Feeding strategies during fresh cow period in pasture-based dairy systems: metabolic adaptation to lactation and resumption of ovarian cyclicity in primiparous and multiparous cows

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Key Words:	Transition period, Metabolism, Parity, Metabolomics

Checklist for REFLECT statement: Reporting guidelines For randomized control trials in livestock and food safety. Bold text are modifications from the CONSORT statement description (Altman DG et al . Ann Intern Med 2001; 134(8):663-694).

Paper section and topic	Item	Descriptor of REFLECT statement item	Reported on Page #
Title & Abstract	1	How **study units** were allocated to interventions (eg, "random allocation," "randomized," or "randomly assigned"). **Clearly state whether the outcome was the result of natural exposure or was the result of a deliberate agent challenge.**	1-2
Introduction Background	2	Scientific background and explanation of rationale.	3-5
Methods Participants	3	Eligibility criteria **for owner/managers and study units at each level of the organizational structure,** and the settings and locations where the data were collected.	6-7
Interventions	4	Precise details of the interventions intended for each group, **the level at which the intervention was allocated,** and how and when interventions were actually administered.	6-9
	4b	**Precise details of the agent and the challenge model, if a challenge study design was used.**	NA
Objectives	5	Specific objectives and hypotheses. **Clearly state primary and secondary objectives (if applicable).**	6
Outcomes	6	Clearly defined primary and secondary outcome measures and the levels at which they were measured, and, when applicable, any methods used to enhance the quality of measurements (eg, multiple observations, training of assessors).	13-18
Sample size	7	How sample size was determined and, when applicable, explanation of any interim analyses and stopping rules. **Sample-size considerations should include sample-size determinations at each level of the organizational structure and the assumptions used to account for any non-independence among groups or individuals within a group.**	6-7
Randomization -- Sequence generation	8	Method used to generate the random allocation sequence **at the relevant level of the organizational structure**, including details of any restrictions (eg, blocking, stratification)	7
Randomization -- Allocation concealment	9	Method used to implement the random allocation sequence **at the relevant level of the organizational structure**, (eg, numbered containers ~~**or central telephone**~~**),** clarifying whether the sequence was concealed until interventions were assigned.	7

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Randomization -- Implementation	10	Who generated the allocation sequence, who enrolled **study units,** and who assigned **study units** to their groups **at the relevant level of the organizational structure**.	7
Blinding (masking)	11	Whether or not ~~**participants**~~ those administering the interventions, **caregivers** and those assessing the outcomes were blinded to group assignment. If done, how the success of blinding was evaluated**. Provide justification for not using blinding if it was not used.**	NA
Statistical methods	12	Statistical methods used to compare groups for all outcome(s); Clearly state the level of statistical analysis **and methods used to account for the organizational structure, where applicable;** methods for additional analyses, such as subgroup analyses and adjusted analyses.	12-13
Results **Study** flow	13	Flow **of study units** through each stage **for each level of the organization structure of the study** (a diagram is strongly recommended). Specifically, for each group, report the numbers of **study units** randomly assigned, receiving intended treatment, completing the study protocol, and analyzed for the primary outcome. Describe protocol deviations from study as planned, together with reasons.	6-7
Recruitment	14	Dates defining the periods of recruitment and follow-up.	7
Baseline data	15	Baseline demographic and clinical characteristics of each group, **explicitly providing information for each relevant level of the organizational structure. Data should be reported in such a way that secondary analysis, such as risk assessment, is possible.**	7
Numbers analyzed	16	Number **of study units** (denominator) in each group included in each analysis and whether the analysis was by "intention-to-treat." State the results in absolute numbers when feasible (eg, 10/20, not 50%).	7
Outcomes and estimation	17	For each primary and secondary outcome, a summary of results for each group, **accounting for each relevant level of the organizational structure**, and the estimated effect size and its precision (e.g., 95% confidence interval)	13-19
Ancillary analyses	18	Address multiplicity by reporting any other analyses performed, including subgroup analyses and adjusted analyses, indicating those pre-specified and those exploratory.	NA
Adverse events	19	All important adverse events or side effects in each intervention group.	NA
Discussion Interpretation	20	Interpretation of the results, taking into account study hypotheses, sources of potential bias or imprecision, and the dangers associated with multiplicity of analyses and outcomes. **Where relevant, a discussion of herd immunity should be included. If applicable, a discussion of the relevance of the disease challenge should be included.**	19-27

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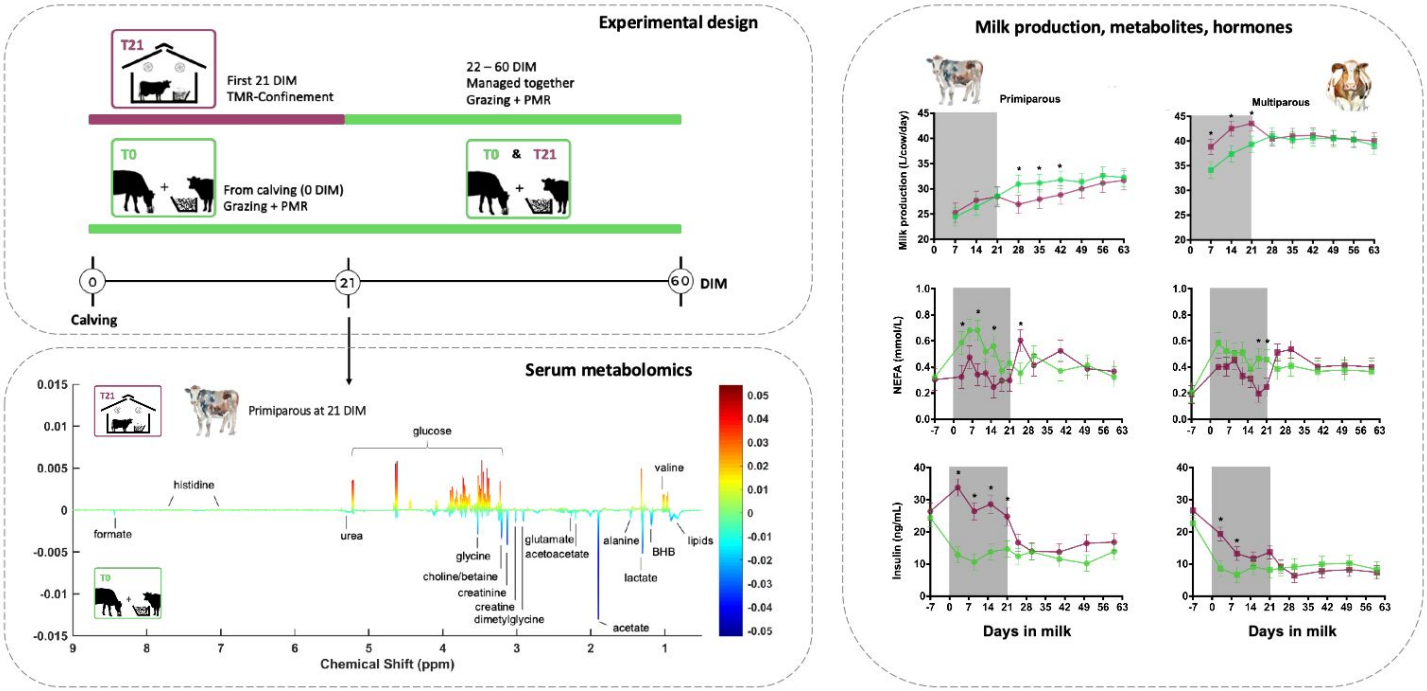
Generalizability	21	Generalizability (external validity) of the trial findings.	NA
Overall evidence	22	General interpretation of the results in the context of current evidence.	26-27

Graphical abstract

Parity-dependent metabolic responses to differential fresh cow management in pasture-based dairy systems:



Highlights

- Strategic short-term TMR-confinement postpartum triggers parity-specific responses
- Milk yield was higher in multiparous cows but not in primiparous cows
- Energy balance benefits are more evident in primiparous cows under TMR-confinement
- Grazing from calving impaired metabolic adaptation in primiparous cows
- Post-TMR shift to grazing altered metabolism and reduced milk production

Interpretive Summary: Strategic TMR-confinement during the first 21 days postpartum in pasture-based dairy systems elicits parity-dependent responses. This strategy improved energy balance and increased milk yield by 12% in multiparous cows, with no production benefits in primiparous cows compared to those grazing with supplementation from calving. Primiparous cows grazing from calving experienced a greater metabolic challenge, with increased lipid and protein mobilization, whereas those fed TMR showed a better energy status. After transitioning to grazing, both parities underwent further metabolic adjustments and reduced milk production. The feeding strategy did not shorten the postpartum anestrus period.

Running head: Metabolic adaptation to fresh cow feeding management

Feeding strategies during fresh cow period in pasture-based dairy systems: metabolic adaptation to lactation and resumption of ovarian cyclicity in primiparous and multiparous cows

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Abstract

The study aimed to determine whether confinement with total mixed ration (TMR) during the first 21 days in milk (DIM), followed by grazing supplemented with partial mixed ration (PMR), alleviates negative energy balance, enhancing productive performance and accelerating the resumption of ovarian cyclicity in primiparous and multiparous dairy cows, relative to a control group managed on grazing supplemented with PMR after calving. Following calving, 16 primiparous and 24 multiparous Holstein

dairy cows were blocked and randomly distributed in two treatments: one included grazing plus supplementation with PMR after calving (T0), while the other one involved confinement with TMR ad libitum during the first 21 DIM and the same feeding management of T0 from day 22 onwards until 60 DIM (T21). Primiparous cows showed no significant differences between treatments in milk production. However, T21 primiparous cows displayed lower non-esterified fatty acids (NEFA), greater glucose, and greater insulin and IGF-1 concentrations compared to T0 primiparous cows during the 21 DIM of the feeding management. In contrast, multiparous T21 cows achieved greater milk production during the first 21 DIM, with no differences in NEFA and BHB levels but greater insulin and IGF-I concentrations than multiparous T0 cows. Both parity groups in T21 underwent an extra metabolic adaptation following the management change at 22 DIM, increasing NEFA and BHB concentrations, and decreasing milk production during this period. Despite the improved endocrine-metabolic profile observed in T21 during the first days postpartum, no differences were found in the resumption of ovarian cyclicity which was shorter in multiparous than primiparous cows. Untargeted metabolomics supported evidence that primiparous cows grazing from calving had greater lipid and muscle mobilization than other groups, reflected by lower glucose and greater creatinine, dimethylglycine, and formate. Strategic feeding management during the fresh cow period affects the metabolic adaptation to lactation, but milk production responses were observed only in multiparous cows, reflecting parity-specific homeorhetic priorities.

Keywords: Early lactation, Transition period, Metabolism, Parity, Metabolomics.

Author-defined abbreviations: NEB = negative energy balance; NEFA = non-esterified fatty acids; PMR = partial mixed ration; T0 = Cows grazing in one session and supplemented with PMR from calving to 60 DIM; T21 = cows fed a TMR ad libitum in confinement from calving until 21 DIM, and managed as T0 cows from 22 to 60 DIM.

Introduction

The transition period, defined as the 21 days before and after calving (Grummer, 1995), represents a critical phase during which dairy cows undergo profound endocrine-metabolic shifts and are subjected to new management conditions. Particularly, the early postpartum period (Cardoso et al., 2020) is characterized by a rapid increase in metabolic and nutrient demands essential for the onset of lactation (Bell, 1995). Successful adaptation to the lactating state, along with the early resumption of ovarian cyclicity to facilitate conception, ultimately determines cow's longevity in the herd and farm profitability (Drackley, 1999). The well-documented negative energy balance (NEB) during this period, marked by elevated concentrations of non-esterified fatty acids (NEFA) and BHB along with reduced insulin and IGF-1, has been associated with compromised productive and reproductive outcomes (Patton et al., 2007; Butler, 2014). This endocrine-metabolic response and NEB are primarily genetically regulated to support milk production via homeorhetic pathways (Bauman and Currie, 1980; Friggens et al., 2004), yet physiological homeostasis is also responsive to nutritional management (Friggens et al., 2004; Roche et al., 2009). Maximizing dry matter and energy intake in the early postpartum could mitigate the severity and duration of NEB, and has been extensively studied to enhance both productivity and reproductive performance (Cardoso et al., 2020).

Pasture-based systems offer advantages from an economic perspective (White et al., 2002) and are well-received by consumers (Cardoso et al., 2019); however, NEB is generally more pronounced compared to confinement systems (Kolver and Muller, 1998). This is due to the morphologic and chemical characteristics of pasture as well as increased energy expenditure related to walking and grazing activities (Kolver and Muller, 1998; Talmón et al., 2025). Various strategies, including increased supplementation and combinations of grazing with partial mixed ration (PMR), have been evaluated (Bargo et al., 2002; Meikle et al., 2013; Fajardo et al., 2015; Mendoza et al., 2016; Méndez et al., 2023). Nonetheless, even when grazing accounted for only one-third of the diet alongside PMR, energy status indicators in early lactation were still poorer compared to fully confined cows (Astessiano et al., 2015). Thus, a short-term confinement during the initial critical postpartum days may serve as a tool to enhance nutrient intake and reduce energy expenditure, though research on this approach remains scarce and inconclusive (Al Ibrahim et al., 2013; Brady et al., 2021). Feeding strategies based on total mixed ration (TMR) during the first 21 (Al Ibrahim et al., 2013) or 30 days in milk (DIM; Brady et al., 2021) did not increase milk production compared to cows grazing after calving, but some carry-over effect on greater milk yield was suggested (Brady et al., 2021). Although this management resulted in some improved endocrine-metabolic indicators, no meaningful differences in the resumption of ovarian cyclicity were found (Al Ibrahim et al., 2013; Brady et al., 2021).

The adaptation to lactation and the response to early postpartum feeding depend on parity. Multiparous dairy cows prioritize milk production while primiparous cows calving for the first time at young age (e.g., 24 months of age) must allocate nutrients for calf growth and milk production while still completing their growth (Wathes et al., 2007; Meikle et al. 2018). Moreover, primiparous cows, which constitute about 30% of the

herd, face additional challenges in adapting to first lactation due to interactions with the remaining two-thirds multiparous cows (Wathes et al., 2006; Proudfoot and Huzzey, 2022). Reports on the energy status of primiparous versus multiparous dairy cows are mixed, showing either better (Wathes et al., 2006; Morales Piñeyrúa et al., 2018) or worse outcomes (Meikle et al., 2004; Berry et al., 2006). Such discrepancies can be attributed to factors like feeding management, age at first calving, and body condition score, among others. In a recent study (Rivoir et al., 2025), we have shown that the response to contrasting TMR vs grazing plus PMR feeding systems at the onset of lactation (21 DIM) was dependent on parity. Indeed, while multiparous TMR cows increased their milk production when compared to PMR+grazing cows, no differences were observed in primiparous cows (Rivoir et al., 2025). However, no studies have compared the endocrine-metabolic adaptation to lactation in primiparous and multiparous cows under contrasting TMR vs grazing plus PMR feeding systems at the onset of lactation (21 DIM), and their transitioning from the indoor TMR-based diet to that pasture-based system.

This study hypothesizes that TMR confinement during the first 21 DIM modifies the metabolic adaptation to lactation and, in consequence, the productive and reproductive performance, compared to cows managed on grazing with PMR supplementation after calving, and that the response to the management is dependent on parity. Thus, this study aimed to determine whether TMR-confinement during the first 21 DIM, followed by grazing with PMR supplementation, endocrine-metabolic indicators, and their association with milk production and resumption of ovarian cyclicity in primiparous and multiparous dairy cows, relative to a control group managed on grazing plus PMR supplementation after calving.

