

—Technology Report—

Positive correlations of age and parity with plasma anti-Müllerian hormone concentrations in Japanese Black cows

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Abstract. Anti-Müllerian hormone (AMH) is secreted from the preantral and small antral follicles. It regulates follicle development and inhibits follicular atresia. This study examined how age, parity, and time after parturition affect plasma AMH concentrations in Japanese Black cows. We measured plasma AMH concentrations in primiparous, secundiparous, and multiparous (third parity or higher) cows at four time points: day 2 (day 0 = parturition), day 8, 2 days before first postpartum ovulation (pre-1stOv), and 12 days after first ovulation (post-1stOV). We observed a positive correlation between plasma AMH concentration and age (in months) and parity on day 2, day 8, and post-1stOV, but not on pre-1stOv. The multiparous cows had higher AMH concentrations than primiparous cows throughout the postpartum period ($P < 0.05$). Our results indicate that age and parity significantly influence plasma AMH concentrations in Japanese Black cows during the voluntary waiting period.

Key words: Aging, Müllerian inhibiting substance, Ruminant

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Beef prices are steadily rising in Japan owing to a number of factors, including the increasing price of Japanese Black calves. In addition, the costs of introducing young Japanese Black heifers are steadily increasing, so many cattle breeders need to breed old cows. Old age is well known to decrease fertility in beef cows [1], but little is known about the exact mechanism underlying this phenomenon in domestic ruminants.

One promising direction of research to help understand why fertility decreases with age is the study of the anti-Müllerian hormone (AMH), a dimeric glycoprotein in the transforming growth factor (TGF)- β family. It is produced mainly by granulosa cells of the preantral and small antral follicles in humans and animals [2] to regulate follicle development during the gonadotropin-responsive phase [3] and inhibit follicular atresia [4]. Several studies in humans have linked aging to plasma AMH concentrations. For instance, blood AMH concentration is highest in pubertal girls and then gradually decreases starting at age 25 until AMH is undetectable after menopause [5], suggesting that low AMH is indicative of ovarian aging [2]. In contrast, information on the relationship between age and plasma AMH concentration in adult female ruminants is lacking, although one study [6] found a positive correlation between pregnancy rate and plasma AMH concentration in dairy cows. Furthermore, the concentration of circulating AMH can predict the number of high-quality embryos produced by a donor goat or cow [7, 8]. These data

suggest that there must be an optimal blood AMH concentration for proper reproductive function in ruminants.

On the basis of existing data, the present study was conducted to evaluate the hypothesis that age, parity, and time after parturition affect plasma AMH concentration. Specifically, we tested whether older multiparous Japanese Black cows have lower AMH levels than younger primiparous cows.

All experiments were performed according to the Guiding Principles for the Care and Use of Experimental Animals in the Field of Physiological Sciences (Physiological Society of Japan), and the study was approved by the Committee on Animal Experiments of the School of Veterinary Medicine, Yamaguchi University (approval No. 300). The Japanese Black cows used in the study were housed in a barn and had had normal parturition (day 0). Primiparous cows ($n = 5$, 22.0 ± 0.2 months old on day 0), secundiparous cows ($n = 7$, 41.8 ± 1.6 months old on day 0), and multiparous cows (third parity or more; $n = 8$, 81.1 ± 7.9 months old on day 0; the mean and maximum parity were 5 and 8, respectively) were included. The feed volume per cow per day met the nutrient requirements set by the Japanese Feeding Standard for Beef Cattle [9]. The daily diet comprised 5.5 kg rice silage (41.0% dry matter [DM], 1.94 Mcal metabolizable energy [ME] kg^{-1} DM, 5.5% crude protein [CP]), 3.3 kg dried rice straw (42.4% DM, 1.45 Mcal ME kg^{-1} DM, 5.4% CP), and 2.1 kg concentrate (88.0% DM, 3.82 Mcal ME kg^{-1} DM, 22.0% CP) on average. Water and mineral blocks were provided *ad libitum*. Absence of disease, including reproductive disease, was confirmed by daily observation. At least four times per week from parturition to 3 weeks after the second postpartum ovulation, blood samples were collected at approximately 1030 h from the jugular vein of all cows into a 5-ml tube containing heparin. Calves were separated from the cows within 2 days of parturition, so none of the calves was in the presence of the cows when blood samples were collected

