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The thermographic monitoring in early pregnancy detection in Holstein cows and heifers

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ABSTRACT

This study aimed to conduct a comprehensive investigation to demonstrate early pregnancy detection using thermography in heifers and cows. A total of 60 heifers (*n*: 30) and cows (*n*: 30) were divided into two groups as pregnant (*n*: 15 heifers, *n*: 15 cows) and non-pregnant (*n*: 15 heifers, *n*: 15 cows) according to the day 28 of gestation. Thermographic images were taken from the vulvar and anal regions on alternate days from D0 to D20. Blood samples were collected to determine estrogen and progesterone concentrations. The mean temperature difference between the anal and vulvar regions (ΔT °C) was used in the statistical analyses. Based on the hormonal profiles, no abnormalities were observed for follicular waves or luteal profiles in heifers and cows. The ΔT °C values between heifers and cows and between days were statistically significant ($P < 0.001$). In thermographic analyses, the differences observed in other main effects and interactions of the group, sampling time, and pregnancy were not statistically significant ($P > 0.05$). In receiver operating characteristic (ROC) analyses, it was concluded that the ΔT °C value of $\leq$ 2.9 °C (100% Se - 61.9% Sp) was highly correlated with pregnancy diagnosis in cows on day six after artificial insemination (AI) ($P < 0.001$). In conclusion, it was determined that the clinical application of thermography can be used for the detection of pregnancy on day six after AI in cows. However, further studies are needed to determine heifers' thermographic characteristics and profiles.

1. Introduction

Achieving pregnancy at the end of the voluntary waiting period is a valuable gain in cattle breeding. However, the most crucial problem is embryonic mortality occurring in the early stages of pregnancy immediately after fertilization (Lee and Kim, 2007). Over

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half of the early embryonic deaths in high-yielding dairy cows occur within the first three weeks of gestation (Sartori et al., 2016; Scully et al., 2014). Therefore, it is vital to identify the pregnancy status during this period. Besides, the specificity of pregnancy diagnosis by classical methods such as ultrasonography, which is based on the observation of embryo or embryo-derived structures, and rectal palpation remains below 70% before the 20th day after artificial insemination (AI) (Boyd et al., 1988; Cengiz et al., 2017; Ribadu and Nakao, 1999; Silva and Ginther, 2010). In addition to classical methods, faster and more accurate results can be obtained with the help of various biomarkers measured from maternal body fluids during very early pregnancy, such as early pregnancy factor protein (EPF), microRNAs (miRNAs), preimplantation factor protein (PIF), pregnancy-associated glycoproteins (PAGs), and circulating nucleic acids (CNAs). However, the fact that these methods are expensive laboratory analyses limits their practical use in a cattle herd (Ioannidis and Donadeu, 2016; Mayer et al., 2013; Ramu et al., 2013; Silva et al., 2007). Thus, researchers are searching for practical and inexpensive methods that can be used for pregnancy detection, providing rapid and real-time results in the early stages. Imaging techniques such as ultrasound and thermography stand out among the others because they are both non-invasive and have the desired characteristics. In particular, thermography has been used in the reproductive field in recent years due to its reduced contact with the animal, the examined area, and its suitability for mechanization.

