รายงานโครงการวิจัยเงินทุนคณะสัตวแพทยศาสตร์

(ฉบับสมบูรณ์)

การตรวจวัดระดับของฮอร์โมนโปรเจสเตอโรนในอุจจาระเพื่อประเมินการทำงานของรังไข่ในสุกรสาว

(THE USE OF FECAL PROGESTERONE PROFILE TO DETERMINE OVARIAN FUNCTION IN GILTS)

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THE USE OF FECAL PROGESTERONE PROFILE TO DETERMINE OVARIAN FUNCTION IN GILTS

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บทคัดย่อ

วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อวัดระดับของฮอร์โมนโปรเจสเตอโรน (P4) ในอุจจาระสุกรสาว เพื่อบ่งชี้การทำงานของรังไข่ร่วมกับการสังเกตพฤติกรรมการเป็นสัด และศึกษาความสัมพันธ์ระหว่างระดับ P4 ในเลือดและในอุจจาระ การทดลองครั้งนี้ใช้สุกรสาวก่อนวัยเจริญพันธุ์จำนวน 5 ตัว ลักษณะของรังไข่ในสุกรทุกตัวถูกตรวจด้วยลาพาโรสโคป สุกรสาวได้สัมผัสกับพ่อสุกรทุกวันและถูกตรวจการเป็นสัดทุกวัน ทั้งอุจจาระและเลือดถูกเก็บทุก 7 วัน ก่อนที่สุกรสาวจะแสดงอาการเป็นสัดครั้งแรก หลังจากเป็นสัดอุจจาระและเลือดถูกเก็บทุก 3 วัน จนกระทั่ง 40 วัน หลังแสดงอาการเป็นสัด การเก็บอุจจาระทำโดยการเก็บจากก้นโดยตรงหรือเก็บทันทีที่สุกรขับถ่าย อุจจาระถูกเก็บประมาณ 10 กรัมและถูกแช่แข็งทันทีที่อุณหภูมิ –20^{0}C เลือดจะถูกเก็บจากเส้นเลือดบริเวณคอและถูกแยกพลาสม่าภายใน 20 นาทีหลังเก็บและแช่แข็งที่อุณหภูมิ –20^{0}C อุจจาระถูกนำมาสกัดโดยใช้สารละลายฟอตเฟตบัฟเฟอร์ ทำการปั่นแยกและนำส่วนใสมาตรวจระดับฮอร์โมนP4 ด้วยวิธี Radioimmunoassay (RIA) เช่นเดียวกับพลาสม่า ความสัมพันธ์ระหว่างระดับฮอร์โมนในพลาสม่าและในอุจจาระถูกวิเคราะห์ด้วยวิธี Sperman's correlation โดยเฉลี่ยระดับของ P4 ในอุจจาระสูงสุดในวันที่ 13 หลังสุกรสาวยืนนิ่ง ในขณะที่ระดับของ P4 ในพลาสม่าสูงสุดในวันที่ 10 หลังยืนนิ่ง อย่างไรก็ดีระดับสูงสุดของ P4 ในอุจจาระที่ตรวจพบแตกต่างกันระหว่างสุกรโดยพบได้ตั้งแต่ 143-2303 นาโนโมล/ลิตร ในขณะที่ระดับต่ำสุดพบได้ตั้งแต่ 0.45-5.7 นาโนโมล/กิโลกรัม ระดับของ P4 ในอุจจาระกับ P4 ในพลาสม่ามีความสัมพันธ์กันในเชิงบวกอย่างมีนัยสำคัญ ($r=0.73$, $P<0.001$, $n=65$) ความสัมพันธ์ของระดับฮอร์โมน P4 ในเลือดและในอุจจาระเป็นรายตัว คือ $r=0.63$ ($P<0.05$), $r = 0.64$ ($P<0.05$), $r = 0.92$ ($P<0.001$), $r = 0.81$ ($P<0.001$) และ $r = 0.69$ ($P<0.01$) ระดับของ P4 ในอุจจาระของสุกรสาวก่อนวัยเจริญพันธุ์อยู่ในระดับต่ำกว่า 1.5 นาโนโมล/กิโลกรัม ระดับของ P4 ในอุจจาระสูงขึ้นประมาณ 7 วันหลังสุกรเป็นสัด และอยู่ในระดับสูงจนกระทั่งวันที่ 16 หลังยืนนิ่งจึงเริ่มลดลง โดยสรุประดับของ P4ในอุจจาระมีความสัมพันธ์ในเชิงบวกกับระดับ P4 ในพลาสม่าในสุกรทุกตัว และระดับของ P4 ในอุจจาระสามารถใช้บ่งชี้ถึงการทำงานของรังไข่ในสุกรสาวได้