Material and methods

This study was performed at the Estación Experimental Mario A. Cassinoni, Facultad de Agronomía, Universidad de la República, Paysandú, Uruguay (32° 23'07.6 "S 58° 03'17.9" W). The protocol was approved by the ethics committee of the Universidad de la República (Comité de Ética en el Uso de Animales de Experimentación, CEUA-CHEA ID 1344, exp. 020300-501632-21).

Animals and treatments

The study was performed with 16 primiparous and 24 multiparous Holstein dairy cows that calved in winter. Sample size calculations were performed through Proc POWER (SAS OnDemand for Academics, v. 3.1.0, SAS Institute Inc., Cary, NC, USA), assuming an expected mean difference of 10% for all variables, with a significance level of 0.05 and a power of 0.8, based on previous reports (Kolver and Muller, 1998; Bargo et al., 2002; Vibart et al., 2008; Meikle et al., 2013; Mendina et al., 2024). Sample size results ranged from 8 to 12 cows per treatment, depending on the variable. Before calving, cows were blocked according to the number of lactations, expected calving date, body weight (BW), and BCS, and then randomly distributed into two treatments which started immediately after calving until 60 DIM. One incorporated grazing plus supplementation with PMR after calving (T0), and the other one involved confinement with TMR ad libitum during the first 21 DIM and the same feeding management as T0 from day 22 onwards (T21). Productive variables and ingestive behavior of the cows used in this experiment have been previously published (Rivoir et al., 2025). All the animals underwent a clinical examination by a veterinarian between 5 and 10 days postpartum to check their health status and ensure their continuity in the experiment. Because of calving complications or serious illnesses (caesarean section, downer cow

156 syndrome, metritis), some animals were removed from the experiment. The final
157 number of animals enrolled in the experiment included 15 primiparous (8 and 7 in the
158 T0 and T21 groups, respectively) and 19 multiparous (8 and 11 in the T0 and T21
159 groups, respectively). The range of the calving date was of 25 days between first and
160 last calving, the mean number of lactations of multiparous was 2.7 ± 1.1, BW at calving
161 was 566 ± 48 for primiparous and 665 ± 89 kg for multiparous, and BCS at calving was
162 3.2 ± 0.2 and 3.3 ± 0.2 for primiparous and multiparous, respectively.
163 All animals had the same prepartum management in separate paddocks according to
164 parity and were fed a TMR with anionic salts. During the postpartum period,
165 primiparous and multiparous cows were managed together within each treatment,
166 reflecting commercial practices. After calving, cows in T0 went out to graze between
167 morning and afternoon milking (7:00 to 14:00 h) and stayed in an outdoor soil-bedded
168 yard where supplementation with PMR was offered, during the rest of the day. The
169 outdoor yard had natural shade and automatic water troughs. Cows in T21 were
170 housed in a compost-bedded pack barn during the first 21 DIM with ad libitum TMR,
171 coming out only for milking. The compost barn consisted of a roofed barn, with
172 ventilation (both natural and with fans) and sprinklers for cooling the animals in the
173 feed alley. The area of compost-bedded pack was 13.5 m²/cow. There was an adjacent
174 feeding area of concrete with 6.75 m²/cow with automatic water troughs and feed bunks
175 with a linear space of 0.77 m/cow. The bedded pack management is described by
176 (Mendina et al., 2024). From 22 DIM onwards, cows of T21 joined T0 treatment and
177 were managed together until 60 DIM.
178 The amount of TMR offered to T21 was adjusted to obtain an ad libitum consumption
179 (approximately 10% of feed refusal). A PMR was supplied for cows under T0 treatment
180 to complement pasture intake, according to the weekly pasture allowance (assuming

50% of pasture utilization). The daily herbage allowance was at least three times higher than the expected DMI (25 to 30 kg DM/cow/day at ground level). Forage resources were paddocks with a third-year mixed pasture of *Medicago sativa* and *Dactylis glomerata*, a first-year pasture of *Lolium multiflorum, Cichorium intybus,* and *Trifolium pratense,* second-year pasture of *Festuca arundinacea*, and annual grass of *Avena sativa* or *Lolium multiflorum*. Grazing was carried out in a weekly occupation rotating throughout grazing paddocks. Pasture supply was adjusted weekly based on pasture growth rate and herbage condition at the start of grazing (number of expanded leaves for grasses or nodes for lucerne), and the herbage mass in the grazing area. The ingredients used in TMR and PMR are shown in Table A1, and the average chemical composition is shown in Table A2. The characteristics and average composition of the pasture are shown in Table A3.

Routine and sample collection

The cows were milked twice a day at 4:00 and 15:00 h. A complete milking routine was performed every milking, as described by Mendina et al. (2023). Milk production was registered daily by an automatic recording system (GEA Farm Technologies, Inc) from 7 to 60 DIM. Milk samples were collected weekly during both daily milking for determination of fat, protein, and lactose and analyzed using near-infrared spectroscopy (Milkoscan Minor, Foss, Hillerød, Denmark). Total milk solids were calculated as the sum of daily kilograms of fat, protein, and lactose from 7 to 56 DIM. Weekly, from prepartum to 60 DIM, BCS was assessed and recorded by the same observer using a 5-point scale (Edmonson et al., 1989). Blood samples were taken from the coccygeal vein using an evacuated tube system (Vacuette 8 mL Serum Beads Clot Activator, Greiner Bio-One GmbH) from one week before calving until 60 DIM.

Blood samples were centrifuged at 1,680 x g for 10 minutes at room temperature and serum was stored at -20 °C until further analysis. Postpartum measurements of NEFA, BHB, and cholesterol, were carried out two times a week from calving to 30 DIM and then every 10 days until 60 DIM. Insulin and IGF-1 were analyzed once a week until 30 DIM, and then every 10 days until 60 DIM. Serum progesterone (P4) was determined two times per week from 12 DIM until detection of resumption of ovarian cyclicity, defined as the first day in which the concentration of serum P4 was greater than 1ng/mL, remaining high in the following sampling (Adrien et al., 2012). Serum samples from 21 and 60 DIM were collected for metabolomics analysis.

Metabolite and hormone determination

Serum NEFA, BHB, cholesterol, insulin, IGF-1, and progesterone concentrations were determined at the Laboratorio de Endocrinología y Metabolismo Animal, Facultad de Veterinaria, Universidad de la República, Montevideo, Uruguay. Metabolites were measured by colorimetric assays on spectrophotometry (BA200, Biosystems S.A, Barcelona, Spain) using commercial kits: NEFA-HR 2 (Fujifilm Wako Pure Chemical Industries Ltd.), BHB, cholesterol (Biosystems S.A, Barcelona, Spain). The controls used were those included in the kit and internal laboratory controls. The interassay and intraassay CV for all determinations was less than 10%. Serum insulin concentrations were determined by solid-phase radioimmunoassay (RIA) in a single assay using the INS-IRMA kit (DIA Source Immune Assays S.A., Belgium). The assay sensitivity was 1.3 µIU/mL, and the intra-assay CV for control 1 (19.4 µIU/mL) was 6.3%, and for control 2 (65.6 µIU/mL) 4.0%. Serum IGF-1 analysis was performed using an automated solid-phase chemiluminescent immunoassay kit in conjunction with an IMMULITE 1000 System (Siemens Healthcare Diagnostics, United Kingdom)

calibrated according to the instructions provided by the manufacturer. The interassay CV for control 1 (142 ng/mL) and control 2 (224 ng/mL) were 8.9 and 6.9%, respectively. The limit of detection for IGF-1 analysis was 14.4 ng/mL. Serum progesterone was analyzed by a solid-phase radioimmunoassay (RIA) using a commercial kit (MP Biomedicals, Los Angeles, CA, USA), as reported by Ruprechter et al. (2020). The assay sensitivity was 0.11 ng/mL and the intra- and inter-assay CV for Control 1 (0.5 ng/mL) were 19 and 21%, respectively, and for Control 2 (5 ng/mL) 8.9 and 16.6%, respectively.

Sample preparation and proton nuclear magnetic resonance (^{1}H-NMR) spectral acquisition

Blood serum samples were allowed to thaw at room temperature, and 200 µL of aliquots were mixed with 400 µL of a deuterium oxide buffer solution pD 7.4 and transferred to 5 mm NMR tubes (NE HL5 7, New Era Enterprises Inc., Vineland, NJ, USA) as reported by Dona et al. (2014). Quality control samples were prepared by pooling equal aliquots of all serum samples and were analyzed intermittently throughout the analytical sequence. Their spectra were also acquired, and a principal component analysis (PCA) was performed at the end of the run to verify that QC samples clustered tightly at the center of the dataset, indicating analytical stability (Fig. A1). All NMR spectra were recorded at 25 °C on a Bruker AVANCE III 500 NMR spectrometer operating at ^{1}H and ^{13}C frequencies of 500.13 and 125.76 MHz, respectively (López-Radcenco et al. 2021). 1D ^{1}H free induction decays were zero-filled to 64 K points and apodized with a 0.3 Hz exponential window function before Fourier transformation. ^{1}H NMR spectra were manually phased and baseline corrected using MNova (version 10.0, MestreLab Research, S.L., Santiago de Compostela,

Spain) and referenced to the α-glucose resonance at 5.22 ppm present in all serum samples. Manually-selected spectral regions were aligned, and the data was normalized to the total spectral area after excluding the residual water resonance signal (4.60–5.00 ppm). The resulting data matrix was then exported as a text file for multivariate analyses.

Metabolite identification and quantification

Metabolites were identified by comparison of 1H NMR data against spectral repositories, including the Biological Magnetic Resonance Bank (BMRB) (Hoch et al., 2023), the Human Metabolome Database (HMDB) (Wishart et al., 2022), and Chenomx (version 9, Chenomx, Inc., Edmonton, Canada). When required, metabolite identification was confirmed with data from 1D-TOCSY and HSQC spectra. Variations in the levels of unambiguously identified serum metabolites were estimated using relative concentrations. This figure was computed as the ratio between the area from individual metabolite ^{1}H NMR signal and the total area of the spectrum.

Statistical analysis

For milk production, TMS, BCS, metabolites, and hormones, the assumption of normality was determined by the Shapiro-Wilk statistic. All those variables were analyzed using a generalized linear mixed model (GLIMMIX procedure; SAS Studio®), with fixed effects defined as treatment, DIM, parity, and their interactions. When the interaction was not significant, the term was removed from the model, as described by Brady et al. (2021). The block was considered a random effect. The cow was used as the experimental unit. The covariance structure was compound symmetry for milk and total milk solids production, and autoregressive order 1 for BCS, metabolites and

hormones, as they better fitted the Akaike information criterion (AIC) value. Variables with a single data point such as delta BCS (ΔBCS) at 21, ΔBCS at nadir, and days at nadir were analyzed using GLIMMIX procedure of SAS (SAS Studio®) as described in Brady et al. (2021). For these analysis, fixed effects were defined as treatment, parity, and the interaction between treatment and parity. The BCS at calving was used as a covariate in all BCS related analyses. Significance was considered with alpha ≤ 0.05, and a tendency between 0.05 and 0.10. Post hoc comparisons were performed with Tukey–Kramer test. The probability of resumption of ovarian cyclicity analysis was carried out using Cox's proportional hazards regression models (PHREG procedure; SAS Studio®), including as fixed effects the treatment and parity. When significant, the outputs are presented as a hazard ratio (HR) set to the reference group, where a HR >1 means that an event occurs sooner, whereas a HR <1 means that an event will occur later (Cox, 1972). Survival curves illustrating the evolution of resumption of ovarian cyclicity per parity and treatment are presented. Multivariate statistical analyses, including principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA), were carried out with the PLS_Toolbox package (version 8.5, Eigenvector Research Inc., Manson, WA, USA) implemented for MATLAB (revision 2017b, The MathWorks Inc., Natick, MA, USA). For all models, the data was mean-centered and scaled using a Pareto factor (van den Berg et al., 2006). Cross-validation of all OLPS-DA models was achieved using the random subset method, which involved 20 iterations over data split into 8 equally sized parts. Receiver operating characteristic (ROC) curves were plotted, and area under the curve (AUC) values were calculated to ensure the goodness of fit of the resulting models. A permutation test with 50 iterations was also performed to determine the degree of over-fitting and further validate the discriminant analyses (Ni et al., 2008). The results from

these validations are provided in Appendix (Figures A2–A3). Metabolomics univariate analyses were performed as described for metabolites.

Results

Milk production, total milk solids, and probability of resumption of ovarian cyclicity

Milk production was affected by parity (Table 1) as multiparous cows produced 36.5% more milk than primiparous cows (40.0 vs 29.3 L/cow/day). The triple interaction of treatment, parity and DIM was also significant. During the differential management period, no differences in milk production were observed between treatments in primiparous cows, while T21 multiparous cows produced more milk than T0 multiparous cows. Following the shift to grazing, T21 primiparous cows exhibited lower milk production than T0 primiparous cows between 28 and 42 DIM, and then milk production converged between treatments (Fig. 1A). In T21 multiparous cows, milk production dropped following the management change, reaching levels comparable to those of T0 multiparous cows and remained stable until the end of the study (Fig. 1B). Multiparous cows produced more milk solids than primiparous cows (4.94 vs 3.65 kg/cow/day, respectively, Table 1). The triple interaction was significant (Table 1), as primiparous T21 cows showed lower total solids than primiparous T0 cows at 42 DIM (Fig. 1C), while in multiparous cows T21 yielded more milk solids than T0 at 7 and 14 DIM (Fig. 1D).

There was no significant effect of treatment on the days to resumption of ovarian cyclicity (32.7 $\pm$ 2.7 and 31.1 $\pm$ 3.1 days, for T0 and T21, respectively; P = 0.9038). Parity had a significant effect as primiparous showed a lower hazard of resumption than multiparous cows (HR = 0.42; P = 0.0380, Fig. 1E, F). Multiparous cows exhibited

an earlier resumption of ovarian cyclicity (28.5 ± 2.2 days) compared to primiparous cows (36.4 ± 3.8 days).

Body condition score, metabolites and hormones

The BCS was affected by treatment, as T21 cows showed a greater BCS on average than those in the T0 group, and BCS decreased over DIM (Table 1). The T0 cows showed a greater ΔBCS at 21 DIM compared to T21 cows (-0.22 ± 0.04 vs -0.10 ± 0.04, P = 0.0280), and tended to have a greater ΔBCS at nadir than T21 cows (-0.53 ± 0.05 vs -0.40 ± 0.05, respectively, P = 0.0683). The ΔBCS at nadir was lower for primiparous than multiparous cows (-0.37 ± 0.06 vs -0.56 ± 0.05, P = 0.0368) and primiparous also reached the nadir earlier (31.5 ± 4.7 days) than multiparous cows (50.9 ± 3.9 days; P = 0.0110).

The concentrations of NEFA were significantly affected by the treatment, with T0 cows exhibiting greater concentrations than T21 cows (Table 1). Additionally, NEFA concentrations were significantly affected by the triple interaction of treatment, parity, and DIM. In T0 primiparous cows, NEFA concentrations were greater than those of T21 primiparous cows during the first 21 DIM (Fig. 2C). On the other hand, multiparous cows showed no differences in NEFA concentrations until 15 DIM, after which T21 multiparous cows exhibited a more rapid decline, resulting in lower concentrations than T0 multiparous cows from 18 to 21 DIM (Fig. 2D). In the first week after the management change (day 25), T21 primiparous cows had greater NEFA concentrations than T0 primiparous cows, with no differences observed thereafter. In multiparous cows, no differences in NEFA concentrations were detected between treatments following the management change. However, T21 multiparous cows experienced a rise in NEFA concentrations between days 25 and 30, surpassing

prepartum levels, with no differences observed thereafter. Concentrations of BHB were significantly affected by the triple interaction of treatment, parity, and DIM (Table 1). No differences were observed in primiparous cows between treatments, while after the transition to grazing T21 multiparous cows exhibited greater BHB concentrations than T0 multiparous cows (Fig. 2F).