Thermography is an imaging technique based on detecting infrared radiation beams emitted from a surface and converting radiation levels into temperature values. Due to these characteristics of thermographic imaging technology, its use in human and animal health has become widespread in the last 20 years (Bowers et al., 2009; Eddy et al., 2001; Mazur et al., 2006; Smaga et al., 2003; Topalidou and Downe, 2016). In this context, studies have been conducted on the use of thermography in the monitoring and diagnosis of superficial injuries, infectious diseases, inflammatory symptoms, joint and bone disorders, and in reproduction (Rekant et al., 2016). The examination of udder health in cows (Çolak et al., 2008; Polat et al., 2010), estrus detection in bitches and gilts (Olğaç et al., 2017; Sykes et al., 2012), determination of ovulation time in cows (Talukder et al., 2015), pregnancy detection in ewes (Yazlik et al., 2020) and evaluation of the pregnancy process in mares (Bowers et al., 2009) are among these procedures. Variations in hormone concentrations during reproductive events, such as cyclic activity and pregnancy, change the blood flow in the genital organs and tissues of females. This information has led to the majority of studies designed to determine estrus and the time of ovulation (Stelletta et al., 2017). In a few studies conducted on pigs, mares, sheep, and wild species, thermographic imaging can also be used to detect pregnancy (Bowers et al., 2009; Domino et al., 2021; Hilsberg et al., 1997; Simões et al., 2014; Yazlik et al., 2020). On the other hand, it was reported that thermographic imaging could not indicate the pregnancy status in bitches (Durrant et al., 2006). Similarly, it has been concluded that using vulvar thermography in cows and heifers is unsuitable for pregnancy detection (Jones et al., 2005; Radigonda et al., 2017).

Previous studies have reported conflicting results regarding the clinical use of thermography in pregnancy assessment. This study is known to be the first on the use of anal and vulvar thermographic temperature differences (ΔT) in pregnancy diagnosis in cows and heifers. Using the ΔT value in thermographic studies is more appropriate than using the body or vulvar temperature alone. This is because surface temperatures can vary among individuals under the influence of internal or external conditions. Therefore, surface temperature differences in relation to body temperature at the time of imaging may provide more accurate and clinically meaningful results. Furthermore, no study evaluates thermographic changes according to early pregnancy development in cows and heifers for the first 20 days after AI. The study hypothesizes that pregnancy can be detected by evaluating ΔT °C during this period, which is affected by critical events such as hormonal regulation, embryonic development, pregnancy recognition, and implantation. The aim was to perform a comprehensive study that reveals the differences and similarities in terms of thermographic characteristics between Holstein heifers and cows.

2. Materials and methods

All applicable international, national, and/or institutional guidelines for animal care and use were followed. Furthermore, all procedures involving study animals in the experiment were approved by Ankara University Animal Experiments Local Ethics Committee (No: 2021–20–180).

2.1. Animals

A total of sixty Holstein cows ($n = 30$) and heifers ($n = 30$) were included in the study. They were fed according to NRC recommendations for ad libitum intake and water. Animals were housed in free-stall barns on the same farm, with similar care and feeding conditions. Animals were evaluated by general examination, body condition scoring, and reproductive examination to determine health status before and during the study. The body condition scores of the animals ranged from 2.5 to 3. The heifers included in the study were 15 months of age and had never been inseminated/mated before. Cows were 2–3 years old, and the mean milk yield was 35 kg/day. Cows were at the end of the voluntary waiting period (90 DIM) after parturition.

2.2. Experimental design

This study was conducted in a commercial dairy farm located in Ankara province (N39.425217, E32.846051) in the spring season of 2022. Healthy animals without reproductive disorders were allocated to the present study. Animals were inseminated using a fixed-time AI followed by a presynch-ovsynch synchronization program (Giordano et al., 2016; Gordon et al., 2010). Briefly, animals received PGF2α (Gestavet-PROST, Hipra, Türkiye), while fourteen days later, a second PGF2α was injected. Twelve days after the second PGF2α, the first GnRH (Buserin, Alke, Türkiye) was injected. Seven days after the GnRH, the animals received another PGF2α, and the synchronization program was performed with the GnRH injection 56 h after the last PGF2α. Fixed-time AIs were performed 16 h after the last GnRH injection. To eliminate the bull/sperm effect on pregnancies, heifers and cows were inseminated with commercial frozen bull semen of a Holstein bull with known fertility.

