Total motility, VAP (μm/s): Velocity average path, LIN (%): Linearity (VSL divided by VCL), VCL (μm/s): Velocity curved line, VSL (μm/s): Velocity straight line, ALH (μm): Amplitude of lateral head displacement

Table 3 Least square means (LSM)±standard error of the mean (SEM) of sperm parameter post-thaw (PT) in semen collected from boar. Samples examined for sperm motility were examined immediately T_0 and after a thermal resistance test (T_{60}). (n = 41; number of ejaculation)

Parameter	T_0	T_{60}
MS-CASA	13.1±1.9[a]	9.3±1.9[a]
VAP	59.7±2.9[a]	38.8±2.9[b]
VSL	35.2±1.9[a]	29.6±1.9[b]
VCL	113.3±6.3[a]	74.3±6.3[b]
ALH	7.0±0.5[a]	4.3±0.5[b]
LIN	44.1±3.2[a]	48.4±3.2[a]

[a,b] Means with different superscripts in the row are significantly between breed ($P<0.05$)

3.1.5 Discussion

From the chapter II, we found that the seasons and boar breeds influenced the sperm production. In this chapter described the post-thaw quality of FT boar semen from different breeds and evaluated its quality using CASA. The present study described the post-thaw quality of FT boar semen from different breeds and evaluated its quality using computerized assessed sperm. CASA motility was assessed by computer program to detect the size and movement of spermatozoa which could reduce the variations of sperm motility estimation by evaluators and the morphology parameters of the same or different ejaculates assessed by different observers. It is suggested that the system offers an accurate and rapid calculation of different sperm parameters, such as total and progressive motility, slow, medium and rapid moving sperm, linearity of sperm movement, the beat cross frequency, the amplitude of the lateral head displacement, and several velocity parameters (Rijsselaere et al., 2005). The CASA system is also indicative of sperm capacitation or hyperactivation.

The thermal resistance tests were used to depict the ability of spermatozoa to sustain incubation at temperatures close to the female body temperature with the assumption that they would describe the vitality of the spermatozoa (Koonjaenak et al., 2007). It appears that spermatozoa change motility, becoming less linear and progressively less vigorous, a process known as a "hyperactivated movement" (Mortimer et al., 1995). Hyperactivated motility occurs in parallel with the attainment of the capacitated state in the female reproductive tract (Yanagimachi, 1970). Kaul et al. (2001) studied capacitation in buffalo and bull spermatozoa and indicated that the percentage of spermatozoa that exhibit capacitated characteristics increases following incubation, which is in agreement with the present findings.

In pigs, AI by frozen semen is not popular due to a high sensitivity to cold shock (Holt, 2000). There are many factors influencing boar semen freezing namely breed, individual, ejaculate manipulation, extenders and freezing techniques (Eriksson et al., 2002). It was found that there are variations in sperm quality among boars and ejaculations in the same boar. Individuality seems to be main factor influencing ejaculated variability in sperm cryosurvival (Barbas and Mascarenhas, 2009).

In present study we found that sperm motility varied among the breeds and there was higher percentage of motility in Yorkshire than in Duroc and Landrace breeds. Most parameters, except LIN, significantly decreased after the thermal resistance test. According to results of Kaeoket et al. (2008) it was found that there is no significance in

breed difference among Landrace, Pietrain, Duroc and Yorkshire. This might come from the male-to-male differences in sperm cryo-tolerance after freezing and thawing. Although the motility (MS-CASA) of Duroc is increased in T_{60} when compared with T_0, it has also composition in different breeds of boars which can explain the major difference in post-thaw survival and fertility breeds (Waterhouse et al., 2006). In addition, Jame et al. (1999) reported the composition of sperm plasma membrane presents highly specific lipid composition, which has a very high level of phospholipids, sterol, saturated and PUFA. The composition of the sperm membrane or the factors influencing post-thawed boar motility spermatozoa among breeds boars spermatozoa and seminal plasma should remain to be investigated in the future. Saravia et al. (2007) revealed a significant difference among breeds in the size of morphologically normal spermatozoa, which were significantly larger and more elliptic in the Duroc breed. In all species, differences among individuals seem to be of genetic origin and it has been suggested that there are differences in specific DNA sequences identified among boars in which thawed semen quality was classified poor or good (Thurston et al., 2001). An increase in LIN has been previously observed when motility was assessed in fresh semen and then later after undergoing freeze-thaw procedures.

Pen˜a et al. (2003) suggested that capacitation-like changes occur in sperm motility after freeze-thaw procedures. Thawed sperm is characterized by an increase in LIN and a decrease in VCL and ALH. However VSL and VAP do not increase after freeze-thawed procedures.

In conclusion, some motion characteristics of FT boar semen i.e., VAP, VCL and ALH significantly decreased after an hour post-thawing, while the LIN remained unchanged. Conventional microscopic methods for sperm evaluation in combination with CASA have allowed us to obtain precise information about sperm quality in pigs.

3.2 Experiment 2 A study on the effect of semen freezability and the reproductive performance after insemination with fresh and frozen-thawed boar semen

3.2.1 Abstract

The aims of the study were to the effect of semen freezability and to investigate the influence of 50% heterologous seminal plasma (HSP) in the thawing medium on frozen-thawed (FT) semen and fertility data after intra-uterine insemination (IUI) with heterospemic frozen-thawed (HFT) boar semen in field. One hundred-fifteen boars including purebred (Birkshire, N=4; Duroc, N=26) and crossbred (Landrace x Yorkshire, LY, N=53; Pietrain x Duroc, PD, N=29) were included in the study. Boars were classified as 3 groups by aging, as 1 yr, >1-2 yr and > 2 yr. Semen was frozen and evaluated to post-thawed motility(%), viability(%) and concentration ($x10^6$ sperm/ml). The classification of boar (good or poor freezability) was performed by thawing FT semen with and without presenting 50% HSP and evaluated by computer-assisted sperm analysis (CASA). For insemination, eight straws from good freezabiltiy were thawed (to achieve at least 2.0×10^9 motile sperm) and diluted with 20 ml of BTS extender. Eighty-six sows were divided in two groups by control (N=43) and treatment (N=43). All sows in treatment group were induced estrus by PG600® injection and inseminated via intra-uterine deposition compared to those sows at the same lot which were inseminated with fresh semen intracervically served as control. The data of farrowing rate (FR), return rate, sow death/culling, total piglet born (TB) and born alive (BA) were collected. The result showed that the percentage of subjective motility of crossbred was higher significant difference than purebred ($P<0.05$). In addition, post thawed motility in boar, age >2 yr, in crossbred (LY,PD) was higher significant difference ($P<0.05$) while Duroc was lower significant difference ($P<0.05$). For thawing, of ten from fifty-three boars were classified as good freezability. In addition, the parameters of good freezability boar were significant difference in motility, MS-CASA, VAP, VSL, LIN and viability ($P<0.05$) between BTS and 50%HSP groups. However, the beneficial effect of seminal plasma did not present in our study, the sperm agglutination and semen precipitations have been found evidently in presenting 50%HSP group. Moreover, the control and treatment groups were no significant difference in FR (81.4 vs 69.7), TB (9.63±5.46 vs 7.23±5.88) and BA (8.49±5.13 vs 6.72±5.56) ($P>0.05$), respectively. In summary, FT semen insemination is possible and has merit for application as a reproductive management tool in pig genetic industry scale. The classification of boar to preserve their genetic with good freezability semen should be done to present an acceptable fertility data.

3.2.2 Introduction

Artificial insemination in pig was longtime developed for male genetic propagation and disease transmission limitation. Moreover the swine genetic industry currently relies on well established breeding techniques for preserving their own genetic as long as possible. Most of them, the use the liquid chilled semen diluted in short term semen extenders which can not be preserved longtime. Generally, a boar can be used for insemination within 2-3 years after mature and later culling. This will limit the acceleration of genetic improvement by superior boar (Bailey et al., 2008). The frozen-thawed semen (FT) then has more advantage to keep for long-term genetic banking and can be easily exported to elsewhere (Hofmo and Grevle, 2000; Bailey et al., 2008). Nevertheless, the post-thawed semen quality was one of the critical factors for using frozen semen for in situ fertilization. Roca et al. (2006) found that boar was the most important factor explaining the variability among ejaculates in sperm survival in cryopreservation. As reported from Buranaamnuay et al. (2012) found a large variation of boar semen freezability. Then a good selection of boar possessing a freezability resistance confirms the success of insemination by frozen semen. Not only boar semen freezability in order to improve post-thawed motility can be done by using various thawing media such as seminal plasma is one of choices (Okazaki et al., 2009). According to the study in autologous or heterologous supernatant from freezing process as thawing solution during thawing process which had a beneficial effect on post-thawed progressive motility of frozen boar semen (Kaeoket et al., 2011). Hormone is one of the technique to stimulate gilts and sows presenting the estrus. In this study, Due to Tummaruk et al. (2011) have reported about follicular cyst when using exogenous gonadotropin releasing hormone but it did not found this evident in human chorionic gonadotrophin, in our study, we select to use PG600® in order to stimulate some sows turning to estrus within 4-5 day after weaning. PG600® which is a combination of two hormones, eCG 400 i.u. and hCG 200 i.u. is a hormone usually used for estrus induction in gilts and sows by inducing follicle growth, estrus and ovulation. Although the hormone has been used both intramuscular and subcutaneous administration, Knox et al., (2000) has been demonstrated that subcutaneous administration of PG600® is more effective than intramuscular administration. The combination of this hormone injection could induce >90% of prepubertal gilts to express estrus within 3 to 7 days, with high percentages conceiving (80%) and farrowing rate (76%).

The objective of this present study was to study the freezability of boar semen related to breeds and age of boar, to improve the frozen semen quality using heterologous seminal plasma as a diluter and to evaluate the sow fertility related to the effect of boar breed using heterospermic-FT semen from which were classified as good freezability boar under field condition.

