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Dynamic changes on progesterone concentration in cow's milk determined by inline milk analysis system Herd Navigator

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Simple Summary: According to the literature, the inline progesterone monitoring system Herd Navigator (Lattec I/S. Hillerød. Denmark) was used in combination with a DeLaval milking robot (DeLaval Inc., Tumba, Sweden). That works automatically and provides real-time physiological information about lactating dairy cows. For making farm management decisions it is not only a novel tool for scientific research but also a mechanism for improving productivity, food safety, animal well-being, the environment, and the public perception of the dairy industry. It has been hypothesized that the progesterone concentration determined by inline milk analysis system and changes in its dynamics correlate with the number of lactations, reproductive status, and milk yield of cows. The aim of the instant study was to evaluate relative inline milk progesterone (mP4) dynamic changes according to parity and status of reproduction and to estimate the relationship with productivity in dairy cows. Frequent automated mP4 sampling can help identify characteristics of mP4 profiles associated with successful pregnancies, pregnancy losses, and potential differences in mP4 dynamics among parity groups, which have not been studied previously.

Abstract: The aim of the instant study was to evaluate relative inline progesterone dynamic changes according to parity and status of reproduction and to estimate the relationship with productivity in dairy cows by inline milk analysis system (IMAS) Herd Navigator. According to a progesterone assay, cows were divided into three periods: postpartum, after insemination, and pregnancy. In the first stage of the postpartum period (0-29 days), the progesterone level in milk was monitored every 6 days. The second stage of the postpartum period (30-65 days) lasted until cows were inseminated. In the third period (0-45 days) after cows were inseminated, progesterone scores were distributed according to whether or not cows became pregnant. The stability of progesterone dynamics was monitored in the last study period (45-90 days). For milk progesterone detection, the fully automated real-time progesterone analyzer Herd Navigator (Lattec I/S. Hillerød. Denmark) was used in combination with a DeLaval milking robot (DeLaval Inc., Tumba, Sweden). The highest progesterone concentration in multiparous cows ranged from 1.08% (11-17 days postpartum) to 34.89% higher than that in cows of the first parity. The lowest progesterone concentrations in the milk of all cows were estimated during the first 5 postpartum days and between 18 and 23 days after calving. Peak milk progesterone concentrations were evaluated in the first stage of the experiment on days 24-29 after calving. In the 30-65-day period after calving, the level of milk progesterone was 2.02-2.08 times higher than that in the 24-29-day postpartum period. After insemination, the level of progesterone in milk increased by 10.77-22.54% compared with the level from cows on days 30-65 after calving. A higher (12.88%) concentration of progesterone in milk was evaluated in multiparous cows compared with that from cows of the first parity. In pregnant cows, milk progesterone within 0-45 days after insemination was 23.88%

(in multiparous cows) and 32.54% (in primiparous cows) higher than that in non-pregnant cows. On days 31–35 after insemination, pregnant cows had higher milk progesterone levels, which can predict pregnancy success. According to our study results, we can suggest that an inline progesterone concentration determined by inline milk analysis system Herd Navigator and changes in its dynamics correlate with different reproductive statuses and milk yield of cows. Pregnant cows 11–15 days after insemination have higher milk progesterone levels, what positively, associated with a successful pregnancy.

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1. Introduction

The corpus luteum (CL) is a transient endocrine organ in the mammalian ovary. The main function of the CL is to produce the steroid hormone progesterone, which is necessary for establishing and maintaining pregnancy in all domestic animals, including cattle [1]. Milk progesterone (mP4) is considered the gold standard for the evaluation of the reproduction status for research purposes [2]. Studies in dairy cattle have reported an association between insufficient maternal progesterone production during the early stages of pregnancy and early embryo loss [3]. Exposure to progesterone is a prerequisite to the first postpartum behavioral estrus of suckled calves [4]. For several years, the presence of progesterone in milk or blood has been recognized as a valid indicator of different reproductive stages within cattle [5,6]. However, progesterone determinations require specific equipment methods and time [7,8]. A system automatically takes representative milk samples from individual cows at specific milking points during milking and, through a specific algorithm called 'biomodel', automatically selects which cattle should be monitored and sampled in each milking session and which parameters should be measured when the animals arrive at the milking parlor [9]. In addition to progesterone, this sensor provides real-time measurements of lactate dehydrogenase for the detection of (subclinical) mastitis, milk urea to assess the efficiency of protein feed rations and β-hydroxybutyrate to reveal (subclinical) ketosis and/or secondary metabolic disorders. This is not truly 'inline', as samples are taken away from the milk-line and analyzed by an adjoining instrument. Furthermore, the system is expensive and can only be used with DeLaval milking systems [10]. The analysis of reproductive performance has revealed that ketosis, cystic ovaries and postpartum anestrus affect conception rates and the length of the breeding period irrespective of the length of the waiting period. With the use of Herd Navigator, inferior cattle performance can be easily monitored on a real-time basis, and factors contributing to improper ketosis and reproductive management can be identified and corrected to improve farm performance [11]. Although it requires a considerable investment during the installation stage, which would be costly for small farms, the tool is profitable for large farms when compared with the frequent manual collection of progesterone information [12]. An inline sampler inside the milking robot automatically takes a representative sample of several milliliters of milk from an individual cow during milking. The sample is then submitted to the Herd Navigator, which measures progesterone using a dry-stick technique based on an immunoassay [13]. The Herd Navigator has resulted in a reasonably accurate system, although more work is needed to validate the system [14]. Interpretation of progesterone profiles is not always straightforward [15]. Studying actual progesterone profiles should improve our knowledge of the effects of profile features and can indicate the chance of insemination success. Moreover, if cow-specific data (e.g., milk yield) can be linked to the profiles, the effects of cow and environmental traits on progesterone profile features can be made visible [15]. According to Friggens et al. [16], reproductive status can be predicted from milk progesterone values using a biological model, and such a model is robust to reductions in the sampling frequency number and to a doubling of the random variation in raw progesterone values. It therefore has the potential to provide the basis for a useful reproductive