Animals were divided into two subgroups according to pregnancy status as pregnant (cow, $n = 15$; heifer, $n = 15$) and non-pregnant (cow, $n = 15$; heifer, $n = 15$) on day 28 (D28) after AI (D0). Thermographic images were taken from the vulvar and anal regions on days 0 (D0), 2 (D2), 4 (D4), 6 (D5), 8 (D8), 10 (D10), 12 (D12), 14 (D14), 16 (D16), 18 (D18) and 20 (D20). Moreover, blood samples were collected from the coccygeal vein or artery into Vacutainer tubes without anticoagulant (10 mL, Hema&Tube®, Türkiye) to determine estrogen and progesterone concentrations immediately after thermographic imaging. After clotting, the blood samples were stored at + 4 °C. Then the samples were centrifuged at 4500 x rpm at 4 °C for 10 min within one h after collection. Serum was collected in 2 mL tubes and frozen at − 80 °C until analysis.

2.3. Thermographic imaging

All thermographic images were taken during the same time period (09:00 a.m. to 11:00 a.m.) on the mentioned days following fixed-time AIs. The thermography device (FLIR E60, Teledyne FLIR LLC, USA) was set to 0.95 emissivity and a temperature range of 15.0–40.0 °C. Prior to thermal imaging, the tail was held aside, and the vulva and anus were gently wiped with dry paper towels to remove feces and dirt. Imaging was performed indoors to avoid wind and daylight to minimize external conditions at a similar time of day during the sampling procedure. Images with the vulva and anus in the same view were taken at a 90° angle and from a distance of 50–100 cm to obtain optimal resolution. Multicolor thermographic images were analyzed with a temperature scale of 15.0–40.0 (<0.05 °C sensitivity), 0.95 emissivity, related environmental humidity, and temperature using ResearchIR software (ResearchIR 4, Teledyne FLIR LLC, USA). The first region of interest (Polygon 1) was determined to include the vulvar labia and upper and lower commissura labiorum, and the second region of interest (Polygon 2) was determined to include the anal region (Fig. 1). The ΔT °C (Polygon 2 – Polygon 1) value was used in the statistical analyses.

2.4. Measurement of blood estrogen and progesterone concentrations

Serum samples stored at 80 °C were gradually thawed by placing them at + 4 °C and then at room temperature. Subsequently, they were centrifuged at 4500 x rpm at + 4 °C for 5 min to proceed with the ELISA application. The levels of estrogen and progesterone in the serum samples were determined using commercial ELISA kits (for estrogen: CK-BIO-18476 and for progesterone: CK-BIO-18626, Coon Koon Biotech, Shanghai, China) according to the kit protocol using an ELISA Reader (Allsheng AMR-100, China) at a wavelength of 450 nm. The obtained absorbance values were converted to concentration values based on the standard graphs generated using the standards provided in the kits. All ELISA applications were performed at the Molecular Genetics Laboratories of the Faculty of Veterinary Medicine in Hatay Mustafa Kemal University, Türkiye.