3.2.3 Materials and methods

3.2.3.1 Boar selection for semen freezability

A total of 115 boars of purebred free PRRS; Birkshire (N=4), Duroc (N=26) and crossbred Landrace x Yorkshire (n=53) and Pietrain x Duroc (N=29) were included in the study. Boars were classified to 3 groups by aging, 1 yr, >1-2 yr and > 2 yr, were selected from a commercial boar stud where is located in Chonburi province, eastern part of Thailand. The boars were routinely served for insemination in three grand parent farms possessing 10,000 sows a year. They are fed twice a day with a commercial feed, 2.2-3.0 kg/day, according to the boar's body condition and raised in an individual pen (9 m^2 per boar). Water is given *ad libitum* via individual nipple.

3.2.3.2 Semen collection, cryopreservation and 50% seminal plasma processing

Sperm-rich fraction was collected from every boar only one ejaculate. Fresh semen with a minimum of 70% sperm motility and normal characteristic sperm was used for freezing. The process of semen freezing was followed as mentioned in 3.1.3.1. The semen was later diluted with isothermal Beltsville thawing solution (BTS; Minitüb, Abfüll- und Labortechnik GmbH & Co. KG, Germany) extender at a ratio of 1:1 (v/v). The diluted semen was kept at 15°C for 2 hrs. and centrifuged at 800 g for 10 min. After centrifugation, diluted semen was separated into 2 parts

- Part 1: the supernatant, recentrifuged twice at 1,500 g for 10 min and kept at -20°C for further use as post-thawing solution as 50% heterologous seminal plasma (50%HSP)
- Part 2: the pellet, resuspended with lactose-egg yolk (LEY) extender (11% lactose solution and 20% egg yolk). After further cooling at 5°C for 90 min per time, both were mixed with one part of extender III (LEY extender and 9% glycerol with 1.5% Equex STM® paste; Nova Chemical Sales Inc., Scituate, MA, USA).

The frozen sperm at a approximately final concentration was $1x10^9$ sperm/ml with 3% of glycerol and filled in 0.5 ml straws. The straws were sealed with PV powder at the open end of 0.5 ml straws before being placed in liquid nitrogen (LN_2) vapor at 4 cm (-120°C) above the level of LN_2 for 15 min. Then, these were plunged into LN_2 (–196°C). The semen from boars possessing a high survival after post-thawing was selected for semen donors. The semen was thawed by immersing the straws in 50°C of water for 12 sec. After thawing, the semen is divided in two parts. The first part was diluted (1:4) with BTS while the other was diluted (1:4) with 50% HSP. Both of the extended-thawed semen samples were incubated in a 38°C water-bath for 10 min.

The semen of boars were classified as good freezability when the motility of FT semen was greater than 35% while that of poor freezability when the motility of FT semen was lower than 35% (Chanapiwat et al., 2009). Then, the frozen-thawed semen from selected boars in same breed classified as good freezability was prepared in each batch of insemination in order to produce the heterospermic frozen-thawed semen (HFT). Thawing was performed immediately by immersing all straws at 50°C of water for 12 sec. The final volume (approximately 25 ml) (Buranaammnuay et al., 2010) and final total number of sperm (approximately 2 $x10^9$ sperm/dose) consisted of semen with warm BTS after thawing. The extended-thawed semen was incubated in a 38°C water-bath for 10-15 min. Then, before insemination process, the post-thaw subjective motility was evaluated.

3.2.3.3 Sperm evaluation

Volume and concentration

A volume was measured by using digital weight. Fresh semen concentration was evaluated manually by using by Spermacue® (Minitube, Tiefenbach, Germany). On the contrary, FT semen diluted to 1:100 with 5% formaldehyde was evaluated by using haemocytometer chamber.

Viability

The percentage of viable sperm from small drop of fresh semen was determined separately with mixing by eosin-nigrosin dyes (Fluka Chemie GmbH, Sigma-Aldrich, Switzerland). Evaluation was undertaken by using a bright-field microscope at 1000X magnification with oil immersion and 200 sperm being examined for each smear. Spermatozoa with an unstained head were regarded as live spermatozoa.

Subjective motility assessment and Computer-assisted sperm analysis (CASA)

The percentage of motile spermatozoa was evaluated by two assessments. The first assessment is light microscopy to evaluate both fresh and FT semen and the second is CASA to assess FT semen only with and without presenting of seminal plasma. The protocol of CASA assessment was followed as described in experiment 1.

3.2.3.4 Sow preparation

The frozen semen were tested by insemination of sows in a commercial pig farm, located in Eastern part of Thailand during June - August 2012. Forty three purebred free PRRS sows (25 Landrace; L and 18 Yorkshire; Y) from parity of 1st to 6th with weaning-to-estrus interval 4-6 days were selected for insemination. The weaned sows were stimulated by vasectomized-boar contact and were fed with a commercial diet according to nutritional requirements and water was given *ad libitum*. An S/C injection of 400 IU of PMSG and 200 i.u. of eCG (PG600® Intervet ®, The Netherlands) was given on the next day after weaning (Day1). .

The estrous sows were intra-uterine inseminated twice with HFT semen (Landrace; n=25, Yorkshire; n=18) at 12 and 24 h after standing heat. The semen and the sows are the same breed. The data consisted of farrowing rate (FR), return rate, sow death/culling, total piglet born (TB) and born alive (BA) were collected and compared to those sows at the same lot which were inseminated intracervically served as control.

3.2.3.5 Statistical analysis

The data was analyzed using the Statistical Analysis Software (SAS; version 9.0 Institute Inc., Cary, NC, USA). Descriptive statistic in each breed and all data were used to describe semen quality before freezing and after thawing in each ejaculate. Pearson's correlation was used to evaluate the correlation among sperm parameters. The general linear model procedure (GLM) was used to calculate as general linear mixed model procedure (MIXED). The model included breed, age and boar's id as fix effect. Mean percentages were calculated for each sperm quality and summarized by semen parameters. For fertility data, TB and BA were presented as mean ± standard deviation (SD) while FR was presented as percentage. The statistical model was included the effect of sow breed (L vs Y). The unpair *t*-test was used to determine differences between fertility parameters of intracervical and intrauterine insemination in same breed with HFT. The differences with $P<0.05$ were considered statistically significant.

3.2.4 Results

3.2.5.1 A study of age and line-breed on post-thawed motility

On average (mean±SD), descriptive data of fresh and frozen-thawed semen of one hundred and twelve ejaculates were presented in Table 4. The summary of each parameters were evaluated between fresh vs frozen thawed semen; concentration (392.05±141.41 vs 773.63±310.27 ($x10^6$ sperm/ejaculate)), percentage of subjective motility (74.90±6.06 vs 25.59±12.93) and percentage of sperm viability (81.00±7.20 vs 81.00±7.20), respectively.

The percentage of subjective motility of crossbred was significant different higher than purebred ($P<0.05$) whereas there were no significant different in concentration and the percentage of viability ($P>0.05$) (Table 5). The average percentage of the FT semen motility in the different age range was presented in Table 6. There was the significant different ($P<0.05$) in higher post thawed motility in boar group showing age range more than 2 years in crossbred LY and crossbred PD while the lower percentage of frozen-thawed motility in Duroc was significant different lower ($P<0.05$)

Table 4 Descriptive statistical analysis presenting as Mean ± SD of semen parameters of fresh and frozen-thawed boar semen with different breed

Parameter	Breed of boars				Sum
	Purebred		Crossbred		
	Berkshire	Duroc	LY	PD	
Number of boars	4	26	53	29	112
Age (months)	23.5±6.95	16.65±6.89	20.75±9.43	17.66±5.65	18.88±7.88
Fresh semen					
Volume (ml)	218±94.28	142.50±59.31	282.18±101.24	225.97±77.68	226.43±100.56
Concentration (x10^6 spz/ml)	379.50±51.20	441.23±93.04	320.60±67.06	448.24±77.08	392.05±141.41
Motility (%)	70.0±8.16	76.92±7.88	75.13±5.13	73.45±4.45	74.90±6.06
Viability (%)	78.75±4.99	81.85±6.58	84.28±6.28	73.45±4.45	81.00±7.20
Frozen semen					
Concentration (x10^6 spz/ml)	641.00±26.87	859.61±333.98	649.36±189.69	810.11±351.26	773.63±310.27
Motility (%)	20.00±14.85	20.42±14.44	29.32±11.78	27.50±11.43	25.59±12.93
Viability (%)	20.50±14.85	26.54±14.27	32.05±13.37	36.64±10.59	31.70±13.30

(LY: Landrace x Yorkshire; PD: Pietrain x Duroc)

Table 5 Mean ± SD of FT semen parameters of purebred and crossbred

[a] letter in each row indicates significant different among line-breed in same parameter (P<0.05)

Parameter	Purebred (N=30)	Crossbred (N=82)
Concentration (x10^6spz/ml)	859.61±333.98	605.10±225.13
Motility (%)	20.42±14.44	27.50±9.38[a]
Viabiltiy (%)	26.54±14.27	28.90±13.05

Table 6 Mean ± SD of age associated to motility of FT boar semen with different breed

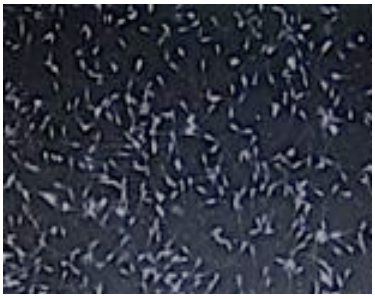
[a] letter in each column indicates significant different among age range in same column (P<0.05)

Age of boar	Breed			
	Birkshire	Duroc	LY	PD
< 1 yr (N=24)	ND	24.91±5.55	28.10±6.02	23.86±5.05
1-2 yrs (N=50)	ND	21.57±3.53	30.62±4.99	26.58±3.09
> 2 yrs (N=25)	20.15±8.83	17.38±7.29[a]	32.12±2.31[a]	33.22±7.79[a]

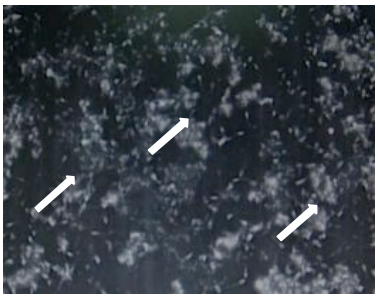
ND = No data in this study

3.2.5.2 Effect of 50% HSP as thawing media on post thawed motility

FT semen with or without presenting of 50% HSP (supernatant after centrifugation step in cryopreservation process) was depicted in Fig 7ab. We found the obvious precipitation as showed in Fig 7b on all of frames when using 50% HSP as semen diluant. The parameters of post-thawed boar semen motility and viability categorized as good or poor freezability by were presented in Table 7. The parameters of good freezability boar were significant difference in motility, MS-CASA, VAP, VSL, LIN and viability (P<0.05) between BTS and 50%HSP groups, while the parameters of poor freezability boar were only significant difference in ALH and viability (P<0.05).



a) without 50 %HSP



b) with 50%HSP

Figure 7ab Sperm with or without presenting of 50% heterologous seminal plasma (HSP) as supernatant from cryopreservation step. Only sperm with presenting of 50%HSP showed the precipitation like as protein (white arrow).

