# Single fixed-time artificial insemination with fresh or frozen thawed semen in pig

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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# การผสมเทียมแบบกำหนดเวลาครั้งเดียวโดยใช้น้ำเชื้อสดหรือน้ำเชื้อแช่แข็งในสุกร

นายพชระ เพียรอดวงษ์



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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การผสมเทียมแบบกำหนดระยะเวลาครั้งเดียวเป็นวิธีการผสมเทียมสุกรที่ทำการผสมเทียมเพียงหนึ่งครั้ง โดยใช้น้ำเชื้อสดหรือน้ำเชื้อแช่แข็ง ณ เวลาที่กำหนดหลังทำการเหนี่ยวนำการตกไข่โดยไม่อาศัยการตรวจสัด วิทยานิพนธ์นี้ศึกษาปัจจัยที่ส่งผลกระทบต่อประสิทธิภาพของการผสมเทียมแบบกำหนดระยะเวลาครั้งเดียวเพื่อพัฒนาการผสมเทียมแบบกำหนดระยะเวลาครั้งเดียวให้ประสบผลสำเร็จในประเทศไทย นอกจากนั้นยังทำการศึกษาการใช้เลซิทินเป็นส่วนผสมในสารละลายน้ำเชื้อเพื่อเพิ่มคุณภาพน้ำเชื้อแช่แข็งเพื่อนำไปใช้ในการผสมเทียมแบบกำหนดระยะเวลาครั้งเดียวด้วยน้ำเชื้อแช่แข็ง จากผลการทดลองพบว่าการฉีดบูเซอรีลิน ในปริมาณ 10 ไมโครกรัม ที่เวลา 72 ชั่วโมง หลังหย่านมในแม่สุกร สามารถกระตุ้นให้แม่สุกรหย่านมตกไข่เร็วขึ้นกว่าการตกไข่ตามธรรมชาติ (127.6 และ 157.2 ชั่วโมง) นอกจากนี้การตอบสนองต่อบูเซอรีลินยังขึ้นกับฤดูกาลและสภาพของแม่สุกรหย่านมด้วย ฤดูร้อนทำให้การตกไข่ช้าลง แม่สุกรหย่านมที่ 20 วันขึ้นไป หรือ มีน้ำหนักลูกหย่านมน้อยกว่า 67 กิโลกรัม หรือ มีคะแนนรูปร่าง 3 ขึ้นไป หรือ มีไขมันสันหลังสะสมสูงจะมีการตอบสนองต่อบูเซอรีลินที่ดี การผสมเทียมแบบกำหนดระยะเวลาครั้งเดียว ที่ 32 ชั่วโมง หลังการเหนี่ยวนำการตกไข่ด้วยการฉีดบูเซอรีลิน (10 ไมโครกรัม) ที่ 72 ชั่วโมง หลังหย่านมในแม่สุกรในเขตร้อนครั้งนี้ประสบผลสำเร็จและให้จำนวนลูกต่อครอกในจำนวนที่ยอมรับได้เทียบกับการผสมเทียมปกติ (12.0 และ 12.8 ตัว) แต่จำนวนลูกต่อครอกลดลงเมื่อการผสมเทียมแบบกำหนดระยะเวลาครั้งเดียวทำในฤดูร้อนหรือใช้การผสมเทียมโดยการปล่อยน้ำเชื้อที่มีปริมาณอสุจิลดลงสู่โพรงมดลูก นอกจากนี้ยังพบว่าคุณภาพของน้ำเชื้อแช่แข็งที่เก็บรักษาในสารละลายที่มี ไข่แดงร้อยละ 20 หรือ ในสารละลายที่มี เลซิทิน ร้อยละ 3 ผสมกับ ไข่แดง ร้อยละ 10 มีคุณภาพไม่ต่างกันและมีคุณภาพสูงกว่าน้ำเชื้อแช่แข็งที่เก็บรักษาในสารละลายที่มีเลซิทิน ร้อยละ 6 การศึกษาครั้งนี้จึงสรุปได้ว่าฤดูร้อนลดประสิทธิภาพการเหนี่ยวนำการตกไข่โดยบูเซอรีลินทำให้สุกรตกไข่ช้าลง ลดประสิทธิภาพทางระบบสืบพันธุ์ภายหลังการผสมเทียมแบบกำหนดระยะเวลาครั้งเดียว แม่สุกรหย่านมที่ 20 วันขึ้นไป หรือ มีน้ำหนักลูกหย่านมน้อยกว่า 67 กิโลกรัม หรือ มีคะแนนรูปร่าง 3 ขึ้นไป หรือ มีไขมันสันหลังสะสมสูงจะมีการตอบสนองต่อบูเซอรีลินที่ดี การผสมเทียมแบบกำหนดระยะเวลาครั้งเดียวโดยการปล่อยน้ำเชื้อที่มีปริมาณอสุจิลดลงสู่โพรงมดลูกให้จำนวนลูกต่อครอกลดลง การทดแทนไข่แดงด้วยเลซิทินในสารละลายน้ำเชื้อแช่แข็งไม่สามารถเพิ่มคุณภาพน้ำเชื้อแช่แข็งได้ดังนั้นจึงควรมีการศึกษาปริมาณความเข้มข้นของเลซิทินในสารละลายน้ำเชื้อแช่แข็งที่เหมาะสมเพิ่มเติมเพื่อนำมาใช้ร่วมกับการผสมเทียมแบบกำหนดระยะเวลาครั้งเดียวด้วยน้ำเชื้อแช่แข็งที่เก็บรักษาในสารละลายที่มีเลซิทินเป็นองค์ประกอบในอนาคต



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KEYWORD: 789, Fixed-time AI, GnRH agonist, Ovulation induction, Semen cryopreservation

Pachara Pearodwong : Single fixed-time artificial insemination with fresh or frozen thawed semen in pig. Advisor: Prof. PADET TUMMARUK, D.V.M., M.Sc., Ph.D. Co-advisor: Dr. CHANYUTH TRETIPSKUL, D.V.M., Ph.D.

Single fixed-time AI is the breeding protocol inseminating the sows once with either fresh or frozen-thawed (FT) semen at fixed time after ovulation induction without estrus detection. To develop the success single fixed-time AI protocol in Thailand, factors affecting the efficacy of single fixed-time AI were evaluated. Additionally, we also evaluated the effect of using lecithin in cryopreservation extender on FT semen qualities in order to find the possibility to perform single fixed-time AI using sperm preserved in lecithin based extender. It was found that administration of 10 ug buserelin at 72 h after weaning decreased weaning-to-ovulation interval compared to spontaneous ovulation (127.6 vs. 157.2 h). However, the response of buserelin injection was also affected by season and weaned sows conditions. Hot season prolonged ovulation time. Weaned sows with lactation length (LL) ≥20 days, litter weight (LW) <67 kg, or body condition score (BCS) ≥3, or high backfat reserve after weaning had better buserelin responses. The single fixed-time AI using 10 ug buserelin injection at 72 h after weaning and insemination the sows at 32 h later was successfully performed under tropical climate with acceptable litter size compared to conventional AI (12.0 vs. 12.8 piglets per litter). A decreased litter size was also observed when single fixed-time AI was performed in the hot season or using intrauterine insemination (IUI) with a reduced number of sperm. Finally, the FT semen qualities of sperm preserved in cryopreservation extenders containing 20% egg yolk or 3% lecithin and 10% egg yolk were higher than those in 6% lecithin extender. In conclusion, the present study found that hot season decreased response of buserelin injection, delayed ovulation time and decreased sows reproductive performances after single fixed-time AI. Weaned sows with LL ≥20 days, LW <67 kg, BCS ≥3 or high backfat had better responses to buserelin injection. Single fixed-time AI using the IUI with a reduced number of sperm decreased litter size. Replacing of egg yolk with lecithin in cryopreservation extender did not increase FT semen qualities. The precise concentration of lecithin should be further investigated to perform single fixed-time AI using FT semen preserved in lecithin-based extender.



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| | |
|---|---|
| ℃ | degrees Celsius |
| µg | microgram |
| µm | micrometer |
| AI | artificial insemination |
| ALH | mean lateral head displacement |
| ANOVA | analysis of variance |
| BCF | beat cross frequency |
| BCS | body condition score |
| BF | backfat thickness |
| CASA | computer assisted sperm analysis system |
| cm | centimeter |
| CV | coefficient of variation |
| DMSO | Dimethyl sulfoxide |
| eCG | equine chorionic gonadotropin |
| EOI | estrus-to-ovulation interval |
| EthD-1 | Ethidiumhomodimer |
| FITC | fluorescein isothiocyanate |
| FSH | follicle stimulating hormone |
| g | gram |
| GnRH | gonadotropin releasing hormone |
| h | hour |
| hCG | human chorionic gonadotropin |
| IEI | injection-to-estrus interval |
| IGF-1 | insulin-like growth factor-1 |
| IOI | injection-to-ovulation-interval |
| IU | international unit |
| IUI | intra-uterine insemination |
| JC-1 | tetraethylbenzimidazolylcarbocyanine iodide |
| kg | kilogram |
| LH | luteinizing hormone |
| LIN | linearity |
| LL | lactation length |
| LPO | lipid peroxidation |

| | |
|---|---|
| LSD | least significant differences |
| LW | litter weight at weaning |
| m | meter |
| Mcal | mega calorie |
| mg | milligram |
| MHz | mega hertz |
| ml | milliliter |
| mM | millimolar |
| mm | millimeter |
| mOsmol | milliosmole |
| NS | not significant |
| PBS | phosphate buffer saline solution |
| pLH | porcine luteinizing hormone |
| PNA | peanut agglutinin |
| PUFA | polyunsaturated fatty acids |
| ROS | reactive oxygen species |
| SD | standard deviation |
| sec | second |
| SEM | standard error |
| sHOST | hypo-osmotic swelling test |
| STR | straightness |
| v/v | volume by volume |
| VAP | average path velocity |
| VCL | curvilinear velocity |
| vs. | versus |
| VSL | straight-line velocity |
| WEI | weaning-to-estrus interval |
| WOB | wobble coefficient |
| WOI | weaning-to-ovulation interval |

# CHAPTER I

# INTRODUCTION

## 1.1 Importance and rationale

Nowadays, an increasing of pig breeding farm leads to an increase of a batch management system instead of traditional continuous system (Vermeulen et al., 2017). Performing of batch breeding management system enables all-in/all-out procedures to be implement (Bown, 2006). Batch weaning can lead to natural estrus synchronization of the sows (Kirkwood and Kauffold, 2015). However, the natural estrus onset after weaning still occurs over several days after weaning (Kemp and Soede, 1996). A result from a previous study implied that the varied weaning-to-estrus intervals resulted in varied estrus durations and ovulation times of the weaned sows (Kemp and Soede, 1996). The spontaneous ovulation of the sows cannot be predicted accurately. Ovulation can take place at varied time from 33 up to 99 h after standing estrus (Wongkaweewit et al., 2012). From this reason, the conventional artificial insemination (AI) protocol, sows are inseminated 2-3 times within 12-24 h interval during standing estrus (Knox, 2016), by depositing large amount sperm per dose (2-3x$10^9$ sperm/dose) in a large volume (80-100 ml) are deposited in the cervix (Roca et al., 2006). Thus, the ovulation time of the sows should be controlled to achieve the correct insemination time relative to time of ovulation (Brüssow et al., 2009; Kirkwood and Kauffold, 2015).



Since almost 20 years ago, a technology for manipulating of estrus and ovulation which allows fixed-time insemination had been developed (Brüssow and Wähner, 2011). In sows, earlier fixed-time AI protocols stimulate the follicle development using equine chorionic gonadotropin (eCG) and using of human chorionic gonadotropin (hCG), luteinizing hormone (LH) or gonadotropin releasing hormone (GnRH) agonist to induce ovulation (Brüssow et al., 2009). The ovulation is occurred within 36-42 h after treatment and it was recommend to inseminate the sows at fixed times (Brüssow and Wähner, 2011). Lately, fixed-time AI protocols have been developed that only GnRH

use (Martinat-Botte et al., 2010; Driancourt et al., 2013). Based on previous studies, the impaired reproductive outcome from the sows submitted to single fixed-time AI compared to conventional AI had been observed (Driancourt et al., 2013; Ulguim et al., 2014; Baroncello et al., 2017). Results from previous studies implied that the efficacy of fixed-time AI could be mainly affected by factors that influence the follicle response and ovulation time after GnRH administration, i.e. timing (Martinat-Botte et al., 2010) and dosage (Driancourt et al., 2013) of GnRH administration. In addition, the other factors including season (Belstra et al., 2004), parity (Vesseur et al., 1994), lactation length (Knox and Zas, 2001), weaned sows condition (Vesseur et al., 1994; Tantasuparuk et al., 2001; Gourdine et al., 2006b) also enable to affect the ovulation of sows. Moreover, the seasonal effect on fertility and reproductive performances of the sows is well-established (Belstra et al., 2004; Tummaruk et al., 2010). In Thailand, inferior farrowing rates and poor litter sizes at birth are observed in sows inseminated during the hot season (Tantasuparuk et al., 2000a; Tummaruk et al., 2010).

To establish the single fixed-time AI protocol in the tropical country like Thailand, the study on factors that may affect the response of GnRH administration and may affect the sow fertility and reproductive performance including season, lactation length and sow conditions need to be investigated. In the conventional AI, large amount of sperm per dose are inseminated to compensate the loss from semen back-flow (Kirkwood and Kauffold, 2015). Intra-uterine insemination (IUI) in pigs with a reduced number of sperm cells per dose has been developed to decrease semen back-flow (Watson and Behan, 2002; Sumransap et al., 2007). However, it was reported that semen back-flow (>5 ml) was observed and reduced the farrowing rate and litter size at birth in the single fixed-time AI sows inseminated using IUI catheter (Fontana et al., 2014). The effect of performing the single fixed-time AI using different insemination techniques (i.e. AI and IUI) on reproductive performance of sows should be investigated.

The principles of AI are to ensure that at the time of ovulation, there is a number of sufficient sperm are in the sperm reservoir or utero-tubal junction (Hunter, 2002). In the swine breeding, the most efficient method for long-term preservation of boar sperm is cryopreservation. However, boar semen cryopreservation is still not

completely successful due to poor frozen-thawed semen qualities (Knox, 2015a). After insemination, the number of inseminated of frozen-thawed semen will be dramatically decreased which may result in the insufficient number of sperm in the utero-tubal junction (Pursel et al., 1978; Sumransap et al., 2007). A previous study reported that the using of frozen-thawed sperm to inseminate the sows submitted to fixed-time AI program resulted in decreased litter size (Chanapiwat et al., 2014). Thus, the additional study on finding the novel technique to improve post-thawed sperm quality should be investigated. Due to the using of egg yolk as a semen extender ingredient has been considered a risk in terms of bacterial contamination and varies concentration (Bousseau et al., 1998). Lecithin and other unsaturated lipids were considered as an alternative ingredients to replace egg yolk (Aires et al., 2003; Pillet et al., 2012; Moraes et al., 2015). Only a previous study found the success of replacement of egg yolk with lecithin which extracted from soybean (Zhang et al., 2009). However, up to date, the number of research on replacement of egg yolk with lecithin in boar cryopreservation extender is limited. This may be due to the lack of clearly defined concentration of lecithin in soybean lecithin used (Zhang et al., 2009). From this reason the additional information on the exact concentration of lecithin used to replace egg yolk should be investigated.

To reach the success of performing single fixed-time AI in Thailand, the factors from both female and male that enable to affect reproductive performance should be investigated. Thus the present study aim to investigate the effect of season, insemination technique and the other potential factors on ovulation time after GnRH administration and reproductive performances after performing single fixed-time AI. Additionally, the effect of replacing of egg yolk with lecithin on frozen-thawed semen quality will be also investigated.



**1.2 Keywords**

Fixed-time AI, GnRH agonist, Ovulation induction, Semen cryopreservation

**1.3 Research coherence**

In the last decade, fixed-time AI has been established in swine industry worldwide with an impaired or comparable results compared to conventional AI (Driancourt et al., 2013; Baroncello et al., 2017; Knox et al., 2017). To reach the better result, the fixed-time AI has been researched in many aspects in order to evaluate the factor affecting the efficacy of fixed-time AI performed in commercial farms. However, most studies were done in Europe (Martinat-Botte et al., 2010; Driancourt et al., 2013), USA. (Knox et al., 2017) and Brazil (Ulguim et al., 2016; Baroncello et al., 2017), which the weather is different from Thailand. To perform the successful single fixed-time AI in tropical country, the factors from both female and male sides that enable to affect the efficacy of single fixed-time AI should be investigated. For the female side, study on controlling of ovulation time using GnRH administration will be investigated in Chapter II. It was reported that timing of GnRH administration affected the ovulation time of the sows (Martinat-Botte et al., 2010). Thus, this chapter is aimed to study the suitable timing of GnRH administration in sows after weaning. Once the suitable timing is selected, we will further investigate on the onset of ovulation time after weaning in the sows treated with GnRH agonist in different seasons. This may be resulted from the reason that hot climate depressed follicle development (Lopes et al., 2014) ovarian steroidogenic function of sows (Bertoldo et al., 2012) and also delayed weaning-to-estrus interval of the sows (Tummaruk et al., 2000). The hot climate may influence the response of the follicle or ovulation time of the sows after GnRH treatment. Moreover, we also study the effect from other factors including parity, lactation length and sows conditions after weaning on the ovulation time. Thus, this chapter will answer about when to administrate the GnRH agonist after weaning and the ovulation time after ovulation induction. This chapter also answer what the factors that should be aware before performing ovulation induction for fixed-time AI.

The principles of AI are to ensure that at the time of ovulation, a number of sufficient sperm are in the utero-tubal junction (Hunter, 2002). The most efficient method for long-term preservation of boar sperm is cryopreservation. However, the frozen-thawed boar semen quality is still poor (Knox, 2015a). The AI using frozen-thawed semen may result in the insufficient number of sperm in the utero-tubal junction (Pursel et al., 1978; Sumransap et al., 2007) and decreased litter size of sows

submitted to fixed-time AI (Chanapiwat et al., 2014). For the male side, we aim to develop the cryopreservation extender that improve the post-thawed sperm quality in order to increase the efficiency of single fixed-time AI using frozen-thawed semen in the future. Only a previous study found the success on improving post-thawed semen qualities by replacing egg yolk in the cryopreservation extender with lecithin (Zhang et al., 2009). Up to the date, the number of using lecithin in boar cryopreservation extender is still limited. Thus, in Chapter III, we will evaluate the use of lecithin to replace egg yolk in order to improve frozen-thawed boar sperm quality.

Finally, in Chapter IV, we aimed to establish the single-fixed time AI protocol that suitable to Thailand. In this chapter the timing of GnRH administration after weaning, type of semen and the insemination time after GnRH treatment will be selected from the knowledge obtained from Chapter II and III. Additionally, to perform single fixed-time AI in the tropical country, the factors that may affect the sow reproductive performance including season (Tummaruk et al., 2010), will be investigated. In the conventional AI, large amount of sperm per dose are inseminated to compensate the loss from semen back-flow (Kirkwood and Kauffold, 2015). Up to date, the Intra-uterine insemination (IUI) in pigs with a reduced number of sperm cells per dose has been developed to decrease semen back-flow (Watson and Behan, 2002; Sumransap et al., 2007). Performing of single fixed-time AI with IUI using reduce number of sperm per dose may decrease the sows reproductive performance. Thus, in this chapter, we will perform single fixed-time AI in different seasons and by using the different AI techniques (AI and IUI).



## 1.4 Literature review

### 1.4.1 Hormonal pattern from late lactation until post-weaning period

After farrowing the sows are normally anestrus and anovulartory due to the insufficiency of follicle stimulating hormone (FSH) and luteinizing hormone (LH) releasing during lactation leading to the suppression of follicle growth, anestrus and anovulatory (Crighton and Lamming, 1969; Cole et al., 1972). The insufficient of LH and FSH level are proposed from the reason that the GnRH level in the hypothalamus and

hypophyseal portal area are low during lactation (Cox and Britt, 1982). The secretion of GnRH from hypothalamus is suppressed by suckling reflexes induced by the piglets during lactation (De Rensis et al., 1993). After weaning, the suckling reflex is disappeared and weaned sows will return to cycle quickly (Quesnel and Prunier, 1995). The LH concentration and frequency increase significantly within 12 h and the LH magnitude increases again during 36 to 48 h after weaning (Shaw and Foxcroft, 1985; Foxcroft et al., 1987). The FSH concentration increases significantly during 12 h after weaning and remains elevated until 32 h after weaning (Shaw and Foxcroft, 1985; Foxcroft et al., 1987). The biding of released LH and FSH to the receptors on theca cell and granulosa cells can induce estrogen biosynthesis (Magoffin, 2005). The increasing level of estrogen acts at the hypothalamus and pituitary to induce the LH surge and expression of estrus sign (Henricks et al., 1972; Elsaesser et al., 1998). The estrogen level starts to increase at 72 h and then rises to the highest level at 36 h before LH and FSH surge (Cox and Britt, 1986). The mean of LH and FSH level are low from 96 to 12 h before LH and FSH surge. Both LH and FSH levels surge at 92 to 116 h (109 ± 5 h) after weaning (Cox and Britt, 1986). Changing of hormonal profile after weaning results in a modification of ovarian activities and sows behaviors.