คำสำคัญ : สุกร โปรเจสเตอโรนในอุจจาระ วงจรการเป็นสัด การทำงานของรังไข่

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ABSTRACT

The objectives of the present study were to measure level of fecal progesterone (P4) metabolite in gilts in order to determine the ovarian function in relation to oestrous behaviour. In addition, correlation between plasma and fecal P4 metabolite were also analyzed. Five prepubertal crossbred gilts were included in the experiment. Laparoscopy was used to determine ovarian status in all gilts. Oestrus detection was performed daily and all gilts were allowed to have a direct boar daily. About 10 g of feces was collected either directly from rectum or immediately after defecation and then kept at –20 ^{o}C until analysed. Blood was collected from a jugular vein and immediately put into heparinized tube. Plasma was separated within 20 minute after collection and then kept at –20 ^{o}C until analysed. Both fecal and blood samples were collected every 7 days before the gilts showed signs of first oestrus. After first oestrus was observed the samples were collected every 3 days starting from day 1 (standing heat) until day 40. P4 metabolite in feces was extracted by using phosphate buffer solution. The measurement of P4 level in blood (plasma) and feces were performed by a solid-phase ^{125}I-radioimmunoassay and its correlation was calculated by Spearman's correlation. On average, fecal P4 metabolite was highest on day 13 after standing heat, whereas plasma P4 was highest on day 10 after standing heat. The maximum levels of P4 metabolite in feces varies among gilts from 143 to 2303 nmol/kg, whereas the minimum levels of P4 metabolite in feces varies from 0.45 to 5.7 nmol/kg. Level of plasma P4 and fecal P4 metabolite was positively correlated (r=0.73, P<0.001, n=65). The correlation coefficient within animal between plasma P4 and fecal P4 metabolite in 5 gilts were 0.63 (P<0.05), 0.64 (P<0.05), 0.92 (P<0.001), 0.81 (P<0.001) and 0.69 (P<0.01). The level P4 metabolite in feces was lower than 1.5 nmol/ml during prepubetal period. Levels of fecal P4 metabolite increased around day 7 after standing heat and remained in a high level (>20 nmol/kg) until day 16 after standing heat. In conclusions, levels of fecal P4 metabolite significantly correlated with P4 in plasma in all gilts and fecal progesterone profile can be a useful measurement to determine luteal function of ovaries in gilts.

Keywords: Pig, Fecal progesterone, Oestrous cycle, Ovarian function

INTRODUCTION

During a recent year, the measurement of steroid hormone concentration in feces has been reported as a useful diagnosis tool for wild animals and pig, which frequent blood samplings are rather difficult to be collected (Sanders et al., 1994; Hamasaki et al., 2001). It is known that progesterone (P4) is inactivated by the liver and the metabolites are partly excreted via bile into the intestinal canal (Taylor, 1971). Therefore, P4 metabolites occur in feces. In sows, the level of plasma progesterone is highly correlated with fecal progesterone concentration both during normal oestrus cycle (Hultén et al., 1995) and during pregnancy (Moriyoshi et al., 1997). Plasma P4 profile has been used as a useful diagnostic tool for measuring age at puberty and anoestrus both in pig and cattle (Son et al., 2001; Chung et al., 2002; Patterson et al., 2002). Most studies on fecal P4 assay were performed in wildlife species. Few studies have determined the level of fecal P4 metabolite in sows and gilts (Hulten et al., 1995; Patterson et al., 2002) and none have been performed in Thailand.

The objectives of the present study were to measure level of fecal progesterone (P4) metabolite in gilts in order to determine the ovarian function in relation to oestrous behaviour and to determine degree of correlation between plasma and fecal P4 in gilts.

