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# Effects of season on plasma progesterone profiles in repeat breeding cows

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**ABSTRACT**: Forty six Holstein Friesian repeat breeding cows (the average AI/conception was 5.2 ± 0.2) were investigated using the progesterone assay after AI to determine possible differences in plasma progesterone profiles between summer and winter seasons. Twenty eight (60.9%) and 18 (39.1%) cows were followed in summer (June–August) and winter (December–February), respectively. In the summer season, the total progesterone concentrations were higher ($P < 0.05$) in pregnant cows with normal luteal function compared to those in non-pregnant animals with abnormal luteal function. In contrast, in the winter season, there was no difference ($P = 0.12$) in total progesterone concentrations between pregnant and non-pregnant cows with normal or abnormal luteal functions. When the progesterone concentrations were compared, the pregnant and non-pregnant cows with normal luteal functions exhibited no difference ($P = 0.92$) in summer and winter seasons. Thus, the present study indicates that there is no effect of season on plasma progesterone profiles in repeat breeding cows; however in the summer season, the total progesterone concentrations were considerably higher in pregnant cows with normal luteal function compared to non-pregnant cows with abnormal luteal function.

**Keywords**: progesterone; embryonic death; luteal function; season; cows

Repeat-breeder cows are commonly referred to subfertile animals without any anatomical or infectious abnormality that do not become pregnant until the third or subsequent breeding or remain infertile after numerous services. Repeat breeding is one of the major problems affecting reproductive efficiency and is a major source of economic waste in dairy herds (Bartlett et al. 1986; Canu et al. 2010; Yusuf et al. 2010). The syndrome contributes to lower dairy profit due to the wastage of semen, insemination costs, increasing intervals to conception, increasing culling and replacement costs and loss of genetic gain through increased generation intervals (Bartlett et al. 1986) and reduced fertility (Garcia-Ispierto et al. 2007).

Katagiri and Takahashi (2004) stated that the causes of infertility in repeat breeder cows are usually unclear, but probably include environmental, management, and animal factors. Therefore, it is important to identify causes of repeat breeding in order to deal with this problem. The incidence of repeat breeding increases in response to inadequate oestrus detection (Heuwieser et al. 1997; Pursley et al. 1998), resulting in errors in timing of insemination in relation to the onset of standing oestrus, or insemination of cows not in oestrus (Yusuf et al. 2010). Other potential factors have also been suggested, such as quality of semen and insemination technique (Hallap et al. 2006; Morrell 2006), uterine and/or cervical/vaginal infections (Moss et al. 2002), endocrine disorders (Gustafsson 1998; Bage et al. 2002; Lopez-Gatius et al. 2004), ovulation failures (Kimura et al. 1987; Silvia 1994), obstructed oviducts, defective ova, anatomical defects of the reproductive tract (Silvia 1994), and early embryonic death (Gustafsson 1998; Bage et al. 2002). Lower parity, abnormal resumption of postpartum ovarian cycles, and shorter days in milk

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at first AI were identified as risk factors for repeat breeding (Yusuf et al. 2010).

Barlett et al. (1986) reported that there was no association between breeding season and repeat breeding syndrome. However, ovarian follicular growth and development of the dominant follicle can be altered during the summer months, and heat stress exerts an inhibitory effect on endocrine function (reduced intensity of oestrus, decreased preovulatory LH peak), thereby reducing fertility. However, it was also observed that higher progesterone concentrations were present in fertile females during warm periods. These were attributed to increased activity of the CL or the adrenals in response to heat stress (Gonzalez 1981). Photoperiod length and temperature variations are linked to the season, and could influence the endocrine regulation of the oestrous cycle. Other factors such as body condition at calving, feeding level and calving to insemination interval are also affected by the season, and can be modified through management practices (Fulkerson and Dickens 1985). It has been demonstrated that repeat breeding syndrome and infertility increase during summer months (BonDurant et al. 1991; Gonzalez-Stagnaro et al. 1993).