Cholesterol concentrations were affected by the triple interaction (Table 1). Although both treatments exhibited similar profile evolution, cholesterol levels were greater in T0 cows at 21 DIM for primiparous (Fig. 2G), and between 18 and 25 DIM for multiparous cows (Fig. 2H), compared to their parity counterparts in the T21 group.

Greater insulin concentrations were found in T21 cows compared to those in the T0 group, and in primiparous compared to multiparous cows (Table 1). The interaction between treatment and parity was significant ($P = 0.0007$). Average insulin concentrations were greater in T21 primiparous cows than in T0 primiparous cows (21.8 ± 1.3 vs 13.8 ± 1.23 ng/mL; $P < 0.0001$), and in T21 multiparous compared to T0 multiparous cows (12.3 ± 1.0 vs 10.1 ± 1.2 ng/mL; $P = 0.0482$). Insulin concentrations was also affected by the interaction among treatment, parity and DIM (Table 1). While T21 primiparous cows had greater insulin concentrations during the first 21 DIM compared to T0 primiparous cows, with similar values thereafter (Fig. 3A), T21 multiparous cows showed greater insulin concentrations at 3 and 9 DIM but reached comparable levels to T0 multiparous cows thereafter (Fig. 3B). This interaction also revealed differences between parities within treatment, as primiparous cows in T21 had consistently greater insulin concentrations than multiparous T21 cows across most days. In contrast, no significant differences between parities were observed over time in the T0 group.

373 On average, greater IGF-1 concentrations were found in T21 cows compared to T0,
374 and in primiparous compared to multiparous cows (Table 1). The concentration of IGF-
375 1 was affected by the interaction among treatment, parity and DIM (Table 1). Both
376 primiparous (Fig. 3C) and multiparous (Fig. 3D) T21 cows had greater IGF-1
377 concentrations from 3 to 21 DIM compared to their counterparts in T0, with no
378 differences thereafter. In both treatments, primiparous cows showed consistently
379 greater IGF-1 concentrations than multiparous over time.

380 ***Serum metabolomics***

381 To identify potential clustering based on treatment, time point, and parity, a PCA was
382 performed as an unsupervised classification method using the data obtained from the
383 ^{1}H NMR spectra of all serum samples, including quality controls. The score plot
384 suggested a separation between treatments in primiparous cows at 21 DIM (Fig. 4A).
385 In contrast, the distinction between treatments was not clear in multiparous cows at 21
386 DIM (Fig. 4B), as well as for primiparous (Fig. 4C) and multiparous cows (Fig. 4D) at
387 60 DIM.
388 These results were further confirmed using supervised classification (OPLS-DA),
389 which showed a clear divergence between treatments only in primiparous cows at 21
390 DIM (Fig. 5A). The accuracy of discrimination was tested, yielding an AUC of 0.96 in
391 the ROC analysis, and was also statistically significant in the permutation test. The
392 corresponding loading plot indicated that glucose and valine were more abundant in
393 T21 primiparous cows, whereas urea, acetoacetate, BHB, lactate, glycine,
394 dimethylglycine, choline, betaine, creatine, creatinine, alanine, acetate, and lipids were
395 present at greater concentrations in T0 primiparous cows (Fig. 5B). Although the
396 OPLS-DA model for multiparous cows at 21 DIM showed a similar trend (Fig. 5C, D),

it had a lower AUC (0.77) and did not pass permutation test validation. Likewise, the OPLS-DA models for both parity groups at 60 DIM failed to pass the permutation tests, with AUC values of 0.71 and 0.61 for primiparous and multiparous cows, respectively. After conducting the univariate analysis based on the relative concentrations of individual metabolites obtained from spectral integration, metabolites affected by the treatment, or its interaction were included for description. The relative concentrations of glucose differed according to the interaction between treatment and parity (Table 2), as they were greater in T21 than in T0 primiparous cows but showed no differences for multiparous cows (Figure 6A, B). Also, primiparous T21 cows showed greater glucose concentrations than multiparous T21 cows, but no differences between parities were found in T0 cows. The interaction between parity and day was significant, as glucose concentrations were lower for multiparous than primiparous cows at 60 DIM, without differences between parities at 21 DIM. The relative concentrations of BHB differed according to the interaction between treatment and day, as T0 cows had greater concentrations than T21 cows at 21 DIM ($P = 0.0215$, Fig. 7C), while T21 cows at 60 DIM showed greater concentrations of this metabolite than T0 cows ($P = 0.0378$). Acetate differed according to the interaction between treatment and parity, as its relative concentration in the T21 group was greater in multiparous than in primiparous cows ($P = 0.0029$), without differences between parities in T0 cows. Valine and histidine concentrations were affected by the interaction between treatment and day, as T21 cows had greater concentrations of these amino acids than T0 cows at 21 DIM, but no differences were found at 60 DIM. Creatinine concentrations were greater in T0 than T21 cows and in primiparous compared to multiparous cows. A significant interaction between treatment and day was observed, with T0 cows showing greater creatinine concentrations than T21 cows at 21 DIM ($P = 0.001$), without differences

between treatments at 60 DIM. Creatinine concentrations increased from 21 to 60 DIM in T21 cows (P = 0.0009), whereas no changes over time were observed in T0 cows. A tendency in the interaction between treatment and parity was detected, as creatinine concentrations were greater in primiparous T0 than in primiparous T21 (P = 0.0049) and multiparous T0 cows (P = 0.0028). However, no differences were found between treatments in multiparous cows or between parities in T21 cows. Allantoin concentrations tended to be greater in T0 than T21 cows (Table 2). There was a tendency for the interaction between treatment and day as T0 cows had slightly greater concentrations of this diureide than T21 animals at 21 DIM (P = 0.0563). Allantoin concentrations tended to decrease from 21 to 60 DIM in T0 cows (Table 2). The interaction between parity and day was significant as multiparous showed greater allantoin concentrations than primiparous cows at 21 DIM (P = 0.0032), decreasing at 60 DIM compared to their 21 DIM levels (P = 0.0039). Dimethylglycine concentrations tended to be greater in T0 than in T21 cows. There was a significant interaction between treatment and parity, as primiparous T0 had greater concentrations of this metabolite than both primiparous T21 (P = 0.0028) and multiparous T0 (P = 0.0049). Formate concentrations were greater in T0 than in T21 cows. There was a tendency for the triple interaction, as at 21 DIM, primiparous T0 cows had greater formate concentrations than primiparous T21 (P < 0.0001, Fig. 7P) and also compared to multiparous T0 (P = 0.0043), decreasing at 60 DIM compared to their 21 DIM levels (P < 0.0001).

Discussion

To the best of our knowledge, this is the first study to investigate the parity-specific responses in dairy cows to a differential feeding management (indoor-fed TMR ad

libitum for the first 21 days postpartum vs grazing with PMR supplementation after calving) in terms of endocrine-metabolic adaptation to lactation which links to productive and reproductive indicators. While TMR-confinement yielded a 12% greater milk production in multiparous cows, no differences in milk production were detected in primiparous cows during the first 21 DIM. However, the effect of the treatment on the endocrine-metabolic indicators was more marked in primiparous than in multiparous cows. The resumption of ovarian cyclicity was not affected by treatment, but multiparous cows had a shorter anestrus than primiparous cows. Thus, the present results suggest that strategic feeding management during the fresh cow period elicits different metabolic and productive responses depending on parity.

In the same experiment but under two calving seasons, we have reported that this dietary intervention did not enhance the productive performance of primiparous cows and suggested that an extended TMR period may be needed to reveal their full productive potential (Rivoir et al., 2025). Indeed, greater milk production in primiparous cows under TMR vs similar pasture-based systems was observed only after 35 DIM (Meikle et al., 2013). For multiparous cows, the differences between treatments were detected already at the first week postpartum, averaging 12% greater milk production in T21 compared to T0 multiparous cows during the first 21 days postpartum. This response aligns with reports of up to 15% greater milk production in Holstein multiparous under similar feeding regimes during early lactation (Fajardo et al., 2015; Mendina et al., 2024), and increases of 5–20% during mid-lactation (Bargo et al., 2002; Mendoza et al., 2016; Salado et al., 2018, 2020). Previously, two studies evaluated short-term TMR-confinement management during the first 21 (Al Ibrahim et al., 2013) or 30 days postpartum (Brady et al., 2021), followed by two grazing sessions and concentrate supplementation. Al Ibrahim et al. (2013) included only multiparous cows,

whereas Brady et al. (2021) adjusted parity in the model as a covariate. In these studies, no differences were found in terms of milk production, which has been attributed to the small magnitude of differences in DMI, as well as the lower starch and CP content of the TMR. Nonetheless, Brady et al. (2021) suggested a carryover effect on milk production as it tended to be greater in TMR-fed cows after the change of management.

In the present study, the T21 management was insufficient in generating carryover effects on milk yield after the shift to grazing, which could be attributed to the short duration of the feeding treatment (see review: Jørgensen et al., 2016) and the greater milk production achieved during the differential period. Despite the drop in milk production when switching to pasture plus PMR, the multiparous T21 cows were able to adapt and maintain their milk production at the same levels as cows that were already adapted. Similarly, no differences were observed in a longer intervention (first 10 weeks postpartum), when multiparous Holstein cows fed TMR ad libitum were switched to grazing, compared to cows that grazed from calving (Fajardo et al., 2015). In the present study, as in Fajardo et al. (2015), high levels of PMR were offered, which combined with favorable pasture conditions, enabled cows to maintain a high DMI. This contrasts with other studies where the transition to grazing, along with reduced supplementation, was accompanied by marked declines in production (Schären et al., 2016; Hartwiger et al., 2018). In primiparous cows, the shift from TMR to grazing, resulted in a maintenance of the milk production, while T0 primiparous cows continue to increase milk production as part of the physiological lactation curve. This plateau in T21 cows led to an 11% difference in milk yield during the first three weeks following the management change. After that, milk production was no longer different between T21 and T0 primiparous cows, coinciding with the time for behavioral, metabolic, and

ruminal adaptation to the shift from TMR to grazing previously reported (Schären et al., 2016, 2017).

Regarding metabolic indicators, the similar NEFA concentrations in multiparous T21 and T0 cows in the first two weeks postpartum suggest that the greater milk production observed in T21 was sustained by the extra dietary energy. On the other hand, the lower NEFA concentrations in primiparous T21 cows compared to T0 cows –and the similar milk production mentioned above– is consistent with the better energy status due to the TMR diet. These findings align with the reported lower capacity for nutrient partitioning towards milk production in primiparous cows (Wathes et al., 2006; Friggens et al., 2007; Morales Piñeyrúa et al., 2018; Ruprechter et al., 2018), in contrast to its prioritization in multiparous cows (Wathes et al., 2006, 2007). This aligns with BCS findings, as primiparous cows showed a lower loss of BCS and earlier nadir than multiparous cows, which likely prioritized the partitioning of body reserves to support milk production over a longer period (Wathes et al., 2006; Friggens et al., 2007; Morales Piñeyrúa et al., 2018; Ruprechter et al., 2018). Nevertheless, the mobilization phase was still shorter for T21 than T0 multiparous cows, as evidenced by the lower NEFA concentrations by 18–21 DIM in the former. Both parities in T21 experienced a metabolic readaptation when starting to graze, which was reflected by the increase of NEFA and BHB concentrations. This finding could be attributed to the lower DMI and energy density of the diet, as well as to the new energy expenditure in walking and grazing activities (Kolver and Muller, 1998; Bargo et al., 2002), and behavioral and social adaptation (Chilibroste et al., 2012). However, multiparous T21 cows maintained greater BHB concentrations until the end of the study compared to T0 multiparous cows, which seemed to be decreasing the activity of ketogenic pathways in this period.

Near the end of the differential management period (18 to 21 DIM), T0 cows exhibited greater cholesterol concentrations compared to T21 cows. Cholesterol profiles in this stage have been described as somehow controversial (Cavestany et al., 2005). Positive associations with dry matter intake have been reported (Drackley et al., 2014; Mendina et al., 2024), but unexpected lower concentrations of cholesterol in TMR-fed cows have been attributed to nutrient-specific characteristics of the diet (Meikle et al., 2013). The elevated cholesterol concentrations in T0 cows could be attributed to the greater ether extract content in the pasture and PMR compared to the TMR used in this study (Supplementary Table 1 and 2). This difference appeared earlier and persisted longer in multiparous T0 than in primiparous cows, likely due to the greater intake capacity of the former animals.

The greater glucogenic sources in TMR-based diets were evidenced by the greater insulin and IGF-1 concentrations during the differential feeding management period in both parities of T21 compared to T0 cows (Kolver and Muller, 1998; Lucy et al., 2013). Insulin and IGF-1 levels were greater over time in primiparous than in multiparous T21 cows, in line with the literature (Taylor et al., 2004; Wathes et al., 2007; Morales Piñeyrúa et al., 2018; Ruprechter et al., 2020; Cattaneo et al., 2023), and the uncoupling of the somatotrophic axis in favor of milk production in multiparous cows (Bauman and Currie, 1980; Wathes et al., 2007). However, the difference in insulin concentrations between parities was not expressed in T0 cows over time. Insulin has been proposed as a more immediate indicator of daily nutrient intake, whereas IGF-1 profiles better reflect changes in BCS (Adrien et al., 2012). In this context, despite being raised on pasture, primiparous cows seemed to struggle more with grazing behavior (Chilibroste et al., 2012), potentially explaining their comparable insulin concentrations along time to those of multiparous cows in the T0 group. These findings

545 could suggest differences between parities adapting to reach their potential DMI under
546 grazing conditions, in line with Chilibroste et al. (2012). Unfortunately, in the present
547 study we do not have precise measurements of individual pasture and PMR intake and
548 can thus only speculate based on PMR group feeding records and pasture intake
549 through energy balance or pasture disappearance estimations.
550 Even with several endocrine-metabolic differences between treatments in primiparous
551 cows, no effect on cyclicity was detected in this parity group. It has been suggested
552 that primiparous cows are more sensitive to endocrine-metabolic signals which can
553 delay the resumption of ovarian cyclicity (Santos et al., 2009). Factors limiting earlier
554 reproductive stimulation may include the brief treatment duration before peak lactation
555 and maximum feed intake capacity (Ingvartsen and Andersen, 2000), and the
556 metabolic re-adaptation after switching to grazing. Although it potentially alleviates the
557 NEB in the first three weeks postpartum, the feeding management was insufficient to
558 impact the resumption of ovarian cyclicity in both primiparous and multiparous cows.
559 This is consistent with reports indicating that early lactation NEB and nutritional
560 strategies have limited effects on reproductive outcomes, as nutrients are primarily
561 directed toward milk production (De Vries and Veerkamp, 2000; Butler, 2014; Berry et
562 al., 2016). Nevertheless, and in agreement with previous findings (Meikle et al., 2004;
563 Santos et al., 2009; Adrien et al., 2012; Ruprechter et al., 2020), multiparous cows
564 exhibited a shorter postpartum anestrous period compared to primiparous cows. This
565 difference is likely attributable to the additional growth requirements of primiparous
566 cows (Coffey et al., 2006).
567 Serum metabolomic analyses revealed more significant differences between
568 treatments for primiparous cows at 21 DIM, than for multiparous cows. Multivariate
569 analyses did not detect significant differences between treatments at 60 DIM in either

