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Original Research Article

Associations of transition cow metabolism with subsequent manifestation and intensity of estrous behavior in cyclic cows

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ABSTRACT

This retrospective cohort study investigated the associations between transition cow metabolism and estrous behavior in 226 cyclic Holstein cows. Body weight, feed intake, and milk yield were monitored to calculate energy balance. Blood samples were collected on days −21, −14, −10, −7, −3, 0, 3, 7, 10, 14, 21, and 65 relative to calving. Following the second $PGF_{2\alpha}$ injection of a Presynch program, cows were monitored for estrous behavior over an 11-day period using activity sensors. Estrus duration, peak activity, and area under the curve (AUC) were calculated from raw activity data. Cows not detected in estrus were grouped as no increase in activity (NIA; n = 90). Those detected in estrus were classified based on AUC as below (moderate increase in activity or MIA; n = 68) or above (large increase in activity or LIA; n = 68) the median. By design, AUC was 147 % greater, peak was 67 % higher, and duration was 74 % longer in LIA compared with MIA cows.

Compared with NIA, cows that expressed estrus had higher body condition and lower serum concentrations of nonesterified fatty acids (NEFA; 0.43 vs. 0.49 ± 0.02 mmol/L), ceruloplasmin (49.9 vs. 52.9 ± 1.0 mg/L), superoxide dismutase (2.06 vs. 2.28 ± 0.06 U/mL), and peak haptoglobin. Among cows that expressed estrus, metabolic differences during the transition were limited to higher NEFA in LIA than in MIA cows (0.46 vs. 0.40 ± 0.02 mmol/L). These findings indicate that transition metabolism may influence the subsequent manifestation of estrous behavior in cyclic cows.

1. Introduction

Reproductive efficiency in dairy cows relies on timely breeding. Delays in first service or rebreeding of eligible cows reduce the insemination rate, ultimately compromising both reproductive performance and herd profitability [1]. Many herds rely heavily on estrus detection to guide breeding decisions; therefore, the efficiency of estrus detection is critical to achieving timely insemination and maintaining overall reproductive performance.

Extended postpartum anovulation represents a major challenge to achieving optimal insemination rates, particularly in herds that rely on estrus detection. Approximately 20 % of dairy cows remain anovular by the end of the voluntary waiting period (VWP), with prevalence exceeding 40 % in some herds [2–5]. The etiology of prolonged anovulation has been extensively studied, and several risk factors have been identified. They include high milk yield, number of lactations, calving complications (e.g., dystocia and stillbirth), substantial loss of body weight (BW) after calving, severe negative energy balance (as indicated by elevated concentrations of nonesterified fatty acids [NEFA] or extensive loss of body condition score [BCS]), uterine inflammation, and poor overall postpartum health [3,5,6].

Nevertheless, even in cyclic cows, the expression of estrous behavior is highly variable. Some cows fail to exhibit any behavioral signs of estrus during the estrus stage of the cycle, while others display only subtle or short-lived signs of estrus, complicating estrus detection and breeding based on behavioral cues. In contrast to the relatively well-understood biology of postpartum anovulation, the factors contributing to variability in estrous behavior among cyclic cows are poorly understood. Given the lasting influence of transition cow biology on reproductive outcomes (e.g. resumption of estrous cyclicity, embryonic

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development, pregnancy per AI, pregnancy loss) [6,7], investigating the association between transition cow metabolism and subsequent estrous behavior in cyclic cows is warranted.

The adoption of automated activity monitors (AAM) for estrus detection has increased consistently in recent years [8–10]. Alongside recent improvements in reproductive efficiency, these technologies have enabled the development of programs that prioritize breeding based on estrus detection, thereby reducing reliance on synchronization programs [11–13]. Notably, research using AAM has showed that estrous behavior in lactating cows is highly variable and closely associated with fertility outcomes. Specifically, estrous characteristics such as duration and intensity are positively associated with pregnancy success following breeding [14–18]. Therefore, longer and more intense estrus events are considered desirable reproductive traits, as they facilitate estrus detection and are linked to greater fertility. A better understanding of the physiological factors that regulate estrous behavior in cyclic cows is, therefore, essential to support sustainable reproductive management and enhance overall efficiency.

The objective of this study was to investigate the associations between estrous behavior measured by AAM in cyclic cows at the onset of the breeding period and metabolic variables measured during the preceding transition period. We hypothesized that both manifestation and intensity of estrus behavior at the onset of the breeding period are associated with energy balance and circulating metabolites during the preceding transition period, indicating that variability in estrous behavior may reflect another carryover consequence of transition biology.

2. Material and methods

This study is a retrospective analysis of data generated in a previous study that evaluated the effects of the source of supplementary trace minerals (organic vs inorganic forms) on the reproductive biology of cows [19]. As such, a convenience sample of 273 Holstein cows (100 primiparous cows and 173 multiparous cows) was initially considered, of which 226 met the inclusion criteria described below and were used for the present study. The experiment was conducted at the Ontario Dairy Research Centre (**ODRC**; Elora, ON) between December 2018 and September 2020. Enrollment of cows into the study occurred over 15 months and was divided into five periods of three consecutive months each to account for possible seasonal effects in the statistical analyses. All research procedures were approved by the University of Guelph Animal Care Committee (Animal Utilization Protocol #4064).

2.1. Animal housing, feeding management, and energy balance calculation

Cows were housed in free stall pens equipped with automated feed bins (Insentec B. V., Marknesse, the Netherlands) to measure feed intake, and mattress stalls bedded with chopped wheat straw. Prepartum diets were delivered once a day, and postpartum diets were delivered twice daily as total mixed rations. Dry matter (**DM**) of all rations was evaluated weekly, and the formulation and chemical composition of diets are described in detail by Mion et al. [20].

The prepartum diet consisted of corn silage, wheat straw, alfalfa haylage, and a dry cow supplement, with a total energy density of 1.45 Mcal of NE_L/kg of dietary DM. The postpartum diet consisted of corn silage, alfalfa haylage, wheat straw, and a lactating cow supplement, with a total energy density of 1.68 Mcal of NE_L/kg of dietary DM. Energy intake was calculated as DM intake (**DMI**) multiplied by the average energy density of the diet. Throughout the study, cows in the inorganic trace minerals group were supplemented with Co, Cu, Mn and Zn sulfates and Na selenite, while cows in the organic trace minerals group were supplemented with Co, Cu, Mn and Zn proteinates and selenized yeast.

