ELSEVIER

USE OF GROUP-SPECIFIC ANTIBODIES TO DETECT FECAL PROGESTERONE METABOLITES DURING THE ESTROUS CYCLE OF COWS

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ABSTRACT

Progesterone is metabolized to pregnanediones and hydroxylated pregnanes prior to its fecal excretion. Therefore, use of progesterone antibodies underestimates the actual amount of fecal metabolites. To improve the methodology of noninvasive fecal progesterone metabolite analysis, enzymeimmunoassays (EIA) using group-specific antibodies against 5-reduced 20-oxo-pregnane-C3-conjugates were developed. Fecal and milk samples were collected at 1- to 2-d intervals during the morning and evening milking throughout 1 estrous cycle in dairy cows (n = 12). Six immunoreactive metabolites were detected in the feces with high performance liquid chromatography (HPLC), eluting as 5α- and 5ß-reduced pregnanes containing a 20-oxo-group (20-oxo-pregnanes). Fecal samples of 3 cows were analyzed by 3 EIAs using antibodies against 4-pregnene-6α-ol-3,20-dione 6HS:BSA (6HS-progesterone), 5α-pregnane-3ß-ol-20-one 3HS:BSA and 5ß-pregnane-3α-ol-20-one 3HS:BSA, respectively. The follicular and luteal phases were identifiable with each EIA. Luteal phase values and the differences between mean follicular (Days 0 to 2 and 19 to 21) and luteal phase (Days 10 to 16) values obtained with the 5-pregnane EIAs were 3- to 4-fold higher than with the 6HS-progesterone EIA. Since results with the former 2 EIAs were almost identical, the remaining samples were only analyzed by the EIA for 5ß-pregnane-3α-ol-20-one. Fecal 20-oxo-pregnane concentrations were parallel to milk progesterone values, but had a lag time of about 0.5 d; the coefficient of correlation ($P < 0.001$) was 0.76 ($y = 155.2 x + 37.2$). Fecal 20-oxo-pregnane concentrations during the follicular and luteal phase were 39.5 ± 2.2 and 341 ± 15.2 ng/g feces, respectively. In conclusion, fecal 20-oxo-pregnanes are significantly correlated to milk progesterone concentrations. They consist of several metabolites and compared to a 6HS-progesterone antibody, their evaluation was improved using antibodies against 5-reduced pregnanes.

Key words: 20-oxo-pregnanes, noninvasive, feces, luteal function, milk progesterone

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INTRODUCTION

Radioinfusion studies using ^{14}C-progesterone in domestic ruminants have shown that for this hormone the predominant route of excretion of radioactive metabolites is through the bile and feces (5, 19, 29, 31). Therefore, the analysis of fecal progesterone metabolites emerged as an appropriate method for monitoring ovarian function and/or for detecting pregnancy, as it has already been shown in cattle (2, 4, 11, 13), bison (9, 12, 15), muskox (4), caribou (16), moose (17) and the scimitar-horned oryx (26). Although useful biological data were reported in these studies, fecal progesterone metabolites were only characterized by antibodies specific for 4-pregnene-3,20-dione (progesterone; 4, 9, 11-13, 15-17), 4-pregnene-20α-ol-3-one (20α-dihydroprogesterone; 26) and 4-pregnene-20ß-ol-3-one (20ß-dihydroprogesterone; 2).

Recent studies on the metabolism of radioactively-labeled progesterone (3, 27, 30), and studies using HPLC separation of fecal extracts and subsequent immunoassay analysis (3, 7, 8, 10, 21-25, 27, 30) have indicated that in several animal species progesterone is metabolized before its fecal excretion. In most cases there was no single, but rather a group of progesterone metabolites, which were described as pregnanediones and mono- and dihydroxylated pregnanes. Intact, unmetabolized progesterone was not, or only to a limited extent, detectable in the feces. Therefore, assays using progesterone antibodies, some of which are very specific, considerably underestimate the actual amount of fecal progesterone metabolites.