570 primiparous or multiparous cows. Overall, the OPLS-DA loading plot for primiparous
571 cows at 21 DIM is consistent with insulin data, indicating greater circulating glucose in
572 T21 cows, while markers of lipid and protein mobilization were more abundant in T0
573 cows.
574 Consistently, univariate analysis revealed that glucose concentrations differed
575 between treatments in primiparous cows, but not in multiparous cows. This finding
576 aligns with milk production and endocrine-metabolic profile results, as primiparous T21
577 cows did not respond to the feeding management with increased milk production but
578 rather exhibited improved energy balance indicators. Conversely, multiparous T21
579 cows likely directed the extra circulating glucose towards the mammary gland to
580 support greater milk yield, which could explain the lack of glucose differences between
581 multiparous T0 and T21 cows despite the dietary contrast. Similar to insulin profiles,
582 relative glucose concentrations did not differ between primiparous and multiparous
583 cows in the T0 group but did in the T21 group. The relative glucose concentrations
584 observed at 21 DIM declined at 60 DIM, suggesting a greater glucose uptake to meet
585 the greater milk production (Lucy et al., 2014).
586 Metabolomics data also detected greater BHB levels in T0 cows at 21 DIM, despite no
587 differences were detected by spectrophotometry on that day (Fig. 2E and F). These
588 data is consistent with greater NEFA concentrations in T0 cows and reflect the greater
589 NEB. Interestingly, BHB concentrations at 60 DIM were greater in T21 cows, which is
590 consistent with BHB profiles at this time and, as mentioned before, could be the result
591 of increased energy expenditure and nutrient intake levels upon transitioning to grazing
592 (Kolver and Muller, 1998; Meikle et al., 2013). The lower relative concentration of
593 acetate observed in primiparous T21 compared to T21 multiparous cows could reflect
594 the greater DMI capacity in the later, with a greater influx from the rumen (Drackley et

595 al., 2014), while this difference was not expressed in grazing cows with a greater
596 proportion of acetate precursors (Ishler et al., 1996).
597 The greater concentrations of the glucogenic amino acids valine and histidine
598 observed in T21 cows at 21 DIM may indicate a better protein balance status (Jorge-
599 Smeding et al., 2021), which aligns with their improved energy status at this stage
600 compared to T0 cows. Moreover, there was a decrease in both amino acids at 60 DIM
601 in T21 cows, suggesting the limitation of the grazing plus PMR diet to maintain high
602 milk production through its contribution to lactose and milk protein synthesis.
603 The level of creatinine, an indicator of body muscle mass and muscle protein
604 breakdown (Megahed et al., 2019; Sadri et al., 2023), was greater in T0 than in T21
605 cows at 21 DIM, a difference mainly originated from the greater concentrations of this
606 metabolite in primiparous T0 cows. Data suggest that primiparous T0 cows mobilized
607 protein reserves to sustain similar milk production levels than T21 cows (Megahed et
608 al., 2019). The greater creatinine levels in grazing could also be due to the higher
609 physical activity on walking and grazing. Our results also showed greater
610 concentrations of creatinine for primiparous T0 compared to multiparous T0 cows, but
611 this difference was not detected for T21 cows. Overall, it seems reasonable to suggest
612 that, under grazing conditions, primiparous cows struggle to meet their metabolic
613 requirements during early postpartum (as further indicated by their lower glucose
614 concentrations), leading to increased muscle mobilization. Allantoin was greater in T0
615 than in T21 cows at 21 DIM, while no differences were observed at 60 DIM. This
616 metabolite has been linked to oxidative stress in cattle (Liao et al., 2018) and humans
617 (Kand'ár and Žáková, 2008), which may be particularly relevant during the immediate
618 postpartum period (Sordillo and Mavangira, 2014). Moreover, allantoin is also
619 associated with triglyceride metabolism and has been reported to be greater in cows

620 with subclinical ketosis (Wang et al., 2016), which, as previously described, may better
621 align with the metabolic profile of T0 cows at this time.
622 Dimethylglycine and formate relative concentrations were greater in T0 vs T21
623 primiparous cows. Dimethylglycine is generated as a by-product in the conversion from
624 betaine to methionine. This process releases a one-carbon unit, which can be oxidized
625 to formate, contributing to the one-carbon pool for nucleotide synthesis and
626 methylation reactions (McFadden et al., 2020). Dairy cows are unable to synthesize
627 methionine in sufficient quantities to meet the demands for fetal growth and milk protein
628 synthesis (Arshad and Santos, 2024). With the conversion from betaine to methionine,
629 cows rely heavily on folate metabolism to support methylneogenesis during the
630 peripartum period and early lactation (Xue and Snoswell, 1986), which may explain the
631 greater abundance of both dimethylglycine and formate observed in primiparous T0
632 cows, particularly at 21 DIM, coinciding with their more pronounced NEB. Although
633 knowledge of one-carbon metabolism in dairy cows remains limited, the marked
634 differences observed in these metabolites reinforce their relevance not only in milk
635 production, but also in hepatic health and immune response, making this a growing
636 area of interest as previously reported (McFadden et al., 2020).
637 In conclusion, strategic feeding management during the fresh cow period elicits distinct
638 endocrine-metabolic and productive responses, reflecting parity-specific homeorhetic
639 priorities. Feeding TMR ad libitum during the first 21 days postpartum allowed
640 primiparous cows to preserve body reserves without increasing milk yield, whereas
641 primiparous cows managed under grazing and supplemented with PMR after calving
642 appeared to struggle to meet their metabolic requirements, relying on greater lipid and
643 muscle mobilization. In contrast, multiparous cows fed TMR exhibited greater milk
644 production and similar lipid mobilization levels in the first two weeks postpartum

compared to their counterparts in a pasture-based system after calving. The transition from TMR to grazing management triggered a metabolic re-adaptation in both parities, and no carryover effect on milk production was observed. Despite the productive and metabolic benefits, the differential fresh cow feeding strategy with TMR during first 21 days postpartum did not influence the time to the resumption of ovarian cyclicity.

Notes

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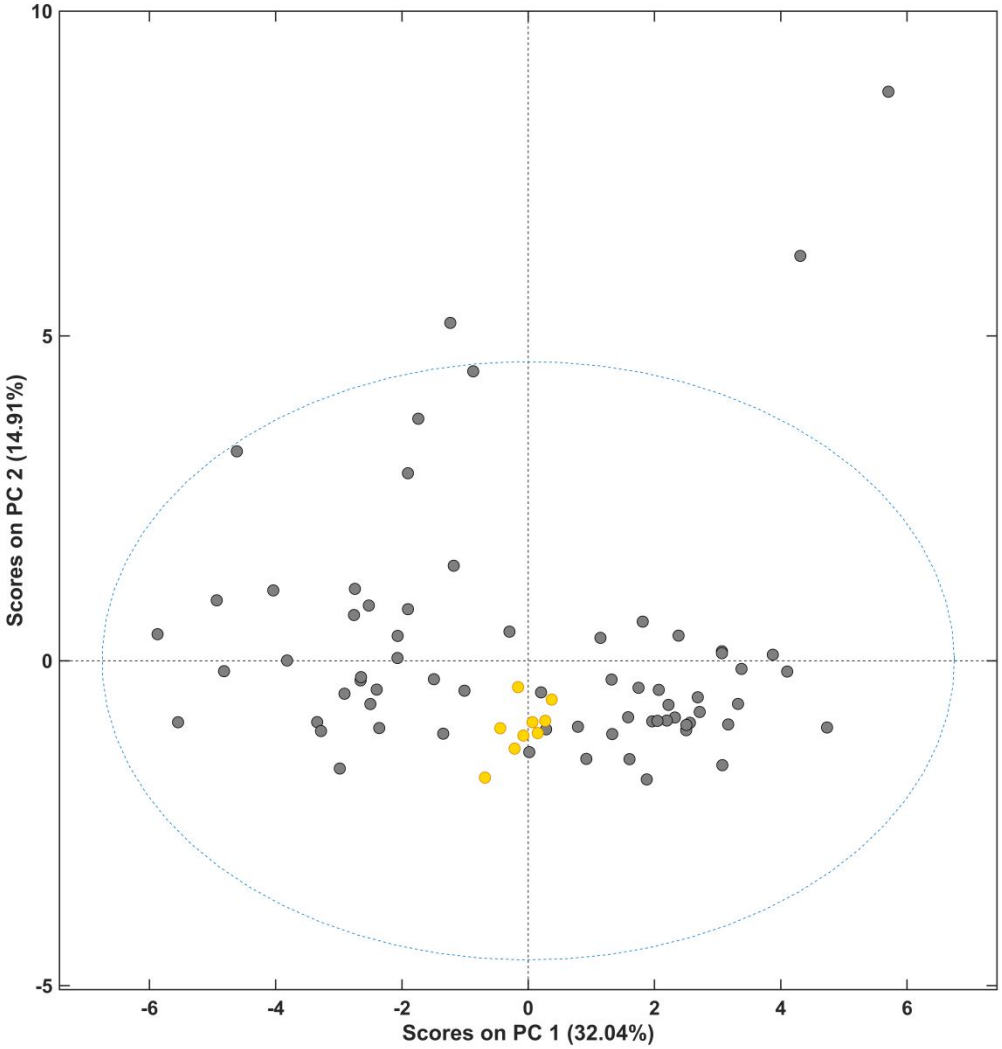
916 **Appendix**



917

918 Figure A1. PCA score plot obtained from ¹H-NMR spectral data of serum samples
919 collected at 21 and 60 DIM from primiparous and multiparous cows in T0 and T21
920 groups, and quality control (QC) samples (yellow circles).

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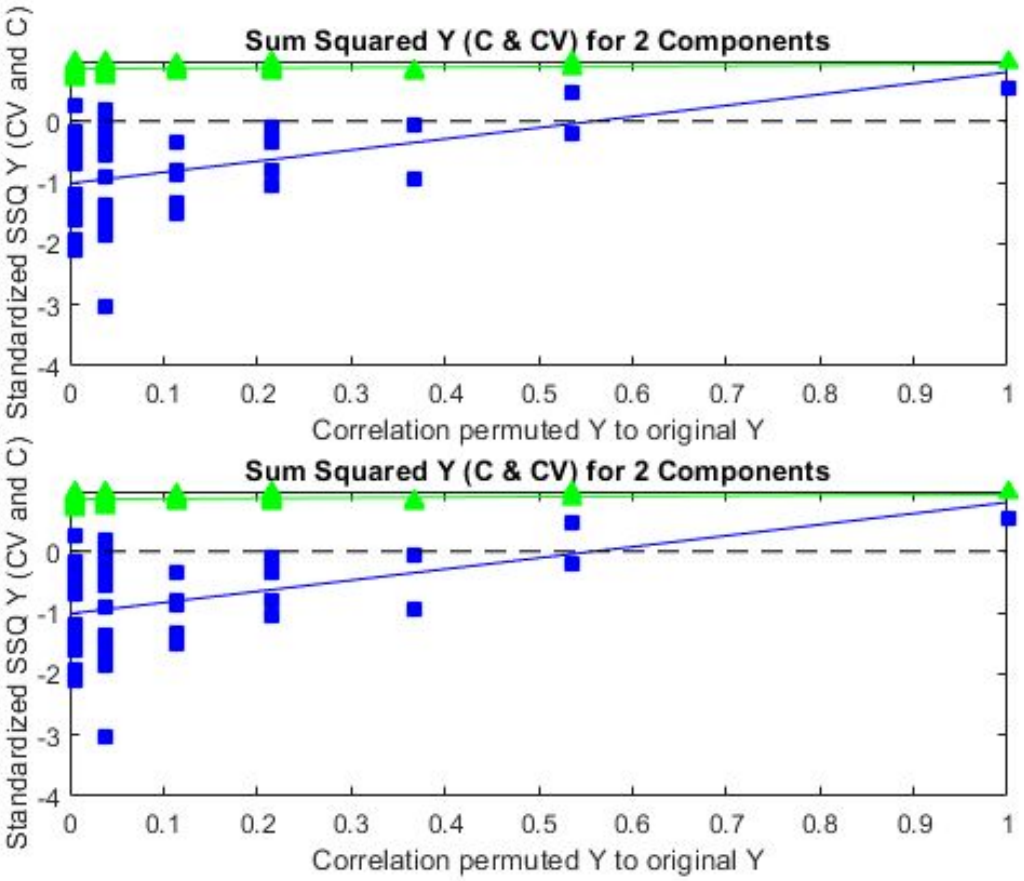
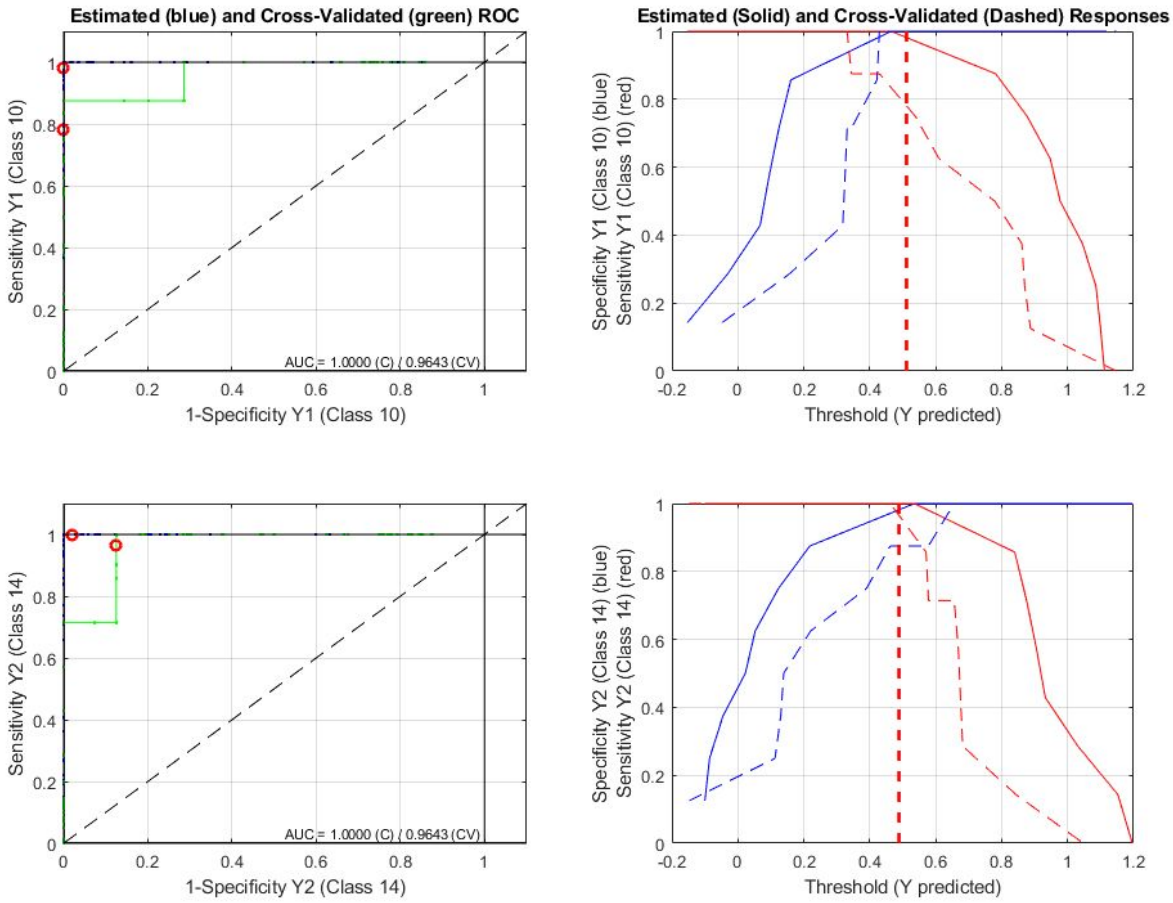


922

923 Figure A2. Permutation test plots for the OPLS-DA model comparing primiparous T0

924 and T21 at 21 DIM (R2Y = 0.92 and Q2Y = 0.48).

925



926

927 Figure A3. ROC analysis curves derived from the OPLS-DA model comparing

928 primiparous T0 and T21 at 21 DIM (AUC = 0.96).