management tool. The testing confirmed the validity of the equation and demonstrated an increased predictive ability with the inclusion of a farm effect. This equation is the first step in moving the focus away from the current paradigm associated with poorer estrus detection, where each detected estrus is automatically inseminated regardless of the situation of near-perfect estrus detection, and it is unknown which cows in estrus are worth inseminating. These results should be extended by incorporating the effects of important factors that were outside the scope of this study and should, where possible, be validated using reproductive examinations [15]. Using the inline milk analysis system (IMAS) data, significant differences in mP4 profiles among primiparous and multiparous cows and among cows with different AI outcomes are observed. A wider adoption of this precision technology will undoubtedly improve our understanding of the factors affecting reproductive physiology of the modern dairy cow, facilitating informed decision making to enhance fertility in dairy herds [17]. The main features of the IMAS for reproductive management include monitoring of resumption of postpartum luteal activity and subsequent luteal phases, declaring imminent estrus based on cessation of a luteal phase, and estimating early nonpregnancy or pregnancy status, all based on mP4 profiles [18].

According to this, we hypothesized that the inline progesterone concentration and changes in its dynamics correlate with the number of lactations, reproductive status, and milk yield of cows. This study aimed to provide information in progesterone dynamic changes determined by inline milk analysis system Herd Navigator, according to parity and status of reproduction and to estimate the relationship with productivity in dairy cows.

2. Materials and Methods

2.1. Location and experimental design

The study was carried out on a dairy farm in the eastern region of Europe in the northern part of Lithuania. Lithuanian Black and White dairy cows were selected according to those fitting a clinically healthy profile after an exact general clinical examination (an average rectal temperature of 38.3-38.8 °C, rumen motility of five to six times per 3 minutes without signs of a disease like mastitis, laminitis, or metritis). The study was performed on 624 dairy cows from a herd of 855 cows. The cows were kept in a loose housing system and fed total mixed ration (TMR) throughout the year at the same time, balanced according to their physiological needs. Cows were fed a TMR consisting of 30% corn silage, 10% grass silage, 4% grass hay, 50% grain concentrate mash and 6% of mineral mixture? Diets were formulated according to meet or exceed the requirements of a 550 kg Holstein cow producing 35 kg/d. Composition of ration – DM (%) 48.8; NDF (% of DM) 28.2; ADF (% of DM) 19.8; NFC (% of DM) 38.7; CP (% of DM) 15.8; NEL (Mcal/kg) 1.6 Feeding took place every day at 06:00 and 18:00. The cows were milked an average of 2.8 times per day. During the study, contact with the animals was minimal, and animal welfare issues were avoided. The average weight of the cows 550 kg +/- 45 kg.

2.2. Measurements

For milk progesterone detection, the fully automated real-time progesterone analyzer Herd Navigator (Lattec I/S. Hillerød. Denmark) was used in combination with a DeLaval milking robot (DeLaval Inc., Tumba, Sweden). The Herd Navigator™ is programmed to collect milk samples automatically and analyze mP4 in individual cows through a dry-stick biosensor technology and enzyme immunoassay [19], based on a bio-model that establishes frequency and quantification of mP4 samples. The system modulated the frequency of assays at an average of six to seven progesterone analyses per cycle according to the postpartum period and the stage of the estrous cycle. An inline sampler in the milking robot automatically took a representative sample of several milliliters of milk from an individual cow during milking. The sample was then submitted to the Herd Navigator. There, progesterone was measured using a dry-stick technique based on an immunoassay. After this, the analyzer sent the data to a user interface. For estrous cycling cattle, a heat alert was displayed as soon as the progesterone level dropped below 5 ng/mL. Together with the heat alert, the algorithm also displayed the percentage of success of prospective artificial

insemination according to the span of the previous luteal phase and the dynamics of the decrease in progesterone values.