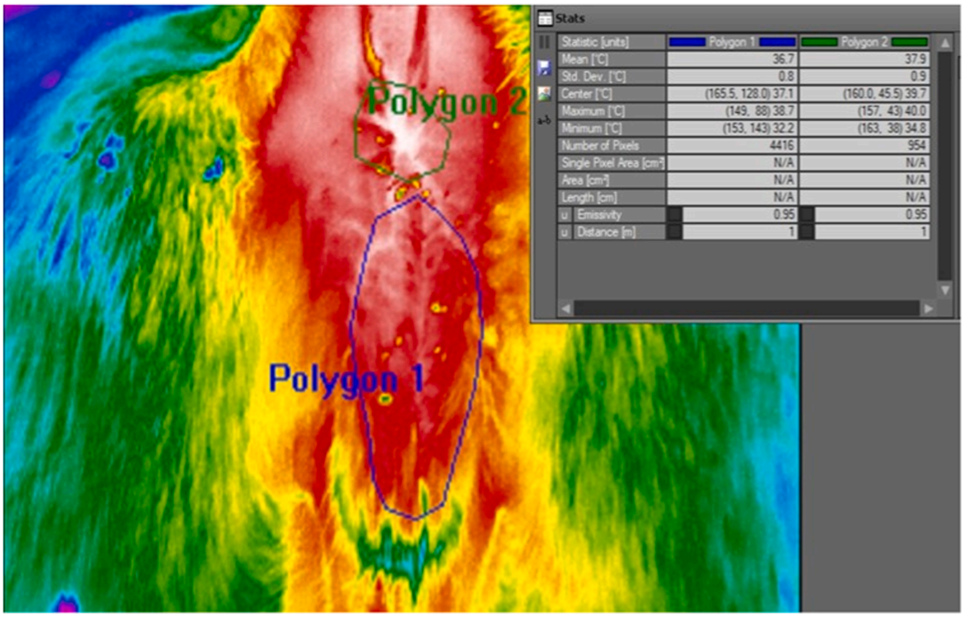

Fig. 1. Analysis of thermographic images. Multicolor thermographic images were analyzed with a temperature scale of 15.0–40.0 (<0.05 °C sensitivity), 0.95 emissivity, related environmental humidity, and temperature. Polygon 1 represents the vulvar region of interest, and Polygon 2 represents the anal region of interest. The results of the thermographic analysis of the selected areas are shown at the top right of the image. ΔT °C was calculated as the mean [°C] value of Polygon 2 minus the mean [°C] value of Polygon 1.

2.5. Pregnancy diagnosis

Pregnancy status was determined on day 28 and day 60 after AI using trans-rectal ultrasonography to identify the presence of an embryonic heartbeat, using a 6.5–9.5 mHz linear rectal probe connected to a portable B-mode ultrasonography device (CTS 800, SIUI, China), and the results were recorded. Ultrasonographic pregnancy assessments were performed by the same staff member throughout the study, using the standard settings of the ultrasound device.

2.6. Statistical analysis

This was a retrospective cohort study. The sample size was calculated with G*Power software version 3.1.9.2. The result of the sample size calculation showed that the minimum number of animals was 60, considering an effect size of 0.31, an alpha value of 0.05, and a power of 0.80. Statistical analyses were performed using IBM SPSS Statistics software version 23.0 and MedCalc version 9.2.0.1. The statistical significance level was set at $P < 0.05$. Descriptive statistics were calculated and presented as "Mean ± Standard error of the Mean (SEM)". Variables were first examined to determine whether the normal assumptions for parametric tests were met. A linear mixed model for repeated measures was performed to test the effects of groups, pregnancy status, and sampling times. Animals (heifers and cows) were included in the model as random effects, while group, pregnancy status, and sampling times were included as fixed effects. Besides, the two-way and three-way interactions were also evaluated in the model. When a significant difference was obtained, all significant terms were compared using a simple effect analysis with Bonferroni correction. The model shown below was used for statistical analysis.

$$Y_{ijklmno} = \mu + G_i + P_j + T_k + (G*P)_l + (G*T)_m + (P*T)_n + (G*P*T)_o + e_{ijklmno}$$

($Y_{ijklmno}$, dependent variable; μ, the overall mean; G_i, the effect of the group (heifer and cow); P_j, the effect of pregnancy status (pregnant and non-pregnant); T_k, sampling time (D0 to D20); $(G*P)_l$, the interaction of group and pregnancy status; $(G*T)_m$, the interaction of group and sampling time; $(P * T)_n$, pregnancy status and sampling time; $(G*P * T)_o$, group, pregnancy status and sampling time; and $e_{ijklmno}$, the residual error).

Receiver operating characteristic (ROC) analysis was performed to determine the thermographic critical threshold (cut-off) points for predicting the cows and heifers most likely to conceive at each sampling time. The area under the curve (AUC) values related to each variable was determined as the most predictive critical threshold for the identification of cows and heifers with the highest probability of conception. Furthermore, sensitivity, specificity, and 95% confidence interval (CI) were calculated. AUC values of = 1, > 0.9, 0.7–0.9, and 0.5–0.7 indicated excellent accuracy (Se = Sp = 1), high level of accuracy, moderate accuracy, and poor accuracy, respectively.