MATERIALS AND METHODS

Animals

Six crossbred (Landrace x Yorkshire) gilts were brought from a commercial herd and kept at the research station of Farm Animal Hospital, Chulalongkorn University, Nakornpathom. The gilts entered the experimental unit at an average age of 175.5 days with an average bodyweight of 92.2 kg (Table 1). Laparoscopic examination was used to determine ovarian function in all gilts. The procedure is performed under general anesthesia as previously described by Kunavongkrit and Lohachit (1988).

A mature boar were used to check oestrus daily. The first day of standing oestrus was definded as day 1 in the oestrous cycle. Oestrous induction was performed in gilts that did not show standing oestrus until approximately 2 months after the gilts had entered the experimental unit by using a combination of PMSG 400 IU + HCG 200 IU (PG600®) subcutaneously (Table 1).

One gilt did not show standing oestrus after injection of PG600® and it was excluded from the experiment. Therefore, data from five gilts were included in the present study.

Blood and fecal collection

Blood samples were collected from jugular vein every 3 days from the first day of standing oestrus (day 1, 4, 7, 10, 13, 16 and 19). The samples were immediately put into heparinized tubes after collection and were centrifuged within 20 min after collection at 3,000 rpm for 10 min. The plasma was stored at –20 °C until assay.

Fecal samples were collected shortly before blood samplings were taken. About 10 g of feces was collected directly from the rectum or immediately after defecations. The fecal samples were stored at –20 °C until extraction.

Hormonal analyses for plasma samples

Plasma progesterone levels were determined by a solid-phase ^{125}I-radioimmunoassay (coat-A-Count®, Diagnostic Product Corporation, Los Angeles, CA, USA). The analyses were performed at the department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Sciences, Chulalongkorn University. Briftly, 100 ul of serum was put in tubes coated with P4 antibody in duplicated. 1.0 ml of ^{125}I-Progesterone was added to every tube and incubated for 3 hours at room temperature (15-28 °C). The liquid and moisture was removed from all tubes and the tubes were count for 1 minute in gamma counter. According the manufacture's instruction, the P4 antiserum is highly specific for P4, with low cross reactivity to other naturally occurring steroids. The sensitivity of the assay was 0.06 nmol/l. The intra-assay coefficients of variation were 5.7% and 3.8% for low and high progesterone concentration, respectively. The inter-assay coefficient of variation were 2% and 5.2% for low and high progesterone concentrations, respectively.

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Hormonal analyses for fecal samples

The extraction procedure was modified after Hulten et al. (1995). Briftly, one gram of feces was suspended in 10 ml 0.01 M phosphate buffer with 0.15 M NaCl (pH 7.0). The sample were shaken for 12 h at 25 °C, centrifuged at 2700 x g for 15 min, and the supernatant was collected and either frozen (-20°C) or immediately used in the assay. The analyses in porcine species have been previously validated (Hulten et al., 1995).

The recovery rate was determined by adding a known amount of I^{125}-Progesterone (approximately 40000 cpm) to 14 samples before the extraction procedure. The radioactive agents after extraction were determined in order to calculate the extraction rate.

A solid-phase ^{125}I-radioimmunoassay (coat-A-Count®, Diagnostic Product Corporation, Los Angeles, CA, USA) was used to determine progesterone concentrations in feces. The kit provides reagent and tube coated with antibodies to progesterone. The calibrators, provided in processed human serum, represented 0, 0.3, 1.6, 31.8, 63.6 and 127.2 nmol/l. A 0.1-ml aliquot of calibrators and undiluted fecal extract samples, followed by 1.0 ml of ^{125}I-Progesterone, were pipetted into appropriate tube in duplicate. After 3 h incubation at room temperature, the incubate was removed by simple decantation and each tube was counted for 1 min in a gamma counter.

Statistical analyses

Spearman's correlation between levels of progesterone in blood and in feces was performed. Analysis of variance (ANOVA) was used to evaluate the differences between plasma P4 concentration and fecal P4 metabolite concentration at various stage of oestrous cycle (day 1, 4, 7, 10, 13 and 19). Mean concentrations of either plasma P4 or fecal P4 metabolite concentration at different stages of oestrous cycle were compared by using Duncan's multiple range tests. The values were considered as statistical significant when P-value less than 0.05.