Lactating cows were milked twice a day in a rotatory parlor (DeLaval, Tumba, Sweden), and milk composition was evaluated once a month using a composite sample representing both the morning and the afternoon milking. Energy-corrected milk (**ECM**) was calculated using the following equation: $ECM = [(0.327 \times \text{kg of milk}) + (12.95 \times \text{kg of milk fat}) + (7.20 \times \text{kg of milk protein})]$.

Body weight was recorded using a walk-through scale (DeLaval, Tumba, Sweden) at enrollment (45 ± 3 days before the expected calving date), once a week until calving, and twice daily after milking during the postpartum period. Energy balance (**EBAL**) was estimated for both prepartum (i.e., net energy intake minus energy requirements for maintenance and pregnancy) and postpartum (i.e., net energy intake minus energy requirements for maintenance and lactation) periods according to NRC equations [21]. Body condition score (**BCS**) was visually assessed by trained researchers on days -45 ± 3, -21 ± 3, 3 (exact), 23 ± 3, and 65 ± 3 relative to calving, using a 1 to 5 scale with increments of 0.25 as described by Ferguson et al. [22].

2.2. Ovarian cyclicity and estrous behavior monitoring

Resumption of cyclicity was evaluated weekly on Tuesdays, starting at 21 ± 3 days in milk (**DIM**), via transrectal ultrasonography (Easy-Scan, BCF Ultrasound, Auckland, New Zealand) using a 7.5 MHz linear-array rectal transducer to examine the ovaries. Once a mature corpus luteum (**CL**; $\geq$20 mm) was observed for the first time, cows were considered to have resumed cyclicity postpartum, and weekly examinations were ceased. Anovulation was defined as the absence of a mature CL in the most recent and all previous ultrasonography examinations. Cows classified as anovular by 56 ± 3 DIM were excluded from the study, as this investigation was designed to assess risk factors for estrus expression in cyclic cows only.

All cows were enrolled in a Presynch program and received two intramuscular injections of 500 μg cloprostenol (**$PGF_{2\alpha}$**; Estrumate, Merck Animal Health, Desoto, KS), one at 42 ± 3 DIM and the other at 56 ± 3 DIM. Estrous behavior was monitored for 11 days following the second $PGF_{2\alpha}$ injection of the Presynch program using an **AAM** consisting of an electronic activity tag fitted to a collar and placed around the neck of the cow (DelproTM, DeLaval, Tumba, Sweden). The AAM measured physical impulses generated by changes in acceleration due to head and neck movements, and expressed it in counts per hour. Increases in physical activity were detected by the software through comparison with each cow's basal activity level. Elevated activity was flagged as a potential estrus event according to the company's proprietary algorithm.

Regardless of the software alerts, raw activity data from all eligible cows was extracted and analyzed for the following characteristics of estrous behavior: duration of estrus, peak of activity during estrus, and area under the curve (**AUC**) for the relative increase in activity. Raw hourly counts, a sum of the binary registrations (0 = inactive; 1 = active) for every 14.1 s, were exported from the monitoring system into an Excel file, and a baseline activity level for each cow was calculated as the average of hourly counts recorded during the seven days preceding the second $PGF_{2\alpha}$ injection. For the observation period, 6-h moving averages were used to smooth the variability of raw activity values and to resemble the internal processing of the monitoring system used to flag cows in estrus. Relative increase in activity was calculated by subtracting the baseline from the 6-h moving average, dividing the result by the baseline, and multiplying by 100.

As described by Mion et al. [19], the beginning of an estrus event was defined as a relative increase in activity $\geq$100 % sustained for at least three consecutive hours. The end of an estrus event was defined as a relative increase in activity <100 % sustained for at least five consecutive hours. Estrus duration was defined as the time interval, in hours, between the beginning and end of the event. Peak activity was defined as the highest relative increase observed during the estrus event. The AUC was calculated using the trapezoid method based on relative activity values during the event.

2.3. Estrus classification and experimental design

All cyclic cows that reached the end of the VWP were considered eligible for the analysis of behavioral estrus, regardless of their history of clinical or subclinical health problems. Cows not detected in estrus were classified as having no increase in activity (**NIA**) during the evaluation period. Within each parity group (primiparous or multiparous), cows detected in estrus were ranked according to their AUC and classified as either below or above the median. Cows below the median were considered to have a moderate increase in activity during estrus (**MIA**), whereas those above the median were considered to have a large increase in activity during estrus (**LIA**), forming three experimental groups for comparison. Orthogonal contrasts were used to evaluate associations of dependent variables of interest (e.g. blood metabolites, energy balance) with subsequent behavioral estrus (NIA vs. MIA + LIA) or the intensity of behavioral estrus (MIA vs. LIA).

2.4. Blood metabolites

Blood samples were collected from the coccygeal vessels into polyethylene terephthalate tubes for separation of plasma (BD Vacutainer TM with sodium heparin) and serum (BD Vacutainer TM for Trace Element Testing with serum clot activator). Samples were collected on days −21 ± 3, −14 ± 1, −10 ± 1, −7 ± 1, −3 ± 1, 0, 3, 7, 10, 14, and 21 ± 3 relative to calving, to characterize circulating metabolites during the transition period, and on day 65 ± 3 relative to calving, to characterize the same metabolites at the onset of the breeding period. Blood tubes were placed in a refrigerator at 4 °C immediately after collection, and were centrifuged at 2860×*g* for 15 min at 4 °C within 3 h of collection. The resulting plasma and serum were transferred to 2-mL tubes and stored at −20 °C until laboratory analyses.

A detailed description of laboratory analyses and coefficients of variation is provided by Mion et al. [19,20]. Briefly, serum metabolites were analyzed in all samples, except those collected on days −14 and −7 due to budget restrictions, using an automated chemistry analyzer (Cobas 6000 c^{50}1, Roche Diagnostics) at the University of Guelph Animal Health Laboratory (Guelph, ON, Canada). Cholesterol and glucose assays (Roche Diagnostics GmbH), as well as nonesterified fatty acids (**NEFA**) and beta-hydroxybutyrate (**BHB**) assays (Randox Laboratories, Canada Ltd.) had a limit of quantification of 0.1 mmol/L. Haptoglobin concentration in serum was measured using the hemoglobin binding capacity method described by Skinner and Roberts [23]. The assay was performed at the University of Guelph Animal Health Laboratory, and the methemoglobin reagent was prepared in-house. The limit of detection was 0.03 g/L.