Table 7 Mean ± SD of semen parameters of two groups of semen freezability of FT boar semen with or without presenting of 50% heterogous seminal plasma (HSP)

Parameter	Group1; Good (N=10) (Landrace, n=4; Yorkshire, n=6)		Group2; Poor (N=43) (Landrace, n=20; Yorkshire, n=23)	
	BTS	50%HSP	BTS	50%HSP
Motility (%)	36.88±10.94[a]	21.44±8.12	20.00±12.19	18.73±11.33
MS-CASA (%)	43.75±6.71[a]	24.5±8.99	11.25±8.57	13.14±2.85
VAP (μm/s)	51.48±15.97[a]	42.83±10.26	49.61±15.29	47.12±31.93
VSL (μm/s)	42.08±11.87[a]	31.36±8.41	39.96±10.79	40.22±27.57
VCL (μm/s)	80.59±30.27	75.24±39.98	64.50±27.64	65.64±43.00
LIN (%)	61.75±11.75[a]	44.73±17.10	50.43±12.14	45.36±17.21
ALH (μm)	6.51±1.06	6.39±1.77	6.33±1.53[b]	5.11±2.45
Viabilit y (%)	38.69±11.79[a]	28.19±13.49	24.34±12.02[b]	19.34±12.02

[ab] Different letters in each group indicate significant different between thawing solution in same group ($P<0.05$)

According to MS-CASA from Table 7, the result showed only ten boars (4 Landrace boars and 6 Yorkshire boars) which were included for semen collection for freezing and insemination further.

3.2.5.3 Heterospermic FT semen and i*n vivo* fertility

The fertility data was presented in Table 8 and Table 9. Of forty-three of the fresh semen group, 23 Landrace sows and 18 Yorshire sows were inseminated twenty four hours interval, while all sows in IUI group were inseminated twelve hours interval. Although the trend of result will decrease in group of using heterospermic FT semen, there were no significant difference in FR,TB and BA ($P>0.05$) (Table 8). In addition, FR in Landrace was no significant difference ($P>0.05$) while Yorkshire showed the significant difference ($P<0.05$) (Table 9).

Table 8 Fertility data of fresh and heterospermic FT semen with presenting of BTS as thawing solution by intracervical and intrauterine insemination, respectively

[a] letter in each group indicate significant different between group in same breed ($P<0.05$)

Parameter	Fresh semen	Heterospermic FT semen
No. of sows	43	43
Parity average (mean±SD)	3.77±1.23	3.90±1.61
Farrowing rate (FR; %)	81.40	69.77
Total piglets born/litter (TB; mean±SD)	9.63±5.46	7.23±5.88
Piglets born alive/litter (BA; mean±SD)	8.49±5.13	6.72±5.56

Table 9 Fertility data (Mean±SD) by breed with fresh semen by intra-cervical insemination and heterospermic FT semen (HFT) with presenting of BTS as thawing solution by intrauterine insemination

[a] Letter in each group indicate significant different between group in same breed ($P<0.05$)

Parameter	Landrace		Yorkshire	
	Fresh semen (IC)	HFT semen (IUI)	Fresh semen (IC)	HFT semen (IUI)
No. of sows	25	25	18	18
Parity average (mean±SD)	3.60±1.66	3.77±1.23	3.28±1.23	3.56±1.67
Farrowing rate (%)	84.00±7.48	80.00±8.16	77.78±10.08	55.56±12.05[a]
Total piglets born/litter (mean±SD)	10.52±5.4	7.72±4.92	8.39±5.44	6.56±7.10
Piglets born alive/litter (mean±SD)	9.68±5.00	7.17±4.81	6.83±4.97	6.11±6.57

3.2.5 Discussion

Unlike in cattle, frozen semen in boar is not so popular due to a low viability after freezing and thawing and low piglet born after insemination. However, recently with a small number of sows inseminated, Buranaumnuay et al. (2008, 2010) reported that around 9 to 10 piglets born alive per litter after intra-uterine insemination (IUI) using FT boar semen. Then, this study aimed to apply FT semen in a commercial pig industry by semen collection and freezing from a boar stud possessing a big number of boars served for grand parent stock. Our study showed the first point that the selection of boars by evaluating the semen quality after freezing are neccessary to discrimininate a good or poor freezability semen in order to get the optimal fertility which relates to reproductive performance in genetic pig farm.

3.2.5.1 A study of age and line-breed on post-thawed motility

The semen freezability is the primary importance to improve the efficiency of utilization of frozen-thawed boar semen for pig AI, particularly for commercial use. One of factor againsts a more extensive use of FT boar semen is the inherent low freezability that spermatozoa from many boars show (Thurston et al., 2002). Some boars produce spermatozoa with low quality of structure (plasma membrane composition, metabolic capacity, etc.) that are insufficiently developed or no good characteristics that make spermatozoa sensitive to cryopreservation (Saravia et al., 2008). It was reported that there is an individual boar effect on semen freezing (Hofmo and Grevle, 2000; Roca et al., 2006; Buranaamnuay et al., 2009). Differences in freezability that is most likely universal, as males from other species than the boar also show such variation, often in relation to the relative phospholipid composition of the plasma membrane of their spermatozoa (Buhr et al., 1994). Moreover, Roca et al. (2006) also reported that the semen collection and transportation variable were not useful to predict the good post-thawed motility. In addition, increasing sperm concentration, together with the greatest proportions of sperm with normal morphology and motility before freezing, did not necessarily guarantee that good sperm could be survival after freezing and thawing. As we refered to result from chapter II, the sperm production of the boars differed among the breeds and seasonal influence (Tretipskul et al, 2012). On the other hand, each ejaculate with low sperm concentration and moderate sperm quality immediately after collection or before freezing could have good sperm quality post thaw (Roca et al., 2006).

The significant influence of breed on post-thawed sperm motility but there is likely to be considerable variability among ejaculates within breed (Roca et al., 2006). Purebred Duroc showed decreasing post-thawed motility turn around with age increasing which according to Joyal et al. (1986). In addition, the effect of boar's age on sperm output was seen in many studies (Jankeviciute and Zilinskas, 2002; Marchev et al., 2003). The reverse corresponding between age and post-thawed motility might relate to fresh semen production (Kennedy et al., 1984). Normally , semen production increased rapidly in the first two years of age until 3.5 years old (Smital, 2009; Huang et al., 2010). Nevertheless, we found a higher post-thawed motility of crossbred LY and PD during age at more than 2 years-old when collection their semen than purebred Bershire and Duroc which might related to the effect of heterosis (Joyal et al., 1986). Wolf and Smital, (2009) has reported that semen traits are heritable traits with heritabilities between 0.06 and 0.24. To get the good quality of FT semen even purebred or

crossbred, frequency of collection should be reminded due to most studies has been agreed that with the increasing frequency of collections semen volume and sperm concentration and thereby total sperm output decrease (Frangež et al., 2005; Pruneda et al., 2005). As boar individually was found to be the primary factor explaining variability in sperm cryosurvival among boar even in same breed, it might be effect from different ejaculate in same boar also. From this study, we can conclude that mature boar aged >2 yrs and and line-breed effect on post-thawed motility in frozen-thawed boar semen motility.

3.2.5.2 Effect of 50% HSP as thawing media on post thawed motility

To order to improve the FT sperm viability, seminal plasma was added in boar semen extender in the experiement. Seminal plasma is the combination of liquid composition during ejaculate which was secreted by the male accessory sex glands (i.e., mainly the seminal vesicle). In addition, more than 90% of seminal plasma proteins are in the spermadhesin family, which may be heparin-binding or non heparin-binding (PSP-I and PSP-II). These seminal plasma protein will coat the sperm and act to among another things to stablize the acrosome (Caballero et al., 2008). PSP-I/PSP-II is 50% of the total seminal plasma proteins which play a major role in the modulation of uterine immune activity by preventing the possible infections of the lower genital tract and providing the optimal uterine environment for the early embryo attachment (Rodrigurez-Martinez, 2005; Chanapiwat et al., 2012). It was reported that a thawing semen with a mixture of semen extender and seminal plasma improve sperm motility in many species such as ram; by protein adsoption onto capacitated spermatozoa suface to revert to a decapacitated state (Barrios et al, 2000) and dog; by postponing the acrosome reaction by prostatic fluid (Rota et al., 2007). However, in boar, a removal of seminal plasma improve motility of FT semen and *in vitro* fertility, moreover seminal plasma protect sperm against a spontaneous capacitation-like reaction during the thawing process (García Herreros et al., 2005).