The normality, skewness and kurtosis of the residual of the data were measured. The normality test revealed that the concentration of fecal P4 metabolite was far from normal distribution. A natural log transformation was then applied to fecal P4 metabolite data to obtain more normal distribution before ANOVA was used.

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RESULTS

Of 6 gilts, 5 gilts showed oestrus at 225 ± 21 days of age (200-245 days). One gilts did not showed any signs of estrus after estrus induction and therefore was excluded from the experiment. Of 5 gilts showed estrus, 2 gilts showed standing oestrous few days after enter the experimental unit, 1 gilt showed standing estrus after fasting for 2 days and other 2 gilts showed standing oestrus after induction by PMSG 400 IU+HCG 200 IU (PG600®). Descriptive data for all gilts and ovarian status as determined by laparoscopic examination are presented in Table 1.

The recovery rate of progesterone metabolite from feces was 27.1±1.2% (25.0-29.2). Means concentration of plasma P4 concentration and fecal P4 metabolite concentration on day 1, 4, 7, 10, 13, 16 and 19 of oestrous cycle were presented in Table 2. However, variations in the levels of P4 metabolite in feces as well as P4 in plasma were relatively high among the gilts (Table 2, Fig. 1). On average, fecal P4 metabolite was lowest on day 1 and highest on day 13 and plasma P4 was lowest on day 19 and highest on day 10 of oestrous cycle. For all gilts, fecal P4 metabolite remained in a high level from day 7 to day 16 of oestrous cycle. Relationship between plasma P4 and fecal P4 metabolite within animal was showed in Fig. 1. Gilts showed estrous after injection with PMSG+HCG (number 2 and 5) had a relatively high level of P4 during luteal phase compared with gilts showed oestrous without any induction (number 1,3 and 4) (Fig. 1). However, the results could not be compared statistically due too low number of observation in each group.

Level of plasma P4 and fecal P4 metabolite was significantly correlated ($r=0.73$, $P<0.001$, $n=65$). The correlation coefficient within animal between plasma P4 and fecal P4 metabolite in 5 gilts were 0.63 ($P<0.05$), 0.64 ($P<0.05$), 0.92 ($P<0.001$), 0.81 ($P<0.001$) and 0.69 ($P<0.01$). The level P4 metabolite in feces was lower than 1.5 nmol/ml during prepubetal period. Levels of fecal P4 metabolite increased around day 7 after standing heat and remained in a high level ($>$ 20 nmol/kg) until day 16 after standing heat.

DISCUSSIONS

The present study demonstrated that P4 metabolite in pig feces could be extracted and analysed by using a solid-phase ^{125}I-radioimmunoassay. Similar findings have been demonstrated in many wildlife species, pig, sheep, goat, dog and cat (Shideler et al., 1993; Brown et al., 1994; Sanders et al., 1994; Hirata and Mori, 1995; Hulten et al., 1995; Borjesson et al., 1996; Gudermuth et al., 1998; Hamasaki et al., 2001; Takahashi et al., 2002). However, the extraction procedure of fecal P4 metabolite as well as hormonal assayed method differed between studies. The extraction procedure used in the present study was modified after Shildeler et al. (1993) and Hulten et al. (1995), in which only phosphate buffer solution was used as a solvent and then shakes for 12-24 hours. The supernatant was collected after centrifugugation and was kept at –20 °C or immediately analyses. The hormonal assay can be apply to the samples in the same way as analyzing plasma samples either by using radioimmunoassay (RIA) (Hulten et al., 1995) or

enzyme immunoassay (EIA) (Shildeler et al., 1993). Recently, time-resolved fluoroimmunoassay (TR-FIA) kit, a product for human medicine, has also been successfully used for analyzing fecal P4 in doe (Takahashi et al., 2002).

In pig with a normal oestrous cycle, the significant positive correlation between plasma and fecal P4 metabolite found in the present study is in agreement with earlier findings (Hulten et al., 1995). It has also been demonstrated that the levels of P4 metabolite in feces was delayed for 2 days after the levels of P4 in plasma (Moriyoshi et al., 1997). In the present study, the samples were collected every 3 days and the result was in agreement to the previous report (Fig. 1).