Ceruloplasmin concentration in plasma was measured in all samples according to the methodology described by Hussein et al. [24]. Briefly, the assay measured the ability of ceruloplasmin to catalyze the oxidation of p-phenylenediamine yielding a purple-colored product whose rate of formation is proportional to the concentration of ceruloplasmin in the sample. Plates were read using an absorbance reader (Cytation 5, BioTek, Winooski, VT), and the concentration was calculated by multiplying the oxidase activity by the constant factor of 0.752.

The activities of superoxide dismutase (**SOD**) and glutathione peroxidase (**GPx**) were analyzed using commercial kits (Cayman Chemical, Ann Arbor, MI) in all plasma samples except those collected on days −14, −10, and −7 due to budget restrictions. Assays were run following the manufacturer's instructions and SOD plates were read once at 450 nm and GPx plates were read five times, once each minute for a total of 5 min at 340 nm both using an absorbance reader (Cytation 5, BioTek, Winooski, VT). Samples were evaluated in duplicate.

For analyses of ferric reducing ability of plasma (**FRAP**), the protocol described by Benzie and Strain [25] was used. All plasma samples were mixed with a working solution of acetate buffer (300 mmol/L, Fisher Chemicals, Saint-Laurent, QC, Canada), 2,4,6-tripyridyl-s-triazine (TPTZ, 10 mM in 40 mM of HCl, Fisher Chemicals) and FeCl3 buffer (20 mmol/L, MP Biomedicals, Solon, OH) in a 10:1:1 ratio and incubated at 37 °C in the dark for 30 min. Plates were read at 593 nm using an absorbance reader (Cytation 5, BioTek, Winooski, VT).

2.5. Statistical analyses

Data were analyzed by ANOVA using general linear mixed models (GLIMMIX) procedure of SAS version 9.4 (SAS Institute Inc.). Statistical models included the fixed effects of estrus classification (i.e., NIA, MIA or LIA), parity (i.e. primiparous or multiparous), the interaction between estrus classification and parity, dietary treatment (i.e. inorganic or organic trace minerals), the interaction between dietary treatment and parity, and period of the study (i.e. 1 to 5, each representing 3-mo interval of enrollment).

For repeated measures, the fixed effect of time (e.g. day of blood sampling, week of observation) and the interaction of time with estrus classification, parity, and diet, and the random effect of cow nested within estrus classification were added in the statistical models. The covariance structure that resulted in the lowest Bayesian Information Criterion was selected.

For every model, residuals were tested for normality and homogeneity of variance, and data were transformed when needed to meet the assumptions of the statistical methods. Post-hoc adjustments of probability values for pairwise comparisons were performed using the Tukey method. The reported values represent the LSM and the SEM. Probability values ≤ 0.05 were considered significant, and those between 0.05 and 0.10 were considered tendencies.

3. Results

A total of 273 Holstein cows were enrolled in the original study. Of these, 33 were excluded from the present analysis because of early culling (n = 16), anovulation by the end of the VWP (n = 15), or technical problems with the AAM (n = 2). Therefore, estrous behavior data from 240 Holstein cows were analyzed. Of these 240 cows, 147 (61.3 %) were alerted as having an estrus event by the AAM system, and 139 (57.9 %) were identified as having an estrus based on our calculations from the raw data.

A disagreement in classification between the two methods occurred for 14 cows. Specifically, 11 cows were flagged as in estrus by the AAM system but not by our raw data calculations. Moreover, 3 cows that were classified as having an estrus by our calculations were not flagged by the AAM system. All 14 cows with inconsistent classification were excluded from the study to avoid any potential misclassification.

Of the remaining 226 cows, 90 (39.8 %) were not detected in behavioral estrus and were classified as NIA, and 136 (60.2 %) were detected in behavioral estrus. Of the latter group, half was classified as MIA (n = 68) and half was classified as LIA (n = 68). The proportion of primiparous and multiparous cows did not differ among groups (NIA: 28 primiparous and 62 multiparous; MIA: 26 primiparous and 42 multiparous; LIA: 26 primiparous and 42 multiparous).

The AUC was highly correlated with both peak activity and duration of estrus, but the latter two variables showed only a moderate positive correlation (Fig. 1). For this reason, we chose to use AUC to classify cows as MIA or LIA. As a consequence of the experimental design, estrous behavior characteristics were substantially different between MIA and LIA cows. Duration, peak, and AUC were 74 %, 67 %, and 147 % greater ($P < 0.01$) in LIA cows than in MIA cows (Table 1).

The median time to resume estrous cyclicity tended ($P = 0.10$) to be 2 days longer for cows detected in estrus compared with those not detected in estrus (adjusted hazard ratio [95 % CI] = 0.79 [0.60–1.04]), but did not differ ($P = 0.19$) between MIA and LIA (adjusted hazard ratio [95 % CI] = 0.80 [0.57–1.12]). However, when the extreme groups were compared, the median interval to resume estrous cyclicity was 5 days longer ($P = 0.04$) in LIA than in NIA cows (adjusted hazard ratio [95 % CI] = 0.72 [0.52–0.98]).

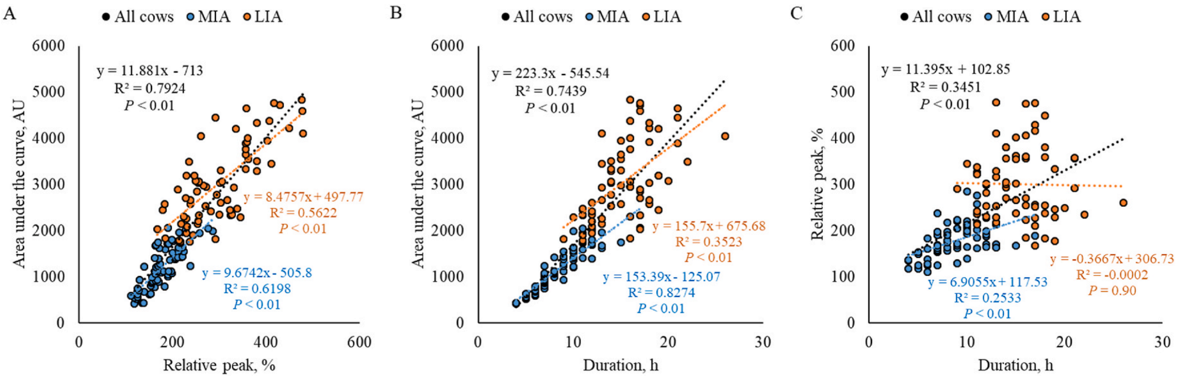

Fig. 1. Correlation and regression analyses between behavioural characteristics of estrus events of 136 cyclic cows detected using automated activity monitors during 11 days following the Presynch program. Half of the cows were classified as having a moderate increase in activity (**MIA**; represented with blue dots; n = 68) and the other half were classified as having a large increase in activity (**LIA**; represented with orange dots; n = 68). Regression equations, coefficients of determination, and probability values are presented in each panel for all cows (text in black), for MIA cows only (text in blue), and for LIA cows only (text in orange). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1

Characteristics of estrus according to classification based on the area under the curve of relative increase in activity during estrus.