### 1.4.2 Action of FSH and LH on the porcine ovaries after weaning until ovulation



The ovary contains various stages of follicles. These follicles develop from primordial follicle to the stage that have fluid-filled cavity adjacent to the oocyte called “antral follicle”. The mature follicles composted of oocyte, granulosa cells enclosed with basement membrane. Between the follicles, there are the theca cells present in the interfollicular stroma (Homburg, 2014). In the pig ovary, there are the continuous growth and atresia of follicle in both luteal and follicular phase without follicular wave or dominant follicle (Evans, 2003). During the estrus cycle, the recruitment and selection of ovulating follicle are occurred during late luteal phase to early follicular phase. During the late luteal phase, there are reduction of small and medium size follicles. The pool of medium size follicle are selected for ovulation (Knox, 2005). Thus, the number of large follicles are increased during follicular phase

until ovulation (Noguchi et al., 2010). The rising of FSH after weaning stimulates follicular growth, proliferation and differentiation of granulosa cells. FSH is important for development of the small antral follicles to medium size. In addition, FSH also induces LH receptor on granulosa cell of dominant follicle, therefore LH can directly stimulate estrogen synthesis (Hillier, 1994). The cooperation between granulosa cells and theca cells to produce estrogen is called two-cell, two-gonadotropin concept (Magoffin, 2005). The circulatory LH trigger the cyclic adenosine monophosphate (cAMP) pathway to stimulate the steroidogenic enzymes including cholesterol side-chain cleavage cytochrome P450 (CYP11A), 17-alpha-hydroxylase/C17–20 lyase cytochrome P50 (CYP17) and 3-beta -hydroxysteroid dehydrogenase (3-beta -HSD) in theca cell. The steroidogenic enzymes in theca cells involve with androstenedione synthesis from cholesterol. The androstenedione is transported to granulosa cell for synthesis of estradiol afterward. Binding of FSH to FSH receptor on granulosa cell also stimulates the cAMP signaling pathway. The cAMP signaling pathway induces expression of CYP19 and 17-beta-HSD enzyme that metabolize androstenedione to estradiol (Magoffin, 2005). The high estradiol level suppresses the FSH level. The selection of follicles for ovulation is also started when follicular development is shifted from dependence on FSH to LH (Hillier, 1994; Knox, 2005). According to the period of gonadotropins dependence suggests that FSH is importance for increasing of number of follicle, while LH plays role in diameter increasing (Soede et al., 2011). The high estradiol level also triggers LH surge by the positive feedback pathway. The LH surge induces the rupture of follicle and ovulation after the surge (Homburg, 2014). Naturally, the sows will ovulate between 24-68 h after standing estrus (Soede et al., 1992). In Thailand, a previous study has reported that the ovulation time in weaned sows ranged between 33 and 99 h after standing estrus (Wongkaweewit et al., 2012).



**1.4.3 Ovulation induction in pigs**

The variation of ovulation time can be caused by the variation of estrus to LH surge interval (Cox and Britt, 1986; Brandt et al., 2009). To control the variation of ovulation time after weaning, various exogenous hormones can be applied into breeding program. The hormones can be classified into two main effects including; first

is to control of follicular development and estrus, the second is to control the ovulation of the sows (De Rensis and Kirkwood, 2016). Earlier protocols suggested to induce follicle development using eCG after weaning and induced ovulation hCG at 72 to 84 h later (Brüssow et al., 2009). The effect of eCG is considered as FSH, so it can be used to stimulate follicular development and estrus of weaned sows (Brüssow et al., 2009; Cassar et al., 2010). The injection of eCG 500-750 IU within 24 h after weaning to induce follicular growth was suggested by previous study (De Rensis and Kirkwood, 2016). The ovulation of the weaned sows can be induced by using hCG which considered as LH agonist (Soede and Kemp, 1993; Wongkaweewit et al., 2012). After injection of hCG, the ovulation is occurred between 35 and 49 h after injection (Soede and Kemp, 1993; Abad et al., 2007). Lately, protocols have been developed that only use GnRH agonist (Driancourt et al., 2013; Baroncello et al., 2017) or only LH agonist (Ulguim et al., 2014) given at a fixed-time after weaning. Administration of 10 μg of GnRH agonist at 94 or 104 h after weaning results in 100% and 66.7% ovulation within 24 h after injection, respectively (Martinat-Botte et al., 2010). A previous report showed that the injection of GnRH agonist at 77 h after weaning resulted in 100% ovulation of the sows within 32-44 h after injection (Driancourt et al., 2013). In Thailand, weaned sows received 50 μg of GnRH agonist at estrus ovulate at 37.5 ± 3.2 h later (Wongkaweewit et al., 2012).



**1.4.4 Fixed-time artificial insemination in pig**

Ovulation induction in weaned sows is being investigated by number of researchers worldwide during recent years in order to develop fixed-time AI in swine industry (De Rensis and Kirkwood, 2016). Fixed-time AI is the breeding protocol allowing insemination to be performed at a fixed-time independent of estrus detection (De Rensis and Kirkwood, 2016). The fixed-time insemination in pig established in swine industry worldwide has been performed in various protocols. The initial protocol is based on two hormone administrations i.e., eCG to induce follicle development and hCG or pLH to induce ovulation (Brüssow et al., 1996; Cassar et al., 2005; Degenstein et al., 2008; Pelland et al., 2008). Lately, the modern protocols have been developed that only use single hormone injection i.e., GnRH (Martinat-Botte et al., 2010; Driancourt

et al., 2013; Baroncello et al., 2017) or pLH (porcine luteinizing hormone) (Fontana et al., 2014; Ulguim et al., 2014; Ulguim et al., 2016) after weaning. The research on the efficacy of fixed-time AI protocols have been investigated in many aspects in commercial farms. Fixed-time AI using 50 ug of GnRH analogue has better fertility than 25 ug of GnRH agonist or 300 ug GnRH plus 300 IU hCG (Brüssow et al., 1996). The number of insemination (double vs. single fixed (eCG and hCG or pLH) (Cassar et al., 2005) also has been investigated. There are no significant differences of farrowing rate and litter size between double or single fixed-time inseminated sows (Gooneratne et al., 1989; Cassar et al., 2005; Fontana et al., 2014). Additionally, the effect of timing of GnRH treatment on reproductive performances of sows after fixed-time-AI is also studied. Treatment of GnRH at 94 h after weaning, 100% of sows ovulate over a 24 h time window, while the treatment of GnRH at 104 h after weaning, only 66.7% of the treated sows ovulate during a 24 h period (Martinat-Botte et al., 2010). Thus, the administration of 10 μg of GnRH at 94 h after weaning in sows enable to establish single artificial insemination at a predetermine time (Martinat-Botte et al., 2010).

A previous study found that performing of single fixed-time AI in the sows received intra-vaginal GnRH gel administrations at 72, 84 or 96 h after weaning affected the weaning-to-ovulation interval (133, 135 and 145 h, respectively) and farrowing rate (29, 50 and 79 %, respectively) (Knox et al., 2014). The single fixed-time AI females received either intramuscular or vulvar submucosal routes have similar farrowing rate (92 vs. 93 %) and litter size (13.3 vs. 12.3) (Ulguim et al., 2014). The effect of the number of sperm per dose or the insemination techniques on efficacy of fixed-time AI have been investigated (Pelland et al., 2008; Knox et al., 2017). The deposition of sperm at different sites (intra-cervix vs. intra-uterine insemination) with $1x10^9$ or $3x10^9$ sperm per dose in sows submitted to single fixed-time AI does not affect the farrowing rate and litter size (Pelland et al., 2008). In contrast, single fixed-time AI sows received intra-uterine insemination with $2.5x10^9$ sperm per dose has higher litter size than those received $1.5x10^9$ sperm per dose (Knox et al., 2017). Presence of semen back flow reduces the number of total born piglet of the sows after performing single fixed-time AI compared to the sow without semen back flow (10.4 vs. 12.5) (Ulguim et al., 2016). Up to date, a number of study on the effect of sow condition in relation with efficacy

of single fixed-time AI is still limited. There is only a previous study demonstrated that parity, lactation length or back fat loss during lactation did not affected the farrowing rate and litter size of single fixed-time AI sows (Driancourt et al., 2013). Even though, up to date, the single fixed-time insemination has been successfully established. However, the reproductive performances of single fixed-time AI sows are compromised when compared to conventional AI. The farrowing rate of single fixed-time AI sows was lower than conventional AI sows (82.2 vs. 93.7 %) (Baroncello et al., 2017). Thus, to reach the better reproductive performances of single fixed-time AI, more studies of factors affecting the efficacy of fixed-time AI need to be investigated.

**1.4.5 Artificial insemination techniques**

AI is the most common reproductive technology in swine production. Due to the high variation of estrus-to-ovulation intervals of the sows (Soede et al., 1992), the weaned sows are normally inseminated for 2-3 times during estrus duration (Knox, 2015b). The conventional AI procedure is expected to deposit sperm at the optimal time related to ovulation (at 0-24 h before ovulation) (Kemp and Soede, 1996). In general, the AI is performed by cervical insemination using the high number of sperm cells (2-3x$10^9$ sperm) in a large volume of semen extender dose (70-100 ml) (Roca et al., 2011; Knox, 2016). The large volume of semen is used to compensate the semen back flow which can be found in 98% of sows within 2.5 h after AI (Steverink et al., 1997). The reduction of sperm cells used per insemination is a challenging concept to increase the efficacy of boars. The post-cervical insemination or IUI allows insemination with a reduced number of sperm cells, namely from 3x$10^9$ to 1x$10^9$ sperms per dose in the lower volume of semen (45 ml) without significant differences of farrowing rate and litter size compared to cervical AI with 2x$10^9$ or 3x$10^9$ sperm per dose (Watson and Behan, 2002; Sumransap et al., 2007). The semen is deposited at the 20 cm beyond from the catheter cervix fixation into the uterine body (Watson and Behan, 2002). The advantages of IUI include allowing sperm cell to be reduced, minimal requirement of training, reduce time consuming for insemination and no welfare implication (Bortolozzo et al., 2015).

The AI in pig using frozen-thawed semen also has been developed using a high number of 5-6x$10^9$ frozen-thawed sperm per dose to deposit at the cervix with a large volume of extender (80-100 ml) (Almlid and Johnson, 1988; Johnson et al., 2000). Despite performing AI with a large number of sperm, the impaired reproductive outcomes are still obtained, 20-30% farrowing rate and 2-3 piglets lower than those inseminated with fresh semen (Almlid and Johnson, 1988; Johnson et al., 2000). This might be resulted from a short longevity of frozen-thawed boar sperm in the female reproductive tracts, which is approximately 6 h after AI (Pursel et al., 1978). Moreover, the optimal timing of AI using frozen-thawed semen related to ovulation is very narrow, 0-4 h before ovulation (Waberski et al., 1994). This indicates that the ovulation induction seemed to be necessary to be incorporated with insemination using frozen-thawed semen.

**1.4.6 Effect of season on sow reproductive performances**

In pigs, it is well documented that season affects the reproductive performances. This phenomenon is often referred as infertility in summer (Love et al., 1993; Peltoniemi et al., 1999). The seasonal infertilities include delayed puberty, anestrus, prolonged weaning-to-estrus interval, reduced farrowing rate and litter size (De Rensis et al., 2017). This is associated with various factors high environmental temperature and relative humidity (Auvigne et al., 2010) or photoperiod (Peltoniemi et al., 1999). Day length in Thailand is almost constant throughout the year, with duration between sunrise and sunset at approximately 11-13 h. Thus, seasonal effect in Thailand is mainly related to high temperature in combination with humidity (Tantasuparuk et al., 2000a). High temperature enables heat stress triggering (De Rensis et al., 2017). It is hypothesized that heat stress has both direct and indirect effects (via reduced feed intake) on animal productivity (Ross et al., 2015). Stressful stimuli leads to activation of hypothalamo-pituitary-adrenal (HPA) system and activation of the systempathetic adreno-medullary system (Einarsson et al., 2008). The activation of HPA system leads to releasing of corticotropin releasing hormone which stimulates the release of cortisol which stimulates lipolysis (Mersmann, 1986). The other hormones i.e., progesterone or inhibin are also released (Madej et al., 2005; Brandt et al., 2007). The activated

systempathetic adreno-medullary system causes the release of adrenaline and noradrenaline lead to increase metabolism (Einarsson et al., 2008). The reduced feed intake results in the negative energy balance of the pig (De Rensis et al., 2017). Negative energy balance of the pig consequences the reduced GnRH-LH-FSH secretion and reduced follicular development (De Rensis et al., 2017). It was found that sows weaned in summer had a smaller follicle size both at weaning and at the onset of estrus than sows weaned in winter (Lopes et al., 2014). These scenarios cause the reduced quality of oocyte and embryo, adverse effect on luteinization, reduced CL quality leading to embryonic loss and delayed wean-to-estrus interval (De Rensis et al., 2017). In Thailand, sows inseminated with conventional AI in hot season have a significantly lower litter size compared to sows inseminated during the cool season, mainly because of the high ambient temperature and/or high humidity during gestation in hot seasons (Tantasuparuk et al., 2000a; Tummaruk et al., 2010). Up to date, the study on the effect of seasons on the efficacy of ovulation induction or fixed-time AI has not been comprehensively investigated.

### 1.4.7 Replacing of egg yolk with lecithin in boar semen cryopreservation

In natural, the plasma membrane of boar sperm have high level of polyunsaturated fatty acid (PUFA) (Maldjian et al., 2005). Not only the PUFA, but the steroid lipid (cholesterol) also plays important roles in the plasma membrane fluidity (van Meer et al., 2008). One of important steroid lipids is cholesterol (van Meer et al., 2008). In the mature boar sperm membrane, the phospholipid, sterol and glycolipid proportion were 76.2%, 12.7% and 5.4%, respectively (Nikolopoulou et al., 1985). When the temperature is decreased, the transformation of liquid-crystalline phase of plasma membrane to gel phase is increased (Stubbs and Smith, 1984). The level of phospholipid can effects the membrane fluidity (Stubbs and Smith, 1984). The high level of polysaturated/saturated fatty acid ratio in membrane can reduce the membrane cold resistance (Darin-Bennett and White, 1977). The cholesterol/phospholipid is also the important parameter to determine the cooling susceptibility. The cholesterol/phospholipid ratio in bull (0.38) was higher than boar (0.12) (De Leeuw et al., 1990).

Up to date, the boar semen cryopreservation is still not successful because of the poor post-thawed semen quality caused by cold shock and cryoinjury (Kumar et al., 2003). There are many techniques have been developed and used to deal with cold shock and cryoinjury (Bwanga, 1991; Yeste, 2015). Low temperature during chilling process damages the sperm membrane that contains high level of PUFA by cold shock and lipid peroxidation (Maldjian et al., 2005). Cold shock reduces membrane fluidity which makes cell membrane more rigid, lost their integrity, motility and intracellular enzyme (De Leeuw et al., 1990; Johnson et al., 2000). Normally, spermatozoa can produce amount of reactive oxygen species (ROS), which is important for sperm capacitation, acrosome reaction and fertilization. Excessive ROS can damage the sperm by causing the lipid peroxidation (LPO) and DNA damage. LPO also reduces the level of PUFA (Marmunti et al., 2012). Egg yolk (15% to 20%) has been used for over 40 years as a standard membrane-modifying agent that is added to traditional semen cryopreservation extender (Phillips and Lardy, 1940; Visser and Salamon, 1974). Egg yolk provides the cold shock protection to the sperm (Benson et al., 1967; Holt, 2000). Egg yolk is an integral component of semen extender that acts as a non-penetrating cryoprotectant. The action of egg yolk may be attributed to phospholipids, cholesterol (Moce et al., 2010), which afford successful protection to the sperm plasma membrane against cold shock and cryoinjuries (Moussa et al., 2002). However, the use of egg yolk as a semen extender ingredient has been considered a risk in terms of bacterial contamination. Moreover, the composition of egg yolk also varies depending on its initial sources (Bousseau et al., 1998). In general, yolk is made up of 62% triglycerides, 33% phospholipids and 5% cholesterol. Phosphatidylcholine (PC) is 76% of total phospholipid. Phosphatidylethanolamine (PE) represents 22% of total phospholipids. Phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM) are present in very low amounts. One gram of egg yolk consist of 12.8 mg of cholesterol (Keum et al., 2018), and the concentration of cholesterol can be varied between breed or diet type of the hens (Yin et al., 2008). Thus, alternative ingredients, e.g. lecithin and other unsaturated lipids, have been considered as the replacements for egg yolk (Aires et al., 2003; Pillet et al., 2012; Moraes et al., 2015). Lecithin (phosphatidylcholine) is one of the phospholipids that is extracted from soybean (Layek et al., 2016). Soybean seed

contains 1-3% phospholipid, which 35% of total phospholipid is PC (lecithin), 25% PE, 15% PI and 5-10% minor phospholipid (Layek et al., 2016). The use of lecithin as a substitution for egg yolk has been developed for many species, i.e. bucks (Forouzanfar et al., 2010; Salmani et al., 2014), bulls (Aires et al., 2003), tom cats (Vick et al., 2012), male dogs (Dalmazzo et al., 2018) and boars (Zhang et al., 2009). In boars, only one report on use of 6% soybean lecithin to replace egg yolk in cryopreservation extender has been reported since 2009 (Zhang et al., 2009).

## 1.5 Research objectives

1. To find a suitable time of GnRH agonist injection, first the effects of timing of buserelin injection (72 vs. 84 h after weaning).
2. To determine ovulation time after GnRH agonist (buserelin) administration at differences times after weaning in sows.
3. To evaluate the effects of season on estrus onset and ovulation time in GnRH agonist (buserelin) treated and non-buserelin treated sows, in a tropical climate.
4. To evaluate the effects of some sow factors, including parity, body condition, lactation length and litter weight at weaning on estrus and ovulation time.
5. To investigate the effects of lecithin in combination with egg yolk in semen extender on frozen-thawed boar sperm qualities.
6. To evaluate reproductive performances of sows after single fixed-time AI under a tropical climate.
7. To investigate the influences of season and insemination technique on the efficacy of single fixed-time AI in sows.

# CHAPTER II

## Factors affecting estrus and ovulation time in weaned sows with induced ovulation by GnRH administration in different seasons

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### 2.1 Abstract

Follicle development in post-weaning sows is influenced by various factors. To control ovulation time using hormone, factors that influence ovulation should be investigated. The present study was performed to evaluate the effect of GnRH (buserelin) administration in relation to season and sow parameters on ovulation time in weaned sows. Seventy-seven weaned sows were divided into the following groups: control (hot season, n=21; cool season, n=16) and treatment (hot season, n=22; cool season, n=18). Sows were kept in a close house equipped with an evaporative cooling system. Ovulation time was determined every 6 h using transrectal ultrasonography. Administration of 10 μg buserelin at 72 h after weaning affected estrus-to-ovulation interval (EOI) and weaning-to-ovulation interval (WOI) in sows ($P$<0.05). The percentage of sows that ovulated between 44-56 h after injection was higher in the cool season than in hot season ($P$<0.05). Weaning-to-estrus interval (WEI) and injection-to-estrus interval (IEI) were affected by season ($P$<0.05). Body condition score (BCS) of sows influenced EOI ($P$<0.01). Sows with low backfat thickness, lactation length <20 days, or litter weight ≥67 kg, had delayed injection-to-ovulation interval (IOI) ($P$<0.05). In conclusions, buserelin administration (10 μg, at 72 h after weaning) advanced ovulation. Hot season prolonged ovulation time. Sows that were weaned with lactation length of at least 20 days, litter weight less than 67 kg, or BCS of at least 3, had better responses to buserelin injection. High backfat reserve after weaning is important for ovulation induction response by buserelin injection.

## 2.2 Introduction

To optimize the efficiency of AI in pigs, the timing of ovulation after weaning should be controlled. Because of this, ovulation induction protocols have been developed (Driancourt et al., 2013). Earlier protocols induced both follicle development and ovulation time (e.g. using eCG after weaning and hCG 72-84 h later) (Brüssow et al., 2009), but lately, protocols that use only GnRH (Gooneratne et al., 1989; Driancourt et al., 2013; Baroncello et al., 2017) or LH agonist have been developed (Cassar et al., 2005; Ulguim et al., 2014). These products are administered at a fixed time from weaning (Cassar et al., 2005; Baroncello et al., 2017) or from estrus (Gooneratne et al., 1989; Ulguim et al., 2014). Such protocols using GnRH agonist have been found to be successful in minimizing weaning-to-ovulation interval (WOI) or estrus-to-ovulation interval (EOI) (Martinat-Botte et al., 2010; Wongkaweewit et al., 2012; Baroncello et al., 2017). However, the variation in response after GnRH agonist administration among animals i.e., interval from GnRH administration to onset of ovulation, or to the end of ovulation can be quite large (Brüssow et al., 2009). The variations in results can be due to factors related to induction protocols, such as dosage used, type of agonist, and timing of administration in relation to follicle development (Knox, 2015b). In nature, post-weaning, follicle development is influenced by various factors, such as season, parity (Vesseur et al., 1994; Belstra et al., 2004), lactation weight loss (Vesseur et al., 1994; Tantasuparuk et al., 2001), lactation length (LL) (Knox and Zas, 2001), backfat thickness (BF), and litter weight at weaning (LW) (Gourdine et al., 2006b). These factors may affect the onset of estrus and ovulation time after GnRH treatment. Thus, to achieve control over ovulation time using hormonal stimulation, factors influencing ovulation should be investigated. The objectives of the present study were to evaluate the effects of season on estrus onset and ovulation time in GnRH agonist (buserelin) treated and non-buserelin treated sows, in a tropical climate, and to evaluate the effects of some sow factors, including parity, body condition, LL, and LW at weaning on estrus and ovulation time.