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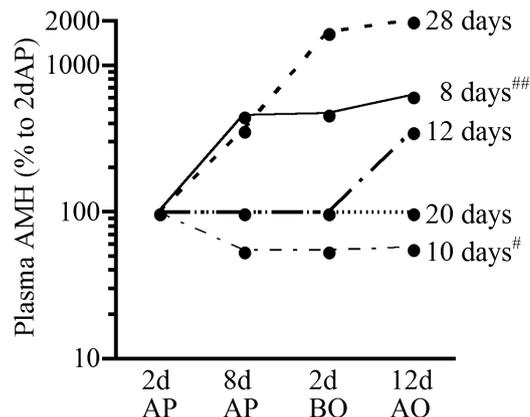
on day 2. Immediately after collection, the tubes were centrifuged at 800 g and 4°C for 15 min and the obtained plasma samples were stored at -20 °C until they underwent enzyme immunoassays (EIAs) of progesterone and AMH. To determine postpartum ovulation and verify normal resumption of ovarian cyclicity, all cows underwent rectal palpations at least four times a week from day 7 to 3 weeks after the second postpartum ovulation. Before each blood sample was collected, the number of follicles ≥ 5 mm and the sizes of the largest follicles in the ovaries of all cows were also obtained using a real-time linear array ultrasound scanner equipped with a 5-MHz rectal probe (Tringa linear VET; Esaote, Genoa, Italy).

We measured progesterone concentrations in all plasma samples to confirm the days of first and second postpartum ovulation. Assay costs prevented a full assessment of plasma AMH; instead, measurements were made only on day 2, day 8, 2 days before first postpartum ovulation (pre-1stOv), and 12 days after first postpartum ovulation (post-1stOv). We selected these time points for the following reasons: First, the first postpartum ovulation is an important factor in the resumption of reproductive function. Second, although little is known about the plasma AMH concentration in postpartum cows, Monniaux *et al.* [10] reported no significant change in AMH concentration in plasma samples collected from dairy cows at 10-day intervals from day 8 to the day of the first postpartum artificial insemination and to the following early pregnancy. Therefore, we speculated that plasma AMH concentration may not change significantly from day 8 to early pregnancy in Japanese Black cows. Third, there are no data on plasma AMH concentration immediately after parturition, e.g., day 2, in any species. Fourth, AMH promotes preantral follicle growth but inhibits antral follicle maturation and dominant follicle selection in primates [11]. Therefore, the level of AMH may be lower 2 days before first postpartum ovulation in cows. Fifth, with the aim of selecting the best cows for embryo production, Rico *et al.* [12] reported that the optimal period of the estrous cycle at which to measure AMH concentrations is 12 days after ovulation.

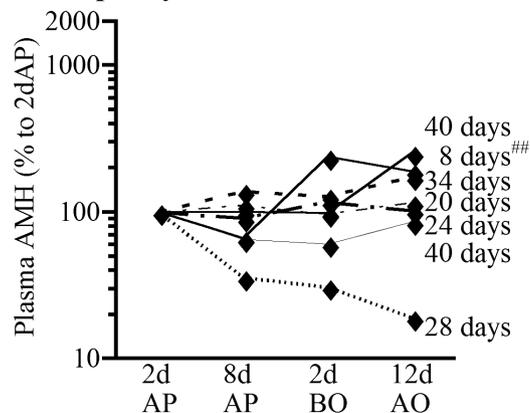
Neither preterm nor delayed delivery was observed, and the difference between the estimated and the actual date of delivery was less than 8 days for all cows. The first postpartum ovulation was earlier in primiparous cows (15.6 ± 3.7 days) than in secundiparous (27.7 ± 4.4 days, $P < 0.05$) and multiparous cows (25.0 ± 2.9 days). There was no significant difference in the number of days from parturition to second postpartum ovulation between the three parity groups (primiparous: 32.6 ± 3.5 days, secundiparous: 44.4 ± 5.4 days, and multiparous: 40.5 ± 2.7 days).