3. Results

None of the animals used in this study showed signs of embryonic mortality. The mean temperature differences between the anal

Table 1
Means and interactions of ΔT °C in pregnant (P) and non-pregnant (NP) cows and heifers evaluated from D0 to D20 (Mean ± SEM).

Sampling Time	Group	
	Cow	Heifer
D0	2.63 ± 0.16	1.30 ± 0.15
D2	2.98 ± 0.20	2.05 ± 0.31
D4	2.49 ± 0.17	1.46 ± 0.12
D6	2.80 ± 0.19	1.75 ± 0.23
D8	3.03 ± 0.22	2.18 ± 0.26
D10	3.11 ± 0.18	2.51 ± 0.30
D12	3.41 ± 0.19	3.50 ± 1.16
D14	3.28 ± 0.21	1.91 ± 0.22
D16	3.27 ± 0.21	2.07 ± 0.22
D18	3.18 ± 0.18	1.71 ± 0.23
D20	3.18 ± 0.18	1.77 ± 0.75
Pregnancy Status		
Non-Pregnant	3.10 ± 0.07	1.88 ± 0.16
Pregnant	2.89 ± 0.10	1.97 ± 0.11
***P* Values**		
Group (G)	< 0.001	
Sampling Time (T)	< 0.001	
Pregnancy (P)	0.941	
G * T	0.582	
G * P	0.316	
T * P	0.217	
G * T * P	0.173	

and vulvar regions (ΔT °C) between heifers and cows and between days were determined to be statistically significant ($P < 0.001$). The ΔT °C changes according to pregnancy did not show statistical significance in the main effect analyses ($P > 0.05$). The differences determined in the interactions of the group, sampling time, and pregnancy in the thermographic analyses were not observed to be statistically significant ($P > 0.05$; Table 1).

Receiver operating characteristic (ROC) analyses were performed to determine a cut-off point for use in pregnancy detection in Holstein cows and heifers (Table 2; Fig. 2). It was concluded that the ΔT °C value of ≤ 2.9 °C (100% Se - 61.9% Sp) had a high correlation with a positive pregnancy in cows on day six after AI ($P < 0.001$). Conversely, in heifers, it was determined that temperature differences on any day during the first 20 days after AI were not found to be predictive ($P > 0.05$).

Based on hormonal profiles, no abnormalities were observed for follicular waves or luteal profiles in heifers and cows. Mean estrogen and progesterone concentrations are shown in Fig. 3.

4. Discussion

In the study, the thermographic monitoring of pregnant and non-pregnant cows and heifers was evaluated, and the cut-off points for predicting pregnancy in cows and heifers were determined. Thermography is known as a technology used to examine the reproductive cases in mammals, such as andrological examinations, udder health, ovulation, estrus detection, pregnancy status, etc. However, in contrast to previous studies, the present study has novelties in terms of the evaluated value (ΔT °C), the imaging frequency and period (repeated on alternate days from days 0–20 after AI), and it compared both cows and heifers. Furthermore, the study has a unique design to simulate thermographic monitoring in clinical applications on a cattle herd.