The effect of seminal plasma on frozen semen quality is controversial. According to Okazaki et al. (2009) revealed that the exposure to 10% (v/v) seminal plasma before cooling and freezing process seriously damaged sperm from poor freezability boar but it can maintain the oocyte penetration activity in *in vitro* fertilization following the thawing process, while Gacia et al., (2010) used the various concentration 10, 20 and 50% (v/v) of seminal plasma and found a significantly higher percentage of progressive motility. Not only the concentration but also source of seminal plasma has been discussed for a

while to the positive benefit to use as thawing solution (Okazaki et al.; 2009; Garcia et al., 2010; Kaeoket et al., 2011). Nevertheless, the beneficial effect of seminal plasma as supernatant from cryopreservation step did not present in our study. The significant difference was obviously observed following to these parameters; MS-CASA, VAP, VSL, LIN and viability in boar as good freezability. On the other hand, boars as poor freezability did not found obviously positive effect of presenting 50%HSP. In our study, the sperm agglutination and semen precipitations have been found evidently in presenting 50%HSP group. These effects might be caused by the reaction among egg yolk and some kinds of protein (heparin-binding and non-heparin-binding) (Caballero et al., 2008). Recently, Yeste et al., (2013) reported that poor freezability boar ejaculate was less resistance than good freezability boar ejaculate to cryopreservation not only in term of motility but also in the integrity of nucleoprotein structure. Lastly, a dilution in normal BTS is more preferable in our test for in vivo fertiltiy.

3.2.5.3 Heterospermic FT semen and *in vivo* fertility

As we discussed in 3.2.4.1, boar semen cryopreservation quality has been affected by several factors. Approximately seventy percentage of total variance among ejaculates in post thaw semen quality was explained by individual boar variability. The assumption for boar selection should be the most important criterion for selecting ejaculates for cryopreservation (Roca et al., 2006). To our knowledge, the insemination by using FT semen in farm where is the industry scale has not been reported in Thailand. The application of FT semen is useful to improve the genetic outcome for the great grand parents (GGP) or grand parents (GP) level. In this study, we studied about the reproductive performance of HFT semen from good boar freezability which was refered to result from Table 3.6. Although overall result from control and treatment groups was not significant difference in TB (9.63±5.46 vs 7.23±5.88) and BA (8.49±5.13 vs 6.72±5.56), respectively, the group inseminated with HFT tended to have fewer piglets per litter than the group inseminated with control group. As basic knowledge, the optimal time for insemination and the longitivity of sperm in female reproductive tract is most important to get the achievement fertilization (Vazquez et al., 2005).

Recently, Didion et al. (2013) has been reported the fertility data with FT semen insemination by high percentage of FR 78.7% and TB were 12.5±3.9. Although, our result was lower than Didion et al. (2013), it might be caused by the frequency of insemination per estrous by using three times with twelve hours interval per estrus while we used only two times per estrus. In spite of the interval between insemination and

ovulation is necessary to get the achievement of fertilization, the interval between first and second time of insemination might effect on TB due to the life span of FT semen is quite low about 8 hr (Matthijs et al., 2003; Mezalira et al., 2005). However, Garcia et al. (2007) studied to the relation of sperm numbers and time of insemination on sow fertility was not different due to single insemination of fewer sperm may compromise sow fertility. In addition, Casas et al., (2010) revealed that the number of pregnant and farrowing sows in intracervical insemination by FT semen from good boar freezabiltiy did not significantly differ from fresh semen. However, the probabilities of pregnancy were absolutely significant difference lower after inseminations with FT semen from poor freezabiltiy compared to FT semen from good freezabiltiy.

The use of hormone can enhance the fertility data by shorten gap of ovulation time. Wongkaweewit et al., (2012) studied to use of hormone by gonadotropin releasing hormone to shorten time of ovulation after estrus detection but there were some follicular cysts development. However, the administration in reproductive tract has been still administrated to improve their own positive result either FT semen insemination (Knox et al., 2000; Chanapiwat et al., 2012; De rensis et al., 2012; Ringwelski et al., 2013). In vivo testing, the hormonal administration was induced ovulation only FT semen insemination group as package program due to the comparison between treatment and normally routine estrus induction in farm.

A lower farrowing rate and lower piglet born were found in Yorkshire group than Landrace group in this study. The fertility by breed with Landrace and Yorkshire has been reported by Huang et al. (2003) that the reproductive data of Landrace and Yorkshire were 10.77±0.08 vs 10.73±0.06, repectively. Although our result was lower than Huang et al. (2003), it might be presumed by many factors which were concerned the success of FT semen for insemination; insemination technique (Roca et al., 2003; Bathgate et al., 2008[ab]), insemination dose, an induction of ovulation (De Rensis et al., 2012; Wongkaweewit et al., 2012). According to, Bathgate et al. (2008[a]) reported the field fertility with low doses of sperm ($150x10^6$ sperm/ml) with deep intrauterine insemination could be used but the uterine bleeding caused by insertion of the inner tube should be reminded.

Future research is needed to increase number of TB. Time of insemination with closely to ovulation with FT semen should be the first authority factor to considering. In this study, we used fixed time AI insemination with PG600® administration for insemination to sows showing standing heat during 4-5 days after weaning. In addition, hormonal using with ultrasonography can coordinately perform to control ovulation time

should be the next study for improve our result. A report has noted that 12.4 hr after IUI using FT semen, spermatozoa can be found in all parts of reproductive tracts of sows according to using of fresh semen insemination (Chanapiwat et al., 2012). Moreover, a major goal of extensive use of FT semen by the pig industry is to design a freezing procedure that allows the use of a unique AI dose with a high sperm concentration and, hopefully, a low volume that would help easy storage and handling under field conditions.

CHAPTER IV

BOAR SEMEN SEXING USING PERCOLL GRADIENT AND *IN VIVO* FERTILITY TESTING AFTER INSEMINATION

4.1 Abstract

Sperm sexing technology is useful for sex pre-selection of offspring by allowing for the production of male and female crossbred lines as desire of domestic animal producers. The objective of this study aimed to study the possibility to use discontinuous percoll-gradient centrifugation to sex boar spermatozoa and test by *in vivo* fertilization. To evaluate the separation efficiency of discontinuous percoll-gradient centrifugation for sperm sexing, semen samples from 15 ejaculates of 5 boars were studied. Eight layers of percoll-gradient concentration was used to separate semen as 90, 80, 75, 60, 55, 45, 30 and 20%, respectively. The fresh semen was placed on the top and was centrifuged. Before (fresh semen) and after centrifugation (upper part, 45% and 55%; lower part, 80% and 90%) were collected into each aliquot; one, unprocessed initially semen while the other two aliquots, upper and lower part, respectively. All samples were extracted DNA then processed by modified quantitative PCR to calculate the percentage of X- and Y- bearing spermatozoa with standard curve. Two sets of primers were designed on specific AMELX and Y-chromosome (SRY) genes with SYBR green. The percentage of difference in each X- and Y- spermatozoa population was compared to initial fresh semen. *In vivo* testing, two boars were included in this study. The control group (unsex semen) was processed as normal routine semen preparation. While the treatment group (sexed semen) was processed by discontinuous percoll gradients centrifugation. Seventy-eight sows were inseminated by IUI as control (N=39) with unsex semen and treatment groups (N=39) with the lower part of semen identified as X dominance. The 21-day conception rate, farrowing rate, total born and sex ratio of total born (female:male) were collected. The result of the difference of percentage of X- and Y- bearing spermatozoa population showed significant different ($P<0.05$) in lower part which was compared to each individual fresh semen. Unfortunately, the result for fertility data did not relate to the significant difference all of the parameters ($P>0.05$), however, the sex ratio of total born (female:male) in treatment seems higher than control (1.09 vs 0.93), respectively. In conclusion, we found that the significant difference in percentage of X- spermatozoa populations can be enhanced by discontinuous percoll-gradient centrifugation, nevertheless, the effect of sex ratio from in vivo fertilization did not relate closely to the insemination with the increasing of the difference of X- or Y- spermatozoa populations.

4.2 Introduction

A sex predetermination in livestock production is in great demand such as dairy cattle and pig productions (Johnson, 2000). The benefit from sex pre-selection was distingly if it was economically available by allowing for the production of male and/or female crossbred lines (Johnson et al., 2005). The ideal application of pig producers is the use of sex-sorting technology in nucleus herds, particularly for producing female lines. If pig producers focus on gilts' market, the male pigs produced are ''by-products" (Vazquez et al., 2009). The sex predetermination can be done before fertilization by semen sexing or after fertilization by embryo sexing (Seidel, 2007). There are several methods for sex pre-selection before fertilization, for example, flow cytometry, swim-up technique (Yan et al., 2006) and discontinuous percoll-gradient centrifugation (Kobayashi et al., 2004). The successful method is based on the difference in X- and Y-sperm in the amount of DNA presenting after processing. The separation of X- and Y-bearing spermatozoa can be accomplished on the basis of physical differences such as detected by flow cytometry, or by detection of chemical differences in membrane characteristics. These difference between of X- and Y- bearing spermatozoa is including the size, density, motility, surface protein and DNA content (Gledhill, 1988; Gledhill and Edwards, 1993). The techniques should satisfy three criterias , a significant shift in the proportion of the Y- and X-bearing sperm population, no interference on fertilization and a producing pregnancy (Jafar and Flint, 1996). The first criterion may be satisfied by analysis of the populations by flow cytometry; the second and third criteria require artificial insemination or *in vitro* fertilization and embryo transfer. Although, flow cytometry is being applied in livestock, zoo animals and humans with a success rate of 90-95% in shifting the sex ratio of offsprings (Johnson et al., 2005) and the efficiency of the sorting procedure has increased with development of improved nozzle designs and adaptation to high speed cell sorting, the usefulness remains limited in pig industry (Vazquez et al., 2009). While, percoll solution consists if polyvinylpyrrolidone (PVP) coated silica beads and it is used to purify the cells with widely used for a selection of mammalian spermatozoa (Avery et al., 1995). According to Kobayashi et al. (2004) showed the using of discontinuous percoll gradient separation in bull semen could sort the X- and Y-bearing spermatozoa which the bottom part was significantly different in X-bearing spermatozoa. Moreover, Suzuki and Nagai (2003) revealed that percoll-separated spermatozoa had increased the higher percentage of motile and progressively motile spermatozoa than those that were not separated. The use of sexed

semen in domestic animals depends on the purity and accuracy of X and Y sperm cells. The method to identify the selection accuracy is by producing offspring, but in some domestic industry is expensive and time-consuming (Joerg et al., 2004). Thus, validation techniques such as embryo sexing (Joerg et al., 2001), re-analysing sorted sperm for DNA content (Welch and Johnson, 1999) or the fluorescence in situ hybridization (Rens et al., 2001) have been established. Quantitative real-time polymerase chain reaction (PCR) has been developed to detect the amount of DNA or RNA from the sample. This method has a very large dynamic range of starting target molecule determination. A SYBR green is used to quantify the relative amount of DNA content in the rest of semen after sexed by discontinuous percoll-gradient centrifugation.