929 Table A1. Ingredient composition (% of DM) of TMR and PMR.

Treatments	T0	T21	T0 & T21
Period	0-21 DIM		22-60 DIM
Ingredients, % of DM			
Corn silage	15	44.3	16.4
Sorghum silage	27.6	-	24.3
Moha hay	1.7	4.6	-
Corn grain	19.1	17.4	21
Soybean meal	10.5	11.7	7.2
Canola meal	-	5.8	-
Sunflower expeller	-	5.8	-
Soybean hulls	-	7.7	-
Wheat bran	22.7	-	24.1
Corn DDGS	-	-	3.1
Minerals and vitamins	3.7	2.5	3.9
Forage:concentrate ratio	44:56	49:51	41:59

930 Abbreviations: DM: Dry matter, DDGS: Dried distillers grains with soluble.

931 Table A2. Chemical composition and offer of TMR (T21) and PMR (T0 and both
932 treatments after 22 DIM)[1]

Treatments	T0	T21	T0 & T21
Period	0-21 DIM		22-60 DIM
% of DM[2]			
DM	40.1 ± 2.1	50.2 ± 2.4	45 ± 4.1
CP	14.3 ± 0.05	17.1 ± 0.3	14.3 ± 0.4
NDF	39.2 ± 1.6	36.5 ± 1.8	40.0 ± 1.7
ADF	17.6 ± 1.0	18.9 ± 1.6	17.4 ± 0.5
Ash	9.1 ± 0.1	7.5 ± 0.4	9.9 ± 0.9
Ether extract	2.4 ± 0.3	1.9 ± 0.1	2.5 ± 0.3
NEL (Mcal/kg DM)	1.64 ± 0.02	1.62 ± 0.05	1.65 ± 0.01
Offer (kg DM/d)*	13.3 ± 0.5	29.9 ± 3.5	13.1 ± 0.8

933 [1] Values expressed as the average ± the standard deviation.

934 [2] DM: Dry matter, PC: Crude protein, NDF: Neutral detergent fiber, FDA: Acid detergent fiber, NEL:
935 Estimated net energy of lactation using NEL (Mcal/kg) = 2.149 − (0.0223 × ADF), (NRC, 2001).

936 * The amount of offered PMR corresponded to the intake, as indicated by the empty feeders in the
937 morning.

938 Table A3. Characteristics and chemical composition of the pasture according to period[1]

Treatments	**T0**	**T0 & T21**
Period	**0-21 DIM**	**22-60 DIM**
Herbage allowance, kg DM/cow/d	27.6 ± 0.6	27.1 ± 4.5
Pre-grazing herbage mass, kg DM/ha	2090 ± 535	2128 ± 372
Post-grazing herbage mass, kg DM/ha	1533 ± 714	1481 ± 363
Pre-grazing sward height, cm	24.2 ± 7	21.1 ± 4.1
Post- grazing sward height, cm	13.5 ± 1.3	14.5 ± 1.3
% of DM[2]		
DM	23.7 ± 3.3	22.4 ± 4.1
CP	20.6 ± 4.3	18.7 ± 4.1
NDF	34.1 ± 7.1	36.1 ± 2.6
ADF	16.4 ± 3.0	17.6 ± 2.0
Ash	11.3 ± 1.4	11.3 ± 1.4
Ether extract	3.2 ± 0.7	3.2 ± 0.6
NEL (Mcal/kg DM)	1.66 ± 0.03	1.64 ± 0.03

939 [1] Values expressed as the average ± the standard deviation.

940 [2] DM: Dry matter, CP: Crude protein, NDF: Neutral detergent fiber, FDA: Acid detergent fiber, NEL:
941 Estimated net energy of lactation using (2.301 − (0.0289 × %ADF)) × 4.1868 × 0.239 (Acosta, 2004).

942 Tables and Figures

943 Table 1. Milk production, total milk solids, metabolites and hormones for T0 and T21

944 treatments

	Treatments			P-value			
	T0	T21	SEM	Treat	DIM	Parity	Treat*Parity*DIM
Milk production, L/cow/day	34.6	34.7	1.2	0.9133	<0.0001	<0.0001	<0.0001
Total milk solids, kg/cow/day	4.2	4.4	0.2	0.5357	0.1144	<0.0001	0.0047
BCS[1], 1-5 scale	3.0	3.1	0.03	0.0370	<0.0001	0.6869	0.9027
NEFA[2], mmol/L	0.45	0.37	0.02	0.0209	<0.0001	0.3947	0.0376
BHB[3], mmol/L	0.58	0.60	0.02	0.3780	0.0003	0.2937	0.0201
Cholesterol, mmol/L	3.62	3.39	0.12	0.1048	<0.0001	0.9637	0.0497
Insulin, ng/mL	12.0	17.0	0.8	<0.0001	<0.0001	0.0005	<0.0001
IGF-1[4], ng/mL	68.7	80.7	3.2	0.0011	<0.0001	<0.0001	<0.0001

945 [1] Body condition score.

946 [2] Non-esterified fatty acids.

947 [3] Beta-hydroxybutyrate.

948 [4] Insulin-like growth factor – 1.

949 Table 2. Univariate analysis of metabolites relative concentration determined by NMR
950 spectroscopy. Fixed effects are treatment, parity, day (21 vs 60 DIM), and their
951 interactions

	P-value						
	Treat	Parity	Day	Treat x Parity	Treat x Day	Parity x Day	Treat x Parity x Day
α-glucose	0.0308	0.0118	<.0001	0.0076	0.9614	0.0307	0.5444
β-glucose	0.0128	0.0233	<.0001	0.0071	0.2553	0.0107	0.8755
BHB	0.8470	0.1330	0.8617	0.4175	0.0029	0.2676	0.6444
Acetate	0.9435	0.0419	0.4155	0.0256	0.3007	0.8465	0.1749
Valine	0.2840	0.7499	0.0020	0.8279	0.0393	0.0039	0.5739
Histidine	0.1648	0.8922	0.0008	0.7774	0.0007	0.0705	0.7252
Creatinine	0.0146	0.0068	0.0097	0.0731	0.0287	0.0889	0.7586
Allantoin	0.0559	0.0562	0.0931	0.9808	0.0915	0.0221	0.7703
Dimethylglycine	0.0640	0.1479	0.7705	0.0068	0.3526	0.0242	0.1790
Formate	0.0005	0.9768	0.0001	0.0813	0.0061	0.0078	0.0959

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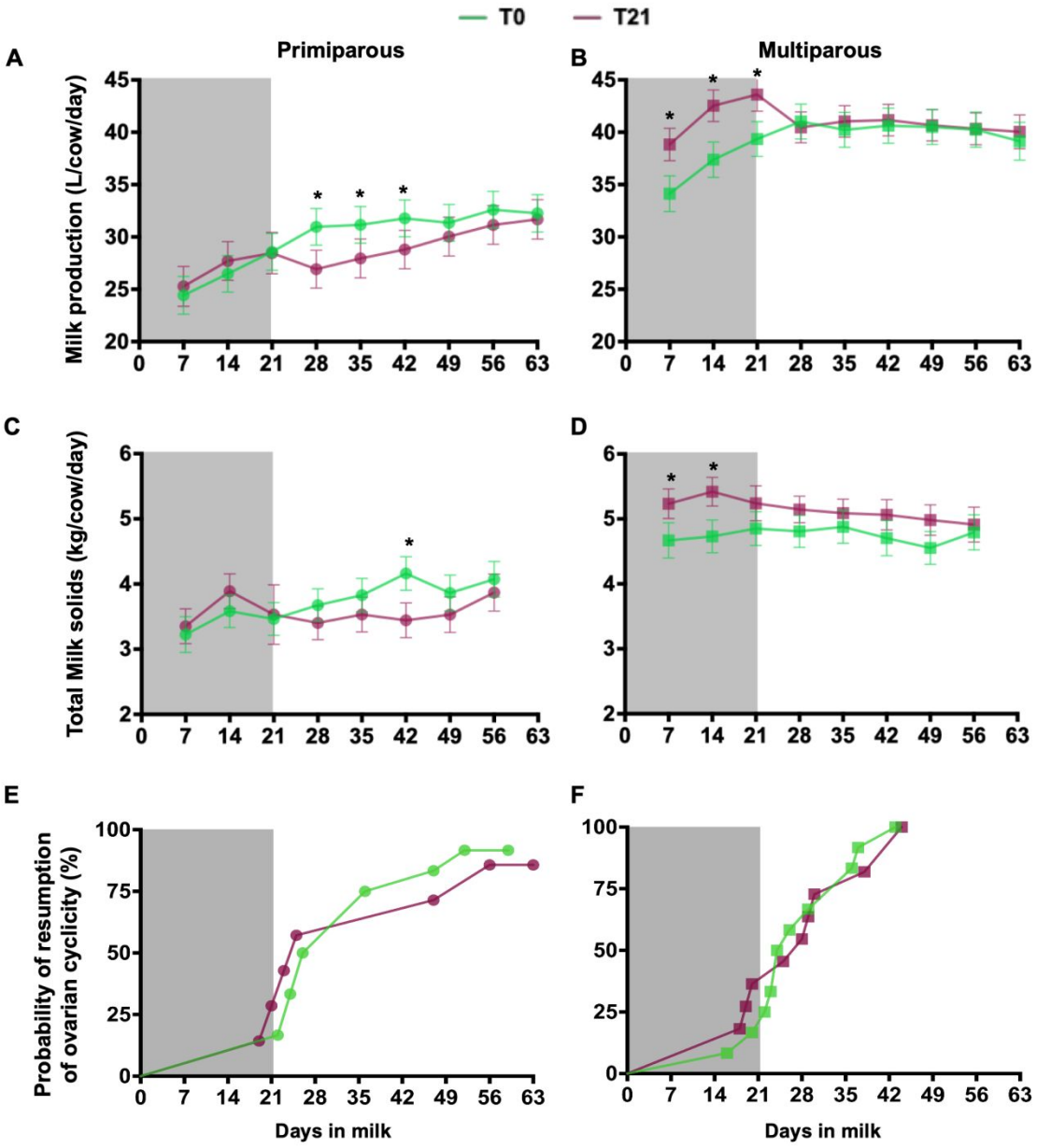
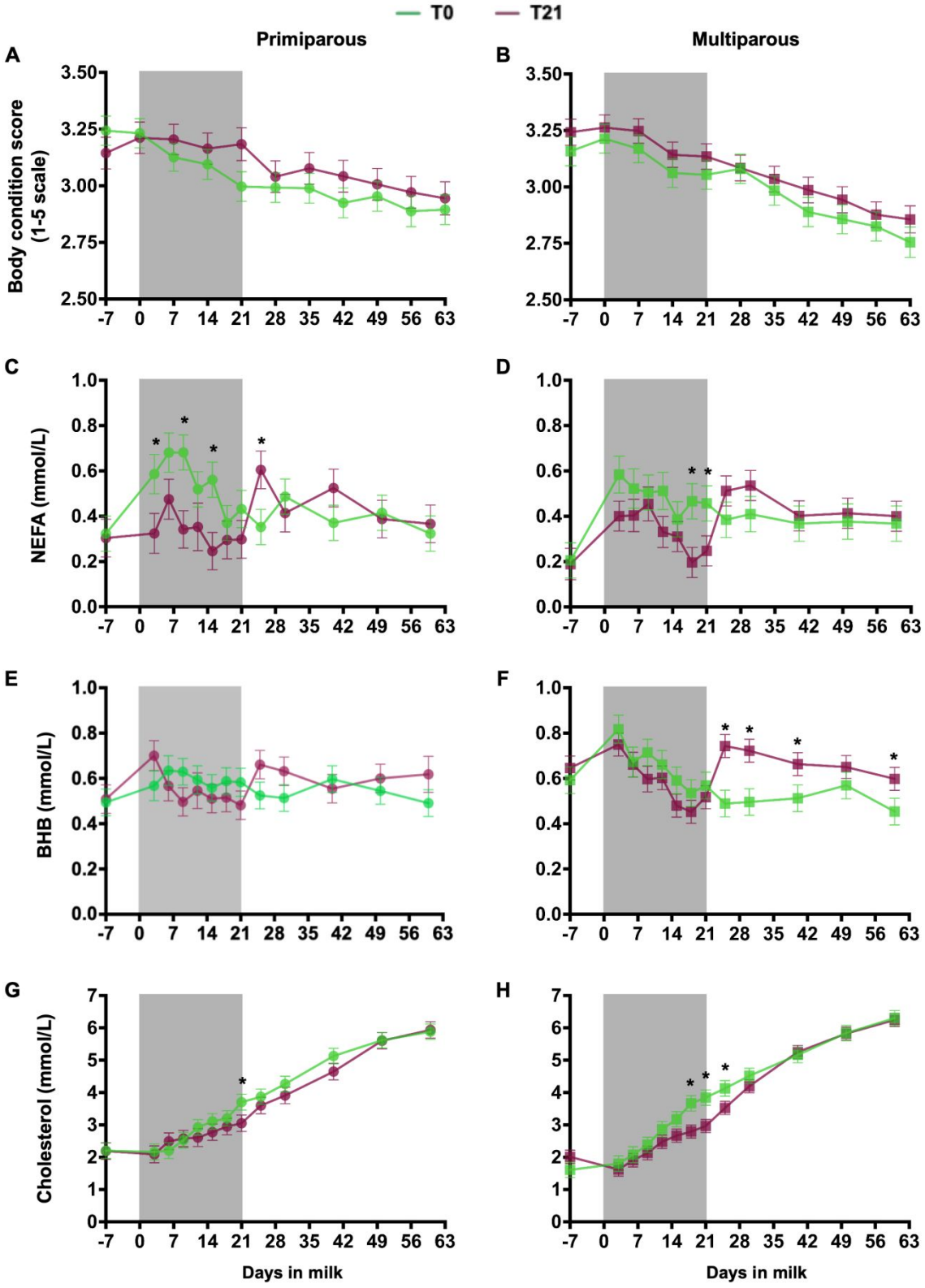


954

955 Figure 1. Milk production (L/cow/day; A, B), total milk solids (kg/cow/day; C, D), and
956 survival curves of the probability of resumption of ovarian cyclicity (E, F) in the first 63
957 days in milk for primiparous (left) and multiparous (right) of T0 (green) and T21 (purple)
958 cows. Grey area represents the period of the differential feeding management in T21.