Amongst various factors associated with the occurrence of repeat breeding syndrome, asynchronous hormonal interplay is one of the major factors causing fertilisation failure and early embryonic mortality (Kimura et al. 1987; Lafi and Kaneene 1988). Delayed formation of the corpus luteum (CL) either alone or in combination with lowered secretion of progesterone during the luteal phase has been identified as one of the major causes of repeat breeding syndrome (Kimura et al. 1987; Thatcher et al. 1994).

Information related to seasonal variations in plasma progesterone and luteal function in repeat breeding animals is scarce. Therefore, the objective of the current study was to investigate the effect of season on progesterone profiles in pregnant and non-pregnant cows with normal and abnormal luteal functions.

## MATERIAL AND METHODS

**Animals**. This study was carried out on 46 Holstein Friesian cows at three commercial dairy farms in the Hiroshima prefecture in Japan. Twenty eight (60.9%) and 18 (39.1%) cows were investigated in summer (June–August) and winter (December–February), respectively. The cows were kept in roofed structures with open sides. They were fed a total mixed ration consisting of alfalfa, timothy and oat hay, corn, tofu ground wet, beet pulp, cotton seed and soybean with approximately 17.5% CP and 73% TDN for lactating cows. Cows were machine-milked twice daily and the average 305 days milk production was approximately 10 400 kg. A voluntary waiting period of 40 days was generally maintained, and cows detected in oestrus after this period were artificially inseminated. Oestrus detection was carried out by visual observation by the herdsmen. When signs of oestrus were noticed, AI was performed by experienced technicians.

**Plasma progesterone profiles**. Ten ml of blood were collected from the cows three times/week into heparinised vacuum tubes. The blood was transported within 2 h to the laboratory in an ice box and centrifuged at 1700 × g for 15 min. Plasma progesterone concentrations were determined using a double antibody enzyme-linked immunosorbent assay as described (Isobe et al. 2005). The intra-assay and inter-assay coefficients of variation were 8.6% and 12.2%, respectively. The sensitivity of the assay was 0.01 ng/ml. Based on plasma progesterone profiles, different luteal dysfunctions in repeat breeding cows were defined (Kimura et al. 1987; Hommeida et al. 2004; Ghanem et al. 2010). Briefly, normal luteal function was defined by progesterone concentrations elevated to 1 ng/ml or more at or before Day 6 post-AI and which reached 2 ng/ml or higher during the mid-luteal phase. Abnormal luteal function was defined as a delayed (progesterone concentrations was rising to 1 ng/ml after Day 6 post-AI) or insufficient (progesterone remained below 2 ng/ml during the luteal phase) rise in progesterone. Moreover, a cow is considered pregnant when the progesterone concentration remained at 2 ng/ml or higher for more than 42 days post-AI. On the other hand, the cow was considered non-pregnant when the progesterone concentration fell below 0.5 ng/ml between Day 20 and Day 42 post-AI (Santos et al. 2004; Ghanem et al. 2006). In addition to the determination of progesterone concentrations, the cows were palpated per rectum starting four weeks after AI to confirm the pregnancy.

**Experimental design**. The cows were distributed into two main groups (summer and winter). Within each group and based on the concentration

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of progesterone, the animals were classified into cows with normal luteal or abnormal luteal functions. The cows with normal or abnormal luteal functions were followed, and then categorised as either pregnant or non-pregnant cows.