2.3. Animals and experimental conditions

293 primiparous and 331 multiparous cows (2+ lactations) have been selected.

According to cows' reproductive status, the cows were classified (Table 1) as belonging to the following nine groups.

During this data acquisition period, cows in were subdivided into groups of target progesterone levels depending on the period of their reproductive cycle.

Table 1. Creation of experimental groups.

Group number/days post partum	Number of cows	
	Primiparous	Multiparous
(1) 0-5	20	18
(2) 6-10	17	19
(3) 11-17	19	22
(4) 18-23	18	21
(5) 24-29	20	23
(6) 30-65 days postpartum until insemination	47	53
(7) 0–45 days after insemination	30	37
(8) Cows that did not become pregnant (45 days after insemination)	44	52
(9) Pregnant cows (45 days after insemination)	78	86

According to the progesterone assay, experimental animals were divided into three periods: postpartum, after insemination, and pregnancy. In the first stage of the postpartum period, progesterone levels in milk were monitored every 5 days. This period of reproductive cycle recovery was followed for 30 days (days 0-29). The second stage of the postpartum period (30-65 days) lasted until cows were inseminated. In the period (0-45 days) after cow insemination, progesterone levels were distributed according to whether or not cows became pregnant. We continued evaluating the stability of progesterone dynamics in the last study period (45-90 days).

The progesterone profiles in the current study had an average of one sample every day (SD = 0.7), and 95% of the cycles had at least one measurement every 7 days.

Additionally, pregnancies were examined on day 46 and confirmed after 60 d. after insemination with a digital diagnostic ultrasound scanner (Draminski iScan, Draminski S.A., Olsztyn, Poland) at a frequency of 7.5 MHz using a linear rectal transducer.

The study was carried out in compliance with the EU legislation. The procedures complied with the criteria given by the Lithuanian animal welfare regulations (No. B1-866, 2012; No. XI-2271,2012) and decree of the director of State Food and Veterinary Service, the Republic of Lithuania No. B6 (1.9) - 855, 2017.

2.4. Data analysis and statistics

The statistical analysis of data was performed using the SPSS 20.0 (SPSS Inc., Chicago, IL, USA) program package. Using descriptive statistics, normal distributions were assessed using the Kolmogorov–Smirnov test. The results were expressed as the mean ± standard error of the mean (M ± S.E.M). The Pearson's correlation (r) was calculated to define the statistical relationship between milk progesterone, highest milk yield (HMY), and average milk yield (AMY). Differences in the mean values of normally distributed variables were analyzed using Student's t-test (the independent samples t-test was used for evaluation of differences between primiparous and multiparous cows, and between become pregnant and did not become pregnant cows). A probability of less than 0.05 was considered significant ($P < 0.05$).

A binary logistic regression technique was performed using pregnancy as the dependent variable (where 1 denotes pregnancy and 0 denotes its absence) to predict the relationship with AMY and milk progesterone levels in cows. AMY values between groups differed statistically significantly from 31-35 days after insemination, and progesterone values in milk - from 11-15 days after insemination (Figure 5). Predictors for logistic regression were considered class variables in the analyses. Values of milk progesterone, estimated 11-15 days after insemination, were divided into two groups: ≤14.73 ng/mL (class 0) and >14.73 ng/mL (class 1). Values of AMY, estimated 31-35 days after insemination, were divided into two groups: ≤19.04 kg (class 0) or >19.04 kg (class 1); milk progesterone values were also divided into two groups: ≤38.35 ng/mL (class 0) and >38.35 ng/mL (class 1). The classification threshold for these indicators was selected on the basis of its arithmetic mean. Estimates and Wald 95% limits were used to calculate odds ratios (ORs) and 95% confidence intervals (CIs).