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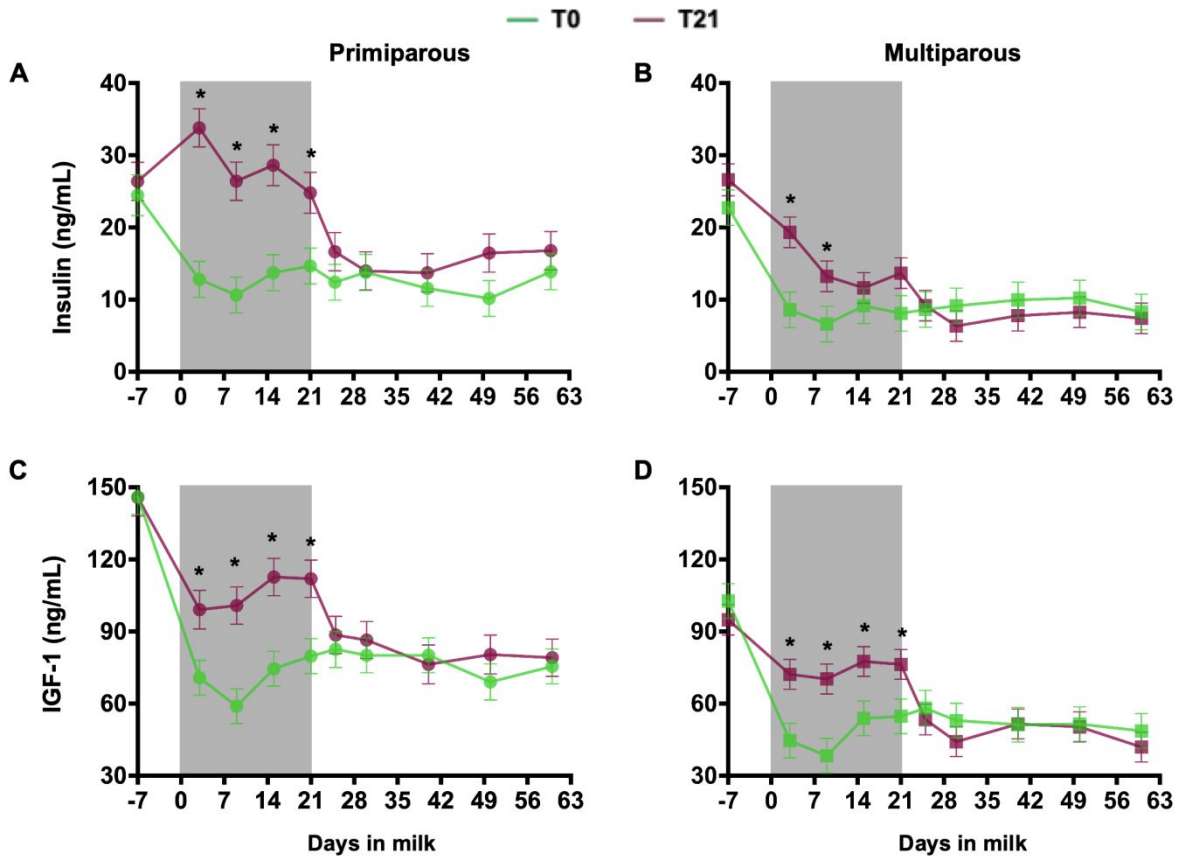
961

962 Figure 2. Body condition score (1-5 scale; A, B), non-esterified fatty acid (NEFA; C,

963 D), beta-hydroxybutyrate (BHB; E, F), and cholesterol (G, H) concentrations in the first

964 60 days in milk for primiparous (left) and multiparous (right) of T0 (green) and T21
965 (purple) cows. Grey area represents the period of the differential feeding management
966 in T21. Asterisks indicate significant differences between treatments.
967

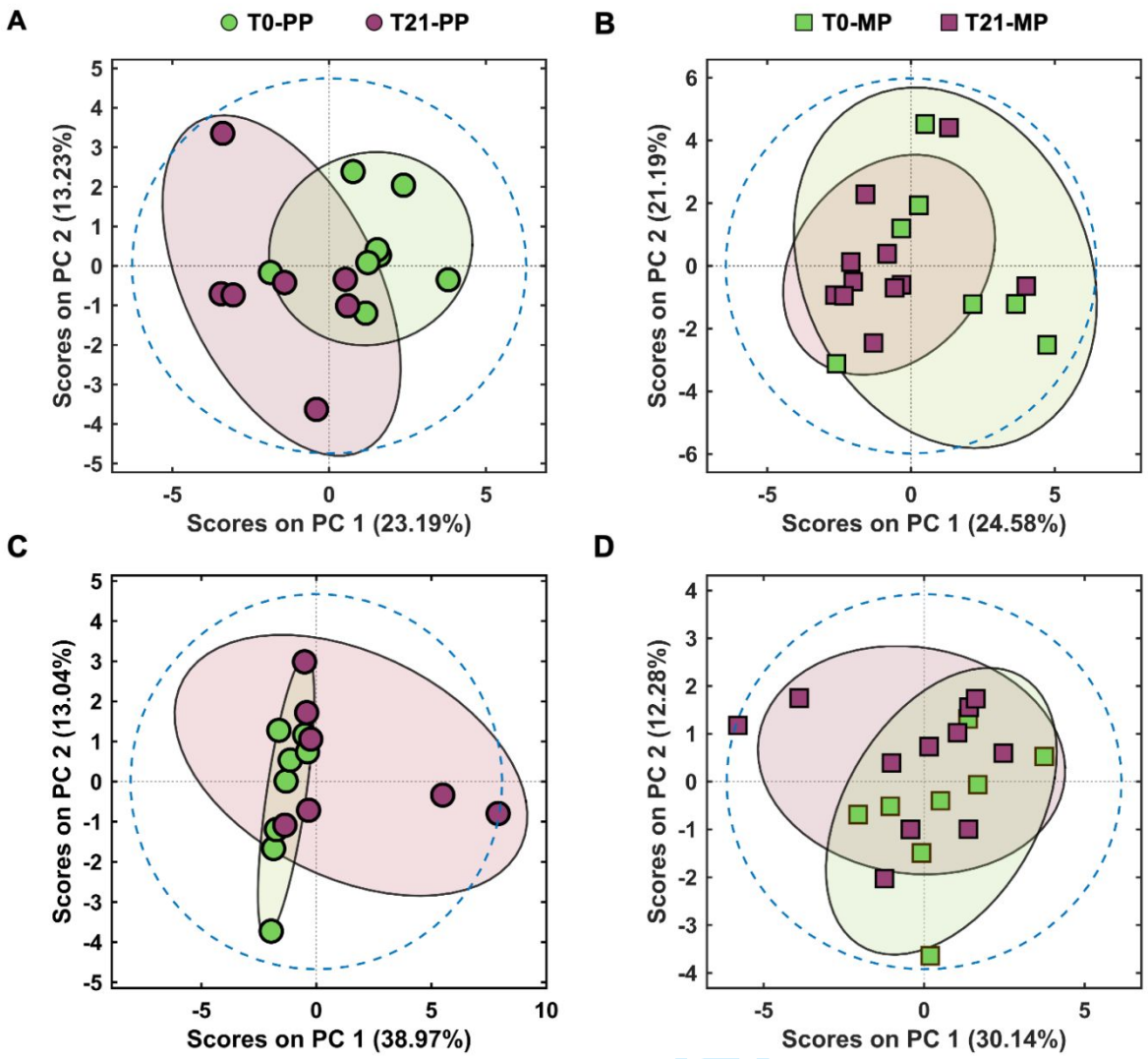
968



969

970 Figure 3. Insulin (A, B), and insulin-like growth factor (IGF-1; C, D) concentrations in
971 the first 60 days in milk for primiparous (left) and multiparous (right) of T0 (green) and
972 T21 (purple) cows. Grey area represents the period of the differential feeding
973 management in T21.

974



975

976 Figure 4. PCA score plot obtained from ^{1}H-NMR spectral data of serum samples from
977 primiparous T21 (purple circles) and T0 (green circles) at 21 DIM (A) and 60 DIM (C)
978 and multiparous T21 (purple squares) and T0 (green squares) at 21 DIM (B) and 60
979 DIM (D).

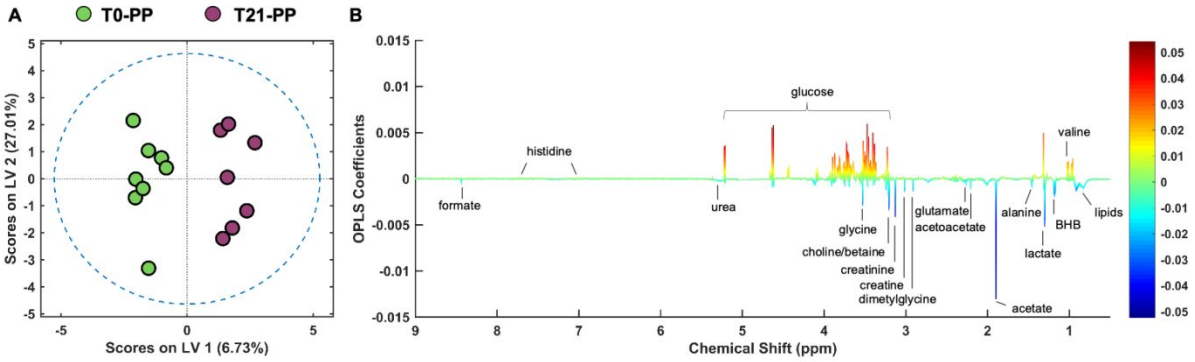
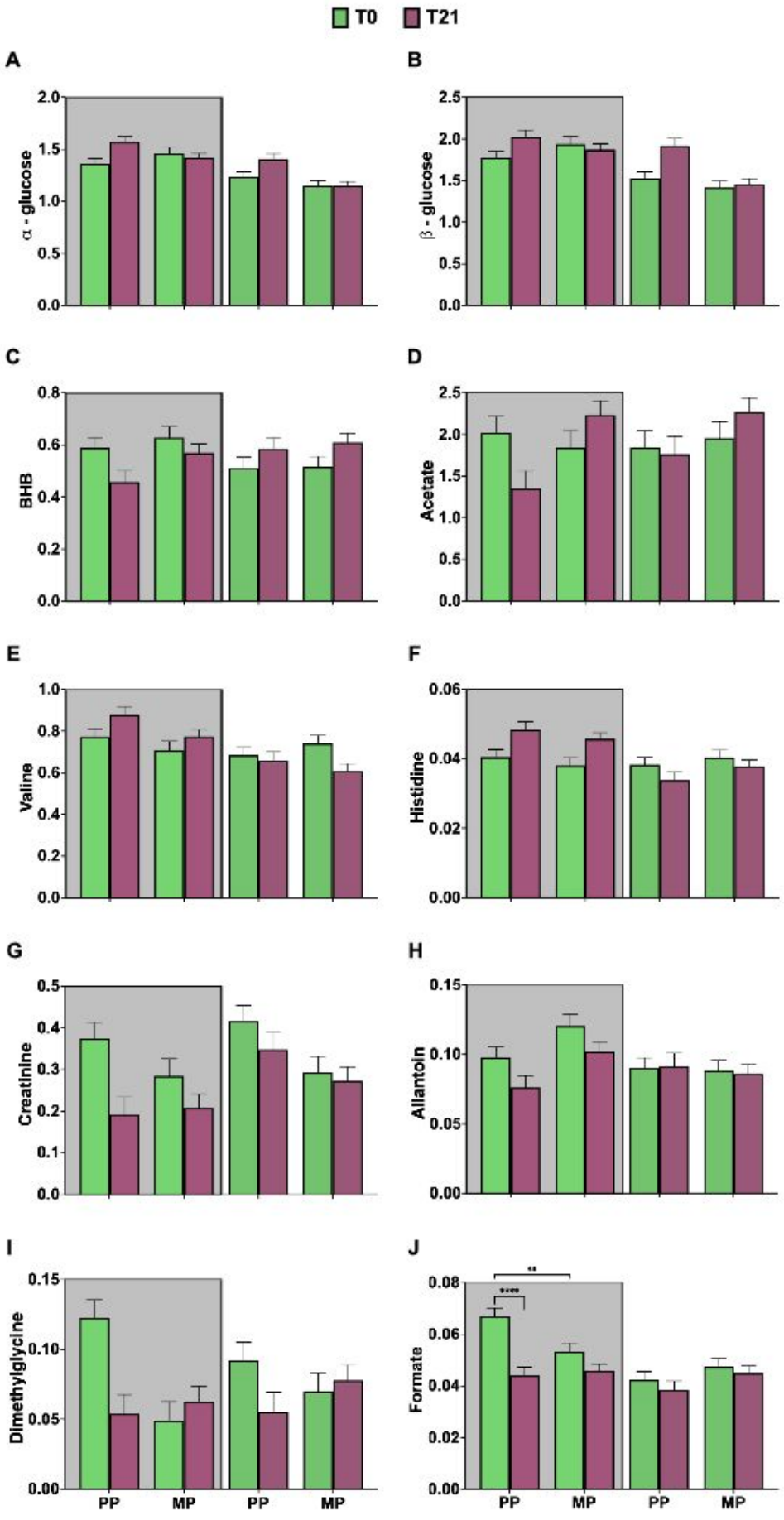


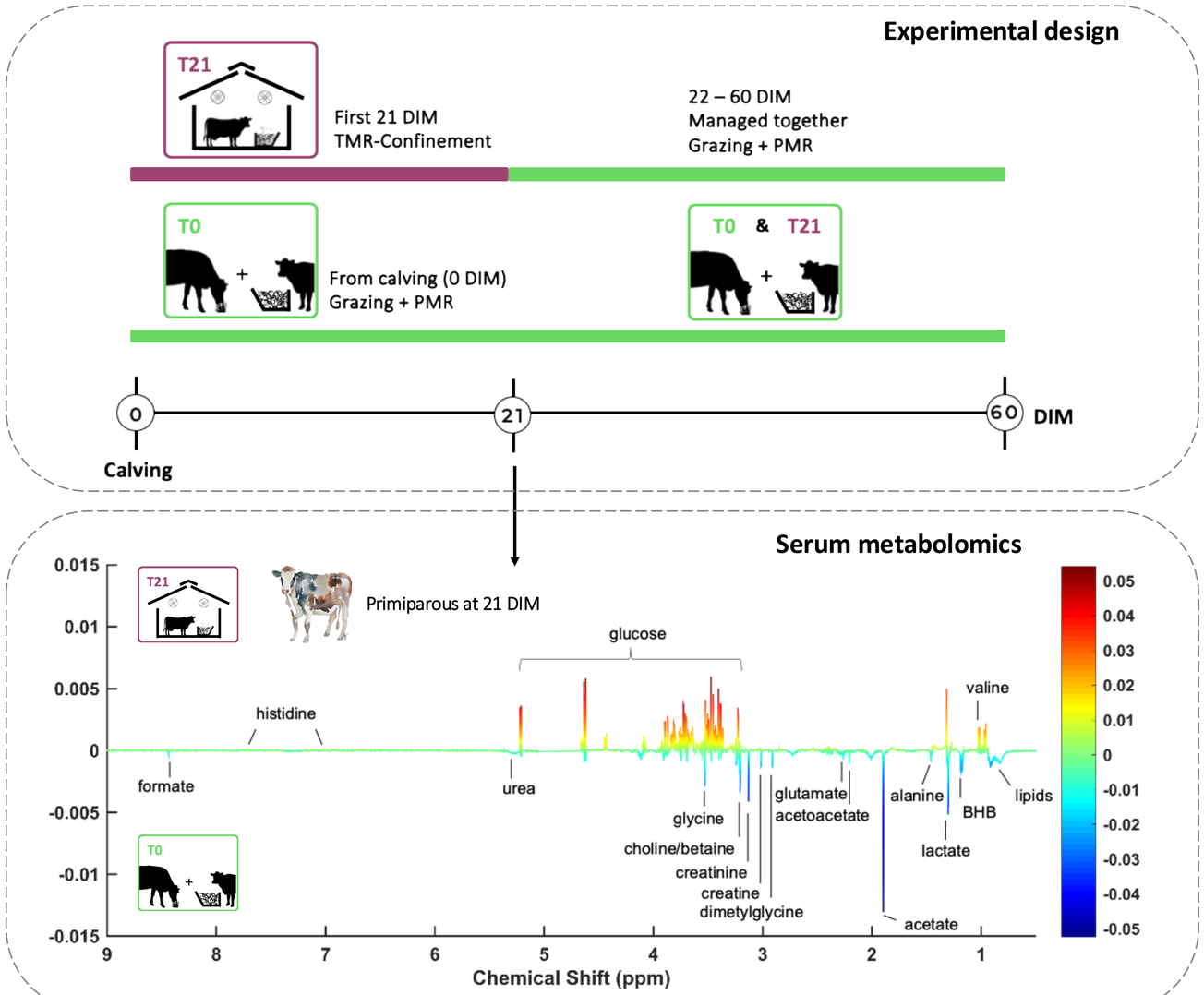
Figure 5. Score (A) and loading factor (B) plots obtained from the OPLS-DA between primiparous T21 and T0 cows at 21 DIM. The metabolites that differentiate the treatments are annotated in the loading factor plot. The R^2Y and Q^2Y coefficients were 0.92 and 0.48, respectively, and the ROC curve had an AUC value of 0.96 (see Figures A2–A3).