**Statistical analysis**. Data were analysed using general linear models, repeated measures mixed analysis of variance (ANOVA). The dependent continuous variable was the progesterone concentration in all analyses. Analysis was done for summer and winter separately, and then combined together in one analysis again to study the seasonal variations. For separate analyses, the first independent factor was the time (days after insemination) which had different repeated measures levels (e.g. Day 1, Day 3, Day 6), while the second independent factor was the condition or treatment type with three levels in summer (pregnant with normal luteal function, non-pregnant with normal luteal function and non-pregnant with abnormal luteal function), and four levels in winter (the three levels as in summer, in addition to pregnant with abnormal luteal function). The time factor was used as the within-subjects factor, while the condition was used as the between-subjects factor. Another analysis was done for the effect of season on progesterone concentrations together with the time factor in all conditions, separately. Testing of homogeneity and sphericity were done using Levene's test for homogeneity of variances and Mauchly's test of Sphericity, respectively. The results suggested homogeneity of variance in most analyses, while violation of sphericity due to the large number of level combinations was observed. We relied on the Greenhous-Geisser correction for the assumption of sphericity. The results were discussed as the main effects and the interaction effects of independent factors on progesterone. Data of parity, BCS, No. of AI/conception and calving to last AI were analysed using the general linear model procedure according to the three-way analysis of variance (ANOVA). Three independent factors were studied; season (summer and winter), progesterone status (normal and abnormal luteal functions) and pregnancy status (pregnant and non-pregnant) as main effects together with their interaction. Data were analysed using SPSS software (version 16.0; SPSS Inc., Chicago, IL, USA) for the main effects, post-hoc tests, followed by the interaction effects using Mstat. The results were considered significant at $P \leq 0.05$.

Table 1. The effect and interaction of season, progesterone and pregnancy status on parity, body condition score and calving to AI (means ± SEM)

| Main and interaction effects | Parity | BCS | Number of AI/conception | Calving to AI (days) |
|---|---|---|---|---|
| Seasons | $P$ = 0.45 | $P$ = 0.62 | $P$ = 0.63 | $P$ = 0.16 |
| Summer | 2.5 ± 0.3 | 3.5 ± 0.1 | 5.1 ± 0.3 | 213.5 ± 17.1 |
| Winter | 2.0 ± 0.4 | 3.9 ± 0.2 | 5.3 ± 0.5 | 177.8 ± 17.9 |
| Progesterone status | $P$ = 0.81 | $P$ = 0.35 | $P$ = 0.24 | $P$ = 0.43 |
| Normal luteal function | 2.4 ± 0.3 | 3.5 ± 0.1 | 5.3 ± 0.3 | 193.10 ± 12.9 |
| Abnormal luteal function | 2.4 ± 0.5 | 3.5 ± 0.2 | 4.6 ± 0.4 | 229.4 ± 39.5 |
| Pregnancy status | $P$ = 0.36 | $P$ = 0.09 | $P$ = 0.56 | $P$ = 0.56 |
| Pregnant | 2.2 ± 0.4 | 3.5 ± 0.1 | 5.5 ± 0.3 | 200.2 ± 13.6 |
| Non-pregnant | 2.5 ± 0.3 | 3.4 ± 0.1 | 4.9 ± 0.3 | 199.7 ± 23.2 |
| Season * pregnancy status * progesterone status | $P$ = 0.6 | $P$ = 0.47 | $P$ = 0.87 | $P$ = 0.98 |
| Summer pregnant with normal luteal function | 2.5 ± 0.6 | 3.5 ± 0.1 | 5.3 ± 0.4 | 207.8 ± 14.8 |
| Summer non-pregnant with normal luteal function | 2.7 ± 0.5 | 3.5 ± 0.01 | 4.9 ± 0.5 | 189.0 ± 48.0 |
| Summer non-pregnant with abnormal luteal function | 2.4 ± 0.7 | 3.6 ± 0.2 | 5.2 ± 0.4 | 252.8 ± 52.9 |
| Winter pregnant with normal luteal function | 1.5 ± 0.3 | 3.7 ± 0.1 | 5.8 ± 0.5 | 186.1 ± 28.5 |
| Winter non-pregnant with normal luteal function | 2.4 ± 0.8 | 3.0 ± 0.3 | 5.5 ± 1.0 | 168.8 ± 34.6 |
| Winter non-pregnant with abnormal luteal function | 2.3 ± 0.6 | 3.4 ± 0.1 | 3.7 ± 0.3 | 171.0 ± 3.9 |

Within the same column, means of the main and interaction effects were non-significant ($P$ > 0.05), according to standard 3-way analysis of variance (ANOVA)

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## RESULTS

### Effect and interaction of seasons, progesterone and pregnancy status on parity, body condition score and calving to AI in cows

Twenty eight cows were investigated in the summer season (June–August) and 18 cows in the winter season (December–February). The average parity of the cows, their body condition score (BCS) at the day of last artificial insemination, the average number of AIs per conception and the interval from calving to last AI are listed in Table 1. There was no effect of season, progesterone or pregnancy status on parity, body condition score, number of inseminations required for conception and interval from calving to AI in repeat breeding cows. All *P*-values indicated the non-significance of main and interaction effects among these parameters (Table 1).