After setting the day 2 plasma AMH concentration of each cow at 100%, the plasma AMH concentrations of each cow on day 8, the day of pre-1stOv, and the day of post-1stOv were expressed as percentages of the day 2 value. Then, the changes in AMH concentration in each primiparous (Fig. 1A), secundiparous (Fig. 1B), and multiparous (Fig. 1C) cow were evaluated. Note that the day of pre-1stOv was the same as day 8 in a primiparous cow, in which first postpartum ovulation occurred on day 10, and day 8 occurred after the day of pre-1stOv in a primiparous and a secundiparous cow, in which first postpartum ovulation occurred on day 8. We identified three patterns of AMH changes in cows: increased, stable, and decreased. However, we did not detect any relationship between such changes and the days from parturition to first ovulation.

(A) First parity



(B) Second parity



(C) Third or more parity

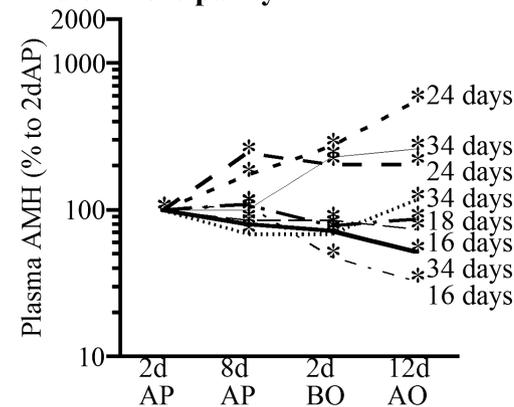


Fig. 1. Changes in plasma AMH concentration at 2 days after parturition (2dAP), 8 days after parturition (8dAP), 2 days before first postpartum ovulation (2dBO), and 12 days after first postpartum ovulation (12dAO) in primiparous cows (A; $n = 5$), secundiparous cows (B; $n = 7$), and multiparous cows (C; $n = 8$). The plasma AMH concentration on day 2 in each cow was set at 100%, and that on day 8, the day of pre-1stOv, and the day of post-1stOv was expressed as a percentage of the day 2 value. In the explanatory notes, numbers followed by “days” are the days from parturition to first postpartum ovulation. # 2dBO was the same as 8dAP in the cow in which the first postpartum ovulation occurred on day 10. ## 8dAP occurred after 2dBO in the cows in which the first postpartum ovulation occurred on day 8.

