

1 **Metabolic Profiles in Ovulatory and Anovulatory Primiparous Dairy Cows during**
2 **the First Follicular Wave Postpartum**

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19 Running head: Metabolic profile and first ovulation in primiparous cows

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1 Abstract

2 Metabolic hormones affect ovarian function in the cow. However, the relationship between
3 metabolic factors and ovarian function is not clear in the postpartum primiparous cow because they are
4 still growing. The aim of the present study was to investigate in detail the time-dependent profile of the
5 metabolic hormones, metabolites, and milk yields of ovulatory and anovulatory primiparous cows during
6 the first follicular wave postpartum. We used 16 primiparous Holstein cows and obtained blood samples
7 for the profiles of metabolites (glucose; non-esterified fatty acid, NEFA; ketone body; total cholesterol;
8 and aspartate aminotransferase), metabolic hormones (growth hormone, GH; insulin-like growth factor-I,
9 IGF-1; and insulin), and progesterone every other day from 1 to 21 days postpartum. In addition, all
10 ovaries were observed using ultrasound. Dairy milk yield was recorded during the experimental period. In
11 all cows, the first follicular wave postpartum was observed and 6 of the cows ovulated. The plasma
12 glucose ($P<0.0001$) and IGF-1 ($P<0.001$) concentrations were lower and the plasma NEFA ($P<0.0001$)
13 and ketone bodies ($P<0.0001$) concentrations and daily milk yield ($P<0.0001$) were higher in the
14 anovulatory cows compared to the ovulatory cows. However, the GH levels, which enhance lipolysis for
15 milk production, insulin and other metabolites did not differ between the two groups. In conclusion, the
16 present study suggests that anovulation of the dominant follicle during the first follicular wave
17 postpartum in primiparous cows is induced by low IGF-1 levels that are similar to those of multiparous
18 cows. In addition, anovulatory cows are likely to mobilize body fat stores for milk production more easily
19 than ovulatory cows.

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1 **Introduction**

2 It is generally accepted that changes in metabolic hormones affect ovarian function in cattle.
3 Insulin and insulin-like growth factor-I (IGF-1) stimulate estradiol production in the granulosa cells [1, 2]
4 and the proliferation of follicular cells [3, 4]. IGF-1 from the liver is a factor that regulates the final
5 maturation of the dominant follicle during the first follicular wave postpartum. That is the plasma IGF-1
6 levels in ovulatory cows at the first follicular wave postpartum are higher than those of anovulatory cows
7 [5]. Moreover, we have previously shown that IGF-1 is an essential factor for growth of the dominant
8 follicle , and together with insulin, it stimulates the dominant follicle to mature and reach ovulation
9 during the first follicular wave postpartum in multiparous cows [6]. Postpartum dairy cows enter a state
10 of serious negative energy balance at the onset of lactation [7, 8]. The metabolic condition shifts toward
11 to catabolic metabolism that in turn causes an elevation in growth hormone (GH) secretion and a decrease
12 in IGF-1 and insulin [9-11]. Consequently, the serious negative energy balance that accompanies catabolic
13 metabolism affects the onset of the first ovulation after parturition [8, 12].

14 Although the milk yield of primiparous cows is lower than that of multiparous cows [13, 14],
15 primiparous cows are still growing. Thus, the metabolic and endocrine responses of their first lactation
16 may be additionally compromised by the partitioning of nutrients towards somatic growth. However, in
17 primiparous cows, the relationship between the first ovulation after parturition and metabolic status or
18 milk yield remains unclear. Thus, the aim of the present study was to investigate in detail the
19 time-dependent profiles of metabolic hormones, including GH, IGF-1, and insulin; metabolites, such as
20 glucose, non-esterified fatty acid (NEFA), ketone body, total cholesterol (T-cho), and aspartate
21 aminotransferase (AST); and milk yields of ovulatory and anovulatory primiparous cows during the first
22 follicular wave postpartum.

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1 **Materials and methods**

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3 All animals received humane care as outlined in the Guide for the Care and Use of Experimental
4 Animals (Animal Care Committee, National Agricultural Research Center for Hokkaido Region).