In earlier studies done in our laboratory, antibodies against 6HS-progesterone (11) and 20ß-dihydroprogesterone (2) were used to determine fecal progesterone metabolites during the estrous cycle of cows. Although the follicular and the luteal phases were identifiable, differences between follicular and luteal phase values were too small to make this methodology suitable for routine analysis. Since the presence of immunoreactive fecal pregnanes (progesterone metabolites) containing a 20-oxo-group was confirmed in a variety of animal species (3, 7, 10, 22, 24, 25, 27, 30), antibodies against 5α-pregnane-3ß-ol-20-one 3HS:BSA and 5ß-pregnane-3α-ol-20-one 3HS:BSA (5-reduced 20-oxo-pregnane-C3-conjugates) were produced and enzymeimmunoassays developed (25). These antibodies are considered to be group-specific and show considerable cross-reactivities with pregnanes sharing a similar C20 configuration (20-oxo-pregnanes). Compared to an assay using a 6HS-progesterone antibody, highly accurate values of fecal 20-oxo-pregnanes were reported (25). Hence, these assays could help to improve the methodology of fecal progesterone metabolite analysis in cattle and the results of this study could become a useful model for the study of reproductive patterns in females of nondomestic bovine species (Bovidae).

The purpose of the present study were 1) to compare measurements of fecal 20-oxo-pregnanes in cattle using 3 EIAs with different degrees of specificity, 2) to select the most suitable assay for measuring fecal 20-oxo-pregnanes in cattle, 3) to determine assay specificity and to obtain indications on the possible structure of the immunoreactive 20-oxo-pregnanes using HPLC separations, and 4) to compare fecal 20-oxo-pregnane concentrations in cyclic dairy cows with milk progesterone concentrations and to determine their correlation.

MATERIALS AND METHODS

Animals and Sample Collection

Twelve cows (Brown Swiss, n = 6; Simmenthal, n = 6), 3 to 6 y of age, were housed at the dairy farm of the Veterinary University of Vienna. The cows were fed with hay and water ad libitum, and their diet was supplemented with grain, minerals and vitamins in accordance with their individual milk production levels.

The ovaries of the cows were examined repeatedly by ultrasonography to determine the presence of preovulatory follicles, the day of ovulation (Day 0), and the presence of corpora lutea as described previously (28). After ovulation had occured, ultrasonographic examinations were performed at 2-d intervals from Days 0 to 12, and daily from Day 14 to the day of the next ovulation. Simultaneously with ultrasonographic examination, samples of milk and feces were collected during the morning and evening milking. Sampling started 41.8 ± 3.8 d post partum. Feces were collected from the rectum and the milk samples were gained by stripping milk. The samples were stored at -20° C until assay.

Extraction of Fecal Samples

Fecal samples (0.5 g), H_2O (2.5 ml) and methanol (3.0 ml) were mixed and vortexed (30 min) as described previously (11). After centrifugation (1500 g x 15 min), 0.01 ml of the methanol-extract was diluted 1:10 with assay buffer and analyzed in the EIAs. Recovery of [^{3}H]-progesterone from a pool of cow feces (n = 6) was 89.7 ± 2.1% .

High Performance Liquid Chromatography (HPLC) of Fecal Extracts

To determine assay specificity and to obtain indications on the possible structure of the immunoreactive progesterone metabolites, fecal extracts from the mid luteal phase of 2 cows were separated by HPLC as described previously (25).The samples were mixed with [^{3}H]-progesterone and [^{3}H]-20α-dihydroprogesterone and extracted; the extracts were separated on a straight phase HPLC system (silica 60 column) using a linear solvent gradient of 0 to 6 % methanol in n-hexane/chloroform (75/25 v/v). Fractions were analyzed by 3 EIAs using antibodies against 4-pregnen-6α-ol-3,20-dione 6HS:BSA (6HS-progesterone), 5α-pregnane-3ß-ol-20-one 3HS:BSA, 5ß-pregnane-3α-ol-20-one 3HS:BSA, respectively (25). The HPLC elution profiles of the immunoreactive fecal steroids were compared with those of [^{3}H]-progesterone and [^{3}H]-20α-dihydroprogesterone and with different 5α- and 5ß-pregnanes containing a 20-oxo-group, which cross-reacted in the 3 EIAs. In essence, pregnanediones (5α/ßpregnane-3,20-dione) eluted in front of [^{3}H]-progesterone, while monohydroxylated 20-oxo-pregnanes (5α- and 5ß-pregnane-3α/ß-ol-20-one) eluted between [^{3}H]-progesterone and [^{3}H]-20α-dihydroprogesterone.

