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4.2.4. Частная зоотехния, кормление, технологии приготовления кормов и производства продукции животноводства (сельскохозяйственные науки)

ИММУФЕРМЕНТНЫЙ АНАЛИЗ ДЛЯ ОПРЕДЕЛЕНИЯ ПРОГЕСТЕРОНА В МОЛОКЕ КОРОВ: ОЦЕНКА РЕПРОДУКТИВНОГО СТАТУСА И ПОДТВЕРЖДЕНИЕ ТОЧНОСТИ ШАГОМЕРОВ, ИСПОЛЬЗУЕМЫХ В СТАДЕ

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Целью данного исследования было изучение репродуктивных характеристик молочных коров на основе анализа уровней прогестерона, а также оценка надежности устройств для мониторинга активности, в частности шагомеров, для обнаружения охоты в стаде. На основании индивидуальных профилей прогестерона была определена продолжительность овуляторных циклов в стаде, произведена оценка циклических паттернов. Также было проведено исследование для подтверждения точности определения половой охоты в стаде на основании сравнения уровня прогестерона и сигналов, получаемых с автоматических устройств. В целом исследование подразумевает, что шагомеры имеют потенциал для определения охоты у молочных коров в определенной степени и предоставления ценной информации о их репродуктивных характеристиках. Однако важно проявлять осторожность при обобщении этих результатов на более широкий контекст, поскольку исследование проводилось на одном стаде и могло подвергаться влиянию различных факторов, влияющих на проявление охоты и работоспособность устройства

Ключевые слова**:** РЕПРОДУКТИВНЫЙ ЦИКЛ, ПРОГЕСТЕРОН, ПОЛОВАЯ ОХОТА,

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4.2.4. Private animal husbandry, feeding, technologies of feed preparation and production of livestock products (agricultural sciences)

PROGESTERONE IMMUNOASSAY IN BOVINE MILK: ASSESSING REPRODUCTIVE STATUS AND VALIDATING PEDOMETER ACCURACY IN THE HERD

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The aim of this study was to investigate the reproductive characteristics of dairy cows based on progesterone level analysis and to assess the reliability of activity monitoring devices, specifically pedometers, for detecting estrus in the herd. The study involved determining the duration of estrous cycles in the herd based on individual progesterone profiles and evaluating cyclic patterns. Additionally, research was conducted to validate the accuracy of detecting sexual heat in the herd by comparing progesterone levels with signals obtained from automatic devices. In summary, the study suggests that pedometers have the potential to detect estrus in dairy cows to a certain extent and provide valuable information about their reproductive characteristics. However, caution should be exercised when generalizing these results to a broader context, as the study was conducted on a single herd and may have been influenced by various factors affecting estrus expression and device performance

Keywords: REPRODUCTIVE CYCLE, PROGESTERONE, ESTRUS, DAIRY COWS,

МОЛОЧНЫЕ КОРОВЫ, ШАГОМЕРЫ PEDOMETERS

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INTRODUCTION

Efficient reproductive management is crucial for maximizing profitability in dairy farming. Timely identification and monitoring of a cow's fertility status play a pivotal role in minimizing fertility-related losses, such as reduced conception rates, embryonic loss, and extended calving intervals (Bruinje *et al.* 2017). Traditional methods relying on external estrous symptoms alone often fail to detect silent estruses or provide comprehensive insights into the cow's reproductive health. Therefore, there is a growing need for reliable and objective tools to assess accurately the reproductive status of dairy cows (Adriaens *et al.* 2018).

Milk progesterone (P4) analysis has emerged as a valuable tool for obtaining a comprehensive and direct image of a cow's reproductive status. Measuring P4 levels in milk over time provides essential information about the onset of cyclicity, estrus detection, successful inseminations, pregnancy, and the occurrence of ovarian abnormalities causing fertility problems (Blavy *et al.* 2016; Gaude *et al.* 2021).

Furthermore, automated systems for on-farm milk P4 measurement are now commercially available, enabling real-time monitoring of reproductive parameters.