5

6 ***Animals***

7 The experiment was carried out in the National Agricultural Research Center for Hokkaido Region
8 (Sapporo, Japan). We used 16 primiparous Holstein cows that calved between February and June 2001.

9 All cows were housed in the same free-stall barn. Throughout the experimental period, the cows were fed
10 a diet to meet their maintenance, growth, and lactation requirements in accordance with the Japanese
11 feeding standard (Agriculture, Forestry and Fisheries Research Council Secretariat, 1999). From May to
12 July, the cows were pastured for 3 to 4 h/day, and the amount of diet was reduced to meet their nutritional
13 requirements during this period. The cows were milked twice daily (0900 and 1900 h), and milk yield was
14 recorded daily.

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16 ***Sampling***

17 Body condition score (BCS) and body weight (BW) were measured at 1 week prepartum, on the
18 day of parturition, and 1 week postpartum. Average BCS was assigned by two or three independent
19 observers and recorded using a 0 to 5 scale in 0.25 intervals, with 0=thin and 5=very fat [15].

20 Blood samples were obtained by jugular venipuncture every other day from day 1 to day 21
21 postpartum. EDTA was used to prevent coagulation. Tubes were centrifuged at 3000 rpm for 20 minutes
22 at 4 C and the plasma samples were kept at -30 C until analysis for biochemical and hormonal analyses.

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24 ***Ultrasound examinations of the ovary and uterus***

25 Ultrasound examinations were applied to monitor the first ovulation and uterine involution [16].

1 The ovaries of all cows were scanned 3 time/week using a real-time linear array ultrasound scanner
2 (SSD-620; ALOKA, Tokyo, Japan) equipped with a 5-MHz rectal probe (UST-580U-5; ALOKA)
3 beginning between days 6 and 8 postpartum and continuing until the first ovulation. Furthermore both
4 uterine horns were scanned three times weekly by ultrasonography, and the maximum diameters of the
5 stratum vasculare were measured at the base of each horn (approximately 5 cm anterior to the uterine
6 body). Uterine involution was defined to be complete (days postpartum) when the diameters of both horns
7 were < 30 mm, and the difference in the diameters between the previously gravid and nongravid horns
8 diminished to within 5 mm.

9

10 ***Measurement of P4, GH, IGF-1 and insulin***

11 Determination of P4 in plasma was performed by enzyme immunoassay (EIA) after extraction by
12 diethyl ether as described previously [17]; the extraction efficiency was 93%. The standard curve ranged
13 from 0.05 to 50 ng/ml, and the ED50 of the assay was 3.2 ng/ml. The intra- and interassay coefficients of
14 variation (CVs) averaged 6.7 % and 7.2 %, respectively.

15 Determination for plasma GH, IGF-1, and insulin was performed by EIA using the
16 biotin-streptavidin amplification technique.

17 The GH concentration was measured by the EIA technique described by Roh with slight
18 modifications [18]. A diluted rabbit antibody to ovine GH (100 μ l, \times 100,000, NIDDK, AFP-0802210) was
19 distributed in all wells of a microplate coated with anti-rabbit γ -globulin antiserum, incubated for 24 h at
20 room temperature, and then the plate was decanted. After chicken serum diluted in assay buffer (100 μ l,
21 1%) was added to each well, 15 μ l of GH standard (0.78 to 100 ng/ml, NIDDK-bGH, AFP-9984C)
22 dissolved in assay buffer or plasma was incubated in the wells for 24 h. After decanting the plate,
23 biotin-labeled GH was distributed into all the wells and then incubated for 3 h. Finally, colorimetric
24 treatments were carried out. The intra- and interassay CVs were 8.1 % and 8.5 %, respectively, and the
25 ED50 of this assay system was 6.2 ng/ml.

1 Determination of IGF-1 in plasma was performed by the EIA after protein extraction using an
2 acid-ethanol (87.5% ethanol and 12.5% 2N hydrochloric acid) to obtain IGF-1 free from the binding
3 proteins [19]. Thirty μ l of human IGF-1 standard (Roche, Indianapolis, IN, USA; 0.39 to 50 ng/ml)
4 dissolved in assay buffer or sample was added to each well coated with anti-rabbit γ -globulin antiserum.
5 In addition, 100 μ l of biotin-labeled hIGF-1 ($\times 10,000$) and rabbit anti-hIGF-1 ($\times 40,000$, NIDDK,
6 AFP-18111298) diluted in assay buffer were distributed into all the wells and then incubated for 72 h at 4
7 C. Finally, colorimetric treatments were carried out. The intra- and interassay CVs were 5.7 % and 6.6 %,
8 respectively, and the ED50 of this assay system was 2.5 ng/ml.