3. Results

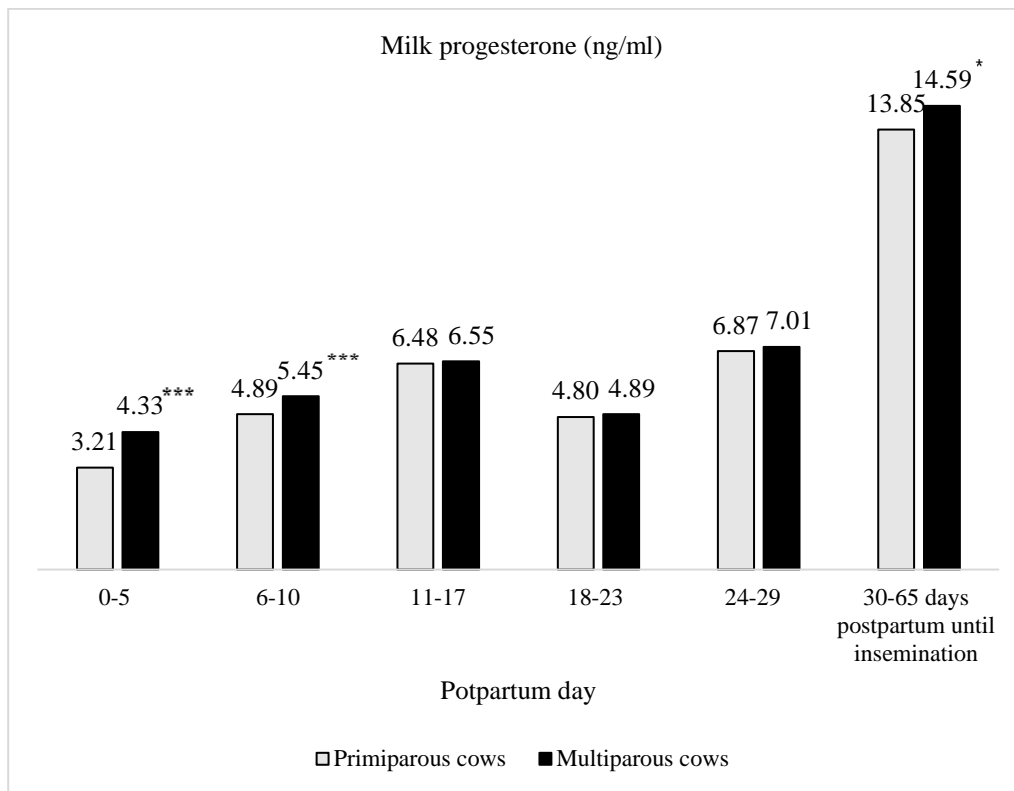*3.1. Changes in the concentration of milk progesterone according to status of reproduction in dairy cows.*

Figure 1. Changes in the concentration of milk progesterone during the postpartum period (*P <0.05, ** P<0.01, *** P<0.001 - average values of progesterone in milk between primiparous and multiparous cows are statistically significantly different)

In the first stage of the postpartum period (Fig. 1), due to the persistence of the CL in pregnancy, the basic progesterone concentration in the cows' milk ranged from 3.2 to 4.3 ng/mL in primiparous and multiparous cows, respectively. In the subsequent period (6-10 days), with gradually recovering reproductive cycles, progesterone concentration increased to 4.9 and 5.4 ng/mL in the two groups, respectively. In the third period, progesterone levels increased significantly to an average of 6.48 and 6.55 ng/mL in both experimental groups. These data are typical of dairy cows during the reproductive cycle.

We also determined that the highest progesterone concentration in multiparous cows ranged from 1.08% (11-17 days postpartum) to 34.89% (0-5 days postpartum; $P < 0.001$) higher than that in cows of the first parity. From 30-65 days postpartum until insemination, the concentration of milk progesterone in multiparous cows was 5.35% higher ($P < 0.05$) than that in the first-parity cows.
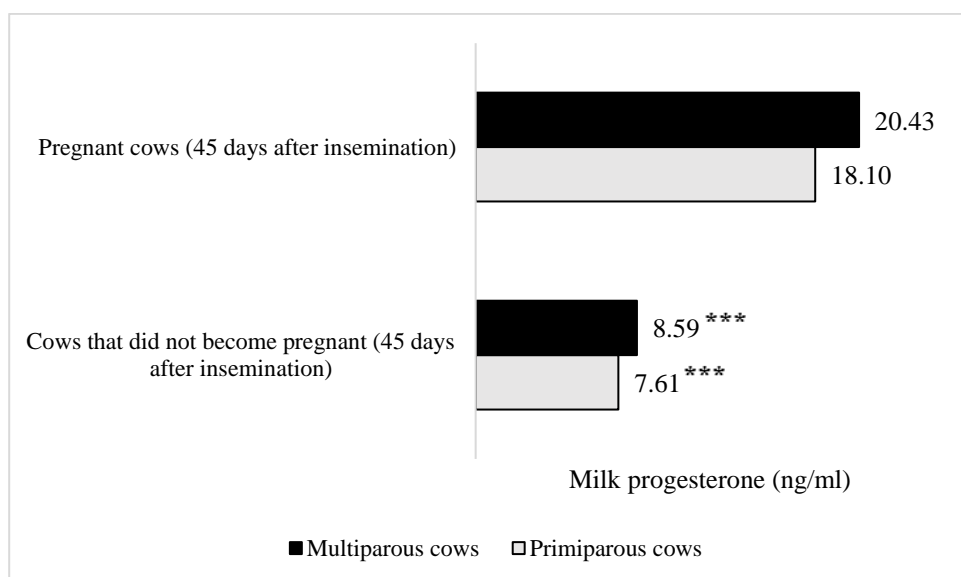
Figure 2. Changes in the level of milk progesterone (ng/mL) after insemination and during the pregnancy period (***- average values of progesterone in milk between become pregnant and did not become pregnant primiparous and multiparous cows with different superscripts differ significantly at P<0.001)