### Progesterone concentrations of cows in the summer season

In summer, progesterone concentrations varied considerably on different days post-insemination [F (3.28, 81.86) = 41.37, $P = 0.001$]. The main effect of the group (pregnant with normal luteal function, non-pregnant with normal luteal function and non-pregnant with abnormal luteal function) on the progesterone concentration was significant, [F (2, 25) = 8.099, $P = 0.002$]. The time * group interaction was significant, [F (6.55, 81, 86) = 3.23, $P = 0.005$], indicating that the changes in progesterone concentrations in the three groups of cows was differed significantly over time. The total progesterone concentrations (ng/ml) were considerably higher ($P < 0.05$) in pregnant cows with normal luteal function (2.2 ± 0.2) compared to non-pregnant cows with abnormal luteal function (0.9 ± 0.3) throughout the oestrous cycle (Figure 1).

### Progesterone concentrations of cows in the winter season

In winter, the differences in progesterone concentrations among the four groups (pregnant with normal luteal function, non-pregnant with normal luteal function, pregnant with abnormal luteal function and non-pregnant with abnormal luteal function) was non-significant, although a marked difference [F (3.09, 43.38) = 22.76, $P = 0.001$] was observed between the different time points (days after insemination). The interaction of time * group was also non-significant [F (9.30, 43.38) = 1.99, $P = 0.06$], indicating that there was no influence of time on pregnant and non-pregnant cows with normal or abnormal luteal functions (Figure 2).

### Progesterone concentrations of cows in summer and winter seasons

When the progesterone concentrations in both seasons were compared to each other, no difference was observed between the pregnant and non-pregnant cows with normal luteal functions [F (1, 18) = 0.021, $P = 0.886$], [F (1, 13) = 0.009, $P = 0.927$];

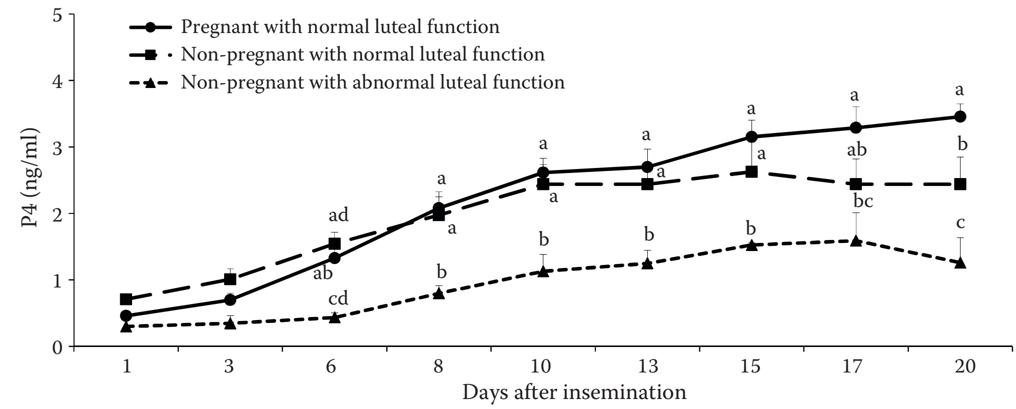


Figure 1. Progesterone (mean ± SEM) concentrations (ng/ml) in pregnant cows with normal luteal function, non-pregnant cows with normal and abnormal luteal functions in the summer season. Letters a–d, significant differences among treatment groups due to interaction effect of time * group ($P < 0.01$)