However, the interpretation of milk P4 data poses challenges due to the inherent variability in measurements. Factors such as measurement techniques, calibration methods, sampling procedures, and milk sample composition can introduce variability in P4 profiles (Blavy *et al.* 2016; Ealy *et al.* 2019; Domingues *et al.* 2023). Furthermore, each cow's progesterone levels can be quite different, making it challenging to interpret the data. These variations include the actual progesterone values, how they change over time (the slopes), how long they last, and irregular patterns. Despite these complexities, studying

milk progesterone profiles provides a reliable way to understand how dairy cows' ovaries are functioning after calving (Wiltbank *et al.* 2014).

Normal milk P4 profiles typically show a low concentration after calving, followed by a gradual increase indicating the first postpartum ovulation. This is followed by a cyclical pattern of rising and falling progesterone levels until pregnancy is established. Deviations from this normal pattern have been associated with decreased fertility. Early resumption of postpartum ovarian cyclicity has been linked to improved reproductive performance, including shorter calving-to-conception intervals, higher conception rates, and reduced costs per conception (Fricke *et al.* 2014; de Bruijn *et al.* 2023). Moreover, factors such as parity, calving-related issues, negative energy balance, uterine inflammation, and prolonged luteal phases have been identified as risk factors affecting reproductive function and influencing the resumption of postpartum cyclicity (Opsomer *et al.* 2000; Royal *et al.* 2000; Gorzecka *et al.* 2011; Bretzinger *et al.* 2023).

In addition to milk progesterone analysis, the integration of data obtained from activity monitoring sensors provides a valuable comparison for assessing reproductive status in dairy cows. Activity monitors detect changes in behavior, such as increased physical activity and mounting behavior, which are indicative of estrus. This activity monitoring offers a complementary approach to milk progesterone analysis, as they provide real-time information on the timing of estrus events (Mazeris 2010; Saint□Dizier *et al.* 2012; Rutten *et al.* 2013; Lardy *et al.* 2023)

By comparing the profiles of progesterone levels obtained through milk analysis with the data collected from activity monitoring sensors, dairy farmers can gain a more comprehensive understanding of the cow's reproductive status. This combined approach allows for the identification of cows that may exhibit silent estrus, in which no observable behavioral signs are detected, but progesterone levels indicating ovulation are increased (Ranasinghe *et al.* 2010; Gaude *et al.* 2021).

Conversely, this approach also helps identify cows that may exhibit estrus behaviors without a corresponding increase in progesterone levels, suggesting anovulation or other reproductive abnormalities (Roelofs *et al.* 2010).

Therefore, the combination of milk progesterone analysis and activity monitoring provides a powerful toolset for assessing the reproductive status of dairy cows. The integration of these data sets enables dairy farmers to obtain a holistic view of reproductive performance, considering both hormonal and behavioral aspects. By leveraging this comprehensive approach, farmers can optimize reproductive management strategies, leading to improved reproductive efficiency, increased conception rates, and enhanced profitability in dairy operations. In this study, we combined two analytical methods (progesterone analysis and activity monitoring) to achieve a more accurate assessment of the presence of heat in cows.

MATERIALS AND METHODS

Animals and housing. The study was conducted at the Livestock Research Center of the Niigata Prefectural Agricultural Research Institute, Japan as part of the exchange of experience. The study was conducted from April to January 2021. The herd were consisting from 58 Holstein-Friesian cattle. The average BCS of 25 milking cows (13 primiparous cows, 12 multiparous cows) was 3.0 [3.1/3.0]. The herd had an average 305-day corrected milk yield 9661 kg. Milking cows were housed in a free stall lined with rice husk with a walkway lined with rubber mats, and milking was performed automatically using an AMS (DairyRobot R9500, GEA Orion Farm Technologies). Cows were fed a partially mixed ration (PMR) consisting mainly of grass and corn silage (38.0% Italian ryegrass silage, 28.0% compound feed, 8.0% corn silage, 8.0% sorghum silage, 1.0% soybean meal), additives (vitamin mix, mineral mix, mineral salt), and formulated feed in AMS. Artificial insemination (frozen semen) was performed by a veterinarian.