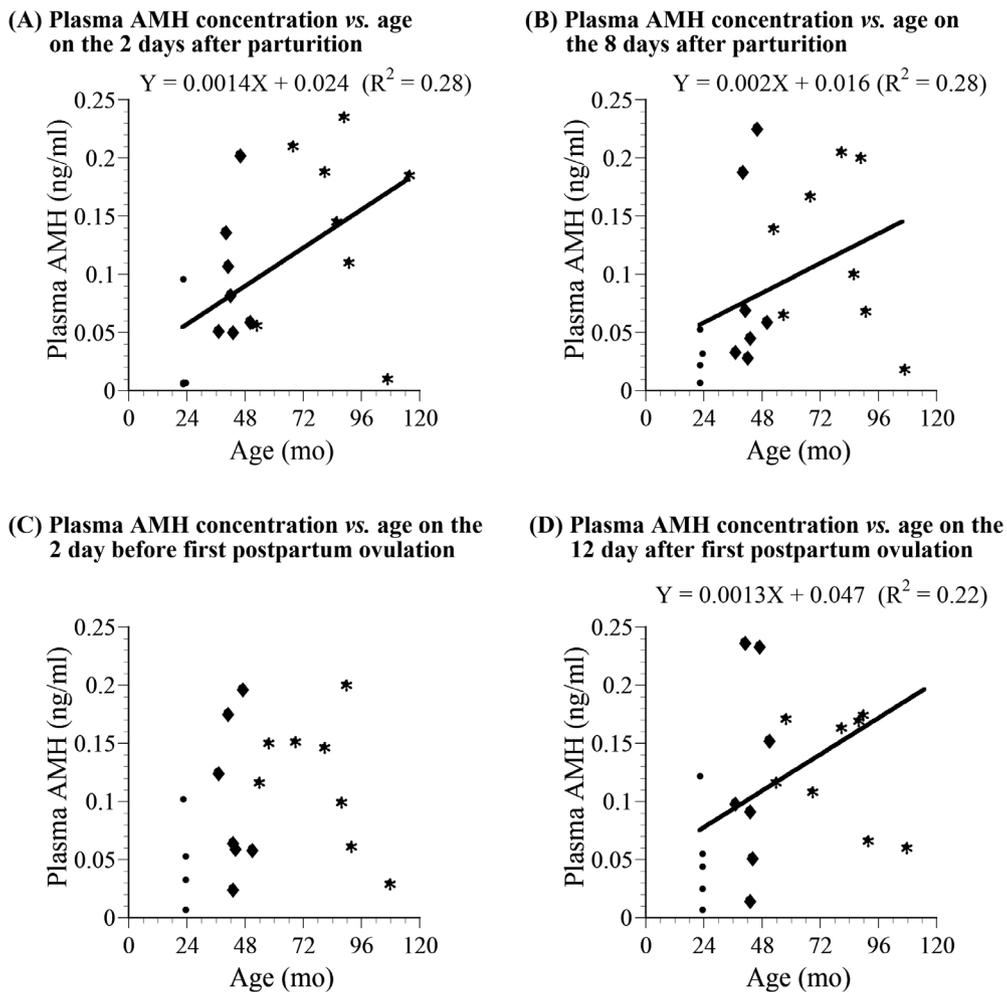


Fig. 2. Relationship between plasma AMH concentration and age (in months) at (A) 2 days after parturition, (B) 8 days after parturition, (C) 2 days before first postpartum ovulation, and (D) 12 days after first postpartum ovulation: (●) primiparous cows ($n = 5$), (◆) secundiparous cows ($n = 7$), (*) multiparous cows ($n = 8$).

Five cows were in estrus [2 multiparous cows (67 and 90 months old at parturition)] or experienced a second ovulation 1 day before post-1stOV [2 secundiparous cows (42 and 45 months old at parturition) and 1 multiparous cow (85 months old at parturition)]. In humans, plasma AMH concentration correlates negatively with plasma estradiol concentration [13], and estradiol suppresses AMH synthesis in cultured human granulosa cells [14]. Thus, estradiol may affect plasma AMH on the day of pre-1stOV, so we statistically analyzed the data with and without the data of the five cows in estrus.

Plasma AMH concentration in all 20 cows positively correlated with age on day 2 ($r = 0.53$, $P < 0.05$; Fig. 2A), day 8 ($r = 0.53$, $P < 0.05$; Fig. 2B), and the day of post-1stOV ($r = 0.48$, $P < 0.05$; Fig. 2D). When we excluded the five cows that were in estrus or ovulated a second time before post-1stOV, the correlation between plasma AMH concentration and age on the day of post-1stOV remained significant for the 15 cows and was, in fact, greater ($r = 0.58$, $P < 0.05$). However, we found no correlation ($P > 0.1$) between plasma AMH concentration and age on the day of pre-1stOV for all 20 cows

(Fig. 2C). This remained the case when the analysis was repeated without the data of the five cows that were in estrus or ovulated a second time before post-1stOV.

Plasma AMH concentration positively correlated with parity on day 2 ($r = 0.45$, $P < 0.05$), day 8 ($r = 0.49$, $P < 0.05$), and the day of post-1stOV ($r = 0.45$, $P < 0.05$) in all 20 cows. When we excluded the data of the five cows, the correlation on the day of post-1stOV remained strong ($r = 0.58$, $P < 0.05$) in the remaining 15 cows. However, plasma AMH concentration did not correlate ($P > 0.1$) with parity on the day of pre-1stOV, regardless of whether the five cows were excluded.

Figure 3 shows the differences in the plasma AMH concentrations of the primi-, secundi-, and multiparous cows at each of the four postpartum time points; these data include all 20 cows. Parity exerted a significant effect on AMH concentration ($P < 0.05$, analysis of variance [ANOVA]) at each time point in all 20 cows. Primiparous cows had lower plasma AMH concentration than multiparous cows ($P < 0.05$) at each postpartum time point, regardless of whether the five