Item	Estrus classification [a]			
	MIA	LIA	SEM	*P*
AUC, AU	1200	2969	85	<0.01
Relative peak, %	177	295	7.3	<0.01
Duration, h	8.7	15.1	0.4	<0.01
Interval from $PGF_{2\alpha}$ to estrus, h	94.8	92.4	7.0	0.81
DIM at estrus	58.0	58.1	0.5	0.98

[a] Within parity, cows were ranked based on the area under the curve for relative increase in activity during estrus and classified as below (moderate increase in activity [MIA]; n = 68) or above (large increase in activity [LIA]; n = 68) the median.

There were no differences in prepartum BCS, BW, DMI (as kg/d), and EBAL among estrus classification groups (Table 2). However, when DMI was evaluated as percentage of BW, a tendency ($P = 0.10$) for a group effect was observed, and cows with LIA had lower DMI than cows with NIA (Table 2; Fig. 2). For prepartum BCS, there was a tendency for an interaction ($P = 0.09$) between estrus classification and day of evaluation. On average, cows with NIA lost 0.04 ± 0.03 BCS from day −45 to −21 relative to calving, whereas cows detected in estrus gained 0.05 ± 0.03 BCS during the same period ($P = 0.02$). No difference ($P = 0.92$) in change of prepartum BCS was observed between MIA and LIA (Fig. 2).

Differences in DMI as % of BW were also detected in the postpartum period (Table 3; Fig. 2). Cows with LIA had lower DMI as % of BW than cows with NIA. Cows with MIA had intermediate values that did not differ from the other two groups.

Cows detected in estrus tended ($P = 0.06$) to have greater postpartum BCS than those not detected in estrus (Table 3; Fig. 2). Although BCS was similar between groups on day 3 after calving, cows detected in estrus had higher BCS on days 21 and 60 after calving than cows classified as NIA (Fig. 2). No differences between groups were observed for postpartum BW, DMI as kg/d, EBAL, and ECM (Table 3).

Regarding blood metabolites, cows detected in estrus had lower ($P < 0.04$) concentrations of NEFA, ceruloplasmin, and SOD during the transition period than cows in the NIA group, and these differences were primarily driven by postpartum values (Table 4; Figs. 3 and 4). The concentrations of NEFA during the transition period also differed according to intensity of estrus, with higher concentrations for LIA than MIA cows (Table 4; Fig. 3). For ceruloplasmin, an interaction ($P = 0.02$) between estrous detection and parity was observed. The difference between groups was observed in primiparous cows (Estrus = 51.9 *vs.* No estrus = 57.6 ± 1.6 mg/L; $P < 0.01$) but not in multiparous cows (Estrus = 48.7 *vs.* No estrus = 48.6 ± 1.1 mg/L; $P = 0.93$). No differences between groups were observed in concentrations of haptoglobin, cholesterol, glucose, BHB, GPx, and FRAP during the transition period (Table 4). When the days of peak in haptoglobin concentration (d 3 and 7 after calving) were analyzed separately, cows detected in estrus presented lower concentrations values ($P < 0.05$) than cows in the NIA group (Fig. 4).

During the early breeding period, the only difference observed in blood metabolites was on concentrations of NEFA. Cows detected in estrus had greater ($P = 0.02$) concentrations of NEFA in serum than cows not detected in estrus, and cows with LIA tended ($P = 0.10$) to have greater concentrations of NEFA in serum than cows with MIA (Table 5).

Table 2

Prepartum (weeks −5 to −1) body weigth (BW), body condition score (BCS), dry matter intake (DMI), and energy balance (EBAL) according to classification of estrus behavior in the subsequent breeding period.

Item	Estrus classification [a]				Orthogonal contrasts [b]	
	NIA	MIA	LIA	*P*	C1	C2
BCS, 1–5 scale	3.61 ± 0.03	3.62 ± 0.03	3.67 ± 0.03	0.33	0.32	0.26
BW, kg	762 ± 7.5	771 ± 8.2	776 ± 8.2	0.45	0.23	0.70
DMI, kg/d	13.1 ± 0.20	13.2 ± 0.22	12.7 ± 0.22	0.26	0.53	0.13
DMI, % of BW	1.72 ± 0.03^{a}	1.71 ± 0.03^{ab}	1.64 ± 0.03^{b}	0.10	0.17	0.09
EBAL, Mcal/d	3.94 ± 0.30	3.92 ± 0.33	3.25 ± 0.33	0.23	0.34	0.15

[a] Cows were monitored for estrous behavior for a period of 11 days after the second $PGF_{2\alpha}$ injection of the Presynch program using automatic activity monitors. Cows not detected in behavioral estrus were classified as no increase in activity (NIA; n = 90). Within parity, cows detected with behavioral estrus were ranked based on the area under the curve for relative increase in activity during estrus and classified as below (moderate increase in activity [MIA]; n = 68) or above (large increase in activity [LIA]; n = 68) the median.

[b] C1 = effect of detection of behavioral estrus (NIA *vs.* MIA + LIA); C2 = effect of the intensity of behavioral estrus (MIA *vs.* LIA).