9 Determination of insulin in plasma was carried out by EIA. Insulin standard (Sigma, St. Louis,
10 MO, USA) was diluted with charcoal-treated serum (insulin-free). Thirty μ l of insulin standard (39 to
11 5000 pg/ml) or plasma were added to each well coated with anti-guinea pig goat γ -globulin antiserum. In
12 addition, 100 μ l of anti-bovine guinea pig insulin ($\times 150,000$, D. Schams) dissolved in assay buffer was
13 distributed into all the wells and then incubated for 24 h at 4 C. After decanting the plates, 100 μ l of
14 biotin-labeled bovine insulin ($\times 50,000$) was distributed into all the wells and then incubated for 2 h at 4
15 C. Finally, colorimetric treatments were carried out. The intra- and interassay CVs were 9.7 % and 14.5 %,
16 respectively, and the ED50 of this assay system was 800 pg/ml.

17

18 ***Biochemical analyses***

19 In each sample, the concentration of glucose, NEFA, ketone body, T-cho, and AST were measured
20 using a clinical chemistry automated analyzer (Hitachi 7250, Hitachi High-Technologies Corporation,
21 Tokyo, Japan).

22

23 ***Calculation of the ratio of increase in daily milk yield***

24 We calculated the linearity of the ratio of increase in daily milk yield from the onset of lactation to
25 a significant increase in daily milk yield as follows. The ratio of increase in milk yield = $(MY_s - MY_f)/D$,

1 where MYs = milk yield on D, MYf = milk yield on the 1st day postpartum, and D = the day postpartum
2 that the significant increase in dairy milk yield stopped.

4 ***Statistical analyses***

5 All data were evaluated by repeated measures ANOVA. A significant interaction between group
6 and time was observed in AST ($P < 0.01$). Therefore, mean values were calculated for each cow for the
7 sampling period, and the significant differences were analyzed by Student's t test. For other data, no
8 significant interaction between group and time was observed. So, significant differences between
9 ovulatory and anovulatory cows were analyzed using Student's t test during the experimental period. In
10 addition, when a significant effect of time was observed within a group, the change of factor in each
11 group was analyzed using Fisher's least square difference test for BCS and BW or Tukey-Kramer test for
12 other factors. Results were expressed as means \pm standard error of the mean (SEM). Differences of
13 $P < 0.05$ were considered significant.

16 **Results**

18 ***The first ovulation and recovery of uterine size after parturition during the experimental period***

19 In all cows, the first follicular wave postpartum was observed during the experimental period. The
20 numbers of cows showing ovulation and anovulation of a dominant follicle during the first postpartum
21 follicular wave were 6 (37.5%) and 10 (62.5%), respectively. The plasma concentrations of P4 in each
22 group are shown in Figure 1. The interval between calving and first ovulation was 17.2 ± 1.2 days in the
23 ovulatory cows and 35.9 ± 3.7 days in the anovulatory cows ($P < 0.01$, Table 1). The age of the cows and
24 uterine involution did not differ between the two groups (Table 1).

1 ***Metabolites, metabolic hormones, and milk yields of the ovulatory and anovulatory cows during the***
2 ***experimental period***

3 The significant increase in milk yield during the experimental period in the anovulatory and
4 ovulatory cows stopped on 10 days postpartum and 13 days postpartum, respectively. The ratio of
5 increase in milk yield in the anovulatory cows was higher than that in the ovulatory cows (1.907 ± 0.186
6 kg/day vs. $1.208 \pm 0.154 \text{ kg/day}$, $P < 0.05$). In addition, the daily milk yield during the experimental period
7 in the anovulatory cows ($26.7 \pm 0.4 \text{ kg/day}$) was higher than in the ovulatory cows ($22.7 \pm 0.5 \text{ kg/day}$,
8 $P < 0.0001$, Table 1).