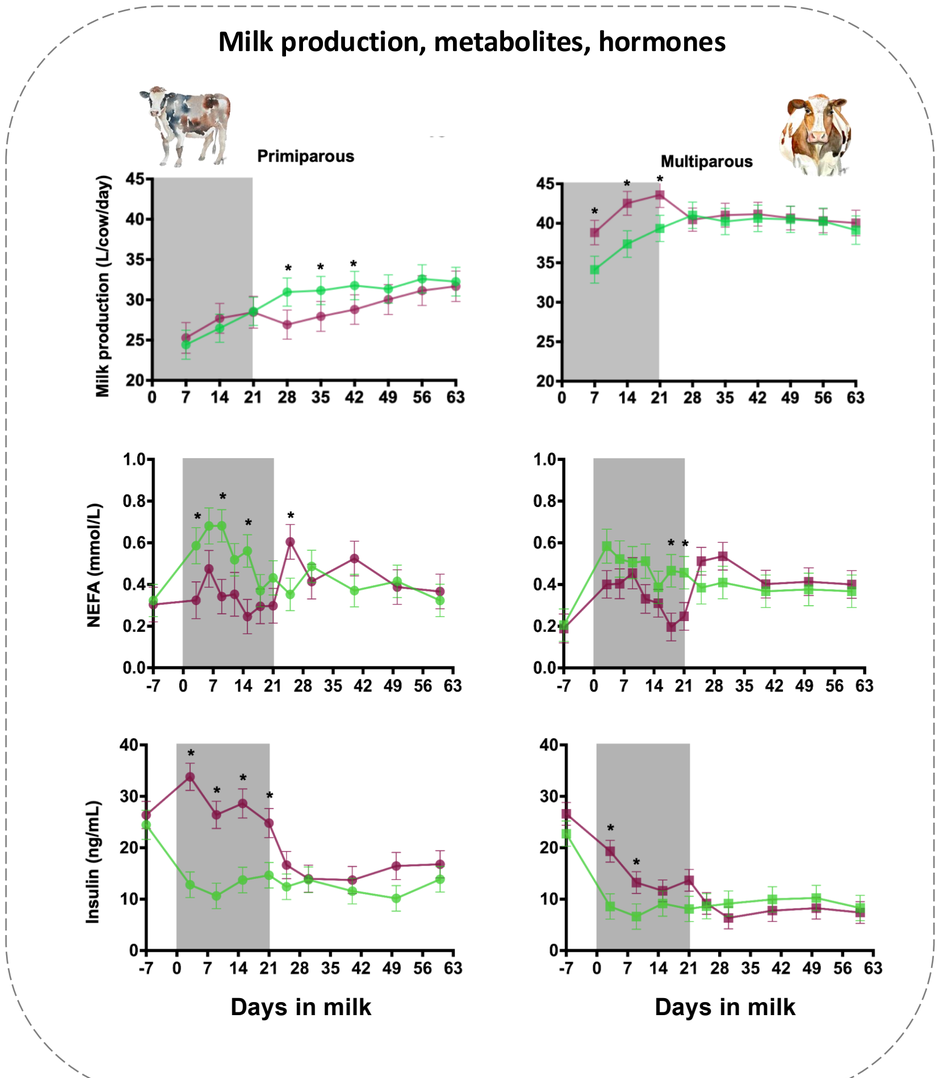


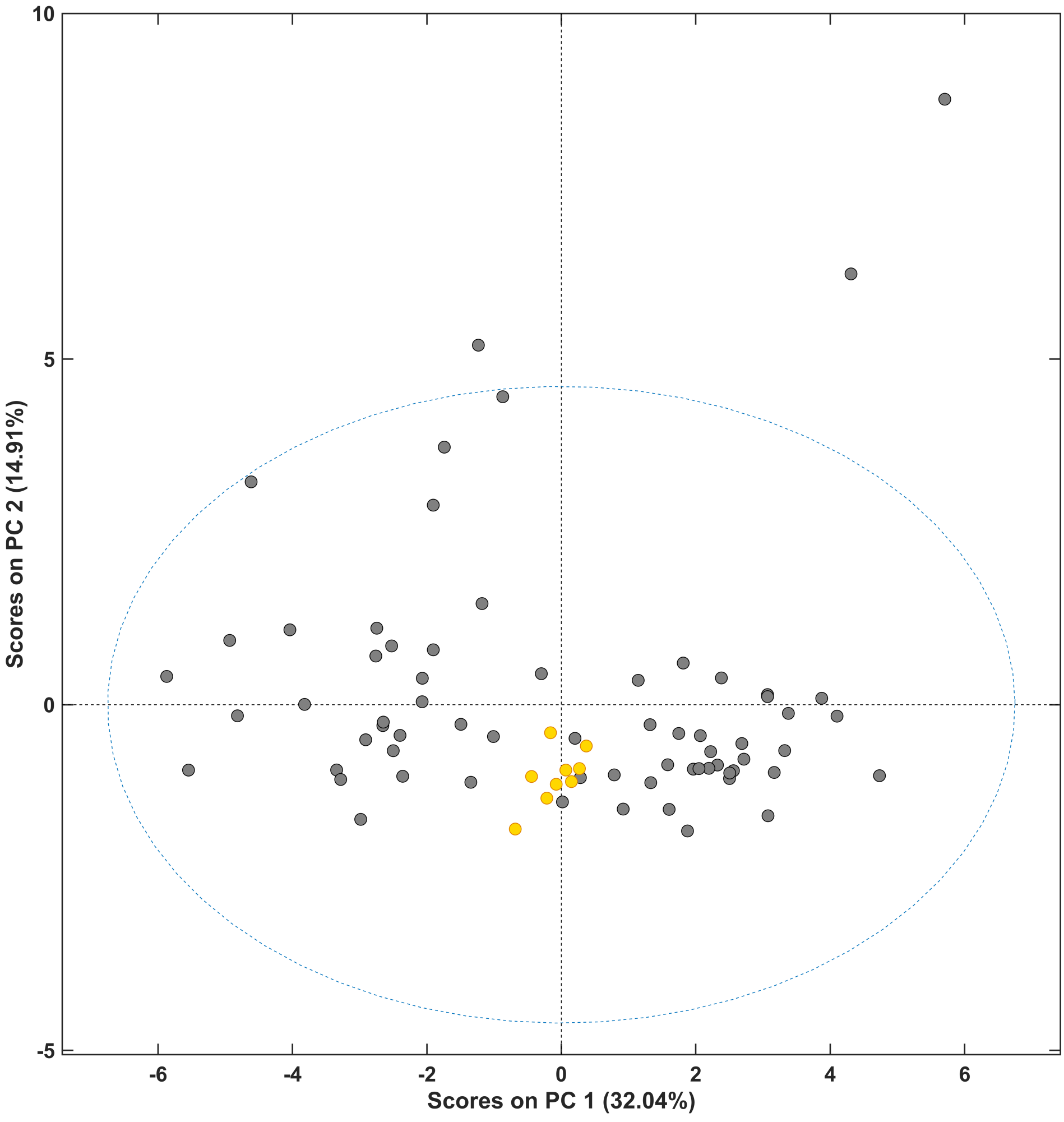
987

988 Figure 6. Relative concentrations of serum metabolites in primiparous (PP) and
989 multiparous (MP) of T0 and T21 groups at 21 (grey area) and 60 DIM. Data are
990 presented as mean ± SEM. Asterisks indicate significant differences (**** = P ≤ 0.0001,
991 *** = P ≤ 0.001, ** = P ≤ 0.01, and * = P ≤ 0.05).

Parity-dependent metabolic responses to differential fresh cow management in pasture-based dairy systems:






10
5
0
-5
-6
-4
-2
0
2
4
6
Scores on PC 2 (14.91%)
Scores on PC 1 (32.04%)

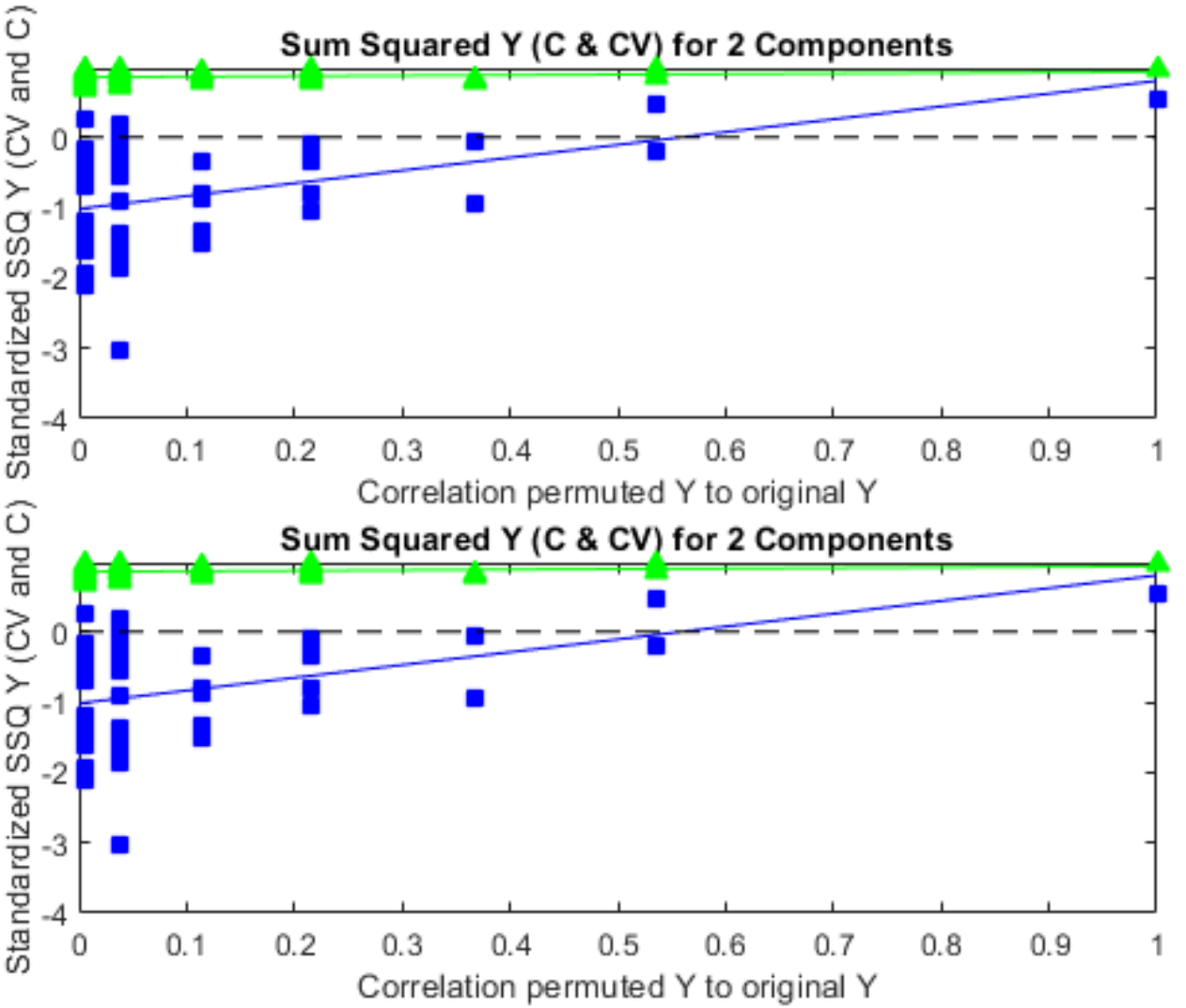
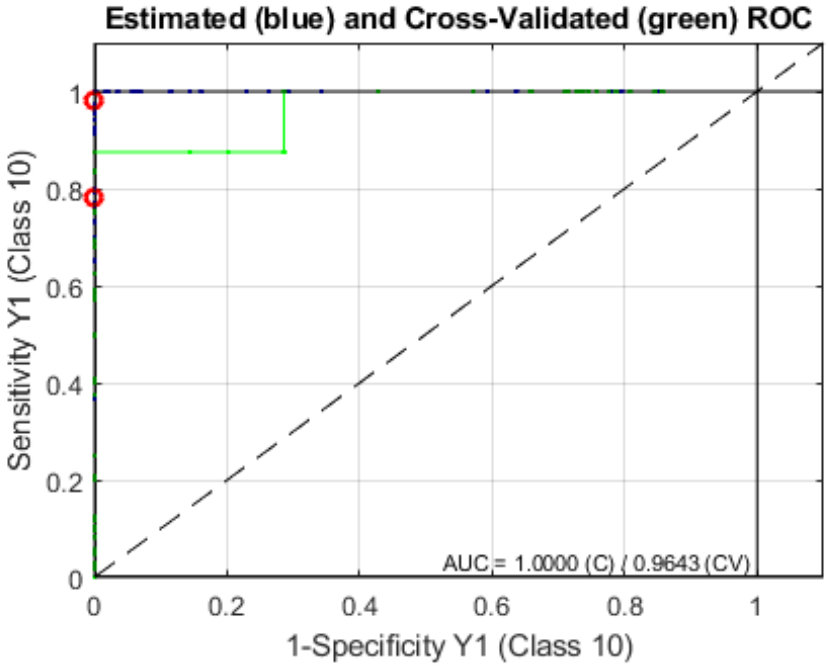
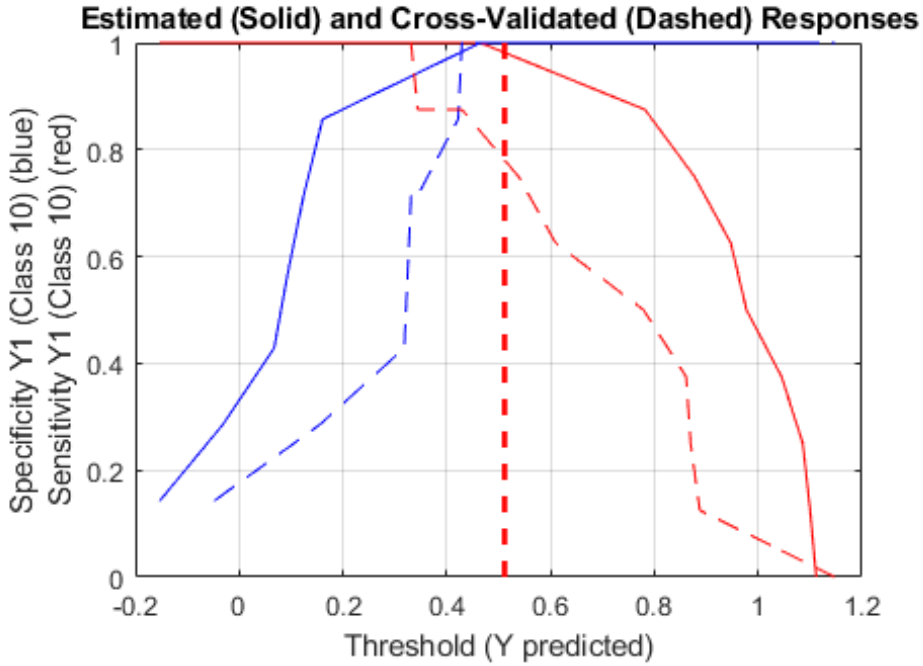
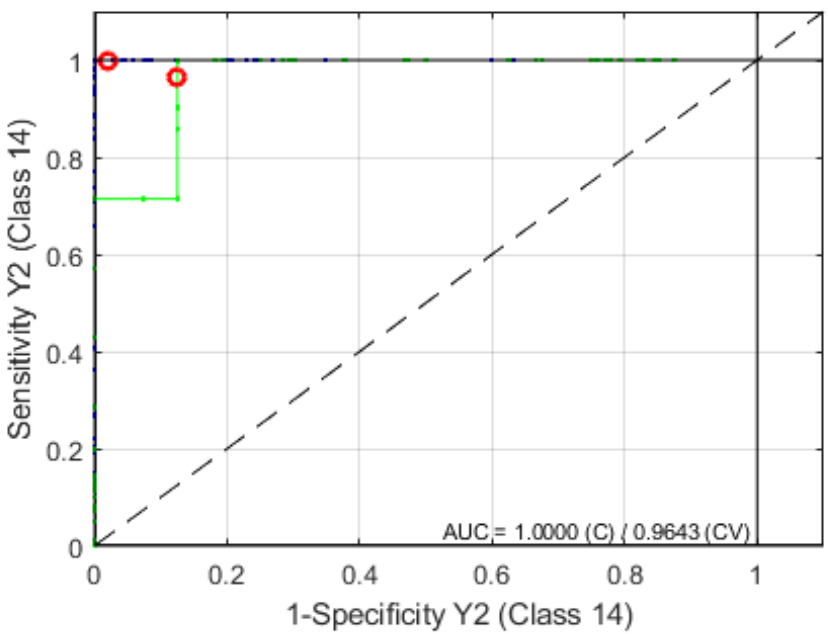


Figure S1. Permutation test plots for the OPLS-DA model comparing primiparous T0 and T21 at 21 DIM (R_2Y = 0.92 and Q_2Y = 0.48).