## 2.3 Materials and methods

### 2.3.1 Animal care, housing and management

The experimental procedures were performed according to the Ethical Principles and Guidelines for the Use of Animals, National Research Council of Thailand. The experimental procedure was approved and licensed by the Chulalongkorn University Animal Care and Use Committee (protocol number 1731012). The study was conducted on a commercial Landrace x Yorkshire crossbred swine herd containing 2,500 sows, with a weekly weaning system, in the southern part of Thailand during April (hot season) and December (cool season). During lactation, sows and their piglets were housed in individual farrowing pens. Piglet cross-fostering was performed within 4 days after farrowing, to standardize piglet numbers (10-12 piglets) considering the size of piglet and the number of sow teats available. The lactating sows were fed twice per day (total about 5-6 kg) a diet containing 3.2 Mcal energy/kg and 18.0% crude protein. On average, 10.6 ± 0.8 piglets with a litter weight of 66.6 ± 5.7 kg were weaned after lactation length of 20.1 ± 2.3 days. The weaned sows (parity numbers 1-5) were moved to the mating house, where they were kept in stalls equipped with an evaporative cooling system with a concrete slatted floor, and provided with 2.5 kg of feed per day (3.0 Mcal energy/kg and 15.0% crude protein). Water was provided on demand via a drinking nipple. Temperature and relative humidity inside the barn were measured using digital thermo-hygrometer (Micro and Sensor Application Company, Bangkok, Thailand). The device was placed at 1.5 m above the floor in the center of the barn. The records included daily minimum and maximum as well as current actual temperatures and humidity. Current actual temperature and humidity were recorded at 0100 h, 0700 h, 1300 h, and 1900 h. The average temperature and humidity in hot and cool seasons were calculated and they are presented in Table 1.

**Table 1** Temperature and relative humidity in cool and hot seasons (Means ± SD)

| Variables | Cool | Hot | *P* value |
|---|---|---|---|
| Average temperature (°C) | 26.2 ± 0.2 | 28.0 ± 0.6 | <0.001 |
| Minimum temperature (°C) | 24.8 ± 1.0 | 26.2 ± 0.7 | <0.001 |
| Maximum temperature (°C) | 27.3 ± 0.9 | 29.8 ± 1.0 | <0.001 |
| Average humidity (%) | 74.0 ± 4.6 | 77.8 ± 1.3 | <0.001 |
| Minimum humidity (%) | 71.7 ± 5.9 | 75.6 ± 2.2 | 0.006 |
| Maximum humidity (%) | 75.9 ± 3.9 | 79.5 ± 1.0 | <0.001 |

**2.3.2 Experimental design**

To find a suitable time for GnRH agonist injection, the effects of timing of buserelin injection (72 h vs. 84 h after weaning) were first evaluated. A pilot study was conducted on 24 weaned sows (parity numbers 2.6 ± 1.0, mean ± SD). The sows were divided into 3 groups: control (n=8; parity numbers 2.5 ± 1.0, mean ± SD), treatment72 (n=8; parity numbers 2.7 ± 0.9, mean ± SD), and treatment84 (n=8; parity numbers 2.5 ± 1.3, mean ± SD). The control sows did not receive the hormone, whereas treatment72 and treatment84 sows received intramuscular injections of 10 μg (2.5 ml) buserelin (Receptal®, Merck Animal Health, Madison, NJ, U.S.A.) at 72 h and 84 h after weaning, respectively, to induce ovulation.

To induce ovulation in weaned sows, previous studies recommended treatment with GnRH between 83-89 h after weaning (Brüssow et al., 2009; Driancourt et al., 2013; Baroncello et al., 2017). However, the weaning-to-estrus interval (WEI) can vary among herds (Tummaruk et al., 2000). In the present study, WEI of the sows was quite short (averaged 3.9 ± 0.2 days); hence the onset of GnRH injection must be carefully selected. In the pilot study, the administration of GnRH at either 72 or 84 h after weaning could effectively induce ovulation. Therefore, in the main experiment, the timing of GnRH injection at 72 h after weaning was chosen to obtain the major population of weaned sows that mostly exhibited standing estrus between 3-4 days post-weaning.

In the main experiment, GnRH injections were used to induce ovulation in weaned sow. Seventy-seven weaned sows were divided into the following groups: control (hot

season, n=21; cool season, n=16) and treatment (hot season, n=22; cool season, n=18). Treatment sows received an intramuscular injection of 10 μg (2.5 ml) buserelin at 72 h after weaning to induce ovulation, while the control sows did not receive hormone. In the control group, sows were inseminated twice at 12 h and 36 h after the onset of estrus. In the treatment group, sows were inseminated at 32 h after buserelin administration. Reproductive performance data including farrowing rate, total number of piglets born per litter, number of piglets born alive per litter, number of stillborn piglets per litter, number of mummified fetuses per litter, piglet body weight at birth, number of piglets at weaning, and piglets' body weight at weaning were compared between control and treatment groups.

**2.3.3 Sow body condition score (BCS) and backfat thickness (BF)**

On the day of weaning, BCS and BF were assessed. The BCS of the sows was based on a previous reported scoring system (Maes et al., 2004) that combines visual assessment and palpation of the body. The BCS varied from 1-5: score 1 (emaciated), score 2 (thin), score 3 (ideal), score 4 (fat), score 5 (overly fat). Body condition was scored by the same person. BF thickness was determined using A-mode ultrasonography (Renco Lean-meater®, Renco Corporation, Golden Valley, MN, U.S.A.) at the level of P2 position (6-8 cm length from body midline at the level of the last rib) on both sides. The average of the left and right measurements is a representative of BF. On average, BF (mean ± SD) of weaned sows was 15.4 ± 3.2 mm (range 10-23 mm).

**2.3.4 Estrus and ovulation detection**

Standing estrus response of the sows was monitored twice a day, at 0700 h and 1700 h, from the beginning of the day after weaning onwards using a back-pressure test in the presence of a mature boar. The sows that responded to a standing reflex including arched back, cocked ears, and immobilized legs, were considered in estrus. The onset of standing estrus was defined as the time the sows showed first standing reflex minus 5 h when the sows exhibited first standing estrus at 1700 h or minus 7 h when the sows exhibited first standing estrus at 0700 h.

In sows that showed estrus, the timing of ovulation was assessed using transrectal ultrasonography (HS-2000, Honda Electronics Co. Ltd., Oiwa-cho Toyohashi, Aichi, Japan) equipped with 7.5 MHz linear probe transducer at 6 h intervals. A gloved hand, lubricated with liquid paraffin and holding the transducer, was gently inserted into the rectum. Once the urinary bladder was seen on the screen, the ovaries were located by rotating the probe to the left and the right sides of the sow. Both sides of the ovaries were examined and the diameters of 4-8 biggest follicles in each ovary were measured. Ovulation time was defined as the time when a noticeable reduction in the numbers of follicles was observed minus 3 h (Belstra et al., 2004). A follicle with diameter ≥15 mm that remained visible for 5 days was defined as cystic ovary (Tummaruk and Kesdangsakonwut, 2012). The sows that showed multiple cysts were excluded from ovulation time analysis.

**2.3.5 Statistical analysis**

The data were analyzed using the Statistical Analysis System (SAS version 9.0, SAS Institute Inc., Cary, NC, U.S.A.). In the pilot study, WEI, EOI, WOI, injection-to-estrus interval (IEI), and injection-to-ovulation-interval (IOI) were considered as dependent variables. The treatment groups (control, treatment72, and treatment84) were considered as the main effect. To evaluate the effect of the main effect on dependent variables (WEI, EOI, WOI, IEI, and IOI), one-way analysis of variance (ANOVA) was performed using GLM procedure for each dependent variable. The qualitative data for follicle cyst detection were analyzed using Chi-squared or Fisher's exact tests with FREQ procedure. In the main study, data including WEI, EOI, WOI, IEI, and IOI, were considered as dependent variables. The independent variables including groups (control and treatment) and seasons (cool and hot) were considered as the main effects. The data were analyzed by using multiple ANOVA. Parity, BCS, BF, LL, and LW were considered as covariates. Univariate analysis was performed to check normality of the variables. Spearman's rank correlation test was performed to analyze the correlation among continuous parameters. The BF of sows was distributed normally. The independent variables that were not distributed normally were categorized as follows: parity number [1 (n=12), 2 to 3 (n=48), >3 (n=17)], BCS [<3 (n=27) and ≥3

(n=50)], LL (days) [<20 (n=45) and ≥20 (n=32)], and LW (kg) [<67 (n=42) and ≥67 (n=35)]. To investigate the independent variables and the interactions that may influence the dependent variables (WEI, EOI, WOI, IEI, and IOI), the multiple linear regression analysis model was developed by backward selection method and was analyzed using GLM procedure. Bonferroni test was used to adjust the multiple analyses. Non-significant independent variables were excluded to avoid over parameterization. The significant independent variables or the interactions that were left from the selection were obtained to fit the model. The average of the largest follicle diameter during 24 h after buserelin injection and from 24 h after buserelin injection to ovulation (pre-ovulatory follicle diameter), was calculated by MEANS procedure. The differences in follicle diameter between groups of sows with different IOI were also analyzed with linear regression using GLM procedure. The percentage of sows that ovulated between seasons was analyzed using chi-squared tests with FREQ procedure. Pearson's correlation was used to determine the relationship between maximum follicular sizes at 24 h after buserelin injection and preovulatory size and estrus/ovulation time (i.e., IEI, IOI, EOI and WOI) in hot and cool seasons. The reproductive performances were compared between control and treatment groups using GLM procedure of SAS. Additionally, reproductive performances were compared between sows inseminated during hot and cool seasons using GLM procedure of SAS. Least-squares means±SEM were obtained from each class of the variables and were compared using least significant differences (LSD) test. For all analyses, the alpha level for the determination of significance was 0.05.



## 2.4 Results

### 2.4.1 Pilot study

All the sows in control, treatment72, and treatment84 showed estrus after weaning, and WEI was not different among groups ($P$=0.63) (Table 2). One sow in treatment84 developed multiple cysts and it was excluded from ovulation time analysis. The EOI in control sows (68.1 h) was longer than that in treatment72 and treatment84 sows (41.3 h and 43.8 h, respectively, $P$<0.01). The WOI was not different

between treatment72 and treatment84 sows (129.9 h and 137.8 h, respectively) and both intervals were lower than that in control sows (162.8 h, $P<0.001$). In addition, IEI and IOI were not different between treatment72 and treatment84 (Table 2).

**Table 2** Effect of different induction timing on the onset of estrus and ovulation time in the pilot study (Least-squares means ± SEM)

| Variables | Control | Treatment | |
|---|---|---|---|
| | | 72 h | 84 h |
| Number of sows | 8 | 8 | 8 |
| WEI (days) | 3.9 ± 0.2 | 3.7 ± 0.1 | 3.8 ± 0.1 |
| EOI (h) | 68.1 ± 5.9[a] | 41.3 ± 3.7[b] | 43.8 ± 5.9[b] |
| WOI (h) | 162.8 ± 4.5[a] | 129.9 ± 4.5[b] | 137.8 ± 2.8[b] |
| IEI (h) | - | 16.8 ± 1.4 | 9.8 ± 3.9 |
| IOI (h) | - | 58.1 ± 4.5 | 54.4 ± 2.8 |

WEI, Weaning-to-estrus interval; EOI, Estrus-to-ovulation interval; WOI, Weaning-to-ovulation interval; IEI, Injection-to-estrus interval; IOI, Injection-to-ovulation interval. [a,b] Different superscripts within row differ significantly ($P<0.05$).

### 2.4.2 Main study

All the sows in both control (37/37) and treatment (40/40) groups showed estrus onset at 3.8 ± 0.1 days after weaning. The WEI was not affected by GnRH treatment at 72 h post-weaning (Table 3), but was longer in hot season than in cool season ($P=0.04$). Two out of 37 control sows (1 in hot, 1 in cool season) and 2 out of 40 treatment sows (2 in cool season) were excluded from ovulation time analyses, due to multiple cysts. The incidence of sows with multiple cysts did not differ between control and treatment groups or between seasons ($P>0.05$).

The EOI was not affected by season, but was shorter for sows in the treatment groups than those in the control group (38.3 vs. 64.2 h, $P<0.001$). The WOI was not affected by season, but was longer in control sows than in treatment sows ($P<0.001$) (Table 3 and Figure 1). Among the treatment sows, the IEI was longer in hot season than in cool season (19.7 vs. 14.3 h, $P<0.001$).

**Table 3** Effect of groups and seasons on estrus and ovulation times (Least-squares means ± SEM)

| Variables | Group | | Season | |
|---|---|---|---|---|
| | Control | Treatment | Hot | Cool |
| Number of sows | 37 | 40 | 43 | 34 |
| WEI (days) | 3.9 ± 0.1 | 3.7 ± 0.03 | 3.9 ± 0.1$^{x}$ | 3.6 ± 0.1$^{y}$ |
| EOI (h) | 64.2 ± 3.6$^{a}$ | 38.3 ± 2.1$^{b}$ | 49.3 ± 3.2 | 52.8 ± 4.1 |
| WOI (h) | 157.2 ± 4.2$^{a}$ | 127.6 ± 2.0$^{b}$ | 142.9 ± 3.4 | 140.2 ± 4.9 |
| IEI (h) | - | 17.2 ± 0.8 | 19.7 ± 1.2$^{x}$ | 14.3 ± 0.5$^{y}$ |
| IOI (h) | - | 55.7 ± 2.0 | 57.6 ± 2.3 | 53.0 ± 3.6 |

WEI, Weaning-to-estrus interval; EOI, Estrus-to-ovulation interval; WOI, Weaning-to-ovulation interval; IEI, Injection-to-estrus interval; IOI, Injection-to-ovulation interval. $^{a,b}$ or $^{x,y}$ Different superscripts within row differ significantly ($P$<0.05); no interaction effects of group and season on estrus and ovulation.

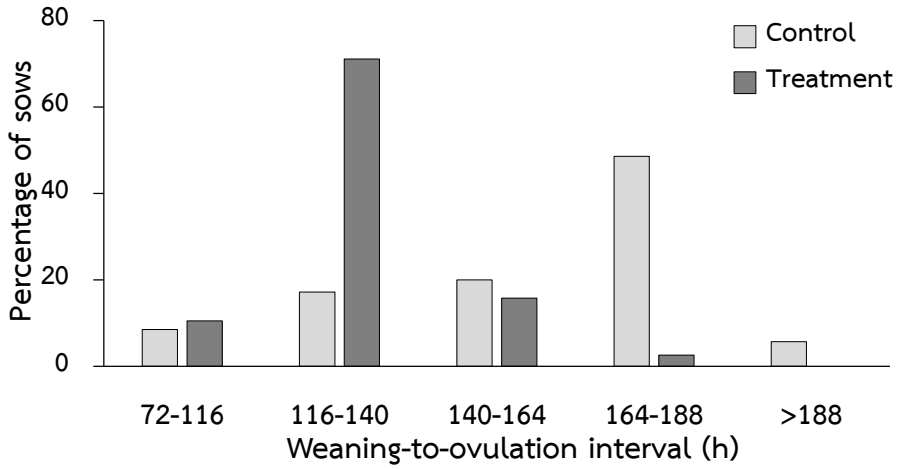


**Figure 1** Distribution of weaning-to-ovulation interval (WOI) in control sows and in sows injected with buserelin at 72 h post-weaning

The IOI was not affected by season ($P$=0.27); nevertheless, the percentage of sows that ovulated 44-56 h after injection was higher in cool season than hot season (69 vs. 32%, $P$=0.02), while the percentage of sows that ovulated 56-68 h after injection was lower in cool season than in hot season (6.2 vs. 36.4%, $P$=0.03) (Figure 2).

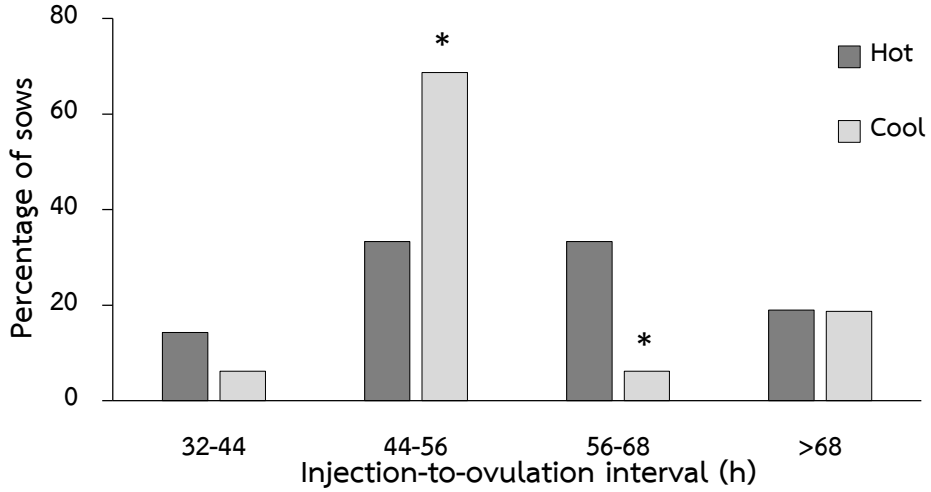


**Figure 2** Distribution of injection-to-ovulation interval (IOI) in treatment sows in hot and cool seasons *) $P<0.05$

The follicle diameters after buserelin injection in treatment sows with different IOI are presented in Figure 3. At 24 h after buserelin injection, the treatment sows with IOI of 32-44 h had a higher follicle diameter than those with IOI of 44-56 h or >68 h (10.1 ± 0.8 vs. 8.1 ± 0.3 and 7.8 ± 0.5 mm, respectively, $P<0.05$).

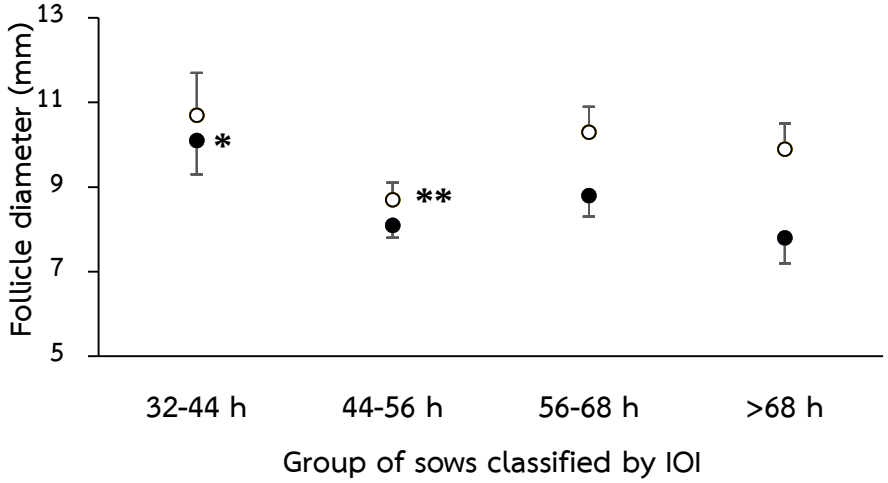


**Figure 3** Maximum follicular size (mm) at 24 h after buserelin injection (●) and preovulatory follicular sizes (○) in treated sows classified by different injection-to-ovulation interval (IOI). *) $P<0.05$ vs. ● in treatment sows with IOI 44-56 h or >68 h, **) $P<0.05$ vs. ○ in treatment sows with IOI 56-68 h

At 24 h after injection, there were no differences in follicle diameter among sows with IOI of 44-56 h, 56-68 h, or >68 h ($P$>0.05). Pre-ovulatory follicle diameter was smaller in sows with IOI of 44-56 h than in sows with IOI of 56-68 h (8.7 ± 0.4 vs. 10.3 ± 0.6 mm, $P$=0.03).

In the treatment group, the relationship between maximum follicular sizes at 24 h after buserelin injection and preovulatory size and estrus/ovulation time (i.e., IEI, IOI, EOI, and WOI) are presented in Table 4. In hot season, the follicular size at 24 h after buserelin injection was negatively correlated with WOI, IEI, and IOI (Table 4). In cool season, the follicular size at 24 h after buserelin injection was negatively correlated with IEI (Table 4).