Thermographic imaging was previously used for pregnancy determination in cows (Radigonda et al., 2017). It was reported that vulvar temperatures measured on the day of AI showed significant changes in determining follicular activity. The follicular activity was determined by the presence of the corpus luteum on day eight after AI. While the vulvar temperature of cows with a corpus luteum was approximately 34 °C, it was around 35.5 °C in cows without a corpus luteum. Likewise, the presence of corpus luteum was determined in all cows in the present study. Thus, no abnormalities in follicular activity were observed. Radigonda et al. (2017) stated that the pregnancy status of the cows on the 30th day after AI and the vulvar temperatures of pregnant and non-pregnant cows measured on the day of AI were similar and between 34 and 34.5 °C. Similar results were also obtained in heifers (Vicentini et al., 2020). The present study seems to support previous research. Although the thermographic data at the time of insemination and within the following 20 days showed significant differences between heifers and cows, similar values were observed between the days of measurement for the main effects among all relevant interactions. This situation is consistent with other studies. While ΔT °C values obtained from heifers and cows vary according to follicular activity, a precise temperature value indicating pregnancy status in the first 20 days after AI could not be determined. However, the present study clearly showed that the thermographic profiles of cows and heifers differ. The ΔT°C values were below 2 °C in heifers and above 3 °C in cows. This means that different thermographic profile characteristics for cows and heifers were also observed in the ROC analyses. According to the cut-off point evaluation, ΔT °C

Table 2
Receiver operating characteristic (ROC) curve analysis for the determination of critical threshold for ΔT °C as a predictor of most likely to be pregnant cows and heifers.

Group	Sampling Day	ΔT°C	Se	%95 CI for Se	Sp	%95 CI for Sp	AUC	P
Cow	**D0**	> 3.1	50.0	18.9 – 81.1	80.95	58.1 – 94.4	0.600	0.374
	D2	≤ 3.6	90.0	55.5 – 98.3	38.10	18.2 – 61.5	0.595	0.377
	D4	≤ 2.3	60.0	26.4 – 87.6	59.09	36.4 – 79.3	0.570	0.516
	D6	≤ 2.9 *	100.0	69.0 – 100.0	61.90	38.5 – 81.8	0.788	< 0.001
	D8	≤ 2.3	50.0	18.9 – 81.1	77.27	54.6 – 92.1	0.550	0.649
	D10	> 4.1	30.0	7.0 – 65.2	95.45	77.1 – 99.2	0.493	0.951
	D12	≤ 3.4	80.0	44.4 – 96.9	42.86	21.9 – 66.0	0.640	0.175
	D14	≤ 2.7	70.0	34.8 – 93.0	63.64	40.7 – 82.8	0.555	0.619
	D16	> 3.8	50.0	18.9 – 81.1	77.27	54.6 – 92.1	0.620	0.278
	D18	≤ 2.7	55.6	21.4 – 86.0	76.19	52.8 – 91.7	0.524	0.837
	D20	≤ 3.4	88.89	51.7 – 98.2	55.00	31.6 – 76.9	0.653	0.153
Heifer	**D0**	≤ 1.1	46.15	19.3 – 74.8	71.43	29.5 – 95.5	0.544	0.752
	D2	≤ 1.1	33.33	5.3 – 77.3	100.0	40.2 – 100.0	0.708	0.246
	D4	≤ 1.9	100.0	71.3 – 100.0	50.0	8.3 – 91.7	0.659	0.353
	D6	≤ 1.8	100.0	40.2 – 100.0	50.0	8.3 – 91.7	0.594	0.659
	D8	> 1.6	66.67	38.4 – 88.1	57.14	18.8 – 89.6	0.514	0.916
	D10	> 1.7	71.43	41.9 – 91.4	57.14	18.8 – 89.6	0.556	0.676
	D12	≤ 3.0	66.67	22.7 – 94.7	100.0	40.2 – 100.0	0.667	0.371
	D14	≤ 0.8	15.38	2.4 – 45.5	100.0	58.9 – 100.0	0.489	0.937
	D16	> 2.4	35.71	12.9 – 64.8	85.71	42.2 – 97.6	0.541	0.763
	D18	≤ 2.3	100.0	71.3 – 100.0	33.33	5.5 – 88.4	0.515	0.938
	D20	> 1.3	100.0	48.0 – 100.0	50.0	8.3 – 91.7	0.675	0.350

The results of the ROC curve analysis of critical ΔT °C thresholds for predicting cows most likely to be pregnant are tabulated and ranked by their respective area under the curve (AUC). Data about the levels of ΔT °C show sensitivity (Se), specificity (Sp), and 95% confidence intervals (CI) are also provided.
* : Highlighted cut-off points are statistically significant ($P < 0.001$).