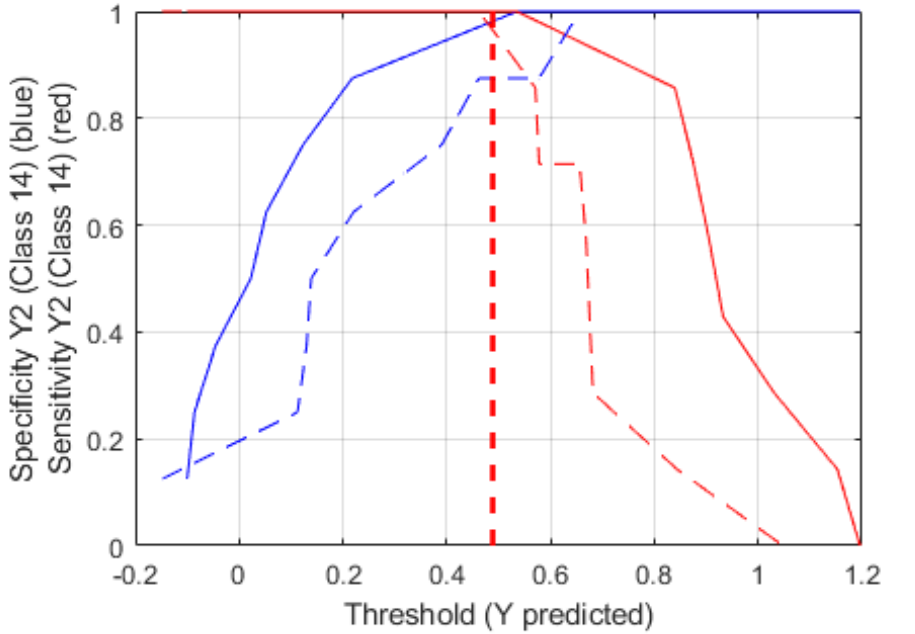
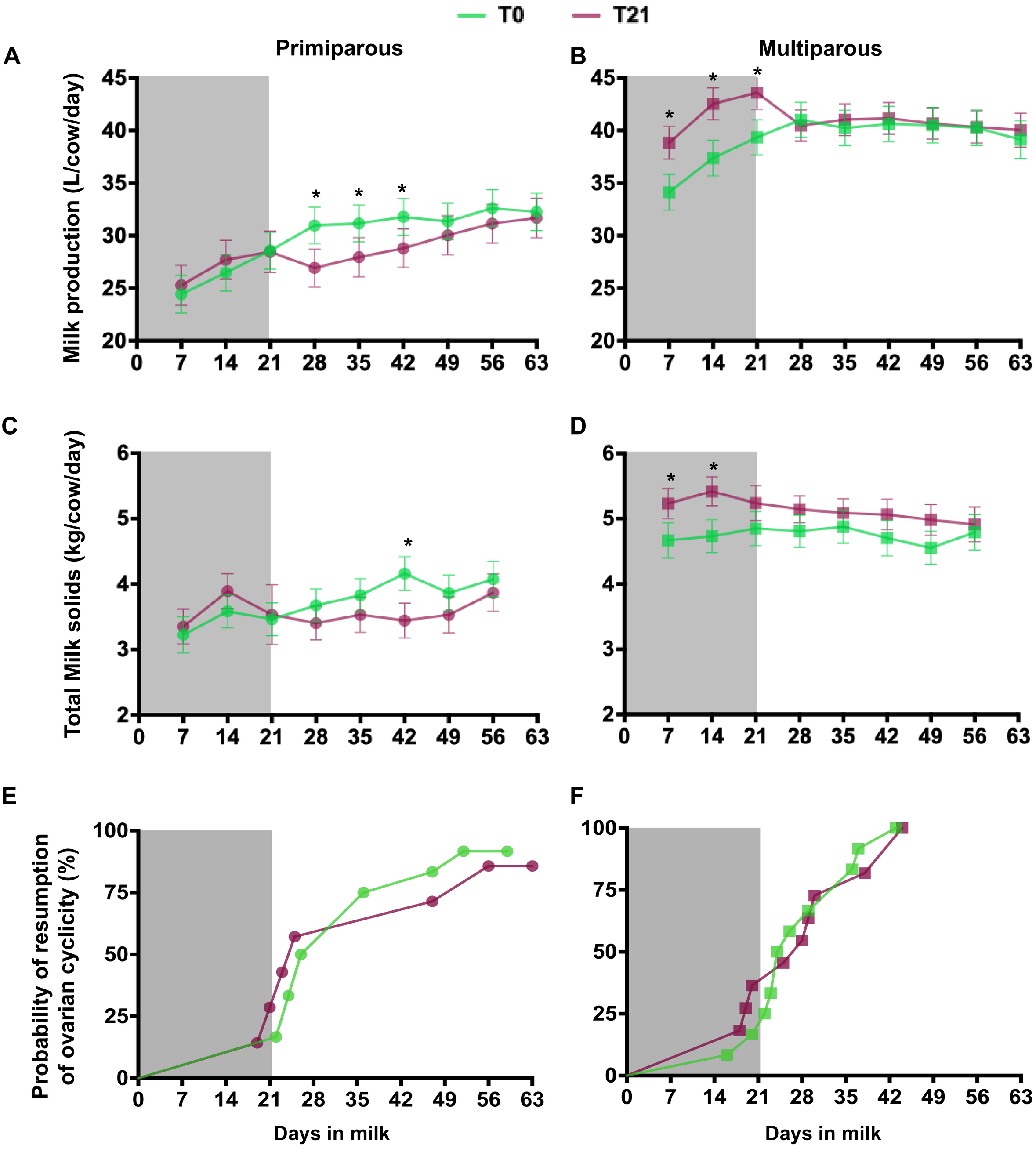
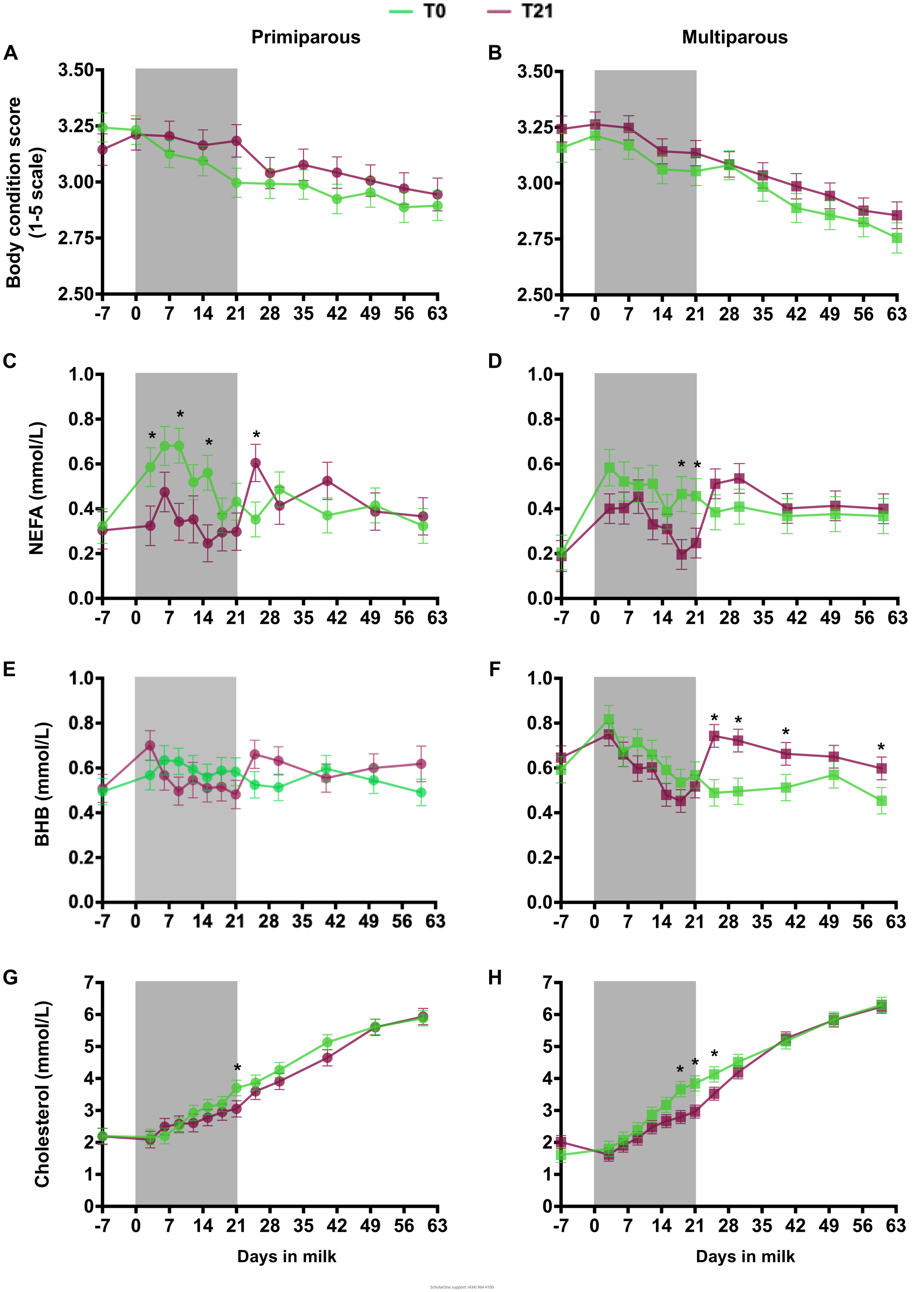
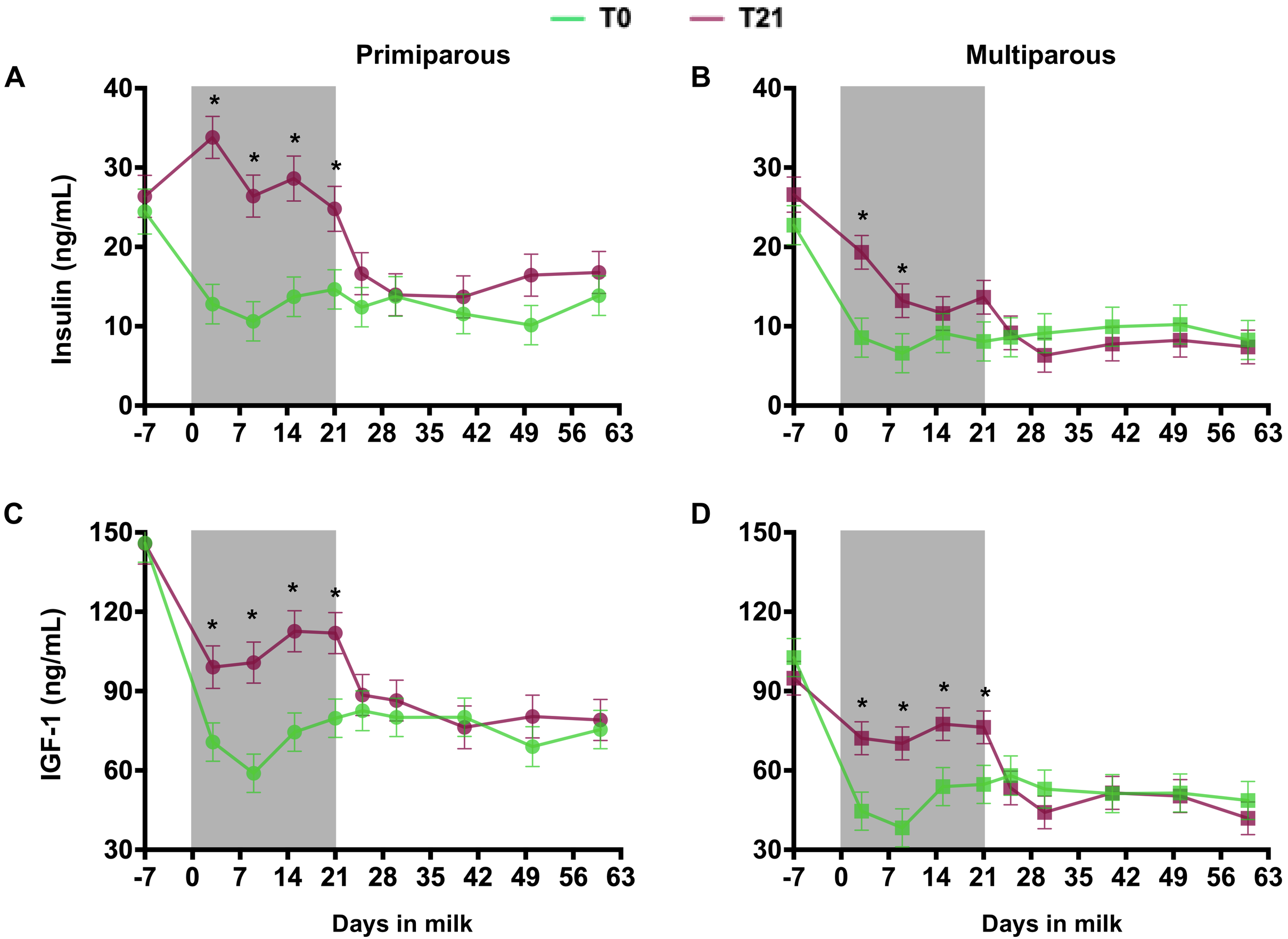


Figure S2. ROC analysis curves derived from the OPLS-DA model comparing primiparous T0 and T21 at 21 DIM (AUC = 0.96).


T0
T21
A
Primiparous
B
Multiparous
Milk production (L/cow/day)
C
D
Total Milk solids (kg/cow/day)
E
F
Probability of resumption of ovarian cyclicity (%)
Days in milk
Days in milk


T0
T21
Primiparous
Multiparous
A
B
C
D
E
F
G
H
Body condition score (1-5 scale)
NEFA (mmol/L)
BHB (mmol/L)
Cholesterol (mmol/L)
3.50
3.25
3.00
2.75
2.50
1.0
0.8
0.6
0.4
0.2
0.0
7
6
5
4
3
2
1
0
-7 0 7 14 21 28 35 42 49 56 63
*
Days in milk


T0
T21
A
Primiparous
B
Multiparous
Insulin (ng/mL)
40
30
20
10
0
-7
0
7
14
21
28
35
42
49
56
63
*
C
D
IGF-1 (ng/mL)
150
120
90
60
30
Days in milk