The project and all procedures were reviewed and approved by the Care and Use Committee of the Livestock Research Center of the Niigata Agricultural Research Institute (approval no. 210104).

Automatically milking system and sensors of activity. At the initiation of the milking process, dairy cows were equipped with state-of-the-art reproductive management instruments readily accessible to farmers (Cowscout, GEA Orion Farm Technologies). The activity of the herd was assessed on an individual cow basis utilizing specialized motion sensors integrated into both the neck and foot devices. These sensors recorded metrics such as step counts, feeding durations, lying periods, and standing intervals. This collected data was wirelessly transmitted from the devices to a computer on an hourly basis. The computerized activity records were then processed through a proprietary system to establish specific criteria for estrous activity. When the system identified that an animal was displaying signs of estrus, it emitted a signal, signifying the commencement of the estrous phase.

Determining the Accuracy of Activity Monitors. An estrus was expected around an ovulation, that is, at a low P4 level just before a luteal phase (high P4). Signals from the devices were recorded as “correct” if it occurred during a period when estrus was expected according to the analysis of progesterone level, or “false” if it occurred during a period when estrus was not expected because the level of progesterone was high. If the progesterone level was low but there was no signal from pedometers during the period of anticipation of estrus, then this period was characterized as a “missed” response.

Milk Sampling. Milk samples were taken daily from 1 to 5 times during the day during milking using an automated milking system (Cowscout, GEA Orion Farm Technologies). Immediately after sampling, the milk was stored at -20 °C. Sampling began at least 7 days after calving and continued until at least day 83 of postpartum.

ELISA for determination of progesterone level. Progesterone concentrations in whole milk were measured by direct enzyme immunoassay (Isobe *et al.* 2004).

Coating the microplates. The P4 antibody was diluted 10,000 times with carbonate buffer, 150μl of the diluted antibody solution was added to each well of the microplate. The microplate was wrapped and incubated for 4 h at 20°C in an incubator. The wells were washed with saline (0.9% NaCl) three times and the microplate was patted dry. 200μl of Assay Buffer was added to each well, and the microplate was incubated for 30 min, then the Assay Buffer was disposed of from the microplate.

ELISA. Initially, HRP-P4 was diluted by 500 times and divided into separate doses for storage in the 4 °C until use. Then, it was further diluted by 50 times during the assay, resulting in a final dilution of HRP-P4 by 25,000 times. The sample was warmed up for 30 min at 37 °C and stirred before being it to the wells of the microplate. P4-free milk was also sufficiently stirred. 10μl of each standard solution (concentrations: 0.1, 1.0, 3.0, 10, 30, 100, respectively) was added in duplicate to the wells containing P4-free milk. Before using the standard solutions, they were thoroughly stirred. 10μl of Assay Buffer was added to all wells, except for the wells containing the standard solutions. 180μl of 25,000 times diluted HRP-P4 was added to all wells, including the standard and sample wells. The microplate was sealed with tape and vortexed at 600 rpm for approximately 1 min. Then the microplate was wrapped in foil and incubated in the incubator at 20□ for 3 h. The solution was disposed of from the microplate. The plate was washed three times with PBS solution and patted dry on paper towels. TMB solution was prepared, and 150 μl of the TMB solution was added to each well. The microplate was sealed with tape, wrapped in foil, and incubated in the incubator at 20□ for about 30 min. The incubation time was adjusted according to the reaction. Approximately 5 min before finishing the incubation, the microplate reader was turned on. After incubation, 50 μl of 6N H_2SO_4 was added to

each well to stop the reaction. The microplate was sealed with tape and vortexed at 600 rpm for about 1 min. The absorbance at 490 nm was measured using the microplate reader.

A standard curve was constructed with progesterone concentrations (ng/mL) plotted on the logarithmic scale on the x-axis and optical density on the y-axis. The concentration of progesterone in the milk was determined based on this curve.