**Table 4** Relationship between maximum follicular sizes at 24 h after buserelin injection, and preovulatory size and estrus/ovulation time

| | Hot | | Cool | |
|---|---|---|---|---|
| Follicle size | At 24 h | Preovulation | At 24 h | Preovulation |
| Number of sows | 22 | 22 | 16 | 16 |
| Means±SD | 8.9 ± 1.5 | 9.8 ± 1.8 | 7.9 ± 1.3 | 9.3 ± 2.0 |
| Pearson's correlation coefficient (r) | | | | |
| EOI (h) | -0.060 $^{NS}$ | 0.206 $^{NS}$ | 0.154 $^{NS}$ | 0.490 $^{*}$ |
| WOI (h) | -0.363 $^{**}$ | -0.103 $^{NS}$ | 0.050 $^{NS}$ | 0.432 $^{NS}$ |
| IEI (h) | -0.540 $^{**}$ | -0.561 $^{**}$ | -0.597 $^{**}$ | -0.317 $^{NS}$ |
| IOI (h) | -0.360 $^{**}$ | -0.106$^{NS}$ | 0.053 $^{NS}$ | 0.435 $^{NS}$ |

*$P$<0.05, ** $P$<0.01, NS=not significant

BCS, BF, LW, and LL affected ovulation time (Table 5 and Figure 4). BCS influenced EOI ($P$<0.05). Weaned sows with BCS<3 had longer EOI than those with BCS≥3 ($P$=0.01). Backfat thickness at weaning was negatively correlated with IOI (IOI=77.6-1.42(BF), $P$=0.04, Figure 4). Litter weight at weaning was associated with ovulation time ($P$<0.05). Sows weaned with heavy LW (LW≥67 kg) had longer IOI than sows weaned with light LW (LW<67 kg) ($P$=0.02). Litter weight x season interaction affected EOI and WOI. In hot

season, sows weaned with heavy LW (≥67 kg) had longer EOI than sows weaned with light LW (<67 kg), (*P*=0.02).

**Table 5** Effect of body condition score (BCS, n=73), backfat thickness (BF, n=73), lactation length (LL, n=73), and litter weight at weaning (LW, n=73) on estrus and ovulation times

| Variables | Covariates | *P* value | Classification of covariates | Least-squares means ± SEM |
|---|---|---|---|---|
| EOI (h) | BCS | 0.01 | <3 | 56.9 ± 5.0$^{a}$ |
| | | | ≥3 | 46.4 ± 2.6$^{b}$ |
| | Season x LW | 0.03 | Hot, <67 | 43.7 ± 3.2$^{A}$ |
| | | | Hot, ≥67 | 59.9 ± 4.1$^{B}$ |
| | | | Cool, <67 | 54.4 ± 4.9$^{AB}$ |
| | | | Cool, ≥67 | 52.4 ± 3.9$^{AB}$ |
| WOI (h) | Season x LW | 0.002 | Hot, <67 | 136.4 ± 3.5$^{a}$ |
| | | | Hot, ≥67 | 155.3 ± 4.5$^{b}$ |
| | | | Cool, <67 | 146.9 ± 5.4$^{ab}$ |
| | | | Cool, ≥67 | 137.0 ± 4.3$^{a}$ |
| IEI (h) | LL | 0.03 | <20 | 18.6 ± 1.1$^{a}$ |
| | | | ≥20 | 14.7 ± 0.5$^{b}$ |
| | Season x LL | 0.05 | Hot, <20 | 21.3 ± 1.5$^{A}$ |
| | | | Hot, ≥20 | 15.3 ± 0.1$^{B}$ |
| | | | Cool, <20 | 14.3 ± 0.6$^{B}$ |
| | | | Cool, ≥20 | 14.2 ± 0.8$^{B}$ |
| IOI (h) | LL | 0.01 | <20 | 59.9 ± 2.6$^{a}$ |
| | | | ≥20 | 48.5 ± 2.0$^{b}$ |
| | LW | 0.02 | <67 | 52.5 ± 2.7$^{A}$ |
| | | | ≥67 | 58.1 ± 2.9$^{B}$ |
| | BF | 0.04 | n.c. | - |

WEI, Weaning-to-estrus interval; EOI, Estrus-to-ovulation interval; WOI, Weaning-to-ovulation interval; IEI, Injection-to-estrus interval; IOI, Injection-to-ovulation interval. $^{a,b}$ or $^{A,B}$ Least-square means with different superscripts within each factor differ significantly (*P*<0.05); Non-significant effects are not shown in the table. n.c., not classified due to normal distribution. Group and season were included in WEI, EOI, and WOI analysis, but for IEI and IOI, only season was included

Sows weaned with heavy LW in hot season had longer WOI than sows weaned with light LW in hot season (*P*=0.009) and those weaned with heavy LW in cool season (*P*=0.020). Lactation length affected both estrus onset and ovulation time. IEI was affected by LL and LL x season interaction. Short lactating sows (LL<20 days) had longer IEI than long lactating sows (LL ≥20 days) (*P*=0.04). In hot season, short lactating sows had longer IEI than long lactating sows (*P*=0.03). Short lactating sows in hot season had longer IEI than sows weaned in cool season, with either short or long LL (*P*=0.002). In addition, sows with short LL had longer IOI than sows with long LL>20 days (*P*=0.01).

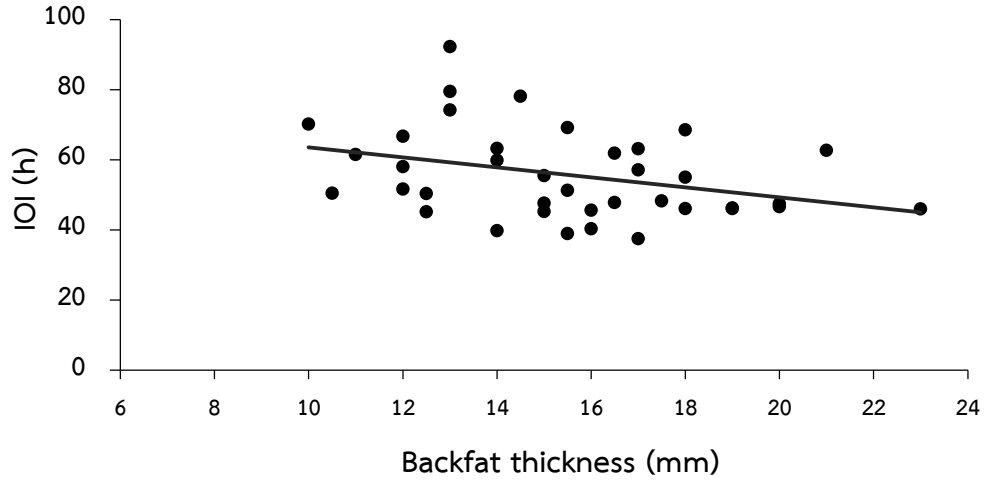


**Figure 4** Relationship between backfat thickness at weaning and injection-to-ovulation interval (IOI) in treatment sows; IOI=77.6-1.42 (BF) ($R^2$ =0.11, *P*=0.04)



Reproductive performance of sows did not differ significantly between control and treatment groups (Table 6). However, some reproductive traits differed between cool and hot seasons (Table 7). For instance, piglet body weight at birth in sows inseminated during cool seasons was higher than that in sows inseminated during hot season (*P*<0.001).

**Table 6** Reproductive performance of the sows in control and treatment groups (Least-squares means ± SEM)

| Variable | Control | Treatment | *P* value |
|---|---|---|---|
| Number of sows | 37 | 40 | - |
| Farrowing rate (%) | 83.3 | 82.5 | 0.923 |
| Total number of piglets born per litter | 12.2 ± 0.6 | 11.4 ± 0.6 | 0.344 |
| Number of piglets born alive per litter | 11.5 ± 0.6 | 10.5 ± 0.6 | 0.220 |
| Number of stillborn piglets per litter | 0.34 ± 0.11 | 0.36 ± 0.11 | 0.862 |
| Number of mummified fetus per litter | 0.37 ± 0.21 | 0.61 ± 0.21 | 0.452 |
| Piglet body weight at birth (kg) | 1.61 ± 0.03 | 1.69 ± 0.03 | 0.153 |
| Lactation length (days) | 20.9 ± 0.4 | 20.1 ± 0.4 | 0.126 |
| Number of piglets weaned per litter | 10.4 ± 0.12 | 10.3 ± 0.13 | 0.400 |
| Piglet body weight at weaning (kg) | 6.0 ± 0.02 | 6.1 ± 0.03 | 0.166 |

**Table 7** Reproductive performance of sows inseminated in hot and cool seasons (Least-squares mean ± SEM)

| Variable | Hot | Cool | *P* value |
|---|---|---|---|
| Number of sows | 43 | 34 | - |
| Farrowing rate (%) | 83.3 | 82.3 | 0.910 |
| Total number of piglets born per litter | 11.9 ± 0.5 | 11.8 ± 0.6 | 0.899 |
| Number of piglets born alive per litter | 10.9 ± 0.6 | 11.1 ± 0.6 | 0.824 |
| Number of stillborn piglets per litter | 0.34 ± 0.10 | 0.36 ± 0.11 | 0.926 |
| Number of mummified fetus per litter | 0.64 ± 0.19 | 0.33 ± 0.22 | 0.312 |
| Piglet body weight at birth (kg) | 1.53 ± 0.03 | 1.76 ± 0.03 | <0.001 |
| Lactation length (days) | 20.9 ± 0.3 | 20.0 ± 0.4 | 0.074 |
| Number of piglets weaned per litter | 10.8 ± 0.1 | 9.9 ± 0.1 | <0.001 |
| Piglet body weight at weaning (kg) | 6.32 ± 0.02 | 5.86 ± 0.03 | <0.001 |



## 2.5 Discussion

The present report incorporates a pilot study that investigated the effects of GnRH agonist, administered at 72 or 84 h after weaning on estrus and ovulation time, and subsequently investigated the effects of season and other factors on estrus and

ovulation time in sows injected with GnRH agonist at 72 h after weaning. On average, WOI decreased by 30 h in treated sows compared to control. Moreover, the variation in ovulation timing among individual sows was reduced. For instance, the standard deviation of WOI was reduced from 24.7 h to 12.6 h. The proportion of sows that ovulated later than 140 h after weaning (68 h after buserelin injection) reduced from 74.3% (control) to 18.4% (treatment). These results are in agreement with a number of previous studies demonstrating that WOI in sows could be shortened by the administration of 50 μg buserelin at weaning (Wongkaweewit et al., 2012), 10 μg at 77 h after weaning (Driancourt et al., 2013), or 10 μg at 94 h or 104 h after weaning (Martinat-Botte et al., 2010). This is because, buserelin injection at the optimal time point after weaning could effectively induce LH surge (Driancourt et al., 2013). However, in the present study, the percentage of sows that ovulated within the expected period (i.e., 32-56 h after GnRH injection) was relatively low (58%) compared to that in the previous studies (Martinat-Botte et al., 2010; Baroncello et al., 2017). The delayed ovulation response in some of these animals might be related to a number of factors, including parity number of sows, BF, BCS, and season. These factors may influence follicle growth and cause a suboptimal follicle size at the time of hormonal injection in these animals. In the present study, pre-ovulatory follicle size influenced IOI. Small follicles in sows after weaning may need more maturation time to reach the pre-ovulatory follicle size before GnRH administration. This finding indicated that delayed ovulation after buserelin injection could be related to poor follicle growth during lactation, poor follicle quality at weaning (e.g., in sows with poor BCS), or follicles that were too small after weaning. Our findings also indicated that, in hot season, follicular size at 24 h after buserelin injection was negatively correlated with WOI, IEI, and IOI. This indicated that sows with larger follicular size at 24 h after buserelin injection had a shorter duration from injection to estrus (IEI) and ovulation (IOI), respectively. In addition, other mechanisms behind poor response to GnRH treatment are discussed below. In the present study, WEI and IEI were delayed in hot season. This is in agreement with previous studies in either tropical (Suriyasomboon et al., 2006) or temperate areas (Yoder et al., 2012). The seasonal effect on sow reproduction was associated with poor follicle steroidogenesis during the hot season (Bertoldo et

al., 2011). This, in turn, may be related to a decreased appetite in the hot season that results in increased lactation weight loss (Gourdine et al., 2004).

Ovulation induction in weaned sows has been investigated by a number of researchers worldwide during recent years to implement a single fixed-time AI in swine industry (Brüssow et al., 2009; Wongkaweewit et al., 2012; Driancourt et al., 2013; Ulguim et al., 2014; Baroncello et al., 2017)A previous study has recommended performing single fixed-time AI at 32 h after buserelin injection (Driancourt et al., 2013). Theoretically, the extended fresh semen of boars shows optimal fertility during the first 24 h after insemination (Sumransap et al., 2007). Therefore, ovulation should take place within 56 h (32 + 24 h) after buserelin injection. In the present study, we presented some novel findings concerning the influence of season on the response of sows to GnRH treatment. For instance, we demonstrated that the percentage of sows that ovulated within 32-56 h after buserelin injection was higher in cool (75%) than hot (48%) seasons. This indicates that high temperature and high humidity during the hot season in Thailand has an effect on GnRH treatment. The reasons could be due to the direct effect of heat stress on the hypothalamus/pituitary function or that hot and/or humid climate compromised follicle growth in post-weaning sows. Lopes et al. (2014) found that the follicles of sows reached the pre-ovulatory size later in hot season than in cool season. To date, the effects of season on ovulation time are still controversial (Weitze et al., 1994; Knox and Zas, 2001; Belstra et al., 2004). However, the study suggested that EOI could vary among farms; therefore, a study based on only one farm was not enough to represent the effect of season on ovulation time (Belstra et al., 2004). In addition, Koketsu et al. (1998) demonstrated that greater average of daily feed intake in sows during lactation was associated with greater concentrations of insulin and glucose, greater LH pulse frequency prior to weaning, and shorter farrowing-to-estrus interval. Johnston et al. (1999) demonstrated that, compared to control environment (mean temperature 20.4 °C), hot environment (mean temperature 29.2 °C) significantly reduced feed intake of sows (6.38 vs. 4.19 kg/day) during lactation, reduced weaning weight of sows (193.6 vs. 176.2 kg), and reduced the percentage of sows displaying estrus by day 15 post-weaning (93.4 vs. 79.2%). Likewise, compared to warm season (mean temperature 23.8 °C), lactating sows in hot season (mean

temperature 26.0 ℃) had a poorer average daily feed intake (−700 g/day), higher body weight loss (17 vs. 12 kg), and a lower growth rate in their piglets (197 vs. 210 g/day) (Gourdine et al., 2006a). In the present study, although an evaporative cooling system is used, the temperature inside the farrowing house averages 28.0 ℃ in hot season, which is 1.8 ℃ higher than that in cool season (26.2 ℃). The high temperature during the hot season may reduce feed intake of sows and increase the proportion of sows with excessive backfat loss during lactation, and subsequently may cause a poor LH pulse frequency and low insulin and glucose concentrations at weaning. Therefore, follicular growth after weaning in these sows might be compromised. Recently, Costermans et al. (2019) demonstrated that a higher weight loss during lactation was also related to a lower percentage of healthy cumulus-oocyte complex. These findings indicate that season or climatic factors may influence feed intake and body weight loss of sows during lactation, and thus, has a significant impact on the response of sows to GnRH treatment.

In the current study, sow conditions, LL and LW affected estrus and ovulation characteristics. In addition, season indirectly affected EOI and WOI by its relationship with LW. After weaning, the BCS of sows has been reported to be associated with follicle size and affected ovulation time (Bracken et al., 2003). Sows with poor BCS at weaning had smaller follicles and longer WOI compared to sows with BCS of more than 2 (Bracken et al., 2003). Backfat is an essential source of estrus cycle-related hormones, such as insulin-like growth factor-1 (IGF-1) (Roongsitthichai et al., 2013) and leptin (De Rensis et al., 2005) which is necessary for the production of reproductive hormone that is associated with granulosa cell proliferation and oocyte development (Silva et al., 2009; Phoophitphong et al., 2017). Moreover, in the present study, we found that sows that were not responding to GnRH treatment (IOI>68 h) had lower BF than those that ovulated before 68 h (13.8 mm vs. 14.9-16.4 mm). Thus, it can be inferred that sows weaned with thick BF are able to respond to ovulation induction better than thin BF sows. In addition, sows nursing a large litter, or those that lost more body protein during lactation had smaller follicles at weaning (Clowes et al., 2003). Clowes et al. (2003) demonstrated that sows that mobilize too much body protein during lactation have a decreased litter growth and ovarian function. Ovarian follicular

development was also the most advanced in sows that lost the least protein, and these sows had the heaviest uterine weight and highest estradiol concentration in the follicular fluid (Clowes et al., 2003). After weaning, small follicles (<6 mm) have lower levels of LH receptor mRNA expression than larger follicles (6 mm) (Liu et al., 2000). In addition, the level or pulse frequency of LH was lower in sows with higher weight loss during lactation (Koketsu et al., 1998). The effect of LL on ovulation time after buserelin injection has not been investigated. However, it was reported that weaned sows with less than 20 days lactation had a longer weaning-to-LH peak than the conventionally weaned sows (Willis et al., 2003) and longer estrus duration than those lactating longer than 20 days (Willis et al., 2003; Belstra et al., 2004). In addition, De Rensis et al. (2005) demonstrated that BF loss in sows during lactation was positively associated with weaning-to-estrus intervals and negatively associated with pregnancy rate. Furthermore, plasma leptin concentrations were higher in sows with high BF thickness (>24 mm) compared to sows with moderate (16-24 mm) and low BF thickness (<16 mm). A recent morphological study has confirmed that leptin exists in different compartments of porcine ovary, including the oocyte, granulosa cells, and corpus luteum, which indicated a close relationship between leptin and ovarian function in pig (Phoophitphong et al., 2017). These findings indicate that the response of sow to GnRH treatment is associated with excessive loss of body weight and BF thickness during lactation.



In conclusion, the use of a 10 μg buserelin injection at 72 h after weaning was able to induce ovulation in weaned sows in a tropical climate. However, the timing of ovulation still varied among sows. Factors significantly influencing the variation of ovulation timing included season, lactation characteristics, and their interaction. Of all the treated sows, 48% and 75% ovulated within the expected period (i.e., 32-56 h after injection) in hot and cool seasons, respectively. Sows that were weaned with lactation length of at least 20 days, litter weight less than 67 kg or BCS of at least 3, had better responses to buserelin injection. High BF reserve after weaning is important for ovulation induction response by buserelin injection.

# CHAPTER III

## Comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of boar semen

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### 3.1 Abstract

The present study investigated the effects of using egg yolk and/or lecithin combinations in semen extender on frozen-thawed sperm qualities in boars. A total of 19 ejaculates from 8 Duroc boars were included. Each ejaculate was aliquoted and cryopreserved in 3 different extenders containing egg yolk (20%) alone (group I), lecithin (6.0%) alone (group II) and a combination of egg yolk (10%) and lecithin (3%) (group III). Frozen-thawed sperm motility, motion characteristics, viability, acrosome integrity, membrane permeability and mitochondria activity were evaluated by a computer assisted sperm analysis system, SYBR14/Ethidiumhomodimer-1, FITC-PNA, sHOST test and JC-1 staining, respectively. The frozen-thawed sperm motility in groups I and III did not differ significantly ($P>0.05$), but both extenders were better than group II ($P<0.05$). The motion characteristics, including straight-line velocity (VSL), linearity (LIN) and wobble coefficient (WOB), were higher in groups I and III than in group II ($P<0.05$). Likewise, the sperm viability, membrane permeability and mitochondria activity were higher in groups I and III than in group II ($P<0.05$). In conclusion, the use of lecithin without egg yolk in cryopreserved boar semen extender impaired frozen-thawed sperm qualities. Thus, using either 20% egg yolk or a combination of 10% egg yolk and 3% lecithin is recommended for cryopreserved boar semen extender.

### 3.2 Introduction

Cryopreservation techniques have been successfully developed to preserve mammalian semen for almost 70 years (Polge et al., 1949). Nevertheless, boar semen cryopreservation is still not completely successful due to poor frozen-thawed semen qualities (Knox et al., 2015). This is mainly due to the fact that the plasma membrane of boar sperm contains a high level of polyunsaturated fatty acids (PUFA), resulting in a high risk of sperm damage after cryopreservation (Maldjian et al., 2005). Therefore, substrates, e.g. egg yolk and/or lecithin, are included in the boar semen cryopreservation extender to protect the sperm plasma membrane from cryo-damage. Egg yolk (15% to 20%) has been used for over 40 years as a standard membrane-modifying agent that is added to traditional semen cryopreservation extender (Phillips and Lardy, 1940; Visser and Salamon, 1974). However, the use of egg yolk as a semen extender ingredient has been considered a risk in terms of bacterial contamination. Moreover, the composition of egg yolk also varies depending on its initial sources (Bousseau et al., 1998). Thus, alternative ingredients, e.g. lecithin and other unsaturated lipids, have been considered as replacements for egg yolk (Aires et al., 2003; Pillet et al., 2012; Moraes et al., 2015).

Lecithin or phosphatidylcholine is one of the phospholipids that is extracted from soybean (Layek et al., 2016). The use of lecithin as a substitution for egg yolk has been successfully developed for many species, i.e. rams and goats (Forouzanfar et al., 2010; Salmani et al., 2014), bulls (Aires et al., 2003), cats (Vick et al., 2012), dogs (Dalmazzo et al., 2018) and pigs (Zhang et al., 2009). However, the use of lecithin to replace egg yolk in the cryopreserved boar semen extender is still rare (Zhang et al., 2009). One reason for its underuse could be due to a limited number of references for pigs and the lack of clearly defined optimal concentrations of lecithin. Theoretically, egg yolk is a source of cholesterol (Faitarone et al., 2013), which can assist the cell membrane in resisting phase transition temperatures (Muller et al., 2008; van Meer et al., 2008). In boar sperm, up to 34% of the total membrane lipids consist of cholesterol, which is necessary for sperm viability, motility and morphology (Am-In et al., 2011; Zaja et al., 2016b). Therefore, the total replacement of egg yolk with other ingredients might

diminish the protective effect from cholesterol. To our knowledge, studies on the effect of the combination of lecithin and egg yolk on frozen-thawed boar semen quality have not been conducted. Therefore, the present study aimed to investigate the effects of lecithin in combination with egg yolk in semen extender on frozen-thawed boar sperm qualities.