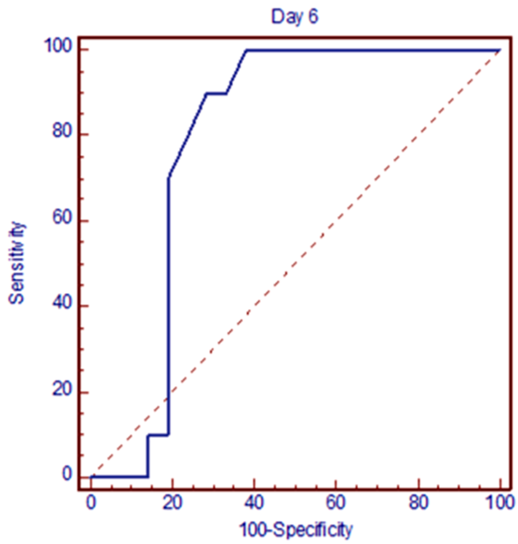

Fig. 2. Display of ROC curves of the cows on D6 that determined the clinical threshold for ΔT °C (≤ 2.9) that predicts cows most likely to be pregnant. The diagonal line represents the specificity (61.90%) and sensitivity (100.0%) levels at which the test is informative.

temperatures measured as ≤ 2.9 °C in cows on day six could be diagnosed as pregnancy with 100% sensitivity ($P < 0.001$). In contrast, no cut-off point was determined for pregnancy diagnosis in heifers within the first 20 days after AI.

In previous thermographic studies, it has been observed that measurements and evaluations are generally performed during the period of estrus and are terminated with ovulation or AI applications. Considering that changes such as edema, inflammation, hyperemia, and anemia in genital tissues and organs are due to estrogenic effects and disappear with ovulation, this methodology seems appropriate. However, in the case of pregnancy after AI in animals of species that are polyestrous, there will be differences in the hormonal regulation of pregnant animals compared to non-pregnant animals due to the presence and continuity of the corpus luteum. Therefore, theoretically, in non-pregnant animals, estrogenic activity will increase in the days close to the next cycle, and temperature may change. Unfortunately, in this study, temperature differences measured in heifers and cows during the first 20 days after AI were not observed to be relevant to pregnancy status on day 28. Moreover, the thermographic cut-off point (≤ 2.9 °C) determined in cows on D6 indicates that the genital organs can respond to the development of pregnancy, which can be evaluated thermographically in cows.

5. Conclusion

In conclusion, it was determined that thermography can be used for the detection of pregnancy in cows on day six after AI. Due to the fact that it is a practical tool to be used in clinical conditions, the integration of thermography in reproductive management applications seems to be suitable for the prediction of pregnant animals. However, further studies are needed to determine the thermographic characteristics and profiles of heifers. There is also a need for more comprehensive studies on the use of thermographic monitoring in cattle reproduction at different ages, breeds, management conditions, and at different times of the year.

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CRediT authorship contribution statement

Kemal Tuna Olğaç: Conceptualization, Methodology, Project administration, Data curation, Formal analysis, Writing – original draft, Visualization. **Murat Onur Yazlik:** Data curation, Investigation, Writing – original draft, Writing – review & editing. **Ufuk Kaya:** Data curation, Formal analysis, Writing – review & editing, Visualization. **Hüseyin Özkan:** Data curation, Formal analysis, Writing – review & editing. **Borga Tırpan:** Data curation, Writing – review & editing, Supervision.

Declaration of Competing Interest

none.