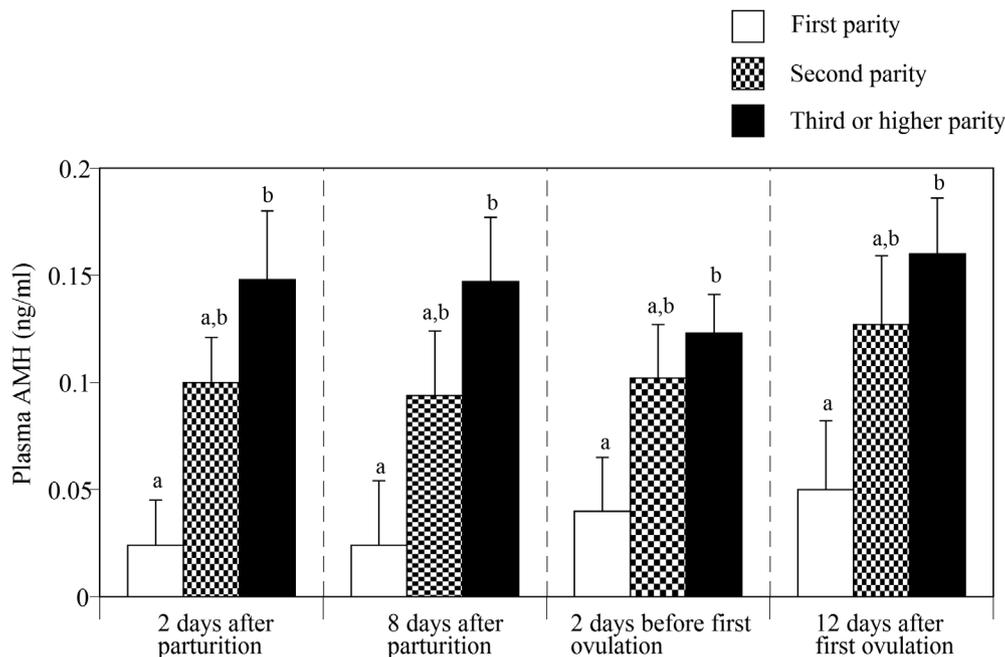


Fig. 3. Mean \pm SEM of plasma AMH concentration 2 days after parturition, 8 days after parturition, 2 days before first postpartum ovulation, and 12 days after first postpartum ovulation in primiparous cows ($n = 5$), secundiparous cows ($n = 7$), and multiparous cows ($n = 8$). Different letters (a or b) indicate a significant difference ($P < 0.05$) between groups. 2dBO was the same as 8dAP in the primiparous cow (shown as # in Fig. 1A) in which the first postpartum ovulation occurred on day 10. In addition, 8dAP occurred after 2dBO in the primiparous cow and the secundiparous cow (shown as # in Figs. 1A and 1B) in which the first postpartum ovulation occurred on day 8.

cows were excluded. Repeated-measures ANOVAs were performed to compare plasma AMH concentration between day 2 and day 8 and between the day of pre-1stOv and the day of post-1stOV. They showed that parity had a significant effect on AMH level ($P < 0.05$) in all 20 cows, but that time and the parity-by-time interaction did not ($P > 0.1$).

We were unable to detect any relationship between plasma AMH concentration and the number of follicles ≥ 5 mm in the ovaries of the 20 cows on day 8 (1.3 ± 0.2), the day of pre-1stOv (1.6 ± 0.2), and the day of post-1stOV (1.3 ± 0.1), and the same was found for the 15 cows at the same time points. In addition, there was no detectable relationship between plasma AMH concentration and the sizes of the largest follicles in the ovaries of all 20 cows on day 8 (10.5 ± 0.1 mm), the day of pre-1stOv (12.2 ± 0.2 mm), and the day of post-1stOV (11.4 ± 0.2 mm), and the same was found for the 15 cows at the same time points. Finally, no relationship was detected between plasma AMH concentration and the size of the corpus luteum in the ovaries of the 15 cows on the day of post-1stOV (21.8 ± 0.1 mm) or between plasma AMH concentration and plasma progesterone concentration on the day of post-1stOV (5.4 ± 0.2 ng/ml).

The surprising result of higher plasma AMH concentration in older, multiparous cows than in younger, primiparous cows contradicts the research in humans that shows that plasma AMH concentration is lower in older women than in young women [5].