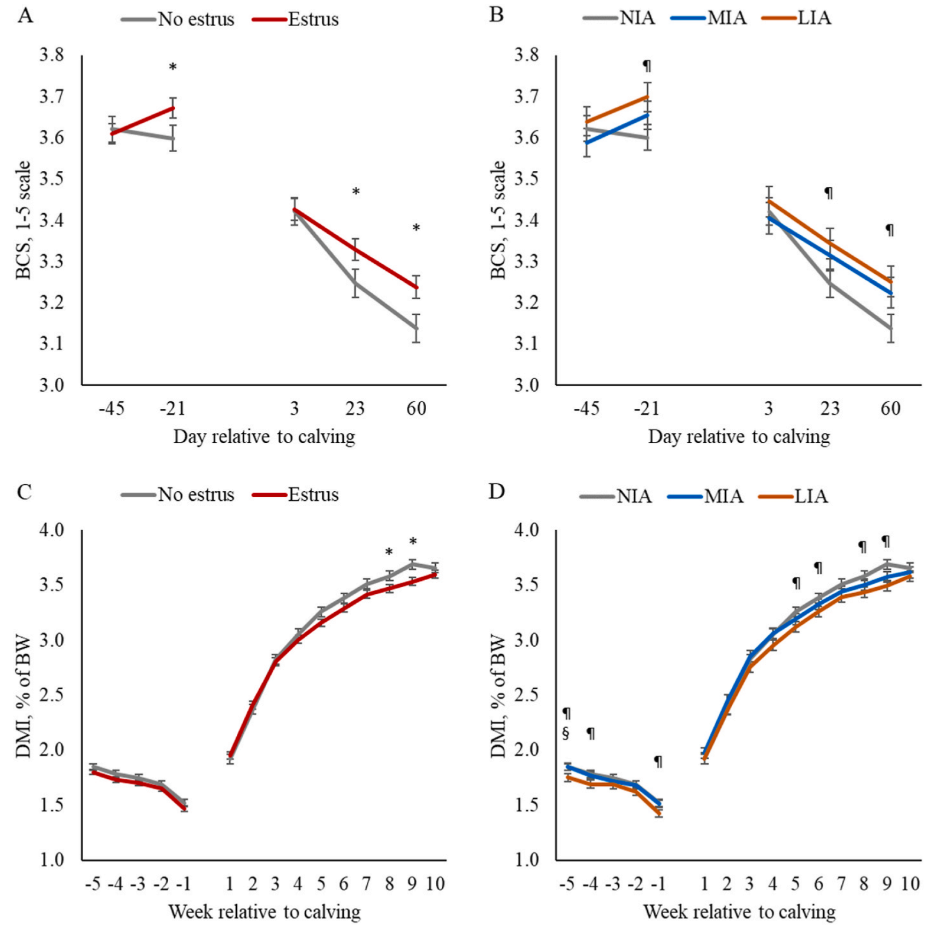

Fig. 2. Body condition score (BCS) and dry matter intake (DMI) as percentage of BW according to detection of behavioral estrus (No estrus *vs.* Estrus; panels A and C) and estrus activity (no increase in activity [NIA] *vs.* moderate increase in activity [MIA] *vs.* large increase in activity [LIA]; panels B and D). Number of cows: No estrus or NIA = 90; MIA = 68; and LIA = 68. Within day of assessment: *$P < 0.05$ between No estrus *vs.* Estrus; ¶$P < 0.05$ between NIA *vs.* LIA; §$P < 0.05$ between MIA *vs.* LIA.

Table 3
Postpartum (weeks 1 to 10) body weigth (BW), body condition score (BCS), dry matter intake (DMI), energy corrected milk (ECM), and energy balance (EBAL) according to classification of estrus behavior in early breeding period.

Item	Estrus classification [a]				Orthogonal contrasts [b]	
	NIA	MIA	LIA	P	C1	C2
BCS, 1–5 scale	3.27 ± 0.03^{b}	3.31 ± 0.03^{ab}	3.35 ± 0.03^{a}	0.13	0.06	0.43
BW, kg	659 ± 6.5	670 ± 7.1	673 ± 7.1	0.26	0.11	0.76
DMI, kg/d	20.6 ± 0.2	20.7 ± 0.2	20.4 ± 0.02	0.65	0.99	0.36
DMI, % of BW	3.13 ± 0.03^{a}	3.10 ± 0.04^{ab}	3.03 ± 0.04^{b}	0.11	0.12	0.15
EBAL, Mcal/d	−2.84 ± 0.38	−3.31 ± 0.42	−3.50 ± 0.42	0.48	0.24	0.75
ECM, kg/d	39.4 ± 0.6	40.1 ± 0.7	39.6 ± 0.7	0.68	0.50	0.58

[a] Cows were monitored for estrous behavior for a period of 11 days after the second PGF$_{2\alpha}$ injection of the Presynch program using automatic activity monitors. Cows not detected in behavioral estrus were classified as no increase in activity (NIA; n = 90). Within parity, cows detected with behavioral estrus were ranked based on the area under the curve for relative increase activity during estrus and classified as below (moderate increase in activity [MIA]; n = 68) or above (large increase in activity [LIA]; n = 68) the median.

[b] C1 = effect of detection of behavioral estrus (NIA *vs.* MIA + LIA); C2 = effect of the intensity of behavioral estrus (MIA *vs.* LIA).

4. Discussion

Estrous behavior is highly variable among estrous cyclic dairy cows [14–18], and the underlying reasons of this variability remain largely unknown. In this study, blood concentrations of NEFA, ceruloplasmin, and SOD during the transition period, as well as peak concentrations of haptoglobin after calving, were higher in cyclic cows that subsequently failed to express estrus following a Presynch program compared with those that were detected in estrus. These findings indicate that transition biology may influence the subsequent manifestation of estrous behavior in cyclic cows.

Importantly, the differences in blood metabolites observed during the transition period were no longer present during the early breeding period, indicating that these changes were not persistent but still exerted carryover effects on estrous behavior. This pattern resembles previously reported carryover effects of postpartum clinical disease on the resumption of estrous cyclicity and fertility, which are observed for months after clinical resolution of the health problem. Although the

Table 4

Concentrations of metabolites in serum during the transition period (days −21 to 23 relative to calving) according to classification of estrus behavior in the subsequent breeding period.

Item	Estrus classification [a]				Orthogonal contrasts [b]	
	NIA	MIA	LIA	P	C1	C2
Cholesterol, mmol/L	2.33 ± 0.06	2.39 ± 0.06	2.38 ± 0.06	0.72	0.42	0.88
Glucose, mmol/L	3.78 ± 0.03	3.75 ± 0.03	3.75 ± 0.03	0.78	0.48	0.96
NEFA, mmol/L	0.49 ± 0.02^{a}	0.40 ± 0.02 ^{b}	0.46 ± 0.02^{a}	<0.01	<0.01	0.02
BHB, μmol/L	582 ± 20	564 ± 21	594 ± 22	0.59	0.88	0.31
Haptoglobin, g/L	0.33 ± 0.02	0.32 ± 0.02	0.29 ± 0.02	0.39	0.28	0.38
Ceruloplasmin, mg/L	52.90 ± 0.9	49.90 ± 1.0	50.57 ± 1.0	0.09	0.03	0.68
GPx, nmol/min/mL	217.5 ± 5.4	219.3 ± 5.9	205.0 ± 5.9	0.16	0.43	0.08
SOD, U/mL	2.28 ± 0.05^{a}	2.20 ± 0.06 ^{ab}	2.06 ± 0.06 ^{b}	0.02	0.03	0.10
FRAP, μmol/L $FeSO_4$	49.90 ± 0.8	49.25 ± 0.89	50.23 ± 0.88	0.72	0.81	0.43

[a] Cows were monitored for estrous behavior for a period of 11 days after the second $PGF_{2\alpha}$ injection of the Presynch program using automatic activity monitors. Cows not detected in behavioral estrus were classified as no increase in activity (NIA; n = 90). Within parity, cows detected with behavioral estrus were ranked based on the area under the curve for relative increase in activity during estrus and classified as below (moderate increase in activity [MIA]; n = 68) or above (large increase in activity [LIA]; n = 68) the median.