9 The plasma concentrations of glucose in the ovulatory cows were higher than in the anovulatory
10 cows during the period of study ($P < 0.0001$, Fig. 2). The plasma concentrations of NEFA and ketone body
11 in the anovulatory cows were higher than in the ovulatory cows during the experimental period ($P < 0.0001$,
12 Fig. 2). The plasma AST levels tended to be higher in the ovulatory cows on day 1 postpartum ($P = 0.07$),
13 but tended to be higher in the anovulatory cows on day 11 postpartum ($P = 0.09$, Fig. 2). The plasma
14 concentrations of T-cho were similar between the ovulatory and anovulatory cows during the period of
15 study.

16 Figure 3 shows the change in metabolic hormones from day 1 to day 21 postpartum. The plasma
17 concentrations of insulin and GH were not significantly different between the ovulatory and anovulatory
18 cows during the experimental period. The plasma concentrations of IGF-1 in the ovulatory cows were
19 higher than in the anovulatory cows during the period of study ($P < 0.001$).

20

21 ***BCS and BW in the ovulatory and anovulatory cows at -1, 0, and 1 week relative to parturition***

22 There were no significant differences in BCS and BW between the ovulatory and anovulatory
23 cows during the experimental period (Fig. 4). BCS and BW after parturition were lower than those before
24 parturition in both the ovulatory and anovulatory cows ($P < 0.05$). Moreover, the BCS and BW of the
25 anovulatory cows 1 week postpartum were lower than during the week of parturition ($P < 0.05$). On the

1 other hand, there was no difference in the BCS and BW of the ovulatory cows between the week of
2 parturition and 1 week postpartum.

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5 **Discussion**

6 The present study investigated the differences in the metabolic profiles and milk production in the
7 ovulatory and anovulatory primiparous cows during the first follicular wave postpartum. The data
8 indicates that the plasma IGF-1 levels were clearly related to the occurrence of ovulation at the first
9 follicular wave postpartum. In addition, the plasma GH concentrations did not differ between the two
10 groups, despite higher milk yields in the anovulatory cows compared with the ovulatory cows.

11 In the present study, the plasma concentrations of IGF-1 in the ovulatory cows were higher than in
12 the anovulatory cows, and this is well consistent with previous studies using multiparous cows [5, 6, 20].
13 IGF-1 stimulates estradiol production [1, 2] and the proliferation of follicular cells [3, 4]. The present data
14 also suggests that hepatic IGF-1 production is a direct influence that regulates the occurrence of ovulation
15 of the dominant follicle during the first follicular wave postpartum in primiparous cows.

16 A decrease in the plasma IGF-1 concentration is induced by decline in the expression of GH
17 receptor 1A at parturition [21, 22], reducing negative feedback on GH [22, 23]. Consequently, plasma GH
18 concentrations are kept high after parturition, and these high GH levels can sustain milk production by
19 promoting lipolysis in adipose tissue and ketogenesis in the liver [24, 25]. The present study showed that
20 the NEFA and ketone body concentrations of the anovulatory cows were higher than in the ovulatory
21 cows. In addition, the BCS and BW of the anovulatory cows continued to decrease from 1 week
22 prepartum to 1 week postpartum, whereas the decrease in these indexes in the ovulatory cows stopped at
23 1 week postpartum. This suggests that mobilization of body fat stores in the anovulatory cows was greater
24 than in the ovulatory cows. Importantly, the ratio of increase in milk yield and the daily milk yields were
25 higher in the anovulatory cows than in the ovulatory cows, but the plasma GH concentrations did not

1 differ between the two groups. Thus, we hypothesize that the partitioning of nutrients induced by GH in
2 anovulatory cows may be slightly different from ovulatory cows. That is, anovulatory cows likely
3 promote more lipolysis for milk production than ovulatory cows, even if they have the same levels of GH,
4 and this may result in a more severe negative impact on liver function and follicular maturation. One
5 peculiar property of primiparous cows is that they are still growing under the control of GH even after
6 their first parturition and first onset of lactation. These particular physiological conditions may affect the
7 complex of GH feedback and feedforward in the postpartum period [26]. Clearly, future studies are
8 necessary to approach the relative contribution of GH secretion on growth and milk production in
9 ovulatory and anovulatory primiparous cows during the first follicular wave postpartum.