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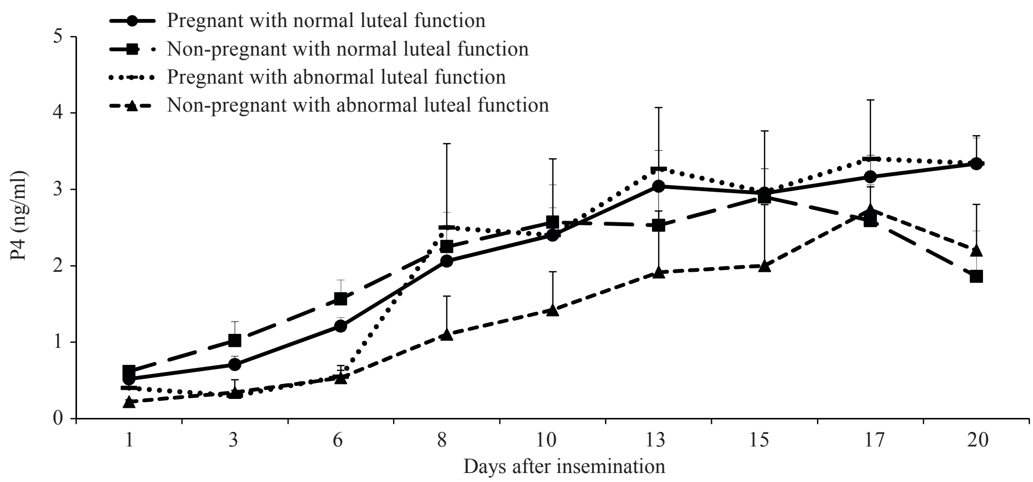


Figure 2. Progesterone (mean ± SEM) concentrations (ng/ml) in pregnant cows with normal and abnormal luteal functions, non-pregnant cows with normal and abnormal luteal functions in the winter season

moreover, the interaction of time * season was also non-significant [F (3.353, 60.35) = 0.351, $P$ = 0.810], F (2.75, 35.78) = 0.511, $P$ = 0.662], in summer and winter seasons, respectively. The progesterone concentrations of non-pregnant cows with abnormal luteal functions revealed no difference [F (1, 6) = 1.72, $P$ = 0.237] in summer and winter seasons. Moreover, the interaction of time * season was also non-significant [F (2.61, 15.64) = 1.15, $P$ = 0.356].

### Pregnancy rates in cows with normal and abnormal luteal functions regardless of the season

Regardless of the season, out of 35 cows with normal luteal function, 20 (57.1%) cows became pregnant. Interestingly, out of 11 cows with abnormal luteal function, three (27.3%) cows became pregnant.

## DISCUSSION

This study was undertaken to clarify the differences in progesterone profiles between different seasons in repeat breeding cattle. In the present study, the number of repeat breeding cows in the farm was higher in summer than that in winter. This might be due to reduced duration and intensity of oestrus, altered follicular development and impaired embryonic development elicited by heat stress (Jordan 2003). The endocrine changes involved in the decline in follicular activity and in the alteration of ovulatory function, might lead to inferior oocyte and embryo quality and a modified uterine environment, thereby reducing the likelihood of embryo implantation (El-Khadrawy et al. 2011).

Repeat breeding cows were identified based on the absence of any other disorders that could explain the pregnancy failure. Reproductive disorders such as cystic ovaries, anoestrus and chronic endometritis increase the risk of pregnancy failure. To avoid these errors, cows treated for these disorders were not included in the present study. Moreover, a weakness of this study was that the cows were allocated by season and then by progesterone profiles so the resulting numbers of cows per group were very limited. This might explain why the progesterone concentrations in pregnant and non-pregnant cows with normal luteal function exhibited similar profiles without any significant differences in both seasons. Moreover, the progesterone profiles in non-pregnant cows with abnormal luteal function were lower in summer compared to winter without reaching a significant difference. In a similar study, corpus luteum growth and function were monitored daily for a complete oestrous cycle in lactating cyclic Holstein cows in summer and spring. The length of the luteal phase and the corpus luteum cross-sectional area were similar for the two seasonal groups. Serum progesterone secreted between Days 6 and 18 was lower during summer. Suppressed luteal function might contribute to low fertility when cows are