[b] C1 = effect of detection of behavioral estrus (NIA *vs.* MIA + LIA); C2 = effect of the intensity of behavioral estrus (MIA *vs.* LIA).

underlying mechanisms are not fully understood, they are thought to involve impaired folliculogenesis, reduced oocyte quality, and altered uterine environment [6,7,12]. Collectively, the existing literature and the current findings suggest that improved transition cow management may not only promote earlier cyclicity and better fertility (as reported in previous studies) but also enhance estrous behavior (based on the present study). This could, in turn, facilitate estrus detection and improve reproductive efficiency in herds reliant on estrus detection for reproductive management.

Additionally, cows detected in estrus experienced smaller losses in BCS after calving and had higher NEFA concentrations during the early breeding period. Unlike the postpartum period, when NEFA concentrations are elevated due to reduced energy balanced and increase rate of lipolysis, NEFA levels during the breeding period are lower and likely reflect basal lipolysis. Thus, the observed difference in NEFA concentrations between groups on day 65 may be attributable to small differences in basal lipolysis, potentially due to greater BCS and larger adipose reserves in cows that exhibited estrus. Fatty acids are known to influence hypothalamic neurons that regulate both energy homeostasis and reproduction [26].

Estrous behavior is triggered by a combination of endocrine events on the uterus ($PGF_{2\alpha}$ pulses that induce luteolysis and reduce progesterone concentrations in circulation), ovaries (rise in estradiol secretion by the ovulatory follicle), and brain (secretion of GnRH, LH, and FSH to support steroidogenesis and ovulation) [27]. Beyond the canonical release of reproductive hormones, the neuroendocrine regulation of reproduction also depends on the hypothalamic sensitivity to estradiol (via estrogen receptors), the pituitary responsiveness to GnRH (via GnRH receptors), and follicular sensitivity to gonadotrophins (via FSH and LH receptors), all of which may be modulated by metabolic signals such as fatty acids and inflammatory mediators [28].

Elevated postpartum NEFA concentration (>0.7 mmol/L) are indicative of a more severe negative energy balance and are associated with altered metabolic and endocrine profiles affecting liver, ovary, and uterus [29]. A more pronounced negative energy balance reduces insulin levels, impairs the expression of growth hormone receptor in the liver, and lowers secretion of insulin-like growth factor-1 (IGF-1) [30]. Circulating insulin and IGF-1 are important regulators of ovarian follicle sensitivity to gonadotrophins and steroidogenesis [31,32]. High concentrations of NEFA have been linked to impaired follicular development, reduced estradiol production, and delayed onset of estrus postpartum [33–35].

In our study, cows detected in estrus were more likely to gain BCS during the prepartum period and had higher BCS during the early postpartum period compared with cows not detected in estrus. Body condition score has consistently been identified as a major factor influencing the occurrence and intensity of estrous expression detected by AAM [16–18,36]. Poor BCS has been linked to delayed expression of estrus, a longer interval to first service and conception, and reduced pregnancy rates after the first AI [5,37,38] and possibly affecting estrous behavior [39].

Cows that were not detected in estrus following the administration of the second $PGF_{2\alpha}$ had higher concentrations of haptoglobin and ceruloplasmin during the transition period. Inflammation around parturition, though physiologically normal, can impair reproductive performance [6,12]. Inflammatory mediators are known to affect the brain [40] and specifically GnRH neurons [41] and could, therefore, affect neuronal signals linked to estrous behavior.

Positive acute phase proteins such as haptoglobin and ceruloplasmin are produced by hepatocytes in response to infection, trauma, or systemic inflammation [42]. Haptoglobin levels can increase up to ninefold in cases of infection [43,44], but elevated levels are also common shortly after calving in otherwise clinically healthy cows [45,46]. Prolonged elevation of haptoglobin has been associated with delayed cyclicity [47] and failed ovulation of the first dominant follicle postpartum [48]. Bruinje et al. [12] found that cows with elevated haptoglobin early postpartum had reduced odds of estrus detection. Ceruloplasmin also functions as an antioxidant, neutralizing reactive oxygen species (ROS) [49], and its levels are increased in cows with subclinical mastitis [50] and endometritis [51].

Oxidative stress, an imbalance between ROS generation and antioxidant defenses, can impair folliculogenesis, steroidogenesis, and early embryonic development [52,53]. In this study, cows with stronger estrous activity had lower SOD levels than those that did not express estrus or had moderate activity, suggesting that optimal oxidative balance may be critical. Lower antioxidant activity could reflect reduced ROS burden. However, contrasting results have been reported. Ghosh et al. [54] for instance found that SOD levels were higher in cyclic buffaloes compared with anestrous animals. The exact role of oxidative stress in reproductive regulation remains unclear, but antioxidants appear to influence reproductive function in ruminants.

Among cows detected in estrus, few metabolic differences between MIA and LIA cows were observed during the transition period. However, cows classified as LIA had higher NEFA concentrations than those classified as MIA, both during transition and at the onset of the breeding period. Although overall levels of NEFA were lower at breeding, this consistent difference may indicate persistent differences in basal lipolysis, which could positively influence hypothalamic reproductive centers as discussed above [26]. Furthermore, cows with LIA had lower DMI as percentage of BW than cows with NIA during both pre- and postpartum periods. These findings suggest that estrous intensity may be influenced by ongoing metabolic differences during the breeding period, rather than by carryover effects from the transition period, as observed for manifestation of estrus.