Enzymeimmunoassays (EIA)

After extraction with petroleum ether, milk progesterone values were analyzed with an EIA using an antibody against 6HS-progesterone as described previously (28).

To select the most appropriate of 3 assays, fecal extracts of 3 cows were analyzed by EIAs using antibodies against 6HS-progesterone (the same EIA as used for milk progesterone analysis), 5α-pregnane-3ß-ol-20-one 3HS:BSA, 5ß-pregnane-3α-ol-20-one 3HS:BSA, respectively. The assays were described previously (25); the antibodies in the latter 2 EIAs

were group-specific, measuring progesterone metabolites containing a 20-oxo-group and the results are considered to be measurements of total immunoreactive 20-oxo-pregnanes. Briefly, the EIAs used a double-antibody technique and were performed on microtitre plates that had been coated with a sheep antibody raised against rabbit IgG. The steroid antibodies for the 3 EIAs were raised in rabbits, and in all 3 EIAs progesterone was used as standard. After overnight incubation of standard or samples (steroid antibody and biotinylated label), the plates were emptied, washed and blotted dry before a streptavidin horseradish peroxidase conjugate was added. After a 45-min incubation, the plates were emptied, washed and blotted dry. The substrate (tetramethylbenzidine) was added and incubated for 45 min (4° C) before the enzymatic reaction was stopped with sulfuric acid (2 mol/l). Optical density was measured at 492 nm.

The fecal samples of the remaining cows were only analyzed by the 5ß-pregnane-3α-ol-20-one EIA (25). In this assay, the antibody crossreacted with 5ß-pregnane-3,20-dione (151 %), 5α-pregnane-3ß-ol-20-one (102 %), 4-Pregnene-3,20-dione (progesterone, 100 %), 5α-pregnane-3,20-dione (75 %), 5ß-pregnane-3ß-ol-20-one (36 %), 5ß-pregnane-3α-ol-20-one (20 %), 5α-pregnane-3α-ol-20-one (8 %). Progesterone was used as the standard, and the assay was validated by demonstrating parallelism between standard curves and serial dilution of fecal extracts. The intra- and inter-assay coefficients of variation for a pool of cattle feces were 8.7 and 12.5%, respectively.

Significant cross-reactivities in the assay using the 5α-pregnane-3ß-ol-20-one 3HS:BSA antibody were the following: 4-pregnen-3α-ol-20-one (390%), 5α-pregnane-3.20-dione (168%), progesterone (100%), 5α-pregnane-3α-ol-20one (89%), 5ß-pregnane-3α-ol-20-one (88%), and 5α-pregnane-3ß-ol-20-one (56%). The assay using the 6HS-progesterone antibody showed significant crossreactivities with progesterone (100%), 5ß-pregnane-3,20-dione (71%), and 5α-pregnane-3,20-dione (40%).

Statistical Analysis

Data are presented as mean ± SEM. The duration of the estrous cycle was calculated as the period between 2 ovulations observed by ultrasonographic imaging. The estrous cycle lengths varied from 19 to 22 d; the hormone values in samples taken on Days 0 to 14 and during the 6 d preceding the day of the next ovulation were standardized to the respective days of ovulation. The coefficient of correlation between milk progesterone and fecal 20-oxo-pregnane concentrations was determined. Mean concentrations of milk progesterone and fecal 20-oxo-pregnanes were calculated during the follicular (Days 0 to 2 and 19 to 21) and the luteal phase (Days 10 to 16). Statistical significance of the differences between the follicular and the luteal phase and between morning and evening samples were tested by the t-test for independent samples.

RESULTS

The HPLC fractionation of fecal samples followed by subsequent analysis with the EIAs detected the presence of 6 and 5 unconjugated immunoreactive peaks in the 5α-pregnane-3ß-ol-20-one EIA and the 5ß-pregnane-3α-ol-20-one EIA, respectively (Figure 1). Quantiative differences in the profiles reflected the crossreactivities in the 3 EIAs, with the 6HS-progesterone antibody detecting the lowest amount of immunoreactivity in the fractions. Most of the immunoreactive material showed polarities that were close to but did not co-chromatograph with [^{3}H]-progesterone. Comparison with corresponding reference steroids