RESULTS

Analysis of cyclicity based on progesterone level. Each normal profile was divided into two phases: ovulatory and non-ovulatory. The phase of interval to commencement of luteal activity postpartum (CLA) was also counted for each individual profiles. Cows were in a non-ovulatory phase if the concentration of progesterone in their milk exceeded 5 ng/mL, and in an ovulatory phase if it was below 5 ng/mL. A physiologically normal reproductive cycle of a healthy cow consists of one ovulatory and one non-ovulatory phase.

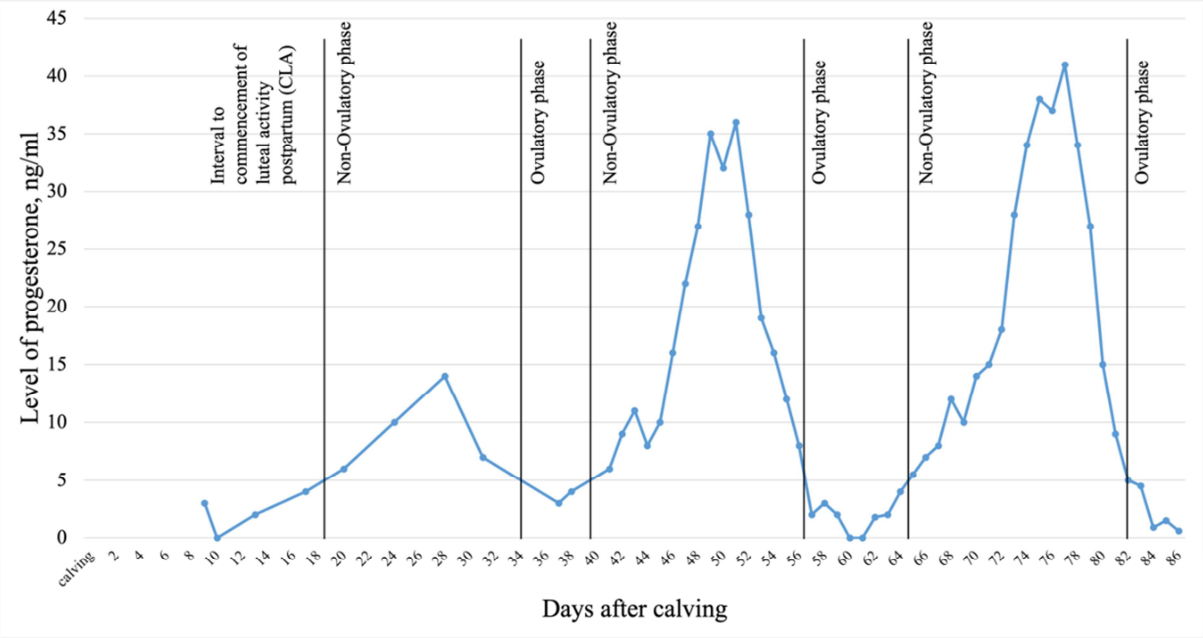
Reproductive cycles were further classified as short (<18 days), normal (18–24 days), and long cycles (>24 days) (Royal *et al.* 2000). Cows with milk progesterone concentrations that never exceeded 5 ng/mL were identified as being in anestrus or the non-ovulatory phase throughout the study.

Representative data of individual progesterone profile shows in the Fig.1.

Figure 1. Progesterone profile for cow number 611.

Figure 1 shows the individual profile of cow number 611 as a representative data. In accordance with this figure, two full cycles are observed, consisting of a non-ovulatory phase and an ovulatory phase. The length of the CLA is 18 days. The length of the first cycle is 22 days (16 days in non-ovulatory phase, 6 days in ovulatory phase). The length of the second cycle is 24 days (16 days in non-ovulatory phase, 8 days in ovulatory phase). In accordance with the classification of ovarian cyclicity, this cow was characterized as an animal with normal cyclicity.