Dry matter intake has been positively associated with hepatic metabolism of steroid hormones, partly due to its relationship with blood flow to splanchnic tissues [55–57]. Greater hepatic clearance of steroid hormones may reduce their circulating concentrations and impair estrous expression, given the role of estradiol and other steroids

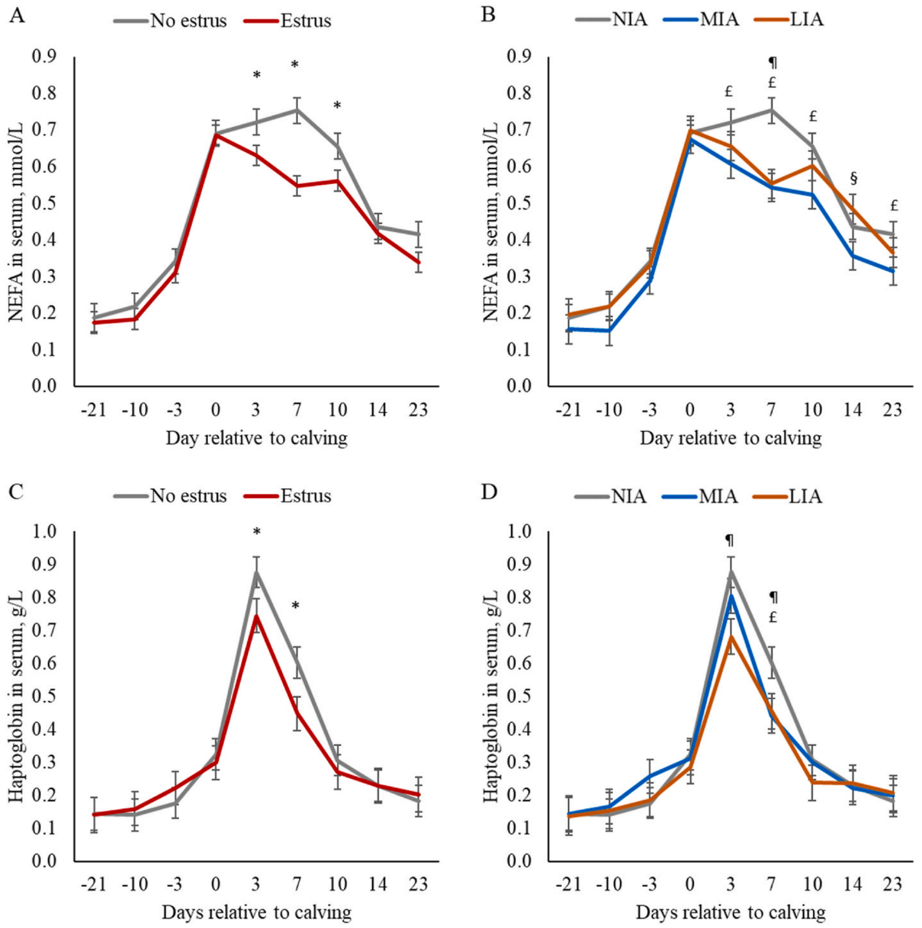

Fig. 3. Concentration of nonesterified fatty acids (NEFA) and haptoglobin in serum according to detection of behavioral estrus (No estrus *vs.* Estrus; panels A and C) and estrus activity (no increase in activity [NIA] *vs.* moderate increase in activity [MIA] *vs.* large increase in activity [LIA]; panels B and D). Number of cows: No estrus or NIA = 90; MIA = 68; and LIA = 68. Within day of assessment: **P* < 0.05 between No estrus *vs.* Estrus; ¶*P* < 0.05 between NIA *vs.* LIA; §*P* < 0.05 between MIA *vs.* LIA; £ *P* < 0.05 between NIA *vs.* MIA.

in modulating behavior [58–60]. Thus, the lower DMI (% BW) observed in LIA cows could be associated with lower hepatic blood flow and reduced steroid clearance, leading to more intense estrus. Notably, we did not observe associations between estrous behavior and DMI measured in kg/day, or with milk production.

Automated activity monitors have substantially advanced the study of estrous behavior by allowing continuous measurement of both estrus duration and peak activity. While these parameters are correlated, they may have distinct implications. Burnett et al. [15] reported that cows with higher peak activity or longer estrus duration had lower ovulation failure rates. Tippenhauer et al. [17] found only a moderate correlation between estrus duration and peak activity ($R^2 = 0.45$), and that only peak activity was associated with pregnancy outcomes. Conversely, Burnett et al. [14] observed associations between estrus duration and pregnancy but not with peak activity. These inconsistencies may reflect differences in sensor technology, data processing, or analytical methods. Nevertheless, both parameters are important and should be considered when studying estrous behavior, as neither has been definitively shown to be superior.

In our study, correlation between peak of activity and duration of estrus was again moderate, thus implying that classification of cows based in one parameter would not necessarily reflect important differences in the other parameter. To address the challenge of selecting the most appropriate parameter to study biological factors associated with estrous behavior, we decided to use the AUC for exploring the variability in intensity of estrous expression among cows. The new variable integrated information from both peak of activity as well as duration of estrus, and it was strongly associated with both variables. By separating cows based on AUC, we created groups of cows that differed both in duration of estrus as well as in peak of activity during estrus; thus, providing a more balanced approach to group cows according to their estrus characteristics and not favoring one parameter over the other. This method may serve as a useful framework for future research into the biological determinants of estrus intensity.

It is important to note that failure to express estrus following Presynch may result from lack of a responsive corpus luteum or incomplete luteolysis, not solely from a behavioral deficit. The absence of progesterone measurements or follow-up ultrasonography in our study limits our ability to confirm this, and may have affected the NIA group classification. Excluding such cows may have increased differences between NIA and the other two groups, but comparisons between MIA and LIA were likely unaffected given the high sensitivity of the methods. It is also noteworthy that the percentage of cows detected in estrus in our study are well within those reported in the literature [61,62].

Another limitation of our study is the possible misclassification of cows due to imperfections in the monitoring system. Although AAM are valuable tools for detecting and characterizing estrus, their accuracy is not absolute. Roelofs et al. [60] reported sensitivity between 76 and 82 %, positive predictive value between 87 and 92 %, and specificity between 99 and 100 % for both leg and neck activity sensors tested both indoors and on pasture.