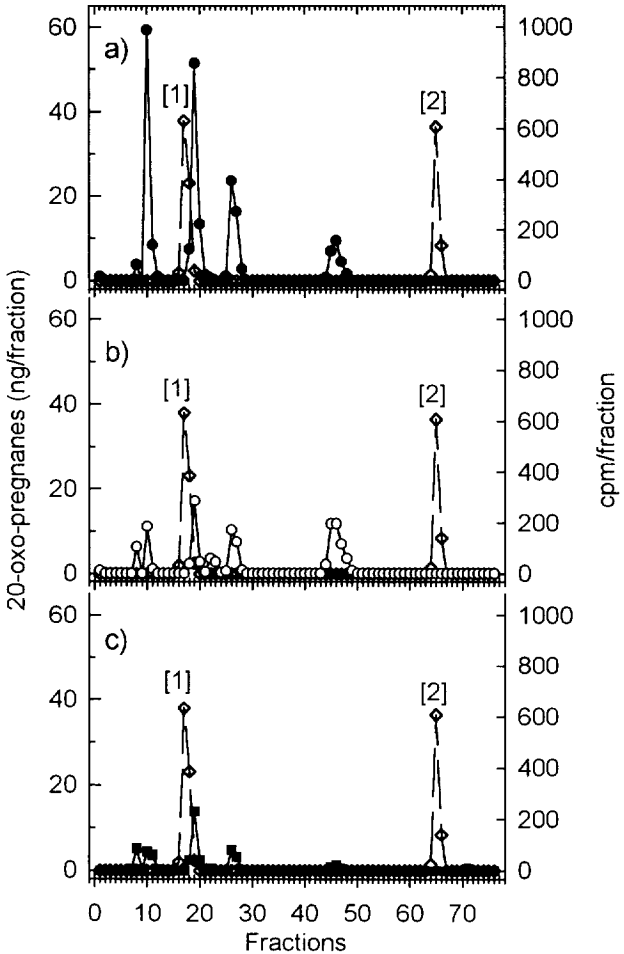

Figure 1. High performance liquid chromatography (HPLC) separations of fecal immunoreactive 20-oxo-pregnanes during the luteal phase of a cow. Samples were mixed with [1] [^{3}H]-progesterone and [2] [^{3}H]-20α-dihydroprogesterone, extracted, and chromatographed on a straight-phase HPLC system. Fractions were analyzed by 3 enzymeimmunoassays using antibodies against a) ● 5ß-pregnane-3α-ol-20-one, b) O 5α-pregnane-3ß-ol-20-one, and c) □ 4-pregnene-6α-ol-3,20-dione 6HS:BSA (6HS:progesterone). Counts per minute (◇ cpm) were measured by liquid scintillation counting. Concentrations (ng/fraction) were calculated for 1 g of feces without correction for methodological losses.

and the different cross-reactivities in the 3 respective EIAs support the concept that fecal immunoreactive peaks are the 5α- and 5ß-reduced 20-oxo-pregnanediones (5α/ßpregnane-3,20-dione) and 5α- and 5ß-reduced monohydroxylated 20-oxo-pregnanes (5α- and 5ß-pregnane-3α/ß-ol-20-one).

Fecal 20-oxo-pregnane values of 3 cows were analyzed by the 3 EIAs and a representative example is shown in Figure 2. The distinct differences between follicular and luteal phases were detectable by each EIA. The luteal phase values and the differences between follicular and luteal phases were 3- to 4-fold higher in the 5α-pregnane-3ß-ol-20-one and the 5ß-pregnane-3α-ol-20-one EIAs than in the 6HS-progesterone EIA. Results from the earlier 2 EIA were almost identical. Hence, fecal extracts of the remaining cattle were only analyzed by the 5ß-pregnane-3α-ol-20-one EIA.

Mean ± SEM values of milk progesterone and fecal 20-oxo-pregnane concentrations during the estrous cycles (n = 12) are shown in Figure 3. Their courses of concentration were parallel, but there was a delay of about 0.5 d in the concentration of fecal 20-oxo-pregnanes as compared with milk progesterone. The coefficient of correlation ($P < 0.001$) between milk progesterone and fecal 20-oxo-pregnane concentrations for the entire estrous cycle was 0.76 ($y = 155.2 x + 37.2$). Mean progesterone concentrations of milk samples and mean 20-oxo-pregnane concentrations of fecal samples were not significantly different between morning and evening samples of the same day, or between evening samples and those of the following morning. Milk progesterone and fecal 20-oxo-pregnane concentrations during the follicular phase and the luteal phase were 0.21 ± 0.01 and 1.64 ± 0.08 ng/ml in milk, and 39.5 ± 2.2 and 341 ± 15.2 ng/g in feces, respectively.