The lowest progesterone concentrations in the milk of all cows were estimated during the first 5 postpartum days and between 18 and 23 days after calving. Peak milk progesterone concentration was evaluated in the first stage of the experiment on days 24-29 after calving, and it was 1.34-1.35 times higher ($P < 0.05$) than the milk progesterone concentration on days 18-23 postpartum and 1.62-2.14 times ($P < 0.001$) higher than that on days 0-5 postpartum. In the 30-65 days after calving, the level of milk progesterone was 2.02-2.08 times higher than that on days 24-29 postpartum ($P < 0.001$). These dynamic progesterone concentration changes between 11-17- and 24-29-day periods illustrate the periodicity of the reproductive cycle, and the average period was 12 days. Additionally, there was no significant difference in progesterone concentration between the two experimental cow groups. After insemination, the level of progesterone in milk increased by 10.77-22.54% compared with that in cows on days 30-65 after calving ($P < 0.01$). A higher (12.88%) concentration of progesterone in milk was evaluated in multiparous cows ($P < 0.01$) compared with that in cows of the first parity. In pregnant cows, milk progesterone within 0-45 days after insemination was 23.88% (in multiparous cows) and 32.54% (in primiparous cows) higher than that in non-pregnant cows ($P < 0.001$). These data are presented in Figure 2.

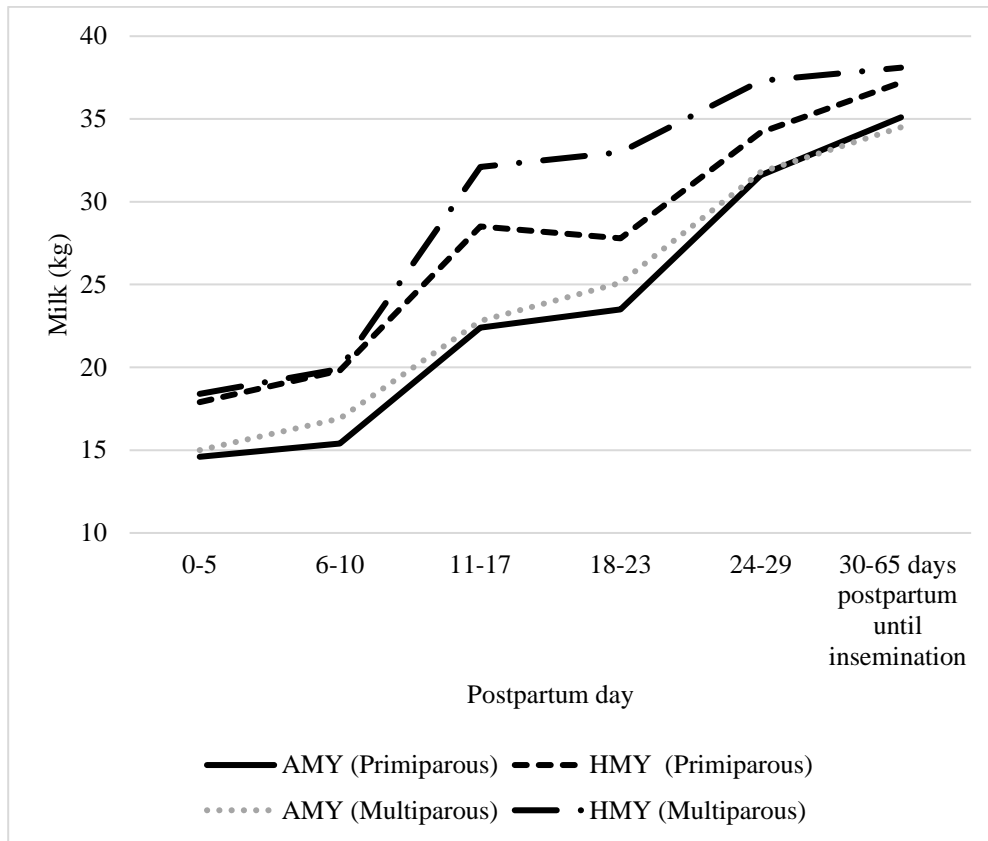*3.2. Changes in milk productivity of cows according to status of reproduction*

Figure 3. The milk yield of cows in the postpartum period until insemination. AMY—average milk yield; HMY—highest milk yield.