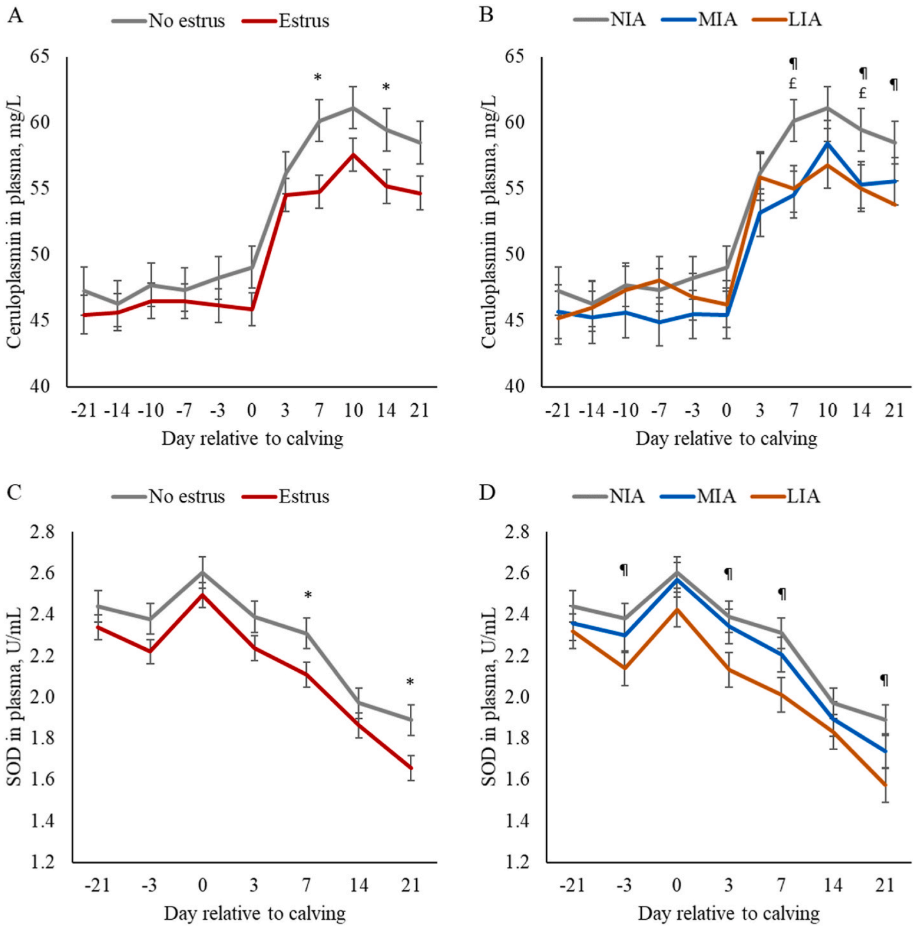

Fig. 4. Concentration of ceruloplasmin and superoxide dismutase (SOD) in plasma according to detection of behavioral estrus (No estrus *vs.* Estrus; panels A and C) and estrus activity (no increase in activity [NIA] *vs.* moderate increase in activity [MIA] *vs.* large increase in activity [LIA]; panels B and D). Number of cows: No estrus or NIA = 90; MIA = 68; and LIA = 68. Within day of assessment: *$P < 0.05$ between No estrus *vs.* Estrus; ¶$P < 0.05$ between NIA *vs.* LIA; £ $P < 0.05$ between NIA *vs.* MIA.

Table 5
Concentration of metabolites in serum during the early breeding period (65 ± 3 DIM) according to classification estrus behavior.

Item	Estrus classification [a]				Orthogonal contrasts [b]	
	NIA	MIA	LIA	*P*	C1	C2
Cholesterol, mmol/L	4.91 ± 0.123	4.97 ± 0.13	5.04 ± 0.14	0.75	0.52	0.69
Glucose, mmol/L	3.72 ± 0.035	3.65 ± 0.04	3.68 ± 0.04	0.41	0.24	0.54
NEFA, mmol/L	0.14 [0.12–0.16]^{b}	0.15 [0.13–0.18]^{ab}	0.18 [0.16–0.21]^{a}	0.02	0.02	0.10
BHB, μmol/L	567 [526–612]	558 [514–606]	566 [521–616]	0.95	0.85	0.80
Haptoglobin, g/L	0.11 [0.09–0.13]	0.12 [0.10–0.14]	0.10 [0.09–0.12]	0.38	0.85	0.17
Ceruloplasmin, mg/L	53.74 ± 1.7	52.90 ± 1.9	51.29 ± 1.9	0.63	0.45	0.55
GPx, nmol/min/mL	166 [156–178]	172 [160–185]	165 [154–177]	0.73	0.75	0.46
SOD, U/mL	1.81 [1.69–1.95]	1.83 [1.69–1.97]	1.67 [1.55–1.80]	0.19	0.41	0.11
FRAP, μmol/L $FeSO_4$	53.39 [50.88–56.02]	55.95 [53.05–59.01]	54.24 [51.49–57.13]	0.43	0.31	0.41

[a] Cows were monitored for estrous behavior for a period of 11 days after the second $PGF_{2\alpha}$ injection of the Presynch program using automatic activity monitors. Cows not detected in behavioral estrus were classified as no increase in activity (NIA; n = 90). Within parity, cows detected with behavioral estrus were ranked based on the area under the curve for relative increase in activity during estrus and classified as below (moderate increase in activity [MIA]; n = 68) or above (large increase in activity [LIA]; n = 68) the median.

[b] C1 = effect of detection of behavioral estrus (NIA *vs.* MIA + LIA); C2 = effect of the intensity of behavioral estrus (MIA *vs.* LIA).

5. Conclusion

In conclusion, our findings provide evidence that transition cow biology – specifically energy status, inflammation, and oxidative balance – is associated with the subsequent manifestation of estrous behavior and estrus detection in cyclic Holstein cows. However, among cows that exhibited estrous behavior, the characteristics of the preceding transition period did not appear to influence the peak intensity or duration of estrus.

CRediT authorship contribution statement

Augusto M.L. Madureira: Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Chantel Van Dorp:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Guilherme Madureira:** Writing – review & editing, Investigation, Formal analysis, Data curation, Conceptualization. **Bruna Mion:** Writing – review & editing, Visualization, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Olivia Chiu:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Tracy A. Burnett:** Writing – review & editing, Visualization, Conceptualization. **José Felipe W. Spricigo:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Eduardo S. Ribeiro:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Eduardo de Souza Ribeiro reports financial support was provided by Natural Sciences and Engineering Research Council of Canada. Eduardo de Souza Ribeiro reports financial support was provided by Food From Thought - Thematic Research Fund. Chantel Van Dorp reports financial support was provided by Dairy Farmers of Ontario. The authors have not stated any conflicts of interest. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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