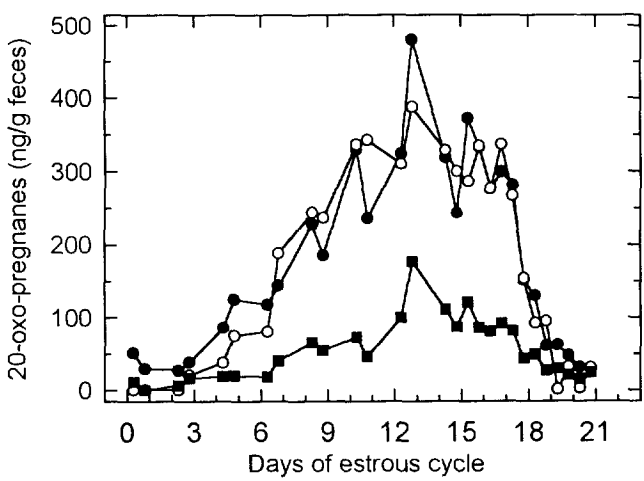

Figure 2. Profiles of immunoreactive 20-oxo-pregnanes in feces of a cow during the estrous cycle. Concentrations were determined by 3 enzymeimmunoassay using antibodies against ● 5ß-pregnane-3α-ol-20-one, O 5α-pregnane-3ß-ol-20-one, and ■ 6HS:progesterone.

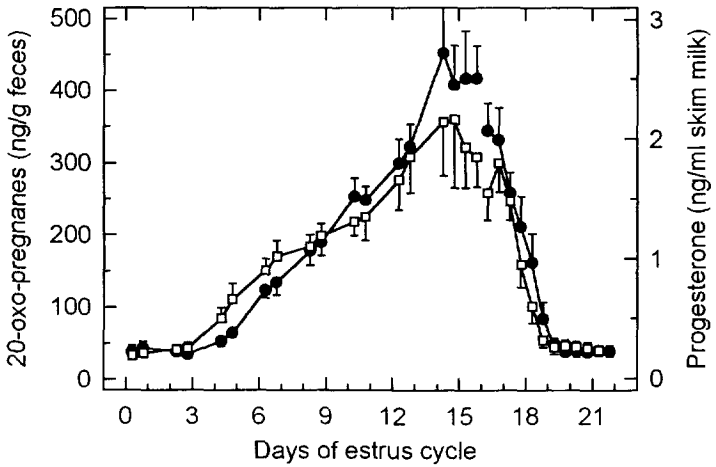

Figure 3. Concentrations (mean ± SEM) of immunoreactive 20-oxo-pregnanes in feces and progesterone in skim milk during the estrous cycle of dairy cows (n = 12). Concentrations were determined by enzymeimmunoassays using antibodies against ● 5ß-pregnane-3α-ol-20-one (feces) and □ 6HS:progesterone (milk).

DISCUSSION

The results of our study have shown that fecal 20-oxo-pregnane analysis is a valid, noninvasive method for determining the function of the corpus luteum in cows; milk progesterone analysis was used as a control for the fecal assays, and the 2 showed parallel patterns. Unlike milk progesterone analysis, which has become a standard procedure in the cattle industry (18, 20), fecal progesterone metabolite analysis is not limited to lactating animals. Therefore, analysis of fecal samples should allow for determining progesterone status in heifers, nursing beef cows and in nondomestic bovine species. Since in all these animals lag time between milk (or blood) progesterone and fecal progesterone metabolite values is difficult to determine, the data from dairy cows can serve as a model for the other species.