Measuring P4 metabolite in feces is a non-invasive, non-stressful and simple technique for evaluating ovarian function in animal. This could be a useful technique for indicating reproductive status in pigs, such as puberty attainment and pregnancy diagnosis (Moriyoshi et al., 1997; Patterson et al., 2002). Measuring P4 level in plasma has been demonstrated as a useful diagnostic tool for identifying ovarian function in anoestous gilts and sows (Chung et al., 2002). Using fecal P4 metabolite measurement in these abnormal cases could also be possible. Fecal estrone sulfate profile in a sow with mummified fetuses has been demonstrated (Ohtaki et al., 2000).

Measuring fecal P4 metabolite have also been applied in many wildlife species to indicate the reproductive status as well as pregnancy diagnosis (Shideler et al., 1994; Hirata and Mori, 1995; White et al., 1995; Takahashi et al., 2002). However, it has been shown and discussed that P4 metabolite was gradually increased after leaving feces at room temperature for several hours and this can be inhibited by leaving fecal sample in cold environment or adding ethanol, antibiotics and silica gel (Hirata and Mori, 1995; Takahashi et al., 2002). To evaluate accurate value of P4 in feces, it is therefore recommend that feces should be collected immediately after defecation and kept at cold environment until extraction.

In the present study, the level of P4 metabolite in feces was almost 10 times higher than P4 level in plasma. This is in agreement with previous report in sows and doe (Hulten et al., 1995; Takahashi et al., 2002). In addition, Takahashi et al. (2002) did calculate the actual amount of P4 metabolite in feces by multiplying the assay values with extraction rate and dry matter ratio. This is obviously an increasing the assay values by adding a constant value to all samples. The degree correlation between plasma and fecal P4 as well as interpretations of ovarian function based on

the same animal will be the same. However, this is still a useful method to calculate a total amount of P4 metabolite in feces.

In conclusion, P4 metabolite in feces can be measured in cycling gilts and well correlated to that in plasmas. This indicate that the extraction and analyze fecal P4 metabolite are feasible alternatives to standard blood progesterone assays. Furthermore, fecal progesterone profile might also be used to determine luteal function of ovaries in gilts.

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Table 1. Descriptive data for gilts included in the present study

Gilts	Age at entry (days)	Body weight (kg)	Breed (Boarx Sow)	Laparoscopic exam[1]	Oestrous induction[2]	Age at first observed oestrus
1	173	100	YL	Ovulating	No	200
2	171	93	YL	Small follicles	Yes	245
3	173	93	LY	Small follicles	No	205
4	167	89	LY	Small follicles	No	234
5	169	86	LY	Small follicles	Yes	243
6	194	92	YL	Small follicles	Yes	-

[1] Laparoscopic exam was performed on day 8th after the gilts enter the experimental unit

[2] Oestrous induction was performed in gilts that did not show standing oestrus at about two months after the gilts enter the experimental unit by using a combination of PMSG 400 IU + HCG 200 IU (PG600®) subcutaneously.

Table 2. Levels of plasma progesterone and fecal progesterone metabolite at different days of oestrous cycle in gilts

Day of oestrus	Plasma progesterone (nmol/l)		Fecal progesterone metabolite (nmol/kg)	
	Means ± SD	Range	Means ± SD	Range
1	1.9 ± 1.8^{d}	0.4 - 4.5	9.6 ± 5.1^{c}	5.2 - 15.6
4	44.0 ± 21.4^{cd}	22.4 - 73.9	17.9 ± 6.3^{bc}	9.6 - 27.3
7	78.5 ± 24.8^{bc}	51.5 - 106.9	63.0 ± 52.7^{b}	21.7 - 122.3
10	131.0 ± 70.0^{a}	66.0 - 216.4	255.6 ± 176.8^{a}	63.6 - 483.0
13	88.5 ± 26.5^{ab}	63.1 - 119.0	1006.6 ± 1033.6^{a}	143.4 - 2303.4
16	20.4 ± 27.0^{d}	3.0 - 65.3	300.0 ± 231.0^{a}	113.9 - 698.2
19	0.8 ± 0.9^{d}	0 - 2.1	71.7 ± 53.0^{b}	11.9 - 141.1