The AMY in primiparous cows increased 2.30-2.40 times from 0-5 days postpartum to 30-65 days, and HMY increased 2.07-2.08 times ($P < 0.001$). A slowdown in productivity growth was observed during two periods: 6-11 days postpartum and 18-23 days (Figure 3).
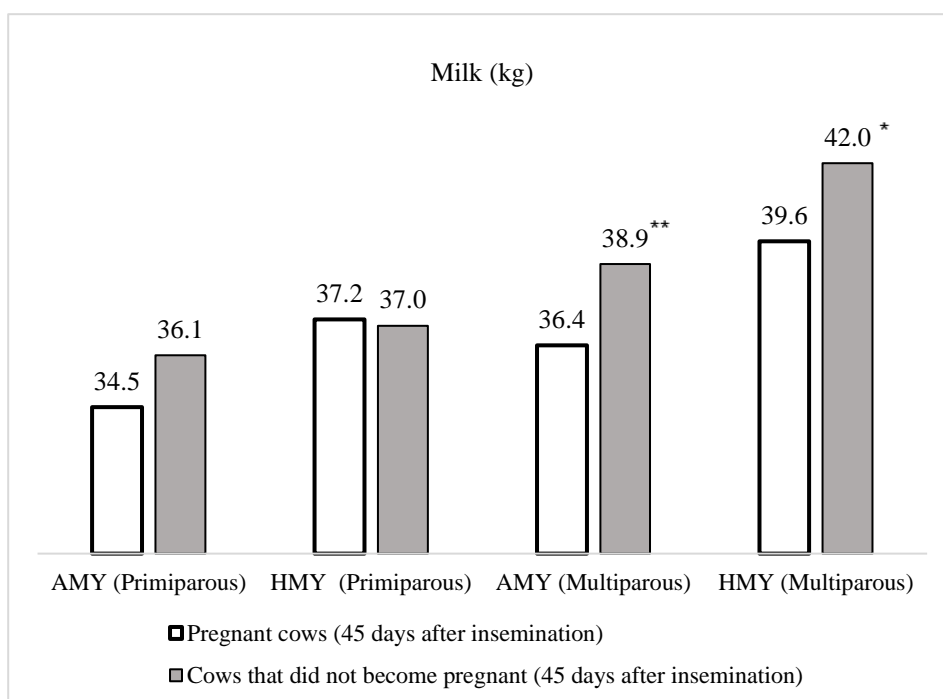
Figure 4. The milk yield (kg) of cows 0-45 days after insemination. (*P <0.05, ** P<0.01, *** P<0.001 - average values of progesterone in milk between primiparous and multiparous cows are statistically significantly different)

Non-pregnant cows within 0-45 days after the insemination period were more productive (Figure 4). The AMY in primiparous cows (34.5 ± 1.72 kg) was 4.64% higher, and in multiparous cows (36.4 ± 1.69 kg), it was 6.87% higher (P < 0.01). We estimated a significantly lower HMY (6.06%, P < 0.05) in pregnant multiparous cows compared with cows that did not become pregnant (39.6 ± 0.62 kg).

3.3. Relationship of progesterone in milk with productivity of cows according to status of reproduction.

Period/status of reproduction			Coefficients of correlation	
			AMY	HMY
Postpartum period	0-30-day postpartum period	0-5 days	0.142	0.072
		6-10 days	0.162	0.130
		11-17 days	0.261	0.207
		18-23 days	0.346*	0.324*
		24-29 days	0.212	0.275
	30-65 days postpartum until insemination		0.092	0.048
Period after insemination	0-45 days after insemination	Became pregnant	0.251*	0.104
		Did not become pregnant	0.082	0.066

Table 2. Correlation of progesterone in milk with milk yield of cows; (*P<0.05 - Pearson's correlation **is** statistically significant)

Correlation coefficients between the progesterone and milk yield of cows are presented in Table 2. The data indicate that cows' AMY and HMY were positively associated with milk progesterone concentration, a statistically significant relationship was found at 18-23 days postpartum (r = 0.324 - 0.346; P < 0.05). The significant coefficient of correlation was estimated between AMY and milk progesterone in pregnant cows 0-45 days after insemination (r = 0.251; P < 0.05).

Logistic regression analysis showed that at 31–35 days after insemination, cows with higher progesterone levels in their milk had a statistically significant higher probability of pregnancy success (OR = 8.53; 95% CI = 1.616–45.061; P = 0.022) compared with that of cows with lower progesterone

levels in their milk. Milk yields in cows did not have a statistically significant effect in predicting the likelihood of their pregnancy.