## 3.3 Materials and Methods

The experimental procedure was performed according to the Ethical Principles and Guidelines for the Use of Animals, National Research Council of Thailand and was approved by the Chulalongkorn University Animal Care and Use Committee (IACUC) (protocol number 1731012).

### 3.3.1 Animals and experimental design

A total of 19 ejaculates from 8 proven Duroc boars (aged 2 to 4 years) were included in the experiment. Each ejaculate was aliquoted and cryopreserved in three different extenders containing egg yolk (20.0%) (Buranaamnuay et al., 2009) (group I) or containing lecithin alone (6.0%) (group II) (Zhang et al., 2009) or containing a combination of egg yolk (10.0%) and lecithin (3.0%) (group III). All ejaculates were frozen and thawed according to our previous protocol (Buranaamnuay et al., 2009). The frozen-thawed sperm qualities were evaluated and compared between the groups. The semen samples were obtained from proven sire routinely used in the Livestock Research and Breeding Center, Nakornratchasima province of Thailand. The experiment was carried out from November to December 2017. On average, the interval between semen collections was 8.2 ± 2.2 days (range: 5 to 9 days). The sperm rich fraction of the boar semen was collected using the gloved hand method. The ejaculate was kept in a thermos flask and the gelatinous fraction was eliminated using filter paper. After semen collection, the sperm were evaluated for subjective motility under a light microscope at 200x magnification. Semen with a subjective motility below 65.0% and/or with the total number of abnormal sperm morphology ≥ 20.0% were excluded.

### 3.3.2 Extender

The cryopreserved boar semen extenders used in the present study consisted of extenders I, II and III. Extender I was a commercial boar semen extender (Duragen®, Magapor, Zaragoza, Spain) 4.23 g in 100 ml of distilled water. The extender II was divided into 3 groups (i.e. group I, II and III), which contained different amounts of egg yolk, i.e. 20.0% egg yolk alone (group I), 6.0% lecithin alone (group II) and a combination of 10.0% egg yolk plus 3.0% lecithin (group III). Group I (20.0% egg yolk alone) was prepared by including 20 ml of egg yolk in 80 ml of 11.0% lactose solution. Group II (6.0% lecithin alone) was prepared by including 6.0 g of lecithin (L-α-Phosphatidylcholine, p3644, Sigma®, MO, USA) and 20 ml distilled water in 80 ml of 11.0% lactose solution. Group III (a combination of 10.0% egg yolk plus 3.0% lecithin) was prepared by including 10 ml of egg yolk and 3.0 g of lecithin in 10 ml distilled water, in 80 ml of 11.0% lactose solution. The preparation of lecithin solution in distilled water was performed at 50.0 °C and mixed using a homogeniser (HG-15A, Daihan, Gangwon-do, Korea). Additionally, the lecithin solution was treated with an ultrasonic machine (Sonorex Super, Bandelin, Berlin, Germany) at 50.0 °C for 3 h. Extender III was prepared by including extender II (89.5%), glycerol (9.0%) and Equex STM Paste (1.5%, Nova Chemical Sales Inc., MA, USA). The osmolarity of extender III in Group I, II and III were evaluated. The average osmolality of extender III were 1971.3 ± 5.1, 1844.6 ± 6.1 and 1906.7 ± 4.1 mOsmol/kg in group I, II and III, respectively.



### 3.3.3 Freezing and thawing procedure

After collection, the semen was diluted (1:1 [v/v]) using extender I (Duragen®, Magapor, Zaragoza, Spain). The diluted semen was transferred to 50 ml centrifuge tubes, equilibrated at 15 °C for 120 min and centrifuged at 800 × g for 10 min to separate seminal plasma from the sperm cells. The supernatant was discarded, and the sperm pellet was re-suspended (about 1-2:1) using extender II to a concentration of $1.5\times10^9$ sperm per ml. The diluted semen was cooled down to 4.0 °C for 120 min. Then, two parts of the semen were mixed with one part of extender III. The final concentration of semen was approximately $1.0\times10^9$ sperm per ml and contained 3.0% glycerol (Chanapiwat et al., 2009). The processed semen was loaded into 0.5 ml straws

(Bio-Vet, Z.I. Le Berdoulet, France). The straws were sealed with PVC powder before placing in liquid nitrogen vapor at 3 cm above the level of liquid nitrogen for 20 min and then plunged into liquid nitrogen. Thawing was achieved by immersing the straws in water at 50 °C for 12 sec. The semen was diluted (1:4) using extender I. Post-thawed sperm qualities were evaluated after incubation in a 37 °C water bath for 15 min.

**3.3.4 Semen evaluation**

The sperm motility and motion characteristics were evaluated using the computer assisted sperm analysis (CASA) system (SCA® CASA System, MICROPTIC S.L., Barcelona, Spain). The images were taken under a phase contrast microscope with a green filter at 100x magnification. The frozen-thawed semen was diluted with phosphate buffer saline solution (PBS) at a ratio of 1:20. The diluted semen (8 µl) was dropped onto a warmed slide (37.0 °C) and covered with a coverslip. A total of 1,500 sperm cells from five different fields of each sample were randomly selected to determine sperm motility and motion characteristics. The motion characteristics of the spermatozoa, including straight-line velocity (VSL, µm/sec), curvilinear velocity (VCL, µm/sec), average path velocity (VAP, µm/sec), linearity (LIN, %), straightness (STR, %), wobble coefficient (WOB, %), mean lateral head displacement (ALH, µm) and beat cross frequency (BCF, Hz), were obtained using the CASA software.

Sperm viability was evaluated with SYBR-14/Ethidiumhomodimer-1 (EthD-1) (Fertilight®, Sperm Viability Kit, Molecular Probes Europe, Leiden, the Netherlands). Thawed semen (10 µl) was diluted with PBS (140 µl). Then, 50 µl of diluted semen was gently mixed with fluorescence solution, composed of SYBR-14 (2.7 µl) and EthD-1 (10 µl). The mixed semen sample was incubated at 37.0 °C for 20 min. Two hundred stained sperm were evaluated under a fluorescent microscope at 1,000x magnification with an oil objective lens. Under the fluorescent microscope, sperm heads with green luminescence were defined as being intact plasma membranes (live), while red luminescence was defined as damaged membranes (dead). Sperm viability was presented as the percentage of live sperm.

Acrosome integrity was determined by using fluorescein isothiocyanate-labeled peanut agglutinin (FITC-PNA). Briefly, the frozen-thawed semen sample (10 µl) was

mixed with 10 µl of working EthD-1 solution (4.65 µM/ml), then incubated at 37.0 ℃ for 15 min. After incubation, the semen sample (10 µl) was smeared on a glass slide and air dried at room temperature. The dried slides were put into 95% ethanol for 30 sec prior to staining with working FITC-PNA solution (100 µg/ml) at 4.0 ℃ for 30 min in a moist chamber. The FITCPNA-stained slides were rinsed with 4 ℃ PBS and air dried at room temperature. A total of 200 sperm were evaluated under a fluorescent microscope at 1,000x magnification using an oil objective lens. The criteria for assessing the acrosome integrity of the sperm was modified from the previous methodology (Cheng et al., 1996). Acrosome integrity was classified as intact acrosome (expressed bright fluorescence of the acrosome, which indicated outer acrosome membrane integrity) or damaged acrosome. Acrosome integrity was presented as percentages.

The hypo-osmotic swelling test (sHOST) was used to evaluate the sperm plasma membrane permeability (Perez-Llano et al., 2001). The hypo-osmotic solution was prepared with fructose and Na-citrate in distilled water until the final osmolality was 75.0 mOsm/kg. The osmolality of the solution was measured by freezing point depression. The frozen-thawed semen sample (100 µl) was mixed with 1,000 µl of hypo-osmotic solution and incubated at 38.0 ℃ for 30 min. Thereafter, the sperm were fixed with 1,000 µl of hypo-osmotic solution with 5.0% formaldehyde (Merck, Darmstadt, Germany). A well-mixed sample (10 µl) was placed on a glass slide with a coverslip. A total of 200 sperm were evaluated under a light microscope with 400x magnification. The coiled tail sperm were defined as sperm with functional sperm membranes. The proportion of sperm with functional sperm membranes was presented as a percentage.

Mitochondria activity was assessed using tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Molecular Probes, Molecular Probes Inc., Eugene, OR, USA). Stock solutions were prepared as follows: 0.153 mM JC-1 in DMSO, 2.4 mM PI in PBS and 0.02 mM SYBR14 in DMSO. Staining solution was made by mixing the stock solution containing JC-1 (2 µl), PI (1.6 µl), SYBR-14 (1 µl) and HEPES-buffer solution (95 µl). The semen sample (12.5 µl) was gently mixed with the staining solution (25 µl), then incubated at 37.0 ℃ for 30 min. One drop (8 µl) of stained sample was placed on a glass slide covered by a coverslip. Sperm mitochondrial activity was examined under

a fluorescent microscope at 1,000x magnification. Green fluorescence of mitochondrial expression was considered low mitochondrial activity, whereas orange fluorescence of mitochondrial expression was considered high mitochondrial activity (Cossarizza et al., 1996; Garner et al., 1997).

### 3.3.5 Statistical analysis

Statistical analysis was performed using Statistical Analysis Systems version 9.0 (SAS Institute Inc., 1996; Cary, NC, USA). Data were presented as means ± SEM. The normality of all variables was evaluated by the UNIVARIATE procedure. Factors affecting frozen-thawed sperm motility, sperm viability, acrosome integrity, mitochondria activity, sperm membrane permeability and motility characteristics were analyzed using general linear mixed models under the PROC MIXED procedure of SAS. The semen extender group (group I, II and III) was included in the model as a fixed effect, and boar identity was included in the model as a random effect. Differences in least squares means between the groups were compared using a least significant difference (LSD) test. A significant difference was defined as $P<0.05$.

## 3.4 Results



### 3.4.1 Sperm motility and motion characteristics

Frozen-thawed sperm motility and progressive motility in the different extenders are presented in Table 8. Frozen-thawed total motility of the boar sperm was higher in groups I and III than in group II (37.8 ± 2.2%, 37.9 ± 2.7% and 26.2 ± 2.8%, respectively; $P<0.01$). Likewise, the progressive motility was higher in groups I and III than in group II (15.7 ± 1.7%, 13.7 ± 1.4% and 8.4 ± 1.3 %, respectively; $P<0.01$). Moreover, the percentage of rapid motile sperm was higher in groups I and III than in group II (10.8 ± 1.4%, 9.5 ± 0.9% and 5.8 ± 0.8%, respectively; $P<0.05$).

**Table 8** Means ± standard errors of frozen-thawed sperm motility (%), sperm viability (%), acrosome integrity (%), membrane permeability (%) and mitochondria activity for three groups (n=19 ejaculates per group).

| Sperm parameter | Group I | Group II | Group III |
|---|---|---|---|
| Sperm motility parameters | | | |
| Total motility (%) | 37.8 ± 2.2[a] | 26.2 ± 2.8[b] | 37.9 ± 2.7[a] |
| Progressive motility (%) | 15.7 ± 1.7[a] | 8.4 ± 1.3[b] | 13.7 ± 1.4[a] |
| Rapid motile (%) | 10.8 ± 1.4[a] | 5.8 ± 0.8[b] | 9.5 ± 0.9[a] |
| Medium motile (%) | 8.2 ± 0.7[a] | 5.9 ± 0.7[b] | 8.9 ± 0.6[a] |
| Slow motile (%) | 18.8 ± 0.6[a] | 15.1 ± 0.9[c] | 21.9 ± 1.0[b] |
| Sperm viability (%) | 44.2 ± 1.8[a] | 32.6 ± 2.0[b] | 42.1 ± 1.9[a] |
| Acrosome integrity (%) | 59.8 ± 2.7 | 52.1 ± 2.6 | 58.3 ± 3.0 |
| Membrane permeability (%) | 31.7 ± 1.3[a] | 23.3 ± 1.3[b] | 31.6 ± 1.3[a] |
| Mitochondria activity (%) | 49.3 ± 3.1[a] | 35.0 ± 3.0[b] | 47.2 ± 2.6[a] |

Values followed by different letters within the same row were significantly different ($P<0.05$).

The frozen-thawed sperm motion characteristics are demonstrated in Figure 5. Sperm VSL was higher in group I than in groups II and III (11.8 ± 0.9%, 6.7 ± 0.7%, 9.2 ± 0.9%, respectively; $P<0.05$). However, the sperm VSL was higher in group III than in group II ($P<0.05$). Sperm VCL, VAP and STR were higher in group I than in group II ($P<0.05$, Figure 5). The sperm LIN and WOB in groups I and III were higher than in group II ($P<0.05$, Figure 5). The sperm BCF was higher in group I than in groups II and III ($P<0.05$).



### 3.4.2 Sperm viability, acrosome integrity, mitochondrial activity

The frozen-thawed sperm quality, assessed by fluorescence staining in different extenders, is presented in Table 8. Frozen-thawed sperm viability was higher in groups I and III than in group II (44.2 ± 7.1%, 42.1 ± 7.2% and 32.6 ± 8.1%, respectively, $P<0.05$). Likewise, the sperm membrane permeability was higher in groups I and III than in group

II ($P$<0.05). The sperm mitochondrial activity was higher in groups I and III than in group II ($P$<0.05). However, the acrosome integrity was not different among groups ($P$>0.05).

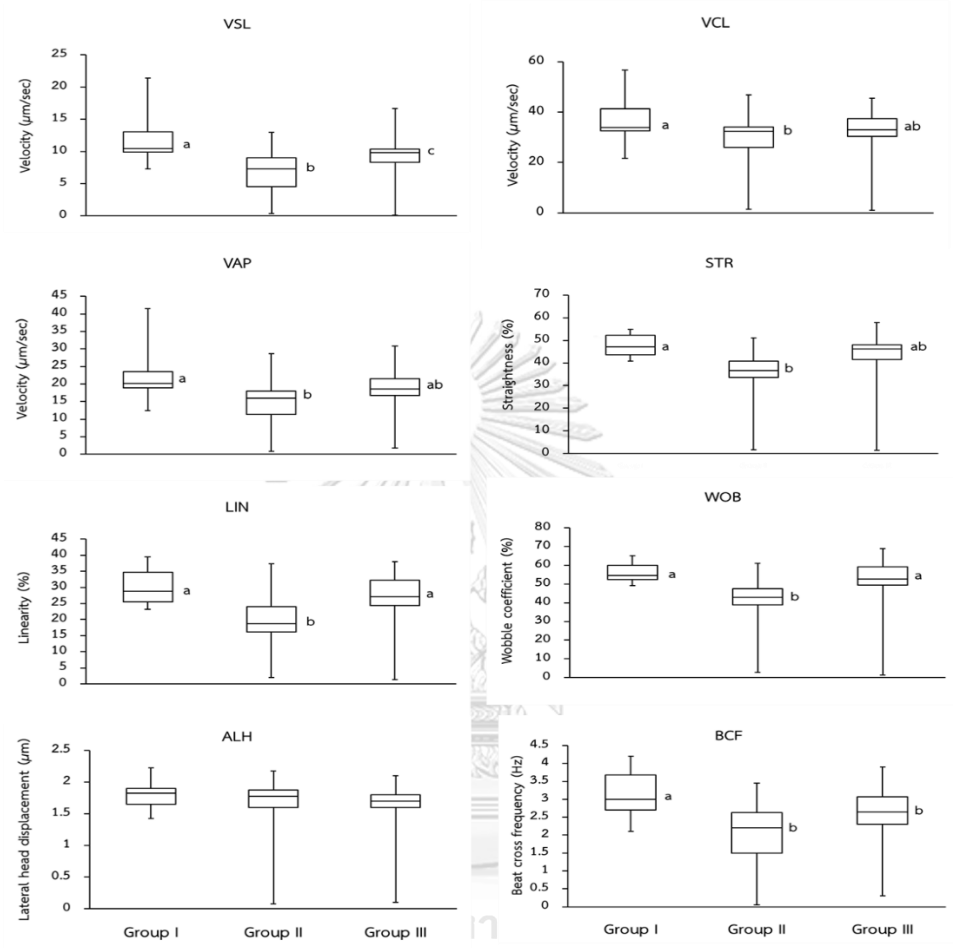


**Figure 5** Motion characteristics of frozen-thawed sperm in group I (20.0% egg yolk), II (6.0% lecithin) and III (10.0% egg yolk and 3.0% lecithin). Different letters within each motion characteristic were significantly different ($P$<0.05).

### 3.5 Discussion

The present study demonstrated that the use of 6.0% lecithin to replace egg yolk in cryopreservation extender significantly compromised the quality of frozen-thawed boar sperm. This is in contrast with Zhang et al. (2009), who found that using 6.0% soybean lecithin for boar semen cryopreservation extender resulted in a higher total sperm motility, higher plasma membrane integrity and higher acrosome integrity than

using 20.0% egg yolk. In fact, the precise mechanism by which soybean lecithin protects sperm during the cryopreservation process remains unclear (Zhang et al., 2009). However, it was hypothesized that lecithin might reduce the cholesterol/phospholipids ratio of the sperm cell membrane by permeating into the sperm membrane (Zaja et al., 2016a). As a consequence, capacitation-like changes during the freezing process are controlled and thus, the freezing ability of the boar sperm increases (Gamzu et al., 1997). Another hypothesis is that phospholipids from egg yolk or soybean lecithin might integrate with sperm membrane to form a protective film against the formation of lethal intracellular ice crystals (Quinn et al., 1980). Therefore, the sperm membrane is protected from mechanical damage during the freezing and thawing process. However, in the previous study (Zhang et al., 2009), the frozen-thawed boar sperm qualities were significantly reduced when the concentration of lecithin was increased from 6.0% to 9.0% or 12.0%. In the present study, the use of 3.0% lecithin in combination with 10.0% egg yolk resulted in better frozen-thawed boar sperm motility, sperm viability, acrosome integrity and mitochondria activity, compared with the use of 6.0% lecithin alone. These data indicate that lecithin can be used as an egg yolk substitution, but the concentration of lecithin should be concerned. Furthermore, the source of lecithin used in the present study was different from the source of lecithin used in the previous study (Zhang et al., 2009). In the previous study, soybean lecithin (obtained from Unicorn Co., Ltd., Beijing, China) was purified through mixing with ethanol, centrifuged and a drying process, while the source of lecithin used in the present study was a purified lecithin purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). Therefore, either the purity or the concentration might be different. These data indicated that the cryo-protective effect of lecithin may also vary depending on the source and the purifying process of the lecithin. Moreover, both the previous study (Zhang et al., 2009) and the present findings indicate that too high of a concentration of lecithin in the semen extender compromises frozen-thawed boar sperm quality.

In the present study, the synergistic effect of egg yolk and lecithin on frozen-thawed sperm quality was not found in boars (i.e. groups I and II did not differ significantly). However, the synergistic effect of lecithin and egg yolk (1.25:2.00) and

lecithin and cholesterol (4:1) on frozen-thawed sperm motility has been demonstrated in stallions (Nouri et al., 2013) and humans (Mutalik et al., 2014). These data indicate that the cryo-protective effect of the egg yolk and lecithin combination may vary depending on species. In boars, we could not demonstrate a synergistic effect of egg yolk and lecithin. Blanch et al. (2014) have demonstrated that the frozen-thawed boar sperm motility did not differ significantly between extenders containing 20.0% or 10.0% egg yolk. Likewise, the present study also found that the frozen-thawed boar sperm quality did not differ significantly between the extenders containing 20.0% egg yolk and 10.0% egg yolk plus 3.0% lecithin. These data indicate that 10.0% egg yolk is a minimum concentration that require for protecting the boar sperm during the cryopreservation. Additionally, the effect of the lecithin and egg yolk ratios on the frozen-thawed boar sperm qualities should be investigated further to determine the optimal concentration of lecithin in combination with egg yolk.

In the present study, lecithin, as well as the egg yolk combined with lecithin extenders, did not improve the motion characteristics of the boar spermatozoa, i.e. VSL, VCL, VAP, STR, LIN, WOB, ALH and BCF, compared to the traditional egg yolk base extender. This is the first report demonstrating the association between lecithin and motion characteristics of the frozen-thawed boar sperm. Interestingly, the use of a high concentration of lecithin (i.e. 6.0%) significantly reduced the motion activities of boar sperm, as indicated by VCL, ALH, STR, LIN and BCF. This could be due to the texture of the lecithin extender, having a higher viscosity than the traditional egg yolk extender. Furthermore, in the previous study, lecithin also had a lethal effect on the inner mitochondria membrane, which interfered with the mitochondria function (Del Valle et al., 2012). Likewise, the egg yolk-based extender also resulted in higher sperm viability and membrane permeability compare to the lecithin-based extender in bulls (Muino et al., 2007; Singh et al., 2018). In the present study, the frozen-thawed sperm viability and sperm membrane integrity was lowest with the lecithin base extender (group II).

In conclusion, the use of lecithin without egg yolk in cryopreserved boar semen extender impaired frozen-thawed sperm qualities. Thus, using either 20.0% egg yolk or

a combination of 10.0% egg yolk and 3.0% lecithin is recommended for cryopreserved boar semen extender.