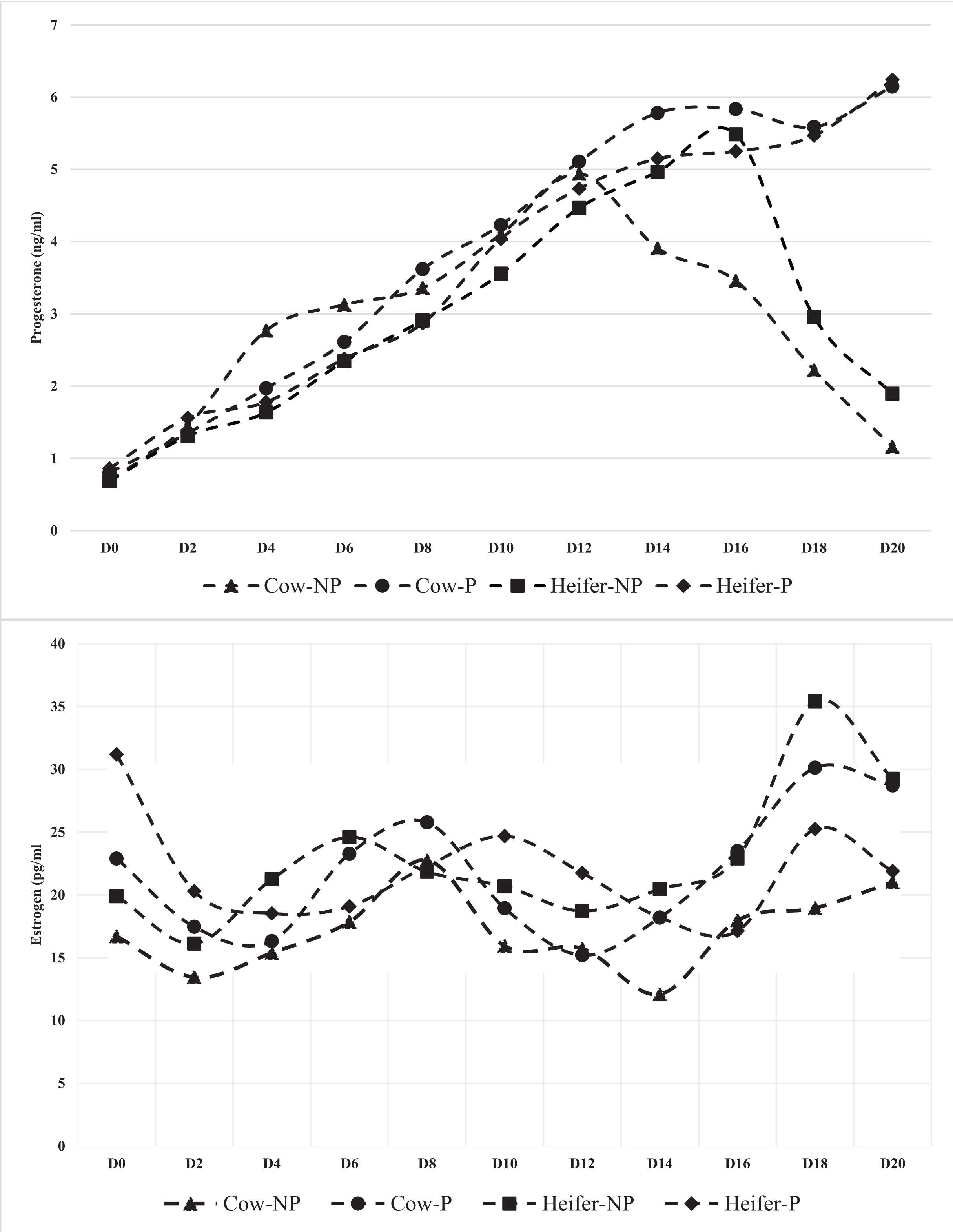

Fig. 3. Estrogen and progesterone concentrations of pregnant cows (Cow-P; $n = 15$), non-pregnant cows (Cow-NP; $n = 15$), pregnant heifers (Heifer-P; $n = 15$), and non-pregnant heifers (Heifer-NP; $n = 15$) on the sampling days (D0 to D20).

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