The study showed that, depending on the concentration of progesterone in milk, pregnancy success in cows can be predicted 11-15 days after insemination, when a significant increase in progesterone is observed in the group of pregnant cows (OR = 7.43; 95% CI = 1.778–31.040; P = 0.006) compared with non-pregnant cows.
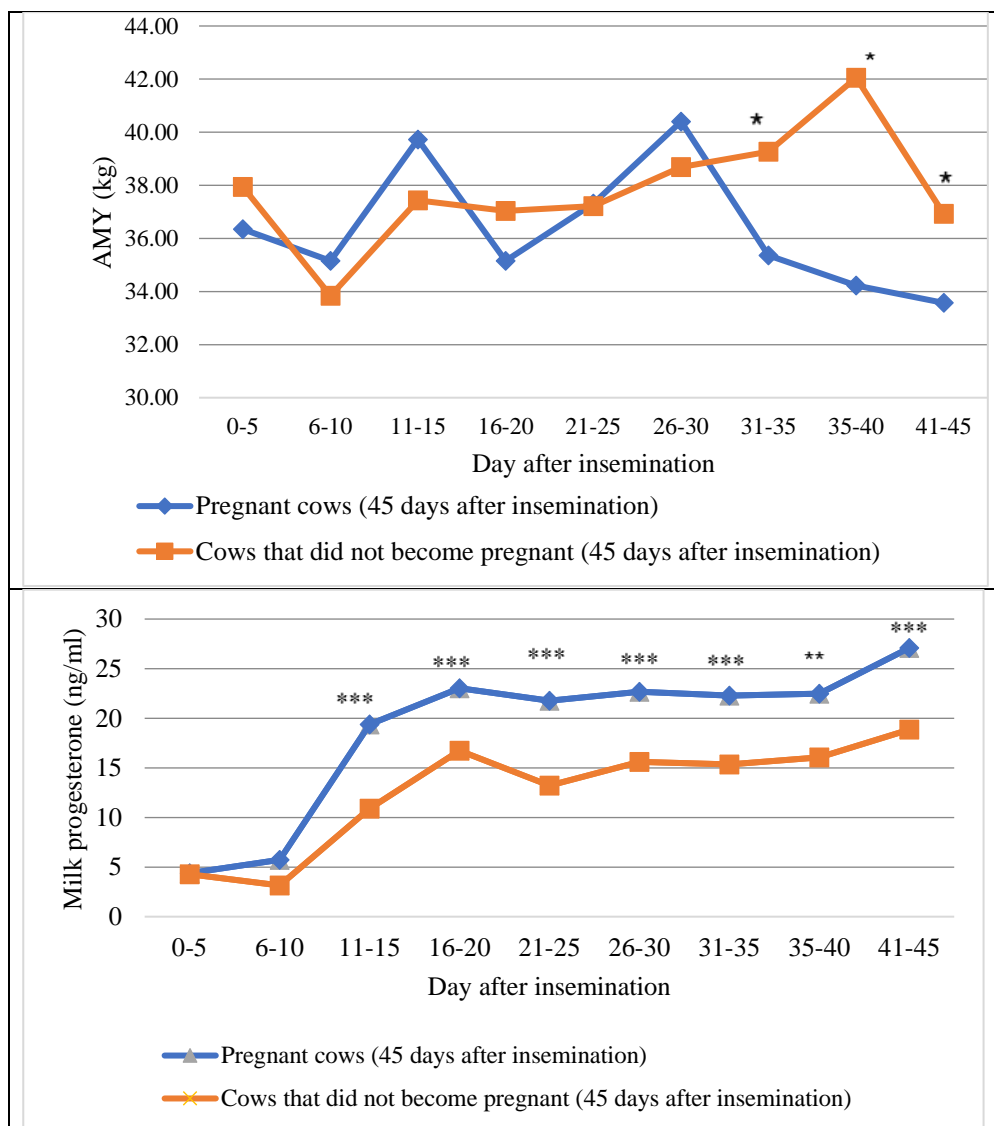

Figure 5. Changes in milk yield and progesterone in cows that became pregnant and did not become pregnant after insemination (*P <0.05, ** P<0.01, *** P<0.001 - average values between become pregnant and did not become pregnant cows are statistically significantly different)

4. **Discussion**

This study investigated inline progesterone dynamic changes according to milk yield, lactation number, and status of reproduction in dairy cows. An inline 'herd navigator' system that works automatically and provides real-time physiological information about lactating dairy cattle for making farm management decisions is not only a novel tool for scientific research but also a mechanism for improving productivity, food safety, animal well-being, the environment, and the public perception of the dairy industry.