The observed plasma AMH concentrations in primi- and secundiparous Japanese Black cows were similar to those reported in postpubertal heifers [15], as well as in postpartum primi- and

secundiparous Holstein cows [7]. However, we were unable to perform cross-study comparisons of AMH concentrations in multiparous cows because ours is the first report of a relationship between age (or parity) and plasma AMH concentration in cows. Why cows show this pattern may be related to the significantly longer period observed from parturition to first postpartum ovulation in multiparous cows rather than in primiparous cows. This slower onset of postpartum ovulation may be linked to the higher AMH concentrations in multiparous cows. Such a mechanism may help to avoid premature exhaustion of the ovarian follicular reserve because AMH inhibits the recruitment of primordial follicles into the pool of growing follicles [16–18] and decreases the responsiveness of growing follicles to follicle-stimulating hormone (FSH) [19]. However, we did not measure the plasma AMH concentration in each cow over several years. In addition, an alternative factor may affect plasma AMH concentration in cows. Indeed, our unpublished data on cows indicated that body condition score may be such a factor. Therefore, further studies are required to clarify the factors that control plasma AMH concentration in cows.

We were unable to detect any relationship between plasma AMH concentration and the numbers of follicles ≥ 5 mm, the sizes of the largest follicles in the ovaries, the size of the corpus luteum, or plasma progesterone concentration. That none of these relationships was observed may be because almost all of the plasma AMH originated from granulosa cells of the preantral and small antral follicles and not from the larger antral follicles and corpus luteum [2]. Accordingly, because of the design of the study and thus its findings, we are unable

to conclude whether estradiol and progesterone control plasma AMH concentration in cows.

In conclusion, age and parity significantly influence plasma AMH concentration in Japanese Black cows during the voluntary waiting period.

Methods

Plasma progesterone concentrations were assayed in duplicate using a competitive EIA, as previously described [20]. Octuplicates of a 0 ng/ml standard were also assayed using EIA, and the lower limits of the 95% confidence interval of the optical density (OD) values (calculated as the average minus 1.96 times the standard deviation) were adapted to a standard curve to calculate the lower detection limit of the assay, which was 4.4 pg/ml. The intra- and interassay coefficients of variation (CV) were 4.6% and 6.6%, respectively, at 7.39 ng/ml.

Plasma AMH concentrations were assayed in duplicate using AMH sandwich EIA kits (AMH Gen II ELISA A73818 and A73819; Beckman Coulter, Brea, CA, USA), following the protocol of the manufacturer with some modifications, i.e., 40 instead of 20 μ l plasma samples were used to improve performance, as in a previous study [21]. Octuplicates of a 0 ng/ml standard were also assayed with EIA, and the lower detection limits were determined by adapting the upper limits of the 95% confidence intervals of the OD values to the standard curve. The detection limit and the intra- and interassay CVs were 0.013 ng/ml and 5% and 9%, respectively.

Data were analyzed using StatView for Windows (ver. 5.0, SAS Institute, Cary, NC, USA). The Shapiro-Wilk *W* and Kolmogorov-Smirnov-Lilliefors tests were used to confirm the normality or log-normality of the distribution of all variables. Differences among the variables across the days from parturition to first or second postpartum ovulation were tested using ANOVA, followed by Fisher's protected least-significant-difference (PLSD) test. Simple regression and Pearson's correlations were used to evaluate the relationships between plasma AMH and (1) age (in months) or parity at each of the four time points; (2) the number of follicles ≥ 5 mm; (3) the sizes of the largest follicles in the ovaries on day 8, the day of pre-1stOV, and the day of post-1stOV; (3) the size of the corpus luteum in the ovaries of 15 cows on the day of post-1stOV; and (4) the plasma progesterone concentration in 15 cows on the day of post-1stOV. ANOVAs followed by Fisher's PLSD tests were used to evaluate the effects of parity (primi-, secundi-, or multiparous) on plasma AMH concentrations at each of the four postpartum time points. Repeated-measures ANOVAs followed by Fisher's PLSD tests were used to evaluate the effects of time, parity, and their interaction on plasma AMH concentrations to compare the values on day 2 with those on day 8 or those on the day of pre-1stOV with those on the day of post-1stOV. Significance was set at $P < 0.05$. Data are expressed as mean \pm standard error of the mean (SEM).

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