The lag time between milk progesterone and fecal 20-oxo-pregnanes in this study was about 0.5 d. The excretion time of progesterone metabolites correlates approximately to the time of the intestinal passage from the bile to the rectum (19). This time is appreciably shorter than the passage time for food through the entire gastrointestinal tract, which depends on the digestibility of the forage (6, 14). Our results are similar to the data from lactating cows, in which 53% of the administered [^{14}C]-progesterone dose was recovered, with 45% apparent in the feces within 12 hours of intake (31). Comparable results were reported in sheep, with peak excretion of [^{14}C]-progesterone metabolites occuring at about 12 h after infusion (19). In another study on the excretion of [^{3}H]-estradiol-17ß in ewes, it was shown that the excretion time is affected by the diet, which influences the rate of passage of digesta and thus of the enterohepatic circulation (1). Excretion was slower in nutritionally restricted than in supplemented groups, however, the final amount of radioactivity excreted in the feces was similar in both groups of animals (1).

In a variety of animal species progesterone is metabolized to several pregnanediones and to mono- and dihydroxylated pregnanes before it is excreted in the feces (3, 7, 8, 10, 21-25, 27, 30), while intact, unmetabolized progesterone is barely present, if at all, in the fecal samples. Therefore, studies reporting fecal progesterone values based on progesterone assays in several ruminant species (9, 13, 15-17), including cattle (4, 11, 13), most likely did not report values of progesterone, but rather of cross-reacting metabolites containing a 20-oxo-group. Our results add to these earlier studies, since we compared 3 EIAs with different degrees of specificity for progesterone metabolites and conducted HPLC separations of fecal extracts.

The HPLC separation of fecal samples and their subsequent analysis with EIAs has shown that the fecal immunoreactive 20-oxo-pregnanes differ from [^{3}H]-progesterone and [^{3}H]-20α-dihydroprogesterone. However, their elution patterns were comparable to those of 5α- and 5ß-reduced 20-oxo-pregnanes. These findings confirm those of previous investigations of fecal progesterone metabolites in several animal species, which also described 5-reduced pregnane metabolites (3, 7, 8, 10, 21-25, 27, 30). In contrast to the 6 immunoreactive 20-oxo-pregnane metabolites in cows, which included 5α and 5ß-pregnanes, we found only 3 immunoreactive 5α-pregnane peaks in fecal samples of black rhinoceroses (25). However, the difference in the metabolism of progesterone in different animal species did not impede the use of group-specific assays across species. Fecal metabolites of cows most probably also include dihydroxylated pregnanes, which have not been analyzed in this study, but which were demonstrated in recent studies using antibodies against pregnanediol, 20α- and 20ß-dihydroprogesterone in fecal samples of several animal species (8, 10, 21-24, 26, 27), including cattle (2).

Selection of relevant antibodies for the analysis of fecal progesterone metabolites is crucial. Our comparison of 3 EIAs with different degrees of specificity and the HPLC data indicated that the most suitable assay(s) show considerable cross-reactivities with several 20-oxo-pregnanes. In contrast, assays using specific progesterone antibodies quantitatively underestimate fecal progesterone metabolites considerably. The use of antibodies against 11-OH-progesterone immunogens (4-pregnen-11α-ol-3,20-dione) should be assessed cautiously, especially since these antibodies are more specific for progesterone than antibodies made to immunogens conjugated at the 3-position, or the 6HS-progesterone antibody used in this study. Hence, antibodies to 3-position 5α- or 5ß-20-oxo-pregnane immunogens are recommended over the use of antibodies against the 11-position immunogen. The 3-position antibodies have high cross-reactivities with 5α- and 5ß-20-oxo-pregnanes and thus are highly specific for these fecal progesterone metabolites. Improved specificity positively affects sensitivity, accuracy, reliability and practicability of the assay.

In conclusion, noninvasive fecal 20-oxo-pregnane evaluations are a useful and valid approach for monitoring corpus luteum function in cattle. Fecal 20-oxo-pregnanes were significantly correlated to milk progesterone concentrations, since fecal steroid and milk progesterone values showed a similar pattern but had a lag time of about 0.5 d. Since specific progesterone antibodies considerably underestimate the actual amount of fecal 20-oxo-pregnanes, antibodies against 20-oxo-pregnane-C3 conjugates are recommended. Evaluation of fecal 20-oxo-pregnanes could be used as an alternative to milk progesterone analysis and as a noninvasive means for monitoring corpus luteum function in heifers, nursing beef cows and nondomestic Bovidae, for which the data from lactating dairy cows could serve as a model.

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