The amelogenin (AMEL) gene has been conserved during the vertebral evolution for example in bear (Yamamoto et al., 1992), sheep (Pfeiffer and Brenig, 2005) and human (Salido et al., 1992). Likewise, SRY gene is the conserve region which was located on the chromosome Y presenting as male phenotype.

Artificial reproductive technology can also enhance the livestock production by producing the genetic and increasing amount of offsprings per one time insemination (Johnson, 2000). By the semen sexing which is desirable to implement in field can improve the genetic and reduce time consuming during gestation period epecially the dairy industry. Pig production would benefit from sex pre-selection if it was economically available by allowing for the production of male and female crossbred lines (Johnson et al., 2005). The ideal application of pig producers is the use of sex-sorting technology in nucleus herds, particularly for producing female lines. If pig producers focus on gilts' market, the male pigs produced are ‘‘by-products” (Vazquez et al., 2009). Controlling the sex of offspring prior to conception permits the livestock industry to produce the desirable sex to take the advantage to sex limited, economically flexible management for the producer and permits faster genetic process, higher productivity, improves animals welfare by decreasing obstetric difficulties in cattle, avoiding castration in pigs, and producing less environmental impact due to the elimination of unwanted sex before they grow up to adulthood (Rath and Johnson, 2008). The use of sexed semen in domestic animals depends on the purity and accuracy of sperm cells. The exact method to identify the selection accuracy is by producing offspring, but in some domestic industry is expensive and time-consuming (Joerg et al., 2004). This fact has made it economically impractical for the pig artificial insemination industry to sex boar sperm in commercial pig production. If we look for general usage at the farm level, we have to try the easy, cheap and convenient method to extend the lifespan and sort boar

spermatozoa for shifting to the desirable sex. Besides, cryopreservation combined with a sex selection, male or female dominance will be interested for preserving desired genetics. Freezing and processing protocols in combination with sex-sorted sperm. However, they are not yet optimal for commercial application in swine, as in other mammalian species (Johnson et al., 2005; Bailey et al., 2008).

The objective of the present study was to investigate the possibility to use discontinuous percoll-gradient centrifugation to sex boar spermatozoa and evaluate by in vivo fertility under field condition.

4.3 Materials and methods

4.3.1 Animals

Boars and sows

Boars was carried out at a boar station in the eastern part of Thailand during the period of May – June 2011. Semen collection from 15 ejaculates of 5 LY crossbred boars (three ejaculates per boar) were included in this study. All boars were trained and classified as proven sire for fertility data in routinely used for insemination to the breeding farms.

For in vivo testing, the experiment was performed in a commercial pig farm where located in middle part of Thailand. Two mature crossbred Duroc and Pietrain boars, aged 1-3 years old which were routinely used for AI in commercial herds, were selected as representative in this experiment by following as good semen production. According to its good semen quality (Motility > 70%), boars' semen was collected for the further investigation. While, seventy-eight crossbred sows (Landrace × Yorkshire) from parity of 1^{st} to 6^{th} with body condition 2.5-3 and weaning-to-estrus interval 4-6 days were selected to be 2 groups by randomization. The weaned sows will be moved to gestation unit after weaning-to-estrus to stimulate the estrus cycle with vasectomized-boar contact and were fed with a commercial diet. All animals included in experiment which were PRRS-free stauts can access to water intake *ad libitum* via nipple.

4.3.2 Semen collection and evaluation

The ejaculates were collected as described in chapter II, briefly, using the gloved-hand method and filtered gel fraction out by gauze. After collection, semen was proceeded to laboratory immediately for evaluation. The gel free semen parameters including volume (ml) measured by weight, sperm concentration ($x10^6$ spz/ml)

measured by Spermacue®, viability measured by eosin-nigrosin staining and subjective motility was evaluated as described in chapter II. Fifty micromilitres was collected in one aliquot as a representative of pre-sex ratio of fresh semen for real-time PCR analysis before separation.

For *in vivo* testing, semen was diluted with the commercial extender and also divided into two groups and kept in dark fridge at 15°C as equal volume for the first and second insemination (24 hours interval). Each group of semen was also divided for the control and treatment group for the first and second insemination.

4.3.3 Discontinuous percoll-gradient and semen preparation

Percoll-gradient stock solution (Solution A) are made by mixing 9 parts of absolute Percoll® (Pharmacia Fine Chemicals, Uppsala, Sweden) with 1 part of 10X commercial boar semen extender (pH 7.2), while the Solution B contains 10% albumin (v/v) in 1X of the same extender. Each percoll-gradient working solution was prepared to these concentrations by mixing ratio of the solution A and solution B; 20%, 30%, 45%, 55%, 60%, 75%, 80% and 90% percoll-gradient solutions, respectively. Each four millilitres of the Percoll-gradient solutions is consecutively layered following by ascending concentration in a 50 ml centrifuge tube . Fifteen milliliters of sperm pellets which were performed by centrifugation fresh semen at 800 g for 10 min are laid on top of percoll-gradients. Then, they are centrifuged at 2200 g for 20 min by refrigerated-centrifuge at 25 °C, after which the top layers containing few sperm are aspirated. Two sperm fractions, the upper part (concentration 45% and 55%) and lower part (concentration 80% and 90%) are collected from the tubes (Kobayashi et al., 2004). Each sperm fraction is resuspended in the same previous extender (10 ml) and centrifuged at 1,000 g for 10 min to remove the residual Percoll. Each aliquot of separated sperm (upper and lower part) is divided to 2 aliquots. One aliquot containing 50 microlitres is collected to real-time PCR procedure to analyse sex ratio after seperation. The rest of upper and lower part were performed the semen evaluation technique as described above (concentration, motility and viability). For in vivo testing, only sperm fraction from lower part, the pellet of sexed sperm was prepared by diluting with the same extender to reach to final total sperm per dose approximately 1,500 sperm in volume 30 ml.

4.3.4 DNA extraction

Blood collection from each male and female piglet as a positive control was extracted DNA by commercial kits (QIAGEN®). Each part of semen samples were extracted DNA by Azupure® modified by exception reagent 1 and adding 2X buffer, Dithiothreitol (DTT) then overnight at 56°C in waterbath before extraction step. After the extraction step, all of DNA samples were determined by measuring the light absorption of the DNA using a photospectrometer ((Nanodrop 2000® ; (ng/µl)) and stored at -20°C.

4.3.5 Primer design

Two pairs of primer were recognized as Y-specific primers to detect Y chromosome and amelogenin (*AMEL*) gene on the X chromosome *(AMELX)*. The Y-specific primers were designed according to oligonucleotide sequences described by Rubeˇs et al. (1999) and Parrilla et al. (2003). PCR amplification resulted in products of 377 bp and 234 bp for Y-specific fragments and X-specific on AMEL gene, respectively. The sequence for Y chromosome (X12696) is 3832 nucleotides in length whereas the sequence for AMELX is 7425 bp (AB091791). The X-specific primers were designed and modified from Sembon et al. (2008). Both of primers were verified by DNA sequencing and rechecked by PCR to certainly specification with blood DNA as positive control described above. Oligonucleotide sequences are as follows:

AMELX	Forward: 5´- TCA GGT TCG TTT GCA CTG AG -3´
	Reverse: 5´- GTC GTT GTC TGT TCC CTG GT -3´
Chromosome Y	Forward: 5´-AAT CCA CCA TAC CTC ATG GAC C-3´
	Reverse: 5´-TTT CTC CTG TAT CCT CCT GC-3´

4.3.6 Quantitative real-time PCR

The primers were designed to be close on the determination logus gene. In the real-time PCR reaction, SYBR green was selected as the quencher dye, FAM as the reporter dye and ROX as the reference dye. Real-time PCR was undergone in a thermal cycler ABI7300 (Applied Biosystems, Foster City, CA, USA). The PCR was carried out in a reaction volume of 10 µl containing 5 ng of genomic DNA, 10 µM of both primers, master mix with KAPA. The standard assays were performed using the thermal cycling parameters: after initial steps of 2 min at 95°C, the PCR profile consisted of a denaturation step at 95°C for 3 s, and an annealing at 60°C for 20 s, elongation at 72°C for 30 s, for a total of 40 cycles.

4.3.7 Insemination

Each of the sows about 6-8 weaned sows in weekly continuous system was divided in two groups as control and treatment groups in order to inseminate by using unsexed and sexed semen. Both of the groups was performed by IUI procedure. After bestriding the AI buddy around on the flank of sows to face with boar, the IUI device (Magapore®, Spain) was used by inserting through the vagina into cervix. The inner tube was gently pushed through the outer catheter and situated presumably in uterine body. A tube contained a predetermined number of sperm about 1.5×10^9 sperm/dose, 30 ml, was connected to the open tip of inner tube of IUI device. All sows were fixed-time inseminated at 12 and 24 h. interval. The data consisted of parity, twenty days conception rate (21d-CR), farrowing rate (FR), abortion and total piglet born (TB); number of male and female piglets were collected.

4.3.8 Statistical analysis

Semen parameters between before and after centrifugation were analyzed by ANOVA of SAS version 9.0. Each spermatozoa population data were analysed using general linear mixed model procedure (MIXED). The models included the different percentage as fixed effect and included boar identification within replication as random adjusted by Tukey-Kramer test. Means and the different value proportion of each spermatozoa population were obtained and compared to baseline within replication by using pair-*t* test. The fertility data was analyzed by using student's *t*-test. $P<0.05$ was regarded as a significant difference.