# CHAPTER IV

## Reproductive performance of weaned sows after single fixed-time artificial insemination under a tropical climate: Influences of season and insemination technique

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### 4.1 Abstract

We evaluated the reproductive performance of sows after single fixed-time AI under a tropical climate and investigated the influences of season and insemination technique on the efficacy of single fixed-time AI. After weaning, the sows were divided into CONTROL (n=212) and FIXED-TIME (n=212) groups. Sows in the CONTROL group were inseminated at 12 and 36 h after the onset of estrus, while sows in the FIXED-TIME group were administered 10 μg of GnRH at 72 h after weaning and were inseminated 32 h later. Reproductive performance parameters, including total born, born alive, mummified foetuses and stillborn piglets per litter, piglet birth weight, variation of piglet birth weight within litter, regular return-to-estrus and farrowing rate, were compared between the two groups. Season was classified into two groups: cool (n = 170) and hot (n=254), and insemination technique was classified into two groups: conventional AI (n=171) and intra-uterine insemination (IUI) with a reduced number of spermatozoa (n=253). On average, regular return-to-estrus (3.3 vs. 5.6%, *P*>0.05) and farrowing rates (92.8 vs. 88.1%, *P*>0.05) did not differ between CONTROL and FIXED-TIME groups. However, the total born and born alive piglets per litter in the FIXED-TIME were lower than in the CONTROL group (12.0 vs. 12.8 piglets/litter; *P*=0.030 and 11.3 vs. 12.2 piglets/litter, *P*=0.007). Interestingly, the number of total born piglets in the FIXED-TIME group was lower than in the CONTROL group only in the sows inseminated in the hot season (11.7 ± 0.32 and 12.9 ± 0.31, respectively, *P*=0.005). Piglet birth weight, variation of piglet birth weight within litter, number of piglets at weaning and body weight of piglets at weaning did not differ between groups, irrespective of the

season ($P$>0.05). The total number of piglets born per litter in the FIXED-TIME group was lower than that in the CONTROL group in sows inseminated via IUI (11.7 ± 0.32 and 12.9 ± 0.31, respectively, $P$=0.013), but not in sows inseminated using conventional AI (12.7 ± 0.42 and 12.5 ± 0.41, respectively, $P$=0.772). Single fixed-time AI could be successfully performed in sows under a tropical climate, with a promising reproductive performance. However, a decreased litter size at birth after single fixed-time AI was observed when insemination was performed in the hot season. Moreover, single fixed-time AI using IUI with a reduced number of spermatozoa also decreased litter size at birth.

## 4.2 Introduction

The use on exogenous hormones to induce estrus and ovulation in sows consists of an administration of eCG at weaning and some other hormones (e.g., GnRH, pLH or hCG) at 72-80 h later (Cassar et al., 2005; Brüssow et al., 2009). In the swine industry (commercial herd), a single treatment of a combination of 400 IU eCG and 200 IU hCG, i.e. PG600® is the preferred medication and has been shown being effective for induction of estrus in both gilt and sow (Brüssow et al., 2009; Tummaruk et al., 2011). In some studies, a single hormone, i.e. GnRH and pLH, has also been used to induce ovulation in sows by administration around the onset of standing estrus (Martinat-Botte et al., 2010; Ulguim et al., 2014). The use of these hormones has been proven to induce the pre-ovulatory LH surge and ovulation (Driancourt et al., 2013; Ulguim et al., 2014), and they have therefore been recommended to be used as a practical tool for fixed-time AI in pigs (De Rensis and Kirkwood, 2016). In the last decade, this approach has been established in the swine industry worldwide (Driancourt et al., 2013; Fontana et al., 2014; Ulguim et al., 2014; Ulguim et al., 2016; Baroncello et al., 2017). However, many aspects associated with the efficacy of fixed-time AI in commercial farms still need to be further investigated.

Martinat-Botte et al. (2010) found that after GnRH treatment at 94 h after weaning, 100% of sows ovulated over a 24 h time window, while only 66.7% of the sows ovulated during a 24 h period when GnRH was applied at 104 h after weaning. The

study concluded that administration of 10 μg of GnRH at 94 h after weaning in sows can tighten the synchrony of ovulation, thereby enabling the establishment of a single fixed-time AI in sows. Thereafter, a clinical study demonstrated that a single fixed-time AI in sows between 30 and 33 h, after 10 μg GnRH administration at 86 ± 3 h after weaning, resulted in a farrowing rate of 87.0% and a total number of 13.6 piglets born per litter (Driancourt et al., 2013). However, the sows that did not exhibit standing estrus after the GnRH treatment (10%) were not inseminated. In addition, Baroncello et al. (2017) found that the farrowing rate of sows that were single fixed-time AI after the GnRH treatment with standing estrus rate was 88.6%, while those inseminated regardless of estrus behaviour accounted for 82.2%. Ulguim et al. (2014) found that administration of 5.0 mg pLH at the onset of estrus and a single fixed-time AI at 16 h later resulted in a farrowing rate of 92.0% and a total number of 13.3 piglets per litter. The farrowing rate of sows after single-fixed time AI was compromised when AI was performed outside the optimal time, i.e. 0-24 h before ovulation (Fontana et al., 2014; Ulguim et al., 2016). Based on previous studies, factors influencing the reproductive performance of sows submitted to single fixed-time AI include the timing and doses of GnRH administration, the number of inseminations after ovulation induction and the timing of insemination in relation to the timing of ovulation (Martinat-Botte et al., 2010; Driancourt et al., 2013; Fontana et al., 2014; Ulguim et al., 2014; Ulguim et al., 2016; Baroncello et al., 2017). However, no study on the efficacy of single fixed-time AI in different seasons has been performed so far.



It is well-established that season influences the reproductive performance of sows (Belstra et al., 2004; Tummaruk et al., 2010). Prolonged estrus durations and estrus-to-ovulation intervals can be detected in some sows during summer (Belstra et al., 2004). A previous study has found that the follicle diameter of sows at weaning, estrus and ovulation during summer was smaller than in winter (Lopes et al., 2014). In Thailand, inferior farrowing rates and poor litter sizes at birth are observed in sows inseminated during the hot season (Tantasuparuk et al., 2000b; Tummaruk et al., 2010). High ambient temperature and high relative humidity during gestation significantly reduce the number of total piglets born per litter (Tummaruk et al., 2010).

The reduction of sperm cells used per AI is a challenging concept to increase the efficacy of the genetic potential of boars. Techniques such as a reduction in the number of AI from 2-3 times to single-time during standing estrus (Cassar et al., 2005) or using post-cervical insemination allow AI with a reduced number of sperm cells, namely from 3.0 to 1.0 billion sperms per dose (Watson and Behan, 2002; Sumransap et al., 2007; Tummaruk and Tienthai, 2010). Intra-cervical AI is the conventional insemination procedure in pigs and involves the deposition of the semen dose into the posterior portion of the cervical canal (Roca et al., 2006). Generally, 1.5 to 4.0 billion sperms, in a volume of 80-100 ml, are used for conventional AI in sows (Roca et al., 2006). Intra-uterine insemination (IUI) in pigs has been developed for almost 20 years to reduce the number of sperm cells per dose and to decrease semen back-flow (Watson and Behan, 2002; Sumransap et al., 2007; Hernandez-Caravaca et al., 2012). Therefore, when performing single fixed-time AI using IUI, the number of sperm cells per dose could be further reduced. A previous study has demonstrated reduced farrowing rate and litter size at birth when performing single fixed-time AI using IUI with semen back-flow (>5 ml) compared to insemination without semen back-flow (78.1 vs. 92.2% and 11.4 vs. 12.6 piglets/litter) (Fontana et al., 2014). However, different AI techniques (i.e. conventional AI and IUI) in sows submitted to single fixed-time AI have not been investigated.

Based on a number of studies, the reproductive performance of sows after single fixed-time AI is rather promising (Driancourt et al., 2013; Fontana et al., 2014; Ulguim et al., 2014; Ulguim et al., 2016; Baroncello et al., 2017). Nevertheless, in Thailand, fixed-time AI has not been performed so far, and the association between single fixed-time AI and the seasonal influences has not been investigated. Therefore, the present study was performed to evaluate reproductive performances of sows after single fixed-time AI under a tropical climate and to investigate the influences of season and insemination technique on the efficacy of single fixed-time AI in sows.

## 4.3 Materials and methods

### 4.3.1 Experimental design

The sows were randomly selected at weaning and were divided into two treatment groups: CONTROL (n=212) and FIXED-TIME group (n=212). The sows in both CONTROL and FIXED-TIME groups had a similar parity number and backfat loss during lactation (see below). The experiment was conducted weekly for 10 weeks in cool season and 13 weeks in hot season and the sows of all groups were included weekly in each season. Sows in the CONTROL group were inseminated at 12 and 36 h after the onset of estrus, while sows in the FIXED-TIME group were administered 10 μg (2.5 ml) of GnRH (buserelin 4.0 μg/ml, Receptal®, Merck Animal Health, NJ, USA) at 72 h after weaning and were inseminated using a single dose of semen 32 h later. Reproductive performance, i.e. number of total-born piglets, live-born piglets, mummified foetuses, stillborn piglets, piglet birth weight, variation of piglet birth weight, regular return-to-estrus, farrowing rate, litter size at weaning and litter weight at weaning in CONTROL and FIXED-TIME groups were compared. Additionally, the efficacy of fixed-time AI, associated with seasons and insemination techniques, was determined. Season was classified into two groups: cool (n=170) and hot (n=254), and insemination technique was classified into the two groups conventional AI (n=171) and IUI (n=253).



### 4.3.2 Animals, general management and backfat thickness measurement

The experiment was conducted in a swine commercial herd with 2,500 sows on production, located in the Southern part of Thailand. The experiment followed the guidelines documented in the Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes, edited by the National Research Council of Thailand, and was approved by the Institutional Animal Care and Use Committee (IACUC) in accordance with the university regulations and policies governing the care and use of experimental animals, approval no. 1731011. Both gestating and lactating sows were housed in an evaporative cooling system housing. In total, 424 Landrace x Yorkshire F1 crossbred weaned sows with parity numbers 1-5 were included in the study. The parity number

of sows was balanced between CONTROL and FIXED-TIME groups. The means ± SD of parity number were 3.0 ± 1.2 and 3.0 ± 1.1 in CONTROL and FIXED-TIME groups, respectively. Backfat thickness of sows at weaning were 11.1 ± 1.7 and 11.8 ± 1.8 mm in CONTROL and FIXED-TIME groups, respectively. Backfat loss during lactation did not differ significantly between CONTROL and FIXED-TIME groups (*P*=0.625). The experiment was performed during the hot (March and April) and the cool (November-December and January-February) seasons. The temperatures inside the barn during cool and hot seasons were 26.2 ± 0.13 (range: 23.3-28.6) and 28.0 ± 0.57 °C (range: 24.9-31.6), respectively. The average humidity levels inside the barn in cool and hot seasons were 77.3 ± 1.99 and 77.7 ± 1.35%, respectively. On average, lactation length was 21.6 ± 2.8 days. The lactation lengths were 21.9 ± 2.8 days and 21.3 ± 2.8 days in cool and hot seasons, respectively. On the day of weaning, the sows were moved to the mating house and kept in individual crates (0.6 x 2.0 m) on a concrete-slatted floor. The sows were fed with 2.5-3.0 kg/day of a corn-soybean-based diet (15.0% crude protein, 0.85% digestible lysine and 3.0 Mcal/kg metabolisable energy). After AI, the sows were moved to the gestation house and kept in the individual crates until 1 week before farrowing. The gestating sows were fed with 1.5-3.5 kg of feed per day with a corn-soybean-based diet (16.0% crude protein, 1.0% digestible lysine and 2.8 Mcal/kg metabolisable energy). The gestation diet was gradually increased to 3.0-3.5 kg/day during 12-15 weeks of gestation, and the amount of feed was gradually declined during the last week of gestation. Gestating sows were moved to the farrowing house approximately 1 week before the expected date of parturition. The sows were placed in individual crates (0.6 x 2.2 m) at the centre of the farrowing pens (2.0 x 2.2 m). Lactating sows were fed with 5.0-6.0 kg/day of a corn-soybean-based diet containing 18.0% crude protein, 1.0% digestible lysine and 3.2 Mcal/kg metabolisable energy, 3-4 times a day. Health was routinely determined by the herd veterinarian. Water was provided ad libitum via water nipples. The backfat thickness of sows was measured via A-mode ultrasonography (Renco Lean-Meater®, Minneapolis, MN, USA) at farrowing, weaning and insemination. Measurements were taken at the level of the last rib at 6-8 cm from the midline, on both sides of the sows. The average between the left and the right sides was calculated. Backfat losses during lactation (i.e. from farrowing to

weaning) were calculated. The relative backfat loss in each sow was defined as the backfat loss (mm) divided by backfat at farrowing and multiplied by 100, expressed as a percentage.

**4.3.3 Estrus detection and artificial insemination (AI)**

Estrus detection of all sows was carried out from the day after weaning by experienced stockpersons and performed twice a day at 0700 h and 1600 h, using the back pressure test in the presence of a mature boar. The sows showing a standing reflex, i.e. accepting the back pressure test, arching their backs, showing cocked ears and immobilised legs, were considered in estrus. In the CONTROL group, sows were inseminated twice at 12 and 36 h after the detection of estrus. In the FIXED-TIME group, ovulation was induced by an intramuscular administration of 10 μg (2.5 ml) of GnRH at 72 h after weaning. Thereafter, the sows were inseminated at 32 h after the GnRH administration. The sows in the FIXED-TIME group were inseminated regardless of exhibiting estrus or not. Additionally, the sows were also divided into two sub-groups: conventional AI (CONTROL 88 sows and FIXED-TIME 83 sows) and IUI (CONTROL 124 sows and FIXED-TIME 129 sows). In the conventional AI group, a standard foam tip catheter was inserted into the vagina and fixed at the cervix to deposit a semen dose of $3.0x10^9$ motile sperms in 100 ml volume. In the IUI group, an intra-uterine catheter (Magaplus S®, Magapor, Zaragoza, Spain) was used to deposit the semen dose of $1.5x10^9$ motile sperms in 50 ml volume. The pooled semen used for all sows was obtained from three Duroc boars aged 1 to 3 years, collected by the gloved hand method; only the ejaculated semen with a subjective motility above 70% after collection was used. Sperm concentration was evaluated using a spectrophotometer (Spermacue®, Minitübe GmbH, Tiefenbach, Germany). The ejaculates were diluted with a semen extender (Bio Pig®, Magapor, Zaragoza, Spain) to obtain a final concentration of $30x10^6$ sperms/ml. Diluted semen was stored at 17 °C and were used within 24 h.

**4.3.4 Data**

The proportion of sows returning to estrus was determined by estrus detection using boar contact twice a day from three days after insemination onwards. The sows

returning to estrus between 18-24 days after insemination were defined as ‘regular return to estrus’, and the sows returning to estrus from 25 days after insemination onwards were defined as ‘irregular return to estrus’. Regular return rate was defined as the number of regular return sows divided by the number of inseminated sows, multiplied by 100. Farrowing rate was defined as the number of farrowing sows divided by the number of inseminated sows, multiplied by 100. At farrowing day, the sows were allowed to farrow naturally. Reproductive data collected included the total number of piglets born per litter, number of piglets born alive per litter, number of mummified foetuses and stillborn piglets per litter, body weight of the piglet at birth and body weight of piglets at weaning. Body weight at birth was measured immediately after the piglet was born, using a digital scale (SDS® IDS701-CSERIES, SDS Digital Scale Co. Ltd., Yangzhou, China). Litter birth weight of the piglet was calculated by summing the individual piglet birth weights. The variation in birth weight within litter was defined as the coefficient of variation (CV) of the body weight at birth of the piglets within the litter. Live-born piglets with a body weight at birth of <1,300 g, 1,300-1,700 g and >1,700 g were classified as light, moderate and heavy piglets, respectively.

**4.3.5 Statistical analysis**

The data were analysed by using the Statistical Analysis System (SAS version 9.0, Cary, NC, USA.). Descriptive statistics on reproductive data of sows in CONTROL and FIXED-TIME groups were calculated by using the MEANS procedure of SAS. Frequency analysis on categorical traits, i.e. birth weight classes, regular return rate and farrowing rate, was carried out using the FREQ procedure of SAS. Categorical data (i.e. regular return-to-estrus rate and farrowing rate) was compared by logistic regression using GLIMMIX macro of SAS. The factors included in the statistical models were group (CONTROL and FIXED-TIME), season (HOT and COOL), insemination technique (AI and IUI) and two-ways interaction. Sow identity was included in the model as a random effect. Least square means were obtained from each class of the variables and compared by using the least significant difference test. Continuous traits (i.e. birth weight of the individual piglets) were analysed by using the general linear mixed model (MIXED) procedure of SAS. The statistical models included fixed effects of group

(CONTROL and FIXED-TIME), season (HOT and COOL), insemination technique (AI and IUI) and two-ways interaction. Sow identity was included in the model as a random effect. Least square means were obtained from each class of the variables and compared by using the least significant difference test.

Reproductive performance of sows, including total number of piglets born per litter, number of piglets born alive per litter, number of mummified foetuses and stillborn piglets per litter, litter birth weight, variation of litter birth weight within litter, number of piglets and body weight of piglets at weaning, were analysed by using the general linear model (GLM) procedure of SAS. Additionally, parity number of sows, gestation length, lactation length, backfat thickness before farrowing and relative backfat loss during lactation were also compared among groups by using the GLM procedure of SAS. The factors included in the statistical models were group (CONTROL and FIXED-TIME), season (HOT and COOL), insemination technique (AI and IUI) and two-ways interaction. Levels of significance for independent variables and interactions included in the statistical models are presented in Table 9. Least square means were obtained from each class of the variables and compared by using the least significant difference test.

## 4.4 Results

Levels of significance for independent variables and interactions included in the statistical models are presented in Table 9.

**Table 9** Levels of significance for independent variables included in the statistical models

| Dependent variables | Group | Season | Technique | G × S | G × T |
|---|---|---|---|---|---|
| Gestation length (days) | 0.784 | 0.649 | <0.001 | 0.094 | 0.463 |
| Regular return-to-estrus (%) | 0.251 | 0.125 | 0.808 | 0.923 | 0.446 |
| Farrowing rate (%) | 0.105 | 0.116 | 0.813 | 0.338 | 0.635 |
| Total born | 0.030 | 0.498 | 0.411 | 0.006 | 0.013 |
| Born alive | 0.007 | 0.484 | 0.598 | 0.002 | 0.005 |
| Born dead | 0.153 | 0.926 | 0.421 | 0.495 | 0.538 |
| - Stillborn | 0.209 | 0.984 | 0.814 | 0.337 | 0.906 |
| - Mummified foetus | 0.380 | 0.898 | 0.412 | 0.711 | 0.557 |
| Piglet birth weight (g) | 0.316 | 0.108 | <0.001 | 0.441 | 0.023 |
| - Low (%) | 0.276 | 0.015 | <0.001 | 0.880 | 0.977 |
| - Moderate (%) | 0.332 | <0.001 | 0.407 | 0.014 | 0.179 |
| - High (%) | 0.859 | <0.001 | <0.001 | 0.008 | 0.194 |
| CV of piglet birth weight (%) | 0.569 | <0.001 | 0.013 | 0.029 | 0.167 |
| Piglets weaned/litter | 0.561 | 0.226 | 0.206 | 0.315 | 0.774 |
| Body weight at weaning (kg) | 0.245 | <0.001 | 0.490 | 0.226 | 0.418 |

Interaction effect of Groups and Seasons (G × S), Groups and Techniques (G × T), level of significance was defined at $P<0.05$



### 4.4.1 Reproductive performances of sows and piglet traits after single fixed-time AI

Descriptive data on sow reproductive traits before and after AI in the CONTROL and FIXED-TIME groups are presented in Table 10. On average, lactation length, backfat loss during lactation and wean-to-service interval did not differ significantly between groups. The percentages of sows that showed standing estrus before insemination were 100 and 94.8% in the CONTROL and FIXED-TIME groups, respectively ($P<0.001$). In the FIXED-TIME groups, 11 sows (5.2%) were inseminated without signs of standing estrus

during insemination. Of these sows (n=11), none of them returned to estrus at a regular interval (0%) and 9/11 sows (81.8%) farrowed. The average numbers of total piglets born per litter and the number of piglets born alive per litter of these sows (i.e. 9 sows) were 12.6 ± 2.5 and 12.0 ± 2.9, respectively.

On average, the sows lost 8.2 ± 8.8% of backfat during the lactation period. The percentages of sows that lost backfat ≥ 20% during lactation were 8.9% (n=19 sows) and 8.0% (n=17) in the CONTROL and FIXED-TIME groups, respectively (*P*=0.727). Across the treatments, the regular return rates and farrowing rates of sows that lost backfat ≥ 20% (n=36) were 5.5 and 91.7%, respectively. Likewise, the total number of piglets born per litter and the number of piglet born alive per litter of the sows that lost backfat ≥ 20% during lactation were 12.6 ± 2.8 and 12.3 ± 2.8, respectively.