During the experiment, 24 cycles were studied in a herd of 9 animals, and one cow was not included in the total sample due anestrus.



Ovarian cycling was estimated to resume 28.7 ± 12.8 days after calving. The overall average value of the interval from calving to the onset of luteal activity, CLA (28.7 days), can be used as an indicator of the interval to the first ovulation, which occurs on average 5 days before CLA. In our case, the interval from calving to the first ovulation was at the level of 23.7 days.

The estimated duration of normal cycles was 20.5 ± 1.7 days. The frequency of a normal, short, and long cycle was 50.0%, 20.9%, 29.1%. The duration of short cycles in this study was 15.4 ± 1.5 days, and long cycles was 27.5 ± 1.9 days.

The first cycles had an average duration of 21.2 ± 4.2 days with a frequency of 44.5% of the normal, 22.2% of the short and 33.3% of the long cycle. The duration of the short and long cycles, in this study the short cycle averaged 15.5 ± 1.5 days, and the long cycle averaged 26.7 ± 1.2 days.

Also, the P4 profiles for each cow separately were classified into 4 categories (Table 1) based on a modified definition (Opsomer *et al.* 2000; Petersson *et al.* 2006). The pattern of cyclicity was characterized for each cow: 66.7% (6/9) were normal, 22.2% (2/9) – prolonged luteal phase, 11.1% (1/9) – delayed cyclicity. Cessation of cyclicity was not observed in this herd.

Table 1 – Description of ovarian cyclicity.

Type of profile	**Description**	**%**
Normal	First rise in progesterone before days 56 postpartum, followed by regular cyclicity	66.7
Delayed cyclicity	Low progesterone for the first 56 days postpartum	11.1
Cessation of cyclicity	Normal start of cyclicity, but interrupted for at least 14 days with low progesterone levels	-
Prolonged luteal phase	Normal start of cyclicity, but with high progesterone levels for at least 20 days	22.2

Analysis of pedometer signal. After the end of the experiment period, 41 signals were received according to level of progesterone and pedometer's signals. 34.1% (14/41) were not detected and classified as "missed", 17.0% (7/41) were false. 48.9% (20/41) estrus signals were identified as "correct" in Fig. 2.

DISCUSSION

In our studies CLA amounted 28.7 days after calving, the interval from calving to the first ovulation was at the level of 23.7 days that is the average statistical indicators of calculation of first ovulation. Previous studies have shown the median interval CLA was 32.9 days (Horan *et al.* 2005) and 27.4 – 27.9 days,

consistent with our findings (Royal *et al.* 2000). (Darwash *et al.* 1997) got shorter results for these intervals. In this study said the interval to the first ovulation was 21.8 days and the mean interval from calving to CLA was 25.6 days. In addition, it claimed that the means for the interval of CLA during the four calving seasons were 25.5, 27.9, 25.1, and 23.1 days for winter, spring, summer, and autumn, respectively. Because of this, a more detailed focus on the relationship between the CLA interval in our herd and the environment is needed.

Regarding the length of normal cycles, some studies show 22.8 days (Nyman et al. 2014), in our study these data were slightly shorter (20.5 days). Our findings are more consistent with another study (Royal *et al.* 2000) which shows that the interovulatory interval increases with time (from 20.2 to 22.3 days). In general, the consensus regarding cycle length in dairy cows is that it lasts 18 to 24 days, with an average of 21 days (Savio *et al.* 1990). The frequency of normal cycle duration in our study was 50.0%, which is also consistent with values in another study (48.5%) (Nyman *et al.* 2014). Regarding the frequency of short estrous cycles, some study showed the percentage of cows with short cycles at the level of 13.6% (Hinshelwood *et al.* 1982). Also, a long cycle rate was found of 24.3% (Olds *et al.* 1951). In our study, the frequency of short and long cycles is higher than expected (20.9% – short cycles, 29.1% – long cycles). The duration of the short and long cycles averaged 15.4 days (short cycle), and 27.5 days (long cycle).