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inseminated during summer (Howell et al. 1994). However, this issue is controversial; various studies have reported progesterone concentrations under heat stress to be higher, lower, or similar to those under cool conditions (Wolfenson et al. 2000). The divergence among these findings from the fact that most short-term, acute experiments did not reproduce the responses obtained in long-term, chronic, seasonal studies.

In another study, plasma progesterone levels were significantly higher in winter compared to summer in Holstein cows at 60 to 80 days postpartum (Wolfenson et al. 2002). No effects of milk yield or parity were detected. Progesterone concentrations in the early days of the cycle were similar in both seasons; however, during the mid-luteal phase they were 1.5 ng/ml higher in winter compared to summer. Jonsson et al. (1997) reported a lower concentration of plasma progesterone in cows in summer compared to winter during the development of the second CL after calving, and the difference was not associated with any differences between seasons, dry matter intake, body condition score or milk yield. The latter indicated that the decreased progesterone concentration in plasma was directly related to the heat load and not necessarily to heat stress-induced nutritional or metabolic changes.

The present study indicated that, in summer, the total progesterone concentrations were considerably higher ($P < 0.05$) in pregnant cows with normal luteal function than in non-pregnant cows with abnormal luteal function throughout the oestrous cycle. Suboptimal progesterone secretion is a possible cause of low fertility of dairy cows during summer heat stress (Wolfenson et al. 2000). Interestingly, the total progesterone concentrations in pregnant and non-pregnant cows with normal luteal function were similar throughout the oestrous cycle during summer. This is in agreement with a recent study (Iwazawa and Acosta 2013), which suggested that elevated temperature did not negatively affect luteal function in cows, and that the low fertility observed during summer was not due to a direct effect of elevated temperature on luteal cells.

Data from the current study support the hypothesis that the abnormal luteal phase could affect the pregnancy rate in repeat breeding cows as 57% of cows with normal luteal function became pregnant compared to 27% of cows with abnormal luteal function, regardless of the season. These results are consistent with the findings of Lamming and Darwash (1995) who reported that delayed formation of the corpus luteum has a major effect in terms of embryo survival and conception. Kimura et al. (1987) observed that 62% of repeat breeder cows had progesterone deficiency during the early phase of the oestrous cycle. Inadequate luteal function may be of prime importance because normal embryonic development depends upon sequential changes in uterine secretion under the influence of progesterone (Wilmut et al. 1986). Larson et al. (1997) postulated that delayed onset of the luteal phase could be associated with abnormal embryonic development and decreased fertility in dairy cows. A total of 37.8% of repeat breeding cows displayed atypical ovarian function, altered progesterone patterns and/or ovarian defects negatively impairing fertility (Perez-Marin and Espana 2007). Interestingly, approximately 43% of the non-pregnant cows in the present study had normal luteal function. This finding is consistent with Linares et al. (1982) who reported that abnormal embryonic development occurs with normal luteal function.

There are other factors that can affect the function of the CL in cows. Dietary energy restriction may decrease IGF-I concentrations, causing altered follicular growth and development of a subfunctional CL (Burns et al. 1997). In addition, metritis was able to impair luteal activity transiently, but did not seem to have a long-term effect on luteal function (Struve et al. 2013). Moreover, the luteal phase and the length of the interovulatory interval were both shorter in heifers with two waves of follicular development compared to those with three (Ginther et al. 1989).

In conclusion, there was no marked effect of season on plasma progesterone profiles in repeat breeding cows, although the total progesterone concentrations were higher in pregnant cows with normal luteal function compared to non-pregnant cows with abnormal luteal function in summer. Data from the current study support the hypothesis that an abnormal luteal phase can affect the pregnancy rate in repeat breeding cows.

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