4.4 Results

The result of sperm assessment between before and after centrifugation was presented in Table 10. The decreasing in all of semen parameters was significant difference from upper part after centrifugation ($P<0.05$) while motility in lower part was still not significant different ($P>0.05$) between before and after centrifugation.

Table 10 Mean ± SD of semen evaluation between before and after percoll-gradient centrifugation

[abc] Letter in each column indicate significant different when compare to each other ($P<0.05$)

Parameter	Before centrifugation	After centrifugation	
	Fresh semen	Upper part	Lower part
Motility (%)	72.0±4.93[a]	18.33±9.39[b]	78.10±12.79[a]
Viability (%)	72.0±1.73[b]	50.4±14.07[c]	85.8±6.75[a]
Concentration ($x10^6$ sperm/ml)	313.07±88.61[b]	64.40±38.12[c]	187.13±85.64[a]

The specific PCR product and DNA sequencing of AMELX were conserved and specificity to porcine genome as 98.97% (Accession number: AB091791.1). The triplicate running was shown same results as Fig 8. The PCR product of SRY (377 bp) and AMELX (234 bp) from blood and semen were demonstrated.

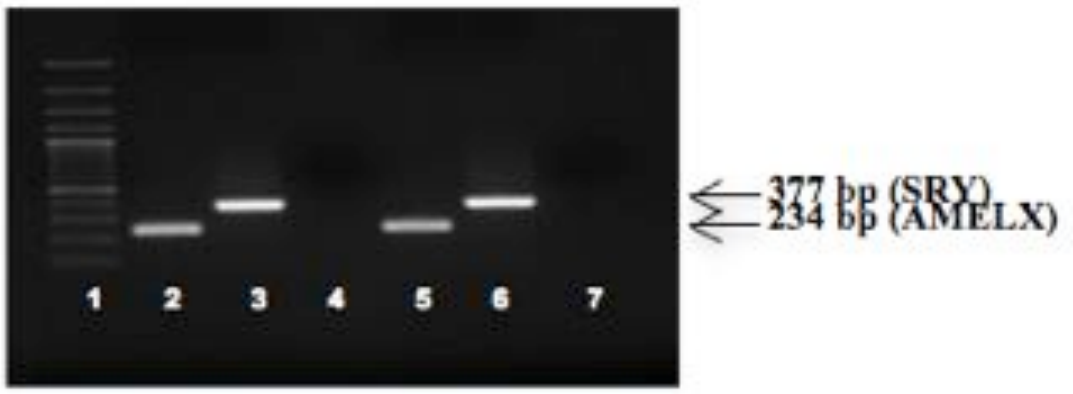


Figure 8 Gel electrophoresis of both primers. (Lane 1: ladder 100bp plus; Lane2,3: blood samples from boar; Lane 5,6: semen samples as same as individual boar's blood; Lane 4,7: negative control)

The summary data of three replicates was described and depicted in Fig 9. Each coloring bar (Mean ± S.E.) was compared to baseline (fresh semen fraction) presenting the percentage of difference X or Y spermatozoa population of each boar. The significant difference of the percentage difference of X and Y spermatozoa population in upper part and lower part were observed in all of replications ($P<0.05$).

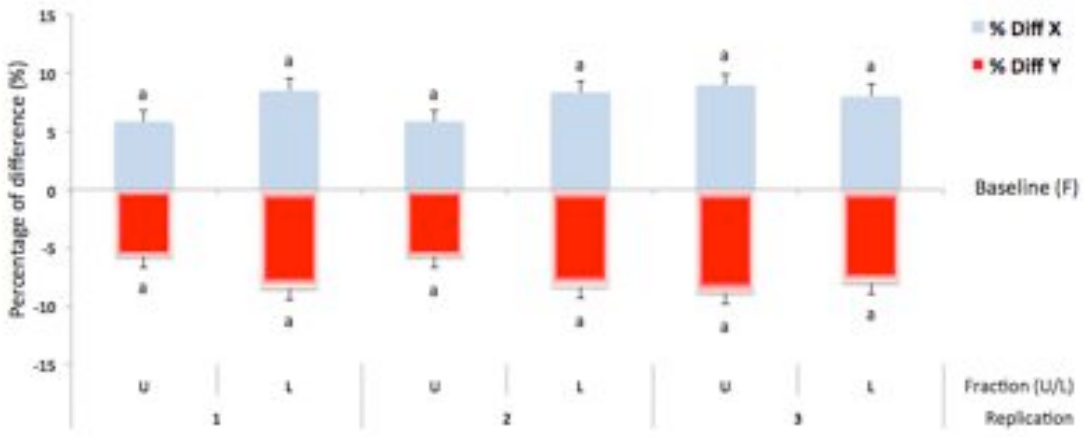


Figure 9 The overall of percentage of difference of X or Y spermatozoa population in each replication comparing to fresh semen as baseline
(F = baseline; fresh semen, U = upper part after percoll-gradient centrifugation,
L = lower part after percoll-gradient centrifugation)
(%Diff X = each percentage of X in each part - each percentage of X in fresh semen;
% Diff Y = each percentage of Y in each part - each percentage of Y in fresh semen)
[a] Letter in each *coloring bar* indicate significant different when compare to baseline ($P<0.05$)

The lower part of semen from two boars which were selected for *in vivo* testing showed more percentage of X- spermatozoa population than the fresh semen to 5 percentage after testing by quantitative PCR. The farrowing data was presented in Table 11. Although there was no significant difference in all of the fertility data in this study, the sex ratio of offspring (Female:Male) in control and treatment groups was 0.93 vs 1.09, respectively. It seems to get higher sex ratio of female in treatment group.

Table 11 Fertility data of least square mean ± standard error of control (unsex semen) and treatment (sexed semen) with intrauterine insemination

[a] Different letter indicates the significant difference at $P<0.05$

Parameter	Control	Treatment
No. of sows	39	39
Parity	4.00 ± 0.20	3.72 ± 0.20
Abortion	0	1
21d-CR (%)	100 (39/39)	97.4 (38/39)
Farrowing rate (%)	100 (39/39)	94.9 (37/39)
Total born (TB)	13.43 ± 0.47	13.11 ± 0.48
Sex ratio of total born	0.93	1.09
(Female:Male) (heads)	(6.50:6.93)	(6.83:6.28)

4.5 Discussion

In this study, the percentage of difference of X and Y spermatozoa population can be separated successfully by percoll-gradient centrifugation combined with a modified quantitative PCR. Our result was slightly difference in the amount of X- and Y-bearing spermatozoa. We found that the lower part of separated boar semen had some different of the percentage of X- and Y- bearing spermatozoa when processing through many layers of discontinuous gradient by showing the higher different in X spermatozoa population when compared to fresh part of semen before centrifugation. These might be resulted from the amount of DNA of X- chromosome whose is more than Y- chromosome approximately 4 percentage and the different of Percoll concentration which we put sequentially on can act as the different vicous substance to obstruct the sperm when they were moving down by centrifugation.

Besides the increasing of population in X spermatozoa was observed also in upper part about 5-10% but presenting the low motility while motility of lower part was still presenting as good. Generally, after centrifugation by Percoll can improve the semen quality (Machado et al., 2009). This situation might be explained by the properties of polyvinylpyrrolidone (PVP) coated silica beads that can purify the cells with for a selection of dead and alive mammalian spermatozoa (Avery et al., 1995). In addition, spermatozoa can be detached their acrosome by the different density of

different concentration of Percoll solution. When Percoll density gradient were prepared in discontinuous fractions, sperm layered on the top naturally penetrate it. The amount of penetration depends on sperm mass and motility (Wolf et al., 2008). However, when a gradient is centrifuged, the effect of sperm motility is minimize and their mass difference effect become maximixed. This makes the heavier spermatozoa reach to the bottom faster. According to Suzuki and Nagai (2003) reported that there was significant difference between spermatozoa separated by Percoll in motility and progressive motility when compared to un separated boar spermatozoa, however, the large difference is the sperm which high percentage motility did not always show high in vitro fertility in their study from two of the four boars.

Normally, when male and female gametes unite randomly to combine with sex chromosome matching, as a consequence the expected primary ratio, defined as the proportion of male at conception, is also expected to be 1:1 with a substantial variance coresponding to binomial distribution (Toro et al., 2006). It is believed that separation occurs as result of X- and Y- bearing spermatozoa density difference. For the in vivo testing, the result showed the increasing of sex ratio to produce female to 1.09 when compared to control as 0.93, about 0.16 unit different, which was related to the higher number of X- bearing spermatozoa population in sexed semen. Not only the higher of female ratio was pleasing but also this technique was practicable in farm. In human, the study from Iizuka et al. (1987) reported that discontinuous percoll-gradient could be used clinically for female sex preselection by 12-step discontinuous percoll-gradient (25-80%) which showed 6 girls born from 6 pregnancies. According to Wang et al. (1994), their study was proven the slight enrichment of X-bearing spermatozoa in the 80% of Percoll fraction (55:41) of 12-step percoll-gradient by using double label fluorescence in situ hybridization (FISH) when compared to neat semen. However, the limited of Percoll-gradient using in this study have to take more time to prepare the number of layers per tube. Normally, it should be easy to prepare. Morrell and Wallgren (2011) have developed the single layer centrifugation with Androcoll-P which is one of the colloid like as Percoll to reduce time for separation per tube. Not only substance can be put all of the semen volume into only one tube for separation but also sex spermatozoa by increasing X-bearing spermatozoa as 10 percent increasing (personal communication).