The duration of the first cycle in this study 21.2 days, this is longer than the duration of cycles in general (20.5 days). Also, our data of the duration of a normal cycle for the first ovulation is less than 23.9 days (Nyman *et al.* 2014). The duration of the short and long cycles averaged 15.5 days (short cycle), and 26.7 days (long cycle). The percentage of normal cycles also was decreased (44.5%), but percentage of the short and long cycle was higher (22.2% for the short and 33.3% for the long cycle). Perhaps this may be due to the recovery of the body after calving because this recovery process should take time and may

affect the length of the first cycle and increase the percentage of long and short cycles.

The pattern of cyclicity was characterized for each cow: 66.7% were normal; 22.2% – prolonged luteal phase; 11.1% – delayed cyclicity. The cessation of cyclicity was not observed in this herd. Information about this pattern in the herd is consistent in that the cessation of cyclicity is quite rare (Opsomer *et al.* 2000). However, it should be noted that the absence of cessation of cyclicity may be due to the small sample of experimental animals. Additionally, a prolonged luteal phase was described such as the most common atypical P4 profile in the herd (Royal *et al.* 2000).

The frequency of false signal given by pedometers in our study was 17.0%. In others study false signals range from 6.5% (Holman *et al.* 2011) to 28.0% (Ranasinghe *et al.* 2010). The missed signal was 34.1% in our studies. Thus, our data is higher than data in other studies which described missed signal rates ranging from 8.0% to 31.0% (Rutten *et al.* 2013). Correct signals were 48.9%, suggesting that activity tracked by the pedometer could be used to detect estrus.

Despite the consistency of the obtained data with existing studies, this inefficiency of the devices can be explained by several factors discussed below. *Suboptimal algorithm* – the reduction in the effectiveness of devices for detecting estrus in cows can be attributed to several attributes. Firstly, some algorithms used in these devices may prove to be insufficient estrus detection. Secondly, inadequate adaptation of algorithms to the individual characteristics of cows, such as their behavior, can diminish their effectiveness. The third f attribute is the unaccounted external environmental factors, such as temperature fluctuations or feeding conditions, which can also influence cow activity and, consequently, estrus detection accuracy. Finally, some algorithms may not align with the actual behavioral traits of cows, leading to false positives or missed estrus signals. Addressing or improving these factors can significantly enhance the efficiency of algorithms in devices for estrus detection for cow’s herd. *Experi-*

mental design: the cows fed in individual raised, and as a result of this, repeated wide movements of the head are possible which reduces the accuracy of devices attached to neck collars. Additionally, our estrus detection was only based on milk P4 assay data but not on visual observe, which quantifies the occurrence of estrus (duration and intensity). *Individual or environmental factors influencing the expression of estrus:* Between 9% and 16% of dairy cows have at least one ovulation without estrus, so some missed signals maybe almost associated with these silent ovulations, and device performance may be underestimated in our study. In addition, false pedometer signals can be caused by cows becoming hyperactive due to an environment, such as temperature changes.

The present results were obtained from single herd observations, so extrapolation should be treated with caution. It's important to note that these devices may not always be entirely reliable due to the possibility of silent ovulation. However, this isn't a significant issue because it's recommended to inseminate within 50 days after the last observed heat (Wiltbank *et al.* 2014). Moreover, the devices in our study only capture part of the animals' behavior. So, combining these devices with visual observations might be a better strategy to improve heat detection, increase conception rates, and reduce culling due to presumed infertility.

Farmers decision to invest in such devices depends on their cost-performers and should be considered in the context of farming practices and specific breeding goals. The benefits of using these devices can vary based on breeding management methods, including calving seasons, herd sizes, and other factors. It's also essential to consider the farmer's lifestyle. For example, if a farmer wants to simplify labor-intensive tasks, have more free time, or allocate time to other activities, that can be a crucial factor in deciding to invest in automated devices. Ultimately, the cost-benefit ratio of these devices requires a more detailed analysis, considering technical, economic, and labor-related aspects for each individual farm.

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