Regular return-to-estrus rate (3.3 vs. 5.6%, *P*>0.05) and farrowing rate (92.8 vs. 88.1%, *P*>0.05) did not differ between CONTROL and FIXED-TIME groups. However, the total number of piglets born per litter and the number of piglets born alive per litter in the FIXED-TIME group were lower than in the CONTROL group (12.0 vs. 12.8 piglets/litter, respectively; *P*=0.030 and 11.3 vs. 12.2 piglets/litter, respectively, *P*=0.007). Figure 6 demonstrates the total number of piglets born per litter in the CONTROL and FIXED-TIME groups by season and insemination type. Total piglet number born per litter was the lowest in sows in the single fixed-time AI group that were inseminated by using IUI in the hot season (*P*<0.05) (Figure 6). The piglet traits at birth, i.e. body weight at birth of the piglets and CV of piglet birth weight within the litters, did not differ significantly between CONTROL and FIXED-TIME groups. Likewise, the number of piglets at weaning and body weight at weaning of the piglets did not differ between groups (Table 10).

**Table 10** Reproductive data of sows in conventional artificial insemination (CONTROL, n=212) and fixed-time artificial insemination (FIXED-TIME, n=212) groups (least square means ± SEM).

| Variables | CONTROL | FIXED-TIME | *P* value |
|---|---|---|---|
| **Before insemination** | | | |
| Parity number | 3.0 ± 0.07 | 3.1 ± 0.07 | 0.541 |
| Backfat loss during lactation (%) | 8.4 ± 0.67 | 8.0 ± 0.65 | 0.625 |
| Sows that lost backfat ≥20% during lactation (%) | 8.9 | 8.0 | 0.727 |
| Lactation length (days) | 21.5 ± 0.19 | 21.6 ± 0.19 | 0.570 |
| Wean-to-service interval (days) | 4.0 ± 0.03 | 3.9 ± 0.03 | 0.052 |
| Sows exhibiting estrus at the moment of AI (%) | 100[a] | 94.8[b] | <0.001 |
| **After insemination** | | | |
| Gestation length (days) | 116.8 ± 0.11 | 116.7 ± 0.11 | 0.743 |
| Regular return-to-estrus (%) | 3.3 | 5.6 | 0.244 |
| Farrowing rate (%) | 92.8 | 88.1 | 0.099 |
| Total born | 12.8 ± 0.24[a] | 12.0 ± 0.25[b] | 0.030 |
| Born alive | 12.2 ± 0.24[a] | 11.3 ± 0.24[b] | 0.007 |
| Born dead | 0.53 ± 0.09 | 0.71 ± 0.09 | 0.153 |
| - Stillborn | 0.25 ± 0.05 | 0.34 ± 0.05 | 0.209 |
| - Mummified foetus | 0.27 ± 0.07 | 0.36 ± 0.07 | 0.385 |
| Piglet birth weight (g) | 1650.9 ± 0.02 | 1643.1 ± 0.02 | 0.783 |
| - Low (%) | 19.7 | 22.5 | 0.054 |
| - Moderate (%) | 39.4 | 36.3 | 0.074 |
| - High (%) | 40.8 | 41.1 | 0.872 |
| CV of piglet birth weight (%) | 23.3 ± 0.90 | 22.8 ± 0.84 | 0.688 |
| Lactation length (days) | 20.2 ± 0.18 | 20.0 ± 0.20 | 0.507 |
| Number of piglets weaned/litter | 10.9 ± 0.08 | 10.8 ± 0.09 | 0.561 |
| Body weight at weaning (kg) | 6.2 ± 0.04 | 6.1 ± 0.04 | 0.127 |

[a,b] Different superscript within row differ significantly ($P<0.05$)

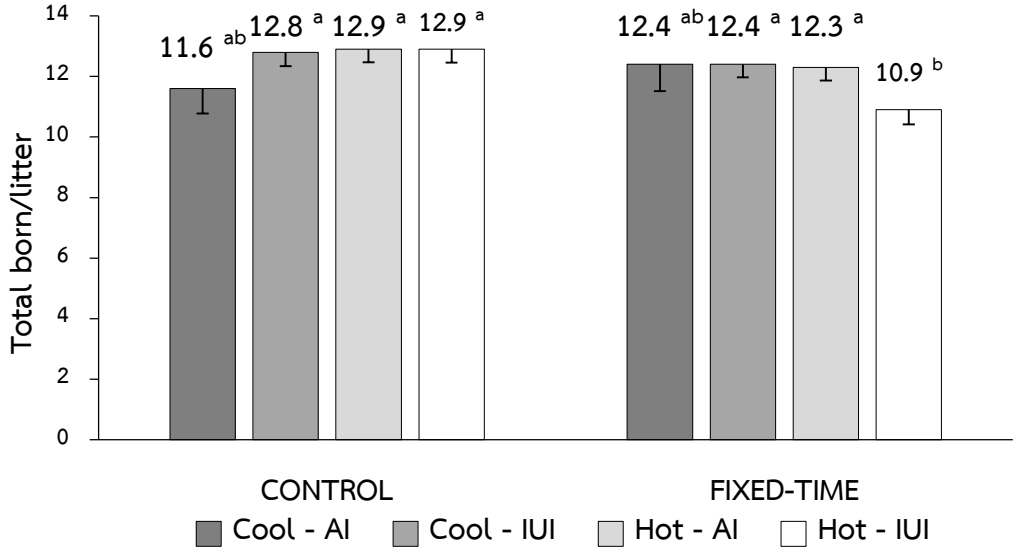


**Figure 6** Total number of piglets born per litter of sows in CONTROL and FIXED-TIME groups in cool and hot seasons by AI techniques (AI and IUI), $^{a, b}$ different superscripts indicate significant differences ($P$<0.05).

### 4.4.2 Seasonal influences on the efficacy of single fixed-time AI

The reproductive performance of sows after single fixed-time AI in cool and hot seasons is presented in Table 11. Both regular return-to-estrus rate and farrowing rate did not differ significantly between CONTROL and FIXED-TIME groups, irrespective of the season. However, the interaction effect between group and season on the total number of piglets born per litter and the number of piglets born alive per litter was significant ($P$<0.05). The total number of piglets born per litter in the FIXED-TIME group was lower than in the CONTROL group in the hot season (11.7 ± 0.32 and 12.9 ± 0.31, respectively, $P$=0.005), but not in the cool season (12.7 ± 0.42 and 12.4 ± 0.42, respectively, $P$=0.621) (Figure 7a). Likewise, the number of live-born piglets per litter in the FIXED-TIME group was lower than in the CONTROL group in the hot season (11.0 ± 0.32 and 12.4 ± 0.30, respectively, $P$=0.003), but not in the cool season (Table 11). The piglet traits, including birth weight and CV of piglet birth weight, did not differ between FIXED-TIME and CONTROL groups in both hot and cool seasons (Table 11). However, the sows that were inseminated during the hot season had a lower CV of piglet birth weight than the sows that were inseminated during the cool season in both CONTROL and FIXED-TIME groups (Table 11).

**Table 11** Reproductive performance of sows in conventional artificial insemination (CONTROL) and fixed-time artificial insemination (FIXED-TIME) groups by seasons (least square means ± SEM).

| Variables | Cool | | Hot | |
|---|---|---|---|---|
| | CONTROL | FIXED-TIME | CONTROL | FIXED-TIME |
| Number of sows | 81 | 89 | 131 | 123 |
| Gestation length (days) | 116.4 ± 0.2[a] | 116.6 ± 0.2[ab] | 116.9 ± 0.1[b] | 116.7 ± 0.1[ab] |
| Regular return-to-estrus (%) | 4.9 | 7.8 | 2.3 | 4.1 |
| Farrowing rate (%) | 88.8 | 86.5 | 95.3 | 89.3 |
| Total born | 12.4 ± 0.42[ab] | 12.7 ± 0.42[ab] | 12.9 ± 0.31[a] | 11.7 ± 0.32[b] |
| Born alive | 12.1 ± 0.40[a] | 11.7 ± 0.38[ab] | 12.4 ± 0.30[a] | 11.0 ± 0.32[b] |
| Born dead | 0.44 ± 0.15 | 0.74 ± 0.14 | 0.57 ± 0.11 | 0.69 ± 0.12 |
| - Stillborn | 0.21 ± 0.08 | 0.38 ± 0.08 | 0.28 ± 0.06 | 0.32 ± 0.07 |
| - Mummified foetus | 0.23 ± 0.12 | 0.35 ± 0.11 | 0.29 ± 0.09 | 0.37 ± 0.10 |
| Piglet birth weight (g) | 1702.0 ± 0.03 | 1661.1 ± 0.03 | 1618.2 ± 0.03 | 1623.4 ± 0.02 |
| - Low (%) | 21.3 | 23.1 | 18.7 | 22.1 |
| - Moderate (%) | 30.3[a] | 32.9[ac] | 45.6[b] | 39.0[c] |
| - High (%) | 48.4[a] | 43.9[ac] | 35.7[b] | 38.9[bc] |
| CV of piglet birth weight (%) | 28.0 ± 1.35[a] | 24.8 ± 1.23[a] | 20.1 ± 1.12[b] | 21.4 ± 1.06[b] |
| Piglets weaned/litter | 10.9 ± 0.14 | 10.9 ± 0.13 | 10.8 ± 0.10 | 10.7 ± 0.12 |
| Body weight at weaning (kg) | 5.7 ± 0.05[a] | 5.7 ± 0.05[a] | 6.4 ± 0.04[b] | 6.4 ± 0.05[b] |

[a,b,c] Different superscripts within row differ significantly ($P<0.05$)



Interestingly, backfat loss during the weaning-to-estrus interval in sows inseminated in the hot season was higher than that in sows inseminated in the cool season (8.8 ± 0.6% vs. 6.0 ± 0.7%, $P$=0.001). Likewise, the proportion of sows losing more than 10% of backfat during the weaning-to-estrus interval in the hot season was also higher than in the cool season (5.9 vs. 0.6%, respectively; $P$=0.005).

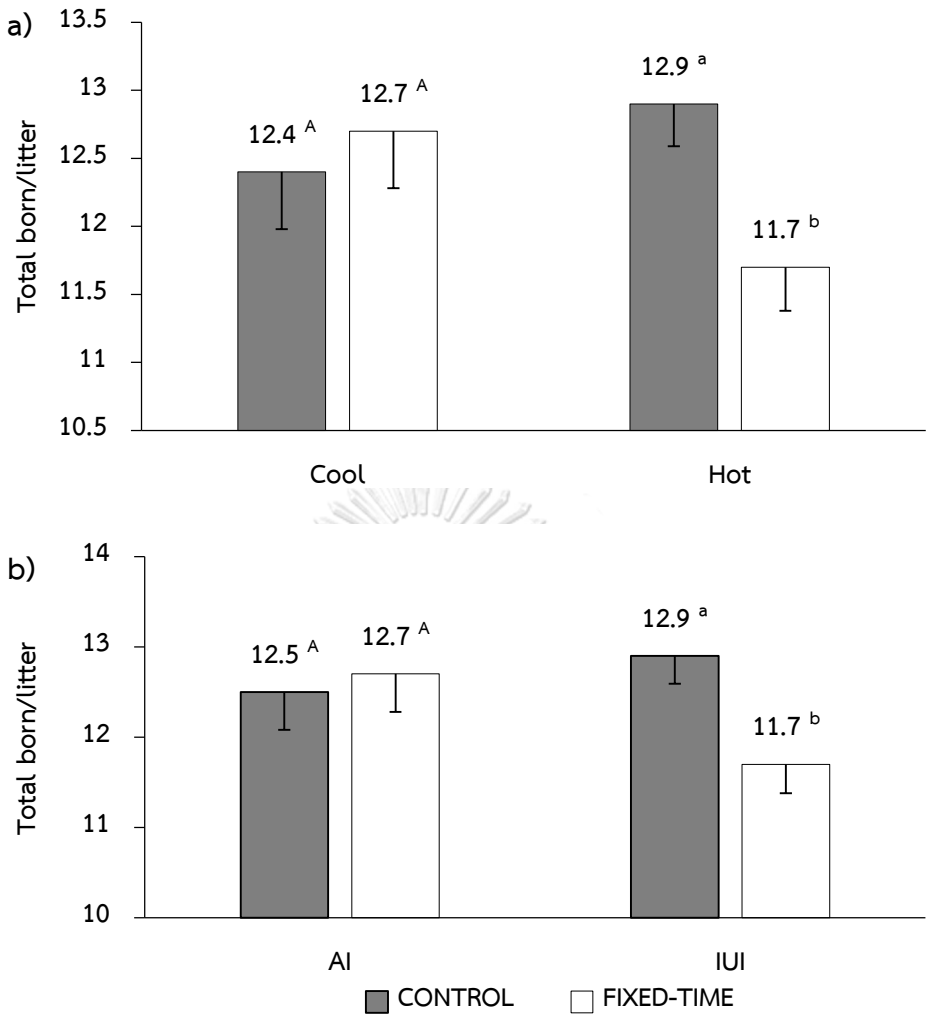


**Figure 7** Total number of piglets born per litter in sows inseminated via conventional artificial insemination (CONTROL) compared with sows inseminated via single fixed-time AI (FIXED-TIME) by season of insemination (a.) and AI technique (b.), $^{A \text{ or } a, b}$ Values with different superscripts within group differ significantly ($P<0.05$).

Backfat loss data during the weaning-to-service interval and the proportion of sows losing more than 10% of backfat during the weaning-to-service interval in hot and cool seasons (CONTROL and FIXED-TIME) are presented in Figure 8, showing that the backfat loss of sows during this period was more pronounced in the FIXED-TIME group than in the CONTROL group (Figure 8).

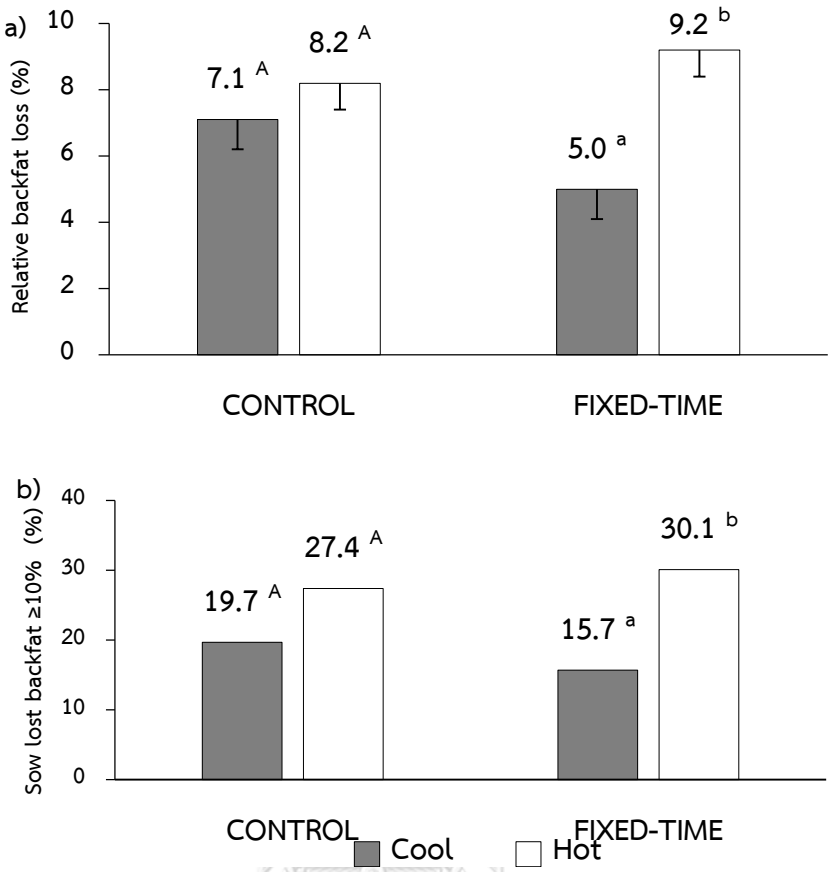


**Figure 8** Average backfat loss during the weaning-to-service interval and the proportion of sows losing backfat ≥ 10% during the weaning-to-service interval in sows inseminated via conventional AI (CONTROL) compared with sows inseminated via single fixed-time AI (FIXED-TIME) by season of insemination, $^{A \text{ or } a, b}$ Values with different superscripts within group differ significantly ($P<0.05$).



### 4.4.3 Influence of insemination technique on the efficacy of single fixed-time AI

The reproductive performance of sows after single fixed-time AI using a conventional AI and IUI with a reduced number of sperm cells is presented in Table 12. Both regular return-to-estrus rate and farrowing rate did not differ significantly between CONTROL and FIXED-TIME groups, irrespective of the use of conventional AI or IUI. However, the total number of piglets born per litter in the FIXED-TIME group was lower than in the CONTROL group in sows inseminated via IUI (11.7 ± 0.32 and 12.9 ± 0.32, respectively, $P$=0.013), but not in sows inseminated using conventional AI (12.7 ± 0.42 and 12.5 ± 0.41, respectively, $P$=0.772) (Figure 7b). Likewise, the number

of live-born piglets per litter in the FIXED-TIME group was lower than that in the CONTROL group in sows inseminated using IUI (11.1 ± 0.32 and 12.3 ± 0.31, respectively, *P*=0.006), but not in sows inseminated using conventional AI (Table 12). The piglet traits, including birth weight, CV of piglet birth weight, number of piglets at weaning per litter and body weight of piglets at weaning, did not differ between the two groups and insemination techniques (Table 12). In the CONTROL group, the CV of the piglet birth weight in the conventional AI was lower than that for sows inseminated by using IUI (19.1 ± 1.7 vs. 24.8 ± 1.0, respectively; *P*=0.005). On the other hand, in the FIXED-TIME group, the CV of the piglet birth weight did not differ between AI and IUI (21.3 ± 1.35 vs. 23.8 ± 1.04, respectively; *P*=0.142).

**Table 12** Reproductive performance of sows in conventional artificial insemination (CONTROL) and fixed-time artificial insemination (FIXED-TIME) by techniques (least square means ± SEM).

| Variables | Conventional AI | | Intra-uterine insemination (IUI) | |
|---|---|---|---|---|
| | CONTROL | FIXED-TIME | CONTROL | FIXED-TIME |
| Number of sows | 88 | 83 | 124 | 129 |
| Gestation length (days) | 117.2 ± 0.17$^{a}$ | 117.2 ± 0.18$^{a}$ | 116.4 ± 0.14$^{b}$ | 116.3 ± 0.14$^{b}$ |
| Regular return-to-estrus (%) | 3.4 | 3.6 | 3.2 | 6.9 |
| Farrowing rate (%) | 93.0 | 89.1 | 92.7 | 87.5 |
| Total born | 12.5 ± 0.41$^{ab}$ | 12.7 ± 0.42$^{ab}$ | 12.9 ± 0.32$^{a}$ | 11.7 ± 0.32$^{b}$ |
| Born alive | 12.1 ± 0.38$^{ab}$ | 11.6 ± 0.39$^{ab}$ | 12.3 ± 0.31$^{a}$ | 11.1 ± 0.32$^{b}$ |
| Born dead | 0.56 ± 0.14 | 0.81 ± 0.14 | 0.50 ± 0.11 | 0.65 ± 0.12 |
| - Stillborn | 0.27 ± 0.08 | 0.35 ± 0.08 | 0.24 ± 0.06 | 0.34 ± 0.06 |
| - Mummified foetus | 0.28 ± 0.11 | 0.45 ± 0.12 | 0.26 ± 0.09 | 0.30 ± 0.09 |
| Piglet birth weight (g) | 1517.8±0.03$^{a}$ | 1614.2±0.03$^{c}$ | 1697.7±0.02$^{b}$ | 1661.3±0.02$^{bc}$ |
| -Low (%) | 25.8$^{a}$ | 27.2$^{a}$ | 17.6$^{b}$ | 19.6$^{b}$ |
| -Moderate (%) | 41.4 | 35.4 | 38.7 | 36.9 |
| -High (%) | 32.6$^{a}$ | 37.3$^{a}$ | 43.7$^{b}$ | 43.5$^{b}$ |
| CV of piglet birth weight (%) | 19.1 ± 1.73$^{a}$ | 21.3 ± 1.35$^{ac}$ | 24.8 ± 1.03$^{b}$ | 23.8 ± 1.04$^{bc}$ |
| Piglets weaned/litter | 10.8 ± 0.13 | 10.6 ± 0.1 | 10.9 ± 0.10 | 10.9 ± 0.11 |
| Body weight at weaning (kg) | 6.3 ± 0.06$^{a}$ | 6.2 ± 0.07$^{a}$ | 6.0 ± 0.05$^{b}$ | 6.0 ± 0.05$^{b}$ |

$^{a,b,c}$ Different superscripts within row differ significantly (*P*<0.05)

## 4.5 Discussion

The present study demonstrates a successful model for performing a single fixed-time artificial insemination in sows under tropical conditions. Single fixed-time AI was performed at 32 h after administration of 10 μg of GnRH agonist. Interestingly, the timing for GnRH administration used in the current study was at 72 h after weaning, which is earlier than specified in a number of previous studies (i.e. 84-96 h after weaning) (Martinat-Botte et al., 2010; Driancourt et al., 2013). Indeed, the optimal time for GnRH administration for ovulation induction depends on the follicle development after weaning. Knox (2015b) has suggested that the follicles responding to GnRH administration should be at least 6.5 mm in diameter. Our preliminary study found that at 78 h after weaning, average follicle diameter was 9.0 ± 1.8 mm (range: 6.4-13.4 mm). Interestingly, the diameter of the follicles in the sow's ovaries at 78 h after weaning were 9.6 ± 0.5 and 7.6 ± 0.7 mm in cool and seasons ($P$=0.034), respectively (Pearodwong and Tummaruk, 2019). Furthermore, the weaning-to-service interval of sows in this herd was as short as 3.9 days, and most of the sows were inseminated within 4 days post-weaning. Therefore, the time for GnRH administration in sows was 72 h after weaning. Indeed, our pilot study has found that the ovulation time in sows after administration of a GnRH at 72 or 84 h after weaning did not differ significantly (i.e., the interval from GnRH administration to ovulation were 58.1 and 54.4 h in 72 and 84 h groups, respectively, (Pearodwong et al., 2019). Furthermore, Knox et al. (2014) have demonstrated that administration of GnRH gel (triptorelin) at 72 or 84 h after weaning significantly reduced the interval from weaning-to-ovulation compared with the administration of GnRH at 96 h after weaning and untreated sow. Using the selected single fixed-time protocol, the percentage of sows with a regular-return-to-estrus rate and farrowing rate did not differ significantly between the control and single fixed-time AI groups. Additionally, neither season nor AI technique influenced the fertility outcome (i.e. conception rate and farrowing rate) of sows after single fixed-time AI. To our knowledge, a comparison of the farrowing rates of sows after single fixed-time AI between hot and cool seasons has not been reported. In the present study, the farrowing rates of sows after single fixed-time AI were 89.3 and 86.5% in hot and cool

seasons, respectively. In Brazil, the farrowing rates of sows after single fixed-time AI during the spring season (March-May) were 82.2% (Baroncello et al., 2017) and 88.0% (Ulguim et al., 2016). In Germany, Spain and France, the farrowing rate of 213 sows from six commercial swine herds after single fixed-time AI was 87.0% (Driancourt et al., 2013). These data indicate that single fixed-time AI in sows is a valid approach at commercial scales, with a promising fertility outcome. Additionally, the present study revealed that the onset of GnRH treatment in sows for single fixed-time AI could be induced as early as 72 h after weaning without compromising the fertility outcome.