According to Machado et al. (2009) whose studied in the effect of Percoll volume, duration and force of centrifugation on sex ratio of bovine spermatozoa that this can increase the motile sperm but it did not sigificantly affect the sex ratio from an

expected 1:1 ratio. they found that the decreasing Percoll volume, reducing duration of centrifugation and using a higher force of centrifugation did not significantly affect the sperm quality, embryo development of in vitro-produced bovine embryos, however, the sex ratio of semen after centrifugation with 4 ml of Percoll at 700g for 20 minutes showed a greater percentage of male embryos when compare to another recipe in their experiment.

However, in our pilot study, we have tried to adjust the volume and force of centrifugation for long time in order to produce the good motility and living spermatozoa after centrifugation. Firstly, the centrifugation by ten layers, at 2500 g for 10 sec made the non-vialble sperm. Then, we found that the duration and concentration especially force of centrifugation should be concerned and related to sperm viability. These might be assumed that the volume and force of centrifugation or the optimal percoll preparation can effect on the sex ratio with different animal species. However, we did not inseminate the upper part of semen after centrifugation eventhough it has showed significant different increasing X-spermatozoa populations. The pattern of separation of the sperm samples by percoll-gradient revealed that most motile spermatozoa concentrate in the layer with high density, whereas immature and damaged and dead cells remain in the top layer (Aitken and West, 1990). We described that the motility of upper part did not be as good motility to insemination.

For conclusion, discontinuous percoll-gradient centrifugation can enrich the X-bearing spermatozoa population and show the positive relation to fertility result in increasing female piglets ratio. To get more different between X - and Y- spermatozoa population, not only force and the percoll volume should be reminded. However, the positive relation did not different so much between *in vitro* and *in vivo*. These might be caused by some factors about seminal plasma with presenting and absence during the sperm transportation in female reproductive tract, is absent between before and after centrifugation due to the seminal plasma contains both estrogenic and non-estrogenic factors that interact with spermatozoa and the surrounding female environment (Rodríguez-Martínez et al., 2005). Another factor is the speed of sperm penetrating Percoll gradient. Heavier spermatozoa should settle down faster than lighter spermatozoa, therefore centrifugation time could positively influence X-bearing spermatozoa moving down the gradient.

CHAPTER V

GENERAL DISCUSSION AND CONCLUSIONS

World pig production has increased markedly due to the dramatical increasing of human population. It is estimated that the meat consumption will increase and South East Asia (SEA) will be one of the important areas of world pork consumption. It is accepted that Thailand is one of the leaders with a pool source of genetic mainly from Europe but already adapted in tropical environment, good management and good biosecurity. AI plays role of genetic improvement and disease prevention in swine herd in the country. With our thesis, we showed the potential of semen production from PRRS free boar in one commercial stud which is the main provider of semen in Grand parent stock. Moreover, it is found that season can relate to the quantity and quality of semen production in crossbred and purebred boar eventhough they were kept in EVAP. While the limitation of boar usage due to overweight, low libido and aging, semen can be collected in certain period and then boar will be culled. A preservation of semen in form of frozen will be useful for genetic distribution, import, export to remote area and genetic conservation. In the thesis, frozen semen was implemented in a commercial swine herd. A semen bank from a group of high genetic boar has been established. The field fertility was tested by insemination with heterospermic frozen-thawed semen from same genetic line. The result showed the possibility to cryopreserve a high genetic boar through an intensive selection semen donor and a moderate reproductive performance. Finally, in the thesis, the semen sexing by using percoll-gradient separation and quantitative PCR technique were elaborated. The X- or Y- spermatozoa predominant part was able to be separated in vitro. Sperm will well motile after separation, however, the result after insemination by showing higher of female sex ratio in treatment group did not significantly different related to that of *in vitro*. It is concluded from the last part of thesis that this technique is not feasible to produce desired sex of piglets as much more seen in *in vitro*. More studies should be paid attention, with a sex pre-selection has still the advantages in the commercial pig competition since the pig producers can produce the desired sex of piglets to get more benefit.

For conclusion, the results of all trials indicated that the best boar sperm production, boar semen cryopreservation and boar semen sexing could be important to swine industry production especially genetic producers. An update reproductive biotechnology is quite the most important in order to outstanding performance. Taking all of them together, these take more advantages and are useful for the conservation and/or production of animals with high genetic merits.

1. Factor influencing boar semen production

The sperm production was affected by many factors such as breed, environment, temperature, humidity (Kunavongkrit et al., 2005; Suriyasomboon et al., 2006; Smital and Fiedler, 2008; Smital, 2010). Boars consistently producing high-quality ejaculates are crucial in AI program. Due to the limited number of insemination doses, that can be produced from one ejaculate (Ciereszko et al., 2000). The European wild pig is a seasonal breeder and does not mate during the summer and fall months (Mauget and Boissin, 1987). For this reason, the lowest reproductive performance of commercial swine is observed in summer (Xue et al., 1994). In general, the first indication of abnormal sperm production is observed after the onset of high temperature and motile sperm do not return to a normal ration until 5 weeks due to spermatogenesis (Yang et al. 2010). As a breed, it was found that crossbred LY produced a higher volume and total sperm per ejaculate than other breeds while Duroc has presented the highest sperm concentration (Park and Yi, 2002). In addition, purebred boars were rather sentitive to the fluctuation of temperature which effect on sperm production (Sonderman and Luebbe, 2008). The values of the heterotic effect for the number of total spermatozoa were generally lower in comparison with semen volume (Smital and Fiedler, 2008). Thailand is located in the tropical area where has the fluctuation of temperature about 10°C between day and night time (Suriyasomboon et al., 2004) while the photoperiod is different only ± 1 hour among seasons. Many other factors may modify semen characteristics of the boar, including high ambient temperature leading to heat stress (McNitt and First, 1970), frequency of semen collection (Strzezek et al., 1995) and age (Kennedy and Wilkins, 1984). Not only breed but also season can effect on sperm production. In this study, rainy season showed significant different in sperm production than other season which may relate to high humidity. However, the sperm production become normal in winter. One of thing which has related between semen quality and fertility was Acrosin. This activity, sperm-specific acrosomal proteinase, is an essential role in the fertilization process. Due to low levels of acrosin appear to be associated with subfertility and infertility in human (Kenedy et al., 1989), and the acrosin activity of spermatozoa may potentially be a useful indicator of semen quality. Ciereszko et al., (2000) reported that the seasonal variation can effect on acrosin activity but it does not affect by breed. However, the fertility data should be investigated if there is a critical effect on sperm production in order to find out the main factors in the next four months. For conclusion, breed and season can effect on sperm produciton even though all boars are kept in EVAP in Thailand.

2. Boar semen freezability and in vivo fertility

The aims of genetic conservation are to long time preserve genetic variation to keep specific genetics of interest by preservation of male (sperm) or female (oocytes) genetic characterization including embryos (Porcue, 1999; Benson et al., 2012; Mara et al., 2013). Sperm cryopreservation is not commonly used in swine farm as in cattle due to a different production system. Using frozen semen for dairy cattle has been done since the 1950s (Xu et al., 2009). Artificial insemination industry provides breeding products and services for both dairy and beef cattle. Normally in dairy cattle, no one raises the bull in the farm. Most of them use AI by frozen semen and the annual genetic will grade up. Semen will be collected in the AI Bull Center, and distributed for many countries or worldwide (Saragusty et al. 2009). While in pig, boar is normally raised in the farm. Semen can be collected and short term preserved in farm in refrigerator. Then, due to easiness to obtain boar semen, the genetic is not so different among farms, this is why frozen semen is not popular. However, in swine genetic breeder, this is different situation, good boar can be used only 1-2 yr maximum and culled due to aging and low libido. Maximum semen production can be achieved once a week with 5 days interval. This might lose opportunity to distribute good genetics from top boars to many sows. On the other hand, in bovine industry, semen from the top bulls have been preserving from time to time. Therefore the bovine genetics improvement is quite higher rate more than in swine industry.

Boar selection in order to be good semen donor is the key factor of frozen semen development since there were some variations in sperm quality among boars and ejaculations in the same boar. Some boars have good semen quality but presenting the poor quality after freezing-thawing (Roca et al., 2006). Individuality is one of criterias to get the good result of FT semen motility and this is the main factor influencing ejaculated variability in sperm cryosurvival (Barbas and Mascarenhas, 2009; Buranaamnuay et al., 2010). In addition, the percentage of motility is usually concerned in order to classify which semen is good or poor. By the truth, the semen evaluation was analyzed by visual examination depending on inspectors, and this is no accuracy or no precision. Using computerized systemic evaluation by CASA is more accuracy and presenting the pattern of motility of spermatozoa. The CASA motility is selected in this study to reduce any biases or variations from inspectors to assess by computer program to detect the motility and movement of sperm (Rijsselaere et al., 2005). Meanwhile, the thermal resistance tests were used to depict the ability of spermatozoa to sustain incubation at temperatures close to the female body temperature with the

assumption that they would describe the vitality of the spermatozoa (Koonjaenak et al., 2007). In pigs, AI by FT semen did not get the good result due to a high sensitivity to cold shock when compared to fresh semen insemination (Holt, 2000). There are many factors influencing boar semen freezing such as breed, individuality, ejaculate, manipulation, type of extenders and freezing techniques (Eriksson et al., 2000; Roca et al., 2006). In our study, we found that FT semen motility varied among the breeds. Most parameters, except LIN from experiment 1, significantly decreased after the thermal resistance test. According to results of Kaeoket et al. (2008) it was found that there is no significant difference in breed among Landrace, Pietrain, Duroc and Yorkshire. However, in the experiment 3.1, this study was conducted to the application of ART by FT semen and insemination technique to apply on genetic pig producer industry in Thailand where have been never done it before. In this study, we have some criterias for selection few boars representing as good freezability depend on the good motility and motility pattern presenting after thawing with or without presenting of 50% HSP by the CASA system. According to Okazaki et al. (2009) revealed that the exposure to 10% (v/v) seminal plasma before cooling and freezing process seriously damaged sperm from poor freezability boar but it can maintain the oocyte penetration activity in *in vitro* fertilization following the thawing process, while Garcia et al, (2010) used the various concentration 10, 20 and 50% (v/v) of seminal plasma and found a significantly higher percentage of progressive motility. Individuality seems to be main factor influencing ejaculated variability in sperm cryosurvival (Tretipskul et al., 2010). For the fertility data, high genetically sows were inseminated by FT semen from boars whose good freezability if this relates to TB and BA in the same breed in filed. Although TB and BA were not different, the decreasing trend of FT semen group with IUI was obviously found in this study. In the meanwhile, the FR in Yorkshire was rather low in FT semen group, the possible reason for explaining included the management or some mistakes occurred during the gestation period. Due to this study used the mixing of seminal plasma from many boars, in this case, differences in seminal plasma composition between good and poor freezability will be mixed. These might be such a factor to reduce in post-thawed motility. Not only insemination technique (Roca et al., 2003; Bathgate et al., 2008[ab]) but also estrus induction with hormone (Wongkaweewit et al., 2011; De Resis et al., 2012) can be applied for the reduction of sperm dose and increasing the conception rate due to the optimal time for insemination. However, Bathgate et al. (2008[a]) reported the field fertility with low doses of sperm ($150x10^6$ sperm/ml) with deep intrauterine insemination could be used but the uterine bleeding caused by insertion of the inner tube should be