10 The ratio of increase in milk yield was higher in the anovulatory cows than the ovulatory cows in
11 this study. In the anovulatory cows, a significant increase in milk yield was synchronized with a decline in
12 nutritional status, which was characterized by low glucose and IGF-1 concentrations and high NEFA
13 levels. In multiparous cows, the ratio of increase in milk yield until peak in anovulatory cows was higher
14 compared with ovulatory cows (our unpublished data). Thus, the high ratio of increase in milk yield may
15 be related to anovulation at the first follicular wave postpartum and may reflect the extent of the negative
16 nutritional condition.

17 Overall, the present study suggests that anovulation of the dominant follicle during the first
18 follicular wave postpartum in primiparous cows is induced by low IGF-1 levels, resembling the condition
19 in multiparous cows.

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Table 1. Ages, dates of first ovulation and recovery of uterine size after parturition, and milk yield of ovulatory and anovulatory cows at the first follicular wave postpartum

	The first follicular wave postpartum		Significance of differences
	Ovulatory cow (n=6)	Anovulatory cow (n=10)	
Age of the cows (month)	23.2 ± 0.7	23.4 ± 0.7	ns
Date of the first ovulation postpartum (days)	17.2 ± 1.2	35.9 ± 3.7	P<0.01
Date of the uterine involution postpartum (days)	17.2 ± 1.6	14.7 ± 0.9	ns
Daily milk yield (1 to 21 days postpartum, kg/day)	22.7 ± 0.5	26.7 ± 0.4	P<0.0001

Values are means ± SEM.

ns: not significant.

1 **Figure legends**

2

3 **Figure 1** The changes in plasma progesterone concentration in ovulatory (n=6) and anovulatory (n=10)
4 cows during the experimental period (mean±SEM). * Indicates differences of P<0.05 between ovulatory
5 and anovulatory cows. The day of ovulation was 17.2±1.2 days postpartum.

6

7 **Figure 2** Metabolites and milk yields from days 1 to 21 postpartum in ovulatory (n=6) and anovulatory
8 (n=10) cows (mean±SEM). A significant interaction between group and time was observed in AST
9 (P<0.01). No significant interaction between group and time was observed for other data. The plasma
10 glucose (P<0.0001) concentrations were lower, and the plasma NEFA (P<0.0001), ketone bodies
11 (P<0.0001), and daily milk yields (P<0.0001) were higher in the anovulatory cows during the
12 experimental period compared with the ovulatory cows. The T-cho and AST levels did not differ between
13 the two groups.

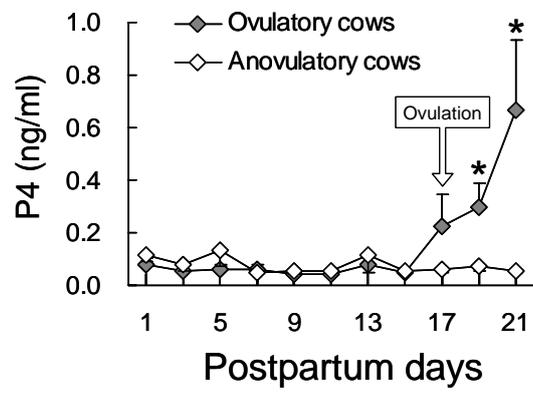
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15 **Figure 3** Metabolic hormones from days 1 to 21 postpartum in ovulatory (n=6) and anovulatory (n=10)
16 cows (mean±SEM). The plasma IGF-1 (P<0.001) concentrations in the anovulatory cows were lower than
17 in the ovulatory cows during the experimental period. The GH and insulin levels did not differ between
18 the two groups.