The new-found results show and confirm that progesterone concentration within pregnant cattle was higher than that in other investigated cattle groups, which coincides with the results reported by Hommeida et al. [20]. Larson et al. [21] noted that progesterone concentrations 5-10 days after insemination were higher in pregnant cattle. After ovulation, an optimal mP4 environment is required for establishment of pregnancy [22] and increasing concentrations of mP4 following AI support embryo development through uterine secretions of proteins and growth factors. In addition to being more sensitive to metabolic changes (e.g. negative energy balance) in the early postpartum period than multiparous cows, primiparous cows are more likely to develop uterine diseases, which are known factors affecting resumption of postpartum cyclicity [22]. According to Berger et al. [23], the luteal area and progesterone concentration were greater in pregnant cattle compared with those in open cattle. However, other studies did not find such a correlation [24]. The overall mean milk progesterone concentration in pregnant cattle did not significantly differ from that in non-pregnant animals, but there was a strong quadratic relationship between both low and high milk progesterone concentrations associated with reduced conception rates. The pregnant animals did not experience an increase in the pulsatile release of PGF2α, and the CL was maintained for a further 200 days of pregnancy [25]. However, a failure to maintain this mechanism was found: it was initiated but not sustained and resulted in early embryonic loss [26]. Herzog et al. [27] reported that embryonic loss did not affect the plasma concentration progesterone or the luteal area, which was similar between pregnant cattle and cattle with apparent embryonic loss. According to Gomez-Seco et al. [28], in the pregnant group, the general correlation (from day 0 to 23) was lower than that in non-pregnant cattle, although, during the developing phase, this value was similar in both groups. In contrast, progesterone concentrations were similar between both groups: they started to show higher values for pregnant cattle after day 13, and this trend was clearly defined after day 17. However, the CL size began to differ earlier (the first significant difference was observed by day 9). We found that 31–35 days after insemination, cows with higher progesterone levels in their milk had a statistically significant higher probability of pregnancy success.

According the results of current study in the period 6-11 days, progesterone concentration increased to 4.9 and 5.4 ng/mL in the two groups, respectively. The faster rise in mP4 from d 0 to 7 was associated with improved pregnancy maintenance. In addition, higher mP4 after d 4 relative to AI was associated with greater trophoblast length at later stages, greater uterine concentrations of interferon-tau and reduced pregnancy losses [28], potentially indicating a direct effect of mP4 on early embryonic development or an indirect effect via a conducive uterine environment. Our results suggest that improved conception is associated with high mP4 beyond d 10 regardless of whether the pregnancy is sustained or lost.

The results of our study show that multiparous cattle had a lower progesterone concentration, which coincides with Pineyrua et al. [29], who found that the mean concentrations of postpartum mP4 were different between multiparous and primiparous cattle. Pineyrua et al. [29] and Caixeta et al. [30] reported limited data to support our hypothesis that metabolic and hormonal changes during the first 4 weeks of lactation possess negative carryover effects on the reproductive performance of Holstein and Jersey dairy cattle later in the lactation period. The higher mP4 levels in primiparous than in multiparous cows were expected as a higher milk yield in multiparous cows would be associated with greater dry matter intake and, consequently, accelerated metabolic clearance of mP4, decreasing its peripheral concentrations. Primiparous cows had distinctly different mP4 profiles (greater levels and more rapid increase early post-AI) than multiparous cows [17]. Multiparous cows

reportedly required greater doses of exogenous PGF2α than primiparous cows to achieve similar luteolysis rate, suggesting potential differences in corpus luteum (CL) responses to PGF2α (greater occurrence of incomplete CL regression in multiparous than in primiparous cows). Recent reports using IMAS in commercial dairy herds or continuous manual milk sampling for mP4 determination in a research herd indicate that differences in ovarian function exist between primiparous and multiparous cows, such as in characteristics of luteal cycles early postpartum [31]. Frequent automated mP4 sampling can help identify characteristics of mP4 profiles associated with successful pregnancies, pregnancy losses, and potential differences in mP4 dynamics among parity groups, which have not been studied previously.

Milk progesterone concentration and a relatively high milk yield were negatively correlated in fresh and inseminated cattle groups and positively correlated in pregnant cattle. Milk yield has been demonstrated to lower concentrations of progesterone as a result of higher clearance in the liver [32]. The milk yield effect may support the clearance of progesterone theory: if progesterone is cleared from the bloodstream faster, the curve of progesterone will be flattened [33].

5. Conclusions

According to our study results, we can suggest that an inline progesterone concentration determined by inline milk analysis system Herd Navigator and changes in its dynamics correlate with different reproductive statuses and milk yield of cows. Pregnant cows 11–15 days after insemination have higher milk progesterone levels what positively associated with a successful pregnancy.

Author Contributions: Ramunas Antanaitis: supervision of the whole study; Vida Juozaitiene: software and algorithm development, design and setup of field experiments, data collection, and analysis; Dovile Malasauskiene: setup of field experiment and data collection, selection, and management of the experimental group of animals; Mindaugas Televicius: setup of field experiment and data collection, selection, and management of the experimental group of animals. Henrikas Žilinskas setup of field experiment and data collection, selection, and management of the experimental group of animals. Walter Baumgartner: Methodology, Writing - review and editing. The manuscript was written by Ramunas Antanaitis and revised by all co-authors

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