Note: Means with different superscripts within column significantly difference ($P<0.05$)

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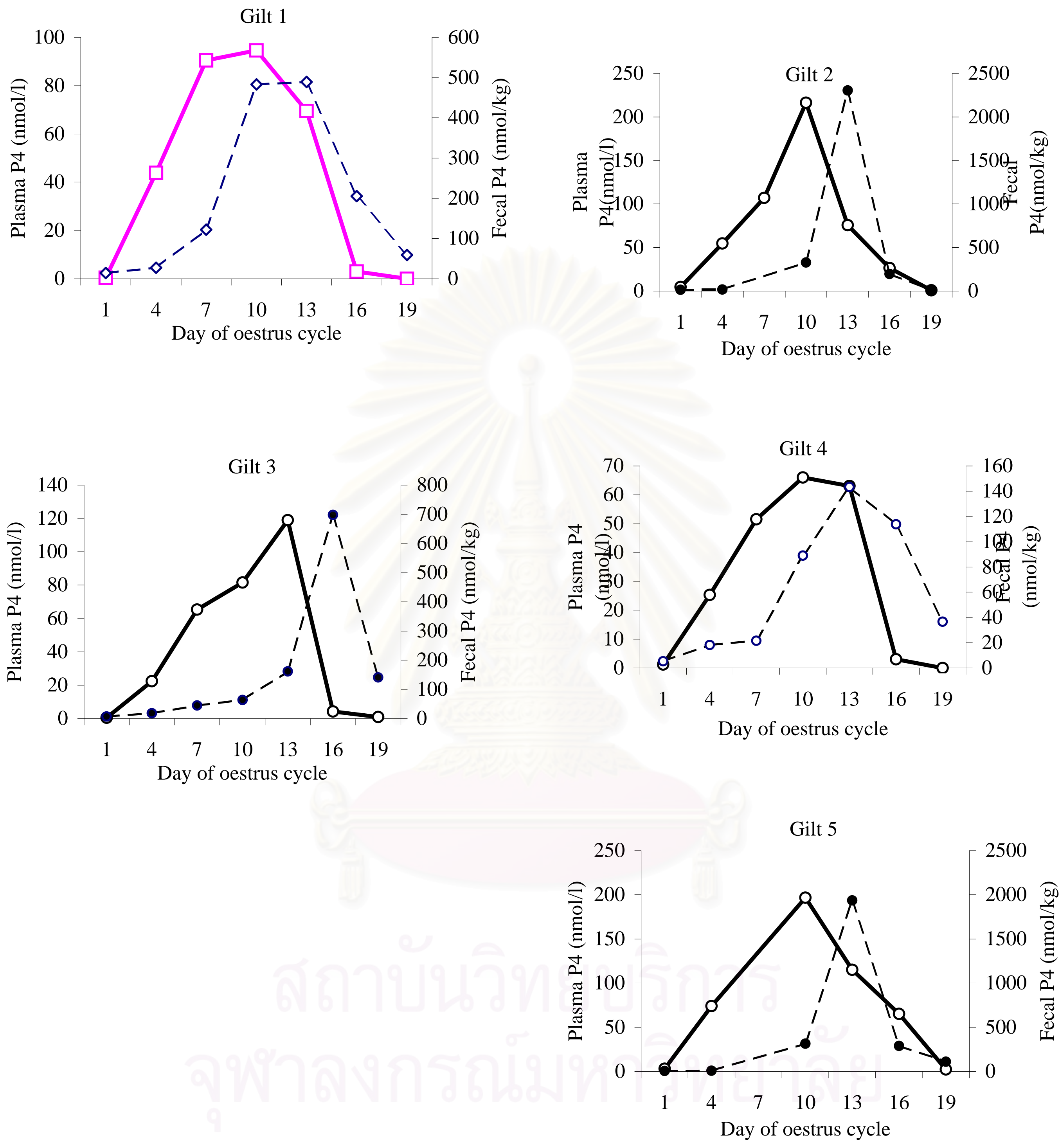

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Fig 1. Relationship between plasma progesterone concentration (dash line) and fecal progesterone metabolite concentration (dot line)