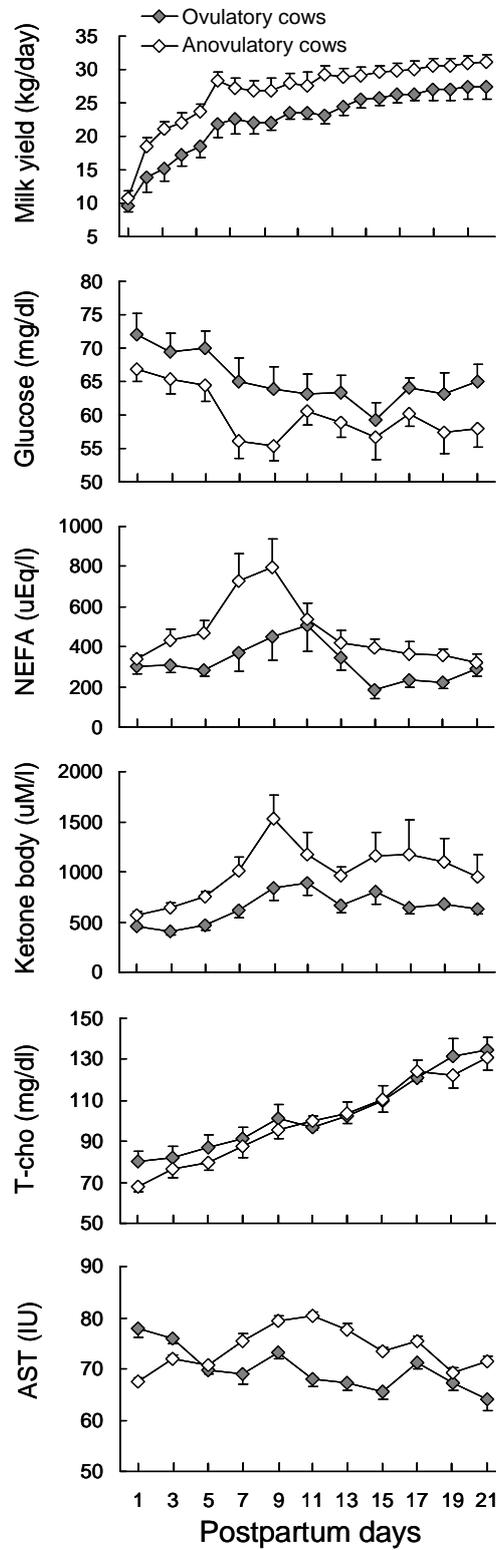
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20 **Figure 4** BCS and BW at 1 week prepartum, on the day of parturition and 1 week postpartum in
21 ovulatory (n=6) and anovulatory (n=10) cows (mean±SEM). a and b indicate differences of P<0.05 within
22 ovulatory cows. x, y and z indicate differences of P<0.05 within anovulatory cows. The BCS and BW
23 levels did not differ between the two groups.

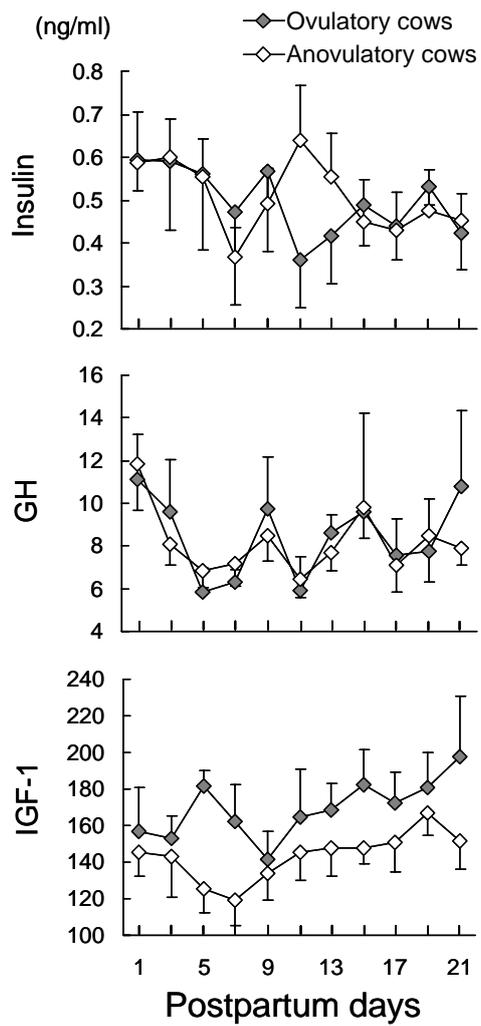
1 Figure 1



1 Figure 2



1 Figure 3



1 **Figure 4**