reminded. In spite of the interval between insemination and ovulation is necessary to get the achievement of fertilization, the interval between first and second time of insemination in our study might effect on TB due to the life span of FT semen is quite low about 8 hr (Mezalira et al., 2003; Buranaamnuay et al., 2010). Besides backflow is the major mechanism of sperm elimination from oviduct, Matthijs et al. (2003) reported that 70% of semen volume could be flushed out during first hour of insemination using intracervical insemination (Hernández-Caravaca et al., 2012).

In the program of boar FT, it is then recommended firstly to select and test for freezability of boar semen, heat detection and interval of time insemination to ovulation should be concerned. The key new finding in successfully high conception rate is that the possible organizational hormone effects on female swine can influence their reproduction (Drickamer et al, 1997). As insemination at/close to the time of ovulation is one of the key roles for achieving high fertility and ovulation time among individuals is variable, control of ovulation time is one of key success which can be manipulated by gonadotropin treatment (Knox, 2000). Implementation of this technique for long-term semen preservation and transfer in swine industry would provide a foundation for effective utilization of the world's most valuable genetic resources on a global basis. It is now time for breeders and producers to adapt pig genetic cryopreservation and transfer into swine production for propagating select and maintain genetics resources for the future (Gerrits et al., 2005).

3. Boar semen sexing using percoll-gradient and *in vivo* fertility testing after insemination

In human, one of assumptions that has been used to justify enrichment of X- and Y-bearing spermatozoa using different preparation procedures is that Y-bearing spermatozoa swim faster than X-bearing spermatozoa and have a greater ability to penetrate viscous solutions and the interfaces between viscous solutions (Flaherty and Matthews, 1996). Nowadays, It was well-known that the best technology is based on the difference in X- and Y- sperm in DNA content whichis about 4% difference of DNA content for X- chromosome more than Y- chromosome (Johnson and Welch, 1999). Flow cytometry is the best technique for semen sexing of mammal sorting with 90% accuracy (Johnson et al., 2005). Since the number of sexed sperm produced per unit time is limited (Seidel, 2003; Seidel, 2007), the technology has not been applied in pig due to the high sperm numbers needed for optimum fertility in the female (Suh et al., 2005). Moreover, the sperm survival after sorting is so poor too insemination to get good reproductive performance by normal AI. This fact has made it economically impractical for the pig artificial insemination in industry to sex boar sperm in commercial pig production. If we look for general usage at the farm level, we have to try the easy, cheap and convenient method to extend the lifespan and sort boar spermatozoa for shifting to the desirable sex. In this study, we adapted the discontinuous percoll-gradient technique to sex boar semen by centrifugation through the different concentrations of this substance. After that, we developed a quantitative PCR to detect the percentage of X and Y spermatozoa population by using the AMELX (Sembon et al., 2008) and SRY gene (Parrilla et al., 2003), respectively. Inseminations in sow herd were performed in order to investigate the sex ratio of piglets after insemination with semen separated by discontinuous percoll-gradient centrifugation. According to our result, we found a significant difference of the percentage of difference of X-spermatozoa populations in lower part from boars in three replications. Therefore, we assumed that if the insemination was performed by lower part of semen to sows, these might be effect on sex ratio of offspring. From our study, we did not inseminate the upper part of semen after centrifugation eventhough in has showed significant different increasing X-spermatozoa populations after analyzing by quantitative PCR. We described that the motility of upper part did not be as good motility to insemination.

Although there were some reports about colloidal centrifugation can enrich the X-bearing spermatozoa populations in in vitro (Flaherty and Matthews, 1996; Kobayashi et al., 2004; Wolf et al., 2008; Morrell et al., 2009), we agreed and found that it can shift

the sperm populations from the baseline of starting point. However the result in our study showed the increasing but no significance of sex ratio to produce female to 1.09 when compared to control as 0.93, about 0.16 unit different, which was related to the higher number of X-bearing spermatozoa population in sexed semen after centrifugation. However, we concluded that discontinuous percoll density gradient centrifugation did not show significant difference on the sex ratio of piglets in this study. In addition, the mechanism of enrichment of X-bearing spermatozoa by discontinuous percoll-gradient is not fully understood (Kobayashi *et al*., 2004). It is believed that separation occurs as a result of X- and Y-bearing spermatozoa density difference. Sperm layered on the top gradient will move down in to the bottom. The amount of sperm depends on mass and motility. However, when a gradient is centrifuged, the effect of sperm motility is minimized and their mass difference effect is maximized (Wolf et al., 2008). It makes the heavier spermatozoa reach the bottom faster; however, the separation threshold between X- and Y-bearing boar sperm is not much due to DNA content is approximately difference about 4% (Johnson, 2000). However, to get more different between X - and Y-spermatozoa population, not only force and the percoll volume should be reminded. The use of larger volume gradients in order to make the movement of sperm more difficult could be an alternative (McEvoy, 1992). Another factor is the speed of sperm penetrating percoll gradient. Heavier spermatozoa should settle down faster than lighter spermatozoa, therefore centrifugation time could positively influence X-bearing spermatozoa moving down the gradient.

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APPENDIX

LIST OF PUBLICATIONS AND PROCEEDINGS

Publications

1. Tretipskul, C., Amin, N., Tummaruk, P. and Techakumphu, M. 2012. Season and breed effects on sperm production in PRRS free boars. Thai J Vet Med. 42(3):267-273.
2. Tretipskul, C., Buranaamnuay, K., Koonjaenak, S., Tummaruk, P. and Techakumphu, M. 2010. The use of computer-assisted sperm analysis for discriminating a series of motility pattern of frozen-thawed boar semen. Thai J Vet Med. 40(1): 15-20.
3. Rienprayoon, C., Klangnak, C., Onton, S., Tretipskul, C. and Tummaruk, P. 2012. A comparative study on the efficacy of four semen extenders and thawing by seminal plasma on the quality of frozen- thawed boar semen. Thai J Vet Med. 42(2): 195-200.

Proceedings

1. Tretipskul, C., Theerawatanasirikul, S., Tummaruk, P. and Techakumphu, M. 2012. A preliminary study: PCR application to detect X and Y population from boar semen in commercial pig farms. The 22nd International Pig Veterinary Society, Jeju, South Korea.
2. Tretipskul, C., Theerawatanasirikul, S., Tummaruk, P. and Techakumphul, M. 2011. Validating percentage of sex in boar semen samples using quantitative PCR. The 1st Joint Symposium of Thai and Japanese Societies for Animal Reproduction, Bangkok, Thailand.
3. Tretipskul, C., Promthep, K., SaiKhun, K., Tummaruk, P. and Techakumphu, M. 2010. Boar semen qualtiies after Percoll-Gradient Separation and Cryopreservation. RGJ: Ph.D. Congress XI, Cholburi, Thailand.
4. Tretipskul, C., Tummaruk, P., Koonjaenak, S. and Techakumphu, M. 2010. Boar semen qualtiies after Percoll-Gradient Separation and Cryopreservation. RGJ Seminar Series LXXI "Perspectives and Innovation in Veterinary Biosciences", Bangkok, Thailand.
5. Tretipskul, C., Tummaruk, P., Koonjaenak, S. and Techakumphu, M. 2010. Sperm production in Duroc, Landrace, Yorkshire, Berkshire and Pietrain boars kept in evaporative cooling system in Thailand. The 21st International Pig Veterinary Society, Vancouver, Canada.

6. Tretipskul, C., Tummaruk, P., Koonjaenak, S. and Techakumphu, M. 2010. Seasonal influence on sperm production in boars kept in evaporative cooling system in Thailand. The 21st International Pig Veterinary Society, Vancouver, Canada.

7. Tretipskul, C., Tummaruk, P., Koonjaenak, S. and Techakumphu, M. 2010. Comparison of fresh and frozen-thawed semen qualities in purebred and crossbred boar kept in evaporative cooling system in Thailand. The 13th Association of Institutions for Tropical Veterinary Medicine, Bangkok, Thailand.

8. Tretipskul, C., Tummaruk, P., Koonjaenak, S. and Techakumphu, M. 2010. Thawing of cryopreserved boar semen with seminal plasma improve the post-thawed sperm quality. The 7th Asian Reproductive Biotechnology Society, Kuala Lumpur, Malaysia.

BIOGRAPHY

Mr.Chanyuth Tretipskul was born on February 17th, 1983 in Bangkok province, Thailand. He graduated with Degree of Doctor of Veterinary Medicine (DVM) (2^{nd} class honour) from the Faculty of Veterinary Science, Chulalongkorn University, in 2006. After he graduated, he has worked for veterinary service department in Pig business, Charoen Phokphand. In 2007, he recieved a scholarship from the Thailand Research Fund through the Royal Golden Jubilee (industrila linked) Ph.D. program (Grant No. 5.V.CU/50/A.2) to perform a Ph.D. program at the Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University.