Interestingly, the litter traits were influenced by the interaction between group (CONTROL and FIXED-TIME) and season (cool and hot). Litter size at birth was decreased in sows that were single fixed-timed-inseminated in the hot season. Generally, in Thailand, sows inseminated during the hot season have a significantly lower litter size at birth compared to sows inseminated during the cool season (Tantasuparuk et al., 2000b; Tummaruk et al., 2010) mainly because of the high ambient temperature and/or high humidity during gestation in hot seasons. However, the specific mechanism of seasonal infertility related to ovarian follicular growth is not clearly known. Lopes et al. (2014) have found that sows weaned in summer had a smaller follicle size both at weaning and at the onset of estrus than sows weaned in winter. Bertoldo et al. (2010) have demonstrated that the blastocyst cell numbers derived from oocytes collected during summer were lower than those from oocytes collected during winter. Moreover, the proportion of sows with delayed of ovulation was higher in summer than in winter (Lopes et al., 2014). In general, the boar semen had an optimal fertility window during 0-24 h after insemination (Steverink et al., 1999). In the single fixed-time AI group, when delayed ovulation was more evident during the hot season, the viable spermatozoa at the time of ovulation might have been decreased. Therefore, some oocytes may not be fertilized. Furthermore, the oocyte of the later ovulating follicles may become a less developed embryo (Xie et al., 1990). In the present study, GnRH administration was performed at 72 h after weaning in both hot and cool seasons. Therefore, it might be possible that the diameter of follicles from sows weaned in the hot season was still too small at the time of GnRH administration. Thus, to improve litter size at birth in sows submitted to single fixed-

time AI in the hot season, follicle size at the time of GnRH administration should be carefully determined. Furthermore, there is also a strong evidence for the hypothesis that reduced embryonic survival in association with summer infertility in sows may be due to a reduction in DNA integrity of the spermatozoa (Pena et al., 2017; Pena et al., 2019). Moreover, boars exposed to a controlled hot-room environment, direct sunlight or ambient temperatures ranging from 30 °C to 40 °C for 3-90 days had a decrease in sperm motility, normal morphology and sperm concentration. Gilts bred with semen from heat-stressed boars had a decreased embryonic survival during the first month of pregnancy (Pena et al., 2017). In the present study, decreased litter size at birth was observed in the single fixed-time AI group only in the hot season; this might be associated with an early embryonic loss due to either heat stress, poor semen quality or poor DNA integrity of the spermatozoa. Thus, increasing the number of spermatozoa per dose for sows inseminated during the hot season might overcome this problem.

In the present study, the interaction between treatment group and insemination technique was significant for both the total number of piglets born per litter and the number of piglets born alive per litter (Table 9). Sows inseminated with a motile sperm dose of $3.0x10^9$, using a single fixed-time AI protocol, had the same litter size at birth as the control group. On the other hand, the single fixed-time AI, using IUI with a reduced number of spermatozoa ($1.5x10^9$ motile sperms per dose), resulted in a lower litter size at birth compared with the control. In previous studies in Brazil, the litter size at birth of sows after single fixed-time AI was not different compared to that of control sows inseminated using conventional AI (Fontana et al., 2014; Ulguim et al., 2016; Baroncello et al., 2017). A previous study has demonstrated that litter size at birth of sows after single fixed-time AI, using IUI with a reduced number of spermatozoa, can be compromised by the amount of semen back-flow (Fontana et al., 2014; Ulguim et al., 2016; Baroncello et al., 2017). Single fixed-time AI sows with semen back-flow (≥5 ml) resulted in a reduced litter size compared to sows without semen back-flow (11.4 vs. 12.6 (Fontana et al., 2014); 10.4 vs. 12.5 (Ulguim et al., 2016)). Additionally, Knox et al. (2017) have demonstrated that a single post-cervical insemination in sows with 2.5 billion motile sperm per dose resulted in a greater farrowing rate and higher litter size at birth than insemination with 1.5 billion sperm

per dose. These data indicate that single fixed-time AI with a reduced number of spermatozoa could result in poor litter size at birth. Fontana et al. (2014) Fontana et al. (2014) have suggested that a second insemination should be performed in sows presenting more than 5 ml of semen back-flow after single fixed-time AI.

Embryonic survival is associated with the energy level in the sow diet during the pre-mating period (Ferguson et al., 2006). This implies that the decreased litter size in the present study may be related to the sow nutritional status after weaning. Here, a certain proportion of sows losing backfat during the weaning-to-estrus interval was detected in the hot season in the control (27.4%) or single fixed-time AI groups (30.1%), possibly because a hot climate decreases sow appetite and therefore leads to weight loss (Gourdine et al., 2006b). Interestingly, in the single fixed-time AI group, the proportion of sows with decreased backfat thickness from weaning to estrus in the hot season was higher than in the cool season (30.1 vs. 15.7%). Additionally, previous studies have found that supplementation of dextrose in the feed during the weaning-to-estrus interval can improve embryo survival and piglet uniformity (Ferguson et al., 2006; Van den Brand et al., 2006). This leads us to infer that the reduced litter size in the single fixed-time AI group during the hot season could be associated with early embryonic loss due to poor oocyte quality in the sows with excessive loss of backfat from the weaning-to-estrus interval. Therefore, energy supplementation and/or an increased feeding level in post-weaning sows during the hot season should be considered to compensate for the backfat loss during lactation in the hot season.

In conclusion, single fixed-time AI was successfully performed in commercial swine herds under a tropical climate, with a promising reproductive performance. However, compromised litter size at birth after single fixed-time could be observed when insemination was performed in the hot season. Moreover, single fixed-time AI, using the IUI technique with a reduced number of sperm cells, also resulted in decreased litter size at birth. These data indicate that the fecundity outcome of sows after single fixed-time AI could be varied according to the season and the insemination technique. Furthermore, the optimal protocol for fixed-time AI in sows during the hot season as

well as associated factors, e.g. the response of sows to GnRH treatment and backfat loss during the weaning-to-estrus interval, should be further investigated.

# CHAPTER V

# GENERAL DISCUSSION

The present study has successfully established the single fixed-time AI protocol by study on ovulation induction of the weaned sows with GnRH agonist incorporated with study on factors influencing on reproductive performances of the sows after single fixed-time insemination. Additionally, the present study has also investigated the possibility to improve frozen-thawed semen quality by using lecithin to replace egg yolk in cryopreservation extender.

## 5.1 Timing of GnRH administration after weaning

Previous studies in Europe (Brüssow et al., 2009; Driancourt et al., 2013) and in Brazil (Ulguim et al., 2014; Baroncello et al., 2017) have recommended to inject GnRH at 83-89 h after weaning. However, in the present study, the wean-to-estrus interval of sows were relatively short (3.9 days). This implies that the timing of GnRH administration should be modified according to the reproductive performance of sows in the herd. Knox (2015b) documented that the minimum diameter of follicle that respond to LH is than 6.5 mm. In our pilot study performed in the present herd, the average diameter of follicle at 90 h after weaning was 8.6 mm (Pearodwong and Tummaruk, 2019). Thus, the present pilot study in Chapter II showed that the administration of GnRH at both 72 or 84 h effectively reduced ovulation. Regarding to the major population of sows showing estrus at 3-4 days post weaning, the timing of GnRH administration at 72 h after weaning was selected. However, to introduce the new ovulation induction protocol to the new herd, we suggested that the GnRH injection timing should be decided based on weaning-to-estrus interval of the selected herd and a preliminary study on follicle size determination after weaning should be considered.

**5.2 Success of ovulation induction**

In the present study, induction of ovulation using GnRH at 72 h after weaning successfully induced ovulation of weaned sows. The ovulation of the treatment sows occurred 30 h earlier than spontaneous ovulation. Also, the variation of ovulation time among sows in treatment group was also smaller than control group. According to recommendation by Kemp and Soede (1996) the insemination should be performed at 0-24 h before ovulation. In the present study, the average ovulation time of the treatment sows was at approximately 56 h after GnRH injection. This indicates that the insemination can be performed at 32 h after GnRH injection similarly to the previous studies (Driancourt et al., 2013; Baroncello et al., 2017). However, in the present study, the proportion of sows that ovulated within the expected time frame (32-56 h) after GnRH injection was relatively low (58%) compared to the previous studies (Martinat-Botte et al., 2010; Baroncello et al., 2017). The delayed ovulation response of some sows in the present study may be influenced by various factors (see below).

**5.3 Seasonal effect on the efficacy of ovulation induction and single fixed-time AI**



The present study demonstrated that high temperature and humidity conditions in hot season (in Thailand) potentially affected the GnRH treatment and efficacy of single fixed-time AI. In the GnRH treated group, the percentage of sow ovulated between 32 and 56 h after GnRH injection in cool season was 27% higher than in hot season. Likewise, in hot season, the total born of single fixed-time AI sows was compromised compared to conventional AI. This indicates heat stress affected the hypothalamus/pituitary function which potentially compromise the follicle growth after weaning (Einarsson et al., 2008). A previous study reported that the follicles at weaning, at standing estrus and at ovulation time of weaned sows in summer were smaller than in winter (Lopes et al., 2014). This implies that follicle of sows weaned in summer may need more time to reach the pre-ovulartory size (i.e., diameter >6.5 mm). The oocyte of delayed ovulating follicles may become a less developed embryo (Xie

et al., 1990). Generally, in Thailand, sows inseminated during the hot season have a significantly lower litter size at birth compared to sows inseminated during the cool season, mainly because of the high ambient temperature and/or high humidity during gestation in hot seasons (Tantasuparuk et al., 2000b; Tummaruk et al., 2010). Hot season also compromises the development of embryo in which blastocyst cell number derived from oocyte collected during summer is lower than those collected during winter (Bertoldo et al., 2010). From these reasons, in the present study, the administration of GnRH at 72 h were done in both hot and cool seasons may result in the different response in follicle development. During the experiment, it might be possible that the follicle of some sows in hot season were still too small at the time of GnRH treatment. Thus, to improve the efficacy of ovulation induction or fixed-time insemination, the follicle diameter at the time of GnRH administration should be carefully determined.

**5.4 Effect of insemination technique on the efficacy single fixed-time AI**

The efficacy of single fixed-time AI was affected by using intrauterine insemination with a reduced number of sperms per dose. It was found that the number of the total born and born alive piglets of the sows submitted to single fixed-time AI using intrauterine AI catheter was affected by the number of sperm inseminated per dose (Knox et al., 2017). Single fixed-time AI sow inseminated with $1.5x10^9$ sperm per dose has total born and born alive piglets lower than those inseminated with $2.5x10^9$ (12.8 vs. 14.1 and 11.6 vs. 12.7, respectively) (Knox et al., 2017). Moreover, a previous study showed that the litter size of single fixed-time AI sows using reduced number of sperm could be compromised if >5 ml semen backflow was present (Fontana et al., 2014; Ulguim et al., 2016). Thus, single fixed-time AI using a reduced number of sperm per dose should be avoid. Fontana et al. (2014) suggested that a second insemination should be performed in sows presenting more than 5 ml of semen backflow after single fixed-time AI.

**5.5 Other factors affecting the response of sows to ovulation induction**

The present study showed that sows weaned with well body conditions i.e., with body condition score at least 3 or with high backfat thickness showed an earlier ovulation and better response to GnRH than those weaned with a lower body condition score and poor backfat thickness. Body condition score of sows has been reported to be associated with follicle size and also affects ovulation time (Bracken et al., 2003). Sows with poor body condition score at weaning have a smaller follicles and a longer weaning-to-ovulation interval compared to sows with a body condition score of more than 2 (Bracken et al., 2003). Backfat thickness is associated with an essential source of estrus cyclicity-related hormones, such as insulin-like growth factor-1 (IGF-1) (Roongsitthichai et al., 2013), which is necessary for the production of reproductive hormone that associated with granulosa cell proliferation and oocyte development (Silva et al., 2009). To induce the ovulation effectively, the body condition and the backfat thickness of sows need to be in the good uniformity. The present results showed that the factors during lactation also affected the response of GnRH administration. However, the effect of lactation length on ovulation time after GnRH administration has not been investigated. The previous studies reported that sows weaned before 20 days of lactation had a longer weaning-to-LH peak interval (Yoder et al., 2012) and had longer estrus duration (Willis et al., 2003; Belstra et al., 2004). The delayed ovulation time was also occurred in the sows nursing large litter (>67 kg weaning weight). Sows nursing large litter may have more risk to lose their condition during lactation. It was reported that sows losing more body protein during lactation had smaller follicle at weaning (Clowes et al., 2003). The small follicles have lower levels of LH receptor mRNA expression than larger follicles (6 mm) (Liu et al., 2000). In addition, the level or pulse frequency of LH are lower in sows with higher weight loss during lactation (Koketsu et al., 1998).

**5.6 Possibility of using lecithin to replace egg yolk for boar semen cryopreservation**

Fixed-time AI can be done using frozen-thawed semen, it was found that the optimal timing of AI using frozen-thawed semen related to ovulation was very narrow, 0-6 h before ovulation (Waberski et al., 1994; Roca et al., 2003). Thus, the longevity of frozen-thawed semen might affect the sow reproductive performances of single fixed-time AI sows. The present study showed that the use of 6% lecithin to replace egg yolk in cryopreservation extender compromised frozen-thawed sperm qualities. However, the comparable result of frozen-thawed semen qualities was found in the extender containing 3.0% lecithin. This finding was in contrast with a previous study found that using 6.0% soybean lecithin for boar semen cryopreservation extender improved frozen-thawed semen qualities than using 20.0% egg yolk. However, it was reported that boar sperm qualities were significantly reduced when the concentration of lecithin was increased from 6.0% to 9.0% or 12.0% (Zhang et al., 2009). These indicate that the concentration of lecithin also plays an important role in its cryo-protective ability. However, the source of lecithin used in the present study was different from the previous study. In the previous study, the soybean lecithin is purified through mixing with ethanol, centrifuged and a drying process, while the source of lecithin used in the present study is a commercial purified lecithin product. Therefore, either purity or the concentration might be different. Furthermore, the present study demonstrated that the frozen-thawed boar sperm quality did not differ between the extenders containing 20.0% egg yolk and 10.0% egg yolk plus 3.0% lecithin. These data indicate that a minimum concentration of 10.0% egg yolk is required for protecting boar sperm during the cryopreservation process. Thus, the effect of the lecithin and egg yolk ratios on frozen-thawed sperm qualities should be further investigated to define the optimal concentration of lecithin that should be combined with 10.0% egg yolk. Moreover, in the present study, the osmolality of the Extender III after diluted with sperm was evaluated, but the osmolality of the Extender II in Group I, II and III were not evaluated. Due to the reason that the Extender II of Group II and III contained 10 ml of distill water more than in Group I. The post-thawed qualities of sperm

preserved in Group II and III may be decreased due to osmotic stress and by lethal ice crystal formation (Okazaki et al., 2009). Thus, to prepare the new cryopreservation extender, the osmolality of Extender should be evaluated before. Interestingly, 5 out of 7 boars that the semen were preserved in 3.0 % lecithin base extender showed better frozen-thawed sperm total motility higher than 20.0% egg yolk based extender. This indicates that the freezability of boar semen with extenders containing lecithin is also deepened on individual boars.

**5.7 Conclusions**

To establish single fixed-time AI successfully in Thailand, the negative influences from hot climate on GnRH treatment and reproductive function need to be overcome. In the present study, the success result of performing single fixed-time AI under tropical climate area resulted from the success ovulation induction. Moreover, the reproductive performances of single fixed-time AI also affected by number of factors. The single fixed-time AI protocol using 10 μg buserelin injection at 72 h after weaning and then inseminate the sows at 32 h later was successfully performed in commercial swine herds under a tropical climate, with a promising reproductive performance. Under tropical climate, hot season affected both response of follicle on GnRH treatment and reproductive performances. Of all the treated sows, 48% and 75% ovulated within the expected time frame (i.e., 32-56 h after injection) in hot and cool seasons, respectively. In addition, compromised litter size at birth after single fixed-time could be observed when insemination was performed in the hot season. Moreover, single fixed-time AI, using the IUI technique with a reduced number of sperm cells, decreased litter size at birth. Therefore, single fixed-time AI with a reduced number of sperm cells should be avoided. Furthermore, there were factors significantly influencing the variation of ovulation time i.e., lactation characteristics and weaned sow condition. Sows that were weaned with lactation length of at least 20 days, with a litter weight under 67 kg, with a BCS at least 3 or with high backfat reserve had better responses to buserelin injection. Lastly, to increase frozen-thawed qualities and longevity of sperm in order to increase sows reproductive performance after single

fixed-time AI with cryopreserved semen, using lecithin in combination with at least 10% egg yolk unable to improve frozen-thawed semen qualities. However the precise concentration of lecithin is need to be further investigated.

**5.8 Research limitations**

For the main experiment, a timing of 72 h was selected. However, in the pilot study, the number of animals included into the experiment was low and it was performed in only summer. So, to define the effect of timing of GnRH agonist injection of the weaned sows in the present herd that had quite short weaning-to-estrus interval more clearly, the number of weaned sows should be increased in the pilot experiment. Moreover, the pilot study should be done in both cool and hot seasons. In the Chapter III, due to the limitation of small number of boar provided by AI center, the individual boar effect on frozen-thawed quality of sperm preserved in extender containing lecithin was quite hard to be demonstrated by statistics. In single fixed-time AI experiment, the animals were collected from 18 different batches. However, 7 batches contained low number of animals per batch (8 sows). Thus, the effect of batch on efficacy of single fixed-time AI is not easy to be investigated.



**5.9 Advantages of the study**

In general, the conventional AI protocol will not inseminate the sow without standing estrus. The result of the present thesis showed that the single fixed-time AI protocol can inseminate the weaned sows that did not show standing estrus. The non-estrous sows was 11 sows (5.2%) and 9 out of 11 sows farrowed which each sow can produce 12 piglet per litter. Therefore, 108 piglets (12x9) can be produced more than conventional AI protocol. The cost of weaned piglet is around 1,400-1,500 Baht per piglet. Thus, the benefit from single fixed-time AI was 151,200-162,000 Baht. Moreover, the cost of semen used, stockperson for estrus detection, for the AI catheter will be

also decreased. Lastly, it can provide the better gilt pool management due to the insemination of the sows or gilt in the farm will be done regularly and continuously.

**6.0 Suggestion and further investigation**

To investigate the effect of factors such as season, sows conditions, backfat thickness on either the response of GnRH treatment or reproductive performances, more information about follicle diameter after weaning is required. Thus, for further investigation, the follicle diameter of the sows from the day following weaning should be observed. In Chapter III, the extender containing 3.0% lecithin and 10.0% egg yolk (Group III) provided the acceptable frozen-thawed semen qualities compared to conventional 20.0% egg yolk based extender. We suggested to add two more experiment groups including extender containing only 3.0% lecithin and containing only 10.0% egg yolk to define that the acceptable result in Group III resulted from 3.0% lecithin or 10.0% egg yolk. In addition, the experiment using gradually decreased concentration of lecithin from 3.0, 2.0, 1.0 and 0.5 % lecithin in 10.0% egg yolk are also interesting to be further investigated.

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# VITA

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1. Pearodwong P, Tretipskul C, Panyathong R, Sang-Gassanee K, Collelle M, Muns R and Tummaruk P 2020. Reproductive performance of weaned sows after single fixed-time artificial insemination under a tropical climate: Influences of season and insemination technique. Theriogenology. 142: 54-61.
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**AWARD RECEIVED**

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